The fibrolytic potential of domestic and wild herbivores microbial ecosystems on maize stover

BY

FABIAN NDE FON

MSc (Agriculture, University of KwaZulu-Natal)

BSc Hons (Biochemistry, University of Natal)

BSc (Biochemistry, University of Buea)

Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

in

Animal Science (Nutrition)

Discipline of Animal and Poultry Sciences

School of Agricultural, Earth and Environmental Sciences (SAEES)

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

2011

DECLARATION

The experimental work described in this dissertation was carried out in the Discipline of Animal and Poultry Science, School of Agricultural Sciences and Agribusiness, Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg Campus, under the supervision of Prof. Nsahlai I.V.

This is to declare that this thesis has been composed by me and has not been presented in any previous application for a degree. All sources of information are shown in the text and listed in the reference and all assistance by others has been duly acknowledged.

FON F.N
UNIVERSITY OF KWAZULU-NATAL
PIETERMARITZBURG CAMPUS

Signed……. Date …….

I, Prof. Nsahlai I.V., Chairperson of the Supervisory committee, approved the release of this thesis for examination

Signed …… Date …….....

ABSTRACT

The growing demand for meat worldwide by the increasing human population (6.8 billion) calls for an increase in livestock production as well as attention to environmental sustainability. Production increases are critical especially in Africa with the highest annual population growth rate (2.5%), where most communities rely on livestock for protein supply. Attempts by intensive livestock farming to optimize production are limited by fibrous quality feeds (roughages) and their unavailability in both developed and developing countries. The overall objective of this study was to scan both domestic and wild herbivores in search for microbial ecosystems with superior fibrolytic potential that can be used as feed additives. It was hypothesized that microbes from wild herbivore can improve fibrous feed breakdown in domesticated ruminants.

Experiment 1 evaluated the use of fresh or *in vitro* cultured faecal inoculum (FF) from two Jersey cows as a potential substitute for rumen fluid (RF). Cultured FF was a better substitute for fresh RF as demonstrated by percentage differences in exocellulase activity (0.4%) and true degradability (TD) (7%), compared to the differences observed between fresh RF and FF for exocellulase activity (33%) and TD (14%). It was applied in subsequent experimentation because it was cost effective (no surgery and reduced sample collection time).

The second experiment compared the fibrolytic competence of cultured faecal inocula from three hindgut fermenters (miniature horse (mH), horse (H) and Zebra (ZB)) in summer and winter grazing in their natural environment. Both cellulase enzyme assays (exocellulase, endocellulase and hemicellulase) and *in vitro* maize stover digestibility study ranked the herbivores according to their fibrolytic competence as ZB > H > mH.

The effect of cultured faecal *inocula* from H, ZB and wildebeest (WB) and its combined systems (N1=H+WB, N2=H+ZB, N3=WB+ZB and N4=H+WB+ZB) on the fermentation of maize stover were also evaluated *in vitro*. Both enzyme assays and MS degradability studies showed that the combined systems were higher (P<0.01) in fibrolytic activities compared to the individual systems. The microbial ecosystems were ranked as N1 > N2 > N4 > H > ZB >

WB >N3; and N3 > N1 > N4 > WB > N2 > ZB >H by their exocellulase activity and degradability parameters, repetitively. The diversity of microbial ecosystems was confirmed by numerous active carboxymethyl cellulase bands present on a carboxymethy cellulose zymograms in experiment 4. The combined microbial ecosystems contain more active and variable bands of cellulases than in the individual microbial ecosystems. Systems N3 and N1 were considered as the best inocula for rumen transinoculation studies.

Experiment 5 assessed the *in vivo* effect of direct-fed microbials from N1 and N3 on MS degradation, ruminal fermentation characteristics and cellulase enzyme profile in sheep. Feed dry matter intake increased ($P<0.03$) in N1 but tended to increase when inoculated with N3. The treatments, N1 and N3 increased ($P<0.05$) rumen exocellulase (9.4 and 33.2%, respectively) and endocellulase (82.1 and 47.1%, respectively) specific activities but not hemicellulase activity. Maize stover degradability parameters for N3 (TD, degradability of the insoluble fraction of MS, effective degradability, total SCFA and propionate) measured after 96 h of incubation tended *($P>0.05$)* to be numerically different (1.1, 5.4, 7.1 and 7.9%, respectively). Increase in propionate for N3 was accompanied by higher total SCFA and lower CH_4. A decrease in CH_4 and no difference in CO_2 allow both systems to be environmentally friendly since they have been associated with global warming.

These studies showed that direct-fed microbials from N1 and N3 inocula have the potential of improving the utilization of maize stover feeds in ruminants, particularly in view of its simplicity and availability which allows it to be implemented at a relatively lower cost compared to other specific strains or microbial cultures. However, more research is required to identify, purify and classify the superior fibrolytic microbes in the most active ecosystems.

ACKNOWLEDGEMENTS

Many people contributed to the success of this research and it may be unjust to single out individuals. However, I will like to express my sincere and profound gratitude to the following persons and institutions:

My supervisor, Prof. Nsahlai I.V. for his guidance, constructive comments and important sources of information provided throughout this study. His patience and support during rainy days inspired me.

Special thanks to the National Research Foundation (NFR) of South Africa for providing the funds that facilitated the smooth running of this project.

The Department of Animal and Poultry Science and the staff (Prof. Chimonyo M. and Ms Marion Young) at the Ukulinga Research farm for providing research facilities, experimental animals and their support throughout this period.

The Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA) for providing most of the experimental animals in this study.

To all my friends, thanks for your help and support especially Dr. Yobo K, Ms. Bame I. Mr. Nasreldin B., Ms Ahmed M., Mr. Fomum S. Mr. Mbu D., Dr. Achilonu I., Norbert N., Dr. Pascaline F. Ms Jessica and Mrs Nsahlai J.

I would also like to appreciate my late dad Mr. Forcob F. and my Brother Fru F. for making my studies possible in South Africa. Special appreciation to my mum (Alias Marie), siblings Neh P., Nanga B., Swiri S., Ngum N., Che S. and Ntseh R.

Finally, I would like to thank my treasured wife, Tessido Anastsie, for the unlimited love, patience and all the moral support she has been offering to me. Thanks for being there for me and Frank when we needed you most.

This thesis is dedicated to my

Mum

Mama Mary Bih Fon

Wife

Anastasie Tessido

and

Children

Frank N. and Shalome K.

Conference proceeding papers published

Fon, F.N. and Nsahlai, I.V. (2009). Activities of microbial fibrolytic enzymes in ten herbivore microbial ecosystems. *In:* XIth International Symposium on Ruminant Physiology. Chilliard, Y., Glasser., F., Faulconnier., Y., Bocquier, F., Veissier, I. and Doreau, M. (eds). Clermont-Ferrand, France: Wageningen Academic, Netherlands, 180 -181.

Conference abstracts

Fon, F.N. and Nsahlai, I.V. (2008). The fibrolytic activity of microbial ecosystems in three hindgut fermenters. *In:* 10th World conference on Animal Production, 23rd to 28th November 2008 WCAP (eds). Cape Town, South Africa: Wageningen Academic, 72.

Fon, F.N. and Nsahlai, I.V. (2011). *In vitro* fermentation using faecal fluids of the horse, wildebeest and zebra. *In:* Southern African Society for Animal Science 44th Biannual Congress. Enhancing Production Efficiency within Current Constraints and Seminar, Stellenbosch University, Western Cape Province, South Africa.

Journal papers submitted for publications

Fon, F.N. and Nsahlai, I.V. (2011). *In vitro* fermentation using faecal fluids of the horse, wildebeest and zebra. **Summitted in** *S. Afr. J. Anim. Sci.* **30 October 2011**

Fon, F.N. and Nsahlaia, I.V. (2011). The effect of direct-fed microbial ecosystems on ruminal fermentation of maize stover in sheep. **Submitted in** *Small Ruminant Res.* Manuscript number Rumin-D-11-3840, **12 September 2011**

Journal papers still working on for submission

Fon, F.N. and Nsahlai, I.V. (2011b). The fibrolytic activity of microbial ecosystems in three hindgut fermenters.

Fon, F.N. and Nsahlai, I.V. (2011b). Laboratory cultured faecal inoculum is a better substitute for fresh rumen inoculum than fresh faecal inoculum for *in vitro* feed evaluation

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Glossary of abbreviations and symbols

°C	Degree centigrade
Acet	Acetic acid
CH4	methane
CO2	Carbon dioxide
DEF	Degradability efficiency factor
DM	Dry matter
DMD	Dry matter degradability
DMI	Dry matter intake
GP	Total gas volume from fermentation
iB	Iso-butyric acid
nB	n-butyric acid
PP	Propionic acid
TD	Truly degradable fraction of feed
NGO	Non-governmental organization
H	Horse
WB	wildebeest
ZB	Zebra
mH	Miniature horse
N1	H+WB
N2	H+ZB
N3	WB+ZB
N4	H+WB+ZB
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PF	Partitioning factor
Ca	Calcium
N	Nitrogen
P	Phosphorus
Mn	manganese
K	Potassium
ATP	Adenosine triphosphate
PF	Partitioning factor
SCFA	Short chain fatty acids

Chapter 1
General Introduction

1 Background

All living systems require some form of energy in order to operate, which may be food, chemical, kinetic or solar energy. Food energy is the most common form of energy utilized by living organisms with carbohydrates being the most abundant and main source of metabolic energy for plants and animals. Carbohydrates are the major substrate through which energy of the sun is harnessed and converted into a form that can be utilized by man and other animals. Therefore, they are the primary products of photosynthesis (Laisk et al., 2006). Photosynthesis is an endothermic reduction condensation of carbon dioxide that harnesses light energy in the presence of the plant pigment chlorophyll to produce carbohydrates and oxygen (Equation 1).

Equation 1.0 $nCO_2 + nH_2O + \text{energy (light)} \longrightarrow C_nH_2O_n + nO_2$

n = natural numbers

Although carbohydrates are known to be the major source of energy for plants and animals, they also play a vital role as structural components in the plant cell wall (cellulose) as well as in the animal cell membrane (glycolipids). They serve as components of recognition sites on cell surfaces (glycoprotein, e.g. immunoglobulins) and as essential components of Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules (ribose sugars) (Freier and Karl-Heinz, 1997; Lindqvist et al., 2000), which are informative molecules.

Carbohydrates are defined as carbon hydrates with respect to their molecular formula $C_n(H_20)_n$. Peter (1990) further separated them chemically into hydroxyl aldehydes or ketones and their derivatives. Simple carbohydrates are called monosaccharides, saccharides or sugars (e.g. glucose, ribose and galactose) while the complex forms include cellulose, starch, glycogen and hemicelluloses. They have also been classified as soluble or non-soluble carbohydrates depending on their interaction with water molecules. Plants are the major source of carbohydrates with cellulose (fibre) being the most plentiful polysaccharide in nature (Schwarz, 2001), contributing 200-400 g/kg of most abundant plant tissue (Van Soest,

1982). Because of cellulose complexity, the rate of consumption varies from one animal to another.

Ruminal microbes are responsible for fibre breakdown in ruminant forestomach. These microbes include bacteria, protozoa and fungi (Flint, 1994; Fonty and Gouet, 1994). They are anaerobic since the rumen environment is known to be oxygen deficient (Kamra, 2005). However, Brooker et al. (1994) reported that the rumen environment is not completely anaerobic, as it is inhabited by facultative bacteria such as *Streptococcus caprznus*, which help to eliminate traces of oxygen entering the rumen.

The distribution of cellulose in plant tissue varies from one plant species to another as well as in different parts of the same species (leaves, stems and fruits) (Ouda et al., 2006; Sun et al., 2009). Due to these differences, animals have evolved to select and consume different components of plants and vegetation depending on their mouth parts and designs of their fore- and hindgut fermentation chambers (Church, 1988). There are a large number of extant species of ruminants found in different ecosystems harbouring a variety of microbes. Hofmann (1998) demonstrated that flora variation among ecosystems as well as seasonal availability of forages influence the nutritional habits of these animals. The extant species were classified according to the physiological evidence obtained from fibre hydrolyses into a flexible system of three overlapping morphological feeding types: concentrate selectors (CS), intermediate selectors (IM) and grass and roughage feeders (GR).

Concentrate selectors (e.g. giraffe) feed on easily digestible forages such as shrubs and fruits while GR graze mainly on grasses (e.g. buffalo and cattle). Intermediate selectors alternate between CS and GR (e.g. goat). According to Hofmann (1998), climatic conditions, habitat pressure, behavioural patterns and ecological opportunities have a great influence on ruminant diversity. Despite the variation of the above factors ruminants have been able to maintain two generic futures such as a complicated morphological master plan of the ruminant digestive system and an incredible flexible adaptation that extends from the level of one animal to another. As one moves from CS to GR, salivary gland regress while the rumen cavity increases in size. These adaptations are flexible, such that seasonal variations with the

availability of forages are common, e.g. increase gland secretion in goats in summer (Hofmann, 1998).

Livestock production is often not cost-effective in the dry season (winter) especially in developing countries where feed quality, quantity and availability of grazing pastures are limited. During this period, feed shortages and poorer feed qualities are the major problems faced by livestock farmers. In developed countries, where resources and technologies are more advanced and available, this is not a major problem as feeds of higher qualities (Leng, 1996; Dominguez Bello and Escobar, 1997) can be stockpiled in preparation for such periods. In developing countries, animals are malnourished and lose body weight due to long journeys in search of pastures (Leng, 1996; IFAD, 2004). Some farmers even sell up to 80% of their livestock during this period at low prices so as to avoid the loss of animal through death. However, during this period crop residues and agro-industrial by-products, which have feed potentials, are often abundant but not exploited (Leng, 1996). The lack of information about the nutritional potential of crop residues limits its application by small scale farmers. Maize is the highest crop fodder production in the world and third cereal produced (Butler, 2007). This implies that maize stover residues are abundant but are not properly utilized, especially in Africa, where it is often burnt, ploughed back into the soil or allowed to be grazed on farm (Yang et al., 2008). Sensitizing small scale farmers on the feed potential of crops residues especially maize stover is important in improving livestock production.

Low hydrolysis of high fibre forages or crop residues by herbivores is a major problem as little of the total intake is converted into feed energy (Leng, 1990; Leng, 1996). Less than 30% of the total fibre consumed by ruminants is converted into chemical energy while the rest is excreted as waste. This implies that metabolic substrates are far less than that which is lost in faeces. The review will be looking at maize stover as the main crop residue, the factors influencing its usage as a forage feed, scans through the complexity of fibre, its association with other components of the plant cell wall, factors influencing its hydrolysis and different fibre additives. At the end of the review, a possible mechanism will be hypothesized, attempting to improve fibre hydrolysis with regards to the available information acquired from the substrates and microbial activity from different ecosystems.

1.1 Overall objective

The broad objective of the study was to identify microbial ecosystems with high fibrolytic activities and investigate their fibrolytic potential when merged both *in vitro* and *in vivo.*

1.2 Specific objectives of the study

The specific objectives of the study were to:

- Investigate the difference between fresh or laboratory cultured inocula for ruminant *in vitro* feed evaluation;
- Compare the fibrolytic activity of microbial ecosystems in three hindgut fermenters;
- Manipulate rumen or hindgut ecology *in vitro* with microbes from the most active ecosystems (wildebeest, horse and zebra);
- Monitor cellulase production from five *in vitro* herbivore microbial ecosystems and their combined systems; and
- Manipulate the sheep rumen ecology with microbes from the two most active *in vitro* microbial synergistic ecosystems.

Chapter 2

Literature review

2 Maize stover as a feed and microbial energy generator in herbivores

Livestock production can be defined as the art of attempting to increase livestock population. Factors such as pasture, fodder production and utilization, improved supplementation of ruminant diet, optimum management techniques, processing of animal products and improved animal techniques influence livestock production. Livestock production accounts for about 30% of the gross value of agricultural production in Africa (Seré, 2004). Seventy percent of the rural poor in Africa own livestock and about 200 million rely on it for income (sales of meat, skin or milk) (Thornton et al., 2002; World Resources Institute, 2005; FAO, 2010). McDowell (1988) also reported that smallholder crop-livestock farming contributes 60-70% of ruminants in sub-Saharan Africa. Although humans are more related to herbivores than carnivores, most herbivores still depends on meat for protein supply (IFAD, 2004). In most Southern African countries, starch is the most abundant foodstuff, while plant protein sources are limited, especially among the poor communities. Given the rising demand for animal protein in diets due to the expanding human population both in developed and developing countries (Thornton et al., 2002; IFAD, 2004), there is an urgent need to increase livestock production.

Livestock production has been classified into four main groups by Otte and Chilonda (2002). These systems are: (I) grassland based systems (Grazing systems), based solely on livestock in which more than 90% of the dry matter (DM) fed to animals comes from range lands, pastures or home grown forages; (II) rain-fed mixed farming of which more than 10% of the DM fed to animals comes from crop by-products or more than 10% of the total value of production comes from non-living farming activities. In these systems, more than 90% of the value of non livestock farm produce comes from rain-fed land use; (III) Irrigated mixed farming systems, which is similar to the above farming system but more than 10% of the value of non-livestock

farm produce comes from irrigated land use; and (IV) landless livestock production systems (industrial systems), that are solely livestock with 10% or less of the DM fed to animals being farm produced.

In most African countries, large-scale systems, such as ranching, commercial farming and co-operative farming, still account for a relatively small proportion of agricultural output whereas small scale farming systems, such as pastoralism, irrigated mixed farming, agro-pastoralism and mixed small holder farming, accounts for most of the agricultural products (IFAD, 2004). However, large scale farming systems such as ranching and irrigated mixed farming systems are rapidly growing in sub-Saharan countries. Therefore, improving policies and available resources to promote such farming systems in these countries will be beneficial.

Modern farming systems are characterized by large capital requirements and employment of a large labour force. These characteristics are prominent in developed countries. Whereas traditional livestock production systems are characterized by family labour and extensive use of land (Wilson and MacLeod, 1991; Wilson, 1991) in developing countries, especially in sub Saharan Africa. In the temperate or developed countries, ruminant production systems mainly use high quality ingredients in formulating feeds, which, in turn, support high production rates close to the genetic potential (Leng, 1996; Hall and Silver, 2001). In traditional livestock production, animals graze predominantly on natural pastures (often of low nutritional value). Often the grazing land is not easily farmed on or too infertile for cropping.

Seasonal variation of the quality, quantity and availability of grazing pastures is also a major problem in tropical countries. In the raining season (summer) the grazing pastures are available and of a higher quality whereas in the dry season (winter) the animals suffer severe nutritional stresses due to the poor nutritional value of pastures and shortages in supply (Smith et al., 1989; FAO, 2010). During this period of nutritional shortages, animals waste a lot of energy as they have to walk long distances in search of food and water. As a result of these adverse conditions and nutritional deficiencies, animals lose weight (Alaku and Moruppa, 1983), poor body condition, low milk yield, low conception rates and increased calf mortalities are also prominent (Zerihun and Oba, 2004). Such factors can contribute to heavy

economic losses to small scale farmers. Although there is a scarcity of quality pastures in winter, crop residues (by-products) and agro-industrial by-products that have feed potentials are often abundant (Saleem, 1995).

2.1 Crop residues

Crop residues are materials generated after harvesting (Dixon and Egan, 1987). They are produced on farm and are wide-spread geographically. They are invariably fibrous, low in digestibility and nitrogen content. The types of crops grown in an area determine the nature of crop residues produced in that region. Table 2.1 shows the common crop residues in sub-Saharan countries.

Table 2.1 Principal crop residues in sub-Saharan countries

Crop residues	**Crop residues**	
Cereals:		
Maize	stover	cobs
Sugar cane	bagasse	Tops
Wheat	straw	
Millet	stover	
Rice	straw	hulls
Sorghum	stover	Threshed heads
Legumes:		
Groundnut	halms	hulls
Soya bean	straw	
Cottonseed	stalks	hulls
Sunflower	heads	hulls

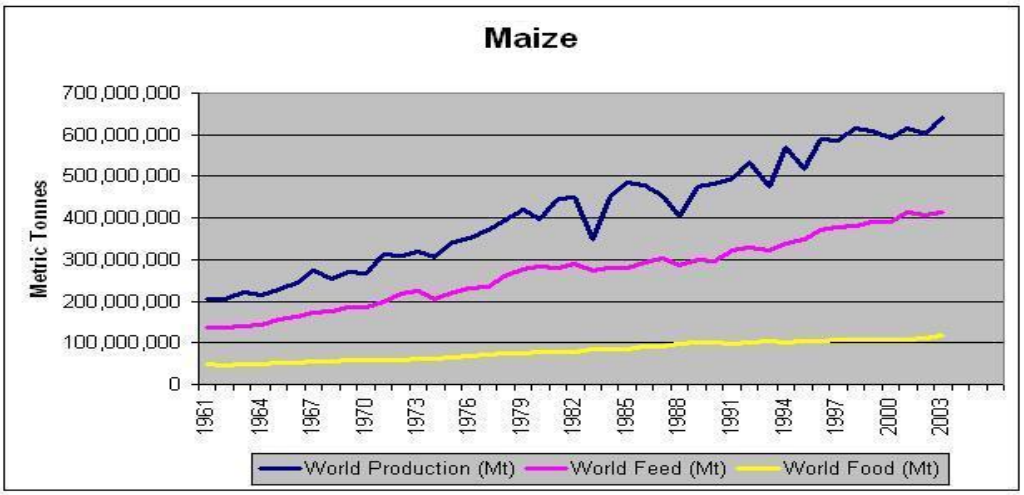
2.2 Agro-industrial by-products

Agro-industrial by-products are produced by agricultural companies indulged in the processing of a specific crop or animal product. Some of these by-products have been studied and classified according to their feed potentials (Bistanji et al., 2000). These by-products vary

from one country to another depending on the availability of industries. Although the type of crop produced in a particular region influences the type of by-product produced, this is not always the case as developed countries with huge industries tend to import such crops from developing countries without processing facilities. Examples of industrial by-products are: bagasse, oilseed cake, molasses, maize milling by-products and brewer's wastes.

Kossila (1988) showed that crop residues account for more than 25% of the total energy suitable for ruminant livestock production in both developed and developing countries. This implies that if crop residues, as well as agro-industrial by-products, are used in the right proportion for feed formulation, production will definitely increase. However, in developing countries where these facilities are limited, an introduction of on farm technologies as well as sensitizing small scale farmers on how to manage crop residues would definitely have a positive influence on livestock production. Maize is one of the world's food-providing crops for human and crop residues both in the developed and developing countries.

2.3 Maize realities



Gramene website (2006).

Figure 2.1 Statistics of production and consumption of maize in the world

After wheat and rice, maize is the third most produced grain in the world (Kossila, 1988; Butler, 2007). Although it is the third in terms of grain production, it tops the chart of crop

fodder production world wide (Kossila, 1988). About 1816 and 340 million tones of maize fodder are produced annually both globally and in Africa, respectively (Reddy et al., 2003). Maize is a staple food source in many countries (e.g. corn flakes, porridge, pap, "fufu" etc.). Porridge and cakes are the common meals made from maize. Industrial products from maize include: maize oil, maize starch, syrup, dextrose, alcohol, ethanol, high fructose corn syrup, grits, flour and additives in paints and explosives. There are about 4000 industrial products of maize. World production of maize has increased tremendously over the past decades but human consumption is increasing slowly (Figure 2.1). This increment has been associated with the increase in demand for feed production.

Maize grain has been fed to both ruminants and non-ruminants as concentrates (Tolera et al., 1998; Dhakad et al., 2002; Garg et al., 2004). However, maize stover, which is the non-grain portion of the maize plant is an important supplement of feed (Gertenbach et al., 1998). Maize stover fractions include the stalk, leaf, cob and husk. It is the most abundant and available crop residue in the world. In most African countries maize stover (MS) is abundant but has not been fully exploited. This is due to the fact that small scale farmers have very little or no knowledge of processing, storage or formulating feed using MS (Aregheore and Chimwano, 1991). The consequence of this is that MS is regarded as an environmental nuisance and is discarded either by burning (Yang et al., 2008) or by ploughing into the soil (Aregheore, 1994). Crop residues are also lost through conservation agricultural practices which involves leaving some crops residues on farm after harvesting as to protect the soil and to help sustain productivity gains over time (Erenstein, 2011).

2.4 Factors affecting the nutritional valuevof maize stover

The chemical components of MS (leaf blade, leaf sheath, stem, husk and tassel) have been analysed by many researchers (Tolera et al., 1999; Tolera and Sundstøl, 1999; Undi et al., 2001; Sun et al., 2009). Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), lignin, cellulase, hemicellulase, total proanthocyanidins (TPA) and tannins are the main chemical components that have been analysed. Table 2.2 shows the chemical

composition of MS morphological fractions harvested at different stages of maturity (Tolera and Sundstøl, 1999).

Table 2.2 Chemical composition of maize stover morphological fractions

Chemical components	Maturity stage	Leaf blade	Leaf sheath	Stem	Husk	Tassel	Whole stover
DM at harvest (g/kg)	I (28%)	804	718	436	761	801	629
	II (20.2%)	895	882	506	816	890	717
	III (9.8%)	935	928	760	925	923	918
	±SE	25	48	91	35	23	78
CP (g/kg)	I	63	42	38	33	62	48
	II	58	39	35	30	60	44
	III	48	38	31	28	52	37
	±SE	5	3	2	2	3	4
NDF (g/kg)	I	639	805	744	872	755	746
	II	656	794	777	882	774	757
	III	694	800	800	887	796	769
	±SE	11	7	17	6	10	7
ADF (g/kg)	I	319	372	414	379	372	371
	II	322	382	465	370	380	393
	III	362	387	475	383	396	408
	±SE	9	5	19	3	5	7
Lignin (g/kg)	I	41	40	51	29	51	46
	II	43	41	65	30	54	49
	III	44	48	64	29	59	52
	±SE	2	3	3	1	3	2
cellulose (g/kg)	I	164	288	352	332	277	275
	II	144	288	385	323	276	307
	III	215	284	397	335	290	320
	±SE	15	4	15	4	6	11
Hemicellulose (g/kg)	I	319	433	330	493	383	375
	II	336	412	312	512	394	364
	III	332	413	325	503	400	362
	±SE	4	9	4	8	7	7
TPA (A_{550} nm/g DM)	I	16	11	10	5	8	12
	II	14	11	9	7	8	12
	III	14	12	8	6	9	11
	±SE	1	1	0.6	0.6	0.6	1

Modified (Tolera and Sundstøl, 1999), maturity stage=grain moisture content, SE = standard error

Table 2.3 Chemical composition of eight varieties of maize stover

Variety	Crop residue yields (t DM ha^{-1})	CP (g/kg)	NDF (g/kg)	ADF (g/kg)	Lignin (g/kg)	Cellulose (g/kg)	Hemicellulose (g/kg)
DTP1	7.96	31	765	511	55	456	254
Guto	5.27	28	837	523	58	464	314
Katumani	5.96	51	758	493	45	448	265
Birkata	5.90	61	731	454	46	407	278
CBF	4.19	59	706	500	66	435	206
Dendane	4.67	46	789	508	53	455	281
A511	8.35	52	768	493	64	429	274
Keroshet	5.78	57	810	476	39	437	335
Mean	6.10	48	770	495	53	441	276

Modified (Tolera et al., 1999)

Analysis of different varieties of MS showed variation in their chemical composition. Tolera et al. (1999) showed that the crude protein (CP) content of eight varieties of MS was not the same though they were cultivated on the same piece of land and treated the same (Table 2.3). This was also true for NDF, ADF, lignin, cellulose and hemicellulose contents. This implies that careful examination of these varieties would assist in the selection of varieties with relatively higher nutritional values for forages.

Since the primary objective of maize production is not for stover production (Tolera et al., 1998), there is a need to select varieties with higher crop residues without compromising grain production if possible. Tolera et al. (1999) found out that drought tolerant population DPT1, produced a high yield of stover without compromising grain yield. Variety A551 produced the highest stover yield but grain production was greatly compromised. Considering the objective of cultivation, DPT1 was considered best for both human and fodder production while A551 will be preferred for silage. Tolera et al. (1999) suggested that DPT1 should be promoted among small scale farmers as well as non-livestock farmers (for commercial purposes) to optimize production.

2.4.1 Shearing force as a tool for estimating the nutritional value of maize stover

Shearing force is a parameter that has been used to estimate forage value. Shearing force is the maximum force required to cut forages in laboratory experiments. It also denotes the required force when animals bite forages (Chen et al., 2007). It is also said to be related to the nutritional value of plant stems. The smaller the shearing force, the richer (nutritional value) the stem while the larger the sharing force the poorer the forage quality. Sheep fed with low shearing force ryegrass leaves showed higher DM intake than those fed with higher shearing force ryegrass (Inoue et al., 1994). Prince et al. (1958) also demonstrated that the shearing force required in cutting *alfalfa* increased with maturity. Iwaasa et al. (1996) later on demonstrated that shearing force is affected by the plants age. Factors affecting the shearing force of a stem are moisture content, diameter of the stem as well as its chemical constituent (Kokubo et al., 1989). This implies that shearing force increases with decreasing moisture content because fibre tensile strength is higher when moisture content is low. Correlation between sharing force and moisture content of maize stems has been found to be significant (Chen et al., 2007). The shearing force required to cut the bottom stem is different to that of the top stem (Iwaasa et al., 1995). Therefore shearing force standards would have to be determined for both the bottom and the top stem in other to obtain reliable results. Due to the simplicity and productiveness of the instrument used in measuring shearing force, it would be right to suggest that an introduction of such technologies to small scale farmers might be important for proper timing of harvest.

2.5 Factors influencing the use of maize stove as a forage supplement

In Africa, the long dry season is characterized by maturity and wilting of grass, inadequacy and poor nutritive value of forages (Nicholson, 1984; FAO, 2010). Feed cost is a major problem as broad animal production and profitability is dependent on low feed cost. However, there is a large area of crop land interspersing with the grazing land. These crop lands produce huge amounts of crop residues with MS being the most abundant. Although MS is abundant, it is not fully exploited as a forage supplement especially in Africa. In countries where land is

used primarily for cash and food crops production with very little available for fodder production, animals are fed in confinement (zero grazing systems). Over grazing is also a major problem in many countries with limited land for fodder productions (Gong et al., 2000). Maize stover could be a potential supplement for forages in such areas. To fully exploit MS as a possible forage supplement especially in the developing world where it is often treated as environmental nuisance, the following points would be important:

2.5.1 Educating the local community of crop residues feed potentials

Most communities are still not aware that MS can be exploited as a forage supplement not to mention commercializing. In most sub-Saharan countries, MS is usually grazed on farm as standing stalks and often by chance (Njie and Reed, 1995; Onwuka et al., 1997). Small scale farmers often consider it as environmental nuisance and are thrown away either by burning or ploughing in the soil (Aregheore, 1994; Onwuka et al., 1997; Shafi et al., 2007). Onwuka et al. (1997) demonstrated that less than 3% of total maize stover was being utilized at Ogun state in Nigeria due to ignorance of its nutritional value. Many sub-Saharan countries including Zambia (Aregheore, 1994), Malawi and Zimbabwe (Manyuchi et al., 1994) are already taking cognizance of MS as a forage supplement due to shortages and high cost of forages during the long dry seasons. Extension programmes educating rural farmers on the importance of MS as a possible forage supplement would be vital in reducing feed cost.

2.5.2 Nutritional value of maize stover

The recognition of MS as a forage is not only based on its relative abundance but on its fast growth, nutritional quality, high palatability and digestibility. The leaves are more palatable and digestibile than the stem. The relatively high digestibility was associated to its relatively high CP and mineral content (Tolera and Sundstøl, 1999) when compared to other fibrous forages. Harika et al. (1995) contributed by stating that the quality of MS also depends on the proportion of leaf and stem fractions. Tolera and Sundstøl (1999) confirmed that the chemical composition did not only vary within varieties but among varieties (Table 2.2 and 2.3). In the same study they also established that varieties A511 and DTP1 produced the highest crop residues among eight varieties. However, production of maize residues was greatly

compromised in A511 for maize residue while grain and stover yields were very high in DPT1. Crude protein content of DPT1 was 246% higher than observed in A511. Therefore, choosing the right verities for fodder production is very important.

An input in technological change (e.g. improved varieties) is important for the transformation of local communities, but it sometimes by-passes some local communities because of production and price risks that could render input use unprofitable (Kelly et al., 2003). Use of improved, high yielding MS varieties by small scale farmers can mean the difference between improve livelihood and staying trapped in poverty and hunger. It is obvious that poor farmers are reluctant to invest in new varieties due to their limited cash resource or access to credit. In Nepal showed that less than 60% of all village development communities used improved varieties while only 15% did in the most remote village development committees (Ransom et al., 2003). Biofortified plants (micronutrient-dense crop varieties developed through conventional plant breeding) are the latest plant varieties in the market. Although such plants are initially engineered to replenish micronutrient undernourishment in human, they can also be important for herbivores. Iron, zinc and provitamin A are the main micronutrients that have been identified by WHO to be limiting in diets of the poor (WHO, 2002). Research is often influenced by micronutrient deficiency with regards to the stable food or forage in a particular region (Ortiz-Monasterio et al., 2007). Although there have been some success in zinc and provitamin A enrichment, the setback on such projects are still enormous (e.g. breeding efficiency and soil deficiency of micronutrients). The lack of resources obligate poor farmers to sell their assets (e.g. motorcycles, bicycles, and televisions) to generate sufficient cash to buy the necessary inputs (Langyintuo and Mungoma, 2008). Therefore government or wealthy group-specific interventions are required to subsidize such varieties and increase their use and adoption among small scale farmers.

2.5.3 Stage of harvest

Maize at between 30 and 25% grain moisture is already physiologically mature but harvest is often delayed to 20-15% grain moisture (Tolera et al., 1999). This is to facilitate the drying process to the conventional storage grain moisture which is 13% (Martin et al., 1976). Harika and Sharma (1994) demonstrated that though 13% grain moisture might be ideal for storage,

stover yield decreases with decrease in grain moisture. The number of leaves per plant and the leaf-stem ratio decreases with delay in harvesting from physiological maturity (30-35%) to the dead ripe stage (10-13%). The loss of leaves affects the declining trend in stover yield with increased stage of maturity. Crude protein content and DM degradability also decreases with decrease in grain moisture. Small increases in NDF and ADF contents of leaf and stem fractions were also observed when harvesting was delayed (Harika and Sharma, 1994). Lower MS yields and decrease in CP and total non-structural carbohydrates are characteristic of late harvest (Russell, 1986; Irlbeck et al., 1993). Russell (1986) illustrated that heifers fed with early-harvested stover silage tended to have greater daily gains and required less feed/kg gain than heifers fed on the late harvested MS silage. Therefore, harvest timing of maize stover is a very important factor to be considered prior to harvesting.

2.5.4 Morphological fractions

Tolera and Sundstøl (1999) studied the morphological fractions of MS harvested at three different stages of grain maturity; stage I (28%), II (20.2%) and III (9.8%) and the nutritive value of different fractions demonstrated that, at late harvest (stage III), stem proportions increased by 20% while leaf sheaths, tassel and leaf blades decreased by 21.5, 41.5 and 44%, respectively. A decrease in CP and an increase in DM, NDF, ADF, lignin and cellulase contents were also characteristic of increased stage of maturity. Similar results were obtained by Harika and sharma (1994), Tan et al. (1995)(wheat straw) or Tolera et al. (1998). The stem is the main contributor of the bulkiness of MS. With enormous information available on the nutritive value of MS harvested at different stages, one can possibly conclude that feeding herbivores with different stover fractions would have an impact on digestibility and weight gain/day.

2.5.5 Maize stover as a supplement

The different morphological fractions of maize stover are stem, leaf sheath, husk, leaf blade and tassel. Pordesimo et al. (2004) reported that 15% of MS dry mass (DM) is cob and that stalks, leaf blades and husks accounted for 51%, 21%, and 13% of the total MS, respectively.

The chemical compositions of these fractions vary within and among varieties as well as the stage at which they were harvested (Tolera et al., 1999; Tolera and Sundstøl, 1999). Sheep and goats supplemented with crop residues were observed to be healthier and heavier (Onwuka et al., 1997) than controls. Leaf blade and tassel of MS are richer in CP than observed in the stem and husk (Methu et al., 2001; Sun et al., 2009). Browne et al. (2005) substituted grass silage with maize silage in a forage mixture and found out that the total DM intake, apparent digestibility and organic matter digestibility increased as compared to the control. Therefore, knowledge about the chemical composition of MS is very important when required as a supplement. Fractions such as the leaves and tassel, with relatively high CP (required by microbes) and palatability, could be a possible supplement in other diets. Maize stover with relatively high CP might reduce the cost on protein supplements required to initiate microbial processes.

2.5.6 Harvesting technology

Maize stover is either grazed *in situ* (on farm) or removed from the field prior to feeding. The practice of grazing on farm is wasteful and insufficient. In the developing countries, especially in Africa, MS is usually grazed "on farm" with very few farmers engaged in harvesting. Even in developed countries, harvesting maize stover using modern technologies is still a sub-standard because harvesting efficiency is still less than 50% (Richey et al., 1982; Sokhansanj et al., 2002; Shinners et al., 2007). Modern equipment such as the shredder, collect less than two-thirds of the available stover, while the baler collects about 50% of what was trimmed by the shredder (Richey et al., 1982). This implies that the harvesting efficiency is approximately 30%. Stover yields are higher when harvested wet than when allowed to dry. The harvest rate of wet stover is higher than dry stover (Shinners et al., 2007). The height of harvest is also an important factor to consider prior to harvesting because it affects yield and quality of MS. Normal-cut (40 cm) stover produces high yields and less water weight than low-cut with high water weight and relatively high stover yield (Table 2.4). Although low-cut yields are relatively high, contamination and transportation are not cost effective because of its high weight (Hoskinson et al., 2007). The height of harvest also affects the quantity of micronutrient available for animal intake (Table 2.5).

Table 2.4 Stover yields for the four harvest scenarios

	Stover		
	Water (mg g^{-1})	**Wet (Mg ha^{-1})**	**Dry (Mg ha^{-1})**
High-cut top	196	6.05	4.86
High-cut bottom	639	4.75	1.71
Low cut	338	10.1	6.68
Normal cut	237	6.67	5.09

Mg ha^{-1} = megagram per hectare, From a Journal paper by Hoskinson et al. (2007).

Table 2.5 Nutrient concentrations in corn collected using different harvest scenarios

	C	**N**	**P**	**K**	**Ca**	**Mg**	**Cu**	**Fe**	**Mn**	**Zn**
	(mg/g)	(µg/g)								
High-cut top	444	7.0	0.79	7.05	3.95	3.07	1.4	42	7	6
High-cut bottom	440	8.1	0.57	19.62	4.05	3.83	0.8	86	8	4
Low cut	444	7.0	0.62	6.52	3.7	3.74	1.1	63	6	4
Normal cut	440	8.0	0.79	6.74	5.4	4.04	1.2	51	13	9

C = carbon, N=nitrogen, P = phosphorus, K = potassium, Mg = magnesium, Fe = iron, Mn = manganese, Zn = zinc. From a Journal paper by Hoskinson et al. (2007)

Sokhansanj et al. (2002) noticed that the weather and season of harvest plays a vital role in the amount of stover collected per unit area. Methu et al. (2001) showed that higher leaf + husk + sheath: stem ration were harvested in the dry season than in the rainy season. Therefore, educating small and large scale farmers on the need of harvesting using appropriate technologies, season and height of harvest might have a positive impact on MS total yields.

2.5.7 Storage conditions

Harvest and storage of MS are often affected by weather conditions, material moisture and storage facilities. There is a great need to conserve forages especially during winter or dry seasons, which are often long and harsh. Maize residues can be stored in different forms such as hay, silage (Sun et al., 2009) or spared veldt (Shinners et al., 2007). However, silage and hay are the most common methods of preservation. If the warehouses are not properly sealed especially during the heavy rains, bale fermentation is very common. Fermented bales in the

presence of oxygen have a negative impact on maize stover quality. In most developed countries or dairy farms, stovers are often harvested wet (45% grain moisture) and preserved by ensiling (Shinners et al., 2007). Ensiling decreases stover losses often observed during bailing and a more uniform product is also achieved when compared to dry stover bales stored outdoors (Shinners et al., 2007). Other advantages that encompass ensiling includes, no fire hazards, silage quality if maintained for longer periods, less dependence on weather, less bleaching by the sun (loss of Vitamin A) and anaerobic fermentation which is known to improve feed palatability. Silage does have an excellent appearance and colour with a familiar and pleasant odour. On-farm grazing of MS appears to be the worst method, because it involves a lot of wastage and exposure to hash weather conditions. Therefore, strategizing on storage options prior to harvest is vital so as to preserve feed quality and minimize cost due to wastages.

2.5.8 Feed formulation

Optimum ration formulation is crucial when MS is required as a supplementary feed for ruminants. Although MS is more palatable than other hays, when fed as a whole, digestion is still low. The low digestibility has been associated to low nitrogen (Siebert and Hunter, 1982) and perhaps mineral in dry MS (Agricultural Research Council 1980). The rate of feed digestion depends on the quality (type of nutrient) and composition (ration) of the feed (Tolera et al., 1998), which is also affected by the number, type of microbes and nutrients that limits microbial growth (Lynd et al., 2002).

The most limiting factor on performance of animals fed on different MS residues appears to be dietary protein **(**Siebert and Hunter, 1982). *In sacco* DM digestibility of the same variety of MS was found to decrease with an increase in grain maturity (Tolera and Sundstøl, 1999). The decrease in digestibility was primarily associated to a decrease in CP (Sun et al., 2009), with an increase in grain maturity, though a decrease in soluble carbohydrates might have played a minimal role. *In vitro* analysis of the degradability of MS fractions showed a lower dry matter degradability (DMD) in stems than in leaves with higher CP content (Tolera and Sundstøl, 1999). This clearly indicates that nutrient deficiencies in certain fractions are the principal

factors. Although nutrient deficiency seems to be the major problem, experimentation on excess feeding with MS has also shown small increments in intake and milk production (Methu et al., 2001). However, the setbacks of this approach are the limiting quantities of MS and an uneconomical use of the refusals.

Genetic engineering has seen the breeding of different varieties of maize with higher crude protein content, foliage, maize grain and MS yields (Bänziger et al., 2006). This was clearly demonstrated by Tolera et al. (1999) in their studies on eight varieties of maize. Though trying to breed maize varieties richer in CP seem to be moving in the right direction, supplementing MS with legumes would be the best option. *In sacco* DMD of MS blended with three different leguminous species (stylo, siratro and centro) showed a higher DMD than observed in MS DMD alone (Undi et al., 2001). Similar experiments on supplementing MS or other hays with legumes have also shown increments in DMD (Ndlovu et al., 1996; Undi et al., 2001; Chakeredza et al., 2002; Aregheore and Perera, 2004). Leguminous supplementation has also been found to increase intake (Preston and Leng, 1987; Chandrasekharaiah et al., 1996). Educating the local communities with the right information and the need to ration animal feed according to their requirements would be beneficial in animal production.

2.5.9 Bioenergy

The increasing demand for bioenergy (Cherubini and Ulgiati, 2010) is going to be a future blow for the utilization of MS as forage. In 2001, about 15 million tons of maize grain was used to produce 5.6 billion liters of bioethanol. A three-fold increase in bioethanol, biodiesel, biopower and other emerging biobased products are envisioned over the next ten decades in the United States (Biomass Research and Development Board, 2001). The high demand for bioenergy has seen alternative feedstock such as MS being proposed as an alternative source for the production of extra ethanol (Perlack and Turhollow, 2003). Interestingly there are a series of projects that has been investigating the use of crop residues to generate electricity (Swanston and Newton, 2005). Although currently there is still a lot of setback in bioenergy technology especially in developing countries, the development of new technologies that are simpler and available would be a huge blow for MS utilization as forage (due to higher

incentives especially in the poorer communities). Establishment of such technologies might divert the attention of many small-scale farmers as the incentives might be higher. Food and feed deficiency would be obvious in such era if nothing is being done to discourage bioenergy production and encourage maize production yields for livestock production.

Maize stover has been clearly shown to have the potential of a forage supplement especially during the period of feed shortages. Plant breeders have shown that the maize plant can be engineered to produce more foliage without compromising grain production. Some of these varieties are already in the market such as A511. Genetically engineered plants with a relatively high foliage production are becoming more available in the market. An introduction of centres all over the world engaged in such research would foster the availability of such varieties in poorer communities. The stage of maturity and harvest timing affects the CP content of MS which is one of the most limiting factors in ruminant digestion. Harvesting MS at 35% grain maturity would be beneficial for both human and animal consumption. Although harvesting techniques have been proven to be less efficient (less than 50%), it is still preferable to harvest and feed indoors because wastages incurred while grazing on farm exceeds that of zero grazing systems. Leguminous supplementation of MS has shown the potential of increasing DMI, DMD, forage palatability, body weight and milk production. Therefore, basic knowledge on feed formulation with MS as the main forage is very important because an animal can only exhibit its full potential with a ration feed.

2.6 Maize stover utilization by herbivores

Ruminants cannot express their full potential if they are not able to harness nutrients from a properly formulated MS feed. Microbes breakdown MS into soluble sugars or cellodextrins (two or more glucose molecules resulting from cellulolysis), which are later metabolized for energy production yielding by-products, such as volatile fatty acids (VFA) or short chain fatty acids (SCFA), carbon dioxide, methane and ammonia. Short chain fatty acids are the most important by-products as they are used to generate energy by the host animals (Armentano, 1992). Undigested MS flows through the abomasum, to the small intestines and then to the large intestine, where it is eventually excreted as faeces through the rectum. Therefore, to fully

understand the fate of MS, it is imperative to study the cell structure of the maize plant (cell wall), MS hydrolysis (both mechanical and microbial), factors affecting MS fermentation and utilization, the origin and fate of SCFA, enzymes responsible for MS hydrolysis (cellulases), MS (cellulose) additives, microbial variation in herbivores and models to improve MS degradability.

2.6.1 Cell wall and cellulose fibre

Plants cell walls vary in their chemical composition and structure (Tolera et al., 1999). plant cell wall constituents increase with maturity, while non-soluble carbohydrate content decreases (Kruse et al., 2008). The strength of the cell wall is attributed to its multicellularity, paving the way for a rigid form by providing a variation in thickness, chemical composition as well as spatial distribution of cells within the tissue. A cell wall can be subdivided into primary, secondary and tertiary cell wall. These layers will be discussed with emphasis on the macromolecules that are involved in or affect digestibility in ruminants. Primary cell walls are formed when expansion of a cell leads to the deposition of components of the cell wall on the plasma membrane. Pectins and hemicelluloses are synthesized in the Golgi apparatus before secretion (Hillman et al., 1986; Doblin et al., 2003; Bárány et al., 2010) while cellulose is synthesized on the plasma membrane by the enzyme complex, cellulose synthase (Saxena and Brown Jr, 2008). Newly synthesized cellulose micro fibrils are deposited at a vertical angle to the direction of cell elongation in a gel-like matrix of interlinked molecules (Doblin et al., 2003; Malcolm and William, 2003). Macro fibrils comprise micro fibrils, which are made up of about 2000 cellulose chains (fibril). The orientation of fibrils in an upright position is said to play a vital role in cell expansion as well as cell growth.

The distribution of celluloses, hemicelluloses and pectins in the matrix is almost equal. Cells, e.g. parenchyma and meristematic cells, embedded in this matrix still have the ability to grow and expand since the matrix is flexible and allows exchange of materials with relative ease. Hemicelluloses are highly branched polysaccharides with saccharide units xylose, mannose and glucoronic acid. They are hydrogen bonded to the surface of cellulose microfibrils, forming a network of tough fibre molecules thus rendering mechanical strength to the plant

cell wall. Pectins are branched polysaccharides with galactose, arabinose and galactorunic acid as saccharide units (Malcolm and William, 2003). They contain a high number of negative charges which enables it to bind with positively charged ions such as those of calcium ions (Ca^{2+}). The presence of such ions traps water molecules, causing the jelly-like nature of primary cell walls which are often observed in meristematic tissues (young shoots and leaves). At this level of development, they are said to be the outermost layer of the cell and are the only layer of protection found in certain cells. Adjacent cells are cemented together by botanic "glues" or pectins in a layer called middle lamellae. Cemented cells communicate through small channels called plasmodesmata. Leaves with only primary cell walls have a relative advantage when it comes to forage availability and digestion, be it mechanical or microbial (thin, flexible and lignin deficient). However, as the plant grows older there is a gradual deposition of secondary thickening that becomes more prominent when the cell growth stops. A rigid secondary thickening is formed between the primary cell wall and the plasma membrane. The secondary cell wall components are similar to that of the primary cell wall in terms of macromolecules but for the introduction of lignin and lack of pectin. Therefore, it is characterized by the deposition of more cellulose and hemicelluloses with very little pectin. The concentration of hemicelluloses and cellulose increases from 50% to 80% in the secondary cell wall (Ghadaki et al., 1975).

Lignin is an insoluble aromatic polymer made from the polymerisation of phenolic alcohols (Okuda et al., 2004) found mainly in mature plant cell walls. Deposition of material into the primary cell wall could either be homogeneous thickening (phloem) or localized thickening (xylem vessel). Further differentiation of the xylem leads to the penetration of lignin from the outside into the secondary thickening. These hydrophobic polymers replace water and encrust all the microfibrils and matrix where the cell walls eventually die. Cell expansion in this layer is practically impossible because it is encrusted with lignin, waterproofing cells, blocking diffusion of water and nutrients into cells. This is the main reason why most lignified cells die on maturity (Boudet, 2003; Guillaumie et al., 2007). However, life is still possible for cells that are able to retain enough plasmodesmata as a means of communication between cells. Lignified cells do not only prevent cell expansion but prevent cell division indirectly; hence meristematic tissues do not contain lignin. Cells of sclerenchyma, collenchyma and xylem

have secondary deposits of lignified cellulose, which provide mechanical strength to the tissue. Deposition of lignified cellulose is highly ordered when compared to that of the primary cell wall with variable orientations forming a laminated structure that significantly increases cell wall strength.

Although cellulose is the major component of plant cell walls, its distribution differs among MS varieties as well as within different parts of the same plant (stem, leaf sheath, leaf blade, husk or tassel) (Tolera and Sundstøl, 1999). In the stem, cellulose is closely blended with other cell wall constituents such as hemicellulose and pectin within the meristematic regions (young stems). In mature stems that have undergone secondary growth the percentage of lignin deposited in the matrix is high. Integration of fibre with other cellular constituents enables it to withstand higher pressure and to support the weight of the plant (Boudet, 2003).

The leaves of monocotyledonous plants and grasses do not undergo secondary growth and therefore do not increase in width (thickness). Although leaf blades do not increase in width they still require strength for support that is provided by lignified cellulose. Lignin is abundant around the xylem vessels, middle lamella and vascular bundles. The amount of fibre deposited in these regions is proportional to the weight to be supported so as to keep the leaves exposed. The cell wall constituents of leaves also vary with age. Deinum and Direven (1971) investigated different leaves from the maize plant and found that digestibility declined with age. They concluded that leaves at a later stage of growth were less digestible than leaves at initial growth of the same plant species at the same stage of development. Similar effects of leaf succession and age were found in Australia with *Pinicum maximum* (Wilson, 1973), Nigeria with *Androgopogon gayanus* (Haggar and Ahmed, 1971) and Puerto Rico with *Digitaria decumbens* (Van Soest, 1982). A sharp drop in digestibility was observed in leaves grown under high temperatures as opposed to low temperatures. Decreased digestibility is due to the fact that higher temperatures cause an increase in cell wall cellulose contents (Deinum, 1976). Generally, leaves have lower quantity of fibre than stems.

Forages, both in the temperate and tropical regions, are covered with a thin waxy cuticle which is the plants first mechanism of protection prior to the cell wall. The waxy cuticle

presents a strong barrier against microbial invasion. Huub *et al.* (1988a) suggested that this layer appears to be totally resistant to microbial digestion within the rumen, however, some fungi may penetrate it. Cutin fractions are 0.2% and 2.4% in wheat straw and alfalfa, respectively (Van Soest, 1982). Cutin forms the structural part of a plant cuticle and it is a polyester of C-16 and C-18 hydroxyl- and hydroxyepoxy fatty acid. However, some pathogenic anaerobic bacteria are able to hydrolyse the ester linkages of cutin. Although cutin has been shown to provide a primary barrier to plant cell wall hydrolysis, the complexity of cellulose is still the major barrier (Malcolm and William, 2003).

2.6.1.1 Cellulose

Partial hydrolysis of cellulose produces cellobiose (disaccharide), which is linked by a beta glycosidic bond between C-1 and C-4. This led to the definition of cellulose as an unbranched polymer of thousands of glucose units (Figure 2.2) linked to each other by beta 1, 4 glycosidic bonds (Levy et al., 2002). They are generally represented by a formula $(C_6H_{10}O_5)_n$.

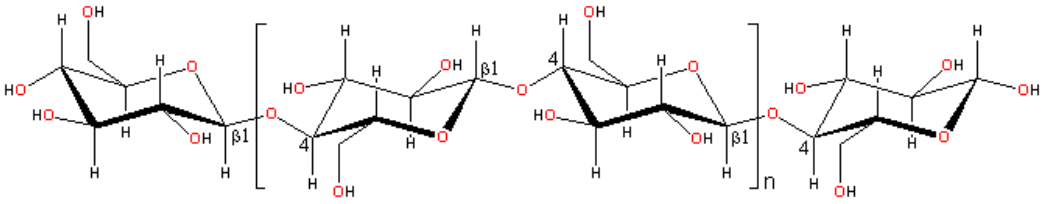


Figure 2.2 The structural unit of cellulose

n= number of disaccharide units in a cellulose molecule [(Martin, 2006) modified]

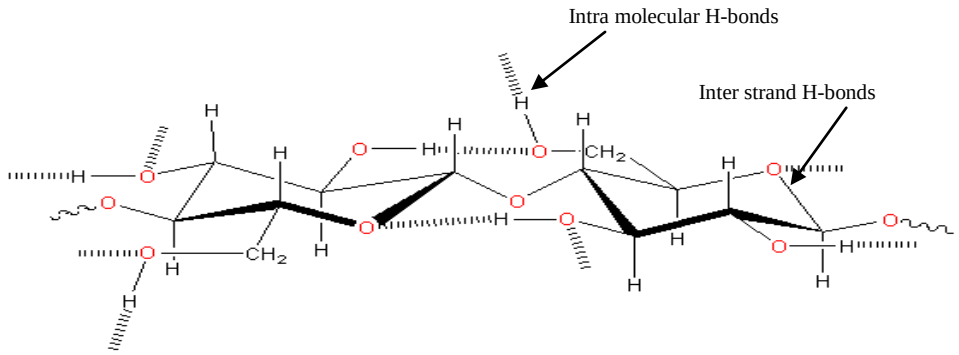


Figure 2.3 Crystalline structure of cellulose (Martin, 2006) modified.

Crystalline celluloses are characterized by the type of hydrogen bonds that are formed between C-3 hydroxyl group and the pyranose ring within the same molecule (inter molecular hydrogen bonding) and those formed between the C-6 hydroxyl group of one molecule and the oxygen of the glycosidic bond of another molecule (intra-strand) (Figure 2.3). The presence of hydrogen bonds between these fibre molecules is responsible for the tightly packed crystalline structure. Celluloses are insoluble due to the inability of water molecules to penetrate the tightly packed crystalline structures (Ross et al., 1991). Amorphous cellulose is relatively soluble in water because it allows the penetration of water molecules due to fewer frameworks of hydrogen bonds.

2.6.2 Maize stover hydrolysis in herbivores

Rumen microbes have the potential of hydrolyzing both structural and soluble carbohydrates into SCFA (butyrate, propionate and acetate), which are then used for energy generation by the host. To understand maize stover (fibre) hydrolysis in ruminants, MS hydrolysis will be examined in two steps, namely, mechanical (physical) breakdown and microbial hydrolysis (fibre fermentation).

2.6.2.1 Mechanical breakdown of maize stover

Mechanical breakdown of MS in ruminants is similar to monogastric animals. However, there are some major differences between monogastric animals and ruminants, such as rumination in ruminants (Aikman et al., 2008), which is absent in monogastric animals. Mechanical breakdown of fibre is a non-enzymatic process where macro-fibres are broken down into microfibres and cellulose units thereby increasing the surface area for enzymatic hydrolysis. Maize stover fermentation increases with surface area. This was confirmed by an *in vitro* experimentation on dry matter digestion rate of alfalfa with variable sizes of feed particles (Robles et al., 1980). Not only did a large surface area increase fermentation, it also increased feed intake. Feed intake was higher in animals fed with ground particles than ungrounded

particles of the same feed. More time was spent on the ungrounded feed during chewing than on the crushed feed (Weston and Hogan, 1967).

The main site for the physiological breakdown of fibre is the buccal cavity with mastication being the main process. The lips, tongue, lower incisors and the dental pad in front of the hard palate are the prehensile organs of the ruminant. These organs are the important features that influence the animal's nutrition. The oral cavity and the lips are used for holding the feed. The size and length of the buccal cavity and lips, respectively, differ from concentrate selectors (CS) through intermediate selectors (IM) to grazers (GR). Grazers have a smaller mouth opening and shorter lips which facilitate cutting and easy manipulation of feed (Hofmann, 1998). Concentrate selectors have longer lips and larger mouth openings for manipulation of forages such as fruit. Molars and premolars are used for grinding and these are affected by the sideways swing of the mandibles. The mandibles of GR offer a larger surface for masticatory muscle attachment and they have bigger masticatory muscles than CS. In GR, chewing is initially very brief when grazing in the fields but particulate size is later reduced by rumination (chewing of the cud) during resting hours. The group of CS is characterized by a shorter rumination period but with an intense initial chewing. The tongue assists in holding and movement of forage material in the oral cavity. The shape and size of the tongue vary among the different feeding types. Concentrate selectors generally possess a plump, piston-like tongue while GR have a pointed slender tongue.

Saliva secreted during mastication by the salivary glands serves: (a) as a rinsing fluid for nutrients released; (b) to dissolve feed particles and allow for flexible movement during chewing; (c) as a fermentation buffer in the rumen (Bowman et al., 2003); and (d) as a transporting fluid. Hofman (1998) showed that the salivary gland varies among the different feeding types. Their sizes regress from CS through IM to GR. Peristaltic movement of chewed material from the oral cavity through the oesophagus to the rumen also plays a minor role in mechanical breakdown. During resting periods ruminants have a special ability to regurgitate the swallowed material for effective chewing. This is a unique feature in ruminants that has been used in their classification. The size of the rumen appears to play a major role in rumen digestion. In grazers, the larger the size of the rumen the longer the time spent by particulate

matter, hence prolonging the time for microbial activity. This implies that mechanical breakdown will be much better in GR with larger rumen than in CS with smaller rumen. Non-rhythmic contractions of the rumen during mixing also assist in the mechanical process of fibre breakdown.

2.6.2.2 Microbial fermentation of maize stover

Microbial fermentation of fibre is also known as enzymatic hydrolysis since enzymes secreted by microbes carry out the fermentation process. Fermentation of fibre occurs mainly in the rumen or hindgut of herbivores (Trinci et al., 1994). Although some herbivores still possess a double fermentation chamber (fore- and hindgut) the foregut fermentation chamber is said to be prominent. The rumen harbours a variety of microbes called symbiotic microbes comprising anaerobic bacteria, protozoa and fungi (Trinci et al., 1994).

These microbes are introduced into the rumen by the host animal during grazing or browsing in the fields. Microbial species vary from one geographical region to another, even within animal species in the same geographical regions (Gonçalves and Borba, 1996). The rumen harbours microbes that have evolved through millions of years of selection with varying nutritional constraints. This is an extraordinary feature that all ruminants possess in order to survive and reproduce while eating high fibre diets (Demeyer, 1981; Dominguez Bello and Escobar, 1997). Survival of microbes within this environment depends on their ability to compete with other microbes as well as adapt to the stringent conditions of these fermentation chambers. The ability to adapt and survive in the fermentation chamber is quite important because an unlimited number of microbes are being introduced each day. The dominant rumen microbes are saccharolytic since carbohydrates are the main metabolic substrates (Leschine, 1995). The rumen is an anaerobic chamber as most, if not all, microbes that it harbours are anaerobic. However, Yokoyama and Johnson (1998) illustrated that these microbes are not absolutely anaerobic. Consequently, these microbes were classified as either obligate or facultative anaerobes.

Obligate anaerobes are those that grow only in the absence of oxygen while facultative microbes are those that are able to grow in the presence or absence of oxygen. Owens and Goestsch (1998) confirmed the presence of these microbes by postulating their locations in the rumen environment. Microbes that associate with particulate matter (Figure 2.4) and free-floating microbes were classified as obligate anaerobes (Bailey and Jones, 1971). Those that attached firmly to the epithelial wall of the rumen were classified as facultative anaerobes. Akin (1980) demonstrated that not all facultative anaerobes adhere to the ruminal wall. Quantitatively, the obligate anaerobes are more than facultative anaerobes (Morris, 1975; Trinci et al., 1994).

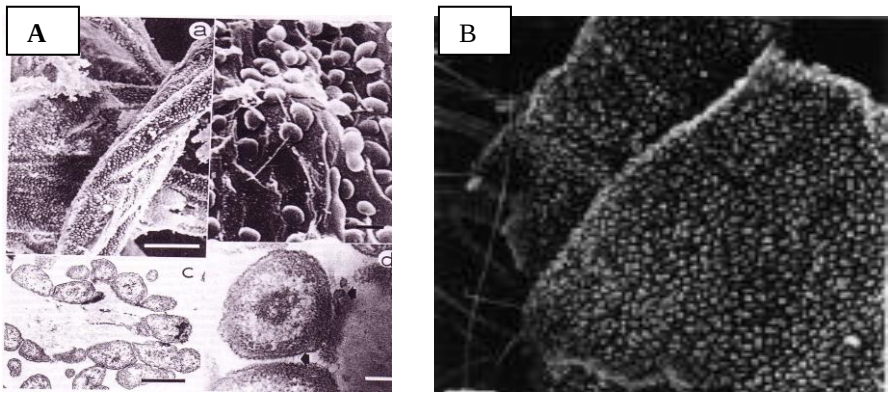


Figure 2.4 Bacteroides succinogenes growing on filter paper cellulose. *(a)* SEM of cells on a cellulose fibre. Bar = 10 um. (b) SEM of cells on a fibre. Bar = 1 um. Groleau and Forsberg (1983) modified.

Bacteria classification is based on their morphological structure, size and shape (cocci, rods and spirilla). Interestingly, some nutritionists have used substrates hydrolysed by microbes as a primary basis of classification (Hobson, 1969). The objective of this approach was to assist in elucidating the contribution of each bacteria species towards the utilization of the different components of feed. Preliminary screening of bacteria by this method led to the classification of bacteria into eight different groups with respect to the following substrates, celluloses, hemicelluloses, sugars, intermediates, proteins, lipids, starch and methane (Yokoyama and Johnson, 1998**).** The shortcoming of this method of classification is the broad spectrum of certain enzymes, which are capable of hydrolysing and fermenting more than one substrate. Cellulolytic microbes are the most important microbes in the rumen based, on the high fibre content of feeds. Bacteria are also known to be the most adaptable species of microbes in the

rumen e.g. *Bacterroides succinogenes, Ruminicoccus flavefaciens, Ruminicoccus albus* and *Butyrivibrio fibrisolvens.* The population of these microbes increases with an increase in substrate availability. Other minor cellulolytic microbes such as *Clostridium lochheadii* do not vary much even with increased concentration of the substrate.

Cellulase secreted by cellulolytic bacteria diffuses through the rumen fluid onto the substrate or stays within the vicinity of the bacteria adhering to a substrate (Owens and Goestsch, 1998). Cellulose is hydrolysed into glucose, which is used as a metabolic substrate by microbes; therefore, yielding by-products such as SCFA, CO_2, methane and hydrogen (Owens and Goestsch, 1998; Tolera and Sundstøl, 1999). Fibre hydrolysis is catalyzed by a complex enzyme system called cellulases. There are three different types of enzymes involved in this system, namely crystalline cellulase (exocellulase), carboxymethyl cellulase (endocellulase) and glucosidase (cellobiase) (Bayer et al., 1998a; Shoham et al., 1999; Desvaux, 2005). The number of enzymes involved in this process clearly indicates that fibre hydrolysis is a stepwise process. This process begins with the breakdown of crystalline cellulose into cellobiose by exocellulase and amorphous cellulose into cellobiose by endocellulase. Cellobiose is hydrolysed into glucose molecules by glucosidase or cellobiase (Teeri, 1997). Glucose molecules are then catabolized through the process of glycolysis yielding SCFA, 2ATP, H and CO_2. Adenosine triphosphate is used as a primary source of energy by the microbes, whereas the by-products are used as metabolic substrates or intermediates by the host.

Hemicellulolytic bacteria secrete hemicellulases that hydrolyse hemicelluloses into monomeric units such as xylose. *Butyrivibrio fibrisolvens* and *Bucroides ruminicola* are top on the list of potent hemicellulolytic bacteria. Besides cellulolytic and hemicellulolytic bacteria, other bacteria that exist in the system ferment monomeric units such as glucose, xylose and galactose (Owens and Goestsch, 1998). Symbiotic bacteria living in this ecosystem utilise the by-products of fermentation as metabolic substrates or monomers for synthesizing macromolecules e.g. methane-producing bacteria such as *Methanobrevibacter ruminantium, Methanobacterium formicicum* and *Methanomicrobium mobile* reduce CO_2 with hydrogen gas

producing methane as an end product. Both CO_2 and hydrogen gas are the by-products of fermentation, which can become harmful if allowed to accumulate.

Protozoa are also associated with fibre breakdown (Huub et al., 1988b) as a number of species have been identified that prefer soluble carbohydrate as a substrate. Fractionation studies by Gijzen et al.(1988) demonstrated that ciliates accounted for 19 to 28% of the total cellulase activity in faunated cultures fed on filter paper cellulose. *Diplodinium diplodinium* and *Diplodinium diploplastron* hydrolyse substrates such as cellulose, hemicellulose and starch, while *Diplodinium polyplastron* hydrolyse cellulose, glucose, starch and sucrose, but not hemicellulose. *Entodinium bursa* utilises starch and hemicellulose, but not cellulose as substrates. *Entodinium caudatum* utilises completely soluble molecules such as cellobiose, maltose, glucose and starch. Some bacterial species adhere to protozoa but the rationale behind this adherence is not clear. Logically one might deduce some kind of proximity effect for the bacteria to their substrate since these protozoa are attached to plant materials.

Anaerobic fungi are found adhering to or within plant materials in cattle and sheep (Mountfort, 1987). They are able to colonize and penetrate the cuticle and cell wall of lignified tissues in the rumen (Bauchop, 1981; Bauchop, 1989). The zoospores attach to fibre and produce hyphae that grow and penetrate deeply into plant tissues. The fungus develops an extensive rhizoid for anchorage and supply of nutrient. Penetration of the cuticle and cell wall by fungi pave way for other anaerobic microbes to gain access to soluble or fibrous material from which their energy is harnessed (Akin and Borneman, 1990). Fungi contribution to fibre hydrolysis in the rumen is minimal (Paul et al., 2004), although limited degradation of fibre and xylan has been observed when incubated with protozoa. Interestingly, protozoa in the rumen may contribute up to 80% of the microbial mass. This high population is yet to be explained as its functional significance is still to be defined.

2.6.3 Factors affecting fibre fermentation and utilisation

Fibre (cellulose) is the most abundant and available carbohydrate polymer in nature (Doblin et al., 2003; Imai et al., 2004) that has been widely researched on as a plentiful source of food or

energy resources. Therefore, studying different factors that affects fibre fermentation is critical for MS utilization by ruminants.

2.6.3.1 Nature of fibre

There are two types of fibre molecules, namely, crystalline and amorphous cellulose (Al-Zuhair, 2008). These two molecules are structurally different due to the type of hydrogen bonding formed within their molecules. The bonding formed within and between these molecules is the primary factor affecting fibre hydrolysis. Intra- and inter-hydrogen bonds formed with crystalline cellulose are more than those formed in amorphous celluloses (Martin, 2006). This implies that crystalline cellulose hydrolysis will be slower than amorphous cellulose hydrolysis upon incubation with microbes. Crystalline cellulose is more resistant to enzyme hydrolysis than amorphous cellulose (Szijártó et al., 2008). The high resistance posed by cellulose to enzyme hydrolysis is due to its crystallinity. Therefore, the crystalline nature of cellulose is one of the major physical parameters that influence its hydrolysis.

2.6.3.2 Hemicelluloses

Hemicelluloses are secondary factors affecting fibre hydrolysis. These molecules interact with fibres through the formation of intra- and inter-chain hydrogen bonds. Niedusznki and Marchessault (1971) reported that hemicelluloses such as xylan are able to form crystalline-like structures rendering the matrix less permeable to cellulases. Association of this complex with lignin renders the matrix even more difficult to hydrolyse as the mesh network will be almost impermeable to cellulolytic enzymes. This implies that the association of these molecules with celluloses makes the complex structure even stronger. Kamra (2005) showed that the degradation of pure fibre when incubated with microbes was higher than that of crude fibre. The study confirms that the interaction of hemicellulose with cellulose slows down the degradation process.

2.6.3.3 Pectin

Pectin are polymers of galacturonic acids and are often referred to as “intercellular cement” (Mohnen, 2008). The term “intercellular cement” was derived from their role in binding two or more cells together. Different roles that have been associated with pectin in plants include; plant growth, development, morphogenesis, defence, cell-cell adhesion, wall structure, signalling, cell expansion, wall porosity, binding of ions, growth factors and enzymes, pollen tube growth, seed hydration, leaf abscission and fruit development (Ridley et al., 2001; Mohnen, 2008). Enzymatic hydrolysis of pectin in a substrate complex was found to separate plant cell walls from each other. Pectins are able to hydrogen bond with celluloses, hemicelluloses and other matrix macromolecules, e.g. proteins. They encrust both celluloses and hemicelluloses, rendering them inaccessible to cellulolytic and hemicellulolytic enzymes, respectively.

2.6.3.4 Lignin

Lignin is formed from the polymerisation of three primary precursors, namely phenylpropanoid precursors, phenylalanine and tyrosine. These primary precursors give rise to p-coumaryl, coniferyl and sinapyl alcohols (Holtman et al., 2003; Guerra et al., 2006). Free radicals are formed from these monomers under the influence of phenol oxidase, which undergoes non-enzymatic reactions to form polyphenols. Lignin polymers are chemically stable structures that are mostly attacked by aerobic bacteria, which therefore limit the possibility of extensive breakdown in an anaerobic rumen (Keford, 1958). Lignin found in plants differs mainly in the proportion of free radicals from which they are formed. Lignin in gymnosperm is made up of 80% p-coumaryl, 14% coniferyl and 6% sinapyl alcohols while angiosperm lignin contain 56% p-coumaryl, 4% coniferyl and 40% sinapyl alcohols. In addition to the phenolic monomers, grasses and herbage lignin is richer in acids such as p-coumaric, ferulic, diferulic, p-hydroxbenzoic and vanillin. A highly condensed phenyl propanoid matrix of lignin is referred to as “core” lignin while the p-coumaric and ferulic fragments are known as “non-core” lignin (Smith et al., 1971).

Non-core lignin fragments (p-coumaric and ferulic acid) are the most important molecules in terms of the cross linkages formed between lignin and structural carbohydrates due to the

presence of hydroxyl and carboxyl groups. Ester bonds are common between "non-core" lignin and hemicelluloses in forages. Therefore, the binding of lignin to hemicelluloses indirectly affects fibre hydrolysis as hemicelluloses are bound to celluloses. However, bonding between lignin and celluloses as well as other carbohydrates is also possible (Henriksson et al., 2007). Variation in the association of lignin to hemicelluloses was substantiated by analysing carbohydrates extracted by alkali and dimethyl sulphur oxide (Morrison, 1974). Alkali analysis of carbohydrates from lignin-carbohydrates liberated 70% xylose, 20% arabinose, 5% galactose and 5% glucose. The high percentage of xylose confirms hemicelluloses as the dominant molecules associated with lignin. Analysis of the carbohydrate moiety extracted by dimethly sulphur oxide indicated that the percentage of glucose (50%) observed was higher than that of xylose (30%), arabinose (12%) and galactose (5%). High glucose concentrations imply that lignin associates with other molecules in the cell matrix.

Casler (1987) and Tolera and Sundstøl (1999) demonstrated that the concentration of lignin increases with increasing maturity for both grasses and legumes. Generally, the concentration of lignin is higher in legumes than in grasses but lignin in legumes is more soluble than lignin in grasses. The solubility of legume lignin is due to higher "core lignin" than "non-core" lignin. Geographical regions from which forages are harvested also play a major role, as lignin content of forages is not stable. The lignin content of grasses from the tropics is higher than those harvested from the temperate regions (Van Soest, 1982; Dominguez Bello and Escobar, 1997). Consequently, digestion of forages from the tropics will be more difficult than those from temperate regions. However, the evolution of microbes in animals that graze in these regions is so magnificent that meeting the nutritional needs of these animals is not problematic. Encrustation is one of the major effects observed on fibre by lignin, which renders fibre inaccessible to cellulolytic enzymes (Camp et al., 1988). The most import mechanism established is the bonding of lignin to other polysaccharides including celluloses forming a polysaccharide complex (Bidlack et al., 1992; Carpita and Gibeaut, 1993). The bonds formed within these complexes are watertight and make the diffusion of cellulolytic enzymes extremely difficult. The effect of hemicellulose and lignin on cellulose conversion into glucose on brewers spent grain shows that the lower the hemicellulose and lignin contents in the sample, the higher the efficiency of cellulose hydrolysis (Van Soest, 1981; Mussatto et

al., 2008). This implies that fermentation will be enhanced in reduced quantities of lignin and hemicelluloses. Since Tolera and Sundstøl (1999) demonstrated that lignin concentration increases with MS maturity, harvest timing is very important so as to reduce the effect of lignin on microbial fermentation.

2.6.3.5 Tannins

Tannins are naturally occurring bio-molecules of high molecular weights synthesized by plants. Notable are the diverse groups of oligomers and polymers of phenols. They are defined as phenolic compounds that precipitate proteins. This definition has some shortcomings as not all tannins bind and precipitate proteins and secondly, not all polyphenols precipitate proteins or form complexes with polysaccharides. However, Horvath (1981) amended the definition by defining tannins as any phenolic compound of high molecular weight containing sufficient hydroxyl groups and other suitable groups to form effectively strong complexes with proteins or other macromolecules under the particular environmental conditions being studied. In the plant kingdom tannins are found both in angiosperms (flowering plants) and gymnosperms (non-flowering plants). In flowering plants dicots are richer in tannin than monocots. Maize is an angiosperm and belongs to the monocot group of plants. An understanding of the distribution of tannins in plant tissues can assist in the enriching or restructuring of the grazing area of ruminants.

Tannins are prominent in plant parts such as barks, stems, fruit, leaves, and roots. They are mostly located in the vacuoles of surface wax in these tissues, where interference with the plant's metabolism is nil (Marty, 1999). This implies that it is only after cell lesion or cell death that these macromolecules can interfere with metabolic activities. The outer part of the bud tissue is believed to have a protective function against freezing in winter while the upper epidermis of plant leaves reduces palatability, thus protecting the plant against predators. However, in the tropics tannins are evenly distributed on the leaf surface compared with plants in temperate regions in which tannins are concentrated in the upper epidermis. Stem tissue, secondary phloem and the xylem, which are active growth areas, are rich in tannins. The role tannins play in this area is not well understood but they have been associated with growth

regulation in these tissues. Functionally, tannins have been associated with the maintenance of plant dormancy due to their allelopathic and bactericidal properties.

Tannins are divided into two main groups: hydrolysable and condensed (proanthocyanidins) tannins (Fahey and Berger, 1998; Waghorn, 2007). They exhibit common characteristics such as being soluble in water (but for some high molecular weight structures), binding to proteins and forming either soluble or insoluble protein-tannin complexes of higher molecular weight and lastly containing oligomeric chains with multiple units having free phenolic groups. Hydrolysable tannins are macromolecules produced by plants with polyol as a backbone. The polyol central core is generally made up of D-glucose. Carbohydrate hydroxyl groups are partially or totally esterified with phenolic groups of acids such as gallic acid or ellagic acid. Quantitative analysis of tannins in plants shows that hydrolysable tannins are usually present in lower concentrations than condensed tannins. Hydrolysable tannins are subdivided into taragallotannins (gallic and quinic acid) and caffetannins (caffeic and quinic acid). They are hydrolysed by mild acids, mild bases or hot water to yield carbohydrates and phenolic acids. Enzymes called tannases, which are specific for ester bond hydrolysis, also hydrolyse them.

Condensed tannins, on the other hand, are polymers of flavanoid units (flavan-3-ol) linked by carbon bonds not susceptible to cleavage by mild hydrolysis. Condensed tannins are widely distributed in legume pasture species such as *Lotus corniculatus*, *Lotus pedunculatus*, *Onobrychis viciifolia*, in several species of acacia (Degen et al., 1995), in sorghum grain (Kumar and Singh, 1984), Zea mays and in many other plant species. The complexity of condensed tannins depends on their flavonoid units which vary among constituents and within sites for interflavan bond formation. The astringent taste of some leaves, fruit and wines is due to the presence of tannin. Solubility of condensed tannins in aqueous organic solvents depends on its chemical structure and degree of polymerization. This implies that the higher the degree of polymerization the less soluble they become. Understanding the interaction of tannins with other macromolecules is vital for designing an approach for negating the harmful effects of these macromolecules or for harnessing tannin properties for a nutritional benefit.

The manner in which tannins affect fibre hydrolysis can be classified as direct or indirect. A direct effect is said to occur when the tannin interacts with carbohydrate molecules while an indirect effect is when it interacts with other macromolecules thus slowing down the fermentation process e.g. reacting with cellulolytic enzymes, dysfunction of cell membrane, deprivation of substrate metal ions and minerals (Scalbert, 1991; Fahey and Berger, 1998). Their interaction with carbohydrates is said to be a direct inhibition process because it interferes with microbial breakdown. Tannins interact with the hydroxyl groups of fibres through hydrophobic bond formation. They form complexes with these insoluble materials, reducing the surface area for microbial fermentation hence inhibiting the fermentation process (Bidlack et al., 1992). However, the degree of inhibition depends on the type of microbes available in that ecosystem as well as the evolutional efficiency of the microbes. Some microbes have evolved in their defensive mechanisms so much so that they are able to tolerate certain amounts of tannins in their diet (Odenyo and Osuji, 1998; Odenyo et al., 1999). They do so by (i) secreting enzymes such as tannases in large quantities to assist in hydrolysing these molecules on fibre surfaces (Vaquero et al., 2004; Sasaki et al., 2005) and (or) (ii) secreting higher quantities of nonsense proteins, which minimize the amount of functional protein complexing with tannins.

Indigestibility of fibre due to the formation of tannin-carbohydrate complexes is not as potent as the indigestibility of tannin-protein complexes (Hagerman and Butler, 1981). Tannins interact with proteins, forming tannin-protein complexes (Bhat et al., 1998.), which can either be classified as soluble or insoluble complexes. Tannin-protein interactions are based on hydrophobic interaction, hydrogen bonding but rarely ionic or covalent bonding. Tannins have a high affinity for proteins hence forming very strong tannin-protein complexes (Goel et al., 2007). These proteins can either be dietary, salivary proteins, endogenous enzymes, cell surface proteins or microbial enzymes. Hydrogen bonding is favoured by the ability of phenolic groups to donate hydrogen to form strong hydrogen bonds with proteins. Protein precipitation is maximal at pH values nearer their isoelectric points. At high pH, tannin-protein complex formation does not occur or occurs very slowly because both the phenolics and protein molecules are ionised with a net negative charge. Same charges imply that these molecules exert a repulsive force on each other. This emphasizes the need for buffering with

large volumes of salivary buffer to higher pH in ruminants fed on tannin-rich diets. Tannin-protein complex formation is said to be the most important anti-nutritional aspect of tannins in ruminants. This is because these complexes are resistant to protease attacks and greatly reduce the availability of cellulolytic enzymes (Kumar and Vaithiyanathan, 1990). However, smaller quantities of tannins in the rumen have also been found to be beneficial as they may bind proteases (which limits ruminal fibrolytic activities) suggesting a possible increase in fibre degradation (Weimer, 1998).

Tannin-protein complexes are either soluble or insoluble, depending on the availability of each component (Rakhmani et al., 2005). Soluble complexes are favoured by high protein concentrations while insoluble complexes are favoured by lower protein concentrations in the reaction medium. Insolubility is due to the exposure of hydrophobic surfaces of many tannin molecules crowding around a single protein molecule hence precipitating it out of solution. Solubility is due to high hydrophilic molecules crowding the surface of the complexes formed. Soluble complexes are a major problem as they are quite difficult to measure in solution. Tannins also react with microbial cell walls (Ahmed, 2010), forming tannin-microbe complexes with a potential of inactivating microbial (bacteriostatic) activity (Kumar and Vaithiyanathan, 1990). Rumen microbes such as *Streptococcus bovis*, *Butyvibrio fibrosolvens* and *Fibrobacter succinogens* are inactivated by tannin complexing (McSweeney et al., 1998). Binding induces morphological changes and deprives microbes of certain metal ions. Bacteriocidal activities are also prominent under high concentrations of condense tannins (Kumar and Vaithiyanathan, 1990).

Sotohy et al. (1997) reported that the total number of bacteria in the rumen of goats decreased significantly when the animals were fed with a tannin-rich plant (*Acacia nilotica*) and the decrease in population was directly proportional to the level of tannin in the diet. Decreasing the microbial population indirectly affects cellulolytic enzyme concentration and hence a decrease in fibre hydrolysis. Therefore, microbial inhibition is said to be an indirect inhibition of fibre hydrolysis. Tannins do not only affect fibre breakdown but also reduce palatability of fibre in ruminants due to their astringent taste. Astringency is due to the precipitation of salivary protein in the mouth. Reducing intake implies decreasing the amount of fibre

available for digestion hence indirectly affecting hydrolysis. Complex formation with proteins reduces the amount of nitrogen in the rumen necessary for microbial growth or lowering the fractional absorption of amino acids from the intestine (Waghorn, 2007). Nitrogen deficiency implies a decrease in the microbial population and a decrease in cellulolytic enzymes, and hence a decrease in fibre breakdown.

2.6.4 Origin of short chain fatty acids in the rumen

Rumen and hindgut microbial organisms depend on each other with respect to their metabolic activity. Microbes depend on the host to harvest and provide fibres, increase surface area for enzyme activity, regulate rumen pH, as well as maintain the internal temperature (38-39˚C) for optimum activity. These microbes in turn break down cellulose and hemicellulose macromolecules from maize plant cell walls into hexoses (glucose) and pentoses (e.g. xylose), respectively. Glycolysis is the main process by which these monosaccharides are metabolized to yield high energy molecules called adenosine triphosphate (ATP) and by-products such as pyruvate, and reduced nicotinamide adenine dinucleotide ($NADH_2$); some pathways of pyruvate metabolism are illustrated in Figure 2.5. Glycolysis is completely anaerobic. Microbes utilise ATP liberated from glycolysis for microbial growth (synthesis of cells) and maintenance (Black et al., 1981). Pyruvate is converted into SCFA, namely acetate, propionate and butyrate. Short chain fatty acids are the essential metabolic molecules of interest to ruminants. The concentration of SCFA produced as end-products of carbohydrate fermentation varies depending on the bacterial species involved and the type of substrate consumed (Owens and Goestsch, 1998). Different pathways have been described for the conversion of carbohydrates into acetate, propionate and butyrate. Figure 2.5 demonstrates the major pathways that are involved in the conversion of pyruvate into SCFA. Fermentation of hemicelluloses to SCFA is very similar to that of celluloses but for the initial conversion stages of hemicellulose. Hemicelluloses are first hydrolysed into pentoses (xyloses) by hemicellulases and then converted into hexoses (fructose phosphates) that can enter the glycolytic pathway. Short chain fatty acid production varies from animal to animal as well as with the type of feed being fed.

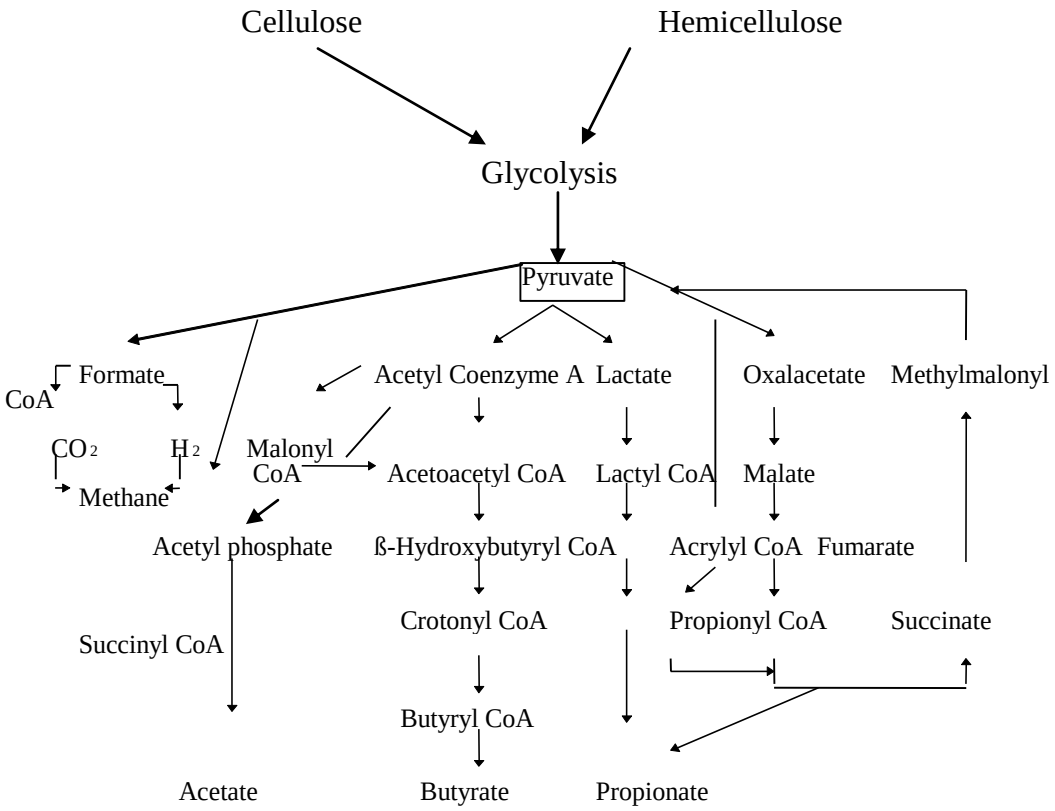


Figure 2.5 The production and conversion of pyruvate to volatile fatty acids in the rumen. McDonald (1995) modified.

2.6.5 Fate of short chain fatty acids

Understanding the fate of SCFA is as important as probing their origin. Short chain fatty acids are essential metabolic substrates in fermenters nutrition since approximately 60% of the energy of feed comes from them. They are absorbed mainly from the rumen by simple diffusion across the rumen wall into the epithelial tissue and then into the blood stream (Bergman, 1990). Acetate and propionate diffuse through the rumen epithelium without any modification while a reasonable amount of butyrate is modified to beta-hydroxybutyric acid (ketone bodies) before diffusion (Leng and West, 1969; Bannink et al., 2008). This accounts for most of the ketone bodies in the blood stream of animals during feeding. Some of the SCFA (e.g. acetate and butyrate) are metabolised in the rumen epithelium to carbon dioxide.

All three SCFAs are involved in the tricarboxylic acid (TCA) cycle. The TCA cycle is a major energy generation pathway in ruminants and can only take place in the presence of oxygen (aerobic process). Short chain fatty acids are modified with coenzyme A (CoA) before introduction at different stages of the TCA cycle. In the cytoplasm of liver cells, free acetate is activated to acetyl-CoA in the presence of 2 ATP molecules and coenzyme A synthetase. Acetyl-CoA is an intermediate in the TCA cycle, which when completely oxidized yields 12 ATP molecules. Butyrate is converted to butyryl-CoA in the presence of butyryl-CoA synthetase and 2 ATPS. The conversion of butyryl-CoA to aceto acetyl-CoA involves two stages with the release of reduced flavin adenine dinucleotide ($FADH_2$) and $NADH_2$. Aceto acetyl-CoA is then hydrolysed into acetyl-CoA, an intermediate in the TCA cycle. This undergoes the same oxidative process as any other acetyl-CoA molecule. The theoretical value of the numbers of moles of ATP released from the oxidation of butyrate is higher than that of acetate. Propionyl-CoA is an intermediate product formed from the reaction between propionate and CoA in the presence of 2 ATPs. Propionyl-CoA is then converted to succinyl-CoA which is an intermediate in the TCA cycle by the introduction of carbon dioxide. The oxidation of succinyl-CoA yields a theoretical value of 20 moles of ATP which is lower than that produced from butyrate but higher than those from acetate.

Propionate is a major substrate in the gluconeogenic pathway. Gluconeogenesis is the generation of glucose from non carbohydrate sources e.g. lactate, glycerol, amino acids and intermediates of the TCA cycle. Gluconeogenesis provides more than 90% of the total glucose needs in fermenters (Young, 1977; Danfær et al., 1995). This process is active immediately after feeding in fermenters. Glucose generated from propionate is the main source of glucose in ruminants (Danfær et al., 1995). Energy required at the tissue level of ruminants is provided mainly by the gluconeogenic pathway as very little or no bypass of glucose is observed except in cases where ruminants are fed with high concentrate feeds. Small quantities of propionate are converted to lactate in the rumen epithelium during absorption while the rest enters the portal blood vein to the liver. In the liver these molecules are converted to lactate, which enters the gluconeogenic pathway for glucose synthesis.

Alternatively, acetyl CoA is used for the synthesis of fatty acids, which are the building units of lipids. However the conversion of glucose molecules to the building units of lipids (acetyl CoA) is limited by the presence of ATP citrate lyase. Butyrate is converted into three major ketones, namely, β-hydroxyl butyrate, acetone and acetoacetate. About 80% of the total ketone produced is β-hydroxyl butyrate with some of it being used in fatty acid synthesis in the adipose and mammary gland tissues. Short chain fatty acids are not only utilised by ruminants for energy generation but also by microbes with limited supply of glucose. However, energy generation for microbial growth from SCFA is lower than that generated from glucose molecules. Therefore, SCFA are the main metabolic energy substrates in ruminants resulting from microbial fermentation of fibre with propionate being the most important in gluconeogenesis.

2.7 Cellulases and cellulosomes

2.7.1 Cellulases

Cellulases are enzymes that hydrolyse β-1,4 glucosidic bonds in fibre into oligosaccharides and monosaccharides (Hoshino et al., 1994). The term cellulase may sound simple but it is a complex enzyme system. There are two complex theories that attempt to elucidate the secretion and functioning of these enzymes. The first one states that cellulases are secreted as separate entities (exocellulase, endocellulase, and cellobiase) into the extracellular medium but functions synergistically on their substrate (Bayer et al., 1998a). Howell and Stuck (1975) revealed that cellulases are complex enzymes made up of different enzymes with specific functions. Reese et al. (1950) incubated cellulose with cellulases and demonstrated that cellobiose and glucose were the main by-products. They also noticed that the concentration of cellobiose decreased with time while that of glucose increased. King and Versal (1969) also showed that cellulases obtained from *Trichoderma viride* operate in a similar manner. They postulated that hydrolysis of cellulose occurs in two basic steps: firstly, hydrolysis of β-1, 4 glycosidic bond by beta-1, 4 exocellulase producing cellobiose as the end product and secondly, the hydrolysis of glycosidic linkage of cellobiose by beta-1, 4 cellobiase yielding glucose units (Equation 2).

Equation 2

$$\text{Cellulose} \xrightarrow[n = \text{natural numbers}]{\beta\text{-1, 4 exocellulase}} n \text{ cellobiose} \xrightarrow{\beta\text{-1, 4 cellobiase}} 2n \text{ glucose}$$

The complexity of cellulose existing in both crystalline and amorphous forms has given rise to a phenomenal diversity of hydrolytic enzymes (cellulases) secreted by microbes with respect to the type present in their environment. This implies that the activity of these enzymes will vary between species. Even within species the cellulolytic activity as well as molecular weight also varies as their genetic material is not identical. Freelove et al. (2001) demonstrated that there was another component of these enzymes that was responsible for the recognition of substrate (carbohydrate binding sites (CBH)). This protein component that recognizes and binds to cellulose surfaces is inert but enhances enzyme activity due to the proximity effect. Figure 2.6 demonstrates the hydrolysis of amorphous and crystalline cellulose by a complexed and non-complexed enzyme system. Endocellulases cut randomly within the amorphous molecule while exocellulases chip off cellobiose molecules from the non-reducing end of crystalline molecules (Teeri, 1997).

2.7.2 Cellulosomes

The second theory explains a greater level of complexity observed in some anaerobic microbes where several cellulases are grouped into an enzymatic complex, called cellulosome (Bayer et al., 1998a). Cellulosomes are extracellular super molecular machines that can efficiently degrade crystalline cellulosic substrates and associated plant cell wall polysaccharides (Shoham et al., 1999). The cellulosomal concept was first established by Lamed et al. (1983), in their study on the antigenic active cellulose-binding factor (CBF) of the bacterium *Clostridium thermocellum*. In an attempt to isolate and characterized the antigenic CBF, also called cellulose binding domain (CBD), they realized that the molecular weight was higher than predicted. Electron microscopic analyses of the CBF complex revealed a particulate, multi-subunit entity of a complicated quaternary structure. This complex molecule was resolved into 14 polypeptide bands on a polyacrylamide gel (Laemmli, 1970). Eight of the polypeptide components exhibited celullolytic activity. They concluded that not

only was the CBF complex molecule responsible for cell adherence to cellulose, but contain polypeptide chains that also hydrolysis cellulose.

The cloning and sequencing of cellulase genes from *Clostridium thermocellum* led to the prediction of the modular structure and multi-subunits of cellulosome (Bayer and Lamed, 1986; Shoham et al., 1999; Madkour and Mayer, 2003; Desvaux, 2005). Although anaerobic clostridial species were the only species with full genetic information available, signal sequences have been identified in other species including anaerobes and fungi (Table 2.6).

The different types of glycosyl hydrolases involved with cellulosome complexes are cellulases, hemicellulases and some carbohydrate esterases. All the enzymes are held

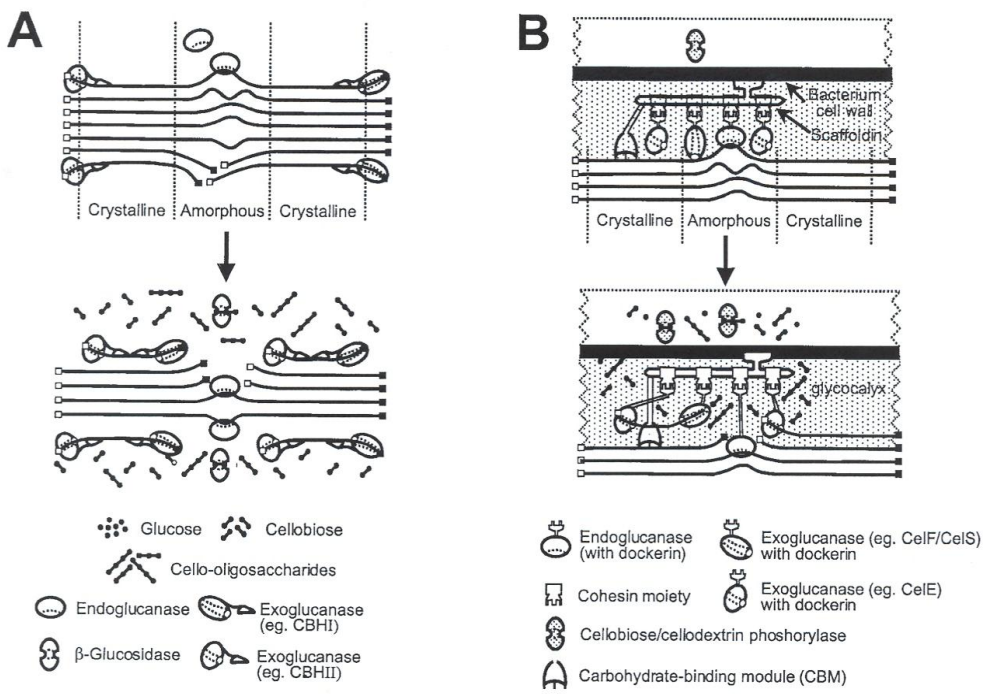


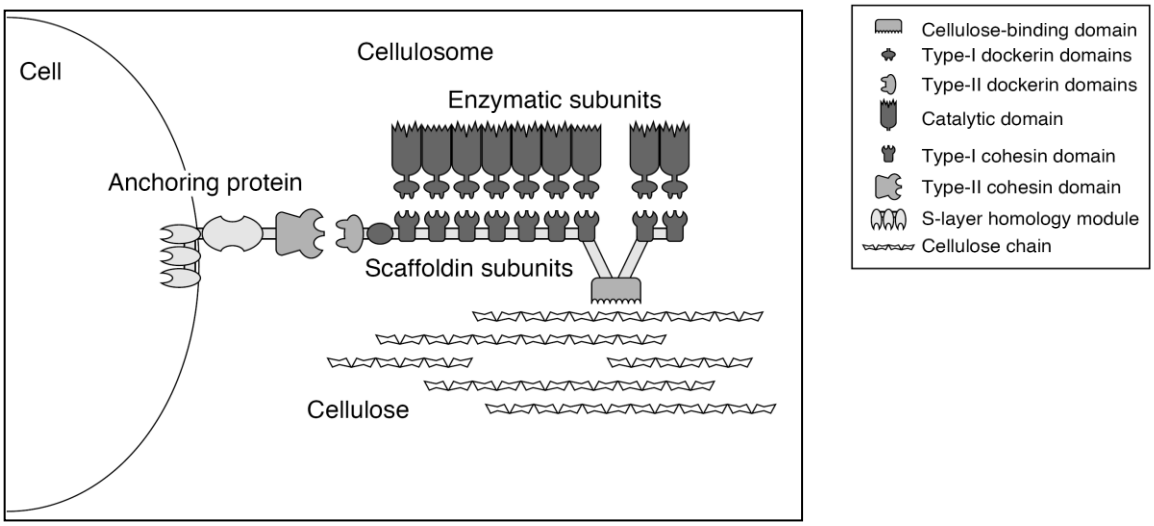
Figure 2.6 Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by a non-complexed (A) and a complexed (B) cellulase system. Solid squares represent reducing ends and open squares represent non-reducing ends (Lynd et al., 2002) modified.

together by a major polypeptide called scaffoldin, also known as cellulosome integrating protein A (CipA). The different activities of scaffoldin are dictated by its functional modules, dokerin domains, cohesion domains and CBD (Bayer et al., 1998a; Shoham et al., 1999; Desvaux, 2005). Scaffoldin interacts with the following processes, cellulose binding, cell anchorage and the organization of enzyme subunits in the complex as well as promoting the cellulosomal enzyme activities. Cohesin facilitates the interaction of cellulosomal complex with enzymes and cell surface. Dockerin allows the interaction of catalytic domain and cohesin domains. The molecular weights of cellulosome complexes range from 40 to 180 KDa. Cell-free cellulosome clusters coating substrate surface and solubilizing celluloses has been reported by Bayer and Lamed (1986), which implies that not all cellulosomes are cell bound.

Table 2.6 Evidence for cellulosomes in cellulolytic microorganisms

Organism	**Cellulosome signature sequence**	
	Protein	**Domain**
Anaerobic bacteria		
Clostridium thermocellum	Scaffoldin	CohI1CBD1DocII
	Surface-anchoring proteins	CohII
	Enzymes	DocI
Clostridium cellulovorans,	Scaffoldin	CohI1 CBD
Clostridium cellulolyticum,	Enzymes	DocI
Clostridium josui		
Acetivibrio cellulolyticus	Scaffoldin and surface-anchoring protein	CohI1CBD1DocII CohII
Bacteroides cellulosolvens	Scaffoldin or surface anchoring protein	CohII1CBD
	Enzymes	DocI
Ruminococcus albus, Ruminococcus flavefaciens		
Aerobic bacteria		
Vibrio sp.	Enzyme	Fungal-type dockerin
Anaerobic fungi	Enzymes	Fungal dockerins
Neocallimastix, Piromyces,		

Abbreviations: CBD, cellulose-binding domain, CohI, type-I cohesin domain; CohII, type-II cohesin domain; DocI, type-I dockerin domain; DocII, type-II dockerin domain, (Shoham et al., 1999).



Shoham et al. (1999)

Figure 2.7 A schematic view of cellulosome subunits and its interaction between cellulose interaction and microbes

The intriguing question is, why is the arrangement of cellulases in a cellulosome complex more efficient than free enzyme systems in the solubilisation of crystalline cellulose? Cellulosomes allow optimum cooperative activity and synergism of cellulase, avoids non-productive adsorption of cellulases, limits competition between cellulases for the site of adsorption and optimal processing of the cellulose all along the cellulose fibre (Schwarz, 2001). Johnson et al. (1982) compared crystalline cellulose solubilisation by cellulosome from *C. thermocellum* to that of free enzyme systems of *Trichoderma reesei* and found a much lesser amount of protein from *C. thermocellum* was required to completely solubilize the same amount of substrate. Boisset et al. (1999) also demonstrated that cellulosomes from *C. thermocellum* were more efficient in solubilizing substrates of high crystalline content. Cell-density dependent growth is affected by the availability of solubles released from cellulose. Cellulosome-mediated attachment of microbial cells to celluloses has an urge over microbes secreting free enzymes with respect to solubles utilization. This is because solubles generated by free enzymes away from the cells are not readily available. Therefore, limiting the concentration of solubles available to support growth at very low cell densities as compared to

cellulosomal systems where cells are available at the site of solubilisation. Cellulose solubilisation is a step wise process involving more than one enzyme. Therefore, the concentration of these enzymes in a cellulosome suggests an even stronger synergism among the catalytic units. Further research on the structural arrangement of cellulosomes and its benefit would be beneficial in the feed industries.

Although there is evidence supporting the cellulosome concept as a major break-through for the solubilisation of cellulose, many questions still need to be readdressed; (i) more evidence on the advantages of cellulosome over free cellulases, (ii) the overall structure of cellulosome, (iii) cellulosome secretion, attachment and assembly on the bacteria surface, (iv) whether a particular microbial species can produce both cellulosome and free cellulases, (v) what are the functions and structures of the accessory domains?, and (vi) its stability and survival in the rumen when applied as feed additives.

2.8 Cellulose additives

More than 70% of forage content consumed by ruminants when grazing on the fields is made up of fibre (Buxton et al., 1995). Fibre hydrolysis is a major problem in these animals as less than 40% is hydrolysed for metabolic purposes and the rest excreted as faeces**.** Agriculturists have found it very difficult to sustain the cost of their forages as much of it is wasted as faeces. The high cost and wastage of fibre intake has instigated many researchers to carry out different studies on the possibilities of improving fibre digestibility. Research directed towards cellulose additives seems to pay a dividend on digestibility. Cellulose additives are substances that are administered to fermenters to enhance fibre breakdown. The different substances include enzymes, antibiotics, probiotics and buffers.

2.8.1 Antibiotics

Although antibiotics over the past decades have shown the potential of improving feed digestibility in herbivores, their application as growth promoting factors in feeds was banned by the European Union in January 2006 (Regulation 1831/2003/EC) due to concerns about the appearance of residues and resistant strains of bacteria (Gustafson and Bowen, 1997).

Antibiotics are chemical substances that halt the growth of micro-organisms e.g. pathogenic microbes. These chemical compounds are very effective when applied in small quantities. Some examples of antibiotics that are believed to reduce liver abscesses includes; tylosin, bacteriocin methylene disalicylate, chlortetracycline, oxytracycline and verhiniamycin (Nagaraja and Chengappa, 1998). Antibiotics are produced by micro-organisms for protection against other microbes. Scientists have exploited these molecules to protect or eliminate undesirable microbial species in a specific environment. The development of advanced biotechnology has made it possible for laboratory synthesis of antibiotics. The rumen is inhabited by a variety of microbes but not all of these microbes are important to the host. The following negative effects have been associated with non-symbiotic microbes in the rumen: production of toxins, competition with the host for nutrients (Wells et al., 1997), decreased absorptive capacity of the ruminal wall and synthesis of unwanted macromolecules and by-products e.g. lactic acids and methane. *Megasphaera elsdenii* has been found to compete with cellulolytic microbes and host for glucose. The presence of *Lactobacillus ruminis* and *Streptococcus bovis* increases the concentration of lactic acid, thereby promoting lactic acidosis (Russell and Hino, 1985). Bacteriocin administered through injection, food or water was associated with a relative decrease in such bacteria and an increase in cellulolytic bacteria activity (Chen and Wolin, 1979). The decrease in ruminal anaerobic fungi population has been associated to bacteriocin toxin to eliminate competition on available substrate (Dehority and Tirabasso, 2000). They observed that in the presence of antibiotics, ruminal fungi population increased by 42-fold in three days while it almost disappeared after a day of incubation in the absence of antibiotics. Due to the continuous resistance exhibited by some microbial strains, most researchers have turn their focus on plant extracts, essential oils, enzymes, probiotics (direct-fed microbials) and buffers for manipulating the ruminal microbial ecosystem.

2.8.2 Ionophores

Ionophores are highly lipophilic (lipid-soluble) molecule usually synthesized by microorganisms to transport ions across the lipid bilayer of the cell membrane. It can also be antibiotic as some have been found to disrupt transmembrane ion concentration gradients, required for the proper functioning and survival of microorganisms (Pressman, 1976). Some

examples of ionophores include; monensin, lasalocid, tetronasin, lysocellin, narasin and laidlomycin. Monesin is the most extensively used ionophore. Its exploitation in countries like the United States has been tremendous with the improvement of feed utilization efficiency being the main benefit. On application of monensin feed intake often decreases while the rate of body weight gain may not or may increase slightly (Rumsey, 1984). Increase in feed utilization efficiency has also been seen as a cumulative effect of an ionophore on different metabolic processes. Monensin in particular has been found to decrease methane production by 30% (Thornton and Owens, 1981; Schelling, 1984), increase the ratio of propionic to acetic acid production (Richardson et al., 1976; de Oliveira et al., 2005) and inhibit the growth of some proteolytic ruminal bacteria (Dinius et al., 1976) hence a decrease in protein deamination to ammonia (Newbold et al., 1988). An increase in ruminal pH and a decrease in lactate concentrations were observed in cattle fed with monensin in their diet (Dennis and Nagaraja, 1981; Nagaraja et al., 1982). This implies that the sustainability of cellulolytic microbes sensitive to lower pH would be higher than when fed without monensin. Although some researchers have reported no or negative effects about ionophores, their beneficial responses seems to outweigh the negative hence requiring more research.

2.8.3 Probiotics

Probiotics are antagonistic to antibiotics. They are chemical compounds or microbes (fungi and yeast) that enhance the growth of certain bacteria (Fuller, 1989). As mentioned in the above paragraph, the rumen harbours a variety of microbes, some of which are very important to the host and others that are of less importance. The elimination of the non-essential bacterial population is also important as this action favours the growth of essential bacteria. Some of the essential microbes in the rumen include *Ruminococcus flavifaciens* (Gram-positive bacterium) and a Gram-negative *Fibrobacter succinogenes (*Weimer, 1996*). R. flavifaciens* has the ability of secreting both hemicellulases and cellulases in the ruminal growth medium. The presence of these two enzymes plays a vital role in fibre breakdown as one digests the hemicellulose mesh network cross-linking cellulose exposing the cellulose for cellulases to act on. *F. succinogenes* ferments glucose releasing by-products such as acetate,

succinate and energy (ATP). Apart from cellulolytic microbes, other microbes in the rumen utilize these by-products to generate energy.

Different cellulolytic microbes hydrolyse fibre with a net positive change being energy production but the by-product depends on the species. The different by-products include methane, fatty acids, lactic acid, succinate, hydrogen (NADH) and carbon dioxide. *Veillonella parvula* is a Gram-negative bacterium that utilizes lactic acid as growth substrate from which it produces end-products such as acetate and propionate. *V. parvula* has a double function because it eliminates lactic acid from the rumen preventing lactic acidosis but at the same time producing propionate which is a gluconeogenic substrate. Therefore, it enhances the growth of both the host and the bacteria. Other microbes that utilize lactate as substrates while producing SCFA as by products are *Megasphaera elsdenii* and *Selenomonas ruminantium*. *Acetaculum ruminis* and *Ruminococcus schinikii* utilize carbon dioxide as a source of carbon and hydrogen from NADH to supply reducing equivalence. The introduction of chemical compounds that enhance the growth of such bacteria is essential in enhancing growth. On the other hand microbes such as *Streptococcus bovis* compete with celullolytic microbes for glucose producing lactic acid as by-product. *In vitro* growth assays demonstrated that *S. bovis* JB1 was sensitive to tannic acid, acacia, and calliandra tannins while *S. gallolyticus* was more tolerant at low levels of tannin (Krause et al., 2005). Hence, chemicals which tend to eliminate such microbes would be beneficial in promoting animal production.

2.8.4 Enzymes

Cellulases secreted by microbes in the rumen are responsible for fibre breakdown. Theoretically, it is possible that an increase in cellulase or cellulosome concentrations will increase the rate of enzyme activity. Biotechnological advancement has made it possible for large quantities of enzymes to be synthesized at moderate cost (Sun et al., 2008). Microbes from different ecosystems have been isolated and chemically engineered to express cellulases (Cavicchioli and Watson, 1991; Morgavi et al., 2000; Wallace et al., 2008). Supplementing roughage feeds with variable quantities of exogenous enzymes showed minimal increment in fibre hydrolysis (Colombatto et al., 2003; Colombatto et al., 2004). This small increase

irrespective of the large amount of enzyme applied was due to the dynamic nature of the rumen physiology. Secondly, these enzymes are protein in nature and are rapidly hydrolysed by the potent microbes in the rumen. Therefore, the stability of these enzymes in the rumen is more of a limiting factor than the addition of enzymes itself (Morgavi et al., 2000). Although the success rate of exogenous enzyme application is minimal, it still possesses the potential of improving stover digestibility.

2.8.5 Buffers

Buffers are solutions that resist minimal changes in pH. Like any other microbes, cellulolytic microbes have an active pH range of 6.2-6.8 (Jouany, 2006). Any alteration of this pH to a very high or lower value will have a negative impact on their activity. Fluctuations in ruminal pH are common especially when forages with high content of soluble carbohydrates are consumed e.g. an increase in lactic acid concentration due to the fermentation of glucose by *Lactobacillus ruminis* decreases the pH leading to a serious clinical condition known as lactic acidosis. Lactic acidosis can either be sub-acute (pH range 6.0-5.0) or acute (pH<5.0) (Krause and Oetzel, 2006). However, sub-acute acidosis in dairy animal can also be caused by temporary accumulation of total SCFA (Oetzel et al., 1999) often due to inefficiency of the ruminal papillae to absorb large quantities (Dirksen, 1985). Acute lactic acidosis implies a reduction of microbial population hence a decrease or an inactivation of enzyme activities (Quinn, 1962). The breakdown of microbial population depends on how long the drop in pH last rather than the actual drop in pH (Maekawa et al., 2002; Beauchemin et al., 2003b). Rumenitis (inflammation of the rumen) is very common in animals with acute acidosis since the epithelial cells are not protected by mucus. Acute acidosis can kill the animal if it is not recognized in time.

In order to regulate the fluctuating pH levels in the rumen, a variety of buffers have been introduced in ruminant feed and investigated e.g. magnesium oxide, calcium carbonate (limestone) and sodium bicarbonate (Erdmann, 1988). Low rumen pH can easily be controlled by adding buffers to the diet at doses of 1-2% dry matter intake (Erdmann, 1988). These buffers regulate the concentration of hydrogen ions as well as the dilution rate of fluid in the

rumen. A decrease in hydrogen ion concentration by these buffers increases the efficiency of microbial activity (Edwards and Poole, 1983). Increased dilutions were found to be relatively advantageous as soluble carbohydrates and proteins were easily flushed from the reticulo-rumen into the abomasum. The shorter the retention time of soluble carbohydrates the smaller the quantity of lactic acid hence a decrease in pH fluctuation. Therefore, proper buffering of the ruminal environment would definitely decrease enzyme inactivation or death of fibrolytic microbes hence an increase in stover digestibility.

2.9 Microbial variations in herbivores

Herbivores cannot digest fibre but depend entirely on microbial fermentation in the rumen and hindgut (Wallace et al., 2008). These microbes supply proteins (90%), vitamins and SCFA to the host animal. The microbes are subjected to a variety of forages and environmental constraints that affects degradability. The efficiency of utilizing such a wide variety of substrates is due to the highly diversified rumen microbial ecosystems consisting of bacteria, protozoa, fungi and even bacteriophages (Krause et al., 1999). Microbial species, dominant microbial population and the type of forage involved may play a vital role in influencing microbial activity and efficiency.

Krause et al. (1999) demonstrated that cellulose fermentation varies among the following microbial species: *Butyrivibris fibriosolvens, R. albus, R. flavefaciens* and *F. succinogens*. When a group of 22 *B. fibriosolvens* strains were assessed for their ability to degrade spear grass few could digest more than 10 % of the DM (McSweeney et al., 1998). A similar study by Krause et al. (1998) also demonstrated that microbial activity varies greatly among strains of *R. albus* and *R. flavefaciens.* They called the most active strains superior fibre degraders. *B. fibriosolvens* strains also showed a potential to digest xylan hence exposing cellulose for fermentation (Hespell et al., 1997).

Microbial species are also said to vary among herbivore hosts of the same or different species coming from the same or different geographical regions. This was demonstrated by Leng (1990) when *R. albus* from the same host species demonstrated variable activity. They also

showed that the activities of isolated strains from the same host as well as different hosts from different geographical regions are even more variable in their activities. This implies that a random combing of different wild lives from different geographical regions could lead to the elucidation of more active strains.

Forage type is said to play a major role in microbial activity as they influence microbial species, population as well as their evolution (Varga and Kolver, 1997). Microbes sense environmental signals and adapt with the nutrient available (Stock et al., 1989; Stock, 1999). Therefore, ecosystems subjected to roughage will switch on microbial fibrolytic systems available in the ecosystem. This was observed when two bacterial membrane proteins (histidine kinase and cytoplasmic regulator protein) were activated and hydrolytic enzyme expression systems were switched on when subjected to soluble polysaccharides (Stock, 1999). Although little is known about how bacteria sense the presence of substrate, and alter gene expression, four major protein phosphatases are said to be involved in this process (Shi et al., 1998).

Digestibility depends on the rate and extent of colonization of fibre and the biomass of adherent organisms (Cheng et al., 1990). Therefore, fibre digestibility may be enhanced with an increase population of superior degraders. Although microbial population and activity is said to play a vital role, Maglione et al. (1997) demonstrated that cellulose digestion is not only limited by microbial population or the activity of cellulolytic microbes but also by the amount of cellulose available for attack. This emphasizes the need for active symbiotic microbes to degrade encrusting substances (e.g. lignin and phenolics), and expose more cellulose (Dehority, 1991).

Screening many ecosystems, both domestic and wild, might lead to the revelation of more superior fibre degraders which when merged can lead to a more superior ecosystem. Secondly, the exposure of more active non-cellulolytic microbes with the potential to degrade anti-nutritional factors will be advantageous to cellulolytic microbes in symbiotic systems.

2.10 A hypothetical model for the improvement of maize stover digestibility

The quantity of MS wasted as by-products from farms or industries is unavoidable. Numerous experiments have been carried out on the improvement of fibre hydrolysis as a source of energy in ruminants but with little success. Earlier attempts included the chopping and grinding of fibre material to increase the surface area for enzymatic hydrolysis (Bourquin et al., 1990). Increasing the surface area showed a very small increase in fibre utilization as there were other inhibiting factors such as hemicellulose and lignin encrusting the cellulose. It is difficult to get rid of these macromolecules in order to expose cellulose during the improvement of MS digestibility. Isolating and purifying the different components of cellulases responsible for fibre breakdown has been the main topic of the millennium. Different research groups have engaged in purifying and investigating the activities of exocellulases, endocellulases or cellulasomes from different strains of bacteria while hoping to find strains with higher activity. Many active fibrolytic enzymes have been found but limited by their stability and hydrolysis by proteases in the rumen. Others research bacteria strains with the capacity to synthesize cellulase, hemicellulase and ligninase.

However, Wanapat (2000) looked at fibre hydrolysis in a different way where he transferred digesta from swamp buffalos into cattle. He established that buffalo digesta were sustained in cattle for up to 14 days. This is an indication that microbes with superior fibrolytic activity can be transferred from endowed into deficient ruminants. With respect to the information available on cellulases and the different research carried out on the improvement of fibre hydrolysis, it is can be hypothesized that if microbes from a variety of rumen ecosystems with higher activities are merged to form a new ecosystem (composite system) MS hydrolysis may be improved (Figure 2.8). The rate of MS hydrolysis might be higher in the new system than in the control systems. Fibrolytic enzyme expression would be a continuous process if transferability is successful. This symbiotic system might also be a more superior enzyme system when compared to exogenous enzyme additives systems.

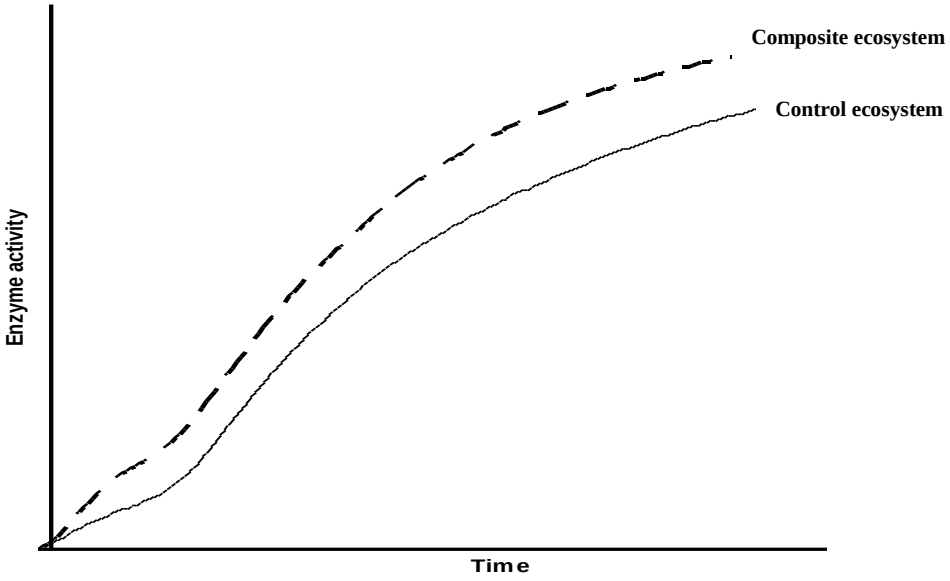


Figure 2.8 A hypothetical graph predicting the activity of a composite ecosystem

2.11 Summary

Feed deficiencies in winter have been identified as one of the many problems in animal production especially in the poorer communities in Africa that own livestock. This problem can be solved by feeding animals with crop residues e.g. maize stover with feed potentials during these periods. Although crop residues are abundant their usage is limited by poor information about its nutritional value and lack of technologies for processing and storage. Therefore, government and NGO interventions in sensitizing and subsidizing such technologies are very important for the utilization and commercialization of crop residues.

Ruminants derive a considerable proportion of their dietary needs from the digestion of cell wall polysaccharides of plants. These polysaccharides comprise crystalline cellulose in a matrix of hemicelluloses, which may associate physically and chemically with lignin. Its nutritional functions are very limited as herbivores depend on microbes for fermentation. The digestibility of fibre is greatly influenced by the amount and distribution of hemicelluloses, pectin and lignin. Ruminants are the only animals that seem to handle fibre breakdown successfully due to the fact that they harbour microbes that synthesize cellulolytic enzymes.

However, hydrolysis by these enzymes is still limited as the fibre concentration lost as faeces is still very high. There have been a number of positive attempts with regards to fibre hydrolysis but there is still a lot that needs to be uncovered. The merging of two ecosystems might pave the way for the improvement of fibre breakdown rather than using purified enzymes. This may be more efficient and cheaper because if cellulolytic microbes are able to compete and survive in the new ecosystem, enzyme secretion will be a continuous process and the species will proliferate.

Chapter 3

Laboratory cultured faecal inoculum is a better substitute for fresh rumen inoculum than fresh faecal inoculum for *in vitro* feed evaluation

Abstract

The objectives of this study were to; (i) preserve whole rumen fluid (RF) or faecal fluid (FF) in the laboratory by culturing using very simple laboratory techniques and (ii) study the effect of substituting fresh RF with fresh or laboratory cultured FF as an alternative inoculum source for *in vitro* dry matter digestibility. Rumen fluid and faeces were collected from two non-lactating fistulated jersey cows (3.5 yr old). Cultured inocula were prepared by incubating RF or FF with salivary buffer, maize stover (MS) and lucerne (1:1) at 39°C for three days. For fresh incubation (FI) or cultured incubation (CI) systems, RF or FF were mixed with salivary buffer containing MS (1g) and incubated at 39°C for 72 h. Apparent degradability (APD), true degradability (TD), total gas produced (GP), total short chain fatty acids (SCFA) and gas kinetic parameters were determined after incubation. Faecal fluid activity was also monitored for 42 d by measuring the GP from fermentation. For enzyme assays crude proteins precipitated by 60% ammonium sulphate from RF or FF were assayed for exocellulase, endocellulase and hemicellulase specific activities (µg reducing sugar/mg crude protein). Exocellulase specific activity was higher (P<0.001) in RF than FF for FI whereas in CI, RF exocellulase specific activity was lower than that of FF. True degradability and GP in both FI and CI systems were higher (P<0.001) in RF than in FF. The rate of MS fermentation (C) did not differ among systems and between incubations. Partitioning factor and degradability efficiency for both RF and FF were similar in FI but differed (P<0.001) in CI. Gas from slowly but degradable fraction of MS was higher (P<0.001) for RF in both FI and CI when compared to FF. Total SCFA was higher (P<0.001) in RF than FF in FI but was lower for RF in CI. Laboratory cultured FF were active (P<0.001) for up to 6 weeks but with better results obtained in week 1. Cultured FF is a better substitute for fresh RF as shown by their percentage differences in exocellulase activity (0.4%) and TD (7%), compared to the differences observed between fresh RF and FF for exocellulase activity (33%) and TD (14%). This study suggests that cultured inocula would reduce the cost of experimentation without compromising the reliability of results.

3.1 Introduction

Most *in vitro* digestibility studies rely on the fermentation of feed using buffered rumen fluid as an inoculum (Tilley and Terry, 1963; Menke et al., 1979; Stern et al., 1997; De Boever et al., 2005; Mirzaei-Aghsaghali et al., 2007; Prates et al., 2010). This is because rumen inoculum has been found to yield results which mimic those from *in vivo* studies (Gizzi et al., 1998; Brown et al., 2002). Despite the reliability of the results obtained from using rumen fluid, there are still some major drawbacks which render it difficult to be exploited by researchers especially those in the developing countries. These drawbacks include; the requirement of animals and surgical operation (fistula), constant care to avoid infection, long term maintenance (expensive), difficulties of traveling when animals are far from the laboratory and ethical issues of using these fistulated animals (Stern et al., 1997; Mauricio et al., 1999; Mauricio et al., 2001).

Finding alternative inoculum (faecal fluid) which does not involve surgical procedures is necessary. Numerous studies have shown positive correlations between rumen and faecal inocula in *in vitro* digestibility studies from either bovine or sheep (Gonçalves and Borba, 1996; Nsahlai and Umunna, 1996; El-Meadaway et al., 1998; Mauricio et al., 2001; Cone et al., 2002; Mould et al., 2005). While many studies engage in comparing rumen and faecal inoculum incubations of the same animals (El-Meadaway et al., 1998; Váradyová et al., 2007), a few extended the research by comparing faecal inocula from ruminants and hindgut fermenters (Denek and Can, 2007; Denek et al., 2008). Denek and Can (2007) reported that buffered faecal inoculum from hindgut fermenters had the highest potential for predicting *in vitro* dry matter degradability than cattle and sheep faecal inocula.

Campbell et al. (2002) and Váradyová et al. (2007) estimated the fermentative capacity among lemur species using faecal inoculum. El-Meadaway et al. (1998) showed that there were minimal differences observed in *in vitro* dry matter degradability between rumen inoculum and 3% fresh faecal samples but values were lower when 6-15% fresh faecal inoculum was used. The results were similar to those obtained by El Shaer et al. (1987) and Nsahlai and Umunna (1996). Collecting fresh samples every day for *in vitro* incubations can be expensive

(Hervás et al., 2005) especially when the experimental animals are far from the laboratory. Studies showing how whole rumen or faecal inoculum can be managed in the laboratory for a few days for further experimentation using simpler techniques are rare in the literature (Akhter et al., 1995; Rymer and Givens, 2000; Hervás et al., 2005; Denek et al., 2010; Prates et al., 2010). The different storage methods that have been investigated include; storage at low temperature or freezing over 24 h interval (Hervás et al., 2005; Prates et al., 2010), liquid nitrogen freezing (Prates et al., 2010) and cryoprotection (glycerol or dimethyl sulphur oxide) (Denek et al., 2010; Prates et al., 2010). The different studies demonstrated that, maximum fermentation was not greatly affected but the fermentation rate was affected and initiation delayed.

Continuous *in vitro* culture systems mimicking rumen fermentation have demonstrated the importance of these systems in elucidating knowledge of the rumen ecosystem. However, the extensive use of these systems is limited by inherent problems in the technique as well as difficulties and complexities specific to each system (Vatthauer et al., 1970a; b). The cost of the system as well as running and maintenance cost is a major limitation especially in the developing countries. Since faecal inoculum from different wild animals will be the main source of inocula in the current study, it is imperative to investigate cheaper methods of storage in the laboratory and their adaptation on a common substrate (maize stover) prior to application.

This study investigated; (i) preserve whole rumen fluid (RF) or faecal fluid (FF) in the laboratory by culturing using very simple laboratory techniques and (ii) the possibilities of substituting fresh RF with fresh or cultured FF as an inoculum source. It is hypothesized that culturing rumen or faecal inoculum in the laboratory would increase fibre fermentation while reducing the cost that would be incurred for daily collection of inocula.

3.2 Materials and Methods

3.2.1 Chemical composition of maize stover

Maize (white maize hybrid PAN6479) was cultivated in spring at Ukulinga, University of KwaZulu-Natal South Africa. The stovers were harvested at grain dry stage, dried and milled through a 1-mm screen. Dry matter (DM) was determined by drying the milled samples in a fan oven at 90°C overnight. Nitrogen was determined by micro-Kjeldahl method and crude protein calculated as Nx6.25 according to AOAC (1990). Neutral detergent fibre (NDF), acid detergent fibre and acid (ADF) detergent lignin (ADL) were determined as described by Van Soest et al. (1991). Hemicellulose was calculated by subtracting ADF from NDF and cellulose by subtracting ADL from ADF.

3.2.2 Experimental animals

Two rumen fistulated Jersey cows (~ 400 kg and 3.8 yr old) were obtained from the Ukulinga Research Farm, University of KwaZulu Natal, Pietermaritzburg. Fistulated cows (non-lactating) were fed *ad libitum* on hay (veld) in the morning before allowing them to graze on an open field where *Pennisetum clandestinum* (Kikuyu grass) and other grass hay were dominant. This study was conducted in autumn (March – May, 2009).

3.2.3 Inoculum collection and preparation

For enzyme assays, a modified procedure previously described by Smith et al. (1974) was employed when collecting rumen samples from fistulated cows. Rumen fluid (200 ml) was collected from both cows at the same time through a fistula before feeding in the morning, strained through four layers of cheese cloth (pre-warmed and flushed with CO_2) and treated immediately with 150 µl of phyenylmethylsulfonyl fluoride (0.1 mM PMSF) to inhibit proteases from lysing enzymes of interest (Tilley and Terry, 1963). Faecal suspensions (faecal fluid) were made by diluting 150 g of faeces (collected before feeding from the rectum) into 150 ml of homogenization buffer (50 mM sodium-acetate buffer, 0.02% (m/v) NaN_3, and 0.1

mM EDTA at pH 5.0) before straining through four layers of cheese cloth. It was treated with 150 µl of 0.1 mM PMSF before being transported in an airtight thermo flask maintained at 38ºC to the laboratory.

For *in vitro* digestibility studies, rumen fluid (RF) and faecal fluid (FF) for fresh incubations (FI) were collected as described for enzyme assays in the previous paragraph but for the exclusion of PMSF as well as replacing homogenization buffer with salivary buffer (buffer C) for faecal inoculum. Buffer C was made by titrating 2*l* of warmed solution A (solution A, 19.60, 7.40, 1.14, 0.94 and 0.26 g of NaHCO3, Na2HPO4, KCl and MgCl.6H2O, respectively in 2 L distilled water) with 2 ml of Solution B (5.3 g CaCl2.2H2O in 100 mL distilled water) just before use while continuously stirring to form a complete buffer solution. 5.8 g of ammonium sulphate was dissolved in buffer C as a nitrogen supplier (Tilley and Terry, 1963). Buffer C was flushed with CO2 till the whitish solution became clear. Carbon dioxide was generated in the airtight thermo flask while in the field by reacting calcium carbonate with sulphuric acid to maintain anaerobiosis.

3.2.4 Rumen fluid and faecal fluid culturing

Rumen fluid or FF for laboratory culturing was collected as described in section 3.2.3. Rumen fluid or faecal filtrate (198 ml) was pipetted into 402 ml of salivary buffer (flushed with CO_2 and placed in an incubator to equilibrate to 39°C for 1 h) containing 6 g lucerne and maize stover (1:1), which had been milled through a 1-mm sieve. The culture samples were flushed with CO_2 and incubated for 72 h at 38.5^{o}C in airtight Duran® bottles (4 L). After 72 h, cultured RF or FF were used as inocula for *in vitro* cultured incubations (CI) as well as analysed for cellulase activities.

3.2.5 Crude Protein extraction

Protein isolation was done using a slightly modified procedure described by Henry et al. (1975). Both RF and FF (100 ml) were used for protein isolation. Sample solutions in sealed centrifuge tubes were placed on a shaker for 30 min to facilitate bacteria detachment from

fibres before centrifuging (6500x*g*, 30min at 4°C) to sediment particulate matter. The supernatant was centrifuged (30 000x*g*, 15 min at 4°C) to sediment bacteria cells. The sedimented particles and bacterial cells were dissolved in 10 ml and 5 ml of homogenization buffer respectively, sonicated (to lyse bacteria cells) and centrifuged (30 000x*g*, 15 min at 4°C). The supernatants of the three different steps were pooled (Figure 3.1) and centrifuged (30 000x*g*, 15 min at 4°C) to sediment any unlysed cells. Ammonium sulphate (60% (m/v) $(NH_4)_2SO_4$) was dissolved in the sample solution to facilitate protein precipitation before centrifuging (7000x*g*, 15 min at 4°C). The precipitate was dissolved in 10 ml of storage buffer (20 mM sodium acetate, 0.02 % (m/v) NaN_3, and 0.1 mM EDTA at pH 5.0) before dialyzing.

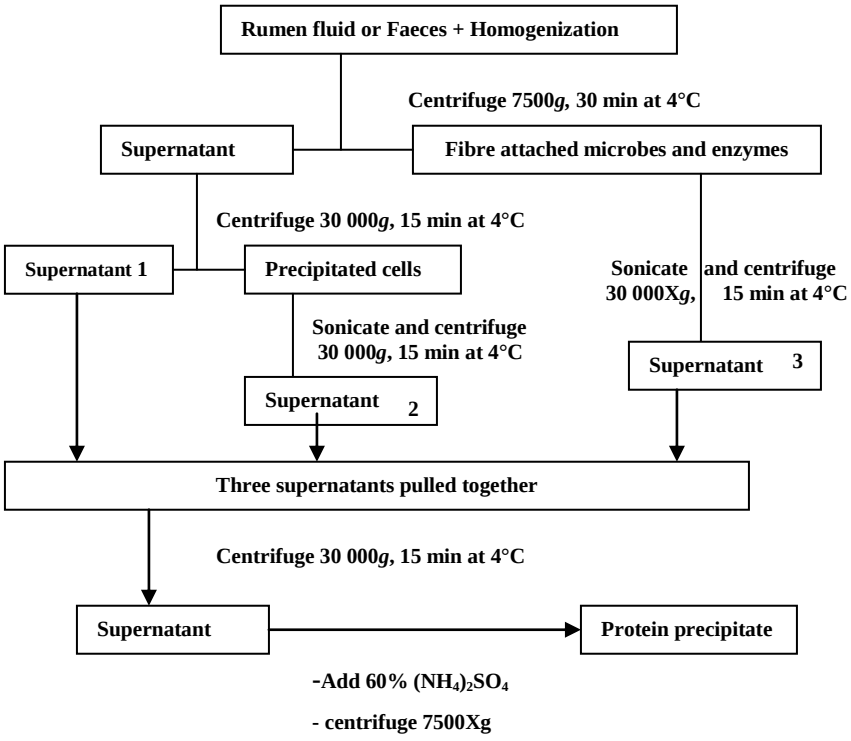


Figure 3.1 A flow chart for the precipitation of cellulase and hemicellulase from rumen fluid and faeces

3.2.6 Protein dialysis

Each sample solution was pipetted into a dialysis membrane and immersed in a 2*l* storage buffer solution overnight (12 h). Dialyzed sample solutions were concentrated using polyethylene glycol 20 000.

3.2.7 Determination of protein concentrations

Bradford (1976) dye-binding assay was used to determine crude protein concentrations. A standard curve for a micro assay was prepared with bovine serum albumin (BSA) concentrations of 0, 5, 10, 20, 30 and 40 µg/100 µl of reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0). Nine hundred microlitres (900 µl) of Bradford reagent (600 mg Coomassie Brilliant Blue G-250 dissolved in 1L of 2% perchloric acid and filtered through Whatman number 1 filter paper) was pipetted onto the standard solution (100 µl) and absorbance read at 595 nm after 3 min. For the unknown protein sample solutions, 5µl was diluted into 100 µl of the reaction buffer before adding Bradford reagent (900µl) and allowed to stand for 3 min. Each assay was performed in triplicates and absorbance was measured at 595 nm. Unknown protein concentrations were determined from the standard curve.

3.2.8 Enzyme assays

3.2.8.1 Exocellulase activity (crystalline cellulase)

Crystalline cellulase activity was assayed following a slightly modified version of the method described by Gerrit et al. (1984) to suit the crude protein assays. Crystalline cellulase activity was measured by pipetting 0.5 ml of 1% (m/v) crystalline cellulose in the reaction buffer into 0.5 ml of crude protein solution obtained from rumen fluid or faeces and incubated for 72 h at 39ºC (standard assay condition). The enzyme reaction was stopped by boiling at 100ºC, following which the reaction mixture was centrifuged (6000xg, 5 min at 4°C) and 400 µl samples analysed for reducing sugars using 3, 5-dinitrosalicylic acid (DNS) method. Each ecosystem was represented by three samples each of which was analysed in triplicates. Specific enzyme activity was measured in µg of glucose/µg protein.

3.2.8.2 Endocellulase (carboxymethyl cellulase) activity

A modified version of the method described by Gerrit et al. (1984) was used to investigate the activity of endocellulase. The reaction mixture contained 0.5 ml of 0.5% (m/v) carboxymethyl cellulose (CMC) in the reaction buffer at pH 5.5 and 0.5 ml of crude enzyme. The enzyme reaction was stopped by boiling at 100ºC after incubating at 39ºC for 2 h. Samples were centrifuged at 6000xg at RT for 5 min, then 400 µl of the reaction mixture was analysed for reducing sugars using DNS method.

3.2.8.3 Hemicellulase (xylanase) activity

A modified procedure described by Khanna (1993) and Seyis and Aksoz (2005) to suit crude protein assays was used to assay xylanase activity. The assay was carried out by pipetting 0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer (pH 5.0) into 0.4 ml of crude protein solution and incubating at 39ºC for 1 h. The reaction was stopped with 100 µl of 200 mM $NaHCO_3$, centrifuged (at 6000xg for 5 min) and analysed for reducing sugars using the DNS method. Each ecosystem was represented by three samples each of which was analysed in triplicates. Specific activities of the above enzymes were defined as µg of xylose/ mg crude protein.

3.2.9 Quantification of reducing sugars

Dinitrosalicylic technique was chosen because of its sensitivity, simplicity and availability. A standard curve of micrograms of reducing sugars (glucose or xylose) against absorbance at 540 nm was plotted as described by Wood and Bhat (1988). Dinitrosalicylic acid reagent, 600 µl (0.001 M sodium metabisulphate, 0.708 M potassium sodium tatrate, 0.25 M sodium hydroxide, and 0.021 M phenol) was pipetted into 400 µl of the sample solution and boiled for 5 min. The reaction mixture was then cooled under running tap water and the absorbance measured at 540 nm. The absorbance values were translated into reducing sugars using the standard curve.

3.2.10 *In vitro* digestibility

3.2.10.1 *In vitro* gas production

The automated gas production method was described by Pell and Schofield (1993), Blummel and Becker (1997) or Ouda (2007) using the computerized pressure transducer system. Maize stover (milled through a 1-mm sieve) was weighed (1 g) and transferred into a 250 ml Duran® bottles containing 67 ml of salivary buffer (buffer C) which had been flushed with CO_2 (to avoid the consumption of initial CO_2 produced from fermentation by the buffer). The substrate-buffer mixture was placed in the incubator to equilibrate to 39°C (for 1 h) before inoculation. Rumen fluid or FF (33 ml) was transferred into Duran® bottles under a stream of CO_2. For FI incubations fresh RF and FF were used while cultured RF and FF were used for CI. After inoculation, the samples were transferred into the incubator and pressure logging was done every 20 min for 72 h at 39°C. The negative control was the same mixture as above with no substrate but the positive control was lucerne and grass hay (1:1) incubated with rumen fluid (to correct for variations observed in repeats if any). Substrate incubation with each inoculum type was replicated three times with five pseudo repeats. The pH of all incubation systems were monitored before and after every experiment. The terminal pressure data was converted to gas volumes (mL) using a predetermined calibration equation (Schofield et al., 1994). *In vitro* gas values were fitted into the exponential equation:

$$GP = \frac{maxp}{1 + e^{[2 + 4C(lt - t)]}} \qquad \text{Eqn. 3.1 (Campos et al., 2004)}$$

$$GP = \frac{A}{1 + \exp[2 + 4a_1 (lt - t)]} + \frac{B}{1 + \exp[2 + 4b_1 (lt - t)]} \qquad \text{Eqn. 3.2 (Soest and Robertson, 1985)}$$

$$PF = TD/maxp \qquad \text{Eqn. 3.3}$$

$$DEF = TD/(T_{1/2} x V_{1/2}) = 2PF/T_{1/2} \qquad \text{Eqn. 3.4 (Ouda and Nsahlai, 2009)}$$

$$\mu = maxp*C$$

where GP is the total gas volume (mL) at time t, maxp is maximum gas production, C is the overall degradable rate, A and B are the gas volume (mL) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, a_1 and b_1 are the degradation rates (h^{-1}) for fast and slowly degradable digestion fractions, respectively, and lt is the bacteria colonization or lag time (h). PF= partitioning factor, TD= truly degradable fraction of feed, DEF = degradability efficiency factor, $V_{1/2}$ = half GP, μ = gas produced at the point of inflection and $T_{1/2}$ = time taken to produce half maximum gas.

3.2.10.2 Monitoring whole faecal inoculum activity over time

The procedure was the same as described for gas production in section 3.2.10.1 with a few modifications. The incubation time was changed to 7 d. After every 7 d, the gas was stopped and 33 ml was used to inoculate another system containing fresh feed. This procedure continued at 7 d intervals until day 42.

3.2.10.3 *In vitro* degradability and volatile fatty acids

True degradability was determined according to Tilley and Terry (1963), and modified by Cottyn and Boucque (1968), in which the second stage (pepsin digestion) was substituted with neutral detergent extraction. After 72 h incubation as described for gas production above (section 3.2.10.1), the samples were centrifuged (8000xg, 15 min, 4°C) and the residue dried for 72 h in a fan oven at 70°C. The difference in mass between the incubated material and the residue equals the apparent degraded fraction (APD) of MS. The residue was refluxed in neutral detergent solution and the residue (NDF) dried (fan oven, 70°C for 72h). Truly degraded fraction (TD) was calculated by subtracting the mass of NDF from that of the incubated material. The difference between APD and TD was recorded as microbial yield.

The filtrate after incubation was prepared for short chain fatty acid analysis (SCFA) as described by Cottyn and Boucque (1968). Five milliliters (5ml) of the filtrate was transferred onto 1 ml of 25% metaphosphoric acid solution and allowed to stand in ice for 30 min before centrifuging (8000xg, 20 min at 4°C). After centrifugation as described above for degradability, the filtrate (2 ml) was filtered through a syringe filter (0.45 µm pore from SUPELCO, USA) into a 2 ml clear ABC Screw Top Vial from SUPELCO, USA. The vials were then transferred into an automatic sampler (HT280 from Brescia, Italy) linked to a Gas Chromatograph (YL6100GC, Young Lin, Korea) for analysis. A temperature-programmed cycle from 90 to 120°C rising by 5°C was applied in the column (BPX70x30x0.25x0.25). The injector block temperature was maintained at 260°C with nitrogen as the carrier gas (60ml/min). Hydrogen and air flow rate to the detector (flame ionization detector) were 60 and 300 ml/min respectively. The injection volume was 1 µl/split 1:80. The theoretical

calculations of CO_2 and methane (CH_4) were estimated as described by Groot et al. (1998) based on the stoichiometric balance of the fermentation of glucose to propionic acid (PP), acetic acid (Acet), butyric acid (nB), iso-butyric acid (iB), CO_2 and methane (Czerkawski, 1978; Van Soest, 1994).

$CO_2 = Acet/2 + PP/4 + 3(nB+iB)/2$ ----------equation 3.5

$CH_4 = Acet + 2(nB+iB) - CO_2$ ----------equation 3.6

3.2.11 Statistical Analysis

In vitro enzyme and digestibility estimates derived from RF and FF inoculum were analysed using the general linear model of SAS (2002) based on the statistical model:

$$Y_{ijk} = \mu + E_i + S_j + (ES)_{ij} + e_{ij}$$

Where Y is the individual observation, μ is the overall mean, E is the effect of the inoculum source, (ES) is the interaction effect of inoculum and pre-incubated inoculum, and e is the random variation.

3.3 Results

3.3.1 Chemical composition of whole maize stover

Chemical analyses of the maize stover showed 5.59% as crude protein content and 87.8% for NDF (Table 3.1). Other values are as shown on Table 3.1.

Table 3.1 Maize stover chemical composition

Nutrient	Whole Maize stover (g/kg)
Dry matter	925
Organic matter	899
Ash	101
Neutral detergent fibre	878
Acid detergent fibre	543
Acid detergent lignin	73
Cellulose	335
Hemicellulose	470
Crude protein	56

3.3.2 Enzyme assays

Exocellulase specific activity differed (P<0.001) between fresh RF and FF. Fresh RF specific activity was higher than that observed in FF (Table 3.2). Cultured RF and FF differed in their exocellulase specific activity (P<0.001). Faecal fluid exocellulase specific activity was higher

Table 3.2 The specific activities of exocellulase, endocellulase and hemicellulase of crude protein extracts from fresh and cultured systems of rumen fluid and faecal suspension

Enzyme source	Hemicellulase Specific activity[1]	Endocellulase specific activity[2]	Exocellulase specific activity[2]
Fresh			
Rumen Fluid	42.91	41.60	23.52
Faecal Fluid	6.98	6.13	15.11
cultured			
Rumen Fluid	8.96	18.65	12.33
Faecal Fluid	18.83	5.65	23.47
SED	2.014	3.123	5.381
P- value	**	**	**

[1] = µg xylose /mg crude protein, [2] = µg glucose/mg crude protein, ** P< 0.001

than that of RF in cultured systems. Endocellulase specific activity differed (P<0.001) in both FI and CI. Endocellulase specific activity of RF was higher than that of FF in both FI and IC. Fresh RF and FF differed (P<0.001) in their hemicellulase specific activities. Rumen fluid specific activity was higher than that observed in FF (Table 3.2). Cultured RF and FF differed (P<0.001) in their hemicellulase specific activity. FF hemicellulase specific activity was higher than that of RF in cultured systems.

3.3.3 *In vitro* digestibility

The fermentation systems (FI and CI) were active and normal as indicated by a small drop in pH after incubations for both FI and CI systems. In the FI systems, the pH drop in RF (7.38-6.62) was slightly higher than that of FF (pH 7.21-6.97) while pH changes between the cultured systems were not significant. The APD of MS did not differ between RF and FF in both FI and CI (Table 3.3a).

Table 3.3a *In vitro* digestibility of Maize stover by *in situ* and cultured rumen or faecal inoculum

Digestibility parameters	Fresh inoculum		Cultured inoculum		SED	P-value
	Rumen Fluid	Faecal Fluid	Rumen Fluid	Faecal Fluid		
APD (g/kg)	524	519	549	549	20.6	ns
TDeg (g/kg)	719	663	745	681	6.3	**
MY (g/kg)	195	144	196	132	29.2	**
GP						
$(ml/g)^{24}$	100	82	60	57	5.1	**
$(ml/g)^{72}$	150	136	144	116	4.2	**
C (h^{-1})	0.027	0.024	0.024	0.028	0.0010	ns
$T_{1/2}$ (h)	21.43	26.59	26.31	24.03	0.720	ns
μ (ml)	4.15	3.15	3.51	3.29	1.213	ns
PF	4.8	4.9	4.2	5.9	0.11	**
DEF	0.46	0.37	0.44	0.50	0.032	ns

* = P< 0.05, ** = P<0.01, ns = non significant, $^{24 \text{ or } 72}$ = Total gas volume (GP) produced after 24 h and 72 h of incubation respectively, nm= not measurable, PF= partitioning factor, DEF = degradability efficiency, $T_{1/2}$= time taken to produced half the total gas, μ = gas at the point of inflection, APD=apparent degradability, TD= True degradability, C= rate of maize stover fermentation, MY = microbial yield.

However, APD was slightly higher in RF than FF of FI, whereas there was no difference between the cultured inocula. True degradability was slightly higher (P<0.001) in RF than FF

in both FI and CI systems. Microbial yield was significantly different (P<0.001) in both FI and CI systems. The highest microbial yield was observed in RF cultured medium. The total gas production (GP) from MS differed (P<0.001) between RF and FF for both FI and CI systems (Table 3.3a). Total gas production from MS was higher in RF than FF for both FI and CI incubations. For the 24 h gas measurement, there was no significant difference between RF and FF in the CI systems. There was no difference in the degradation rate of MS between RF and FF for both FI and CI systems. The time taken to produce half GP ($T_{1/2}$) and the gas produced at the point (hour) of inflection (U) were also not different between RF and FF for both incubation systems. The partitioning factor (PF) differed (P<0.01) between RF and FF. Partitioning factor was highest in cultured FF followed by fresh FF. The lowest PF was observed in cultured RF. Degradability efficiency factor did not differ between RF and FF for both FI and CI systems.

Total SCFA was different (P<0.01) between RF and FF. Total SCFA was highest in FI for RF, followed by FF in CI then RF in CI and lastly FF in FI (Table 3.3b). The Molar proportions of acetic acid (Acet), propionic acid (PP) and n-butyric (nB) acid were different (P<0.01) between RF and FF. Generally, Acet had the highest molar proportions followed by PP and nB in both FI and CI systems. The highest molar proportion of Acet was observed in

Table 3.3b Short chain fatty acid and gas production calculated from VFA

Digestibility parameters	Fresh inoculum		Cultured inoculum		SED	P-value
	Rumen Fluid	Faecal Fluid	Rumen Fluid	Faecal Fluid		
SCFA						
Total (µM)	982	541	570	612	47.7	**
Molar proportion						
Acetic acid	0.732	0.605	0.625	0.609	0.0321	**
Propionic acid	0.204	0.337	0.329	0.37	0.0143	**
n-Butyric acid	0.054	0.056	0.044	0.021	0.0051	**
iso-butyric acid	0.001	nm	nm	nm	0.0020	**
Iso-valeric acid	0.003	0.003	0.001	ns	0.0001	ns
Carbon dioxide	0.497	0.470	0.461	0.428	0.0071	**
Methane	0.342	0.245	0.252	0.222	0.0101	**

** = P<0.01, ns = non significant, SCFA = short chain fatty acids

RF of FI. Propionic acid was highest in FF of CI. Methane and CO_2 productions were higher (P<0.01) in FI than CI systems. The highest methane and CO_2 production were observed in Fresh RF and the lowest in cultured FF.

3.3.4 Gas production kinetics

Gas volumes produced from rapidly degradable fraction (A) of MS differed (P<0.01) between RF and FF for both FI and CI systems while no differences were observed from the gas produced from slowly degradable fraction (B) (Table3.4). The gas produced from B was generally higher than that of A in all incubations. The fermentation rates of A (a_1) were similar between RF and FF for both FI and CI systems. The rate of fermentation of B (b_1) differed (P<0.01) between RF and FF for both FI and CI systems. The fastest b_1 was observed in

Table 3.4 Gas parameters of maize stover when incubated with fresh or cultured inocula

Inoculum	A (ml)	a_1 (h^{-1})	B (ml)	b_1 (h^{-1})	lt (h)
Fresh					
Rumen Fluid	55.6	0.107	94.7	0.028	2.3
Faecal Fluid	45.8	0.095	91.1	0.023	4.6
Cultured					
Rumen Fluid	41.6	0.076	99.4	0.023	4.0
Faecal Fluid	31.1	0.085	85.1	0.027	4.3
SED	0.00	0.0001	3.41	0.0001	0.00
P-value	**	ns	ns	**	***

**=P<0.01, ***=P<0.001, ns= non significant, a and b are the gas volumes (mL) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, a_1 and b_1 are the degradation rates (h^{-1}) for fast and slowly degradable fractions, respectively, and lt is the bacteria colonization or lag time (h).

fresh RF followed by cultured FF and thirdly fresh FF and cultured RF. The lag time (lt) was generally longer (P<0.01) for CI than FI systems. The longest lt was observed in FF for FI and the shortest in RF for FI systems.

3.3.5 Faecal fluid activity over time

Total gas production decreased (P<0.001) with time (Table 3.5). The highest GP was observed after 7 d (191ml) and the least after 21 d. Interestingly, GP at 42 d was higher that that of day 35. The second highest value was recorded on day 14. The overall rate of gas production (C) from MS was highest on day 21 and lowest on day 7. The results showed that the overall rate of MS fermentation increase with time. Apart from day 21, a_1 and b_1 were not different for the different incubation days. The results also showed that lt decreases (P<0.001) with time. The gas produced at the point of inflection (μ) also increased with time. Both A and B from MS fermentation generally decreased over time (P<0.001).

Table 3.5 Gas production from maize stover over a period using faecal inoculum from cow

Days	GP	C	A	a1	B	b1	lt	T1/2	μ
7	191.0	0.020	81.0	0.059	110.0	0.013	10.0	45.0	2.7
14	169.8	0.015	72.5	0.070	97.3	0.013	9.8	43.6	2.5
21	108.2	0.045	25.3	0.124	82.8	0.028	5.4	16.5	4.8
28	122.3	0.025	74.3	0.055	48.0	0.016	7.8	28.2	3.0
35	109.1	0.024	65.0	0.061	44.1	0.016	7.0	28.2	2.5
42	130.0	0.026	77.3	0.063	52.6	0.018	8.5	27.7	3.3
SED	19.80	0.0103	13.71	0.0102	6.34	0.0111	1.21	7.123	0.60
P-value	***	ns	***	ns	***	ns	***	***	ns

***=P<0.001, GP= total gas produced over time, ns= non significant, a and b are the gas volumes (mL) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, C= overall rate of maize stover breakdown, a_1 and b_1 are the degradation rates (h^{-1}) for fast and slowly degradable fractions, respectively, $T_{1/2}$= time taken to produced half the total gas, μ = gas at the point of inflection and lt is the bacteria colonization or lag time (h).

3.4 Discussion

Many studies have shown that FF is a possible substitute for RF but insist on more research to improve on its value (Dixon et al., 1982; Holden, 1999; Mabjeesh et al., 2000; Omed et al., 2000; Mauricio et al., 2001; Mould et al., 2005; Tufarelli et al., 2010). The percentage difference of digestibility parameters obtained when fresh RF was used as an inoculum

compared to fresh FF is still a limiting factor. The time spent on collecting RF or FF for daily incubations is not cost effective. That is why one of the objectives of this study was to preserve whole rumen or faecal inoculum in the laboratory for extended periods using a very simple laboratory technique. The second objective was to investigate the possibilities of substituting fresh RF with fresh or cultured FF for *in vitro* evaluation of forages.

Activities of exocellulase, endocellulase and hemicellulase were higher in FI for RF than FF. This was probably due to the fact that rumen fluid has a higher microbial population in the liquid phase than in the strained faecal inoculum (Mauricio et al., 2001). Although the quantity of crude protein extracted from FF was higher than that of RF, cellulolytic activity remained lower in FI incubations. The higher crude protein in FF was associated with endogenous protein contamination. After culturing FF for 72 h, its cellulolytic (exocellulase and endocellulase) activity was relatively higher than that of cultured RF. This implies that culturing FF with MS increases the initial microbial population, decreases the amount of endogenous contaminants, selects mostly microbes that can survive on MS and allowed for the expression of more active fibrolytic enzymes relative to the type of substrate available. A shorter retention time in the hindgut of cows has always been associated with lower fermentation (Hidayat et al., 1993). However, the rate at which fermentation occurs in the hindgut is unresolved although a comparative study on bovine rumen fluid and faeces by Mauricio et al. (2001) showed higher rates of gas production in RF than FF. If the retention time is very short in the hindgut of ruminants, the microbes that inhabit this chamber must be able to harness energy from the fast moving fibrous feed as fast as possible in order to survive. Therefore, it is possible that hindgut cellulolytic microbes have a higher fibrolytic potential but limited by their numbers as demonstrated by our results when FI was compared with CI systems. Laboratory cultured FF can be a better substitute to fresh RF compared to fresh FF as demonstrated by its exocellulase activity. The difference in exocellulase specific activity between fresh RF and cultured FF (0.4%) was smaller compared to the difference between fresh RF and fresh FF (33%).

Apparent degradability did not differ between RF and FF incubations while TD differed. True degradability correlation between RF and FF was relatively high ($r^2 = 0.71$) for FI incubations. Culturing both inoculum increased TD for both RF and FF by 2.6% and 1.6%, respectively.

This increment in TD was associated to high fibrolytic microbial population influenced by the culturing conditions (high fibre and lucerne providing proteins to boost microbial growth) (Hobson, 1971; Kern et al., 1974; Mauricio et al., 2001). Interestingly, culturing slightly decreased the disparity observed between RF and FF in FI and CI from 4.5 to 4.0%. However, this was contrary to the results obtained by Akhter et al. (1995) who reported a 40% deviation of TD between RF and FF. The difference in TD between fresh RF and cultured FF (7%) is much smaller when compared to the difference between fresh RF and fresh FF (14%), suggesting that, laboratory culturing of FF may reduce the error margin when using FF as a substitute for fresh RF than fresh FF.

Total gas production was generally higher in RF than FF in both FI (Kern et al., 1974) and CI incubations. Although less microbial diversity and absence of protozoa in FF has been associated with low and slower rate of gas production (Hidayat et al., 1993), it is likely that low fibrolytic microbial population initially also plays a major role (Nsahlai and Umunna, 1996; Cone et al., 2002). The GP produced from MS fermentation by RF or FF was higher in the FI systems than CI systems unlike TD which increased in CI systems. The decrease in GP and an increase in TD imply conversion of energy lost in the form of gas into other metabolic substrates such as PP, nB or Acet as shown by the results. The difference in GP produced between RF and FF in the FI incubations after 24 and 74 h were 10.4 and 3.7%, respectively. This implies that fermentation was much slower in the FF system initially but gradually increases as the population of microbes increases with time (Váradyová et al., 2005). These results were similar to those obtained from studies comparing rumen and faecal inoculum in sheep (Grings et al., 2005; Kiran and Krishnamoorthy, 2007). For the CI systems, the difference in GP production between RF and FF at 24 h was much smaller (2.5%) than observed in the FI systems (10.4%). This was linked to an increase in fibrolytic microbial population after culturing, hence increasing the rate of fermentation. The results were similar to that obtained by Rymer and Givens (2000) where FF was pre-incubated overnight before incubation to enrich initial microbial number. They concluded that GP production profile parameters produced by RF can be extrapolated from FF gas production profile. Therefore, the lower fermentation in the hindgut may be associated to low microbial populations, poorer feed

quality, shorter retention time and the absence of protozoa (Blümmel and Lebzien, 2001; Kiran and Krishnamoorthy, 2007) rather than the fibrolytic efficiency of the microbes present.

Although the overall rate of fermentation of MS was not statistically different between RF and FF for both FI and CI system, a small increase in C for FF in CI was noticeable. This implies that the C was enhanced by laboratory culturing. C from FF in CI was 5% higher than that of RF in FI giving more evidence of substituting RF with cultured FF than fresh FF (C for FF was 12% less than that of RF in FI). The gas produced at the point of inflection (U) showed no differences upon analysis. This suggested their similarities in microbial activity after establishment. Therefore, culturing may improve microbial population as well as eliminates dietary differences often observed between the rumen and hindgut. Partitioning factor and DEF were highest for FF in CI systems compared to all the other inocula (RF in CI or RF and FF in FI). This is an indication of a higher efficiency of microbial protein synthesis as demonstrated by other researchers (Jackson et al., 2010; Krishnamoorthy and Robinson, 2010). High PF and DEF showed that FF can generate data that are good enough in formulating ruminant feeds (Váradyová et al., 2005). The high PF for FF in CI shows that culturing improves the fibrolytic competence of cultured inoculum, by concentrating microbes that can digest MS hence increasing fibrolytic microbial population as well as the overall fermentation rate. High PF has also been associated with high feed intake although other studies (Mauricio et al., 2001) have shown that it is not a good parameter to be used solely in feed formulation. The total SCFA difference between fresh RF and cultured FF (37%) is much smaller when compared to the deference observed between fresh RF and fresh FF (48%). This suggests that culturing FF improves MS fermentation. The highest molar proportion of propionic acid was observed in CI systems for FF. This was due to its low GP (CO_2 and methane) and high TD. This implies that acetate: propionate was higher than in all other systems.

There were no differences in the gas produced from A or B between RF and FF in the FI incubations. Therefore, using gas parameters from FF to estimate digestibility is plausible. Váradyová et al. (2005) were not able to establish strong relationship between RF and FF gas parameters as shown in this study. However, the rate at which A or B was fermented was higher for RF than FF in FI incubations. These results were similar to those carried on sheep

by Mauricio et al. (2001). Culturing the inocula saw the production of higher gas from B by RF than FF which surprisingly had a higher fermentation rate. This implies that MS was converted to other metabolic substrates apart from gas. This is an indication that culturing faecal microbes under suitable anaerobic conditions before application as an inoculum helps to improve on initial microbial population, microbial specificity and fermentation rate. It is positive to think that microbes in the hindgut can be very effective in fermenting high roughage feeds at a higher rate but are limited by their initial microbial numbers during *in vitro* incubation. This is because passage rate at the hindgut might be relatively high; therefore microbes have a very limited time to extract nutrients so as to survive in that ecosystem. The lt for FF was longer than that of RF in both FI and CI incubations. However, lt for RF was highly correlated (r^2 = 0.8) to that of FF in cultured incubations. This implies that culturing decreases microbial colonization time.

Monitoring FF microbial activities in the laboratory over a period showed that FF inoculum was active for 42 d as demonstrated by GP measurements. Generally, GP decreases over time. Total gas production was highest after week 1 but decreased by 11%, 43%, 36%, 43% and 31% for week 2, 3, 4 and 5 respectively. Decreased GP has been associated to low a microbial activity which implies that FF fermentation potential decreases over time. However, this is not conclusive as TD increases with decreased GP as seen in CI systems after 72 h. This suggests that the microbial systems may still be efficient in hydrolyzing carbohydrates into metabolites (SCFA) other than gases. The gas parameters, C, a1, b1 and lt were not significantly different between the different weeks. Therefore, laboratory culturing of rumen microbes under suitable condition would minimize the error margin often observed in fresh FF.

3.5 Conclusion

Faecal inoculum is a potential substitute of rumen inoculum for *in vitro* feed evaluation as demonstrated by the small differences observed in their true digestibility and gas production. Faecal inoculum is a better substitute when cultured before application as demonstrated by the smaller differences observed in their degradability parameters. Culturing whole rumen fluid or faecal fluid was successful for three days with an increase in fermentation rate and less than

5% loss in activity. Monitoring inoculum activities over time shows that whole FF was active for six weeks with week 1 being the best inoculum source for feed evaluation. However, the lag time still remains a major problem to resolve as it did increase slightly with culturing. The drop in GP and an increased in TD observed in cultured ecosystems demands for more research on the microbial variety which can possible play a vital role as a feed additive in animal production.

Chapter 4

The fibrolytic activity of microbial ecosystems in three hindgut fermenters

Abstract

This study evaluated the fibrolytic competence of microbial ecosystems in faeces collected fresh from three hindgut fermenters (miniature horse (mH), horse (H) and Zebra (ZB)) in summer and winter grazing in their natural environment. The fresh faecal inocula were cultured in the laboratory on maize stover (MS) and lucerne (1:1) with salivary buffer for 72 h at 38°C before being used as inocula or for crude protein extraction. Fresh faecal sample collection was replicated thrice with five pseudo repeats for each herbivore. Crude proteins were precipitated from cultured faecal inocula by 60% ammonium sulphate and analysed for exocellulase, endocellulase and hemicellulase specific activities (µg reducing sugar/mg crude protein). *In vitro* fermentation was done by transferring 33 ml of laboratory cultured faecal inoculum into 67 ml of salivary buffer containing 1g maize stover (MS) and incubating for 72 h at 38°C. After incubation, MS degradability and fermentation characteristics were determined. Endocellulase and hemicellulase activities differed ($P<0.001$) among animal types and between seasons. The H was the most active endocellulytic system in both seasons with the highest activity observed in winter. Hemicellulase activity was most active in the ZB system in both seasons. Exocellulase activity differed ($P<0.001$) among animal types and between seasons. The most active exocellulase system was that of the ZB in both season followed by H and then mH. The maximum gas produced (GPmax) were similar among system and between seasons. True degradability, partitioning factor and microbial yield were highest in summer for ZB (841 g/kg, 9.10, 341 g/kg respectively) compared to H (787 g/kg, 7.80, 280 g/kg respectively) and mH (816 g/kg, 7.53, 276 g/kg respectively). Total VFA was highest ($P<0.05$) in winter for ZB but lowest in summer when compared to H and mH. Methane and CO_2 were not significantly different among animal types but was shown to be relatively lower especially in summer for ZB than in H and mH. Although these herbivores are all hindgut fermenters they harbour microbes of varied fibrolytic competence with the ZB being the most active, followed by H and lastly mH. This implies that ZB be harbouring microbes that have evolved through millions of years of selection to become more competent in fibre breakdown. Application of microbes or enzymes from ZB as feed additives to domesticated ruminants can be very important in improving forage utilization.

4.1 Introduction

In tropical countries, grazing pastures of higher quality are more readily available in summer (rainy season) than in winter (dry season) (McNaughton, 1985). In winter, animals suffer severe nutritional stresses due to the poor nutritional value of pastures (Leng, 1990; Hindrichsen et al., 2004) and shortages in supply (Elliott and Folkersten, 1961). During this period of nutritional shortage, animals waste a lot of energy as they have to walk long distances in search of high quality forages and water. As a result of these adverse conditions and nutritional deficiency, animals lose weight (Poppi and McLennan, 1995), body condition; their milk yield and conception rates are low; and increased calf mortalities are also prominent.

Beside quality concerns, winter is also a period when feed is scarce yet fibrous crop residues (e.g. maize stover) and agricultural by products are abundant (Ben Salem and Smith, 2008). The question one could possibly ask, is, how can there be forage shortages with plentiful availability of crop residues? Many farmers are still ignorant of the potential of crop residues hence they are being underused in many localities. However, in regions where crop residues are being exploited, livestock producers are still faced with the daunting task of lower digestibility (Ben Salem and Smith, 2008). The high fibre content of crop residues limits the amount of energy harvested by herbivores per kilogram. The difficulties associated with crop residues breakdown tend to reduce the quantity of available feed during the dry periods.

Different approaches for improving this high fibre forage digestion have been proposed and tested, including components of cellulase enzyme systems (Gerrit et al., 1984; Owolabi et al., 1988; Xiao et al., 2000), unfractionated cellulase systems (Ali and Tirta, 2001), pure cultures of cellulolytic micro-organisms (Seyis and Aksoz, 2005) and mixed cultures of cellulolytic micro-organisms. Supplementing diets with fibre-degrading enzymes improved milk production in dairy cows and feed efficiency in growing cattle (Beauchemin et al., 1995; Beauchemin et al., 2003a). The utilization of these enzymes as feed additives is yet to be elucidated as only a minimal increase in fibre hydrolysis has been reported (Beauchemin et al., 2003a). In addition, enzymes are expensive and difficult to manage since they are susceptible

to lyses by ruminal proteases. Strategies to enhance the herbivores ability to utilize crop residues will contribute to improve livestock production especially in winter. One strategy is to fish for microbial ecosystems with a higher potential of improving the digestion of high fibre forages. This study evaluated the fibrolytic competence of microbial ecosystems in faeces collected from three hind gut fermenters (miniature horse (mH), horse (H) and Zebra (ZB)) grazing in their natural environment in summer and winter. The hypothesis was that fibrolytic activity is similar among the three hind gut fermenters in winter and summer.

4.2 Materials and Methods

4.2.1 Materials

The substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany) and xylan from beech wood (high grade) from SIGMA (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose and D-(+)-glucose were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) from MERCK Laboratory supplies, South Africa and 3,5-Dinitrosalicylic acid from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide and ethylene diamine tetra acetic acid (EDTA) were bought locally from Capital Supplies, South Africa. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) was from Sigma-Aldrich. All spectral scans were carried out with Virian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd).

4.2.2 Chemical composition of maize stover

The chemical composition of MS was analysed as previously described in chapter 3 (section 3.2.1).

4.2.3 Experimental animals and nutrition

Samples were collected from animals with no preference to sex in December (summer) and in May (winter). *Equus caballus* (horse) and miniature horse were from the Ukilinga Research farm, University of KwaZulu-Natal, Pietermaritzburg. *Equus quagga boehmi* (zebra) was made available by the Tala Game Reserve, uMbumbulu, KwaZulu-Natal (SA). The zebra was grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) standing hay and other fibres were dominant. At the Ukulinga research farm, horse and miniature horse were also grazing in fields with Kikuyu grass, standing hay and other fibrous forages.

4.2.4 Inoculum collection and preparation for sampling

For enzyme assays, a modified procedure previously described by Smith et al. (1974) was employed for faecal fluid collection from the three study species (miniature horse (mH), horse (H) and Zebra (ZB)). Faecal fluid collection and sample preparations were carried out as previously described in Chapter 3 (section 3.2.3**)**.

4.2.5 Faecal fluid culturing

Faecal fluid from mH, H and ZB were cultured in the laboratory for 72 h on MS and lucerne (1:1) in salivary buffer (anaerobic) before application as inocula for *in vitro* feed evaluation. This was done as previously described in chapter 3 (Section 3.2.4). The activity of microbial ecosystems was determined by monitoring pH changes.

4.2.6 Crude protein extraction

Cultured inocula from mH, H and ZB were used to extract and evaluate crude proteins for exocellulase, endocellulase and hemicellulase specific activity. Crude protein (CPZ) samples for enzyme assays were extracted from cultured inocula and precipitated using 60% ammonium sulphate as described in Chapter 3 (section 3.2.4). Crude protein samples were dialyzed overnight (as described in section 3.2.6) and concentrated before determining the

protein concentration of each sample (see section 3.2.7). Crude protein extraction was replicated thrice with five pseudo repeats.

4.2.7 Enzyme assays

Exocellulase, endocellulase and hemicellulase specific activities were determined by incubating with crystalline cellulose, carboxymethyl cellulose and xylan, respectively, as described in Chapter 3 (section 3.2.8). Reducing sugars liberated from the different enzyme assays were measured as previously described by Miller (1959) (section 3.2.9). Each ecosystem was represented by three replicates each of which was analysed in triplicates. Enzyme specific active was defined as the amount of reducing sugar per mg of CPZ (µg reducing sugar/mg).

4.2.8 *In vitro* digestibility

4.2.8.1 *In vitro* gas production

The automated gas production method based computerized pressure transducer system described by Pell and Schofield (1993) was used as previously described in chapter 3 (section 3.2.10.1). Inocula were from cultured microbial ecosystems of mH, H and ZB. Substrate incubation with each inoculum type was replicated three times with five pseudo repeats. The maximum gas production (GPmax) and the rate (C) of gas production were measured by fitting the raw data to the model described by Campos et al. (2004) and Schofield et al. (1994).

4.2.8.2 *In vitro* degradability and VFA determination

In vitro degradability was determined as previously described in section 3.2.10.2 but for the inocula which were from cultured mH, H and ZB. Apparent (APD), true degradability (TD), microbial yield (MY), total SCFA, CO_2 and CH_4 were the parameters measured in this experiment. The partitioning factor (PF=mg of substrate digestibility divided by the volume of

gas) and degradability efficiency (DEF=the rapidity at which feed is degraded to make nutrients available and the extent to which the degraded material is fermented) were calculated from the fermentation parameters. Substrate incubation with each inoculum type was replicated three times with five pseudo repeats.

4.2.9 Statistical analysis

SAS software was used to perform statistical analysis (SAS, 2002). Samples were analysed to determine the effects of season and microbial ecosystem using the general linear model (GLM) procedure. Means were compared by least significant difference.

$$Y_{ijk} = \mu + E_i + S_j + (ES)_{ij} + e_{ijk}$$

Where Y is the observation, μ is the overall mean, E is the effect of the inoculum source, S is the seasonal effect, ES is the interaction between inoculum source and season, and e is the random variation.

4.3 Results

4.3.1 pH of microbial ecosystems

The pH of the initial microbial incubation systems was higher ($P<0.001$) than values observed after incubation. The mH had the highest drop in pH while the ZB showed the least drop in pH (Figure 4.1). The results also showed that there was a small drop in pH in negative control systems.

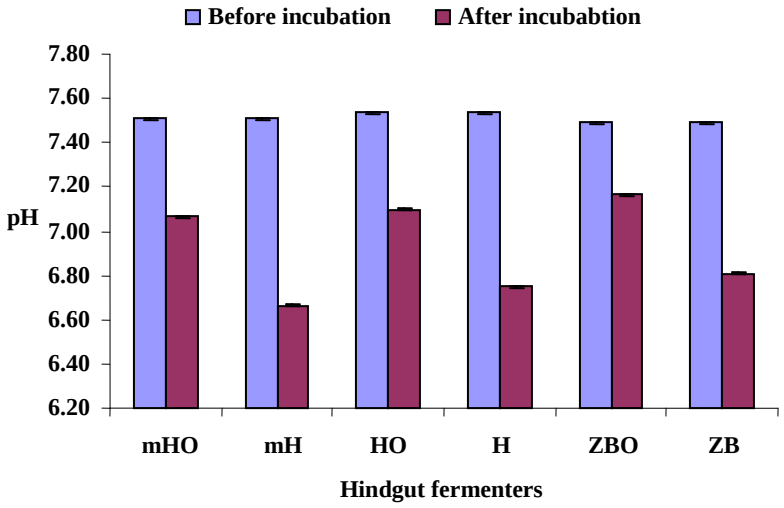


Figure 4.1 Variation in *in vitro* pH in three hindgut microbial ecosystem before and after incubation. mH = miniature horse inoculum with MS, mHO = miniature horse inoculum without MS, HO = horse inoculum without MS, H = horse inoculum with MS, ZBO = zebra inoculum without MS, ZB = Zebra inoculum with MS.

4.3.2 Enzyme assays

Endocellulase specific activity differed (P<0.001) among animal types and between season. The horse was the most active endocellulase system in both seasons with the highest specific activity observed in winter (Table 4.1). Endocellulase specific activity was higher in mH than

Table 4.1 Effect of source of inoculum, animal type and season on enzyme activities from three hindgut fermenters

Enzyme specific activity	Animal type	Season		*P*-value		SED
		summer	winter	Animal	Season	
Endocellulase (µg glucose/mg)	mH	82.6	172.9			
	H	99.5	215.2	0.001	0.001	2.67
	ZB	94.5	152.6			
Hemicellulase (µg xylose/mg)	mH	384.1	121.3			
	H	411.5	119.9	0.001	0.001	6.06
	ZB	455.1	127.3			
Exocellulase (µg glucose/mg)	mH	37.1	74.3			
	H	43.7	87.5	0.002	0.001	3.08
	ZB	56.7	113.4			

H=horse, mH= miniature horse and ZB= zebra

ZB in winter but lower in summer. Hemicellulase activity also varied (P<0.001) among animal types and between seasons. The ZB was the most active hemicellulolytic system in both seasons (Table 4.1). In summer hemicellulase activity was higher in H than mH but showed no difference in winter. The most active (P<0.001) exocellulase system was that of the ZB (Table 4.1) in both seasons while mH was the least active among the three enzyme systems.

4.3.3 *In vitro* degradability

Apparent degradability differed (P<0.01) among animal types but not between seasons. True digestibility differed (P<0.0001) between seasons and among the animal types. True degradability was generally higher in summer than winter (Table 4.2). True degradability was highest in the ZB (841.30 g/kg) microbial system in summer while that of mH (816.60 g/kg) was relatively high compared to H (787.20 g/kg) in the same season. True degradability was also highest for ZB in winter than in mH and H. The maximum gas production (GPmax) was statistically not different among animal types but differed (P<0.004) between seasons. However, the results show that GPmax from mH and H were relatively higher than that of ZB in both seasons. Generally, the rate (C) of MS fermentation increased (P<0.01) from summer to winter. There were no differences in C among animal types within a season. The rate of fermentation tended to be higher in H and ZB compared to H in both seasons. The time taken to produce half GPmax ($T_{1/2}$) was not different among animal types within and between seasons. The time taken to produce half GPmax was relatively shorter for H in both seasons compared to mH and ZB. There was no difference in the gas produced at the point of inflection (U) among animal types within a season but differed between seasons.

The partitioning factor (PF) and degradability efficiency (DEF) differed among animal types (P<0.01 and P<0.001 respectively) and between season (P<0.001 and P<0.01 respectively). Partitioning factor and DEF were highest for ZB followed in order by H and mH in both seasons. Microbial yield (MY) was generally higher in summer than in winter (Table 4.2). Microbial yield varied (P<0.05) among animal species and between season. Microbial yield was highest for ZB in both seasons compared to H and mH. Total SCFA varied (P<0.05)

Table 4.2 Effect of source of inoculum, animal type and season on *in vitro* digestibility of maize stover and gas production

DM Digestibility	Animal type	Season		*P*-value		
		summer	winter	Species	Season	SED
Apparent Digestibility (g/kg)	mH	540	370			
	H	507	353	0.003	NS	10.8
	ZB	500	408			
True Digestibility (g/kg)	mH	817	603			
	H	787	588	0.005	0.005	122.1
	ZB	841	607			
Microbial yield (g/kg)	mH	276	233			
	H	281	235	0.001	0.001	106.2
	ZB	342	199			
GPmax volume (ml/mg)	mH	108.5	103.1			
	H	101.1	95.9	NS	0.004	2.97
	ZB	92.5	90.6			
C (h^{-1})	mH	0.027	0.034			
	H	0.030	0.042	NS	0.01	0.0101
	ZB	0.028	0.037			
$T_{1/2}$(h)	mH	21.0	22.6			
	H	19.31	17.9	NS	NS	0.412
	ZB	23.57	21.7			
U (ml/h)	mH	3.05	3.4			
	H	3.06	3.9	NS	0.02	0.521
	ZB	2.31	3.3			
PF	mH	7.53	5.9			
	H	7.80	6.2	0.007	0.001	1.09
	ZB	9.10	6.8			
DEF	mH	0.13	0.11			
	H	0.15	0.13	0.001	0.01	0.022
	ZB	0.19	0.15			

mH=miniature horse, ZB=zebra, H=horse, PF=partitioning factor, DEF=degradability efficiency, U=gas at point of inflection, $T_{1/2}$= time taken to produce half total gas , C=rate of MS breakdown

between season and among experimental animals within seasons. Generally, total SCFA increased from summer to winter. Total SCFA was highest in ZB when compared to H and mH in winter. Seasonal differences were significant (P<0.001) for molar proportions of SCFA but not significant among animal types within a season. Acetic acid was the highest SCFA produced in both seasons followed by propionic acid and lastly N-butyric acid. Propionic acid molar ratios were slightly higher ZB in winter. Methane and CO2 gases differed between seasons but not significantly different among animal types within seasons.

Table 4.3 Effect of source of inoculum, animal type and season on short chain fatty acids and gas production from maize stover fermentation

Short chain fatty acids	Animal type	Season		*P*-value		
		summer	winter	Species	Season	SED
Total SCFA (mM)	mH	34.1	69.2			
	H	51.7	65.0	0.0001	0.0001	13.27
	ZB	28.6	74.4			
Acetic acid (molar proportion)	mH	0.53	0.58			
	H	0.58	0.53	NS	0.003	0.027
	ZB	0.50	0.60			
Propionic acid (molar proportion)	mH	0.41	0.37			
	H	0.38	0.40	NS	0.0001	0.041
	ZB	0.47	0.33			
N-butyric acid (molar proportion)	mH	0.05	0.05			
	H	0.04	0.07	NS	0.0001	0.0015
	ZB	0.03	0.06			
CO2 (molar proportion)	mH	0.45	0.46			
	H	0.45	0.47	NS	0.0001	0.022
	ZB	0.41	0.48			
Methane (molar proportion)	mH	0.19	0.22			
	H	0.22	0.20	NS	0.0001	0.031
	ZB	0.15	0.25			

4.3.4 Gas production kinetics

No seasonal differences were observed from A and B whereas animal type differences ($P<0.05$) were observed. A was highest in H compared to mH and ZB in both seasons (Table 4.4). The least amount of B was observed in H and the highest in mH for both seasons. There were no differences in the fermentation rate of A (a_1) and B (b_1) between seasons and among animal types. The lag time (lt) was not different among animal types but differed ($P<0.001$) between seasons. The lag time increases from summer to winter. The rate at which all three animals fermented B was very similar as demonstrated by the values of b_1 in both seasons.

Table 4.4 Effect of source of inoculum, animal type and season on mean gas production kinetics of maize stover fermented by faecal fluid from three hindgut fermenters

Digestibility parameters	Inoculum source	Season		*P*-value		
		summer	winter	Animal	Season	SED
A (ml/g)	mH	43.0	38.1			
	H	49.2	51.8	0.01	NS	1.77
	ZB	31.8	41.7			
a_1 (h^{-1})	mH	0.131	0.168			
	H	0.098	0.097	NS	NS	0.0060
	ZB	0.150	0.145			
B (ml/g)	mH	65.5	64.9			
	H	52.1	44.0	0.05	NS	3.99
	ZB	60.7	52.1			
b_1 (h^{-1})	mH	0.027	0.028			
	H	0.025	0.029	NS	NS	0.0021
	ZB	0.024	0.036			
lt (h)	mH	1.8	5.9			
	H	1.9	5.3	NS	0.001	2.11
	ZB	3.1	4.5			

A and B are the gas volumes (mL) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, a_1 and b_1 are the fractional degradation rates (h^{-1}) for A and B, respectively, and lt is the bacteria colonization or lag time (h).

4.4 Discussion

All the *in vitro* incubation systems were active as demonstrated by the small drop in pH. A drop in pH is an indication of the accumulation of fermentation products such as SCFA (Russell and Wilson, 1996; Tripathi et al., 2004) and other acidic metabolites, which contributes in decreasing the pH. The drop in pH for ZB was slightly smaller than that of mH and H which suggest that it might be harbouring microbes with a higher buffering capacity.

Exocellulolytic enzyme specific activities demonstrated that the ZB had the highest ability to digest high roughage diets when compared to that of mH and H in winter and summer. Enzyme activities have been found to be influenced by the type of microbes, evolutionary stage of microbes, forage type, geographical location and climate (Gonçalves and Borba, 1996). The high exocellulase activity of ZB was most likely due to microbes that have evolved to digest fibres more efficiently rather than the forage type since they were grazing on similar pastures. Besides, all inocula were cultured in the laboratory using MS and Lucerne as the

main substrates for 72 h before applying the inoculum so as to minimize the effect of feed type. This was more evident when exocellulase activity of H was observed to be higher than that of mH grazing on the same field. A similar study showed that buffalo and cattle raised under similar condition exhibited a diverse rumen ecology (Wanapat, 2001).

Hemicellulase activity was slightly higher in ZB than in mH and H for both seasons. Generally, hemicellulase activity was higher in summer than winter. This was probably influenced by the relative abundance of hemicellulose in summer than in winter (Varga and Kolver, 1997; Stock, 1999) which might have increased the population of substrate specific microbes. In winter most grazing pastures are mature and their cellulose content increases (Kruse et al., 2008) compared to hemicellulose. The H ability to hydrolyse CMC more than ZB and mH in both seasons was probably due to the presence of microbes that might have evolved in their ability to hydrolyse amorphous cellulose due to high solubles especially in summer. Furthermore, hydrolyzing crystalline cellulose even in winter requires the use of endocellulase since fibre hydrolysis is a stepwise process (Woodward, 1991; Bayer et al., 1994; Reverbel-Leroy et al., 1997; Bayer et al., 1998a; Shoham et al., 1999; Ye et al., 2001; Lynd et al., 2002; Murashima et al., 2002) that requires the use of exocellulase, endocellulase and cellobiase. Therefore, high endocellulase activities are definitely required in both seasons. The results from enzyme assays show that, ZB is the most active system followed in order by the H and mH in fibre hydrolysis.

Microbial responses to artificial substrates (crystalline cellulose, CMC and xylan) incubations are sometimes slightly different from that of natural substrates (Deacon, 1997). This is why *in vitro* dry matter degradability was also carried out with natural substrates (MS) so as to mimic the *in vivo* natural systems and digestibility parameters measured. Apparent degradability was higher for mH and H than ZB in summer but did not differ among animals in winter. This difference is probable due to the accumulation of microbial matter relatively to the abundance of the availability nutrients in summer. True degradability was highest for ZB in summer but was equal to that of the mH in winter. The high TD observed in ZB was expected as previously demonstrated by its exocellulase activity. This provided more evidence that it might be harbouring microbes that have evolved with superior fibrolytic activity. Although

the type of feed on which the animal were grazing on can influence microbial activity (Cone et al., 1999; Stock, 1999), its effect among the animal types in this study was considered minimal but for microbial evolution since all inocula were cultured in the laboratory for 72 h using the same feed prior to application. True degradability was generally higher in summer than in winter as opposed to exocellulase activity which was higher in winter than summer. Summers are characterized by the availability of nutrients in low fibre diets that promote the rapid growth of microbes (Leng, 1990). Therefore, the high TD in summer might not have been affected only by the type of substrate but also by the availability of microbial nutrient (Leng, 1990), initial microbial population mixture with varied microbial fibrolytic potential and their evolution over time. Since fibre hydrolysis is a steps wise process, yielding soluble short chain polysaccharides, opportunistic microbes which are abundant and are not able to hydrolyse crystalline cellulose will feed on these products hence increasing TD in summer.

Interestingly, the GPmax produced by ZB (with the highest TD and exocellulase specific activity) was the lowest in both seasons when compared to that of mH and H. If low gas is an indicator of low digestibility, therefore this result contradicts the results obtained from TD and exocellulase activity which demands for an explanation. This is probably due to the fact that the ZB microbial ecosystem may be harbouring different microbial species (Gonçalves and Borba, 1996), with the ability to conserve energy by fermenting MS into energy rich substrates such as SCFA (e.g. propionic acid) and microbial matter than gases (CO2 and CH4) (Beauchemin et al., 2008). Consequently, the ZB system had low SCFA but more microbial matter in summer, and more SCFA and lower microbial matter in winter than in mH and H. Total SCFA was higher in winter than in summer emphasizing the effect of high fibre feeds on cellulase activity.

Zebra in summer favours lower acetate to propionate ratio hence decreasing methane production (Beauchemin et al., 2008). Horse and mH favoured higher acetate to propionate ratio hence increasing methane production. This was confirmed by the high molar proportion of methane in H and mH in summer. Therefore, this could be one of the possible explanations for the higher GPmax observed in H and mH compared to ZB. The rates of GP were relatively the same among animal types. The similar $T_{1/2}$ among animal types and between seasons is an

indication that culturing before incubation minimizes the error obtained between the growth rate of gas curves. This similarity can be explained by an increase in initial microbial population which minimizes the error often caused by variation in initial microbial population at the beginning of fermentation. The high PF and DEF for ZB in both seasons denote high microbial syntheses, digestion and intake. More than 75% of amino acids and protein absorbed by ruminants are from microbial origin (Dewhurst et al., 2000), therefore high PF suggests more microbial protein to the ruminant. This was clearly demonstrated by the high MY observed for ZB in summer. However, in winter MY was lower in ZB than H and mH. A shift in propionate production to acetate in ZB might have deprived some microbial population (protozoan) that depends on propionate for metabolism. A decrease in protozoan population may affect MY significantly.

The A-fraction of gas production varied among animals within seasons. The high A observed in H in both seasons was associated to the microbial species and population as well as the ease with which solubles are being hydrolysed. The gas from B did not vary between season and among animal types within a season. The major difference seems to arise from the type of end products which they produced. Considering the quantity of gas produced in both seasons, mH would appear to be the best system. Total SCFA and the SCFA molar proportions are also very important factors to be considered when selecting a system. The three most important SCFA in the rumen are acetic acid, propionic and butyric acid (Jan, 1994; Sutton et al., 2003). Although all these acids can be used to generate energy (ATP) in the intermediary metabolism, some of them have been associated with different metabolic processes. Propionic acid is a major precursor for hepatic gluconeogenesis in non-fasting cows (43-73%). Propionic acid also affects milk protein content positively while acetic acid increased milk yield and milk fat content and butyric acid affects milk fat positively (Jan, 1994). Based on the total SCFA ZB will be the best system followed by H and mH as the least active system. For dairy farmers who require high milk yield and high milk protein, the ZB system in summer and winter (highest total SCFA) will be the best system to be considered because of their acetic acid: propionate ratio.

4.5 Conclusion

It was the objective of this study to compare the fibrolytic competence of three hindgut fermenters using cultured faecal inocula as the main source of CPZ for enzyme assays and microbes for *in vitro* fermentation studies. Both winter and summer enzyme assays on crystalline cellulose hydrolysis showed that the ZB microbial ecosystem was the most competent fibrolytic ecosystem, followed in order by H and mH. Although the rate of MS fermentation was not different among the animal types in both season, the results from TD, PF, DE and total SCFA especially in winter shows that ZB was a superior fibre fermenter. Low gas, high TD and total SCFA observed in ZB implies that the ZB possesses microbes that have the ability to conserve energy by reducing the amount of energy wasted a gas during fermentation into energy rich metabolites. It is plausible to conclude that ZB and H may contain microbes with higher fibrolytic potential which can be beneficial to other domesticated ruminants. It will be imperative in future to find out which gases were being compromised for what SCFA or other metabolites. An investigation of the synergistic effect of the most active microbial ecosystems will be very important. Thirdly, the identification of these microbes will be beneficial in animal production as they can easily be exploited as feed additives.

CHAPTER 5

In vitro fermentation using faecal fluids of the horse, wildebeest and zebra

Abstract

An *in vitro* trial was conducted to investigate the effect of different *inoculum* sources, horse (H), wildebeest (WB) and zebra (ZB) and their combinations on fermentation of maize stover (MS). The combined systems (CS) in ratio of 1:1 were: N1) H+WB, N2) H+ZB, N3) WB+ZB and N4) H+WB+ZB. Fresh faecal inocula were cultured in the laboratory on maize stover and lucerne (1:1) with salivary buffer for 72 h at 38°C before application as an inoculum or extraction of crude protein for enzyme assays. Crude proteins were precipitated from cultured faecal inocula by 60% ammonium sulphate and analysed for exocellulase, endocellulase and hemicellulase specific activities (µg reducing sugar/mg crude protein). *In vitro* fermentation study was carried out by transferring 33 ml of laboratory cultured faecal inoculum (squeezed through four layers of cheese cloth) into 67 ml of salivary buffer containing 1g maize stover (MS) and incubating for 72 h at 38°C. Total gas production (GP) and rate (C), apparent degradability (APD), true degradability (TD), microbial yield (MY) and total short chain fatty acids (Total SCFA), were determined, and methane and CO_2 were calculated. Exocellulase specific activities differed (P<0.05) among the seven microbial ecosystems. Exocellulase activity on crystalline cellulose ranked the different microbial ecosystems according to their fibrolytic potential as follows: N1 > N2 > N4 > H > ZB > WB >N3. Total gas, TD, MY and total SCFA were higher (P<0.001) in the CS than in the individual systems (IS). Total SCFA and TD were highest in N1 and N3. The overall fermentation rate of MS did not differ among the systems. The time taken to produce half GP and microbial colonization time were longer (P<0.05) for CS than in IS. Methane, CO_2, partitioning factor and degradability efficiency were higher (P<0.05) in IS than CS. Propionate to acetate ratio was higher in N1 than the rest of CS which were intermediate. True degradability, total gas, total SCFA, PF and degradability efficiency ranked the microbial ecosystems according to their fibrolytic potential as follows; N3 > N1 > N4 > WB > N2 > ZB >H. These results showed that *in vitro* transinoculation is possible and could improve rumen fermentation efficiency, rumen fibrolytic microbes' quantity and ecology.

5.1 Introduction

Milk yield and animal performance are affected by low quality forages, low digestibility and low voluntary intake (Hungate, 1984; van Ackeren et al., 2009). Low digestibility is partly a consequence of the forage property (Wei et al., 2009), host animal adaptation and partly associated with the extent of microbial activity in the rumen and the caecum. Microbial fermentation yields short chain fatty acids (SCFA) and the energy yield during fermentation is used for metabolic processes and microbial growth. The SCFA and microbial proteins (MP) are the main energy and protein sources for herbivores (Armentano, 1992; Weimer, 1998; Wanapat, 2000). Several factors have been associated with a decrease in forage breakdown including: forage quality (fibre content) , intake and passage rate, forage composition and type of forage (Ranilla et al., 2008), microbial species, efficiency of microbes and microbial population (Leng, 1990).

Forage type, microbial species and evolution have been the major focus of most studies (Barrière et al., 1991; Philippeau and Michalet-Doreau, 1997; Barrière et al., 2003; Bänziger et al., 2006). Although forage type is still a major problem, the advancement of plant biotechnology has allowed the modification of plants through the process of genetic engineering, producing forages of higher nutritive values (Tolera et al., 1998; Tolera et al., 1999; Tolera and Sundstøl, 1999). *In vivo* digestibility of NDF from maize hybrids almost doubled from 32.9 to 60.1% (Barrière et al., 1991; Barrière et al., 2003). However, in the tropics where these technologies are limited, herbivores depend largely on low quality roughages and agricultural crop residues which are lower in both fermentable carbohydrate and protein content (Wanapat, 2000; 2009). With the high variation in forage quality and quantity, fermentation depends largely on the efficiency of microbes in the rumen and hindgut of herbivores. Different studies have shown that microbes from the same or different herbivore species grazing on the same or different fields may vary in their ability to ferment fibre (Gonçalves and Borba, 1996; Dalrymple and McSweeney, 1998; Wanapat, 2001; Yang et al., 2010; Kenters et al., 2011). This was also observed in our previous studies (Fon, 2006; Fon and Nsahlai, 2009), where crystalline cellulose hydrolysis by enzymes from a zebra was higher than those of a wildebeest grazing on the same field. Several studies have also

evaluated the effects of dietary enzyme supplements on ruminant feedstuff utilization and predominance with mixed results achieved (Yang et al., 1999; Muwalla et al., 2007; Ranilla et al., 2008; Tang et al., 2008; Moharrery et al., 2009). Some of these studies reported an increase in weight gain, SCFA, milk production or cellulolytic enzymes (Beauchemin et al., 2003a; Giraldo et al., 2008; Miller et al., 2008; Ranilla et al., 2008; Álvarez et al., 2009; Gado et al., 2009) while others observed no differences in enzyme supplementation (ZoBell et al., 2000; Elwakeel et al., 2007; Krueger and Adesogan, 2008). Because these enzymes are often faced with the daunting task of being digested by proteases, microbial (bacteria, protozoan or fungi) supplementation became imperative.

The results obtained from *in vivo* studies have been variable regarding the effects of direct-fed microbials (DFM) on ruminant feedstuff utilization and performance. While increases in milk production, SCFA, live weight gain or total tract digestibility were reported with DFM supplementation by some researchers (Lee et al., 2004; Walsh et al., 2007; Chaudhary et al., 2008), others observed little or no differences (Vaithiyanathan et al., 2005). Application of microbes might just have an urge over enzymes supplementation if they can survive lyses by rumen proteases which imply enzyme supply will be continuous. It is, however, yet to be determined if microbes from these ecosystems can co-exist in the same medium and if such co-existence can confer positive synergism on fibrolysis. This is because there is very limited information in the literature monitoring the synergistic activity of two or more rumen ecology apart from the ones described by Wanapat (2001); Vaithiyanathan et al. (2005); Jones and Lowry (1984) and Dominguez Bello and Escobar (1997) where microbes survived for up to 14 days. Therefore, our objective will be to investigate the synergistic (combine) activities of two or more ecosystems.

In chapter 4, it was found that some herbivore microbial ecosystems were more active than others even when grazing on the same fields. In this study the three most active fibrolytic herbivores microbial ecosystems (horse (H), wildebeest (WB) and zebra ZB) identified from our previous experiments, were used to create four combined microbial systems (CS); N1) H+WB, N2) H+ZB, N3) WB+ZB and N4) H+WB+ZB. Secondly, the effect of each inoculum (microbial ecosystem) on *in vitro* digestibility of maize stover (MS) was determined and used

to compare the individual microbial ecosystems (H, WB and ZB) against the combined microbial ecosystems (N1, N2, N3 and N4).

5.2 Materials and Methods

5.2.1 Materials

The substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany) and xylan from beech wood (high grade) from SIGMA (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose and D-(+)-glucose were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) from MERK Laboratory supplies, South Africa and 3,5-Dinitrosalicylic acid from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide and ethylene diamine tetra acetic acid (EDTA) were bought locally from Capital Supplies, South Africa. Maize stover and lucerne were bought locally. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) was from sigma-Aldrich. All spectral scans were carried out with Virian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd), Australia.

5.2.2 Chemical composition of maize stover

Maize stover (MS) chemical composition was analysed as previously described in chapter 3 (section 3.2.1).

5.2.3 Experimental animals

Samples (faeces) were collected in winter from a group of animals; horses (H), wildebeests (WB) and zebras (ZB) with no preference to sex. *Equus caballus* (H) was from the Ukulinga Research farm, University of KwaZulu-Natal, Pietermaritzburg. *Equus quagga boehmi* (ZB) and *Connochaetes taurinus* (WB) were available in the Tala Game Reserve, umbumbulu, KwaZulu-Natal (SA). The ZB and WB were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) standing hay and other fibres were dominant.

Although the H was grazing on a dry land in an open field where Kikuyu grass standing hay and other fibres were dominant, they were supplemented with hay while in pens.

5.2.4 Faeces collection and inocula preparation

Faeces were collected on farm within 2 min of defecation from H, ZB and WB before transferring into an airtight thermo flask (38°C) which has been flushed with CO_2. While in the laboratory, 300 g of faeces was mixed with 300 ml of warm incubation buffer (buffer C, chapter 3, section 3.2.3) and mixed thoroughly before squeezing through four layers of cheese cloth (pre-warmed and flushed with CO_2) to make faecal fluid (FF). Faecal fluid preparation was done in the shortest possible time in order to minimize the exposure of microbes to oxygen since they are anaerobic.

5.2.5 Faecal fluid culturing

Table 5.1 Faecal fluid proportions for culturing microbial ecosystems

Ecosystems	Faecal fluid proportions (ml)	Salivary buffer (ml)	Substrate (g) MS:lucerne
H	198	402	3:3
WB	198	402	3:3
ZB	198	402	3:3
N1	99:99	402	3:3
N2	99:99	402	3:3
N3	99:99	402	3:3
N4	66:66:66	402	3:3

MS=maize stover, N1= H+WB, N2=H+ZB, N3=WB+ZB and N4=H+WB+ZB.

Faecal fluid was cultured in the laboratory for *in vitro* inoculation as previously described in chapter 3 (Section 3.2.4) with some few modifications. Besides H, WB and ZB as inocula sources, four new combined microbial inocula; N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB) were created (Table 5.1) before incubating with salivary buffer (402 ml) containing 6 g lucerne and MS (1:1) for 72 h at 38°C. The viability of these microbial ecosystems was determined by monitoring pH changes. After 72 h of incubation, the sample fluid for each ecosystem was used for crude protein extraction or as inoculum for *in vitro*

digestibility studies. Horse, WB and ZB were the individual systems (IS) while N1, N2, N3 and N4 were the combined systems (CS).

5.2.6 Crude protein extraction

Crude protein samples were precipitated from cultured faecal fluid using 60% ammonium sulphate (section **3.2.5**), dialyzed overnight (section **3.2.6**) and analysed to determine concentrations (section **3.2.7**).

5.2.7 Enzyme assays

Exocellulase, endocellulase and xylanase specific activities were determined by incubating with crystalline cellulose, carboxymethyl cellulose and xylan respectively, as described in chapter 3 (section 3.2.8). Reducing sugars liberated from the different enzyme assays were measured as previously described by Miller (1959) (section 3.2.9). Enzyme specific activity was defined as µg of reducing sugar/mg crude protein. Each ecosystem enzyme assay was replicated thrice with five pseudo repeats.

5.2.8 *In vitro* digestibility

5.2.8.1 *In vitro* gas production

An automated gas production method using computerized pressure transducer system similar to the one described by Pell and Schofield (1993) was used in this experiment as previously described in chapter 3 (section 3.2.10.1). Inocula were from H, WB, ZB, N1, N2, N3, and N4 cultured systems. Substrate incubation with each inoculum type was replicated three times with five pseudo repeats. Gas and the rate of gas production were measured by fitting the raw data in the model described by Campos et al. (2004). The total gas produced from MS fermentation (GP), the time taken to produce half the gas ($T_{1/2}$), the gas at the point of inflection (µ), the overall rate of MS fermentation (C), the gas produced by soluble (A) and

the insoluble but degradable fraction of MS (B) and their rates a_1 and $b_{1,}$ respectively, as well as the lag time (lt) were measured in this study.

5.2.8.2 *In vitro* degradability and SCFA

In vitro degradability of MS was determined as previously described in chapter 3 (section 3.2.10.3) but for the inocula which were from H, WB, ZB, N1, N2, N3, and N4 cultured systems. *In vitro* degradability was carried out with MS as the main substrate. Maize stover incubation with each inoculum type was replicated three times with five pseudo repeats. Apparent (APD) and true degradability (TD), microbial yield (MY), the partitioning factor (PF), degradability efficiency (DE) and total SCFA were the parameters measured in this experiment as described in chapter 3 section 3.2.10.1 and 3.2.10.3.

5.2.8.3 Stoichiometric calculations of carbon dioxide, methane and microbial yield

The theoretical calculations of CO_2, CH_4 and microbial yield (MY_1) were conducted as described by Groot et al. (1998) based on the stoichiometric balance of the fermentation of glucose to propionic acid (PP), acetic acid (Acet), n- and iso-butyric acid (nB and iB, respectively), CO_2 and CH_4 (Czerkawski, 1978; Van Soest, 1994). Microbial yield was calculated from the total moles of ATP yield from Acet (2), PP (3), nB(2) and CH_4(1) multiplied by the grams of microbial dry matter per mole of ATP (Y_{ATP}) derived from fermentation. Methane, CO_2 and MY_1 were calculated from the following stoichiometric equations (5.1, 5.2, 5.3 and 5.4);

CO_2 = Acet/2 + PP/4 + 3(nB+iB)/2 ----------equation 5.1

CH_4 = Acet + 2(nB+iB) – CO_2 ---------- equation 5.2

Total ATP (moles) = 2*Acet + 3*PP + 2*nB + 1*CH_4 ----------equation 5.3

MY_1 = Y_{ATP}*total ATP ----------equation 5.4

Where MY_1 =calculated microbial yield, Total ATP = sum of ATP from the fermentation products, Y_{ATP}= grams of microbial dry matter per mole of ATP derived from fermentation, Assumed Y_{ATP}=20 g/DM (Czerkawski, 1978; Harrison and McAllan, 1980).

5.2.9 Statistical analysis

SAS software was used to perform statistical analysis (SAS, 2002). Samples were analysed to determine the effects of season and microbial ecosystem using the GLM procedure. The model was:

$Y_{ijk} = \mu + T_i + run_j + e_{ijk}$

Where Y_{ijk} is the individual observation, μ is the overall mean, T_i is the effect of the inoculum source, runj is the effect of run, and e_{ijk} is the random variation.

5.3 Results

5.3.1 Enzyme assays

The specific activities of hemicellulases differed (P<0.001) among the seven herbivore microbial ecosystems (Table 5.2). Hemicellulolytic activity was highest in N4 followed by N2. The H and ZB had the third highest specific activity compared to N1 and N3 while the least hemicellulolytic activity was observed in WB. Endocellulase activity differed (P<0.001) among the microbial ecosystems. The highest endocellulolytic activity was observed in N4 while H and ZB were the second highest. All the other systems demonstrated a moderate

Table 5.2 Effect of source of inoculum on the specific activities of exocellulase, endocellulase and xylanase of crude protein extracts from seven *in vitro* microbial ecosystems

Inoculum source	Enzyme specific activities		
	Hemicellulase (µg xylose/mg)	Endocellulase (µg glucose/mg)	Exocellulase (µg glucose/mg)
H	98.2	92.7	141.8
WB	57.3	72.5	90.5
ZB	88.8	89.2	107.1
N1	81.4	87.7	163.1
N2	104.5	77.6	162.6
N3	68.5	80.5	98.1
N4	183.0	108.5	160.9
SED	2.63	1.96	4.15
P-value	0.001	0.001	0.001

H = horse, WB = wildebeest, ZB = zebra, N1= H+WB, N2= H+ZB, N3=WB+ZB and N4 = H+WB+ZB.

endocellulolytic activity but for N2 and WB which showed the lowest activities. Exocellulase specific activity differed (P<0.001) among the seven microbial ecosystems. The highest specific activities were observed in N1, N2 and N4. The H demonstrated the second highest exocellulase specific activity while the least was observed in WB.

5.3.2 *In vitro* degradability of maize stover and gas production

The results from *in vitro* degradability of MS are presented on Table 5.3. The highest (P<0.001) APD value for MS was observed in N1 while systems WB, N4 and N3 were intermediate when compared to systems H, ZB and N2. The TD was highest (P<0.001) in N1 and intermediate in N3, WB, N4 and N2 when compared to H and ZB microbial ecosystems. Systems N3 and N4 recorded the highest (P<0.001) GP, followed by N1 and WB with intermediate

Table 5.3 Effect of source of inoculum on *in vitro* fermentation of maize stover by seven microbial ecosystems

Digestibility Parameters	Inoculum source							SED	*P*-value
	H	WB	ZB	N1	N2	N3	N4		
APD (mg/g)	356	457	324	485	316	430	402	9.7	***
TD (mg/g)	584	679	589	714	606	680	671	4.2	***
MY (mg/g)	228	222	265	229	290	250	269	9.0	*
MY_1 (mg/g)	139	227	152	240	275	348	160	17	**
GP (ml/g)	97	135	102	132	117	146	148	3.7	***
C(h^{-1})	0.022	0.02	0.025	0.021	0.02	0.02	0.02	0.001	ns
T1/2 (h)	24.6	27.3	21.8	28.3	27.5	28.9	30.6	0.72	**
μ (ml/h)	2.1	2.6	2.5	2.7	2.3	2.9	2.8	0.11	ns
PF	6	5	5.8	5.6	5.2	4.7	4.6	0.23	**
DEF	0.12	0.08	0.12	0.09	0.09	0.07	0.08	0.010	***

APD = apparent degradability, TD=true degradability, MY=microbial yield, MY_1 = microbial yield calculated from fermentation products, H=horse, WB=wildebeest, ZB = zebra, N1=H+WB, N2= H+ZB, N3=WB+ZB and N4 = H+WB+ZB, GP= total gas produced from MS fermentation after 72h, DE=degradation efficiency factor, μ= the gas at the point of inflection, PF=partitioning factor (mg of substrate degradable/maximum volume of gas) , *=P<0.05, **=P<0.01, ***=P<0.001, ns= non significant.

values when compared to N2, ZB and H with the lowest GP. Microbial yield (MY) was highest (P<0.001) in N2 and intermediate in N4, ZB and N3. Microbial yield calculated from fermentation products (MY_1) was higher (P<0.01) in the CS than IS. It was highest in N3, intermediate in N2, N1 and WB and lowest in N4, ZB and H. The lowest MY values were observed in H, WB and N1. The rate (C) of MS fermentation was not significantly different among the microbial ecosystems but the results show that, C was relatively higher in ZB compared to the other systems. The time taken to produce half GP ($T_{1/2}$), varied (P<0.01) among the herbivore microbial ecosystems. Generally, $T_{1/2}$ was longer for the combined systems (CS) than in the individual systems (IS). Gases produced at the point of inflection (U) were similar among the microbial systems. The PF and DEF varied (P<0.01) among the seven herbivore microbial ecosystems. Horse, ZB and N1 recorded the highest PF and DEF values. The PF and DE for N3 and N4 were relatively lower when compared to that of N2 and WB.

Total SCFA was generally higher (P<0.001) in the CS than in the IS (Table 5.4). System N3 recorded the highest total SCFA followed by N1, WB and N2 while the rest of the systems (H, ZB, and N4) were relatively lower. Acetic acid had the highest molar proportions in all systems but for N1. Propionic acid had the second highest molar proportions while nB was the least in all seven microbial ecosystems. Horse and

Table 5. 4 Effect of source of inoculum on short chain fatty acids and gas production from maize stover fermentation

Digestibility Parameters	Inoculum source							SED	*P*-value
	H	WB	ZB	N1	N2	N3	N4		
Short chain fatty acids									
Total SCFA (mM)	29.12	46.81	30.99	47.42	56.74	71.54	33.02	3.343	***
Molar proportions									
Acetic	0.575	0.517	0.498	0.451	0.545	0.498	0.431	0.0081	***
Propionic	0.381	0.427	0.47	0.529	0.425	0.433	0.423	0.0062	***
Butyric	0.044	0.056	0.031	0.019	0.03	0.069	0.114	0.0032	***
CO_2	0.449	0.449	0.414	0.387	0.424	0.460	0.540	0.0031	***
Methane	0.214	0.179	0.147	0.103	0.181	0.175	0.182	0.0041	**

SCFA = short chain fatty acids, H = horse, WB = wildebeest, ZB = zebra, N1= H+WB, N2= H+ZB, N3=WB+ZB and N4 = H+WB+ZB, **=P<0.01, ***=P<0.001.

N2 had the highest molar proportion of Acet followed by WB, ZB and N3 while the least was found in ZB and N4. Propionic acid was highest in N1, intermediate in ZB and low in the rest of the systems. Methane and CO_2 molar proportions varied (P<0.001) among the seven *in vitro* microbial ecosystems. Carbon dioxide was highest in N4 and N3 and lowest in N1 while the rest of the systems were intermediate in CO_2 production. Unlike CO_2, CH_4 was lowest in N1 and highest in H.

5.3.3 *In vitro* gas production kinetics

The kinetic parameters of gas production from MS fermentation are shown on Table 5.5. The gas volumes from A were smaller (P < 0.01) than that of B. The highest gas volume from A was produced by WB, followed by ZB compared to H and N2 with intermediate volumes. The gas from A was relatively lower in N1, N3 and N4. The gas from B varied (P<0.001) among the seven herbivore microbial ecosystems with N4, N3 and N1 producing the highest gas volumes. Gas production from B by N2 and WB was relatively higher compared to H and ZB with the least gas volumes. The rate of gas production from solubles (a_1) was higher (P<0.01) than that of non-solubles (b_1) in all microbial ecosystems. The fermentation rate of solubles

Table 5.5 Effect of source of inoculum on mean gas production kinetics of maize stover fermented in various inocula

Inoculum source	Gas production kinetic parameters				
	A (ml)	a_1 (h^{-1})	B (ml)	b_1 (h^{-1})	lt (h)
H	27	0.100	71	0.022	1.4
WB	52	0.067	83	0.019	1.6
ZB	38	0.180	63	0.023	1.9
N1	10	0.389	121	0.021	3.7
N2	25	0.076	92	0.019	1.5
N3	10	0.345	136	0.021	3.5
N4	11	0.229	136	0.021	4.1
SED	2.0	0.0471	4.0	0.0010	0.33
P-value	***	ns	***	ns	**

A and B are the gas volumes (mL) from fast and slowly degradable fractions of MS, respectively, a_1 and b_1 are the fermentation rates (h^{-1}) of A and B respectively. H = horse, WB = wildebeest, ZB = zebra, N1= H+WB, N2= H+ZB, N3=WB+ZB and N4 = H+WB+ZB, lt=lag time, **=P<0.01, ***=P<0.001.

varied (P<0.001) among the microbial ecosystems. The rate (a_1) was fastest in N1 while N3 and N4 were relatively faster. The slowest a_1 was observed in H, WB and ZB. The fermentation rate of B (b_1) was not different among the microbial ecosystems. Microbial colonization time (lt) was longer (P<0.01) in the CS (N1, N2, N3 and N4) than in the IS (H, WB and ZB).

5.4 Discussion

The rumen is an essential fermentation chamber in herbivores colonized by microbes capable of fermenting forages into SCFA and microbial protein, which serves as a major source of energy and protein, respectively, to the host (Weimer, 1998; Wanapat, 2000). In hindgut fermenters, forages are fermented by microbes yielding SCFA and microbial proteins. The SCFA in hindgut fermenters are utilized for energy by the host while microbial proteins are excreted in faeces unlike in ruminants where it is the main protein source to the host (Wanapat, 2000; Zhu et al., 2005). The more efficient the rumen or hindgut is, the better the fermentation products being synthesized from low quality forages and crop residues. Therefore, strategies directed towards enhancing rumen ecology will be beneficiary in livestock production. This study probed both rumen and hindgut ecology manipulation in an attempt to find combined systems with enhanced fibrolytic activities.

Hemicellulase specific activities showed that microbial synergism in CS had a net positive effect on xylan hydrolysis with higher values observed in N4 and N1 than in the IS. Only N4 had a much higher endocellulase specific activity than the other CS. This implies that the effect of CS on amorphous cellulose solubilisation was only prominent in N4. Exocellulase specific activities (the hydrolysis of crystalline cellulose) in CS were generally higher than those of the IS. Although the exact explanation of the fibrolytic enhancement of these ecosystems are not very clear, it is possible that mixing microbial inoculum from different animal species might have introduce microbes of higher fibrolytic potential, which can compete and survive in the new ecosystem. The survival of such microbes implies an increase in potent exocellulases, hence an increase in fibre breakdown. The total microbial population of such microbes may play a major role in the quantity of cellulases available for crystalline

cellulose hydrolysis. The quantity of cellulases available for fibre hydrolysis may not be affected by microbial population only, but by the efficiency of fibrolytic enzyme secreted and their combined effect. An examination of N3 enzyme assays showed that combining WB and ZB inocula, suppresses substrates hydrolysis by 22.7%, 9.7% and 8.4% for hemicellulase, endocellulase and exocellulase specific activities, respectively. This enzyme activity suppression might be due to microbial protein secretions (proteases, antibiotics) during competition for substrates and niche colonization. This implies that the microbes with higher fibrolytic potential might have been dominated by microbes with a higher niche colonization potential but relatively lower fibrolytic potential. The CS had a higher effect on crystalline cellulose hydrolysis than IS. This may be due to a positive synergistic effect with an increase in enzyme concentration and efficiency. Both *in vivo* and *in vitro* enzyme supplementation experiments have been carried with some of them achieving a positive effect on fibre hydrolysis (Ranilla et al., 2008; Gado et al., 2009; Gallardo et al., 2010; Shekhar et al., 2010) whereas others reported no effect (Pinos-Rodriguez et al., 2008; Ranilla et al., 2008). *In vitro* enzyme supplementation study by Ranilla et al. (2008), showed that fibre hydrolysis was improved only within the first 10 h, but not at long 24 h incubations. They also mentioned that the activity was also dependent on the enzyme concentration used. The result from crystalline cellulose hydrolyses shows that, systems N1, N2 and 4 were the best enzymes systems to be exploited for future enzyme additive studies.

The TD was highest in N1 and N3 when compared to all the other ecosystems. The highest activity was associated to the cumulative effect of microbes from two different species as previously been demonstrated by Wanapat (2001). It was also observed that TD increases in CS when compared to IS. The TD improvement in these new systems might be due to increases in microbial population, increases in a predominant fibrolytic microbial species (bacteria, protozoa, or fungi) and introduction of microbes that might have evolved with their potential in hydrolyzing fibre or the type of fermentation end-products available (Russell and Rychlik, 2001; Calabrò et al., 2008; Wanapat et al., 2009). Although feed type is assumed not to be a major factor influencing TD is this study since all systems where subjected to a common feed for three days before application, it is still possible that the type of forages which these animals have been grazing on in the wild might have played a major role in the

evolutionary development and efficiency of their microbes. This was observed in the study by Xi et al. (2007) who demonstrated that Gayal cattle was able to utilize low quality forages better than Yunnan Yellow cattle due to the continuous exposure and adaptation on low quality forages in their natural environment.

The maximum extent of GP was generally higher in CS than IS. The GP was highest in N4 and N3 intermediate in WB, N1 and N2. If high GP reflects the amount of substrate digested, this means that systems N4 and N3 were the most active systems while H and ZB were the least. Although GP was generally higher for CS as observed for TD, the ranking of these ecosystems were different. The ranking order of these ecosystems from the most active to the least was N1>N3> WB> N4> N2> ZB > H for TD values and N4>N3 > WB>N1 > N2> ZB > H for GP values. Only systems N3 and WB were ranked in the same position by both TD and GP. The microbial ecosystems N4 and N1 switched positions in GP ranking. However, lower GP does not necessary imply lower fibrolytic activities as demonstrated by Gonçalves and Borba (1996) where MS might have been fermented to other energy rich metabolites than gases. The rate of MS fermentation (C) was not significantly different among the seven microbial ecosystems. Because C did not differ among the systems, other cumulative factors of fermentation such as TD, GP, intake and microbial protein synthesis can be used for ranking the different inocula activities.

The lag time and $T_{1/2}$ was generally longer for CS than in the IS. The reason for this delay was not really clear as culturing the microbial inoculum prior application was thought to increase microbial population, hence a decrease in microbial colonization time. However, this delay could have originated from the fact that the CS are quite new and the microbes are still competing for ecological niche dominance (when used as an inoculum hence the set back in colonization time). The established IS competes for substrates rather than ecological niche when used as an inoculum thereby speeding up the microbial colonization time. The gas produced at the point of inflection was not different among the seven ecosystems. Partitioning factor and DEF were significantly higher in the IS than in the CS. High PF values have been associated with high microbial protein synthesis and intake (Blümmel et al., 2003; Darshan et al., 2007). This implies that microbial protein synthesis in the IS was relatively higher than

that of the CS but for N1 and N2. If a higher MY implies a higher digestibility then the PF prediction of higher MY in the IS contradicts the higher TD and GP in CS. Recent studies by Jackson et al. (2010); Krishnamoorthy and Robinson (2010) has rejected the theory of PF being a good predictor of microbial protein synthesis alone. The prediction of microbial protein synthesis from PF did not rank the ecosystems in the same order as MY and MY_1. MY and MY_1 ranking showed that, microbial protein synthesis were generally higher in the CS than IS. Degradability efficiency was also higher in the H, WB and ZB than in N1, N2, N3 and N4 with higher TD and GP. This implies that MS fermentation efficiency was much higher in IS. This does not correspond with the values obtained from TD and GP. A clear explanation for the higher DEF in IS was not known.

Total SCFA was generally higher in CS than in IS but for N4. Total SCFA was highest in N3, intermediate in N2, N3 and WB. These results confirm that CS can improve the rumen ecology, microbial protein synthesis and the total SCFA available for energy generation by the host ruminant. Methane and CO_2 were generally lower in the CS than in the IS. This is an indication that, microbial synergism suppresses the production of gases and promotes the production of other metabolites such as SCFA. The higher CO2 and methane observed in IS could be a possible reason for the decrease in total SCFA in these systems. Therefore, the total SCFA production was compromised for gases production which is disadvantageous to the host ruminant. Lower methane production in CS renders these systems environmental friendly as CH_4 production from ruminants is one of the major greenhouse gases involved in global warming. The molar proportion of SCFA was generally similar to those obtained by different researcher where acetic acid was the most abundant, followed by propionic acid and lastly butyric acid (Solaiman and Shoemaker, 2009). The highest propionic acid to acetic acid ratio was observed in N1 while the lowest in H. This suggests that these ecosystems can be suitable in different livestock production systems.

Gas production from A was generally lower than that of B in all systems. This is due to relatively small amount of solubles in MS for fermentation (Ouda, 2007). The fermentation rates (a_1 and b_1) did not vary among the ecosystems. Similar results were also obtained by Hervas et al. (2005) when comparing *in vitro* digestibility of forages (alfalfa and barley hay)

by rumen inocula from goat and deer. They found no differences in their fermentation rates but they differed in their organic matter digestibility. The quantities of A were limited and were digested rapidly (highly soluble) compared to B. Because B is fibrous (cellulose), its solubility by enzymes is very slow, hence the lower values observed from b_1 values. Systems N4, N3 and N1 were noted for their exceptionally high B values when compared to the rest of the systems. This corresponds with GP values observed in the previous paragraphs.

5.5 Conclusion

Both CS and IS systems had an overall positive effect on crystalline cellulose, carboxymethyl cellulose and xylan hydrolysis. With respect to crystalline cellulose hydrolysis, systems N1, N2, N4 and H were the most active fibrolytic ecosystems. *In vitro* fermentation of MS showed that microbial synergism was positive among the CS. Based on the following digestibility parameters; TP, GP, PF, total SCFA and B, the microbial ecosystems were ranked according to their fibrolytic potential; N3 > N1 > N4 > WB > N2 > ZB >H. The ranking of the microbial ecosystems based on *in vitro* fermentation was preferred to exocellulases ranking because of its application of a natural substrate (MS) during fermentation. This implies that the most active microbial ecosystems were N3, N1 and N4. This shows that *in vitro* transinoculation is possible and could improve rumen fermentation efficiency, rumen fibrolytic microbes' quantity and ecology. Therefore, investigating the *in vivo* effect of these systems would be useful. Further experimentation is required to probe the lower exocellulase activities observed in N3 which was the best systems as demonstrated by its digestibility parameters. Secondly, more research is also required to investigate the suppression of both TD and total gas production by H when mixed with ZB. The delay in microbial colonization observed in the CS requires further investigation as microbial colonization time was expected to decrease with an increase in microbial population.

Chapter 6

Cellulase production from five *in vitro* herbivore microbial ecosystems and their combined systems

Abstract

As the most abundant biomass in nature, cellulose is the main chemical component in forages for herbivores. The energy locked in these complex polymers can only be released by cellulolytic enzymes. Therefore, researches aiming to increase the expression of cellulolytic enzymes or browsing uncultured microbial ecosystem in search of potential fibrolytic enzymes is imperative. The main objectives were to: (a) investigate the variation of cellulase enzymes in cow (CW), horse (H), miniature horse (mH), wildebeest (WB) and zebra (ZB) and (b) identify their presence and activity in combined microbial ecosystems (CS), N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB). Fresh faecal or rumen inocula were cultured in the laboratory on maize stover and lucerne (1:1) with salivary buffer for 72 h at 38°C. Crude proteins (CPZ) were precipitated from both fresh and cultured inocula using 60% ammonium sulphate for enzyme assays and zymography. Endocellulases (Carboxymethyl cellulases) and its activity were identified on 1% (m/v) carboxymethyl cellulose (CMC) zymograms stained with Congo red before rinsing with 5% acetic acid to yield a dark blue coluoration for photographing. All CPZ extracts were active as reducing sugars were produced after incubation with crystalline cellulose, CMC and xylan. Crude protein extracts showed numerous protein bands on both reducing and non-reducing 10% sodium dodecyl sulphate polyacrylamide gels. The number and types of proteins with Carboxymethyl cellulases (CMCases) activity varied (P<0.05) among and within the different animal species CW (15), H (14), mH (14), WB (13) and ZB (13). Combined microbial ecosystems were successfully created with more active CMCases than in the individual microbial ecosystems, N1 (17), N3 (14), N4 (14), and N2 (13). These results show that cellulase enzymes vary among and within herbivore species grazing on the same or different fields. Therefore, identifying specific enzymes and microbes with higher fibrolytic potentials from different ecosystems for transinoculation could play a vital role in improving forage digestibility in ruminants.

6.1 Introduction

Cellulases are enzymes secreted by a large number of free-living and rumen symbiotic microorganisms to catalyze the hydrolysis of cellulose (Ye et al., 2001). Cellulose is a plant polysaccharide composed of β-D-glucopyranosyl units joined together by β-1, 4-glycosidic bonds. It is the major component of plant cell walls and most abundant plant polysaccharide (Imai et al., 2004). The solubilization of cellulose to glucose units occurs in three basic steps involving three major cellulase enzymes, namely exocellulase, endocellulase and cellobiase (Lenting and Warmoeskerken, 2001; Ye et al., 2001; Percival Zhang et al., 2006); (1) endocellulase randomly hydrolyse β-1,4 glycocidyl linkages within the water insoluble cellulose chains, (2) exocellulase hydrolysis β-1,4 glucocidyl linkages at either the non-reducing or reducing ends of cellulose forming cellobiose and (3) β-cellobiase hydrolysis cellobiose into two glucose molecules (Bhat and Bhat, 1997). These glucose molecules are fermented by the microbes for energy generation (ATP) yielding short chain fatty acids (SCFA) as by-products which are the main metabolic substrates for energy production in ruminants (Armentano, 1992).

Cellulases and related polysaccharidases are commonly used in many industrial applications including; food, brewery, paper, animal feed and agricultural research (Bhat and Bhat, 1997; Poutanen, 1997; Pratima, 1999; Beauchemin et al., 2003a). Animal feed will be our main focus as more than 600 million tons of feed worth more than 50 billion US dollars are produced annually (Bhat, 2000). Cellulases and hemicellulase have been shown to have a wide range of feasible applications in both monogastric (Bohme, 1990; Bedford and Classen, 1992; Ponte et al., 2004; Mourao et al., 2006; Yang and Xie, 2010) and ruminants (Lewis, 1996; Bhat, 2000; Beauchemin et al., 2003a; Eun and Beauchemin, 2008; Yang and Xie, 2010). In monogastric animals, cellulases are used either to eliminate anti-nutritional factors present in grains or vegetables, degrade certain cereal components in order to improve the nutritional value of feed or to supplement animals' own digestive enzymes whenever these enzymes are insufficient especially at post-weaning period in piglets (Galante et al., 1998; Ponte et al., 2004; Mourao et al., 2006). In ruminants, it has been used to improve feed digestibility, milk yield, body weight gains and animal performance (Yang et al., 2000; Hainze

et al., 2003; Bala et al., 2009; Peters et al., 2010; Arriola et al., 2011). However, enzyme application in ruminants is much more difficult than observed in monogastric animals. This is primarily due to the complexity of the feed (high cellulose, hemicellulose, pectin, lignin content, tannin, silica and waxy substances (Himmel et al., 2007; Wei et al., 2009)) fed to ruminants. Although many studies on enzyme supplementation have shown substantial improvements in feed digestibility and animal performance (Titi and Tabbaa, 2004; Bala et al., 2009), others reported negative effects or none at all (Titi and Tabbaa, 2004; O'Connor-Robison et al., 2007; Peters et al., 2010).

Enzyme sources can either be pure or crude (but richer in the application enzyme). Pure cellulase enzymes are expressed by specific microorganism using molecular biology techniques in the laboratory while crude cellulase enzymes mixtures are concentrated from *in vitro* incubators. Although cellulase expressions by microbes or genetically modified microbes have been the main sources of enzymes, attempts have also been made to clone cellulase and hemicellulase genes in order to produce transgenic animals. These animals would secrete the required enzymes into the gastrointestinal tract to facilitate its feed digestion efficiency (Hall et al., 1993). Despite reports describing the isolation and characterization of cellulases and hemicellulase from different environmental genomic libraries (Ferrer et al., 2005), the biotechnological potential of novel cellulases from uncultured microbial community has been far from being fully explored. Therefore, exploring a variety of ecosystems to identify the different types (size) of cellulase enzymes present, their fibrolytic potential as well as their combined effect might be beneficiary in ruminant nutrition.

The main objectives were to: (a) to identify the types of cellulase enzymes available in the following individual microbial ecosystems (IS); cow (CW), horse (H), miniature horse (mH), wildebeest (WB) and zebra (ZB); and (b) identify their presence and activeness in combined microbial ecosystems (CS), N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB).

6.2 Materials and methods

6.2.1 Materials

The substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany) and xylan from beech wood (high grade) from SIGMA (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose and D-(+)-glucose were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) from MERK Laboratory supplies, South Africa and 3,5-Dinitrosalicylic acid from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide and ethylene diamine tetra acetic acid (EDTA) were bought locally from Capital Supplies, South Africa. Maize stover (MS) and lucerne (LC) were bought locally. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) and molecular weight markers (150, 100, 75, 50, 35, 25 and 15 kDa) were from sigma-Aldrich. All spectral scans were carried out with Varian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd).

6.2.2 Experimental animals

Samples (faeces) were collected in winter from a herd of animals; cows (CW), horses (H), miniature horses (mH), wildebeests (WB) and zebras (ZB) with no preference to sex. CW, mH and H were from the Ukulinga Research farm, University of KwaZulu Natal, Pietermaritzburg. *Equus quagga boehmi* (ZB) and *Connochaetes taurinus* (WB) were available at the Tala Game Reserve, uMbumbulu, KwaZulu-Natal (SA). The ZB and WB were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) standing hay and other fibres were dominant. Although the CW, mH and H were grazing on a dry land in an open field where Kikuyu grass (standing hay) and other fibres were dominant, they were supplemented with veld hay while in pens.

6.2.3 Faeces collection and inocula preparation

Faeces were collected on farm within 2 min of defecation from H, ZB and WB before transferring into an airtight thermo flask (38°C) which has been flushed with CO_2. While in the laboratory, 300 g of faeces was mixed with 300 ml of warm incubation buffer (buffer C, chapter 3, section 3.2.3) and mixed thoroughly before squeezing through four layers of cheese cloth (pre-warmed and flushed with CO_2) to make faecal fluid (FF). Faecal fluid preparation was done in the shortest possible time in other to minimize the exposure of microbes to oxygen since they are anaerobic. For the cow, rumen fluid (RF) was collected and prepared as described in chapter 3 (section 3.2.3).

6.2.4 Faecal and rumen inocula culturing

Table 6.1 Faecal and rumen fluid proportions for culturing microbial ecosystems

Ecosystems	Faecal fluid proportions (ml)	Salivary buffer (ml)	Substrate (g) MS:LC
CW	198	402	3:3
H	198	402	3:3
mH	198	402	3:3
WB	198	402	3:3
ZB	198	402	3:3
N1	99:99	402	3:3
N2	99:99	402	3:3
N3	99:99	402	3:3
N4	66:66:66	402	3:3

CW=cow, H=horse, WB=wildebeest, ZB=zebra, mH=miniature horse, N1= H+WB, N2=H+ZB, N3=WB+ZB and N4=H+WB+ZB, MS=maize stover, LC=lucerne

Faecal fluid was cultured in the laboratory for *in vitro* inoculation as previously described in chapter 3 (Section 3.2.4) with some few modifications. Besides CW, mH, H, WB and ZB as inocula sources, four new combined microbial inocula were created; N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB) (Table 5.1). The inocula were incubated with salivary buffer (402 ml) containing 6 g of MS and LC (1:1) for three days at 38°C. The viability of each microbial ecosystem was determined by monitoring pH changes. After three

days of incubation, the sample fluid for each microbial ecosystem was used to extract crude protein (CPZ) for enzyme assays and zymography. The individual systems (IS) were CW, mH, H, WB and ZB while the cultured combined systems (CS) were N1, N2, N3 and N4.

6.2.5 Crude protein extraction for enzyme assays

Crude protein samples for enzyme assays were precipitated from both fresh (CW, mH, H, WB and ZB) and cultured (CW1, mH1, H1, WB1, ZB1, N1, N2, N3 and N4) inocula by adding 60% ammonium sulphate (section **3.2.5**), dialyzing overnight (section **3.2.6**) and determining the concentrations (section **3.2.7**).

6.2.6 Enzyme assays

Enzyme assays were carried out to confirm the activeness of each crude protein extract (CPZ) prior to zymogram assays. Exocellulase, endocellulase and xylanase specific activities were determined by incubating with crystalline cellulose, carboxymethyl cellulose and xylan respectively, as described in chapter 3 (section 3.2.8). Reducing sugars liberated from the different enzyme assays were measured as previously described by Miller (1959) (section 3.2.9). Each ecosystem was represented by three samples each of which was analysed in triplicates. Specific activities of the above enzymes were defined as µg of reducing sugar/ mg crude protein.

6.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and cellulase zymography

6.2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most powerful techniques used in biochemistry, molecular biology and genetics to separate proteins mixtures according to their sizes. An ionic detergent (SDS) is applied to the protein mixture to be separated which denatures the secondary and non-disulphide-linked tertiary structures and

applies a negative charge to each protein in proportion to its mass. Therefore, the protein mixture in an electric field will migrate at a speed determined by their charge: mass ratio. There are two types of SDS-PAGE namely reducing and non-reducing. In the reducing SDS-PAGE, 2-mercaptoethanol is included in the sample treatment buffer which is a disulphide reducing agent that breaks proteins into their constituent subunits. These protein subunits bind to SDS and will more efficiently open up the structure giving a better estimate of the molar ratio. The denatured protein structures cannot be re-natured. In the non reducing SDS-PAGE, 2-mercaptoethanol is not included hence the denatured secondary and non-disulphide-linked tertiary structures can be re-natured by the removal of SDS. This implies that the proteins on the gel can still perform their different functions after separation.

Both reducing and non-reducing SDS-PAGE (Laemmli, 1970) was used in this study to monitor the presence of proteins in the CPZ samples. Crude protein extracts (10µg/5µl) were separated on a 10% running gel. The gels were stained with Coomassie blue-250 or by silver staining (when the proteins were too small to be stained by Coomassie blue-250) for the detection of protein bands on the gel (Blum et al., 1987). Crude protein extracts were run alongside a molecular weight marker (150, 100, 75, 50, 35, 25 and 15 kDa) for estimating unknown molecular weights of protein bands. Each ecosystem CPZ (CW, mH, H, WB, ZB, N1, N2, N3 or N4) extracted at three different times of collection, were ran on gels more than three times to established the presence of protein bands.

6.2.7.1 Cellulase zymography

Zymography is a versatile two-staged technique involving protein separation by electrophoresis followed by the detection of cellulase activity. This method is regularly used to identify cellulase activities on polyacrylamide gels under non-reducing conditions. To established zymograms of cellulase activities, CPZ (from CW, mH, H, WB, ZB, N1, N2, N3 and N4) were separated on a 10% non-reducing SDS polyacrylamide gels which have been polymerized with 1% (m/v) CMC substrate. After separation on the non-reducing SDS polyacrylamide gels, re-naturation of proteins were carried out by soaking the gels in two changes of 50 ml of 2.5% (v/v) Triton X-100 (to remove SDS) over 3 h at room temperature

(Heussen and Dowdle, 1980; Dodia et al., 2008). Following this, the gels were incubated in the reaction buffer (chapter 3, section 3.2.7) of pH 5.0 at 33ºC for 48 h. After incubation these gels were stained with 0.2% (w/v) Congo red to identify protein bands with cellulase activity as previously described by Beguin (1983). After staining the gels were rinse with 5% acetic acid to change the background to blue black for easy photographing (Beguin, 1983). Crude protein extracts samples were run alongside a molecular weight marker (150, 100, 75, 50, 35, 25 and 15 kDa) which was cut off after re-naturation and stained with Coomassie blue-250. These markers were used to plot a standard curve (distances migrated versus molecular weights) to estimate the molecular weight of the unknown active cellulase bands.

6.2.8 Statistical analysis

In vitro enzyme specific activities estimates derived from H, WB, ZB, N1, N2, N3 and N4 were analysed using the general linear model of SAS (2002).

$Y_{ij} = \mu + E_i + e_j$

Where Y is the individual observation, μ is the overall mean, E is the effect of the inoculum source and e is the random variation.

6.3 Results

6.3.1 Enzyme assays

Exocellulase, endocellulase and hemicellulase specific activities were higher ($P < 0.001$) in *the in vitro* cultured microbial ecosystems than in the fresh microbial ecosystems (Table 6.2). For exocellulase enzymes, N1, N2, and N4 had the highest specific activities followed by H1 with intermediate activity. Exocellulase specific activity was also relatively high in N3, WB1 ZB1, mH1 and CW1 but lower for the fresh enzyme systems.

Table 6.2 The specific activities of exocellulase, endocellulase and xylanase of crude protein extracts from nine *in vitro* microbial ecosystems

Inoculum source	Hemicellulase (µg xylose/mg)	Endocellulase (µg glucose/mg)	Exocellulase (µg glucose/mg)
CW	38.0 ± 2.34	2.9 ± 0.01	1.8 ± 0.02
mH	30.0 ± 2.14	5.9 ± 0.05	4.5 ± 0.07
H	46.3 ± 3.11	6.4 ± 1.00	5.4 ± 0.20
WB	8.0 ± 2.01	3.7 ± 0.10	3.2 ± 0.06
ZB	43.5 ± 4.10	5.9 ± 0.14	4.4 ± 0.30
CW1	83.2 ± 1.21	78.6 ± 2.11	68.2 ± 3.00
mH1	75.4 ± 1.66	83.3 ± 3.01	94.9 ± 3.08
H1	98.3 ± 1.90	92.7 ± 2.31	141.8 ± 9.03
WB1	57.3 ± 5.76	72.6 ± 0.45	90.5 ± 2.67
ZB1	88.8 ± 1.53	89.2 ± 2.11	107.1 ± 0.04
N1	81.4 ± 1.17	87.8 ± 4.57	163.1 ± 6.67
N2	104.5 ± 2.62	77.7 ± 2.65	162.6 ± 2.73
N3	68.6 ± 2.85	80.5 ± 0.53	98.1 ± 2.76
N4	183.1 ± 4.74	108.5 ± 4.11	160.9 ± 9.33
P-value	0.001	0.001	0.001

±SE=standard error

Endocellulase activity differed ($P < 0.001$) among the microbial ecosystems with systems N4, H1, ZB1, N1 recording the highest specific activities. Systems N3, N2, WB1, mH1 and CW were intermediate in their endocellulase specific activities while enzyme activities were lower for the fresh microbial ecosystems. Hemicellulase activity varied among the microbial ecosystems. System N4 recorded the highest hemicellulase activity while N2, H1 and ZB1 were intermediate. Hemicellulase specific activities were much lower in the fresh systems than in the rest of the cultured systems.

6.3.2 SDS-PAGE analysis

SDS-PAGE demonstrated that all the CPZ extracts from the different microbial ecosystems contain proteins of diverse molecular weights. These CPZ contain numerous proteins bands ranging from 1 to 250 kDa (Figure 6.1A, B, C, D, E and F).

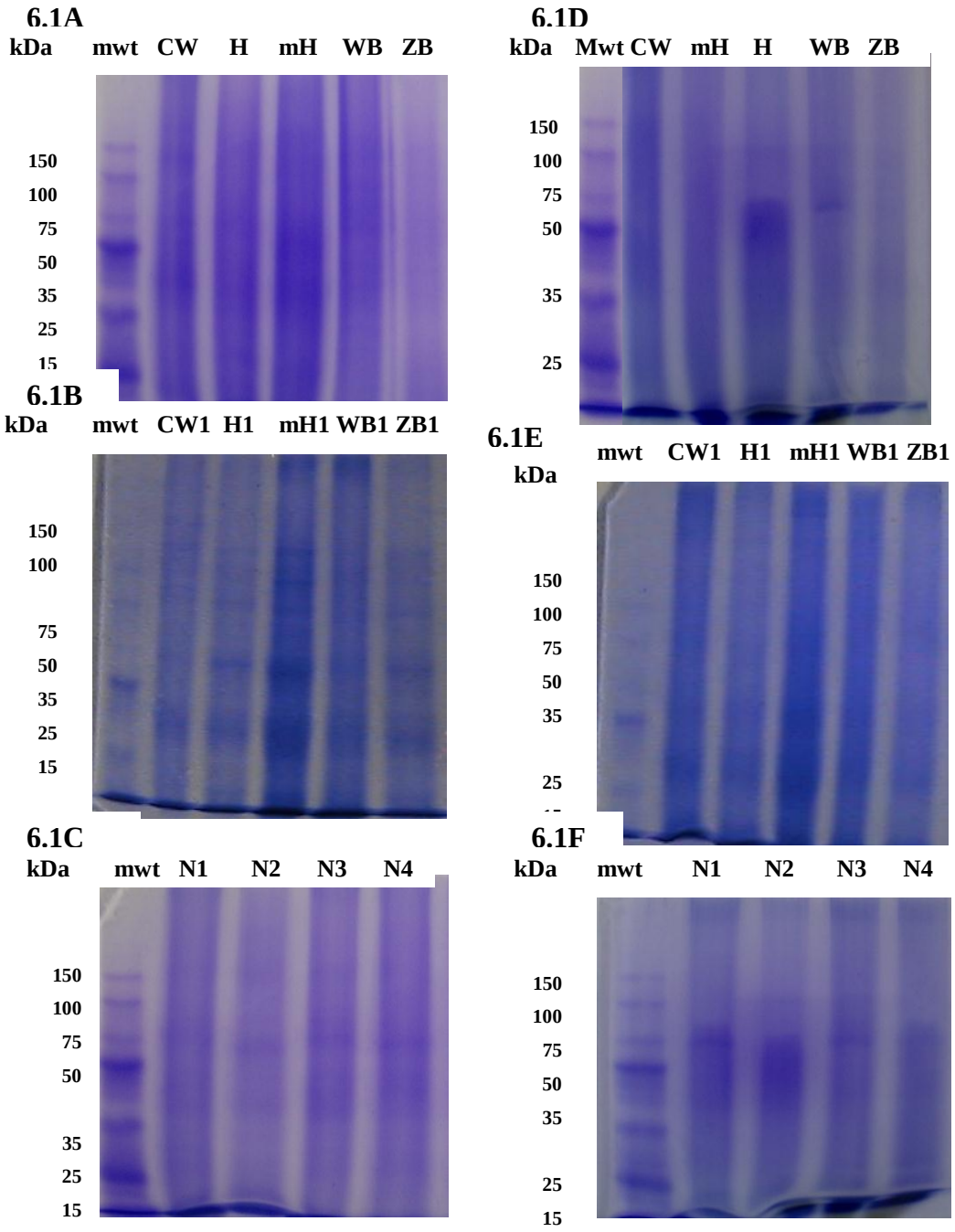


Figure 6.1 Coomassie stained Laemmli reducing and non-reducing 10% SDS-PAGE monitoring the presence of proteins in crude protein extracts from different microbial ecosystems. 6.1 A, B and C are reducing SDS-PAGE while D, E and F are non-reducing SDS-PAGE. mwt=molecular weight markers (150, 100, 75, 50, 35, 25 and 15 kDa), fresh crude protein extract were from cow (CW), horse (H), miniature horse (mH) ,wildebeest (WB) and zebra (ZB) while cultured crude protein extracts were from CW1, H1, mH1, WB1, ZB1, N1 (H+WB), N2 (H+ ZB), N3(WB+ZB) and N4 (H+WB+ZB).

The quantity of each protein in the fresh CPZ was much smaller compared to those of the cultured ecosystems. This was demonstrated by the prominent protein bands observed in cultured CPZ (Figure 6.1A and D) than fresh CPZ (Figure 6.1 B, C, E and F).

Specific protein abundance was variable in the different microbial ecosystems Figure 6.1 C (30, 48, 50 and 91 kDa), Figure 6.1 B (30, 40, 50, 70, 75 and 80 kDa), Figure 6.1E (30 and 38 kDa) and figure 6.1F (75 and 85 kDa).

6.3.3 Cellulase zymography

All nine CPZ extracted from *in vitro* microbial ecosystems exhibited carboxymethyl cellulase (endocellulase) activities as demonstrated by the clear zones (caused by digestion of CMC on gels) observed on the zymograms (Figure 6.2). One major (216 kDa) activity bands was observed in all the CPZ from fresh systems (Figure 6.2A). Besides protein 216 kDa, the protein 180 kDa also exhibited a higher endocellulase activity in WB and ZB. There were many other active bands on the gel that were too small to be captured by the photographic equipment available. These clear bands were read and reported on Table 6.3A. The active band of molecular weight 216 kDa was common in CPZ from all the cultured systems. The enzyme of 191 kDa was present in all the combined systems (Figure 6.2C) including H1 (Figure 6.2B). Not all bands in the cultured systems were captured by the camera. These active bands were read and reported on Table 6.3A and B. The total number of active proteins bands varied among the nine microbial ecosystems. Cultured microbial ecosystems CPZ had more active cellulases than fresh CPZ. In fresh CPZ, the number of active CMCases was 10, 7, 6, 5 and 5 for ZB, CW, WB, mH and H, respectively. In the cultured systems, the highest number of CMCases was found in N1 (16) and followed by N3, N4, and mH with 14 CMCases. Samples N2 and ZB had the least number of CMCases.

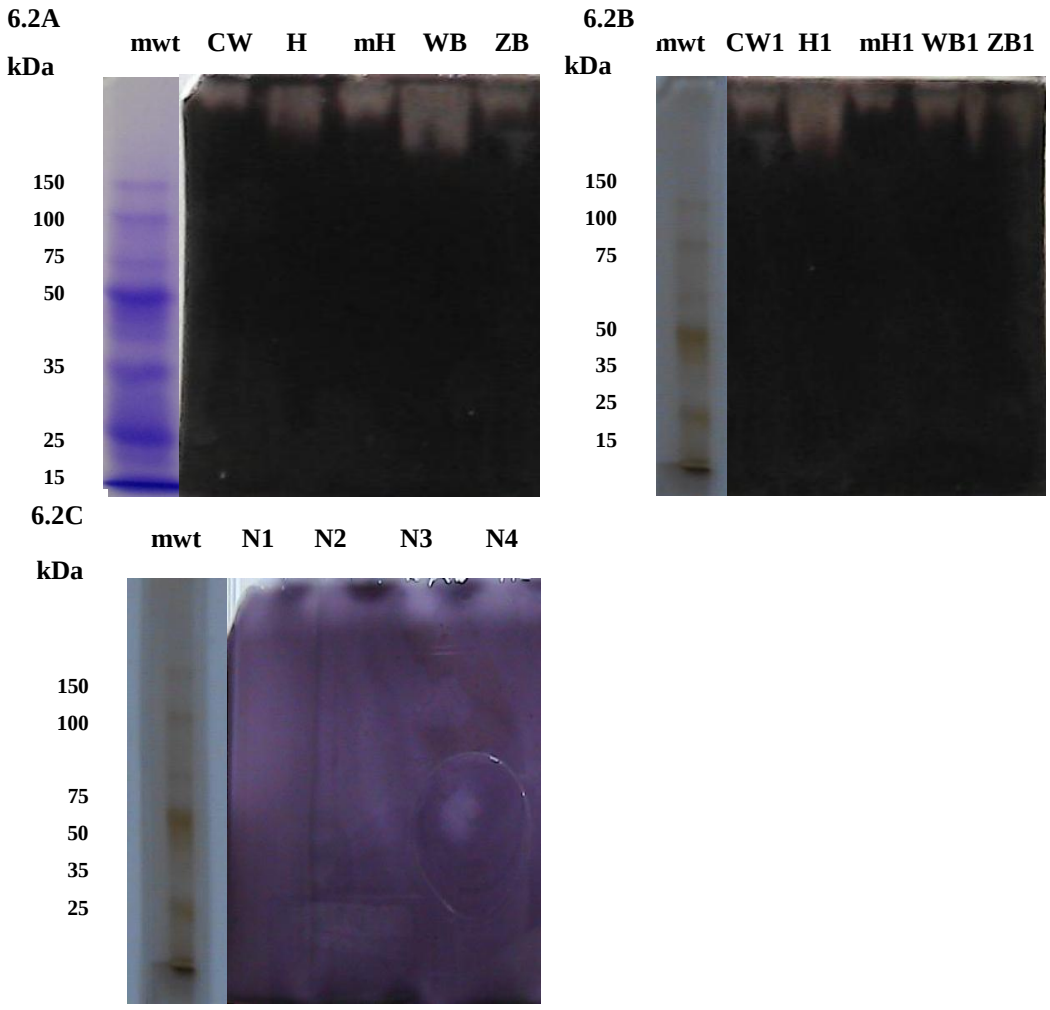


Figure 6.2 Zymograms of cellulase activities from crude protein extracts of nine in vitro microbial ecosystems on Laemmli non-reducing polyacrylamide gel electrophoresis. Endocellulases (Carboxymethyl cellulase) activities detected on non-reducing polyacrylamide gels containing 1% CMC (6.2 A, B, C). The wells were loaded with crude protein samples (10µg/5µl) extracted from both fresh (CW, H, mH, WB, ZB) and cultured (CW1, H1, mH1, WB1, ZB1, N1, N2, N3 and N4) microbial ecosystems and incubated for 48 h at 38°C. The gels were stained with Congo red (Beguin, 1983) and rinse with 5% acetic acid which changed the background to blue-black for easy photographing. The molecular weight marker (mwt) were stained with Coomassie-blue-250 or silver stain (where protein quantities on gel too small to be stained by Coomassie blue-250).

Table 6.3 A. Apparent molecular masses of cellulase enzymes from crude protein extracts of five *in vitro* microbial ecosystems

Enzyme	Enzyme source									
	CW	**mH**	**H**	**WB**	**ZB**	**CWI**	**mHI**	**HI**	**WBI**	**ZBI**
Approximate molecular Weight (KDa)	216	216	216	216	216	216	216	216	216	216
	175	165	175	124	180	185	185	191	206	165
	124	124	150	74	165	180	175	175	195	155
	119	119	135	48	150	175	165	165	185	150
	114	48	48	48	99	150	155	150	175	135
	74	-	-	38	74	145	150	145	160	124
	23	-	-	-	58	124	145	124	150	114
	-	-	-	-	48	114	135	109	145	99
	-	-	-	-	18	99	124	104	135	74
	-	-	-	-	3	84	104	99	124	64
	-	-	-	-	-	74	99	74	64	58
	-	-	-	-	-	64	74	64	23	48
	-	-	-	-	-	33	53	48	-	-
	-	-	-	-	-	23	48	18	-	-
	-	-	-	-	-	18	-	-	-	-

Table 6.3 B. Apparent molecular masses of cellulase enzymes from crude protein extracts of four *in vitro* microbial ecosystems

Enzyme	**Enzyme source**			
	N1	**N2**	**N3**	**N4**
Approximate molecular Weight (KDa)	216	216	216	216
	195	191	191	201
	191	170	185	191
	175	150	175	175
	170	124	150	165
	156	114	135	160
	150	99	114	150
	135	74	109	135
	124	53	74	124
	74	48	48	99
	69	23	33	94
	64	6	13	74
	59	-	6	64
	48	-	3	23
	33	-	-	-
	23	-	-	-
	10	-	-	-

N1 (H+WB), N2 (H+ ZB), N3 (WB+ZB) and N4 (H+WB+ZB).

6.4 Discussion

This study was designed to investigate the presence and variation of cellulases in 5 *in vitro* herbivore microbial systems (CW, H, mH, WB and ZB) and their survival in combined microbial ecosystems (N1, N2, N3, and N4). The results from this study were also going to provide more evidence to explain the variation of cellulase activities within and among herbivore microbial ecosystems examined in the previous chapters. All CPZ extracts were active as demonstrated by their exocellulase, endocellulase and hemicellulase activities when incubated with their specific substrates at optimum pH.

Separation of crude protein extract on both reducing and non-reducing SDS polyacrylamide gels was successful after staining with Coomassie blue G-250 but did not form prominent bands that could be captured by the camera at our disposal. The lack of prominent bands on the gels was due to low individual protein quantity since CPZ extracts contain numerous proteins and only a limited amount can be loaded on the gels at a time (10 µg). Using a more sensitive method (Silver stain) to make these bands more visible failed as the whole gel turned black due to numerous protein bands (Oakley et al., 1980). However, the protein bands were more prominent for cultured CPZ than fresh. This was probably due to the absence of endogenous protein contamination in cultured systems where cellulolytic protein concentrations were being enriched on MS and LC feed. The total number of active CMCases on fresh CPZ (CW, H, mH, WB and ZB) was less than those observed in cultured CPZ (CW1, H1, mH1, WB1 and ZB1). The lack of these CMCases in fresh CPZ was probably due to smaller quantities of these specific enzymes than their absence. Therefore, clear band formation on the gels might have been limited by smaller quantities of specific proteins on gels given the time of incubation. These results show that where cellulase enzyme purification methods are limited, the application of cultured CPZ extracts for enzyme assays and identification would be preferred to fresh CPZ.

The number and type of cellulases (CMCases) vary within and among herbivores species grazing on the same field. Seven of the proteins bands (216, 175, 150, 145, 124, 99 and 74 kDa) with CMCase activities were common in CW, H and mH grazing on the same field while

the remaining 13 cellulases were either present in one or two of the three animals. Five of the proteins (216, 150, 124, 99 and 74 kDa) with CMCase activities from herbivores in the wild were also found in the domesticated herbivores (CW, H and mH). The following proteins with CMCase activities; 206, 195 and 58 kDa were unique for herbivores in the wild. However, the rest of the CMCases found in WB and ZB were either present in CW and mH or H alone. Metagenomic studies of bovine rumen microflora by Ferrer et al. (2005) revealed nine active endo-beta-1,4-glucanases less than observed in our study. This implies that crude method screening is very important to approximate the different types of active enzymes available prior to purification and expression.

The combined microbial ecosystems exhibited CMCase activities as clear bands on zymograms. Clear bands on zymograms are evidence of CMC digestion as previously established by other researchers (Kim et al., 2009). Apart from N2, the total number of proteins with CMCases activities in N1, N3 and N4 were higher than those of the individual systems from which they were formed. The systems were ranked according to the number of CMCases present as follows; N1>N3>N4>N2. Although system N1 had the highest number of CMCases (17), it was formed from individual systems with a combined CMCases number of 20 which was less than that from which N4 (23), N3 and N2 were formed. The variation in cellulase numbers observed in this study goes a long way to confirm the diversity of enzyme and microbes in the same or different microflora (Ferrer et al., 2005; Wang et al., 2009; Duan and Feng, 2010). Nine CMCases from the individual systems H1 and WB1 were absent in N1. There is likelihood that the expression of these missing enzymes might have been inhibited by other microbes in the systems during colonization of the different ecological niches. It is also possible that these enzymes might still be there but in smaller quantities (not detected on the gels) if they are not being expressed by the dominant microbes in the ecological systems. There is also a possibility of proteolysis by the dominant microbial species hence reducing the total quantity of such proteins (Morgavi et al., 2000; Morgavi et al., 2001).

The combined microbial ecosystems N1, N2, N3, and N4 were found with 5, 4, 5 and 2 new CMCases respectively, which were not visible on the individual systems zymograms from which they were formed. These new enzymes may be coming from microbes which might

have proliferated to become dominant in the new system conditions that were probably lacking in the previous individual ecological system. It is also possible to suggest that these enzymes might have been there but in smaller quantities unable to form clear bands on the zymograms given the incubation time.

The molecular weights of proteins with CMCase activities in this study were ascertained online (UniProtKB/Swiss-Prot, 2011) to verify their presence on protein data bases. Almost all the CMCases were matched with cellulases of the same or similar molecular weights with protein names as cellulases or endoglucanase or endocellulase (with CMCase activities). These matching proteins were mainly from ruminant or hindgut microbes. Some of the proteins with CMCase activities (3 and 6 kDa) were not found online from rumen or hindgut microbes but were available from other sources (Jauris et al., 1990; Waksman, 1991; Bronnenmeier et al., 1997; Clarke et al., 1997). Therefore, more research is required to establish these proteins as novel CMCases. The molecular weight of cellulases isolated from rumen or hindgut microbes ranges from 9 to 160 kDa online. This, therefore, calls for an explanation of the proteins bands that were greater than 150 kDa observed with CMCase activities (175, 180, 191, 185, 206 and 216). These proteins of higher molecular weights with CMCase activity were suggested to be cellulosomes (complexes of cellulolytic enzymes). This was confirmed after comparison with a databse (UniProtKB/Swiss-Prot, 2011) and identifying cellulosomes with similar molecular weight (Ahsan et al., 1996; Najmudin et al., 2006). However, some cellulosomes were found with molecular weights lower than 150 kDa (Gal et al., 1997). With the results obtained from zymography, it is possible to conclude that all the proteins that formed clear bands on the zymogram had CMCase activities but could not conclude that each clear band was a CMCase or cellulosome enzyme. These call for more research on the purification of these enzymes before any absolute conclusion can be made.

6.5 Conclusion

This study shows that all seven *in vitro* microbial ecosystems were active as demonstrated by their carboxymethyl cellulase activities. Cellulases activities on zymograms are better studied with cultured than fresh inocula as culturing increases specific protein concentration. CMCase

expressions vary within and among animal species grazing on the same field. CMCase enzymes were higher in CW than mH and H grazing on the same field. Wildebeest and ZB did not differ in terms of the numbers of CMCases expressed but varied with the types of CMCases expressed. Transinoculation was possible as indicated by the activeness of the combined microbial ecosystems. The combined systems were ranked according to the number of cellulases they retained as follows; N1>N3>N4>N2. Because of the variation of cellulases within animal and among animal species and the possibilities of transinoculation, identifying novel enzymes from the wild could be beneficial in animal production as feed additives. Although four proteins of apparent molecular weights 206, 216, 6 and 3 kDa exhibiting CMCase activities seems to be novel, more research is still required for their purification and characterization.

Chapter 7

Effect of direct-fed microbials on ruminal fermentation of maize stover in sheep

Abstract

These experiments were conducted to evaluate the effect of direct-fed microbials (faecal inoculum from; N1 (horse + wildebeest, 1:1 ratio) and N3 (wildebeest + zebra, 1:1 ratio)) on maize stover (MS) degradation, ruminal fermentation characteristics and cellulase enzyme profile. In experiment 1, six fistulated male Marino sheep (44±1.5 kg) receiving 1.3 kg of MS and lucerne (1:0.3 ratio), mineral block and water *ad libitum*, were randomly assigned to two groups of three sheep (control and treatment). The treatment groups were dosed with 50 g faeces (on collection day) or 50 ml (cultured faecal inocula) of N1 every 3.5 d through the fistula. After 15 days of adaptation, the degradation characteristics of MS were measured by incubating 3g in nylon bags for 96, 72, 48, 24, 12, 9, 6 and 3 h in the rumen of the sheep. Rumen fluid was analysed for ruminal fermentation characteristics (pH, short chain fatty acids (SCFA), CO_2, CH_4) and cellulase enzymes (exocellulase, endocellulase and hemicellulase). In experiment 2, experimental animals, feeding and the adaptation period were the same as in experiment 1 but for the inoculum (N3). Exocellulase and endocellulase specific activities (µg glucose/mg) increased (P<0.05) in N1 (1.1 and 1.8 fold) and N3 (1.3 and 1.5 fold) compared to its controls whereas hemicellulase specific activities (µg xylose/mg) were lower in both N1 and N3. Feed dry matter intake (DMI) increased (P<0.03) in N1 but tended to increase (P=0.083) when inoculated with N3. No significant differences were observed for the following degradation parameters of MS; washing loss (A), insoluble but degradable fraction of MS (B), rate of degradation of B (C), potential degradability and effective degradability in both N1 and N3 after 96 h. Although total SCFA and propionic acid were relatively higher in N3 than the control, the differences were not significant. Interestingly, both CH_4 and CO_2 were relatively lower in both N1 (3 and 1.9%) and N3 (9 and 1.9%), respectively. These results show that, direct-fed microbials from N1 and N3 increased cellulase activity with the potential of increasing MS degradation and DMI. Optimization of such systems can improve livestock production at a reduced feed cost as the method is cheaper and will be readily available to all farmers, especially in the developing countries.

7.1 Introduction

Most rumen microbial studies are geared towards the improvement of fibrous feed utilization, animal production and performance, animal health and feed safety (Seo et al., 2010). Numerous feed additives such as enzymes, antibiotics, probiotics and prebiotics have been used to manipulate the rumen microbial ecosystem and its fermentation characteristics (Titi and Tabbaa, 2004; Chaucheyras-Durand et al., 2008; Chaucheyras-Durand and Durand, 2010; Silva et al., 2010; Antonio Facchini et al., 2011; Yang et al., 2011). Exogenous fibrolytic enzymes hold a lot of promise as means of increasing forage utilization, milk production, average daily weight gain and improving the productive efficiency of ruminants (Lee et al., 2000; Wang and McAllister, 2002; Antonio Facchini et al., 2011; Nagpal et al., 2011; Yang et al., 2011) but limited by their hydrolysis in the rumen environment (Lee et al., 2000; Peters et al., 2010). However, some studies demonstrated little or no significant changes in the fermentation parameters measured when supplemented with enzymes (Peters et al., 2010). As the use of antibiotics in ruminant feeds has been banned, supplementing fibrous forages with probiotics (microbes or direct-fed microbials) that can survive in the rumen could be a better approach. If the microbes can colonize and establish (gain stability) in the rumen, then fibrolytic enzyme expression would be continuous. This could probably be a better approach compared to enzyme additives that are usually not cost effective in livestock production systems.

Probiotics (direct-fed microbials) were primarily introduced in herbivore research as therapeutic agents. There are many studies illustrating the therapeutic potential of direct-fed microbials (DFM) e.g. the treatment of goats that were excreting mimosine toxic by-products (3-hydroxy-4 (1H) pyridone) (Palmer et al., 2010), ruminants suffering from acidosis (Fonty and Chaucheyras-Durand, 2006; Chaucheyras-Durand et al., 2008; Chiquette, 2009; Maldonado et al., 2011), oxygen toxicity (Newbold, 1996), animals suffering from diarrhoea caused by enterotoxigenic *Escherichia coli* K88 (Setia et al., 2009) and gastrointestinal nematodes infections in lambs (Eysker et al., 2006). In ruminant nutrition DFM have also been applied to maximize forage utilization and enhance production efficiency (Beauchemin et al., 2006). The different digestibility measurements which have shown marked increased upon

supplementation with DFM includes, milk production (Nocek et al., 2003; Stella et al., 2007), average daily weight gain (Aydin et al., 2009), final weight (Adams et al., 2008), dry matter intake (Nocek et al., 2003; Stella et al., 2007; Sehgal et al., 2008; Aydin et al., 2009), total microbial population (Martin and Nisbet, 1992; Lee et al., 2000), fibre utilization and fibre digestibility (Lee et al., 2000; Guedes et al., 2008; Sehgal et al., 2008; Stephens et al., 2010) and animal performance (Cruywagen and van Zyl, 2008). However, some studies did not observed any significant change in their digestibility parameters when supplemented with DFM (Arcos-García et al., 2000; Elam et al., 2003; Yang et al., 2004; Lee and Seo, 2005; Raeth-Knight et al., 2007).

Although a few experimental studies on DFM supplementation did not improve forage utilization in ruminants, many others have shown that DFM have the potential of improving fibre digestibility. That is why this study will be investigating the effect of DFM from three herbivores (horse, wildebeest and zebra) on maize stover (MS) fermentation in sheep. The novelty of this study is the application of crude microbial inocula (containing bacteria, protozoa and fungi) for inoculation compared to pure and specific microbial strains utilised in most studies (Angeles et al., 1998; Nocek et al., 2002; Lee et al., 2004; Mwenya et al., 2005; Wang et al., 2005; Nocek and Kautz, 2006; Jin et al., 2007; Hagg et al., 2010). In our previous studies 11 herbivore microbial ecosystems were scanned in search of potential fibrolytic ecosystems of which the horse, wildebeest, zebra and elephant showed the highest enzyme specific activities (Fon, 2006). Combined enzyme systems were created from these animals and their *in vitro* fibrolytic enzyme and fermentation potential, using MS as a substrate was investigated. The results obtained from the *in vitro* study showed that the combined microbial ecosystems N1 (horse + wildebeest) and N3 (wildebeest + zebra) holds a lot of promise in improving forage utilization in herbivores. The objective of this study was to investigate the *in vivo* effect of DFM from N1 (H+WB) and N3 (WB+ZB) on ruminal fermentation of MS in sheep. It was hypothesized that *in vitro* transfer of DFM from N1 and N3 would increase MS fermentation in sheep.

7.2 Materials and Methods

7.2.1 Materials

Enzyme assay substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany) and xylan from beech wood (high grade) from SIGMA (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose and D-(+)-glucose were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) from MERK Laboratory supplies, South Africa and 3, 5-Dinitrosalicylic acid from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide and ethylene diamine tetra acetic acid (EDTA) were bought locally from Capital Supplies, South Africa. Maize stover and lucerne were bought locally. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) was from sigma-Aldrich. All spectral scans were carried out with Virian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd), Australia.

7.2.2 Experimental diet, animals and design

7.2.2.1 Feed composition

Table 7.1 Feed compositions for *in vivo* fermentation study

Feed composition	Mass of nutrient (g/kg)
Maize stover	769
Lucerne	231
Mineral block	
Calcium	120
Phosphorus	60
Sulfur	35
Magnesium	30
Manganese	1.2
Copper	0.3
Cobalt	0.003
Iron	0.75
Iodine	0.015
Zinc	1.2
Selenium	0.003
Total crude protein	87

Maize stover and lucerne (ground through a 5 mm sieve) as a supplement were from the Ukulinga Research farm, University of KwaZulu-Natal, Pietermaritzburg. The feed was formulated as shown on Table 7.1. The mineral block (of molasses base, from Voermol Feed Maidstone, Kwazulu-Natal, SA) was richer in calcium, phosphorus, sulfur and magnesium.

7.2.2.1.1 Chemical composition of feed

The rationed feed (comprised of MS and LC in a ratio of 1:0.3) was ground through a 2-mm sieve and DM determined after drying for 24 h at 60°C. The micro-Kjeldahl method was used to determine nitrogen and crude protein (CP) calculated as N x 6.25 according to AOAC (Ref. S585.A8) (1990). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by Van Soest et al. (Van Soest et al., 1991) using the ANKOM Technology Method. Hemicellulose was the difference between NDF and ADF while the difference between ADF and ADL was cellulose. Maize stover (MS) chemical composition was analysed as previously described in chapter 3 (section 3.2.1).

7.2.2.2 Experimental animals, feeding and experimental design

For microbial inoculum preparation, samples (faeces) were collected in winter from the following animals; horses (H), wildebeests (WB) and zebras (ZB) with no preference to sex. *Equus caballus* (H) was from the Ukulinga Research farm, University of KwaZulu Natal, Pietermaritzburg. *Equus quagga boehmi* (ZB) and *Connochaetes taurinus* (WB) were available in the Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA). The ZB and WB were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) standing hay and other fibre-rich grasses were dominant. Although the H was grazing on a dry land in an open field where Kikuyu grass standing hay and other fibre sources were dominant, they were supplemented with hay while in pens.

For *in sacco* digestibility studies, six fistulated male Merino sheep (from Ukulinga Research farm, University of KwaZulu-Natal) with an average mass of 44 (SD = ± 1.5) kg were used. These animals were divided into two groups of three (control and treatment animals) separated

by a distance of 20 m. These animals were housed in individual pens (230 x 203 cm) and fed at *ad libitum*. Each sheep was fed a total of 1.3 kg feed (Table 7.1) per day (0.8 kg and 0.5 kg at 8:00 and 15:30, respectively). Each sheep was given a 12 kg mineral block of trace elements as indicated on Table 7.1 at the start of the experiment. The mineral block residue was only weighed at the end of the trial. Water was provided *ad libitum*. The pens were shaded and properly ventilated. The animals were fed for a total of 21 days which incorporated 15 days of adaptation and 6 days of sample incubation and collection period. All animals were treated for internal parasites before experimentation. All animals' treatments were governed by the UKZN ethical roles (083/10/Animal).

7.2.3 Faecal collection and inocula preparation

Faeces were collected on farm within 2 min of defecation from H, ZB and WB before transferring into an airtight thermo flask (38°C) which had been flushed with CO_2. While in the laboratory, 300 g of faeces was mixed with 300 ml of warm incubation buffer (buffer C, chapter 3, section 3.2.3) and mixed thoroughly before squeezing through four layers of cheese cloth (pre-warmed and flushed with CO_2) to make faecal fluid (FF). Faecal fluid preparation was done in the shortest possible time in order to minimize microbial exposure to oxygen since it is anaerobic (Newbold, 1996).

7.2.4 Faecal fluid culturing

Table 7.2 Faecal fluid proportions for culturing microbial ecosystems

Ecosystems	Faecal fluid proportions (ml)	Salivary buffer (ml)	Substrate (g) MS:LC
N1	99:99	402	3:3
N3	99:99	402	3:3

H=horse, WB=wildebeest, ZB=zebra, N1= H+WB, N3=WB+ZB, MS=maize stover, LC=lucerne

Faecal fluid was cultured in the laboratory for *in vivo* inoculation of sheep as previously described in chapter 3 (Section 3.2.4) with some few modifications. Faecal inocula from the H, WB and ZB were used to create two combined microbial ecosystems (CS); N1 (H+WB) and N3 (WB+ZB) (Table 7.2). These inocula (198 ml) were incubated with salivary buffer (402 ml) containing 6 g of MS and LC (1:1) for 72 h at 38°C. The activeness of these microbial ecosystems was determined by monitoring pH changes and gas pressure. After 72 h of incubation, the sample fluid for each system was used for inoculating the sheep for *in sacco* digestibility studies.

7.2.5 *In sacco* degradability and ruminal fermentation

7.2.5.1 Sheep inoculation and *in sacco* degradability

During the feeding adaptation period as described in section 7.2.2.2 (21 days), only the treatment sheep were being dosed with inocula from N1 or N3 at 3.5 d interval. In experiment 1, three of the six fistulated sheep were dosed (through the fistula) with 50 g of fresh faecal matter from N1 while the other three received no treatment (control). After 3.5 d, the sheep were dosed again with 50 ml of cultured N1. All inocula (N1) were cultured in the laboratory for a maximum of 3.5 d. This implies that after every seventh day of the experiment, fresh samples were collected for inoculation and culturing for subsequent inoculation (after 3.5 d). After adaptation period, sheep dosing continued at the same interval with the same mass or volume till the end of experiment 1. Because of the limitation of fistulated animals only one treatment was tested at a time (3 control and 3 treatment sheep). In the second trial (Experiment 2), three sheep were dosed with N3 while the other three animals received no treatment (control). In experiment 2, the feeding process, inoculation, adaptation, sample incubation and collection were the same as in Experiment 1.

In sacco degradability of MS by N1 or N3 supplemented sheep were investigated in this study as previously described by Kempton (1980) with slight modifications. *In sacco* dry matter degradability (DMD) of MS was determined by incubating approximately 3.0 g (ground through a 3 mm sieve) in nylon bags (41 µm pore size; bag size 6.5 x 14 cm) in the rumen of

fistulated sheep. At each incubation period, one nylon bag of MS was suspended in the rumen of each sheep through the fistula. Sequential addition method was adopted for sampling with a maximum number of 10 bags per sheep. All the bags were withdrawn from the rumen after incubating for 96, 72, 48, 24, 12, 9, 6 and 3 h. The incubated bags were thoroughly washed under running tap water until no coloured liquid could be extruded before transferring into a washing machine (Hoovermatic, model T4350, South Africa) for a final wash. In the washing machine, the incubated bags were washed (5 x 3 min) together with three nylon bags (containing 3.0 g of MS) that were not incubated in the rumen to determine the washing losses. After washing, the bags were dried for 2 d at 60°C, cooled in a desiccator and weighed. The degradability at each time interval was calculated by taking the mean value obtained from the set of bags as well as for the zero hour. For each incubation time, one bag (two for 24 and 48 h) was incubated in each animal for each treatment (control, N1 or N3) that was replicated thrice per treatment. The degradability (Y) of MS at time (t) was estimated by using the non-linear curve proposed by McDonald (1981) and modified by Dhanoa (1988):

$Y = A + B\ (1-e^{-C*(t-lt)})$……..……..equation 7.1

$PD = A + B$……..equation 7.2

$ED = A + B*C/(C + 0.03)$ ……...equation 7.3

where Y= disappearance of DM at time (t), A= readily soluble fraction of MS (washing loss), B = insoluble but degradable fraction of MS, C= rate of degradation of B, lt = lag time, PD= potential degradability, ED= effective degradability and 0.03 h^{-1} was the assumed passage rate (kp) as determined by Bonsi et al. (1994) and Nsahlai et al. (1998).

7.2.5.2 pH and short chain fatty acid measurements

For pH measurements, rumen fluid (RF) was collected from each sheep on day 20 at the following times: 0, 3, 6, 9, 12 and 21 h. The pH of these samples were determined on-farm after which 5 ml was acidified in 1 ml of 25% (v/v) metaphosphoric acid solution (to stop microbial activity) and stored in ice (Cottyn and Boucque, 1968). The samples were stored in ice for a minimum of 30 min (to precipitate contaminating proteins) or in the freezer until when required for analysis. After centrifugation (8000 x g, 20 min at 4°C), the filtrate (2 ml) was filtered through a syringe filter (0.45 µm pore from SUPELCO, USA) into a 2 ml clear

ABC Screw Top Vial from SUPELCO, USA. The vials were then transferred into an automatic sampler (HT280 from Brescia, Italy) linked to a Gas Chromatograph (YL6100GC, Young Lin, Korea) for analysis. A temperature-programmed cycle from 90 to 120°C rising by 5°C was applied in the column (BPX70x30x0.25x0.25). The injector block temperature was maintained at 260°C with nitrogen as the carrier gas (60ml/min). Hydrogen and air flow rate to the detector (flame ionization detector) were 60 and 300 ml/min, respectively. The injection volume was 1 µl/split 1:80. The molar concentrations of SCFA were determined from a linear curve of standards (acetic acid, propionic acid, n- and iso-butyric acid and valeric acid) that were run together with the samples. For pH and SCFA measurements, RF was collected once for each animal at different time intervals per treatment that was replicated thrice.

7.2.6 Rumen fluid collection, crude protein extraction and cellulase enzyme assays

Cellulase enzyme assays were carried out to monitor the fibrolytic enzyme activity of each supplemented systems. Rumen fluid was collected from each sheep per treatment (control, N1 and N3) and the crude proteins were precipitated using 60% ammonium sulphate (section **3.2.5**), dialyzed overnight (section **3.2.6**) and protein concentrations determined (section **3.2.7**).

Exocellulase, endocellulase and xylanase specific activities were determined by incubating with crystalline cellulose, carboxymethyl cellulose and xylan, respectively, as described in chapter 3 (section 3.2.8). Reducing sugars liberated from the different enzyme assays were measured as previously described by Miller (1959) (section 3.2.9). Enzyme specific activity was defined as µg of reducing sugar/mg crude protein. Each sample (N1 or N3) was replicated thrice and was analysed in triplicates.

7.2.7 Statistical analysis

The results from cellulase enzyme activities and MS degradability estimates derived from RF and *in sacco* degradability were subjected to analysis of variance (ANOVA) using the general linear model of SAS (2002). The model was:

$Y_{ij} = \mu + T_i + e_{ij}$

Where Y_i is the individual observation, μ is the overall mean, T_i is the effect of the treatment (N1 or N3) and e is the random variation.

7.3 Results

7.3.1 Chemical composition of whole maize stover and rationed feed

Chemical analyses of diet and whole maize stover showed that DM and CP tended to be higher in feed than whole MS (Table 7.3). Hemicellulose, cellulose, NDF, ADF and ADL tended to be higher in whole MS than feed.

Table 7.3 The chemical composition of feed and whole maize stover

	Chemical composition (g/kg DM)	
Feed constituents	Feed **(MS:LC)**	Whole maize stover
DM	935	925
NDF	723	878
ADF	447	543
ADL	60	73
Hemicellulose	276	335
Cellulose	387	470
Crude protein	87	56

MS:LC=maize stover and lucerne mixed in 1:0.3 ratio, DM=dry matter of feed,

NDF= neutral detergent fibre. ADF= acid detergent fibre and ADL = acid detergent lignin.

7.3.2 Daily feed intake of sheep

The mean feed (dry matter and organic matter (OM)) and crude protein (from OM) intakes are presented in Table 7.4. Sheep that were inoculated with microbes from N1 consumed more (P<0.01) feed than the control sheep (experiment 1). While in experiment 2, sheep that were

inoculated with N3 tended to increase DMI and OMI. The results also showed that crude protein intake were higher (P<0.03) in N3 animals compared to its control whereas crude protein tended to increase in N1 compared to its control.

Table 7.4 Effect of source of inoculum on daily feed intake of sheep

Treatments	Feed intake (g/day)		
	Dry matter	Organic matter	Crude protein (OM)
Experiment 1			
Control	1045	934	83
N1	1161	1043	92
SED	22	21	3
***P*-value**	0.03	0.03	ns
Experiment 2			
Control	966	863	90
N3	1064	957	100
SED	33	31	2
***P*-value**	ns	ns	0.01

OM= organic matter

7.3.3 Degradability characteristics of whole maize stover

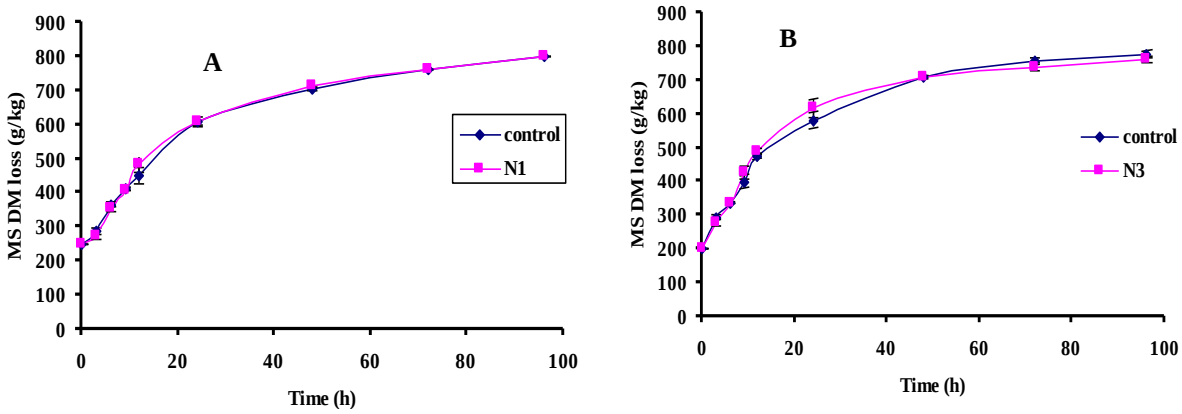


Figure 7.1 Effect of source of inoculum on dry matter disappearance of whole maize stover. Where A represents experiment 1 and B represents experiment 2, N1= Sheep supplanted with microbes from wildebeest and zebra, N3=sheep supplemented with microbes from wildebeest and zebra.

The mean *in sacco* DM degradability and degradability parameters of MS in experiment 1 and 2 are presented on Figure 7.1 and Table 7.5. The washing loss (A), insoluble but degradable fraction (B) and potential degradability (PD) of MS DM did not differ between the treatment and the control in both experiments (1 and 2). The rate (C) of degradation of B by N1 and N3 tended to be higher than that of its controls (Table 7.5). No significant difference was observed for effective degradability (ED) between N1 or N3 and its controls (in experiment 1 and 2). The lag time (lt) did not differ between N1 and N3 and its controls.

Table 7.5 Effect of source of inoculum on dry matter degradation parameters of whole maize stover

Maize stover Degradability	Experiment 1				Experiment 2			
	Ctrl 1	**N1**	**SED**	**P-value**	**Ctrl2**	**N3**	**SED**	***P*-value**
A (g/kg DM)	231	231	0	ns	231	231	0.0	ns
B (g/kg DM)	559	556	6	ns	545	534	14.2	ns
C (h^{-1})	0.044	0.047	0.004	ns	0.047	0.054	0.0101	ns
PD (g/kg DM)	790	787	6	ns	776	775	14.5	ns
ED (g/kg DM)	563	570	11	ns	564	570	11.3	ns
lt (h)	0.1	0.4	0.31	ns	0.5	0.7	0.30	ns

A= washing loss, B = insoluble but degradable fraction of MS, C= rate of degradation of B, lt = lag time, PD= (A+B)= potential degradability, ED= effective degradability, Kp = 0.03 h^{-1} (Nsahlai et al. 1998), N1= Sheep supplanted with microbes from wildebeest and zebra, N3=sheep supplemented with microbes from wildebeest and zebra.

7.3.4 Short chain fatty acid, carbon dioxide, methane and pH changes

The mean production of total SCFA, SCFA molar proportions and the gases calculated from the fermentation products of MS when supplemented with direct-fed microbials (from N1 or N3) are presented on Table 7.5 while the fermentation pattern with time for these products are presented on Figure 7.2.1, 7.2.2 and 7.2.3. The total SCFA, acetic acid and CH_4 produced by N1 at different time intervals were lower ($P<0.05$, $P<0.05$ and $P<0.05$ respectively) than those

observed in the control while differences were quite small for these parameters between N3 and the control (Table 7.6, Figure 7.2.1 and 7.2.3). Total SCFA was highest for N3 at 3 h post feeding and lowest at 9 h pre-feeding. The molar ratios of propionic acid, n-butyric, iso-butyric acid, iso-valeric acid and CO_2 were not different between N1 and the control (Table 7.6, Figure 7.2.1 and 7.2.2). Iso-butyric acid and iso-valeric acid were highest before feeding. No significant differences were observed between N3 and the control for the following parameters; iso-butyric acid, iso-valeric acid and CO_2 measured at different time intervals. Methane production by system N3 was lower (P<0.05) than that of the control. Methane production by N3 was lowest after 6 h of incubation. The mean pH measurements for both N1 and N3 showed no differences when compared with its respective controls (Table 7.6). The pH measurements over time showed a steady drop with time until the 12 h (Figure 7.2.3).

Table 7.6 Effect of source of inoculum on mean production of short chain fatty acids and gases from maize stover fermentation

Fermentation Parameters	Experiment 1				Experiment 2			
	Ctrl1	N1	SED	P-value	Ctrl2	N3	SED	P-value
Short chain fatty acids								
Total SCFA (mM)	51.4	45.5	1.75	0.05	60.7	65.0	1.98	ns
Molar proportions								
Acetic acid	0.74	0.71	0.003	0.05	0.69	0.70	0.003	ns
Propionic acid	0.19	0.21	0.001	ns	0.18	0.22	0.008	ns
n-Butyric acid	0.06	0.07	0.001	ns	0.10	0.08	0.006	ns
Iso-Butyric acid	0.01	0.01	0.001	ns	0.01	0.01	0.001	ns
Iso-valeric acid	0.01	0.01	0.001	ns	0.04	0.01	0.002	ns
CO2	0.52	0.51	0.001	ns	0.53	0.52	0.001	ns
CH4	0.35	0.34	0.001	0.05	0.35	0.32	0.002	0.05
pH	6.70	6.69	0.055	ns	6.67	6.70	0.090	ns

ns=non significant, ctrl=control, N1= Sheep supplanted with microbes from wildebeest and zebra, N3=sheep supplemented with microbes from wildebeest and zebra.

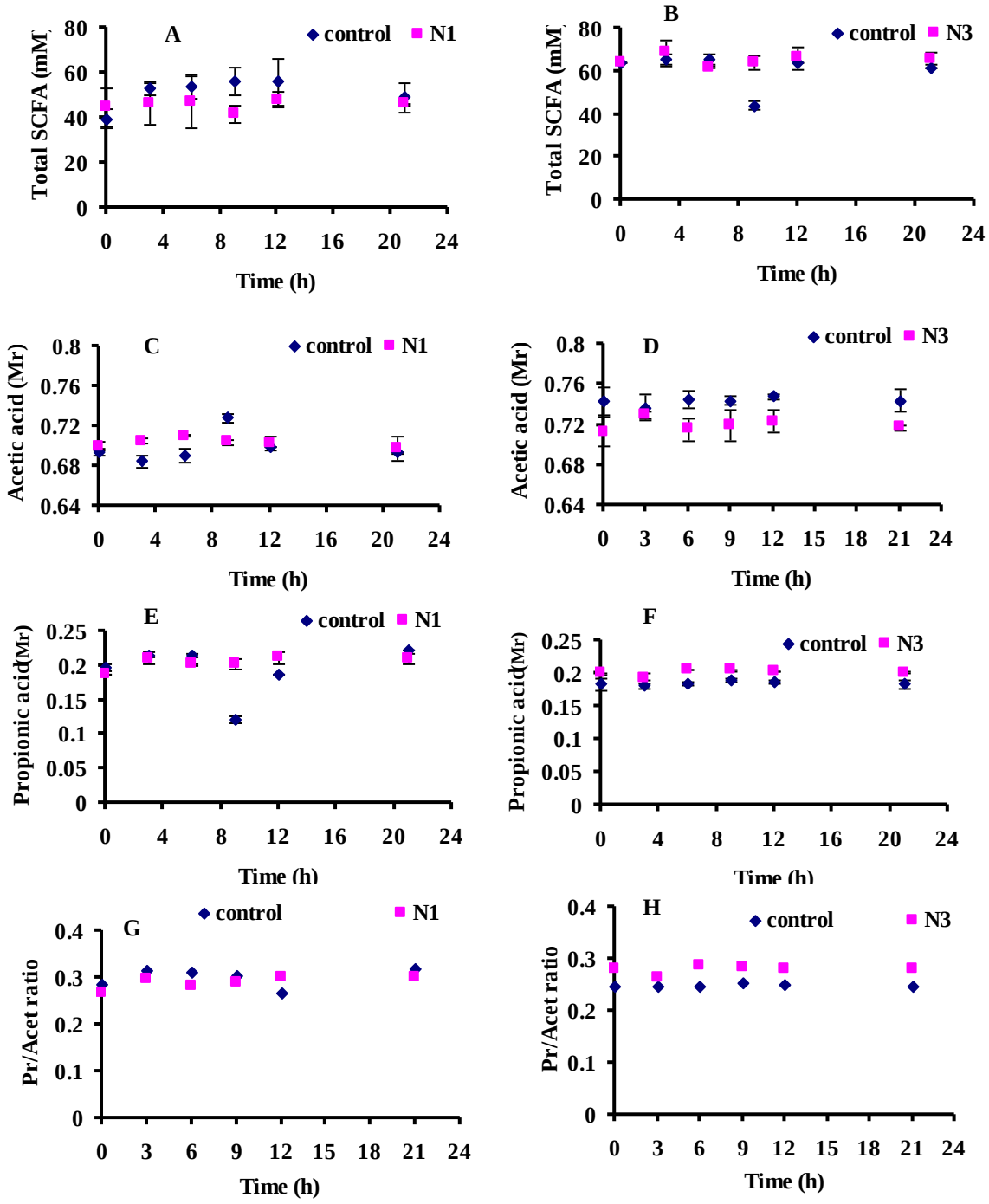


Figure 7.2.1 Effect of source of inoculum on total short chain fatty acids and molar proportions of acetic and propionic acid patterns as a function of time after feeding sheep with maize stover supplemented with microbes from N1 and N3. Where Figure A and B = taotal SCFA, B and C = acetic, D and E = propionate and F and G = Propionate/acetic acid (Pr/Acet) molar ratios.

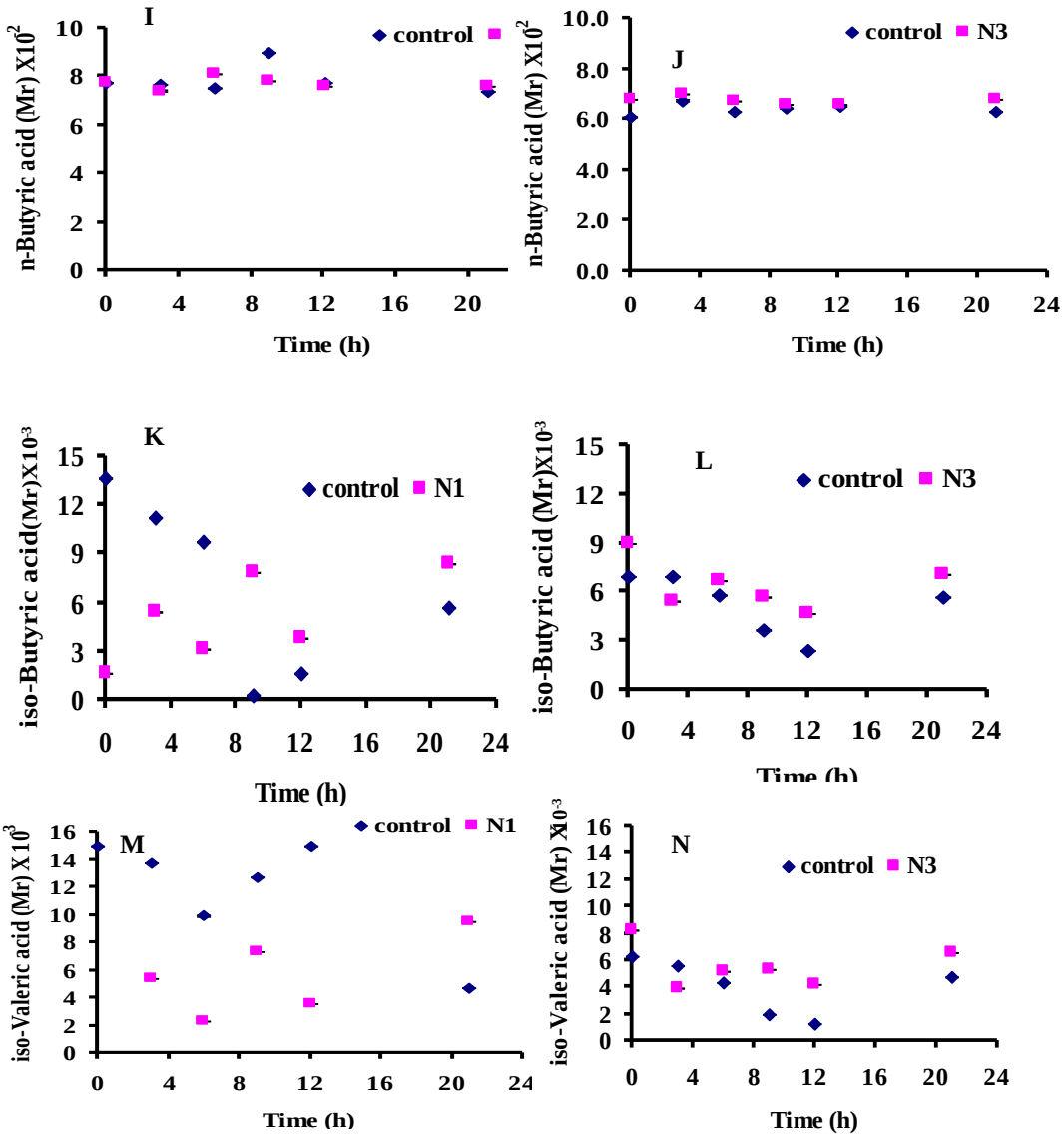


Figure 7.2.2 The effect of source of inoculum on the molar proportions of n-butyric acid, iso-butyric and iso-valeric acid patterns as a function of time after feeding sheep with maize stover supplemented with microbes from N1 and N3. Where Figure I and J = n-butyric acid, K and L= iso-butyric and M and N iso-valeric represent molar ratios.

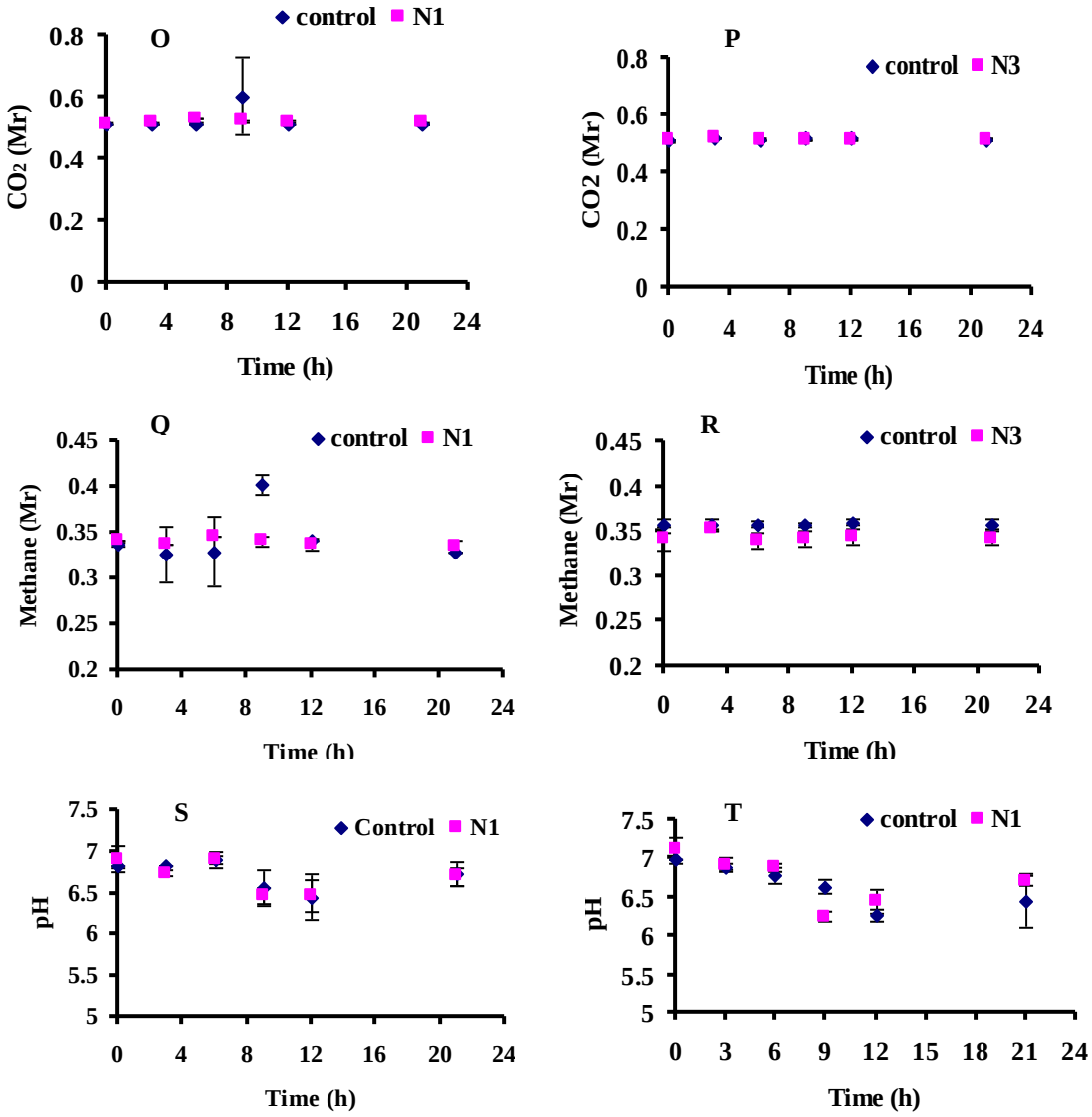


Figure 7.2.3 The effect of source of inoculum on pH, carbon dioxide and methane patterns as a function of time after feeding sheep with maize stover supplemented with inoculum from N1 and N3.

7.3.5 Activities of cellulases in the different systems

The different cellulase enzyme specific activities (exocellulase, endocellulase and hemicellulase) that were tested, varied (P<0.05) among the different experimental systems (Controls, N1 and N3). Exocellulase and endocellulase specific activities from N1 and N3 systems were higher (P<0.0001) than those of its controls (control 1 and control 2 respectively, Table 7.7). Exocellulases from N3 systems hydrolysed crystalline cellulose more than those from N1 and the control systems. Endocellulase activity was highest for N1 than in N3 and the control. Hemicellulase activity was most active in the control systems than in N3 and N1 ecosystems (Table 7.7).

Table 7.7 The effect of inoculum source on exocellulase, endocellulase and xylanase specific activities

Treatments	Enzyme specific activities		
	Hemicellulase (µg xylose/mg)	Endocellulase (µg glucose/mg)	Exocellulase (µg glucose/mg)
Experiment 1			
Control 1	146	56	96
N1	126	102	105
SED	4.2	3.3	1.1
***P*-value**	0.001	0.0001	0.01
Experiment 2			
Control 2	276	51	184
N3	248	75	245
SED	6.3	3.2	10.6
***P*-value**	0.001	0.001	0.001

N1= Sheep supplanted with microbes from wildebeest and zebra, N3=sheep supplemented with microbes from wildebeest and zebra

7.4 Discussion

7.4.1 Chemical composition of whole maize stover and rationed feed

The whole maize stover feed was supplemented with lucerne in order to meet the nutritional requirement for both ruminal microbes and host animals (Aganga and Monyatsiwa, 1999). Supplementing MS with lucerne (1:0.3 ratio) decreased the total amount of NDF, ADF, ADL,

hemicellulose and cellulose by 17.7, 17.8, 17.8, 17.7 and 17.6% respectively. The total amount of crude protein in the lucerne supplemented feed increased by 55%. This increase in crude protein concentration was associated to the relatively high protein content of lucerne which has also been reported in other studies (Aganga and Monyatsiwa, 1999; Dewhurst et al., 2009).

7.4.2 Feed intake and *in sacco* degradability of maize stover

Both DMI and OMI were 11.1 and 11.7 % higher in N3 inoculated sheep whereas crude protein tended to increase (9%). Sheep inoculated with N1 increased DMI and OMI by 10.1 and 10.9% respectively. The result obtained from N1 inoculation are similar to those obtained by Nocek and Kautz, (2006). However, DFM inoculation has been reported as tending to increase DMI as observed in N3 inoculation (Tripathi and Karim, 2010; 2011), no effect on DMI (Corona et al., 1999) or a decrease in DMI with an increase in the efficiency of fibre utilization (Gomez-Basauri et al., 2001). The increasing tendency of DMI was associated to an increase in microbial fermentation (increase in cellulolytic microbial population from inoculant).

During experimentation, it was noticed that sheep receiving DFM from N1 or N3 was drinking more water than the control animals. This was somehow a strange phenomenon as all experimental animals were fed and treated the same. An explanation for this increase in water consumption was not clear but could be associated to slight increase in feed intake or stimuli from N1 and N3 inoculum. An increase in DM intake (DMI) was the most probable reason for this increase as other researches have reported an increase in water consumption with DMI (Singh et al., 1976; Lautier et al., 1988). Previous research by Abraham et al. (1975) shows that water consumption in sheep can be stimulated by intracarotid infusion of angiotensin II. Angiotensin (oligopeptide) is a hormone and a powerful dipsogen (ability to increase thirst). Inocula stimulation of sheep is possible if any of the oligopeptides secreted by microbes due to their interaction resembles angiotensin. The zebra and wildebeest graze in a 30 km game with limited water sources. Therefore, oligopeptides mimicking the angiotensin function may exist so as to force this animal to return to water sources or increase water consumption when they

find a water source. The specific stimuli and animal may not be known but further research from this area can be very interesting as water is an essential nutrient for all animals (Markwick, 2002).

The washing loss (A) and the degradability of B by systems N1 and N3 were not significantly different from that of its control experiments. The washing loss for the controls and that of the treatments (N1 and N3) were expected to be the same since the same feed was used (Nurfeta et al., 2008). Although the degradability of B by N1 and N3 were not significantly different from that of its control, the degradability of B by N3 was 1.08% higher than that of the control. The insignificance of B in this study is similar to the results obtained by Paul et al. (2004). They showed that there was no significant effect of gut fungus from wild blue bulls on rumen fermentation in buffaloes. Similar results have also been observed in other studies (Arcos-García et al., 2000; Lee et al., 2000; Wang and McAllister, 2002; Silva et al., 2010). Lack of differences in potential degradability and C in both N1 and N3 were consistent with other studies (Lee et al., 2000; Paul et al., 2004). Consequently, ED was not affected by both N1 and N3 supplementation probably due to the relatively small increase in the rate of MS breakdown.

7.4.3 Rumen fermentation parameters

Total SCFA, acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid, CH_4, CO_2 and pH values agrees with results obtained by other studies (Markwick, 2002; Raeth-Knight et al., 2007; Chaucheyras-Durand and Durand, 2010; Qiao et al., 2010; Silva et al., 2010; Nagpal et al., 2011). The total SCFA produced by N1 at different time intervals was lower than that observed in the control whereas N3 was similar with the control in total SCFA. The decreased in total SCFA observed in N1 was associated to a relatively lower overall microbial fermentation and small drop in acetic acid production which is usually the major proportion of SCFA (Singh et al., 1976). Changes in pH during incubation were minimal in both systems suggesting that both the treatment and control systems were efficiently buffered. While there was no difference in CH_4 production between N1 and the control, CH_4 production in N3 was significantly lower than observed in its control experiment. The main reason for

this was not clear but could be associated with the increase in propionate production (5.5%) hence limiting the quantity of hydrogen available for CH_4 production (Lautier et al., 1988; Sejrsen et al., 2006). Methane emission is a major environmental concern today as it has been associated with global climate change. About 18 % of global warming has been associated with CH_4 production of which 14% is link to domestic animals. Interestingly, 97% of the 14% methane is produce by ruminants (Sejrsen et al., 2006). It was also noticed that CO_2 was relatively lower in both N1 and N3 systems although their differences (1.9 and 1.2% respectively) were not significant. Therefore, screening different microbial ecosystems in the wild in search of microbes with both fibrolytic and CH_4 reduction potential (N3) would be of a twofold advantage (increase production and environmental friendly).

7.4.4 Activities of cellulases in the different systems

Enzyme specific activities for endocellulase and exocellulase were higher in N1 and N3 compared to its controls. Endocellulase specific activities stimulation was much higher in N1 (182%) than in N3 (147%). For exocellulase activities, N1 and N3 stimulated an increase in enzymes specific activities by 109 and 133 %, respectively. This implies that system N3 has a higher potential in hydrolysing crystalline substrate than systems N1. The result obtained in this study was similar to those reported in other studies (Abraham et al., 1975; Wang and McAllister, 2002; Paul et al., 2004). Chaucheyras-Durand and Fonty (1975) showed that exocellulase and hemicellulases specific activities were higher in the DFM systems than its controls. This was also seen in the study of Paul et al. (2004) who reported that all cellulase enzyme activities were stimulated upon inoculation. However, in this study, hemicellulases specific activities were higher in the controls than in N1 and N3. The reason for hemicellulase specific activity suppression in N1 and N3 was not clear. Although other studies have shown indirect stimulation of cellulase activities by decreasing pathogenic microbes or lactate producing bacteria (Maldonado et al., 2011), such effects in this study were not established. The results obtained from these enzymes assays demonstrate that microbes from N1 and N3 have the potential of stimulating both endocellulose and crystalline cellulose hydrolyses in the rumen. Although both systems have the potential of improving fibre digestibility, N3 seems to

be a better inoculum than N1 due to its relatively high total SCFA, C, ED, and exocellulase specific activities.

7.5 Conclusion

Inoculating sheep with microbial inocula from N1 (horse and wildebeest) and N3 (wildebeest and zebra) increased rumen exocellulase and endocellulase specific activities but not hemicellulase activity. The fibrolytic potential of these rumen microbes were not translated into MS degradation. Dry matter intake increased with N1 inoculation while N3 inoculation tended to increase DMI. This result demonstrates that administration of microbes from N1 and N3 have the potential of improving forage fermentation in the rumen and DMI but may be limited by its dosing frequency. Therefore, more research on the dosing frequency and the mechanism of action as well as a digestibility study with sufficient animals will be very important in establishing the effect of these inocula on nutrient utilization in sheep.

Chapter 8

General discussion, conclusions and recommendations

Livestock contributes about 33% and 16% of the total protein and food energy consumption respectively, in human diets globally (Chadd et al., 2002). The demand for meat as a protein source could double (233 to 300 million tons) by the year 2020 due to the changing food preferences, income growth, urbanization and an increase in population growth (FAO, 2010; United Nations, 2010; WHO, 2011). These predictions show a substantial increase in animal protein demand, needed to satisfy the growth in the human population and the increasing affluence of the emerging economies. The world's human population is about 6.8 billion with Africa contributing 15% of the population. This population is increasing at a rate of 1.2% per year with the highest increase expected to come from Africa. Most African countries rely on livestock as a major source of protein because plant sources are scarce and are unevenly distributed. This implies that livestock production needs to increase in order to sustain this ever increasing human population especially in Africa. Attempts by intensive livestock farming to optimize production are facing daunting challenges with the human population in competition for food sources. Livestock presently consume almost 50% of world cereal grain supplies which is a future problem for the growing human population (Delgado, 1999). Therefore alternative sources of feed (crop residues and agro-industrial by-products) for domesticated herbivores such as cow, goats, sheep, horses and buffaloes will increase the quantity of grains available to humans (Hilali et al., 2011). This is very important in developing countries where maize grain is a staple food.

In most developed and developing countries, livestock are fed on forages, crop residues and agro-industrial by-products which are often poor in energy, protein and vitamins (Powell and Unger, 1998; Krause et al., 2003; Iñiguez, 2011). The actual energy content of these residues are high but are locked up in the complex carbohydrate molecules (carbon-hydrogen bonds) which can only be unlocked by rumen cellulolytic microbes in ruminants (Bhat, 2000; Vuong and Wilson, 2009). Different strategies have been used to improve forage digestibility in ruminants with variable results obtained. Examples of these methods include supplementation

with foliage or legumes (Undi et al., 2001), concentrates (Izadifard and Zamiri, 2007; Cherdthong et al., 2010), urea (Aregheore, 2005; Cherdthong et al., 2011), chemical treatment of forages (Chen et al., 2008), specific or composite cellulases (Hristov et al., 1998; Shekhar et al., 2010; Yang et al., 2011), specific microbial strains (Paul et al., 2011) and microbial inoculum (Singh et al., 1994; 1997; Wanapat et al., 2003). Apart from enzyme supplementation most of these methods work by increasing the rumen fibrolytic microbial population either directly or indirectly of which they intend increases fibre breakdown. Although some fibrolytic enzymes have been shown to increase fibre breakdown, their activities are often limited by rumen proteolytic activity and relatively low stability in the rumen (Morgavi et al., 2000). Therefore, microbial inoculation could be a better substitute if they can colonize and occupy a specific ecological niche in the rumen. That is why the main objective of this study was to scan different ecological systems (both wild and domestic) in search of a potential fibrolytic microbial ecosystem that can be manipulated for the improvement of rumen fermentation. The different hypothesis were: a) Culturing whole rumen or fecal inoculum will increase MS fermentation, b) Microbial fibrolytic activity does not vary between H, mH and ZB, c) *In vitro* microbial fermentation of MS by H, WB and ZB does not differ from that of its combined systems, and d) *In vivo* transinoculation of N1 and N3 does not have an effect of ruminal fermentation of MS in sheep.

8.1 Laboratory cultured faecal inoculum is a better substitute for fresh rumen inoculum than fresh faecal inoculum for *in vitro* feed evaluation

Chapter 3 investigated the management of whole rumen fluid (RF) and faecal fluid (FF) in the laboratory by culturing, using very simple laboratory techniques and the application of FF as an alternative inoculum for fresh RF. The results from this study confirmed that FF is an alternative inoculum for RF, which is in agreement with the results observed by other researches (Can et al., 2009). Although most of the *in vitro* fermentation parameters measured and cellulase enzyme activities obtained from fresh incubations with FF were slightly lower than those obtained from fresh RF, the percentage difference was less than 8%. A similar study by Can et al. (2009) showed that the rate of gas production was highest in sheep RF whereas the best regression curves within 24 to 48 h were obtained from horse and sheep FF.

This suggests that FF can conveniently be used in place of RF in *in vitro* fermentation studies. Culturing FF in the laboratory for 72 h before application in fermentation studies decreased the percentage differences observed between cultured FF and fresh RF fermentation parameters (TD, C and Total VFA). This suggests that culturing FF inoculum before *in vitro* incubation studies would yield values much closer to those of RF. The novelty of the current study is the culturing of whole rumen inocula prior incubation using simple laboratory technique which is available to all compared to other methods of preservation (freezing at -20, freeze-dried or expensive Rusitec fermenters). A 42 d FF incubation in the laboratory also showed that FF cultures were most active within a week although cultures were still alive after 6 weeks. The cost often incurred on animal purchases, surgical procedures and everyday collection of RF for fermentation studies can be minimized by simply using FF which can be preserved for up to 7 d for subsequent inoculations.

8.2 The fibrolytic activity of microbial ecosystems in three hindgut fermenters

Chapter 4 evaluated the fibrolytic competence of microbial ecosystems in faeces collected from three hindgut fermenters (mH, H and ZB) grazing in their natural environment in summer and winter. Zebra was the most active system, followed by H and lastly mH. The differences observed between these hindgut fermenters are suggestive of a fibrolytic competence of microbes that had evolved over time rather than changes in microbial population. This was demonstrated by the difference in exocellulase activity and endocellulase activity observed between mH and H grazing in the same field. The expectation is that herbivores grazing in the same field would be colonised by the same group of microbes due to cross contamination. This implies that cellulase activities would be very similar but this was not the case as exocellulase and endocellulase activities were consistently higher in H than in mH. Although a conclusive response about the type of microbes colonizing these ecosystems could not be reached in this study (microbial species were not investigated), it was suggested the microbial species were similar in herbivores grazing on the same field but vary in their fibrolytic potentials. Therefore, the higher exocellulase, endocellulase, TD and C in H was probably due to microbes that have evolved in the hindgut with their fibrolytic competence (Nagpal et al., 2011). True degradability, exocellulase and endocellulase activities were

highest in ZB than H and mH. The higher activity in ZB could be associated to four different factors; feed type (Varga and Kolver, 1997), microbial species, microbial population or genetically evolved microbes, since ZB was not grazing in the same field with mH and H. Microbial species and genetically evolved microbes with higher fibrolytic potential may be the main contributing factors for the higher activity in ZB. Although feed type is said to play a major role in influencing the type of microbes in an ecological niche (Xi et al., 2007), it was not considered to be a major factor in this study as all microbial inocula were cultured in the laboratory with the same feed before application. Therefore, more research is required to identify the microbial species in ZB with higher fibrolytic activity.

Increased ruminal fermentation of fibre has often been associated with higher fibrolytic microbial population but the factors stimulating this increase in population are not conclusive. Some of the possible mechanisms that could arise from rumen microbial inoculation include; (i) secretion of ionophores e.g. manosin which are lipid soluble ion transporters. Its antibiotic effect on certain microbes in the rumen has been well documented (Schelling, 1984). The effect of manosin on rumen fermentation include increase fibrolytic microbes, forage intake, propionate, fibre digestibility, protein digestibility, and reduced acetate, methane, butyric acid, ruminal deamination and proteolysis (Schelling, 1984), ii) Antibiotics secreted by microbes that might kill or reduce the population non-cellulolytic microbes hence an increase in fibrolytic microbes, iii) introduction of substrate specific microbes e.g. lactate utilizing microbes that will increase rumen pH hence promoting the growth and activity of fibrolytic microbes and iv) introduction of facultative microbes such as yeast which has been reported to utilize oxygen in the rumen hence promoting anaerobiosis which is important for the growth of rumen microbes. Consequently, more research is required to identify microbial species and their mode of action in improving forage fermentation.

8.3 *In vitro* fermentation using faecal fluids of the horse, wildebeest and zebra

Chapter 3 investigated the cheapest sources of inoculum and their preservation in the laboratory without compromising its overall effect on fermentation while chapter 4 applied the results established in chapter 4 to compare MS fermentation in three hindgut fermenters. In

chapter 5, the effect of different *inoculum* sources, horse (H), wildebeest (WB) and zebra (ZB) and their combinations (N1=H+WB, N2= H+ZB, N3=WB+ZB and N4=H+WB+ZB) on MS fermentation was examined. The individual systems were chosen for this experiment based on their high fibrolytic and degradability activities (chapter 4 and Fon (2006)). The results obtained from both enzyme and degradability studies showed for combined systems that the fibrolytic and degradability activities on cellulose and MS, respectively, were generally higher compared to the individual systems. This result confirms that interspecies trans-inoculation of rumen or hindgut microbes is possible *in vitro* and could go a long way in improving forage fermentation. Similar results have been observed by Lee et al. (2004) when they studied the effect of rumen anaerobic fungal culture on cumulative gas production, cellulose digestion, microbial population and enzyme activities *in vitro*. Other *in vitro* fermentation studies have also reported a positive increase in the efficiency of fibre utilization but not on the extent of forage digestibility (Martin and Nisbet, 1992; Lee et al., 2000). Inoculating the H with microbes from WB, ZB and WB+ZB increased TD by 22, 3.7 and 14.9%, respectively, while WB supplementation with H, ZB increased TD by 22.3 and 0.2% respectively. True degradability was suppressed in WB when supplemented with H+ZB. The reason for this suppression was not clear but could be associated to microbial competition during colonization of ecological niche (Chen and Weimer, 2001). If the dominant microbial species in the new system have a relatively lower fibrolytic activity then the overall fibrolytic activity was bound to decrease as observed in H+ZB inoculation. When the ZB system was supplemented with microbial inoculum from WB, H and H+WB TD increased by 15.5, 2.9 and 13.9% respectively. It is tempting to conclude from this experiment that systems H, WB and ZB will be best supplemented with microbial inoculum from WB (N1), H (N1) and WB (N3), respectively. Therefore, the application of this technology *in vivo* can be of a greater benefit to livestock production especially in the developing countries where ruminants are predominantly maintained on low grade roughages and/or graze on degraded range land resulting in poor nutrient utilization and productivity.

8.4 Cellulase production from five *in vitro* herbivore microbial ecosystems and their combined systems

Rumen or hindgut fermenters rely on anaerobic microorganisms (bacteria, protozoa and fungi) to produce fibrolytic enzymes for the degradation of forages (plant cell wall degradation) while the host animal provides the anaerobic fermentation chamber with feed, buffering capacity and regulate the rumen temperatures (Krause et al., 2003). This symbiotic relationship is of critical importance to the productive efficiency of the animal especially under extensive grazing systems. The diversity of rumen microbes (bacteria, protozoa and fungi) is said to be extensive with less that 10% of the extent of ruminal bacteria diversity cultured (Hungate, 1984; Krause and Russell, 1996). Therefore, the suggestion that representatives of all the major functional groups of rumen microbes isolated can give a clear and precise understanding of the rumen is not true. This is because evolution is a slow and continuous process, hence, microbial evolution with better fibrolytic activities is eminent. Although the microbial diversity is extensive, the mechanism of fibre breakdown is similar with the secretion of three major enzymes; exocellulase, endocellulase, and cellobiase (Bayer et al., 1998a; Bayer et al., 1998b). However, a second theory explains a greater level of complexity observed in some anaerobic microbes where several cellulases are grouped into an enzymatic complex called cellulosome (Bayer et al., 1998a). *In vitro* enzyme assay and degradability studies in chapter 5 clearly indicates that rumen transinoculation increased cellulase activity and true degradability compared to the individual systems. Whether this increment was due to increases in cellulase population or evolved cellulase enzymes from the wild, was not clear.

Chapter 6 examined the different individual and combined ecosystems for cellulase diversity. All seven *in vitro* microbial ecosystems were active and demonstrated different types (size) and numbers of carboxymethyl cellulases (CMCases). The number and types of CMCases was generally lower in fresh samples than in the cultured individual systems. The higher number in cultured systems was due to an increased specific microbial population during culturing compared to fresh samples where specific microbial concentrations might be limiting (chapter 5). Limiting microbial population of a particular species implies limited enzyme

concentrations to be detected on the gel. It is possible to conclude that CMCases diversity in these systems is extensive but conclusion on the specific type of enzyme is dodgy. This is because the size (kDa) variation of the different proteins that exhibited CMCase activity was bigger than what was available in the protein data systems hence enzyme affinity purification is required to make conclusion. The overlapping activity of cellulase enzymes and cellulosomes makes it even trickier to draw absolute conclusions on the specificity of these enzymes. Some crystalline cellulases have the potential of hydrolysing both crystalline cellulose and carboxylmethy cellulose (Knowles et al., 1987). This was clearly demonstrated in the study carried out by Vuong and Wilson (2009) where mutant enzymes were capable of hydrolysing up to five substrates (CMC, phosphoric acid-treated cotton, filter paper, bacterial microcrystalline cellulose and phosphoric acid-swollen cellulose). The results obtained from this chapter (6), confirms the extensive diversity of CMCases in all seven five systems despite the existence of common cellulases in all ecosystems. This is a clear indication that transinoculation has the potential of introducing microbes which have evolved with its ability to digest fibre. However, more research is still required to purify these enzymes, identify their specific activity as well as sequencing and characterization before their application can be validated in the feed market.

8.5 The effect of direct-fed microbials on ruminal fermentation of maize stover in sheep

Systems N1 and N3 were chosen as inoculants for *in sacco* degradability of MS in sheep because of high TD and cellulase enzymes diversity (chapter 5 and 6). It could be questioning why N3 was chosen over WB and N2 with a higher exocellulase activity and TD values that were similar. The fermentation parameters GP, total SCFA, and propionic acid were higher in N3 by 8.4 and 24, 52.3 and 26, and 1.8 and 1.4% than in WB and N2 respectively. Higher MY and total SCFA implies more microbial protein and energy metabolites (SCFA) for the host animal (Hungate, 1984; Armentano, 1992; Wanapat, 2000; Krause et al., 2003) which are the main objectives of most research. Inoculating sheep with direct-fed microbials from N1 and N3 increased rumen exocellulase and endocellulase specific activities but not hemicellulase activity. Increased cellulase activity could be associated with three factors; i) increase in cellulolytic microbial population (Martin and Nisbet, 1992; Lee et al., 2000), ii) the

introduction of superior fibrolytic microbes from inoculants and iii) the introduction of cellulolytic microbes growth promoting factors by inoculants (Martin and Nisbet, 1992). Interactions among these factors are also possible e.g. the introduction of a superior cellulolytic microbial strain which has the potential of competing and colonizing an ecological niche would increase its microbial population and exert a greater influence on fibre hydrolysis. Although the exact stimulant for the increase in cellulase activity was not conclusive, one of the objectives of this trial was achieved (increase in cellulase activity).

A lower hemicellulase activity in N1 and N3 was not expected and was very difficult to explain. However, this was probably due to partial inhibition of hemicellulolytic microbes by proteins or anti-growth factors from the game herbivores (WB and ZB). The game herbivores were previously observed with lower hemicellulase activities compared to that of the H (chapter5). Feed dry matter intake increased in N1 as observed by Krehbiel et al. (2003) but tended to increase when inoculated with N3 (Arcos-García et al., 2000; Malik and Bandla, 2010). The feed intake results are promising as most probiotics or direct-fed microbial studies tend to improve the efficiency of feed utilization than intake. *In sacco* degradability parameters of MS (B, PD and C) were similar between the treatments and control. These results were similar to those obtained by Aydin et al. (2009) where direct-fed microbials plus enzyme supplementation on the fattening performance of Holstein young bulls at two different initial body weights were studied. The digestibility parameters (average daily and total weight gains, final weight and DMI) measured were numerically different (1.8, 3.6, 2.9 and 0.2% respectively) but not statistically significant. However, some of the rumen fermentation by-products measured (total SCFA, acetic acid and propionic acid), showed that N1 and N3 have the potential of increasing average daily weight gain (Aydin et al., 2009; Malik and Bandla, 2010), live weight (Adams et al., 2008) and milk production (Nocek and Kautz, 2006). This was further confirmed by a small increase in MS degradability for both N1 and N3 between the 10-25 h of incubation. The indifference in B and PD between the treatments and controls may be due to longer dosing periods. Reducing the dosing time from three days interval to daily interval may improve forage fermentation. A similar study by Paul et al. (2011) also observed a decreased in dry matter degradability and enzyme activities post dosing with direct-fed microbials. Different dosing times have been adopted by different researches; for

instance twice daily through the fistula (Nocek et al., 2002) or rationed feed (Lee et al., 2000; Raeth-Knight et al., 2007; Arthur et al., 2010), once daily through a fistula (Raeth-Knight et al., 2007) and weekly (Paul et al., 2011). The dosing time and method of dosing depends on the research objectives (Malik and Bandla, 2010). Daily dosing was not applied in this study because microbial adaptation over time was being tested. The expectations were that, cellulolytic microbes from the inocula that survive rumen microbial hydrolysis and colonize a particular niche in the rumen would continue to exist and exert its fibrolytic activities.

One of the shortcomings of this research was that the different types of microbes in the inocula (N1 and N3), sheep rumen fluid and rumen inoculated fluid were not identified. Only the overall effect of these microbes on MS fermentation was measured. Knowledge about the different types of microbes especially the cellulolytic microbes and their population would have been very useful in explaining some of the outputs observed (higher exocellulase activity and rate of degradation of MS in N1 but lower total SCFA and PD, and an increase in exocellulase activity and total SCFA in N3 inoculated sheep). A study carried out by Paul et al. (2004) showed that increased in total tract digestibility and SCFA when supplemented with a specific fungal strain was associated with increases in cellulolytic (2.5 fold), hemicellulolytic (2.9 fold), total bacteria (2.5 fold) and fungal counts (4.4 folds). Similar results also associated with increases in microbial population have also been reported (Lee et al., 2000; Paul et al., 2011). It is also speculated that some microbes secrete proteins (peptides, oligopeptides or antibiotics) that stimulate microbial growth (Hernandez-Diaz et al., 2010). The mechanisms of such proteins are very complex but can be explain in very simple terms. Some of the mechanisms include the suppression of lactic acid producing microbes activity, stimulating lactic utilising microbes to use up lactic acid and inhibiting microbes competing for the same ecological niche (Baah et al., 2009). All these factors would contribute to a net increase in the dominant cellulolytic microbes hence an increase in fibre breakdown.

Rumen fermentation products measured after incubation with direct-fed microbials vary from one study to another. In this study, total SCFA increased in N3 (Dey et al., 2004) but decreased in N1 which is similar to those observed by Paul et al. (2010). Short chain fatty acids proportion also varied among the treatment and the control. The variation observed in

molar proportion of SCFA in N3 and N1 were similar to those observed by Paul et al. (2010). This implies that different inocula may affect the rumen differently depending on the type of microbes it harbours. These microbes may affect rumen fermentation by either increasing or decreasing one of the following parameters: total SCFA, acetic acid, propionate acid, butyric acid, CH_4 and CO_2 (Sehgal et al., 2008; Shelke et al., 2009; Mamen et al., 2010; Paul et al., 2010). Inoculating sheep with N3 decreased CH_4 production which is similar to the results obtained by Paul et al. (2010). Improving forage utilization without increasing CH_4 production will be a major achievement as CH_4 production by ruminants has been linked with global warming. Although the total methane contribution of all the factors that leads to global warming is less than 2%, it is very essential as it is 21 times more effective than CO_2 (Song et al., 2011). Therefore, identifying the superior fibrolytic strains and those that secrete factors which promote the proliferation of fibrolytic microbes would improve forage fermentation and utilization in herbivores.

8.6 Conclusion

Laboratory cultured faecal inoculum is a better substitute for rumen fluid than fresh faecal inoculum. *In vitro* fermentation of MS and cellulases enzyme assays by laboratory cultured faecal inocula from H, mH and ZB showed that hindgut microbial species from both wild and domestic herbivores may be different in their fibrolytic potential. These inocula vary in their ability to degrade fibre *in vitro* with the ZB being the most active, followed by H and lastly mH. Further investigation on the fibrolytic competence of microbes in chapter 5 where H, WB, ZB and its combined systems were investigated *in vitro*, showed that the combined systems N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB) fibrolytic competence were higher than those of the individual systems based on their exocellulase activities and true degradability of maize stover. The digestibility parameters TD, GP, PF, total VFA and B, ranked the microbial ecosystems according to their fibrolytic potential as N3 > N1 > N4 > WB > N2 > ZB >H.

The fibrolytic competences of the different inocula (H, WB, ZB, N1, N2, N3 and N4) were confirmed by the variation in carboxymethyl cellulase bands observed on carboxymethyl

cellulose zymograms. Systems N1 had the highest number of proteins with carboxylmethy cellulase activities. Although cultured CW, mH, N2, N4, WB, ZB and H had almost the same number of carboxylmethy cellulase bands, fewer bands were observed with the same molecular weights showing the diversity of these systems. The activities of sheep cellulases increased while hemicellulase did not when inoculated with N1 and N3 systems. Although PD and B were similar between N1 and N3, its C and Total SCFA values shows that, these ecosystems possess the potential of improving fibre utilization in ruminants.

Achievements in this study include; i) the preservation of faecal inoculum in the laboratory using very simple laboratory technique (reduces the cost on daily collection of faecal inocula for fermentation studies), ii) establishing microbial competence variation on MS fermentation between hindgut fermenters grazing in the same field, iii) identification and creation of synergistic (combined) systems with variable potential of digesting forages and iv) the identification of system N1 and N3 with the highest potential of improving fibre breakdown in ruminants. The major advantage of this study is its simplicity and availability to all livestock farmers for the improvement of rumen fermentation especially for straws in the developing countries where quality feeds and resources are limited.

8.7 Recommendations

The results obtained from Chapter 3 showed that whole rumen fluid and faecal inocula can be preserved in the laboratory for 42 days. However, seven days preservation would be recommended for future *in vitro* studies as exocellulase activity and microbial fermentation of MS were higher during this period. Culturing a faecal inoculum is also advisable as the daily cost incurred for transportation to the laboratory was saved. However, more research is still required to explain the low fermentation observed after day 7 when provided with fresh nutrients.

Chapter 4 and 5 showed that herbivores in the wild harbours microbes of different fibrolytic potentials. The numerous cellulase activity bands observed on the zymograms in Chapter 6 confirms the differences that exists between these herbivores systems in the wild. Therefore,

using faecal inocula from the ZB, WB, H, N2 and N3 as a crude sample for screening potential fibrolytic microbes would be recommended. The results from this study also show that N3 inoculum is suitable for screening fibrous feed in *in vitro* fermentation studies.

Attempts to transinoculate inocula from N1 and N3 to sheep was successful as indicated by true degradability and total VFA increases in N1 but tended to increase with N3. Therefore, using N1 for transinoculation study in sheep can be recommended for future studies. However, more research is needed in other to improve on the results obtained in this work. We would need to: i) identify, purify and classify the different cellulolytic microbes; ii) separate superior cellulolytic microbes and identify their mechanism of action; iii) optimize the dosage and the dosing interval; and iv) to use more animals to perform *in vivo* digestibility and animal performance studies to ascertain the effect of feasible systems.

8.8 References

Abraham, S.F., Baker, R., Blaine, E., Denton, D. and McKinley, M. (1975). Water drinking induced in sheep by angiotensin: A physiological or pharmacological effect? *J. Comp. Physiol. Psychol.* **88:** 503-518

Adams, M.C., Luo, J., Rayward, D., King, S., Gibson, R. and Moghaddam, G.H. (2008). Selection of a novel direct-fed microbial to enhance weight gain in intensively reared calves. *Anim. Feed Sci. Technol.* **145:** 41-52

Aganga, A.A. and Monyatsiwa, C.B. (1999). Use of Browses (Terminalia serecia, Combretum apiculatum or Euclea schimperi) as a Supplement for Growing Tswana Goats. *Trop. Anim. Health Prod.* **31:** 295-305

Agricultural Research Council (1980). The Nutrient Requirements of Ruminant Livestock. *In:* Commonwealth Agricultural Bureaux, London.

Ahmed, M.A.A. (2010). Gastrointestinal (nematode) infections in small ruminants : epidemiology, anthelmintic efficacy and the effect of wattle tannins. MSc Thesis, University of KwaZulu-Natal, South Africa.

Ahsan, M.M., Kimura, T., Karita, S., Sakka, K. and Ohmiya, K. (1996). Cloning, DNA sequencing, and expression of the gene encoding Clostridium thermocellum cellulase CelJ, the largest catalytic component of the cellulosome. *J. Bacteriol.* **178:** 5732-5740

Aikman, P.C., Reynolds, C.K. and Beever, D.E. (2008). Diet Digestibility, Rate of Passage, and Eating and Rumination Behavior of Jersey and Holstein Cows. *J. Dairy Sci.* **91:** 1103-1114

Akhter, S., Owen, E., Theodorou, M.K., S.L., T. and Deaville, E.R. (1995). Repeatability of the in vitro digestibility assays of forages when using fresh, frozen or freeze-dried

cow faeces instead of sheep rumen liquor as sources of microorganism. *In:* Proceedings of the Bristish Society of Animal Science. 110.

Akin, D.E. (1980). Attack on lignified grass cell walls by a facultatively anaerobic bacterium. *Appl. Environ. Microbiol.* **40:** 809-820

Akin, D.E. and Borneman, W.S. (1990). Role of Rumen Fungi in Fiber Degradation. *J. Dairy Sci.* **73:** 3023-3032

Al-Zuhair, S. (2008). The effect of crystallinity of cellulose on the rate of reducing sugars production by heterogeneous enzymatic hydrolysis. *Bioresour. Technol.* **99:** 4078-4085

Alaku, O. and Moruppa, S. (1983). Dry season weight losses in Red Sokoto (Maradi) goats reared in the Sahel region of Northeastern Nigeria. *Int. J. Biometeorol.* **27:** 143-156

Ali, M. and Tirta, K. (2001). Correlation between microbial enzyme activities in the rumen fliud of sheep under different treatments. *Reprod. Nutr. Dev.* **41:** 513-529

Álvarez, G., Pinos-Rodríguez, J.M., Herrera, J.G., García, J.C., Gonzalez, S.S. and Bárcena, R. (2009). Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. *Livest. Sci.* **121:** 150-154

Angeles, S.C., Mendoza, G.D., Cobos, M.A., Crosby, M.M. and Castrejon, F.A. (1998). Comparison of two commercial yeast cultures (Saccharomyces cerevisiae) on ruminal fermentation and digestion in sheep fed on corn-stover diet. *Small Ruminant Res.* **31:** 45-50

Antonio Facchini, F.D., Vici, A.C., Amin Reis, V.R., Jorge, J.A., Terenzi, H.F., Reis, R.A. and Teixeira de Moraes Polizeli, M.d.L. (2011). Production of fibrolytic enzymes by Aspergillus japonicus C03 using agro-industrial residues with potential application as additives in animal feed. *Bioprocess Biosystems Eng.* **34:** 347-355

AOAC. (1990). Official Methods of Analysis (S585.A8). Association of Official Analytical Chemists. AOAC,Washington, USA, 69-68.

Arcos-García, J.L., Castrejón, F.A., Mendoza, G.D. and Pérez-Gavilán, E.P. (2000). Effect of two commercial yeast cultures with Saccharomyces cerevisiae on ruminal fermentation and digestion in sheep fed sugar cane tops. *Livest. Prod. Sci.* **63:** 153-157

Aregheore, E.M. (1994). Potential of crop residues in ruminant nutrition. *Zambian J. Agric. Sci.* **4:** 40-41

Aregheore, E.M. (2005). Effect of Yucca schidigera saponin on the nutritive value of urea-ammoniated maize stover and its feeding value when supplemented with forage legume (Calliandra calothyrsus) for goats. *Small Ruminant Res.* **56:** 95-102

Aregheore, E.M. and Chimwano, A.M. (1991). Agro-industrial by-products and crop residues in Zambia:availability,utilization and potential value in ruminant nutrition. *In:* Proceedings of the 1st African Feed Resources Network (AFRNET) Worksop, Gaborone, Botswana.

Aregheore, E.M. and Perera, D. (2004). Effect of supplementation of a basal diet of maize stover with Erythrina variegata, Gliricidia sepium or Leucaena leucocephala on feed intake and digestibility by goats. *Trop. Anim. Health Prod.* **36:** 175-189

Armentano, L.E. (1992). Ruminant hepatic-metabolism of volatile fatty-acids, lactate and pyruvate. *J. Nutr.* **122:** 838-842

Arriola, K.G., Kim, S.C., Staples, C.R. and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. *J. Dairy Sci.* **94:** 832-841

Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Wells, J.E., Shackelford, S.D., Wheeler, T.L. and Koohmaraie, M. (2010). Evaluation of a Direct-Fed Microbial Product Effect on the Prevalence and Load of Escherichia coli O157:H7 in Feedlot Cattle. *J. Food Prot.* **73:** 366-371

Aydin, R., Yanar, M., Kocyigit, R., Diler, A. and Ozkilicci, T.Z. (2009). Effect of direct-fed microbials plus enzyme supplementation on the fattening performance of Holstein young bulls at two different initial body weights. *Afr. J. Agric. Res.* **4:** 548-552

Baah, J., Wang, Y. and McAllister, T.A. (2009). Impact of a mixed culture of Lactobacillus casei and L. lactis on in vitro ruminal fermentation and the growth of feedlot steers fed barley-based diets. *Can. J. Anim. Sci.* **89:** 263-271

Bailey, R. and Jones, D. (1971). Pasture quality and ruminant nutrition. III. Hydrolysis of ryegrass structural carbohydrates with carbohydrases in relation to rumen digestion. *N. Z. J. Agric. Res.* **14:** 847-857

Bala, P., Malik, R. and Srinivas, B. (2009). Effect of fortifying concentrate supplement with fibrolytic enzymes on nutrient utilization, milk yield and composition in lactating goats. *Anim. Sci. J.* **80:** 265-272

Bannink, A., France, J., Lopez, S., Gerrits, W.J.J., Kebreab, E., Tamminga, S. and Dijkstra, J. (2008). Modelling the implications of feeding strategy on rumen fermentation and functioning of the rumen wall. *Anim. Feed Sci. Technol.* **143:** 3-26

Bänziger, M., Setimela, P.S., Hodson, D. and Vivek, B. (2006). Breeding for improved abiotic stress tolerance in maize adapted to southern Africa. *Agric. Water Manage.* **80:** 212-224

Bárány, I., Fadón, B., Risueño, M.C. and Testillano, P.S. (2010). Cell wall components and pectin esterification levels as markers of proliferation and differentiation events during pollen development and pollen embryogenesis in *Capsicum annuum L. J. Exp. Bot.* **61:** 1159-1175

Barrière, Y., Guillet, C., Goffner, D. and Pichon, M. (2003). Genetic variation and breeding strategies for improved cell wall digestibility in annual forage crops. A review. *Anim. Res.* **52:** 193-228

Barrière, Y., Traineau, R., Emile, J. and Hébert, Y. (1991). Variation and covariation of silage maize digestibility estimated from digestion trials with sheep. *Euphytica.* **59:** 61-72

Bauchop, T. (1981). The anaerobic fungi in rumen fibre digestion. *Agricult. and Environ.* **6:** 339-348

Bauchop, T. (1989). Biology of gut anaerobic fungi. *BioSyst.* **23:** 53-64

Bayer, E.A., Chanzy, H., Lamed, R. and Shoham, Y. (1998a). Cellulose, cellulases and cellulosomes. *Curr. Opin. Struct. Biol.* **8:** 548-557

Bayer, E.A. and Lamed, R. (1986). Ultrastructure of the cell surface cellulosome of Clostridium thermocellum and its interaction with cellulose. *J. Bacteriol.* **167:** 828-836

Bayer, E.A., Morag, E. and Lamed, R. (1994). The cellulosome - A treasure-trove for biotechnology. *Trends Biotechnol.* **12:** 379-386

Bayer, E.A., Shimon, L.J.W., Shoham, Y. and Lamed, R. (1998b). Cellulosomes-Structure and Ultrastructure. *J. Struct. Biol.* **124:** 221-234

Beauchemin, K., Rode, M. and Sewalt, H. (1995). Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. *Can. J. Anim. Sci.* **75:** 641-644

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. (2003a). Use of Exogenous Fibrolytic Enzymes to Improve Feed Utilization by Ruminants. *J. Anim. Sci.* **81:** E37-47

Beauchemin, K.A., Krehbiel, C.R. and Newbold, C.J. (2006). Chapter 7 Enzymes, bacterial direct-fed microbials and yeast: principles for use in ruminant nutrition. *In:* Biology of Growing Animals. R. Mosenthin, J.Z. and Zebrowska, T. (eds). Elsevier, 251-284.

Beauchemin, K.A., McGinn, S.M. and Grainger, C.M. (2008). Reducing Methane Emissions from Dairy Cows. *Adv. Dairy Tech.* **20:** 79-93

Beauchemin, K.E., Yang, W.Z., Morgavi, D.P., Ghorbani, G.R., Kautz, W. and Leedle, J.A.Z. (2003b). Effects of bacterial direct-fed microbials and yeast on site and extent of digestion, blood chemistry, and subclinical acidosis in feedlot cattle. *J. Anim. Sci.* **81:** 1628-1640

Bedford, M.R. and Classen, H.L. (1992). Reduction of Intestinal Viscosity through Manipulation of Dietary Rye and Pentosanase Concentration is Effected through Changes in the Carbohydrate Composition of the Intestinal Aqueous Phase and Results in Improved Growth Rate and Food Conversion Efficiency of Broiler Chicks. *J. Nutr.* **122:** 560-569

Beguin, P. (1983). Detection of cellulase activity in polyacrylamide gels using congo red-stained agar replicas. *Anal. Biochem.* **131:** 333-336

Ben Salem, H. and Smith, T. (2008). Feeding strategies to increase small ruminant production in dry environments. *Small Ruminant Res.* **77:** 174-194

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* **70:** 1580-1588

Bhat, M.K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnol. Adv.* **18:** 355-383

Bhat, M.K. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv.* **15:** 583-620

Bhat, T.K., Singh, B. and Sharma, O.P. (1998.). Microbial degradation of tannins—a current perspective. *Biodegradation.* **9:** 343-357

Bidlack, J., Malone, M. and Benson, R. (1992). Molecular structure and component integration of secondary cell walls in plants. *Proc. Okla. Acad. Sci.* **72.:** 51-6

Biomass Research and Development Board (2001). Fostering the bioconversion revolution in biobased products and bioenergy *In:* National Renewable Energy Laboratory. NREL=MP-28950, Golden, CO

Bistanji, G., Hamadeh, S., Hajj Hassan, S., Tami, F. and Tannous, R. (2000). The potential of agro-industrial byproducts as feeds for livestock in Lebanon. Livestock Research for Rural Development.

Black, J.L., Beever, D.E., Faichney, G.J., Howarth, B.R. and Graham, N.M. (1981). Simulation of the effects of rumen function on the flow of nutrients from the stomach of sheep: Part 1-Description of a computer program. *Agricult. Sys.* **6:** 195-219

Blum, H., Beier, H. and Gross, H.J. (1987). Improved silver staining of plant-proteins, rna and dna in polyacrylamide gels. *Electrophoresis.* **8:** 93-99

Blummel, M. and Becker, K. (1997). The degradability characteristics of fifty-four roughages and roughage neutral detergent fibers as described by in vitro gas production and their relationship to voluntary feed intake. *Br. J. Nutr.* **77:** 757-768

Blümmel, M., Karsli, A. and Russell, J.R. (2003). Influence of diet on growth yields of rumen micro-organisms in vitro and in vivo: influence on growth yield of variable carbon fluxes to fermentation products. *Br. J. Nutr.* **90:** 625-634

Blümmel, M. and Lebzien, P. (2001). Predicting ruminal microbial efficiencies of dairy rations by in vitro techniques. *Livest. Prod. Sci.* **68:** 107-117

Bohme, H. (1990). Experiments on the efficacy of enzyme supplements as a growth promoter for piglets. *Landbauforsch Voelkenrode.* **40:** 213-217

Boisset, C., Chanzy, H., Henrissat, B., Lamed, R., Shoham, Y. and Bayer, E.A. (1999). Digestion of crystalline cellulose substrates by the clostridium thermocellum cellulosome: structural and morphological aspects. *Biochem. J.* **340:** 829–835

Bonsi, M.L.K., Osuji, P.O., Nsahlai, I.V. and Tuah, A.K. (1994). Graded levels of Sesbania sesban and Leucaena leucocephala as supplements to teff straw given to Ethiopian Menz sheep. *Anim. Prod.* **59:** 235-244

Boudet, M.A. (2003). Towards an understanding of the supamolecular organisation of lignified wall. *In:* The Plant Cell Wall, Annual Plant Reviews. Jocelyn, K. (eds). New York: Blackwell Publishing Ltd, 55-178.

Bourquin, L., Garleb, K., Merchen, N. and Fahey Jr, G. (1990). Effects of intake and forage level on site and extent of digestion of plant cell wall monomeric components by sheep. *J. Anim. Sci.* **68:** 2479

Bowman, G.R., Beauchemin, K.A. and Shelford, J.A. (2003). Fibrolytic Enzymes and Parity Effects on Feeding Behavior, Salivation, and Ruminal pH of Lactating Dairy Cows. *J. Dairy Sci.* **86:** 565-575

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254

Bronnenmeier, K., Kundt, K., Riedel, K., Schwarz, W.H. and Staudenbauer, W.L. (1997). Structure of the Clostridium stercorarium gene celY encoding the exo-1,4-beta-glucanase Avicelase II. *Microbiology.* **143 (Pt 3):** 891-898

Brooker, D., O'Donovan, L., Skene, I., Clarke, K., Blackall, L. and Muslera, P. (1994). Streptococcus caprznus sp., a tannin-resistant ruminal bacterium from feral goats. *Lett. Appl. Microbiol.* **18:** 313-18

Brown, V.E., Rymer, C., Agnew, R.E. and Givens, D.I. (2002). Relationship between in vitro gas production profiles of forages and in vivo rumen fermentation patterns in beef steers fed those forages. *Anim. Feed Sci. Technol.* **98:** 13-24

Browne, E.M., Juniper, D.T., Bryant, M.J. and Beever, D.E. (2005). Apparent digestibility and nitrogen utilisation of diets based on maize and grass silage fed to beef steers. *Anim. Feed Sci. Technol.* **119:** 55-68

Butler, R.A. (2007). Maize cultivated at least 7,300 years ago in Mexico. *http://news.mongabay.com/2007/0409-maize.html.*

Buxton, D., Mertens, D. and Moore, K. (1995). Forage quality for ruminants: Plant and animal considerations. *Anim. Sci.* **11:** 121-131

Calabrò, S., Moniello, G., Piccolo, V., Bovera, F., Infascelli, F., Tudisco, R. and Cutrignelli, M.I. (2008). Rumen fermentation and degradability in buffalo and cattle using the in vitro gas production technique. *J. Anim. Physiol. Anim. Nutr.* **92:** 356-362

Camp, H.J.M.O., Verhagen, F.J.M., Kivaisi, A.K. and Windt, F.E. (1988). Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by rumen microorganisms. *Appl. Microbiol. Biotechnol.* **29:** 408-412

Campbell, J.L., Williams, C.V. and Eisemann, J.H. (2002). Fecal Inoculum Can Be Used to Determine the Rate and Extent of In Vitro Fermentation of Dietary Fiber Sources across Three Lemur Species That Differ in Dietary Profile: Varecia variegata, Eulemur fulvus and Hapalemur griseus. *J. Nutr.* **132:** 3073-3080

Campos, F.P., Sampaio, A.A.M., Bose, M.L.V., Vieira, P.F. and Sarmento, P. (2004). Evaluation of in vitro gas production of roughages and their mixtures using the curves subtraction method. *Anim. Feed Sci. Technol.* **116:** 161-172

Can, A., Hummel, J., Mobashar, M., Boeser, U. and Sudekum, K.H. (2009). Comparison of Sheep Ruminal Fluid with Sheep and Horse Faeces as Inoculum for In Vitro Gas Production Measurements. *J. Appl. Anim. Res.* **35:** 143-148

Carpita, N.C. and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3:** 1–30

Casler, M. (1987). In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. *Crop Sci.* **27:** 931-934

Cavicchioli, R. and Watson, K. (1991). Molecular Cloning, Expression, and Characterization of Endoglucanase Genes from Fibrobacter succinogenes ARI. *Appl. Environ. Microbiol.* **57:** 359-365

Chadd, A.S., Davies, W.P. and Koivisto, M.J. (2002). Practical production of protein for food animals. *In:* FAO, ed. FAO animal production and health proceedings, Bangkok, ftp://ftp.fao.org/docrep/fao/007/y5019e/y5019e00.pdf.

Chakeredza, S., ter Meulen, U. and Ndlovu, L.R. (2002). Effect of cowpea hay, groundnut hay, cotton seed meal and maize meal supplementation to maize stover on intake, digestibility, microbial protein supply and acetate kinetics in weaner lambs. *Trop. Anim. Health Prod.* **34:** 49-64

Chandrasekharaiah, M., Reddy, M.R. and Reddy, G.V.N. (1996). Effect of feeding urea treated maize stover on growth and nutrient utilization by sheep and goats. *Small Ruminant Res.* **22:** 141-147

Chaucheyras-Durand, F. and Durand, H. (2010). Probiotics in animal nutrition and health. *Benef. Microbes.* **1:** 3-9

Chaucheyras-Durand, F., Walker, N.D. and Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. *Anim. Feed Sci. Technol.* **145:** 5-26

Chaudhary, L.C., Sahoo, A., Agarwal, N., Kamra, D.N. and Pathak, N.N. (2008). Effect of direct fed microbials on nutrient utilization, rumen fermentation, immune and growth response in crossbred cattle calves. *Indian J. Anim. Sci.* **78:** 515-521

Chen, J. and Weimer, P.J. (2001). Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria. *Microbiology.* **147:** 21-30

Chen, M. and Wolin, M. (1979). Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. *Appl. Environ. Microbiol.* **38:** 72-77

Chen, X.L., Wang, J.K., Wu, Y.M. and Liu, J.X. (2008). Effects of chemical treatments of rice straw on rumen fermentation characteristics, fibrolytic enzyme activities and populations of liquid- and solid-associated ruminal microbes in vitro. *Anim. Feed Sci. Technol.* **141:** 1-14

Chen, Y., Chen, J., Zhang, Y. and Zhou, D. (2007). Effect of harvest date on shearing force of maize stems. *Livest. Sci.* **111:** 33-44

Cheng, K., Forsberg, C., Minato, H. and Costerton, J. (1990). Microbial ecology and physiology of feed degradation within the rumen. Paper presented at the International Symposium on Ruminant Physiology, Sendai, Japan.

Cherdthong, A., Wanapat, M., Kongmun, P., Pilajun, R. and Khejornsart, P. (2010). Rumen Fermentation, Microbial Protein Synthesis and Cellulolytic Bacterial Population of Swamp Buffaloes as Affected By Roughage to Concentrate Ratio. *J. Anim. Vet. Adv.* **9:** 1667-1675

Cherdthong, A., Wanapat, M. and Wachirapakorn, C. (2011). Influence of urea calcium mixture supplementation on ruminal fermentation characteristics of beef cattle fed on concentrates containing high levels of cassava chips and rice straw. *Anim. Feed Sci. Technol.* **163:** 43-51

Cherubini, F. and Ulgiati, S. (2010). Crop residues as raw materials for biorefinery systems - A LCA case study. *Appl. Energy.* **87:** 47-57

Chiquette, J. (2009). Evaluation of the protective effect of probiotics fed to dairy cows during a subacute ruminal acidosis challenge. *Anim. Feed Sci. Technol.* **153:** 278-291

Church, D.C. (1988). The ruminant animal digestive physiology and nutrition. Church, D.C. (eds).New Jersey,Uninited States of America, Waveland Press.

Clarke, A.J., Drummelsmith, J. and Yaguchi, M. (1997). Identification of the catalytic nucleophile in the cellulase from Schizophyllum commune and assignment of the enzyme to Family 5, subtype 5 of the glycosidases. *FEBS Lett.* **414:** 359-361

Colombatto, D., Mould, F.L., Bhat, M.K., Morgavi, D.P., Beauchemin, K.A. and Owen, E. (2003). Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms *in vitro*. *J. Anim. Sci.* **81:** 1040-1050

Colombatto, D., Mould, F.L., Bhat, M.K., Phipps, R.H. and Owen, E. (2004). In vitro evaluation of fibrolytic enzymes as additives for maize (Zea mays L.) silage: III. Comparison of enzymes derived from psychrophilic, mesophilic or thermophilic sources. *Anim. Feed Sci. Technol.* **111:** 145-159

Cone, J.W., van Gelder, A.H. and Bachmann, H. (2002). Influence of inoculum source on gas production profiles. *Anim. Feed Sci. Technol.* **99:** 221-231

Cone, J.W., Van Gelder, A.H., Soliman, I.A., De Visser, H. and Van Vuuren, A.M. (1999). Different Techniques to Study Rumen Fermentation Characteristics of Maturing Grass and Grass Silage. *J. Dairy Sci.* **82:** 957-966

Corona, L., Mendoza, G.D., Castrejon, F.A., Crosby, M.M. and Cobos, M.A. (1999). Evaluation of two yeast cultures (Saccharomyces cerevisiae) on ruminal fermentation and digestion in sheep fed a corn stover diet. *Small Ruminant Res.* **31:** 209-214

Cottyn, B.G. and Boucque, C.V. (1968). Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. *J. Agric. Food Chem.* **16:** 105-107

Cruywagen, C.W. and van Zyl, W.H. (2008). Effects of a fungal enzyme cocktail treatment of high and low forage diets on lamb growth. *Anim. Feed Sci. Technol.* **145:** 151-158

Czerkawski, J.W. (1978). Reassessment of Efficiency of Synthesis of Microbial Matter in the Rumen. *J. Dairy Sci.* **61:** 1261-1273

Dalrymple, B.P. and McSweeney, C.S. (1998). Manipulation of rumen function by the inoculation of novel microorganisms. *Outlook Agric.* **27:** 107-113

Danfær, A., Tetens, V. and Agergaard, N. (1995). Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **111:** 201-210

Darshan, K.A., Krishnamoorthy, U., Kiran, D., Bhaskaran, R. and Manjunath, V. (2007). Effect of supplementing finger millet straw with two concentrates differing in their partitioning factor on dry matter intake, organic matter digestibility and nitrogen metabolism in Karan Friesian crossbred heifers. *Anim. Feed Sci. Technol.* **137:** 35-45

De Boever, J.L., Aerts, J.M., Vanacker, J.M. and De Brabander, D.L. (2005). Evaluation of the nutritive value of maize silages using a gas production technique. *Anim. Feed Sci. Technol.* **123-124:** 255-265

de Oliveira, M.V.M., Lana, R.D., Freitas, A.W.D., Eifert, E.D., Pereira, J.C., Valadares, S.D. and Perez, J.R.O. (2005). Effects of different dietary levels of monensin on nutrient digestibility and on ruminal, blood and urinary metabolites in dairy heifers. *Rev. Bras. Zootecn.* **34:** 2143-2154

Deacon, J.W. (1997). Modern mycology. *Wiley-Blackwell* [Online]. Available from: http://books.google.co.za/books?id=WQldeYhfy-EC&printsec=frontcover&source=gbs_ge_summary_r&cad=0#v=onepage&q&f=false].

Degen, A.A., Becker, K., Makkar, H.P.S. and Borowy, N. (1995). Acacia saligna as a fodder tree for desert livestock and the interaction of its tannins with fibre fractions. *J. Sci. Food Agric.* **68:** 65-71

Dehority, B.A. (1991). Effects of microbial synergism on fibre digestion in the rumen. *Proc. Nutr. Soc.* **50:** 149-159

Dehority, B.A. and Tirabasso, P.A. (2000). Antibiosis between Ruminal Bacteria and Ruminal Fungi. *Appl. Environ. Microbiol.* **66:** 2921-2927

Deinum, B. (1976). Effect of age, leaf number and temperature on cell wall and digestibility of maize. *In carbohydrate research in plants and animals Paper number 12.* 29-41

Deinum, B. and Dirven, J. (1971). Climate, nitrogen and grass. 4. The influence of age on chemical composition and in vitro digestibility of maize (Zea mays L.) and tall fescue (Festuca arundinacea Schreb.). *Neth. J. Agric. Sci.* **19:** 264–272

Delgado, C.L. (1999). Livestock to 2020: the next food revolution. International Food Policy Research Institute, http://books.google.co.za/books?id=MqTT1hsfcy0C&printsec=frontcover#v=onepage&q&f=false.

Demeyer, D.I. (1981). Rumen microbes and digestion of plant cell walls. *Agricult. and Environ.* **6:** 295-337

Denek, N. and Can, A. (2007). Use of faecal fluid for dry matter digestibility of ruminant feeds. *J. Appl. Anim. Res.* **31:** 29-32

Denek, N., Can, A. and Avci, M. (2010). Frozen rumen fluid as microbial inoculum in the two-stage in vitro digestibility assay of ruminant feeds. *South Afr. J. Anim. Sci.* **40:** 251-256

Denek, N., Polat, E., Koncagul, Z. and Can, Z. (2008). The Determination of Incubation Time and Amount of Faecal Content of Horse Faeces as an Inoculum Source for Digestibility Determination of Forages with in vitro Procedure. *J. Anim. Vet. Adv.* **7:** 698-702

Dennis, S.M. and Nagaraja, T.G. (1981). Effect of Lasalocid or Monensin on Lactate-Producing or Using Rumen Bacteria. *J. Anim. Sci.* **52:** 418-426

Desvaux, M. (2005). The cellulosome of Clostridium cellulolyticum. *Enzyme Microb. Technol.* **37:** 373-385

Dewhurst, R.J., Davies, D.R. and Merry, R.J. (2000). Microbial protein supply from the rumen. *Anim. Feed Sci. Technol.* **85:** 1-21

Dewhurst, R.J., Delaby, L., Moloney, A., Boland, T. and Lewis, E. (2009). Nutritive value of forage legumes used for grazing and silage. *Ir. J Agr. Food Res.* **48:** 167-187

Dey, A., Sehgal, J.P., Puniya, A.K. and Singh, K. (2004). Influence of an anaerobic fungal culture (Orpinomyces sp.) administration on growth rate, ruminal fermentation and nutrient digestion in calves. *Asian-Australas. J. Anim. Sci.* **17:** 820-824

Dhakad, A., Garg, A.K., Singh, P. and Agrawal, D.K. (2002). Effect of replacement of maize grain with wheat bran on the performance of growing lambs. *Small Ruminant Res.* **43:** 227-234

Dhanoa, M.S. (1988). Research note: On the analysis of dacron bag data for low degradability feeds. *Grass Forage Sci.* **43:** 441-444

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

Dirksen, G.U., Liebich, H.G., Mayer, E. (1985). Adaptive changes of the ruminal mucosa and their functional and clinical significance. *Bovine Pract.* **20:** 116-120

Dixon, R.M. and Egan, R.M. (1987). Strategies for utilizing fibrous crop residues as animal feeds. Paper presented to the 7th AAFARR Workshop. 2-6 July, 1987, Chiang Mai, Thailand.

Dixon, R.M., Nolan, J.V. and Milligan, L.P. (1982). Studies of the large intestine of sheep. *Br. J. Nutr.* **47:** 301-309

Doblin, S.M., Vergara, E.C., Read, S., Newbigin, E. and Bacic, A. (2003). Plant cell wall biosynthesis:making the bricks. *In:* The Plant Cell Wall, Annual Plant Reviews. Jocelyn, K. (eds). New York: Blackwell Publishing Ltd

Dodia, M.S., Rawal, C.M., Bhimani, H.G., Joshi, R.H., Khare, S.K. and Singh, S.P. (2008). Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp AH-6. *J. Ind. Microbiol. Biotechnol.* **35:** 121-131

Dominguez Bello, M.G. and Escobar, A. (1997). Rumen manipulation for the improved utilization of tropical forages. *Anim. Feed Sci. Technol.* **69:** 91-102

Duan, C.J. and Feng, J.X. (2010). Mining metagenomes for novel cellulase genes. *Biotechnol. Lett.* **32:** 1765-1775

Edwards, S.A. and Poole, D. (1983). The effects of including sodium bicarbonate in the diet of dairy cows in early lactation. *Anim. Prod.* **37:** 183-188

El-Meadaway, A., Mir, Z., Mir, P.S., Zaman, M.S. and Yanke, L.J. (1998). Relative efficacy of inocula from rumen fluid or faecal solution for determining in vitro digestibility and gas production *Can. J. Anim. Sci.* **78:** 673-679

El Shaer, H.M., Omed, H.M., Chamberlain, A.G. and Axford, R.F.E. (1987). Use of faecal organisms from sheep for the in vitro determination of digestibility. *J. Agric. Sci.* **109:** 257-259

Elam, N.A., Gleghorn, J.F., Rivera, J.D., Galyean, M.L., Defoor, P.J., Brashears, M.M. and Younts-Dahl, S.M. (2003). Effects of live cultures of Lactobacillus acidophilus (strains NP45 and NP51) and Propionibacterium freudenreichii on performance, carcass, and intestinal characteristics, and Escherichia coli strain O157 shedding of finishing beef steers. *J. Anim. Sci.* **81:** 2686-2698

Elliott, R. and Folkersten, K. (1961). Seasonal changes in composition and yield of veld grass. *Rhodesia Agr. J.* **58:** 186-187

Elwakeel, E.A., Titgemeyer, E.C., Johnson, B.J., Armendariz, C.K. and Shirley, J.E. (2007). Fibrolytic Enzymes to Increase the Nutritive Value of Dairy Feedstuffs. *J. Dairy Sci.* **90:** 5226-5236

Erdmann, R.A. (1988). Dietary buffering requirements of lactating dairy cows: a review. *J. Dairy Sci.* **71:** 3246-3266

Erenstein, O. (2011). Cropping systems and crop residue management in the Trans-Gangetic Plains: Issues and challenges for conservation agriculture from village surveys. *Agricult. Sys.* **104:** 54-62

Eun, J.S. and Beauchemin, K.A. (2008). Assessment of the potential of feed enzyme additives to enhance utilization of corn silage fibre by ruminants. *Can. J. Anim. Sci.* **88:** 97-106

Eysker, M., Bakker, N., van der Hall, Y.A., van Hecke, I., Kooyman, F.N.J., van der Linden, D., Schrama, C. and Ploeger, H.W. (2006). The impact of daily Duddingtonia flagrans application to lactating ewes on gastrointestinal nematodes infections in their lambs in the Netherlands. *Vet. Parasitol.* **141:** 91-100

Fahey, G. and Berger, L. (1998). Carbohydrate Nutrition of Ruminants. *In:* The Ruminant Animal Digestive Physiology and Nutrition. Church, C. (eds). US America: Waveland Press, Inc., 269-297.

FAO. (2010). *Animal genetic resources. Food and Agriculture Organization*.[Online]. http://www.fao.org/docrep/012/al383e/al383e00.pdf.

Ferrer, M., Golyshina, O.V., Chernikova, T.N., Khachane, A.N., Reyes-Duarte, D., Dos Santos, V., Strompl, C., Elborough, K., Jarvis, G., Neef, A., Yakimov, M.M., Timmis, K.N. and Golyshin, P.N. (2005). Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. *Environ. Microbiol.* **7:** 1996-2010

Flint, J. (1994). Degredation of plant cell wall polysaccharides by rumen bacteria. Prins, R. and Stewart, C. (eds). Nottingham University Press.

Fon, F.N. (2006). Fibrolytic enzyme activity of herbivore microbial ecosystems. MSc. Agric Thesis, University of KwaZulu-Natal.

Fon, F.N. and Nsahlai, I.V. (2009). Activities of microbial fibrolytic enzymes in ten herbivore microbial ecosystems. *In:* XIth International Symposium on Ruminant Physiology. Chilliard, Y., Glasser., F., Faulconnier., Y., Bocquier, F., Veissier, I. and Doreau, M. (eds). Clermont-Ferrand, France: Wageningen Academic, Netherlands, 180 -181.

Fonty, G. and Chaucheyras-Durand, F. (2006). Effects and modes of action of live yeasts in the rumen. *Biologia.* **61:** 741-750

Fonty, G. and Gouet, H. (1994). Plant cell wall degredation by anaerobic fungi. Prins, R. and Stewart, C. (eds). Nottingham University Press.

Freelove, A.C.J., Bolam, D.N., White, P., Hazlewood, G.P. and Gilbert, H.J. (2001). A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromyces equi. *J. Biol. Chem.* **276:** 43010-43017

Freier, M. and Karl-Heinz, A. (1997). The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Res.* **25:** 4429-4443

Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* **66:** 365-375

Gado, H.M., Salem, A.Z.M., Robinson, P.H. and Hassan, M. (2009). Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Anim. Feed Sci. Technol.* **154:** 36-46

Gal, L., Pages, S., Gaudin, C., Belaich, A., ReverbelLeroy, C., Tardif, C. and Belaich, J.P. (1997). Characterization of the cellulolytic complex (cellulosome) produced by Clostridium cellulolyticum. *Appl. Environ. Microbiol.* **63:** 903-909

Galante, Y.M., De Conti, A. and Monteverdi, R. (1998). Application of Trichoderma enzymes in food and feed industries. *In:* Trichoderma and Gliocladium-Enzymes, biological control and commercial applications. Harman, G.F. and Kubicek, C.P. (eds). London: Taylor and Francis, 327-42.

Gallardo, I., Barcena, R., Pinos-Rodriguez, J.M., Cobos, M., Carreon, L. and Ortega, M.E. (2010). Influence of exogenous fibrolytic enzymes on in vitro and in sacco degradation of forages for ruminants. *Ital. J. Anim. Sci.* **9:** 34-38

Garg, A.K., Singh, P., Malik, R. and Agrawal, D.K. (2004). Effect of replacing maize grain with de-oiled rice bran on intake and utilisation of nutrients in adult ewes. *Small Ruminant Res.* **52:** 75-79

Gerrit, B., Marjo, F., Frank, M. and Fons, G. (1984). The cellulase of Trichoderma Viride. Purification characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *Eur. J. Biochem.* **146:** 301-308

Gertenbach, W.D., Visser, P.J.L. and van H Henning, P.W. (1998). The utilisation of maize-crop residues for overwintering livestock 2. Strip and continuous grazing of maize-crop residues. *S. Afr. J. Anim. Sci.* **28:** 16-23

Ghadaki, M., Van Soest, P., Mcdowell, R. and Malekpour, B. (1975). Chemical composition and in vitro digestibility of some range forage species of Iran. *In:* Evaluation and mapping of tropical African rangelands Seminar, Bamako-Mali.

Gijzen, H.J., Lubberding, H.J., Gerhardus, M.J.T. and Vogels, G.D. (1988). Contribution of rumen protozoa to fibre degradation and cellulase activity in vitro. *FEMS Microbiol. Lett.* **53:** 35-43

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. (2008). Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *J. Anim. Sci.* **86:** 1617-1623

Gizzi, G., Zanchi, R. and Sciaraffia, F. (1998). Comparison of microbiological and fermentation parameters obtained with an improved rumen in vitro technique with those obtained in vivo. *Anim. Feed Sci. Technol.* **73:** 291-305

Goel, G., Puniya, A.K. and Singh, K. (2007). Phenotypic characterization of tannin-protein complex degrading bacteria from faeces of goat. *Small Ruminant Res.* **69:** 217-220

Gomez-Basauri, J., de Ondarza, M.B. and Siciliano-Jones, J. (2001). Intake and milk production of dairy cows fed lactic acid bacteria and mannanoligosaccharide. *J. Dairy Sci.* **84:** 283

Gonçalves, L. and Borba, A. (1996). Study of gas production capacity by three sources of inocula. *J Agr. Sci. (Cambridge).* **127:** 511-515

Gong, L.S., Harazono, Y., Oikawa, T., Zhao, H.L., Ying He, Z. and Chang, X.L. (2000). Grassland desertification by grazing and the resulting micrometeorological changes in Inner Mongolia. *Agricult. Forest Meterol.* **102:** 125-137

Gramene website. (2006). *Zea mays*.[Online]. http://www.gramene.org/species/zea/maize_nutrition.html.

Grings, E.E., Blümmel, M. and Südekum, K.H. (2005). Methodological considerations in using gas production techniques for estimating ruminal microbial efficiencies for silage-based diets. *Anim. Feed Sci. Technol.* **123-124:** 527-545

Groleau, D. and Forsberg, C. (1983). Partial characterization of the extracellular carboxymethylcellulase activity produced by the rumen bacterium Bacteroides succinogenes. *Can. J. Microbiol./Rev. Can. Microbiol.* **29:** 504-517

Groot, J.C.J., Williams, B.A., Oostdam, A.J., Boer, H. and Tamminga, S. (1998). The use of cumulative gas and volatile fatty acid production to predict in vitro fermentation kinetics of Italian ryegrass leaf cell walls and contents at various time intervals. *Br. J. Nutr.* **79:** 519-525

Guedes, C.M., Goncalves, D., Rodrigues, M.A.M. and Dias-da-Silva, A. (2008). Effects of a Saccharomyces cerevisiae yeast on ruminal fermentation and fibre degradation of maize silages in cows. *Anim. Feed Sci. Technol.* **145:** 27-40

Guerra, A., Filpponen, I., Lucia, L.A., Saquing, C., Baumberger, S. and Argyropoulos, D.S. (2006). Toward a better understanding of the lignin isolation process from wood. *J. Agric. Food Chem.* **54:** 5939-5947

Guillaumie, S., San-Clemente, H., Deswarte, C., Martinez, Y., Lapierre, C., Murigneux, A., Barrière, Y., Pichon, M. and Goffner, D. (2007). Maize wall, Database and Developmental Gene Expression Profiling of Cell Wall Biosynthesis and Assembly in Maize. *Plant Physiol.* **143:** 339-363

Gustafson, R.H. and Bowen, R.E. (1997). Antibiotic use in animal agriculture. *J. Appl. Microbiol.* **83:** 531-541

Hagerman, A.E. and Butler, L.G. (1981). The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **256:** 4494-4497

Hagg, F.M., Erasmus, L.J., Henning, P.H. and Coertze, R.J. (2010). The effect of a direct fed microbial (Megasphaera elsdenii) on the productivity and health of Holstein cows. *S. Afr. J. Anim. Sci.* **40:** 101-112

Haggar, R. and Ahmed, M. (1971). Seasonal production of Andropogon gayanus. III. Changes in crude protein content and in vitro dry-matter digestibility of leaf and stem portions. *J. Agr. Sci. (Cambridge).* **77:** 47-52

Hainze, M.T.M., Muntifering, R.B. and McCall, C.A. (2003). Fiber digestion in horses fed typical diets with and without exogenous fibrolytic enzymes. *J. Equine Vet. Sci.* **23:** 111-115

Hall, H.J. and Silver, S. 2001. Nutrition and Feeding of the cow-calf herd:Digestive system of the cow. *Verginia Cooperative Extension* *http://pubs.ext.vt.edu/400/400-010/400-010.html* [Online].

Hall, J., Ali, S., Surani, M.A., Hazlewood, G.P., Clark, A.J., Simons, J.P., Hirst, B.H. and Gilbert, H.J. (1993). Manipulation of the Repertoire of Digestive Enzymes Secreted into the Gastrointestinal Tract of Transgenic Mice. *Nat. Biotechnol.* **11:** 376-379

Harika, A. and Sharma, D. (1994). Quality and yield differences in maize stover due to varieties and stage of harvesting. *In:* Variation in the Quantity and Quality of Fibrous Crop Residues Joshi, A.L., Doyle, P.T. and Oosting, S.J. (eds). ICAR, New Delhi and Wageningen Agricultural University,: Wageningen,The Netherlands, 20–28.

Harika, A., Tripathi, H. and Saxena, V. (1995). Maize stover. *In:* Handbook for Straw Feeding Systems, New Delhi, India. ICAR, 379-391.

Harrison, D. and McAllan, A. (1980). Factors affecting microbial growth yields in the reticulo-rumen. *In:* Digestive physiology and metabolism in ruminants. Ruckebusch, Y. and Thivend, P. (eds). Clermont-Ferrand: MTP Press Ltd., 205-226.

Henriksson, G., Lawoko, M., Martin, M.E.E. and Gellerstedt, G. (2007). Lignin-carbohydrate network in wood and pulps: A determinant for reactivity. *Holzforschung.* **61:** 668-674

Henry, H., Christou, N., Verma, D. and Maclachlan, G. (1975). Purification and characterization of two cellulases from auxin-treated pea epicotyls. *J. Biol. Chem.* **250:** 1012-1018

Hernandez-Diaz, R., Pimentel-Gonzalez, D.J., Figueira, A.C., Viniegra-Gonzalez, G. and Campos-Montiel, R.G. (2010). Influence of an aerobic fungus grown on solid culture on ruminal degradability and on a mixture culture of anaerobic cellulolytic bacteria. *J. Anim. Physiol. Anim. Nutr.* **94:** 330-337

Hervás, G., Frutos, P., Giráldez, F.J., Mora, M.J., Fernández, B. and Mantecón, Á.R. (2005). Effect of preservation on fermentative activity of rumen fluid inoculum for in vitro gas production techniques. *Anim. Feed Sci. Technol.* **123-124:** 107-118

Hervas, G., Ranilla, M.J., Mantecon, A.R., Tejido, M.L. and Frutos, P. (2005). Comparison of sheep and red deer rumen fluids for assessing nutritive value of ruminant feedstuffs. *J. Sci. Food Agric.* **85:** 2495-2502

Hespell, R., Akin, D. and Dehority, B. (1997). Bacteria, fungi, and protozoa of the rumen. *In:* Gastrointestinal Microbiology. Mackie, R.I., White, B.A. and Isaacson, R.E. (eds). New York: Chapman and Hall Press, 59-141.

Heussen, C. and Dowdle, E.B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.* **102:** 196-202

Hidayat, Hillman, K., Newbold, C.J. and Stewart, C.S. (1993). The contributions of bacteria and protozoa to ruminal forage fermentation in vitro, as determined by microbial gas production. *Anim. Feed Sci. Technol.* **42:** 193-208

Hilali, M., Iñiguez, L., Knaus, W., Schreiner, M., Rischkowsky, B., Wurzinger, M. and Mayer, H.K. (2011). Prospects for using nonconventional feeds in diets for Awassi dairy sheep in Syria. *J. Dairy Sci.* **94:** 3014-3024

Hillman, L., Peters, G., Fisher, A. and Pomare, E. (1986). Effects of the fibre components pectin, cellulose, and lignin on bile salt metabolism and biliary lipid composition in man. *Gut.* **27** 29-36

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W. and Foust, T.D. (2007). Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science.* **315:** 804-807

Hindrichsen, I.K., Osuji, P.O., Odenyo, A.A., Madsen, J. and Hvelplund, T. (2004). Effect of supplementation of maize stover with foliage of various tropical multipurpose trees and Lablab purpureus on intake, rumen fermentation, digesta kinetics and microbial protein supply of sheep. *Anim. Feed Sci. Technol.* **113:** 83-96

Hobson, P. (1969). Rumen Bacteria. *In:* Methods in Microbiology. Norris, J. and Rinbbons, D. (eds). London: Academic Press, 133-150.

Hobson, P.N. (1971). Rumen Micro-organisms. *Progr. Ind. Microbiol.* **9:** 42-77

Hofmann, R. (1998). Anatomy of the gastro-intestinal tract. *In:* The Ruminant Animal Digestive Physiology and Nutrition. Church, C. (eds). USA: Waveland Press, 269.

Holden, L.A. (1999). Comparison of methods of in vitro dry matter digestibility for ten feeds. *J. Dairy Sci.* **82:** 1791-1794

Holtman, K.M., Chang, H.-M., Jameel, H. and Kadla, J.F. (2003). Elucidation of Lignin Structure through Degradative Methods: Comparison of Modified DFRC and Thioacidolysis. *J. Agric. Food Chem.* **51:** 3535-3540

Horvath, P. (1981). The nutritional and ecological significance of Acer-tannins and related polyphenols. Masters of Science Thesis, Cornell University, Ithaca, New York.

Hoshino, E., Kubota, Y., Okazaki, M., Nisizawa, K. and Kanda, T. (1994). Hydrolysis of cotton cellulose by exo-and endo-type cellulases from Irpex lacteus: differential scanning calorimetric study. *J. Biochem.* **115:** 837-842

Hoskinson, R.L., Karlen, D.L., Birrell, S.J., Radtke, C.W. and Wilhelm, W.W. (2007). Engineering, nutrient removal, and feedstock conversion evaluations of four corn stover harvest scenarios. *Biomass Bioenergy.* **31:** 126-136

Howell, J. and Stuck, J. (1975). Kinetics of Solka Floc cellulose hydrolysis by Trichoderma viride cellulase. *Biotechnol. Bioeng.* **17:** 873-893

Hristov, A.N., A. McAllister, T. and Cheng, K.J. (1998). Stability of exogenous polysaccharide-degrading enzymes in the rumen. *Anim. Feed Sci. Technol.* **76:** 161-168

Hungate, R.E. (1984). Microbes of nutritional importance in the alimentary-tract. *Proc. Nutr. Soc.* **43:** 1-11

Huub, J., Verhagen, M., Kivaisi, K. and de Windt, E. (1988a). Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by rumen microorganisms. *Appl. Microbiol. Biotechnol.* **29:** 408-412 .

Huub, J.G., Henk, J.L., Martin, J.T.G. and Godfried, D.V. (1988b). Contribution of rumen protozoa to fibre degradation and cellulase activity in vitro. *FEMS Microbiol. Lett.* **53:** 35-43

IFAD (2004). Livestock Services and the Poor: A Global Initiative. Collecting, Coordinating and Sharing Experiences. Rome: International Fund for Agricultural Development. *Danish International Development Agency, and World Bank. http://www.ifad.org/lrkm/book/english.pdf.*

Imai, M., Ikari, K. and Suzuki, I. (2004). High-performance hydrolysis of cellulose using mixed cellulase species and ultrasonication pretreatment. *Biochem. Eng. J.* **17:** 79-83

Iñiguez, L. (2011). The challenges of research and development of small ruminant production in dry areas. *Small Ruminant Res.* **98:** 12-20

Inoue, T., Brookes, I.M., John, A., Kolver, E.S. and Barry, T.N. (1994). Effects of leaf shear breaking load on the feeding value of perennial ryegrass (Lolium perenne) for sheep. I. Effects on leaf anatomy and morphology. *J. Agric. Sci.* **123:** 137

Irlbeck, N.A., Russell, J.R., Hallauer, A.R. and Buxton, D.R. (1993). Nutritive value and ensiling characteristics of maize stover as influenced by hybrid maturity and generation, plant density and harvest date. *Anim. Feed Sci. Technol.* **41:** 51-64

Iwaasa, A., Beauchemin, K., Buchanan-Smith, J. and Acharya, S. (1996). Effect of stage of maturity and growth cycle on shearing force and cell wall chemical constituents of alfalfa stems. *Can. J. Anim. Sci.* **76:** 321-328

Iwaasa, A.D., Beauchemin, K.A., Buchanan-Smith, J.G. and Acharya, S.N. (1995). Assessment of stem shearing force for three alfalfa varieties grown under dryland and irrigated conditions. *Can. J. Anim. Sci.* **75:** 177-179

Izadifard, J. and Zamiri, M.J. (2007). Effects of supplementary feeding on growth and carcass characteristics of fat-tailed lambs grazing cereal stubble. *Iran J. Veterinary Res.* **8:** 123-130

Jackson, W., Krishnamoorthy, U., Robinson, P.H. and Fadel, J.G. (2010). Effect of changing partitioning factor (PF) and in vitro rate of gas production (k) of diets on intake and digestibility, microbial N production, as well as milk production and composition, of lactating crossbred dairy cows. *Anim. Feed Sci. Technol.* **160:** 128-136

Jan, D. (1994). Production and absorption of volatile fatty acids in the rumen. *Livest. Prod. Sci.* **39:** 61-69

Jauris, S., Rücknagel, K.P., Schwarz, W.H., Kratzsch, P., Bronnenmeier, K. and Staudenbauer, W.L. (1990). Sequence analysis of the Clostridium stercorarium celZ gene encoding a thermoactive cellulase (Avicelase I): identification of catalytic and cellulose-binding domains. *Mol. Gen. Genet.* **223:** 258-267

Jin, G.L., Choi, S.K., Choi, S.H. and Song, M.K. (2007). Effect of microbial additives on metabolic characteristics in sheep and milking performance of lactating dairy cows. *J. Anim. Sci. Tech.* **49:** 819-828

Johnson, E.A., Sakajoh, M., Halliwell, G., Madia, A. and Demain, A.L. (1982). Saccharification of Complex Cellulosic Substrates by the Cellulase System from Clostridium thermocellum. *Appl. Environ. Microbiol.* **43:** 1125-1132

Jones, R. and Lowry, J. (1984). Australian goats detoxify the goitrogen 3-hydroxy-4 (1H) pyridone (DHP) after rumen infusion from an Indonesian goat. *Cell. Mol. Life Sci.* **40:** 1435-1436

Jouany, J.P. (2006). Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Anim. Reprod. Sci.* **96:** 250-264

Kamra, D.N. (2005). Rumen microbial ecosystem. *Curr. Sci.* **89:** 124-135

Keford, N.P. (1958). Lignification of plants in relation to ruminant nutrition. *Journ. Aust. Inst, agric. Sci.* **24:** 297-301

Kelly, V., Adesina, A.A. and Gordon, A. (2003). Expanding access to agricultural inputs in Africa: a review of recent market development experience. *Food Policy.* **28:** 379-404

Kempton, T.J. (1980). The use of nylon bags to characterise the potential degradability of feeds for ruminants. *Trop. Anim. Prod.* **5:** 107-116

Kenters, N., Henderson, G., Jeyanathan, J., Kittelmann, S. and Janssen, P.H. (2011). Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J. Microbiol. Methods.* **84:** 52-60

Kern, D.L., Slyter, L.L., Leffel, E.C., Weaver, J.M. and Oltjen, R.R. (1974). Ponies vs. Steers: Microbial and Chemical Characteristics of Intestinal Ingesta. *J. Anim. Sci.* **38:** 559-564

Khanna, S. (1993). Regulation, purification, and properties of xylanase from Cellulomonas fimi. *Enzyme Microb. Technol.* **15:** 990-995

Kim, D., Baik, K., Park, S., Kim, S.-J., Shin, T.-S., Jung, S.-J., Oh, M.-J. and Seong, C. (2009). Cellulase production from Pseudoalteromonas sp. NO3 isolated from the sea squirt Halocynthia rorentzi. *J. Ind. Microbiol. Biotechnol.* **36:** 1375-1382

King, K. and Vessal, M.I. (1969). Enzymes of the cellulase complex. *Advan. Chem. Ser.* **95:** 7-25

Kiran, D. and Krishnamoorthy, U. (2007). Rumen fermentation and microbial biomass synthesis indices of tropical feedstuffs determined by the in vitro gas production technique. *Anim. Feed Sci. Technol.* **134:** 170-179

Knowles, J., Lehtovaara, P., Penttilä, M., Teeri, T., Harkki, A. and Salovuori, I. (1987). The cellulase genes of Trichoderma. *Antonie Van Leeuwenhoek.* **53:** 335-341

Kokubo, A., Kuraishi, S. and Sakurai, N. (1989). Culm strength of barley: correlation among maximum bending stress, cell wall dimensions, and cellulose content. *Plant Physiol.* **91:** 876-882

Kossila, V. (1988). The availability of crop residues in developing countries in relation to livestock populations. Reed, J., Capper, B. and Neate, P. (eds).Addis Ababa, Ethiopia, ILCA.

Krause, D., Bunch, R., Smith, W. and McSweeney, C. (1998). Diversity of Ruminococcus strains: a survey of plant digesting ability and genetic polymorphisms. *J. Appl. Microbiol.* **86:** 487-490

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

Krause, D.O., Denman, S.E., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T. and McSweeney, C.S. (2003). Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* **27:** 663-693

Krause, D.O., McSweeney, C.S. and Forster, R.J. (1999). Molecular ecological methods to study fibrolitic ruminal bacteria: phlylogeny, competition and persistence. *In:* Proceedings of the 8th International Symposium on Microbial Ecology. Bell, C.R., Brylinsky, M. and Johnson-Green, P. (eds). Halifax, Canada, 15-19.

Krause, D.O., Smith, W.J.M., Brooker, J.D. and McSweeney, C.S. (2005). Tolerance mechanisms of streptococci to hydrolysable and condensed tannins. *Anim. Feed Sci. Technol.* **121:** 59-75

Krause, K.M. and Oetzel, G.R. (2006). Understanding and preventing subacute ruminal acidosis in dairy herds: A review. *Anim. Feed Sci. Technol.* **126:** 215-236

Krehbiel, C.R., Rust, S.R., Zhang, G. and Gilliland, S.E. (2003). Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. *J. Anim. Sci.* **81:** E120-132

Krishnamoorthy, U. and Robinson, P.H. (2010). Prediction of rumen microbial N supply in bovines from dietary values of partitioning factor (PF), in vitro rate of gas production (k), neutral detergent fibre and crude protein: A brief systematic review of studies completed in Bengaluru (India). *Anim. Feed Sci. Technol.* **160:** 167-171

Krueger, N.A. and Adesogan, A.T. (2008). Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Anim. Feed Sci. Technol.* **145:** 84-94

Kruse, S., Herrmann, A., Kornher, A. and Taube, F. (2008). Evaluation of genotype and environmental variation in fibre content of silage maize using a model-assisted approach. *Eur. J. Agron.* **28:** 210-223

Kumar, R. and Singh, M. (1984). Tannins: their adverse role in ruminant nutrition. *J. Agric. Food Chem.* **32:** 447-453

Kumar, R. and Vaithiyanathan, S. (1990). Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. *Anim. Feed Sci. Technol.* **30:** 21-38

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. . *Nature (Lond).* **227:** 680-685

Laisk, A., Eichelmann, H. and Oja, V. (2006). C-3 photosynthesis in silico. *Photosynthesis Res.* **90:** 45-66

Lamed, R., Setter, E. and Bayer, E.A. (1983). Characterization of a cellulose-binding, cellulase-containing complex in Clostridium thermocellum. *J. Bacteriol.* **156:** 828-836

Langyintuo, A.S. and Mungoma, C. (2008). The effect of household wealth on the adoption of improved maize varieties in Zambia. *Food Policy.* **33:** 550-559

Lautier, J.K., Dailey, T.V. and Brown, R.D. (1988). Effect of Water Restriction on Feed Intake of White-Tailed Deer. *J. Wildl. Manage.* **52:** 602-606

Lee, S.H. and Seo, I.J. (2005). Effects of supplementing aqueous direct-fed microbials on in vitro fermentation and fibrolytic enzyme activity in the ruminant nutrition. *J. Anim. Sci. Tech.* **47:** 789-804

Lee, S.S., Choi, C.K., Ahn, B.H., Moon, Y., Kim, C.H. and Ha, J.K. (2004). In vitro stimulation of rumen microbial fermentation by a rumen anaerobic fungal culture. *Anim. Feed Sci. Technol.* **115:** 215-226

Lee, S.S., Ha, J.K. and Cheng, K.J. (2000). Influence of an anaerobic fungal culture administration on in vivo ruminal fermentation and nutrient digestion. *Anim. Feed Sci. Technol.* **88:** 201-217

Leng, R. (1996). Evaluation of tropical feed resources for ruminant livestock. *In:* In: Proceedings of the First FAO Electronic Conference on Tropical Feeds and Feeding Systems http://www.fao.org/ag/aga/agap/frg/econf95/PDF/EVALU.PDF.

Leng, R.A. (1990). Factors Affecting the Utilization of Poor-Quality Forages by Ruminants Particularly Under Tropical Conditions. *Nutr. Res. Rev.* **3:** 277-303

Leng, R.A. and West, C.E. (1969). Contribution of acetate butyrate palmitate stearate and oleate to ketone body synthesis in sheep. *Res. Vet. Sci.* **10:** 57-&

Lenting, H.B.M. and Warmoeskerken, M.M.C.G. (2001). Mechanism of interaction between cellulase action and applied shear force, an hypothesis. *J. Biotechnol.* **89:** 217-226

Leschine, S.B. (1995). Cellulose Degradation in Anaerobic Environments. *Annu. Rev. Microbiol.* **49:** 399-426

Levy, I., Shani, Z. and Shoseyov, O. (2002). Modification of polysaccharides and plant cell wall by endo-1,4-[beta]-glucanase and cellulose-binding domains. *Biomol. Eng.* **19:** 17-30

Lewis, G.E., Hunt, C.W., Sanchez, W.K., Treacher, R., Pritchard, G.T. and Feng, P. (1996). Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. *J. Anim. Sci.* **74:** 3020-3028

Lindqvist, M., Sarkar, M., Winqvist, A., Rozners, E., Stromberg, R. and Graslund, A. (2000). Optical spectroscopic study of the effects of a single deoxyribose substitution in a ribose backbone: Implications in RNA-RNA interaction. *Biochemistry.* **39:** 1693-1701

Lodge, S., Klopfenstein, T.J., Stock, R. and Herold, D. (1996). Use of Direct Fed Microbials to Alleviate Subacute Acidosis. **http://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1477&context=animalscinbcr**

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol. Mol. Biol. Rev.* **66:** 506-577

Mabjeesh, S.J., Cohen, M. and Arieli, A. (2000). In vitro methods for measuring the dry matter digestibility of ruminant feedstuffs: Comparison of methods and inoculum source. *J. Dairy Sci.* **83:** 2289-2294

Madkour, M. and Mayer, F. (2003). Structural organization of the intact bacterial cellulosome as revealed by electron microscopy. *Cell Biol. Int.* **27:** 831-836

Maekawa, M., Beauchemin, K.A. and Christensen, D.A. (2002). Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. . *J. Dairy Sci.* **85:** 1165-1175

Maglione, G., Russell, J.B. and Wilson, D.B. (1997). Kinetics of cellulose digestion by Fibrobacter succinogenes S85. *Appl. Environ. Microbiol.* **63:** 665-669

Malcolm, A.O.N. and William, S.Y. (2003). The composition of plant primary cell wall. *In:* The Plant Cell Wall, Annual Plant Reviews. Jocelyn, K. (eds). New York: Blackwell Publishing Ltd

Maldonado, N.C., de Ruiz, C.S., Otero, M.C., Sesma, F. and Nader-Macías, M.E. (2011). Lactic acid bacteria isolated from young calves - Characterization and potential as probiotics. *Res. Vet. Sci.* **Article in Press, Corrected Proof**

Malik, R. and Bandla, S. (2010). Effect of source and dose of probiotics and exogenous fibrolytic enzymes (EFE) on intake, feed efficiency, and growth of male buffalo (Bubalus bubalis) calves. *Trop. Anim. Health Prod.* **42:** 1263-1269

Mamen, D., Vadivel, V., Pugalenthi, M. and Parimelazhagan, T. (2010). Evaluation of Fibrolytic Activity of Two Different Anaerobic Rumen Fungal Isolates for their Utilization as Microbial Feed Additive. *Anim. Nutr. Feed Technol.* **10:** 37-49

Manyuchi, B., Mikayiri, S. and Smith, T. (1994). Effect of treating or supplementing maize stover with urea on its utilization as feed for sheep and cattle. *Anim. Feed Sci. Technol.* **49:** 11-23

Markwick, G. (2002). Water requirements for sheep and cattle. *New South Wales Department of Primary Industries.* **http://www.livestock-emergency.net/userfiles/file/water-supply/Marwick-2007.pdf**

Martin, C. (2006). *Cellulose*.[Online]. http://www.lsbu.ac.uk/water/hycel.html

Martin, J.H., Leonard, W.H. and stamp, W.I. (1976). Principles of field crop production. . *McMillan,New york.* 1118

Martin, S.A. and Nisbet, D.J. (1992). Effect of Direct-Fed Microbials on Rumen Microbial Fermentation. *J. Dairy Sci.* **75:** 1736-1744

Marty, F. (1999). Plant Vacuoles. *Plant Cell.* **11:** 587-600

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S. and Theodorou, M.K. (1999). A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. *Anim. Feed Sci. Technol.* **79:** 321-330

Mauricio, R.M., Owen, E., Mould, F.L., Givens, I., Theodorou, M.K., France, J., Davies, D.R. and Dhanoa, M.S. (2001). Comparison of bovine rumen liquor and bovine faeces as inoculum for an in vitro gas production technique for evaluating forages. *Anim. Feed Sci. Technol.* **89:** 33-48

McDonald, I. (1981). A revised model for the estimation of protein degradability in the rumen. *J. Agric. Sci.* **96:** 251-252

McDonald, P. (1995). Animal nutrition. Edwards, R., Greenhalgh, J. and Morgan, C. (eds).New York, Longman Scientific and Technical Press.

McDowell, R.E. (1988). Importance of Crop residues for feeding livestock in small-holder farming systems. *In:* Plant Breeding and the Nutritive Value of Crop Residues. Reed, J.D.C., R.S. and Neate, P.J.H. (eds). Workshop at ILCA,Ethiopia, 3-27.

McNaughton, S.J. (1985). Ecology of a Grazing Ecosystem: The Serengeti. *Ecol. Monogr.* **55:** 260-294

McSweeney, C.S., Dulieu, A. and Bunch, R. (1998). Butyrivibrio spp and other xylanolytic microorganisms from the rumen have cinnamoyl esterase activity. *Anaerobe.* **4:** 57-65

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D. and Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agric. Sci.* **93:** 217-222

Methu, J.N., Owen, E., Abate, A.L. and Tanner, J.C. (2001). Botanical and nutritional composition of maize stover, intakes and feed selection by dairy cattle. *Livest. Prod. Sci.* **71:** 87-96

Miller, D.R., Elliott, R. and Norton, B.W. (2008). Effects of an exogenous enzyme, Roxazyme® G2, on intake, digestion and utilisation of sorghum and barley grain-based diets by beef steers. *Anim. Feed Sci. Technol.* **145:** 159-181

Mirzaei-Aghsaghali, A., Maheri-Sis, N., Mirza-Aghazadeh, Z. and Safaei, A.R. (2007). Use of in vìvo, in situ and in vitro Gas Production Techniques to Evaluate Nutritive Value of Alfalfa Hay in Sheep. *J. Anim. Vet. Adv.* **6:** 964-970

Moharrery, A., Hvelplund, T. and Weisbjerg, M.R. (2009). Effect of forage type, harvesting time and exogenous enzyme application on degradation characteristics measured using in vitro technique. *Anim. Feed Sci. Technol.* **153:** 178-192

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* **11:** 266-277

Morgavi, D., Beauchemin, K., Nsereko, V., Rode, L., McAllister, T., Iwaasa, A., Wang, Y. and Yang, W. (2001). Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. *J. Anim. Sci.* **79:** 1621

Morgavi, D.P., Newbold, C.J., Beever, D.E. and Wallace, R.J. (2000). Stability and stabilization of potential feed additive enzymes in rumen fluid[small star, filled]. *Enzyme Microb. Technol.* **26:** 171-177

Morris, J.G. (1975). The Physiology of Obligate Anaerobiosis. *In:* Advances in Microbial Physiology,. Rose, A.H. and Tempest, D.W. (eds). Academic Press, 169-246.

Morrison, I.M. (1974). Structural investigations on the lignin–carbohydrate complexes of Lolium perenne. *Biochem. J.* **139:** 197-204

Mould, F.L., Kliem, K.E., Morgan, R. and Mauricio, R.M. (2005). In vitro microbial inoculum: A review of its function and properties. *Anim. Feed Sci. Technol.* **123-124:** 31-50

Mountfort, D.O. (1987). The rumen anaerobic fungi. *FEMS Microbiol. Lett.* **46:** 401-408

Mourao, J.L., Ponte, P.I.P., Prates, J.A.M., Centeno, M.S.J., Ferreira, L.M.A., Soares, M.A.C. and Fontes, C. (2006). Use of beta-glucanases and beta-1,4-xylanases to supplement diets containing alfalfa and rye for laying hens: Effects on bird performance and egg quality. *J. Appl. Poult. Res.* **15:** 256-265

Murashima, K., Kosugi, A. and Doi, R.H. (2002). Synergistic Effects on Crystalline Cellulose Degradation between Cellulosomal Cellulases from Clostridium cellulovorans. *J. Bacteriol.* **184:** 5088-5095

Mussatto, S.I., Fernandes, M., Milagres, A.M.F. and Roberto, I.C. (2008). Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. *Enzyme Microb. Technol.* **43:** 124-129

Muwalla, M.M., Haddad, S.G. and Hijazeen, M.A. (2007). Effect of fibrolytic enzyme inclusion in high concentrate fattening diets on nutrient digestibility and growth performance of Awassi lambs. *Livest. Sci.* **111:** 255-258

Mwenya, B., Santoso, B., Sar, C., Pen, B., Morikawa, R., Takaura, K., Umetsu, K., Kimura, K. and Takahashi, J. (2005). Effects of yeast culture and galacto-

oligosaccharides on ruminal fermentation in Holstein cows. *J. Dairy Sci.* **88:** 1404-1412

Nagaraja, T.G., Avery, T.B., Bartley, E.E., Roof, S.K. and Dayton, A.D. (1982). Effect of Lasalocid, Monensin or Thiopeptin on Lactic Acidosis in Cattle. *J. Anim. Sci.* **54:** 649-658

Nagaraja, T.G. and Chengappa, M.M. (1998). Liver abscesses in feedlot cattle: a review. *J. Anim Sci.* **76:** 287-298

Nagpal, R., Puniya, A.K., Sehgal, J.P. and Singh, K. (2011). In vitro fibrolytic potential of anaerobic rumen fungi from ruminants and non-ruminant herbivores. *Mycoscience.* **52:** 31-38

Najmudin, S., Guerreiro, C.I.P.D., Carvalho, A.L., Prates, J.A.M., Correia, M.A.S., Alves, V.D., Ferreira, L.M.A., Romão, M.J., Gilbert, H.J., Bolam, D.N. and Fontes, C.M.G.A. (2006). Xyloglucan is recognized by carbohydrate-binding modules that interact with beta-glucan chains. *J. Biol. Chem.* **281:** 8815-8828

Ndlovu, L.R., Francis, J. and Hove, E. (1996). Performance of draught cattle in communal farming areas in Zimbabwe after dry season supplementation. *Trop. Anim. Health Prod.* **28:** 298-306

Newbold, C. (1996). Probiotics for ruminants. *Ann. Zootech.* **45:** 329-335

Newbold, C.J., Wallace, R.J., Watt, N.D. and Richardson, A.J. (1988). Effect of the novel ionophore tetronasin (ICI 139603) on ruminal microorganisms. *Appl. Environ. Microbiol.* **54:** 544-547

Nicholson, J. (1984). Digestibility, nutritive values and intake. *In:* Straw and other fibrous feeds. Sundstol, F. and Owen, E. (eds). Elsevier Publishers Amsterdam Netherlan 85-90.

Nieduszynski, I. and Marchessault, R.H. (1971). Structure of B-D-(1-4)Xylan hydrate. *Nature.* **232:** 46-47

Njie, M. and Reed, J.D. (1995). Potential of crop residues and agricultural by-products for feeding sheep in a Gambian village. *Anim. Feed Sci. Technol.* **52:** 313-323

Nocek, J.E. and Kautz, W.P. (2006). Direct-fed microbial supplementation on ruminal digestion, health, and performance of pre- and postpartum dairy cattle. *J. Dairy Sci.* **89:** 260-266

Nocek, J.E., Kautz, W.P., Leedle, J.A.Z. and Allman, J.G. (2002). Ruminal supplementation of direct-fed microbials on diurnal pH variation and in situ digestion in dairy cattle. *J. Dairy Sci.* **85:** 429-433

Nocek, J.E., Kautz, W.P., Leedle, J.A.Z. and Block, E. (2003). Direct-fed microbial supplementation on the performance of dairy cattle during the transition period. *J. Dairy Sci.* **86:** 331-335

Nsahlai, I.V., Bryant, M.J. and Umunna, N.N. (1998). Utilisation of barley straw by steers: the effect of quantity and source of nitrogen on the degradation of straw fractions, particle outflow rate and intake. *J. Appl. Anim. Res.* **14:** 33-50

Nsahlai, I.V. and Umunna, N.N. (1996). Comparison between reconstituted sheep faeces and rumen fluid inocula and between in vitro and in sacco digestibility methods as predictors of intake and in vivo digestibility. *J Agr. Sci. (Cambridge).* **126:** 235-248

Nurfeta, A., Eik, L.O., Adugna, T. and Sundstøl, F. (2008). Chemical composition and in sacco dry matter degradability of different morphological fractions of 10 enset (Ensete ventricosum) varieties. *Anim. Feed Sci. Technol.* **146:** 55-73

O'Connor-Robison, C.I., Nielsen, B.D. and Morris, R. (2007). Cellulase Supplementation Does Not Improve the Digestibility of a High-Forage Diet in Horses. *J. Equine Vet. Sci.* **27:** 535-538

Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:** 361-363

Odenyo, A. and Osuji, P. (1998). Tannin-tolerant ruminal bacteria from East African ruminants. *Can. J. Microbiol.* **44:** 905-909

Odenyo, A.A., McSweeney, C.A., Palmer, B., Negassa, D. and Osuji, P.O. (1999). In vitro screening of rumen fluid samples from indige nous African ruminants provides evidence for rumen fluid superior capacities to digest tannin-rich fodders. *Aust. J. Agric. Res.* **50:** 1147–1157

Oetzel, G.R., Nordlund, K.V. and Garrett, E.F. (1999). Effect of ruminal pH and stage of lactation on ruminal lactate concentrations in dairy cows. *J. Dairy Sci.* **82** 38

Okuda, K., Man, X., Umetsu, M., Takami, S. and Adschiri, T. (2004). Efficient conversion of lignin into single chemical species by solvothermal reaction in water–p-cresol solvent. *J. Phys.: Condens. Matter.* **16:** 1325-1330

Omed, H., Lovett, D. and Axford, R. (2000). Faeces as a source of microbial enzymes for estimating digestibility. *In:* Forage Evaluation in Ruminant Nutrition. Omed, H., Lovett, D., Axford, R., Givens, D. and Owen, E. (eds). Wallingford, UK: CBI Publishing

Onwuka, C.F.I., Adetiloye, P.O. and Afolami, C.A. (1997). Use of household wastes and crop residues in small ruminant feeding in Nigeria. *Small Ruminant Res.* **24:** 233-237

Ortiz-Monasterio, J.I., Palacios-Rojas, N., Meng, E., Pixley, K., Trethowan, R. and Peña, R.J. (2007). Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *J. Cereal Sci.* **46:** 293-307

Otte, M.J. and Chilonda, P. (2002). Cattle and Small Ruminant Production Systems in sub-Saharan Africa - A Systematic Review. *In:* Food and Agriculture Organization of the United Nations. Green, I.L. (eds). Rome FAO,Viale delle Terme di Caracalla

Ouda, J. and Nsahlai, I. (2009). Relevance and potential use of in vitro gas production measurements to evaluate varying ratios of roughages and protein sources for ruminants. *J. Appl. Anim. Res.* **35:** 9-16

Ouda, J.O. (2007). Nutritive value and manure quality in supplemented maize stover and grass hay diets for ruminants PhD Thesis, University of KwaZulu-Natal.

Ouda, J.O., Nsahlai, I.V. and Mahundu, P. (2006). *In vitro* degradability and gas production parameters of sericea lespedeza (Lespedeza cuneata) mixed with varying types and levels of roughages. *S. Afr. J. Anim. Sci.* **32:** 111-121

Owens, F. and Goestsch, A. (1998). Ruminal fermentation. *In:* The Ruminant Animal Digestive Physiology and Nutrition. Church, C. (eds). Illinois: Waveland Press, Inc., 145-216.

Owolabi, B., Beguin, P., Kilburn, G., Miller, C. and Antony, J. (1988). Expression in Escherichia coli of the cellulomonas fimi structural Gene for Endoglucanase B. *Appl. Environ. Microbiol.* **54:** 518-523

Palmer, B., Jones, R.J., Poathong, S. and Chobtang, J. (2010). Within-country variation in the ability of ruminants to degrade DHP following the ingestion of Leucaena leucocephala-a Thailand experience. *Trop. Anim. Health Prod.* **42:** 161-164

Paul, S.S., Deb, S.M., Punia, B.S., Das, K.S., Singh, G., Ashar, M.N. and Kumar, R. (2011). Effect of feeding isolates of anaerobic fungus Neocallimastix sp CF 17 on growth rate and fibre digestion in buffalo calves. *Arch. Anim. Nutr.* **65:** 215-228

Paul, S.S., Deb, S.M., Punia, B.S., Singh, D. and Kumar, R. (2010). Fibrolytic potential of anaerobic fungi (Piromyces sp.) isolated from wild cattle and blue bulls in pure culture and effect of their addition on in vitro fermentation of wheat straw and methane emission by rumen fluid of buffaloes. *J. Sci. Food Agric.* **90:** 1218-1226

Paul, S.S., Kamra, D.N., Sastry, V.R.B., Sahu, N.P. and Agarwal, N. (2004). Effect of administration of an anaerobic gut fungus isolated from wild blue bull (Boselaphus tragocamelus) to buffaloes (Bubalus bubalis) on in vivo ruminal fermentation and digestion of nutrients. *Anim. Feed Sci. Technol.* **115:** 143-157

Pell, A.N. and Schofield, P. (1993). Computerized Monitoring of Gas Production to Measure Forage Digestion In Vitro. *J. Dairy Sci.* **76:** 1063-1073

Percival Zhang, Y.H., Himmel, M.E. and Mielenz, J.R. (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnol. Adv.* **24:** 452-481

Perlack, R.D. and Turhollow, A.F. (2003). Feedstock cost analysis of corn stover residues for further processing. *Energy.* **28:** 1395-1403

Peter, A. (1990). Carbohydrates of Physiological significance. *In:* Herper's Biochemistry, Prentice Hall International. Daryl, K., Robert, K. and Vector, W. (eds). London, United Kingdom, 124

Peters, A., Lebzien, P., Meyer, U., Borchert, U., Bulang, M. and Flachowsky, G. (2010). Effect of exogenous fibrolytic enzymes on ruminal fermentation and nutrient digestion in dairy cows. *Arch. Anim. Nutr.* **64:** 221-237

Philippeau, C. and Michalet-Doreau, B. (1997). Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. *Anim. Feed Sci. Technol.* **68:** 25-35

Pinos-Rodriguez, J.M., Moreno, R., Gonzalez, S.S., Robinson, P.H., Mendoza, G. and Alvarez, G. (2008). Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. *Anim. Feed Sci. Technol.* **142:** 210-219

Ponte, P.I.P., Ferreira, L.M.A., Soares, M.A.C., Aguiar, M., Lemos, J.P.C., Mendes, I. and Fontes, C. (2004). Use of cellulases and xylanases to supplement diets containing alfalfa for broiler chicks: Effects on bird performance and skin color. *J. Appl. Poult. Res.* **13:** 412-420

Poppi, D.P. and McLennan, S.R. (1995). Protein and energy utilization by ruminants at pasture. *J. Anim. Sci.* **73:** 278-290

Pordesimo, L.O., Edens, W.C. and Sokhansanj, S. (2004). Distribution of aboveground biomass in corn stover. *Biomass Bioenergy.* **26:** 337-343

Poutanen, K. (1997). Enzymes: An important tool in the improvement of the quality of cereal foods. *Trends Food Sci. Technol.* **8:** 300-306

Powell, J.M. and Unger, P.W. (1998). Alternatives to crop residues for sustaining agricultural productivity and natural resource conservation. *J. Sustainable Agric.* **11:** 59-84

Prates, A., de Oliveira, J.A., Abecia, L. and Fondevila, M. (2010). Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation. *Anim. Feed Sci. Technol.* **155:** 186-193

Pratima, B. (1999). Application of Enzymes in the Pulp and Paper Industry. *Biotechnol. Prog.* **15:** 147-157

Pressman, B.C. (1976). Biological Applications of Ionophores. *Annu. Rev. Biochem.* **45:** 501-530

Preston, T.R. and Leng, R.A. (1987). Matching ruminant production systems with available resources in the tropics and sub-tropics. *In:* Penambul Books. Armidale 245.

Prince, R., Wheeler, W. and Fisher, D. (1958). Discussion on energy requirements for cutting forage. *Agr. Eng.* **39:** 638-639

Qiao, G.H., Shan, A.S., Ma, N., Ma, Q.Q. and Sun, Z.W. (2010). Effect of supplemental Bacillus cultures on rumen fermentation and milk yield in Chinese Holstein cows. *J. Anim. Physiol. Anim. Nutr.* **94:** 429-436

Quinn, L.Y., Burroughs, W., Christiansen, W.C. (1962). Continuous culture of ruminal microorganisms in chemically defined medium. II. Culture medium studies. *Appl. Microbiol.* **10:** 583-592

Raeth-Knight, M.L., Linn, J.G. and Jung, H.G. (2007). Effect of direct-fed microbials on performance, diet digestibility, and rumen characteristics of Holstein dairy cows. *J. Dairy Sci.* **90:** 1802-1809

Rakhmani, S., Brooker, J.D., Jones, G.P. and Palmer, B. (2005). Composition of condensed tannins from Calliandra calothyrsus and correlation with in sacco digestibility. *Anim. Feed Sci. Technol.* **121:** 109-124

Ranilla, M.J., Tejido, M.L., Giraldo, L.A., Tricárico, J.M. and Carro, M.D. (2008). Effects of an exogenous fibrolytic enzyme preparation on in vitro ruminal fermentation of three forages and their isolated cell walls. *Anim. Feed Sci. Technol.* **145:** 109-121

Ransom, J.K., Paudyal, K. and Adhikari, K. (2003). Adoption of improved maize varieties in the hills of Nepal. *Agric. Econ.* **29:** 299-305

Reddy, B.V.S., Reddy, P.S., Bidinger, F. and Blummel, M. (2003). Crop management factors influencing yield and quality of crop residues. *Field Crops Res.* **84:** 57-77

Reese, E.T., Siu, R.G.H. and Levinson, H.S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J. Bacteriol.* **59:** 485-950

Reverbel-Leroy, C., Pages, S., Belaich, A., Belaich, J.P. and Tardif, C. (1997). The processive endocellulase CelF, a major component of the Clostridium cellulolyticum cellulosome: purification and characterization of the recombinant form. *J. Bacteriol.* **179:** 46-52

Richardson, L.F., Raun, A.P., Potter, E.L., Cooley, C.O. and Rathmacher, R.P. (1976). Effect of Monensin on Rumen Fermentation in Vitro and in Vivo. *J. Anim. Sci.* **43:** 657-664

Richey, C.B., Liljedahl, J.B. and Lechtenberg, V.L. (1982). Corn stover harvest for energy production. *Trans. ASAE.* **25:** 834–844

Ridley, B.L., O'Neill, M.A. and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry.* **57:** 929-967

Robles, A., Belyea, R., Martz, F. and Weiss, M. (1980). Effect of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchardgrass forages. *J. Anim. Sci.* **51:** 783-790

Ross, P., Mayer, R. and Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. *Microbiol. Mol. Biol. Rev.* **55:** 35-58

Rumsey, T.S. (1984). Monensin in Cattle: Introduction. *J. Anim. Sci.* **58:** 1461-1464

Russell, J.B. and Hino, T. (1985). Regulation of lactate production in Streptococcus bovis: a spiralling effect that contributes to rumen acidosis. *J. Dairy Sci.* **68:** 1712-1721

Russell, J.B. and Rychlik, J.L. (2001). Factors That Alter Rumen Microbial Ecology. *Science.* **292:** 1119-1122

Russell, J.B. and Wilson, D.B. (1996). Why Are Ruminal Cellulolytic Bacteria Unable to Digest Cellulose at Low pH? *J. Dairy Sci.* **79:** 1503-1509

Russell, J.R. (1986). Influence of harvest date on the nutritive value and ensiling characteristics of maize stover. *Anim. Feed Sci. Technol.* **14:** 11-27

Rymer, C. and Givens, D.I. (2000). Predicting the in vitro gas production profile of dried grass with strained rumen fluid from the in vitro gas production profile of dried grass with faeces. *Proceedings of the British Society of Animal Science.* 48

Saleem, M. (1995). 'Fragile East African highlands: a development vision for smallholder farmers in the Ethiopian Highlands'. *Outlook Agric.* **24:** 111-116

SAS (2002). Statiscal Analysis System user's guide (version 8). SAS Institude Inc., SAS CampusDrive, Cary, N.C., USA.

Sasaki, E., Shimada, T., Osawa, R., Nishitani, Y., Spring, S. and Lang, E. (2005). Isolation of tannin-degrading bacteria isolated from feces of the Japanese large wood mouse, Apodemus speciosus, feeding on tannin-rich acorns. *Syst. Appl. Microbiol.* **28:** 358-365

Saxena, I.M. and Brown Jr, M.R. (2008). Biochemistry and Molecular Biology of Cellulose Biosynthesis in Plants: Prospects for Genetic Engineering. *In:* Advances in Plant Biochemistry and Molecular Biology. Pergamon, 135-160.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry.* **30:** 3875-3883

Schelling, G.T. (1984). Monensin Mode of Action in the Rumen. *J. Anim. Sci.* **58:** 1518-1527

Schofield, P., Pitt, R.E. and Pell, A.N. (1994). Kinetics of fiber digestion from in vitro gas production. *J. Anim Sci.* **72:** 2980-2991

Schwarz, W.H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Appl. Microbiol. Biotechnol.* **56:** 634-49

Sehgal, J.P., Jit, D., Puniya, A.K. and Singh, K. (2008). Influence of anaerobic fungal administration on growth, rumen fermentation and nutrient digestion in female buffalo calves. *J. Anim. Feed Sci.* **17:** 510-518

Sejrsen, K., Hvelplund, T. and Nielsen, M.O. (2006). Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Wageningen Netherlands, Wageningen Academic Publishers.

Seo, J.K., Kim, S.W., Kim, M.H., Upadhaya, S.D., Kam, D.K. and Ha, J.K. (2010). Direct-fed Microbials for Ruminant Animals. *Asian-Australas. J. Anim. Sci.* **23:** 1657-1667

Seré, C. (2004). Raising Livestock Production in Africa. *Assuring food and nutrition security in Africa by 2020: Prioritizing Action, Strengthening Actors, and Facilitating Partnerships April 1-3, 2004, Kampala, Uganda*

Setia, A., Bhandari, S.K., House, J.D., Nyachoti, C.M. and Krause, D.O. (2009). Development and in vitro evaluation of an Escherichia coli probiotic able to inhibit the growth of pathogenic Escherichia coli K88. *J. Anim. Sci.* **87:** 2005-2012

Seyis, I. and Aksoz, N. (2005). Investigation of factors affecting xylanase activity from Trichoderma harzianum 1073 D3. *Braz. Arch. Biol. Technol.* **48:** 187-193

Shafi, M., Bakht, J., Jan, M.T. and Shah, Z. (2007). Soil C and N dynamics and maize (Zea may L.) yield as affected by cropping systems and residue management in North-western Pakistan. *Soil Till. Res.* **94:** 520-529

Shekhar, C., Thakur, S.S. and Shelke, S.K. (2010). Effect of exogenous fibrolytic enzymes supplementation on milk production and nutrient utilization in Murrah buffaloes. *Trop. Anim. Health Prod.* **42:** 1465-1470

Shelke, S.K., Chhabra, A., Puniya, A.K. and Sehgal, J.P. (2009). In vitro degradation of sugarcane bagasse based ruminant rations using anaerobic fungi. *Ann. Microbiol.* **59:** 415-418

Shi, L., Potts, M. and Kennelly, P.J. (1998). The serine, threonine, and/or tyrosine specific protein kinases and protein phosphatases of prokaryotic organisms: a family portrait. *FEMS Microbiol. Rev.* **22:** 229-253

Shinners, K.J., Binversie, B.N., Muck, R.E. and Weimer, P.J. (2007). Comparison of wet and dry corn stover harvest and storage. *Biomass Bioenergy.* **31:** 211-221

Shoham, Y., Lamed, R. and Bayer, E.A. (1999). The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. *Trends Microbiol.* **7:** 275-281

Siebert, B.D. and Hunter, R.A. (1982). Supplementary feeding of grazing animals. *In:* Nutritional Limits to Animal Production from Pastures, Proceedings of an International Symposium. Hacker, J.B. (eds). St. Lucia, Queensland, Australia: Commonwealth Agricultural Bureaux Press, 409–426.

Silva, M.L.F., Lima, J.A.D., Cantarelli, V.D., Amaral, N.D., Zangeronimo, M.G. and Fialho, E.T. (2010). Probiotics and antibiotics as additives for sows and piglets during nursery phase. *Rev. Bras. Zootecn.* **39:** 2453-2459

Singh, N.P., More, T. and Sahni, K.L. (1976). Effect of water deprivation on feed intake, nutrient digestibility and nitrogen retention in sheep. *J. Agric. Sci.* **86:** 431-433

Singh, S., Pradhan, K. and Bhatia, S.K. (1994). The effect of trans-inoculation of rumen contents in microflora concentration in the rumen of cattle and buffalo. *Indian J. Anim. Nutr.* **11:** 133-138

Singh, S., Pradhan, K. and Bhatia, S.K. (1997). Effect of transinoculation of rumen contents between cattle and buffalo on feed intake, nutrient digestibility and rumen metabolites. *Indian J. Anim. Nutr.* **14:** 25-30

Smith, L., Goering, H., Waldo, D. and Gordon, C. (1971). In vitro digestion rate of forage cell wall components. *J. Dairy Sci.* **54:** 71-76

Smith, O., Idowu, O. and Asaolu, V. (1989). Comparative rumen degradability of forages, browse, crop residues and agricultural by-products. *In:* African small ruminant and research develeopment. Trevor, W.R. and Azeb, M. (eds). Bamenda, Cameroon: ILCA, Addis Ababa, 204.

Smith, R.C., Moussa, N.M. and Hawkins, G.E. (1974). Utilization of the nucleic acids of Escherichia coli and rumen bacteria by sheep. *Br. J. Nutr.* **32:** 529-537

Soest, P.V. and Robertson, J. (1985). Analysis of forages and fibrous feeds. Cornell University, Ithaca, New York, USA

Sokhansanj, S., Turhollow, A., Cushman, J. and Cundiff, J. (2002). Engineering aspects of collecting corn stover for bioenergy. *Biomass Bioenergy.* **23:** 347-355

Solaiman, S.G. and Shoemaker, C. (2009). Intake, digestibility, rumen metabolism and growth performance of goat kids raised under different production systems. *Trop. Subtrop. Agroecosyst.* **11:** 219-223

Song, M.K., Li, X.Z., Oh, Y.K., Lee, C.K. and Hyun, Y. (2011). Control of Methane Emission in Ruminants and Industrial Application of Biogas from Livestock Manure in Korea. *Asian-Australas. J. Anim. Sci.* **24:** 130-136

Sotohy, S., Sayed, A. and Ahmed, M. (1997). Effect of tannin-rich plant (Acacia nilotica) on some nutritional and bacteriological parameters in goats. *DTW. Deutsche tierärztliche Wochenschrift.* **104:** 432-435

Stella, A.V., Paratte, R., Valnegri, L., Cigalino, G., Soncini, G., Chevaux, E., Dell'Orto, V. and Savoini, G. (2007). Effect of administration of live Saccharomyces cerevisiae on milk production, milk composition, blood metabolites, and faecal flora in early lactating dairy goats. *Small Ruminant Res.* **67:** 7-13

Stephens, T.P., Stanford, K., Rode, L.M., Booker, C.W., Vogstad, A.R., Schunicht, O.C., Jim, G.K., Wildman, B.K., Perrett, T. and McAllister, T.A. (2010). Effect of a direct-fed microbial on animal performance, carcass characteristics and the shedding of Escherichia coli O157 by feedlot cattle. *Anim. Feed Sci. Technol.* **158:** 65-72

Stern, M.D., Bach, A. and Calsamiglia, S. (1997). Alternative techniques for measuring nutrient digestion in ruminants. *J. Anim Sci.* **75:** 2256-2276

Stock, J. (1999). Sensitivity, coopertivity and gain in chemotaxis signal transduction *Trends Microbiol.* **7:** 1-4

Stock, J., Ninfa, A. and Stock, A. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Mol. Biol. Rev.* **53:** 450-490

Sun, X., Liu, Z., Zheng, K., Song, X. and Qu, Y. (2008). The composition of basal and induced cellulase systems in Penicillium decumbens under induction or repression conditions. *Enzyme Microb. Technol.* **42:** 560-567

Sun, Z.H., Liu, S.M., Tayo, G.O., Tang, S.X., Tan, Z.L., Lin, B., He, Z.X., Hang, X.F., Zhou, Z.S. and Wang, M. (2009). Effects of cellulase or lactic acid bacteria on silage fermentation and in vitro gas production of several morphological fractions of maize stover. *Anim. Feed Sci. Technol.* **152:** 219-231

Sutton, J.D., Dhanoa, M.S., Morant, S.V., France, J., Napper, D.J. and Schuller, E. (2003). Rates of Production of Acetate, Propionate, and Butyrate in the Rumen of Lactating Dairy Cows Given Normal and Low-Roughage Diets. *J. Dairy Sci.* **86:** 3620-3633

Swanston, J.S. and Newton, A.C. (2005). Mixtures of UK Wheat as an Efficient and Environmentally Friendly Source for Bioethanol. *J. Indust. Ecol.* **9:** 109-126

Szijártó, N., Siika-aho, M., Tenkanen, M., Alapuranen, M., Vehmaanperä, J., Réczey, K. and Viikari, L. (2008). Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of Melanocarpus albomyces. *J. Biotechnol.* **In Press, Accepted Manuscript**

Tan, Z., Chen, H., He, L., Fang, R. and Xing, T. (1995). Variation in the nutritional characteristics of wheat straw. *Anim. Feed Sci. Technol.* **53:** 337-344

Tang, S.X., Tayo, G.O., Tan, Z.L., Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, X.F. and Shen, S.B. (2008). Effects of yeast culture and fibrolytic enzyme supplementation on in vitro fermentation characteristics of low-quality cereal straws. *J. Anim. Sci.* **86:** 1164-1172

Teeri, T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends Biotechnol.* **15:** 160-167

Thornton, J.H. and Owens, F.N. (1981). Monensin Supplementation and in vivo Methane Production by Steers. *J. Anim. Sci.* **52:** 628-634

Thornton, P., Kruska, R., Henninger, N., Kristjanson, P., Reid, R., Atieno, F., Odero, A. and Ndegwa, T. (2002). *Mapping Poverty and Livestock in the Developing World.Nairobi, Kenya: International Livestock Research Institute.*

http://www.ilri.cgiar.org/InfoServ/Webpub/fulldocs/mappingPLDW/index.htm.[Online] . http://www.ilri.cgiar.org/InfoServ/Webpub/fulldocs/mappingPLDW/index.htm.

Tilley, J.M.A. and Terry, R.A. (1963). A two-stage technique for the in vitro digestion of forage crops *J. Brit. Grass. Soc.* **18** 104-11

Titi, H.H. and Tabbaa, M.J. (2004). Efficacy of exogenous cellulase on digestibility in lambs and growth of dairy calves. *Livest. Prod. Sci.* **87:** 207-214

Tolera, A., Berg, T. and Sundstøl, F. (1999). The effect of variety on maize grain and crop residue yield and nutritive value of the stover. *Anim. Feed Sci. Technol.* **79:** 165-177

Tolera, A. and Sundstøl, F. (1999). Morphological fractions of maize stover harvested at different stages of grain maturity and nutritive value of different fractions of the stover. *Anim. Feed Sci. Technol.* **81:** 1-16

Tolera, A., Sundstøl, F. and Said, A.N. (1998). The effect of stage of maturity on yield and quality of maize grain and stover. *Anim. Feed Sci. Technol.* **75:** 157-168

Trinci, A.P.J., Davies, D.R., Gull, K., Lawrence, M.I., Bonde Nielsen, B., Rickers, A. and Theodorou, M.K. (1994). Anaerobic fungi in herbivorous animals. *Mycol. Res.* **98:** 129-152

Tripathi, M.K. and Karim, S.A. (2010). Effect of individual and mixed live yeast culture feeding on growth performance, nutrient utilization and microbial crude protein synthesis in lambs. *Anim. Feed Sci. Technol.* **155:** 163-171

Tripathi, M.K. and Karim, S.A. (2011). Effect of yeast cultures supplementation on live weight change, rumen fermentation, ciliate protozoa population, microbial hydrolytic enzymes status and slaughtering performance of growing lamb. *Livest. Sci.* **135:** 17-25

Tripathi, M.K., Santra, A., Chaturvedi, O.H. and Karim, S.A. (2004). Effect of sodium bicarbonate supplementation on ruminal fluid pH, feed intake, nutrient utilization and growth of lambs fed high concentrate diets. *Anim. Feed Sci. Technol.* **111:** 27-39

Tufarelli, V., Cazzato, E., Ficco, A. and Laudadio, V. (2010). Evaluation of Chemical Composition and In vitro Digestibility of Appennine Pasture Plants Using Yak (Bos grunniens) Rumen Fluid or Faecal Extract as lnoculum Source. *Asian-Australas. J. Anim. Sci.* **23:** 1587-1593

Undi, M., Kawonga, K.C. and Musendo, R.M. (2001). Nutritive value of maize stover/pasture legume mixtures as dry season supplementation for sheep. *Small Ruminant Res.* **40:** 261-267

United Nations (2010). United Nations Population Information Network. *http://www.un.org/popin/*.

Vaithiyanathan, S., Sheikh, Q. and Kumar, R. (2005). Effect of transinoculation of goat rumen liquor on degradation and metabolism of mimosine in sheep fed with Leucaena leucocephala leaves. *Asian-Australas. J. Anim. Sci.* **18:** 332-339

van Ackeren, C., Steingaß, H., Hartung, K., Funk, R. and Drochner, W. (2009). Effect of roughage level in a total mixed ration on feed intake, ruminal fermentation patterns and chewing activity of early-weaned calves with ad libitum access to grass hay. *Anim. Feed Sci. Technol.* **153:** 48-59

Van Soest, P. (1982). Nutritional Ecology of the ruminant. O & B Books, Corrallis, .

Van Soest, P. (1994). Nutritional Ecology of the Ruminant: Ruminant Metabolism. *Nutritional Strategies, the Cellulolytic Fermentation and the Chemistry of Forages and Plant Fibers, Oregon: O&B Books Inc.*

Van Soest, P., Robertson, J. and Lewis, B. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **74:** 3583-3597

Van Soest, P.J. (1981). Limiting factors in plant residues of low biodegradability. *Agricult. and Environ.* **6:** 135-143

Vaquero, I., Marcobal, Á. and Muñoz, R. (2004). Tannase activity by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* **96:** 199-204

Váradyová, Z., Baran, M. and Zelenák, I. (2005). Comparison of two in vitro fermentation gas production methods using both rumen fluid and faecal inoculum from sheep. *Anim. Feed Sci. Technol.* **123-124:** 81-94

Váradyová, Z., Styriaková, I. and Kisidayová, S. (2007). Effect of natural dolomites on the in vitro fermentation and rumen protozoan population using rumen fluid and fresh faeces inoculum from sheep. *Small Ruminant Res.* **73:** 58-66

Varga, G.A. and Kolver, E.S. (1997). Microbial and Animal Limitations to Fiber Digestion and Utilization. *J. Nutr.* **127:** 819S-

Vatthauer, R.J., Hinds, F.C. and Garrigus, U.S. (1970a). Continuous In Vitro Culture System for Ruminant Research I. Design. *J. Anim Sci.* **30:** 618-623

Vatthauer, R.J., Hinds, F.C. and Garrigus, U.S. (1970b). Continuous In Vitro Culture System for Ruminant Research II. Biological Characterization. *J. Anim Sci.* **30:** 624-633

Vuong, T.V. and Wilson, D.B. (2009). Processivity, Synergism, and Substrate Specificity of Thermobifida fusca Cel6B. *Appl. Environ. Microbiol.* **75:** 6655-6661

Waghorn, G. (2007). Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production--Progress and challenges. *Anim. Feed Sci. Technol.* **147:** 116-139

Waksman, G. (1991). Purification and characterization of 2 endo-beta-1,4-d-glucanases from sclerotinia-sclerotiorum. *Biochim. Biophys. Acta.* **1073:** 49-55

Wallace, R.J., Colombatto, D. and Robinson, P.H. (2008). Enzymes, direct-fed microbials and plant extracts in ruminant nutrition. *Anim. Feed Sci. Technol.* **145:** 1-4

Walsh, M.C., Saddoris, K.L., Sholly, D.M., Hinson, R.B., Sutton, A.L., Applegate, T.J., Richert, B.T. and Radcliffe, J.S. (2007). The effects of direct fed microbials delivered through the feed and/or in a bolus at weaning on growth performance and gut health. *Livest. Sci.* **108:** 254-257

Wanapat, M. (2000). Rumen manipulation to increase the efficient use of local feed resources and productivity of ruminants in the tropics. *Asian-Australas. J. Anim. Sci.* **13:** 59-67

Wanapat, M. (2009). Potential uses of local feed resources for ruminants. *Trop. Anim. Health Prod.* **41:** 1035-1049

Wanapat, M., Nontaso, N., Yuangklang, C., Wora-anu, S., Ngarmsang, A., Wachirapakorn, C. and Rowlinson, P. (2003). Comparative study between swamp buffalo and native cattle in feed digestibility and potential transfer of buffalo rumen digesta into cattle. *Asian-Australas. J. Anim. Sci.* **16:** 504-510

Wanapat, M., Pilajun, R. and Kongmun, P. (2009). Ruminal ecology of swamp buffalo as influenced by dietary sources. *Anim. Feed Sci. Technol.* **151:** 205-214

Wanapat, W. (2001). Swamp buffalo rumen ecology and its manipulation. Proceedings of Buffalo Workshop. www.mekarn.org/procbuf/wanapat.htm

Wang, F., Li, F., Chen, G. and Liu, W. (2009). Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. *Microbiol. Res.* **164:** 650-657

Wang, Y., Baah, J., Yanke, L.J. and McAllister, T.A. (2005). Effect of mixed culture of Lactobacillus paracasei and Lactobacillus lactis and their fermentation products on ruminal fermentation of a barley grain/barley silage diet. *J. Dairy Sci.* **88:** 50-50

Wang, Y. and McAllister, T.A. (2002). Rumen microbes, enzymes and feed digestion - A review. *Asian-Australas. J. Anim. Sci.* **15:** 1659-1676

Wei, H., Xu, Q., Taylor Ii, L.E., Baker, J.O., Tucker, M.P. and Ding, S.-Y. (2009). Natural paradigms of plant cell wall degradation. *Curr. Opin. Biotechnol.* **20:** 330-338

Weimer, P.J. (1996). Why Don't Ruminal Bacteria Digest Cellulose Faster? *J. Dairy Sci.* **79:** 1496-1502

Weimer, P.J. (1998). Manipulating ruminal fermentation: a microbial ecological perspective. *J. Anim Sci.* **76:** 3114-3122

Wells, J.E., Krause, D.O., Callaway, T.R. and Russell, J.B. (1997). A bacteriocin-mediated antagonism by ruminal lactobacilli against Streptococcus bovis. *FEMS Microbiol. Ecol.* **22:** 237-243

Weston, R. and Hogan, J. (1967). The digestion of chopped and ground roughages by sheep. 1. The movement of digesta through the stomach. *Aust. J. Agric. Res.* **18:** 789-801

WHO (2002). World Health Report 2002:Reducing Risks, Promoting Healthy Life. *In:* World Health Organization, Geneva.

WHO. (2011). *World Health Statistics 2011*. Geneva.[Online]. http://www.who.int/whosis/whostat/EN_WHS2011_Full.pdf.

Wilson, A.D. and MacLeod, N.D. (1991). Overgrazing: Present or Absent? *J. Range Manage.* **44:** 475-482

Wilson, J. (1973). The influence of aerial environment, nitrogen suplly, and ontogenetical changes on the chemical composition and digestibility of Panicum maximum Jacq. var. trichoglume Eyles. *Austr. J. Agric. Res.* **24:** 543-556

Wilson, R.T. (1991). Small ruminant production and the small ruminant genetic resource in tropical Africa. *In*. Rome, Italy: Food and Agriculture Organization of the United Nations, 16.

Wood, T.M. and Bhat, K.M. (1988). Methods for measuring cellulase activities. *Methods Enzymol.* **160:** 87-112

Woodward, J. (1991). Synergism in cellulase systems. *Bioresour. Technol.* **36:** 67-75

World Resources Institute (2005). The Role of Livestock. *In:* The Wealth of the Poor: Managing ecosystems to fight poverty. World Resources Institute (eds). http://www.wri.org/publication/content/8063:

Xi, D.M., Wanapat, M., Deng, W.D., He, T.B., Yang, Z.F. and Mao, H.M. (2007). Comparison of Gayal (Bos frontalis) and Yunnan Yellow Cattle (Bos taurus): In vitro dry matter digestibility and gas production for a range of forages. *Asian-Australas. J. Anim. Sci.* **20:** 1208-1214

Xiao, Q., Brent, S., Yanke, J. and Cheng, J. (2000). Isolation and analysis of two cellulase cDNAs from Orpinomyces johonii. *Gene.* **245:** 119-126

Yang, H.E., Son, Y.S. and Beauchemin, K.A. (2011). Effects of Exogenous Enzymes on Ruminal Fermentation and Degradability of Alfalfa Hay and Rice Straw. *Asian-Australas. J. Anim. Sci.* **24:** 56-64

Yang, H.J. and Xie, C.Y. (2010). Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn stalks in in vitro enzymatic and ruminal batch cultures. *Anim. Feed Sci. Technol.* **159:** 110-121

Yang, S., He, H., Lu, S., Chen, D. and Zhu, J. (2008). Quantification of crop residue burning in the field and its influence on ambient air quality in Suqian, China. *Atmos. Environ.* **42:** 1961-1969

Yang, S.L., Ma, S.C., Chen, J., Mao, H.M., He, Y.D., Xi, D.M., Yang, L.Y., He, T.B. and Deng, W.D. (2010). Bacterial diversity in the rumen of Gayals (Bos frontalis), Swamp buffaloes (Bubalus bubalis) and Holstein cow as revealed by cloned 16S rRNA gene sequences. *Mol. Biol. Rep.* **37:** 2063-2073

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. (1999). Effects of an Enzyme Feed Additive on Extent of Digestion and Milk Production of Lactating Dairy Cows. *J. Dairy Sci.* **82:** 391-403

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. (2000). A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *J. Dairy Sci.* **83:** 2512-2520

Yang, W.Z., Beauchemin, K.A., Vedres, D.D., Ghorbani, G.R., Colombatto, D. and Morgavi, D.P. (2004). Effects of direct-fed microbial supplementation on ruminal acidosis, digestibility, and bacterial protein synthesis in continuous culture. *Anim. Feed Sci. Technol.* **114:** 179-193

Ye, X.Y., Ng, T.B. and Cheng, K.J. (2001). Purification and characterization of a cellulase from the ruminal fungus Orpinomyces joyonii cloned in Escherichia coli. *Int. J. Biochem. Cell Biol.* **33:** 87-94

Yokoyama, M. and Johnson, A. (1998). Microbiology of the rumen and intestine *In:* The Ruminant Animal Digestive Physiology and Nutrition. Church, C. (eds). US America: Waveland Press, 25.

Young, J. (1977). Gluconeogenesis in cattle:significance and methodology. *J. Dairy Sci.* **60:** 1-5

Zerihun, H.D. and Oba, G. (2004). Feed scarcity and livestock mortality in enset farming systems in the Bale highlands of southern Ethiopia. *Outlook Agric.* **33:** 277-280

Zhu, C.L., Rademacher, M. and de Lange, C.F.M. (2005). Increasing dietary pectin level reduces utilization of digestible threonine intake, but not lysine intake, for body protein deposition in growing pigs. *J. Anim. Sci.* **83:** 1044-1053

ZoBell, D.R., Wiedmeier, R.D., Olson, K.C. and Treacher, R. (2000). The effect of an exogenous enzyme treatment on production and carcass characteristics of growing and finishing steers. *Anim. Feed Sci. Technol.* **87:** 279-285