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Effects of supplementing different proportions of cellulase and xylanase enzyme mixtures on intake and digestibility of high forage total mixed ration by sheep

by

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DECLARATION

I hereby declare that this thesis submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria is my own work and has not been previously submitted by me for a degree at any other university.

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K. Selzer
Pretoria
March 2017

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ABSTRACT

Effects of supplementing different proportions of cellulase and xylanase enzyme mixtures on intake and digestibility of high forage total mixed ration by sheep

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Exogenous fibrolytic enzymes present one way of improving fibre digestibility. Therefore, the aim of this study was to investigate the effect of adding different proportions of cellulase and xylanase enzyme mixtures on the *in vitro* gas and methane production characteristics, ruminal fermentation and *in vitro* digestibility, as well as the *in vivo* digestibility and rumen fermentation parameters in sheep fed with a high roughage total mixed ration (TMR).

During the *in vitro* study Smutsfinger hay was pre-treated for 24 hours with different enzyme solutions (100% cellulose; 75% cellulase: 25% xylanase; 50% cellulase: 50% xylanase; 25% cellulase: 75% xylanase; 100% xylanase and a no enzyme control). The samples were then incubated in a shaking incubator and the gas and methane production was measured at 0, 2, 4, 8, 12, 24 and 48 hours. Addition of different proportions of cellulase and xylanase plus enzyme mixtures to Smutsfinger hay, especially the 100% cellulase treatment, increased nutrient degradability, total gas and methane production; lowered end fermentation pH and altered the volatile fatty acid (VFA) concentration. Cellulase had a much greater effect than xylanase, and its effects decrease as its concentration in the enzyme mixture decreased below 75%.

During the *in vivo* study a TMR diets were prepared fresh every day starting with a 24hr pre-incubation period of the Smutsfinger hay with the prepared enzyme solution. The treatments were 100% cellulase; 75% cellulase: 25% xylanase; 50% cellulase: 50% xylanase, 25% cellulase: 75% xylanase; 100% xylanase and an untreated TMR (control). Before feeding each day, the pre-incubated Smutsfinger hay was mixed with the appropriate amount of concentrate to form the TMR. A digestibility trial was conducted using a 6 x 6 latin square

design and rumen fluid samples were collected and preserved for VFA analysis. Addition of the mixture of 50% cellulase and xylanase plus enzyme mixture, to Smutsfinger hay increased acetate and total VFA concentrations as well as enhanced the intake and the total tract digestibility of the neutral detergent fibre (NDF) and acid detergent fibre (ADF) of the test feed. Of all the treatments, the 50:50 treatment followed by 75:25 cellulase was most effective in increasing dry matter intake in g/head/unit metabolic weight. The 50% treatment increased digestibility of dry matter (DM), organic matter (OM) and crude protein (CP), but the increase in the digestibility was not significant, the treatment however, showed significantly higher NDF and ADF digestibility, acetate production as well as total VFA production compared to the control. There was no significant difference between the treatments for propionate and butyrate. Although the different proportions of cellulase and xylanase plus enzyme mixtures generally improved TMR intake, fibre digestion and altered the VFA concentration, the best response was obtained with the 50% cellulase and xylanase plus enzyme mixture.

LIST OF ABBREVIATIONS

ADF	acid detergent fibre
ADG	average daily gain
CaOH	calcium hydroxide
CO_2	carbon dioxide
CP	crude protein
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
EFE	exogenous fibrolytic enzymes
FCR	feed conversion ration
GC	gas chromatograph
Hr(s)	Hour(s)
CH_4	methane
N	nitrogen
NDF	neutral detergent fibre
OM	organic matter
OMD	organic matter digestibility
OMI	organic matter intake
NaOH	sodium hydroxide
SNF	soluble non-fat
TMR	total mixed ration
VFA	volatile fatty acid

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GENERAL INTRODUCTION

Forages have always provided the bulk of ruminant feeds (El-Kady *et al.*, 2006) and many ruminant production systems across the world depend on forage as the main nutritional component (Wilkins., 2000). Ruminants have adapted to a variety of ecological niches because of a diverse microbial population residing in the rumen (Varga *et al.*, 1997). This was made possible because of rumen microbes that synthesise and secrete a fibrolytic enzyme complex capable of hydrolysing plant fibre (Varga *et al.*, 1997). Nutrition of ruminants is determined, among other things, by the extent of microbial fermentation that occurs in the foregut or reticulorumen and their adaptation to digest high fibre feeds (Wallace, 2001). The rumen, which is sometimes described as a fermentation vat, is a home for three major groups of micro-organisms namely bacteria, protozoa and fungi. A synergistic relationship exists between the host animal and these rumen micro-organisms where the host provides a constant supply of feed by ingestion as well as a suitable environment in the rumen for their survival and proliferation. The micro-organisms, on the other hand, attack, degrade and ferment the feed, thereby producing volatile fatty acids as an energy source and microbial cells as a protein source for the host animal (France *et al.*, 2005). This fermentation enables ruminants to make maximal use of low quality forages that otherwise cannot be utilised by mono-gastric species (Wallace, 2001). This allows ruminants to convert the low quality feeds to meat, milk, wool etc. which in turn makes the foundation of the animal agricultural industry that served mankind through many millenniums (Weimer *et al.*, 2009).

Even though ruminants can utilize feed of very low quality to survive, production efficiency is not at its best on these low-quality feeds. This is because only a small percentage (10-35%) of the gross energy intake is retained as nett energy in the end, because the animal may not be able to digest 20-70% of the cellulose (Marquardt *et al.*, 1997). A number of feed and animal factors determine the rate and extent of fibre degradation in ruminants (Varga *et al.*, 1997). These include, intake (Tyrrell *et al.*, 1975); physical and chemical nature of the forage (Jung *et al.*, 1995; Spalinger *et al.*, 1986); particle size (Bjornsal *et al.*, 1990; Yang *et al.*, 2001); chemical and biological treatment (Zhong *et al.*, 2011; Sarnklong *et al.*, 2010); as well as feeding strategies and additives (Wenk, 2000) to mention a few.

Despite expected low nett energy intake from the low quality feed, the high fibre content is potentially important to the ruminant animal. Fibre stimulates contraction of the rumen wall and rumination that enhances the breakdown and fermentation of the fibre components. The rumination in turn stimulates salivation that buffers the rumen pH to ensure the effective production of the ruminal fermentation end products (Mertens, 1997). The fermentation process begins with the attachment of the microbes to the feed through the process of adhesion and this is followed by successive microbial colonization, which occurs quite rapidly. Thus, attachment of ruminal micro-organisms to their substrate is a prerequisite for the digestion of forage particles in the rumen (Varga *et al.*, 1997).

Tropical pastures, which often have variable quality, are mostly of poor nutritional value at maturity due to high lignification (Meissner, 1997). Lignification increases with maturity, leading to poorer degradation and lower feed utilization efficiency (Krueger *et al.*, 2008). This

inefficiency can result in an increase in the quantity of feed needed to maintain required levels of animal performance. That in turn increases the feeding cost and the environmental pollution due to increased waste (Sheppy, 2001). In periods of high feed costs, increasing the efficiency of feed utilization will decrease the cost of feeding while reducing the pollution of the environment associated with the excretion of unused nutrients such as urea, and a loss of energy as methane gas.

A ruminant's intake and digestibility is mainly affected by the passage rate and mean retention time of the feed in its rumen. In turn these factors are affected by dietary, animal as well as climatic factors.

Dietary factors such as grinding of feed are well known to affect digestibility. The finer the feed is ground, the faster the digestion will be. This is due to increased surface area of the feed particles for microbial attachment and colonisation which in turn increases passage rate and ultimately feed intake. Fibre content though presumed to decrease intake, in fact increases passage rate when present in relatively small amounts. By stimulating propulsive activity and thus decreasing feed retention time in the digestive tract it decreases its digestion. But when present in excessive amounts it has the reverse action.

Animal factors such as pregnancy or lactation can also affect digestibility by decreasing mean retention time due to increased passage time and thus increasing intake but decreasing digestion. The age of the animal can also affect digestibility. At birth, the reticulum, rumen, and omasum of a ruminant are undeveloped, small in size compared to the abomasum, basically non-functional, and disproportionate to the adult digestive system. The young rumen must undergo physiological changes before they can digest high fibre feeds, and feedstuff that are digested to propionic and butyric acids in the rumen is needed to stimulate the growth of the rumen papillae. Only after 3 to 4 weeks of rumen development will the calf be able to digest substantial amounts of dry feeds. Due to the underdeveloped rumen and rumen micro-organisms, gut fill is reached quick, digestion is slow and intake is decreased (Heinrichs, 2005).

Climatic factors such as temperature can also have an effect. A positive relationship exists between temperature and the mean retention time. This can lead to an increase in mean retention time with an increase in temperature, that in turn leads to a decreased passage rate and decreased intake (Fraichney, 1986).

During ruminant feeding, increasing the roughage proportion in the diet will increase mean retention time which in turn will lead to a decreased intake (Allen, 1996), that might have a negative effect on performance (Gibb, 1999). Thus, supplementation with fibrolyic enzymes may have a positive role by improving the digestibility (Bala *et al.*, 2009; Feng *et al.*, 1996; Geraldo *et al.*, 2008) and intake (Hussain *et al.*, 2008; Pinos-Rodrigues *et al.*, 2002; Bala *et al.*, 2009) of the roughage proportion thus decreasing the retention time.

There are numerous industrial applications for enzymes all across the world. These include:

- The food industry (Galante *et al.*, 1998; Grassin *et al.*, 1996 a & b; Leroy *et al.*, 2004; Gibbs *et al.*, 1999; Godfrey *et al.*, 1982; Minussi *et al.*, 2002; Kirk *et al.*, 2002),
- Textile and laundry industry (Galante *et al.*, 1998; Godfrey *et al.*, 1982; Minussi *et al.*, 2002; Kirk *et al.*, 2002),
- Fuel industry (Godfrey *et al.*, 1982; Kirk *et al.*, 2002),

- Chemical and drug industry (Schmid *et al.*, 2002), as well as,
- Paper and pulp industry (Godfrey *et al.*, 1982; Minussi *et al.*, 2002; Bajpai, 1999; Call *et al.*, 1992).

But of these its application to animal feed (Bhat, 2000; Walsh *et al.*, 1993; Bedfort *et al.*, 2001; Kirk *et al.*, 2002; Galante *et al.*, 1998) is of particular interest. Cellulases, hemicellulases, xylanase and even pectinases are used in ruminant feed biotechnology to improve feed utilization, affect production of milk or meat and to improve the digestibility of certain feed components. The majority of research on fibrolytic enzyme additives in ruminants has been conducted on beef or dairy cattle. Research on its effects in small ruminant diets, like sheep, will be beneficial and will thus be the focus of this study.

CHAPTER 1

LITERATURE REVIEW

1.1. Poor Quality Roughage

In many countries the main feed-stuff for production are pastures and crop residue e.g. roughages. Roughages are bulky materials, which have high fibre content and low nutrient density. Hay, pasture, silage, straw and cottonseed hulls are a few examples of roughages (Birhan, 2014). There is no doubt that during the dry season the main basal feeds for ruminants in warm climate in developing countries are essentially crop residues and poor quality grasses from rangelands either grazed or, even manually collected at a very advanced vegetation stage, when mature, during the dry season. What is less obvious are the ways and means for optimal utilisation of these feed resources at both the nutritional and economic levels (Chenost, 1995).

In countries like Ethiopia, where 85% of the population live in the rural areas and their economy depend on crop-livestock production systems, natural pasture grazing land was the main forage feed source for livestock in previous years. However, the natural grazing land continues to be reduced due to the fast growth of the country's population with increasing land demand for crop cultivation. Forage production on the remaining uncultivated pasture-land is decreasing due to over grazing and reduced soil fertility. Currently the main feed resource for livestock in traditional production systems is crop residue (Birhan, 2014).

There are many constraints to efficient animal production based on these livestock feed resources. For example, poor quality and quantity. Ruminants have evolved in a way that enables them to utilize cellulolytic material. However, agricultural systems based on maximum exploitation of the use of these poor quality and low quantity feed resources result in poor animal production, primarily because of the low intakes coupled with them (Ndlovu, 1985). Poor quality roughages are feedstuffs that are high in fibre content with a low digestibility. The result of feeding these feeds is a decrease in productivity due to malnutrition with reduction of disease resistance (Birhan, 2014). These feedstuffs are often unable to support rumen conditions that are conducive for optimal microbial activity because of their deficiency in total nitrogen, true protein, readily fermentable carbohydrates and minerals. Many methods exist in which farmers may improve the value of their poor quality forages and crop residues. Poor quality constraints can be slightly overcome by certain physical and chemical and biological treatments and one such biological treatment is enzyme pre-treatment.

According to Sergeant (1956), there are different types of low-quality roughages but that all of these are characterised by a low-protein, high-fibre content and can be grouped as follows: (1) Fodder conserved on farm such as cereal straw, poor meadow hay, and poor lucerne hay such as the first-cut lucerne hay with a large weed content, or lucerne that is overgrown before cutting. These all at their best can only be considered to be of poor quality. (2) Native grasses either conserved as hay or the edible shrubs. (3) Mature natural herbage and stems. (4) By-products from farms and factories such as corncobs, corn stalks, cottonseed hulls, old newspapers and sawdust etc. All these vary in physical make-up and appearance, and they are all good sources of energy, but are generally unpalatable to stock. Stock will eat them when

forced to, but their thrift suffers. Even mature stock have difficulty in maintaining condition on such feed and it is very unsuitable for young growing stock and pregnant, lactating or wool producing stock (Sergeant, 1956; Sanson *et al.*, 1990; Hristov, 2015; Khan *et al.*, 2012).

There are six major factors that contribute to the quality of roughages: (1) Maturity: This is the most important factor affecting forage quality. As a plant cell ages the cell wall content increases and causes indigestible lignin to accumulate. (2) Crop species: Grasses and legumes can vary largely in protein content, with legumes usually being higher. (3) Harvest and storage: Improper harvest techniques can lead to loss of leaves that have a higher nutritive value than the stems while storing a hay crop at an incorrect moisture content, or improper ensiling of a forage crop, can lower the quality significantly. (4) Environment: Moisture, temperature, and the amount of sunlight influence forage quality. (5) Soil fertility: This usually affects forage yield much more than it affects the quality. A low, as well as very high soil fertility can reduced forage quality. (6) Variety (cultivar): The variety or type of cultivar can affect the forage quality, but its effect is not as great as the other five factors (Van Soest et al, 1978).

1.2. Roughage and its degradation by ruminants

It is important to recognize the characteristics of plant material as a factor determining its nutritional value (Walters, 1971). Roughage consist of a heterogeneous population of cell types, each one possessing its own degradability characteristics that is determined by three things, (1) their location within the plant, (2) their anatomical features and (3) the chemical composition of their walls. (Travis *et al.*, 1997). This is in agreement with Weimer (1996) who states: that the architecture of the plant cell may be just as important as its chemistry. Furthermore McManus *et al.* (1973) reported that the distribution pattern of lignin, for instance, rather than the total amount of lignin can mask the potentially digestible cell walls of forages (Travis *et al.*, 1997). Marked interactions exist between the cell wall thickness, lignification and other anatomical characteristics of forages and their digestibility (Wilson, 1993). Therefore, investigations on the anatomical structure of forages are of importance in determining the digestibility potential of forages. According to the ARC Centre of Excellence, a plant cell wall is a structure that surrounds the cell and that provides the cell with a number of functions such as strength to support the plant, rigidity to fix cell shape, flexibility, porosity, water-proofing, a barrier to pests, protection against environmental stress, apoplastic transport, signalling and sensing.

Certain plant species have a secondary cell wall interior to the primary cell wall. This lends increased structural strength to the plant and forms a formidable barrier against microbial invasion. A plant cell wall is a highly active and differentiated network formed by a mixture of cellulose, hemicelluloses, pectins and in some cases proteins and phenolic compounds. The cell wall composition in plants on a dry matter basis is approximately 30% cellulose, 30% hemicellulose and 35% of pectin, with 1-5% structural proteins. (Somerville *et al.*, 2004).

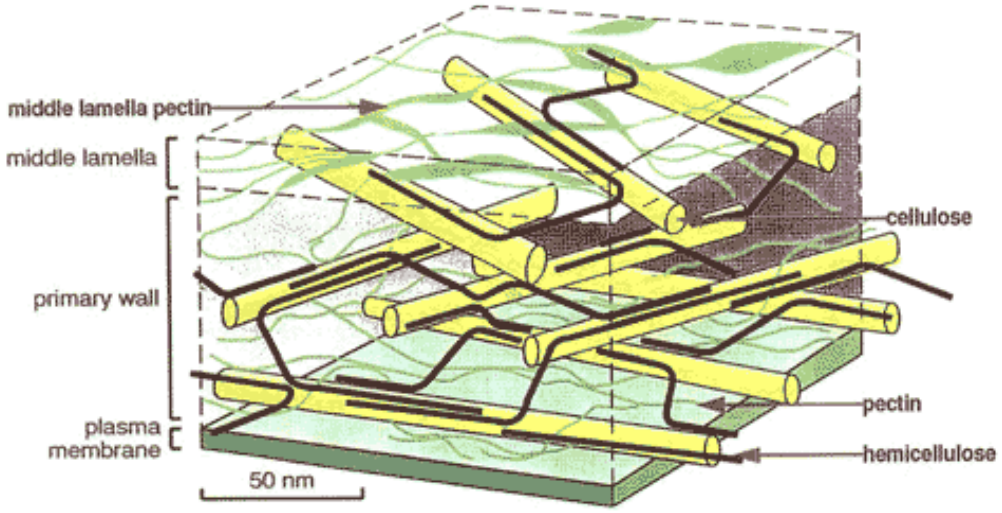


Figure 1.1 Plant cell wall structure (Encyclopedia of Science)

The fibre portion of the the cell wall makes up to 300 to 800 g/kg of forage dry matter and can be a major source of nutritional energy for ruminants, but, unfortunately less than 50 % of this fraction is readily digested and utilized by the animal (Hatfield *et al.*, 1999). Hristov *et al.* (1998) reviewed the function and complexity of digestion of plant cell walls, which contain numerous chemical bonds. The outer layers cuticle and lamellae composed of pectin connecting the cell walls are the plant's first line of defence against microbial degradation in the rumen (Forsberg, 1992). The cuticle, is disrupted by mastication and pre-treatment of the feedstuffs. Pectin itself is readily digestible. Pectin is digested in the rumen by micro-organisms producing pectinases and xylanases (Cheng *et al.*, 1991; Gordon, 1992).

The plant cell wall is comprised primarily of microfibrils of cellulose, a chain of D-glucose monomers (Chafe, 1970) that forms the core of the plant cell wall, which accounts for 20-30% of the dry matter of primary cell walls and one third of the total mass of the wall (McNeill *et al.*, 1984). Cellulose, a linear insoluble unbranched polymer of β-(1,4)-D-glucose residues associated with other cellulose plant cell wall polymers, aggregates together to form microfibrils, that are highly crystalline and insoluble structures. Each microfibril is about 3 nm in diameter, chemically stable and resistant to enzymatic attack and the higher the crystalinity, the more resistant the cellulose is to digestion. Cellobiohydrolase, endoglucanase, cellobiase and cellulase are needed for the breakdown of cellulose (McNeill *et al.*, 1984).

Hemicellulose is another major component of both stem and leaf cell walls and is comprised mainly of a backbone of β-1, 4 linked xylose residues (xylans) a diverse group of polysaccharides (McNeil *et al.*, 1984). The xylan polymer backbone in turn is bonded to the cellulose fibrils and this structure is further complicated by side-chains of α-(1,2) linked glucuronic acid, 4-O-methyl glucuronic acid, acetic acid and arabinose residue known as arabinoxylans and glucuronoarabinoxylans (Ochoa-Villarreale *et al.*,2012). Xylan polymers can then be further cross-linked to other hemicellulose backbones, or to lignin. This structural complexity of hemicellulose obviously requires many enzymes for its digestion (Sorek *et al.*, 2014).

Hemicellulose is bound to cellulose to prevent direct contact among microfibrils, and they are embedded in the amorphous pectin polymers. They are stabilized by proteins and phenolic compounds bringing rigidity to the wall while pectin, bound to hemicellulose forms a gel phase and provides fluidity and extention (Wang *et al.*, 2013).

Ruminant animals have a dynamic array of microbial fibrolytic enzymes to cleave fibrous structures (Hristov *et al.*, 1998). One potential strategy of ruminal bacteria and fungi to support rapid rates of fibre hydrolysis is the synthesis of large amounts of fibrolytic enzymes, particularly cellulase. This is predominantly the strategy of ruminal fungi which will also produce lower amounts of fibrolytic enzymes, but of high specific activity (Wood *et al.*, 1986). Ruminal bacteria on the other hand utilize a strategy in which the enzyme activity is predominantly located at the cell surface that facilitates adhesion to and degradation of the fibre. According to Wilson *et al.* (1995), fibrous cells can only be digested by bacteria from the interior because the middle-lamella primary wall region is indigestible. This is consistent with the "inside-out" theory of plant digestion as described by Cheng *et al.* (1991).
The most readily digestible plant tissues are located inside the plant and therefore intact plants are digested slowly. For micro-organisms to digest the plant cell contents access is only gained via the stomata and therefore mechanical disruption of plant material, such as chewing or grinding improves microbial access. Only as little as 20% of the wall thickness would typically be degraded within the average residence time of fibre particles in the rumen (Wilson *et al.*, (1995).

According to Boon *et al.* (2005) the plant cell wall accessibility to ruminal micro-organisms is rather complex and is described by three components. Accessibility of a tissue particle is the first. This is related to the size of the particle with large particles having only outer cell walls available for fermentation, hence the slow initial rate of fermentation of these often lignified and poorly degradable tissue (Engels *et al.*, 1992). Mastication of course plays a major role in overcoming this limitation (Wilson, 1990), as does processing of forages breaking down the particles and thus increasing the surface area available for fermentation. The second and third components are accessibility of the plant cell wall and the plant cell wall components by ruminal micro-organisms. These components are related to structural factors such as cell wall thickness. For instance, sclerenchyma cells increase their cell wall thickness to such an extent that the lumen diameter of the cell becomes so limited that the space available is only sufficient for one microbe at a time (Boon *et al.*, 2005). Finally, the highly digestible cell wall contents can be encrusted by indigestible lignin, making it almost impossible for the micro-organisms or even their enzymes to find access to such components. Major constraints to fibre digestion are caused by cell-wall structure of the plant by matrix interactions among wall biopolymers and the low substrate surface area, as well as by limited penetration of the non-motile cellulolytic microbes into the cell lumen (Weimer, 1996). In addition to the chemical and architectural composition of fibre, the rumen environment is also an important contributing factor to fibre digestion. With regards to this it is mostly the ruminal pH that can serve as constraint. The optimal pH for ruminal bacteria is near neutrality and ruminal cellulolytic bacteria in particular appear to be sensitive to pH lower than six. Weimer (1996) also discussed microbial interactions as a second environmental factor of importance for fibre digestion. There is significant competition between individual species of ruminal cellulolytic bacteria and other bacteria for nutrients. All these limitations in degradation can theoretically

be overcome by the addition of exogenous fibrolytic enzymes to complement the rumen microbial system.

1.3 Treatment of poor quality roughages

A significant amount of work has been done over the last forty years in both the research and development levels on methods and techniques improving the value of fibrous feeds (Wanapat *et al.*, 1996). High fibre feeds or poor quality roughages consist of many different types, including crops residues, poor meadow or lucerne hay, some native grasses, edible shrubs, mature natural herbage and stems as well as certain by-products from farms and factories that is either grazed or, manually collected at a very advanced vegetation stage during the dry season (Sergeant, 1956; Chenost, 1995). This usually make up the bulk and sometimes the only component of the ruminants' diets in developing countries with a warm climate. In order to optimizing the use of these poor quality roughages, rather than to improving the nutritive value for ruminants, requires not only the use of treatments, that improve the quality of the roughage, but also the improvement of the digestive utilization of these feeds, either untreated or treated, through appropriate supplementation and feeding techniques (Wanapat *et al.*, 1996).

Methods for the improvement of the digestive utilization of poor quality roughages include: (1) Chemical methods that includes treatments with NaOH/CaOH, ammonia, urea and urine (Chenost, 1995). (2) Physical methods such as processing including grinding, pelleting and ensiling (Hull *et al.*, 1974). (3) Genetic methods such as breeding and (Mittler *et al.*, 2010) (4) biological methods such as inoculants and enzymes (Beauchemin *et al.*, 2003).

When choosing a method of use there are a few factors to consider for example: economics, effectiveness, safety aspects for the producer as well as the livestock, availability and feasibility. According to Adesogan (2003) processing such as chopping, grinding and pelleting increases surface area and density resulting in less sorting and higher intakes. It also makes it easier to handle but there are risks associated with over processing such as reducing the effective fibre content and fibre digestibility, heat damaged proteins, dust and dry matter losses. Although less effective than soaking, NaOH is currently sprayed on forages due to polluted waste water disposal issues. It does increase roughage digestibility and intake but it is hazardous and can cause alkali burns. Ammonia is even less effective than NaOH, is also hazardous and rather pricy. Urea is relatively safer than ammonia but has more variable effects that could result in ammonia toxicity if consumed in large quantities. Urine can increase digestibility and it is also cost effective but its collection and health issues is a major problem (Chenost, 1995; Adesogan, 2003). Examples of breeding are cold/short day length tolerant grasses, it is quite effective but has a very slow progress (Mittler *et al.*, 2010; Adesogan, 2003). Fungal treatments can decrease the lignin effect but it degrades desirable carbohydrates in the process and enzyme treatments such as cellulase and xylanase tend to increase digestibility and intake (Beauchemin *et al.*, 2003; Adesogan, 2003).

1.4. Exogenous fibrolytic enzymes

The use of exogenous enzymes as feed additives to improve the utilization of roughage has gained popularity in recent years (Giraldo *et al.*, 2008). For many years, however, researchers were discouraged from using enzymes to enhance the utilization of ruminant

TMRs. This was due to the perception that these enzymes would be rendered ineffective due to the action of ruminal proteolysis (Adesogan, 2005) or deactivated in the feed manufacturing process, and thus addition of enzymes may not enhance the hydrolytic capacity of the rumen. However, these concerns have been disproved in recent studies which showed that supplementing exogenous fibrolytic enzymes could enhance productivity in beef and dairy cattle (Beauchemin *et al.*, 1995; 2003) and even small stock such as sheep (Cruywagen *et al.*, 2004). As indicated by Bhat (2000), many earlier research findings have shown an improvement in feed digestibility and animal production using exogenous enzymes (Burroughs *et al.*, 1960; Rust *et al.*, 1965).

However, only a few examples of such improvements in animal performance are reported, which includes increased milk yield as well as increased fat corrected milk and energy corrected milk for cows fed on enzyme treated diets compared to untreated diets (Mohamed, 2013). Other authors have reported improved live weight gain (Beauchemin *et al.*, 1995), feed intake, higher DM and NDF digestibility in cattle fed enzyme treated diets compared to those fed untreated diets (Feng *et al.*, 1996). But negative effects have also been shown in some of these early studies (Theurer *et al.*, 1963; Perry *et al.*, 1966). Today, renewed research report very similar positive effects (Beauchemin *et al.*, 1995, 2003) but with inconsistencies in research findings still being prevalent. Great strides in our understanding of the enzymes and their application have however been made, as is evident in the host of exogenous fibrolytic enzymes (EFE) commercially available.

Research on exogenous fibrolytic enzymes began in early 1950. Since then their production became easy and economical in the early 1980s due to advances in fermentation technology and biotechnology. Exogenous fibrolytic enzymes revealed their biotechnological potential in various industries including animal nutrition (Bhat, 2000). Currently there are several fibrolytic enzyme supplements commercially available for use to improve fibre degradation (Adesogan, 2005). The main aim of the inclusion of these enzymes is to increase the fibre digestibility of the diets fed, with the subsequent improvements in feed intake and animal production, amongst others. Preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fibre (Feng *et al.*, 1996). Hristov *et al.* (1998) defined the mode of action of exogenous enzymes asproteins that catalyze chemical reactions in biological systems. In the context of animal feeds, exogenous enzymes catalyze the degradation reactions of feedstuffs in order to release nutrients for utilization by the micro-organisms or host animal itself.

Exogenous fibrolytic enzyme additives, primarily xylanases and cellulases, are concentrated extracts resulting from bacterial or fungal fermentation that have specific enzymatic activities (Beauchemin *et al.* 2003). Adding commercially produced exogenous fibrolytic enzymes such as cellulase plus and xylanase plus will offer the opportunity to increase the proportion of the roughage component in the TMR or the use of very low quality roughage in periods of feed shortage or high feed prices. These enzymes normally increase digestion while exhibiting no negative effect on the intake, though improved performance is not always the case due to a variety of interacting factors such as the enzyme type, source, concentration etc. (El-Kady *et al.*, 2006). The nutritional benefit of supplementing ruminant feeds with these exogenous fibrolytic enzymes appears to depend on many factors, most of which are yet to be evaluated. The successful use of enzymes depends on their stability in the feed, the ability of the enzymes to hydrolyse the plant cell wall components and the ability of

the animal to utilize the resultant products efficiently (Bhat, 2000). Since Bhat's review in the year 2000, many research groups have been studying effects of fibrolytic enzymes in ruminant diets. Although the results can still be regarded as inconsistent, the understanding of the action of these enzymes has greatly improved. Some explanations for the variability include the types and activities of enzymes which is caused to a large extent by the organism from which it is produced, the substrate used for its growth and the culture conditions used (Considine *et al.*, 1989; Gashe, 1992). There is evidence that biodegradable substrates such as sugar cane bagasse yield higher enzyme activities than submerged fermentation (Gerardo *et al.*, 2009). The effectiveness of these enzymes varies with the substrate composition, the method of enzyme application and with the component of the diet to which the enzyme additive is added (Wallace *et al.*, 2001; Beauchemin *et al.*, 1996; Hristov *et al.*, 1998).

Cellulase plus, a liquid carbohydrase enzyme with a 1.15-1.25 density and 4.8 ± 1 pH, is of medium to dark amber colour with a slight fermentation odour. Similarly xylanase plus is a liquid enzyme with the same properties as described for cellulase plus, but it has a pH of 4.5 ± 1 pH. The two enzymes have shown good promise when compared to the various exogenous fibrolytic enzymes tested at University of Florida, Florida, USA (Dyadic International, 2009).

Cellulase is responsible for the hydrolysis of cellulose. The main enzymes involved in this hydrolysis are endocellulase, exocellulase and β-glucosidase, while the main enzymes involved in degrading xylan core polymer to soluble sugars are xylanase, β-1,4 xylosidase and endoxylanase. Evidence exists that the mode of action of exogenous enzymes in ruminants is a combination of pre- and post-feeding effects (McAllister *et al.*, 2001; Colombatto *et al.*, 2003). The pre-feeding effects include an enzyme-substrate pre-incubation interaction period. In a review paper by Alvarez *et al.* (2009) it was reported that several researchers had previously suggested that pre-incubation of the diet with the enzyme is of importance (Forwood *et al.*, 1990; Elwakeel *et al.*, 2007; Krueger *et al.*, 2008). Exogenous enzymes are more stable when applied to the feed prior to ingestion as opposed to infusion into the rumen (Beauchemin *et al.*, 2003). Application of the enzymes to the feed allows for absorption and binding time to the substrate, enhances the binding of the enzyme to the substrate making it more resistant to proteolytic breakdown in the rumen thereby prolonging the resident time of the feed and enzymes (Beauchemin *et al.*, 2003; Forwood *et al.*, 1990). The resultant stable enzyme-feed complex can then potentially degrade the relevant plant tissue in the rumen (Kung *et al.*, 2000) and provide a slow-release-mechanism for the enzymes in the rumen.

When enzymes were directly infused into the rumen instead of inclusion via the feed, no improvements in degradation were observed (Kopecny *et al.*, 1987; Lewis *et al.*, 1996) which serves as further justification for allowing a pre-incubation interaction period. In a review paper by Moharrery *et al.* (2009), improved *in-vitro* dry matter digestibility (DMD) and neutral detergent fibre (NDF) digestibility was reported after eight hours incubation in rumen fluid where forages were pre-treated (24h prior to incubation) with exogenous fibrolytic enzyme. When no pre-treatment time of the substrate with the enzyme was allowed, none of the reported effects mentioned earlier were observed (Moharrery *et al.*, 2009), supporting the recommendations of other research groups that a pre-treatment period should be allowed.

However, proteolysis of exogenous enzymes does not seem to be the sole reason why a pre-incubation interaction time should be allowed as several studies have reported that fibrolytic enzymes are resistant to rumen proteolysis for a significant (6hrs) time (Hristov *et*

al., 1998; Morgavi *et al.*, 2000a, 2000b). In the study of Giraldo *et al.* (2008) it was found that direct-fed fibrolytic enzymes positively affected fibrolytic activity in the rumen of sheep and increased the growth of cellulolytic bacteria without a pre-feeding substrate enzyme interaction period. They reported increases in the ruminally insoluble potential degradable fraction of grass hay DM, as well as its fractional rate of degradation. However, the enzyme supplementation did not affect diet digestibility even though molar proportions of propionate were greater and acetate: propionate was lower.

Alvarez *et al.* (2009) reported that the reducing sugars released due to fibrolytic enzyme addition would provide energy that should lead to rapid microbial growth which in turn would increase the ruminal bacteria numbers and could lead to increased microbial colonization of the feed particles. Giraldo *et al.* (2008) also suggested an alteration in the fibre structure due to the enzyme effects. An altered fibre structure in combination with an increased colonization would shorten the lag time (Yang *et al.*, 1999) and the access of the microbes to the potentially fermentable fibre by enzymes acting on the structures of plant cell walls, is enhanced (Sutton *et al.*, 2003; Elwakeel *et al.*, 2007). The beneficial activities obtained from adding exogenous fibrolytic enzymes to a diet are related to their ability to enhance the initial degradation of plant's structural carbohydrates and complement normal enzymatic activities associated with ruminal micro-organisms (Dawson *et al.*, 1999).

1.5. Exogenous fibrolytic enzymes in the nutrition of ruminants

The challenge in successful farming with ruminants, be it dairy cattle, beef cattle or sheep and goats lies in the effective utilization of feed resources, as the feeding costs present the largest component of production costs. Of the feeds typically utilized, forage composes the biggest part of the feeding cost and hence presents a logical area of research for the improvement of its utilization. Exogenous fibrolytic enzymes present one way of improving fibre digestibility (Johnston, 2000).

Fibrolytic enzyme supplementation in small ruminants has received relatively little attention compared to that of large ruminants (Giraldo *et al.*, 2008). Only a limited number of small ruminants have been considered with regards to their responses to fibrolytic enzyme treatments (Marquardt *et al.*, 1997).

Positive effects of supplementing diets of large ruminants with exogenous fibrolytic enzyme have been reported in the past. These results were obtained with pre-treatment of the test feeds with the enzymes, but direct-fed enzymes have also shown positive effects. Some of these effects are increases in NDF digestion in bulls (Murillo *et al.*, 2000) and in dairy cattle (Lopez-Soto *et al.*, 2000); average daily weight gain increases reported for steers (Ware *et al.*, 2002; Beauchemin *et al.*, 1995; Beauchemin *et al.*, 1997; Iwaasa *et al.*, 1997; Beauchemin *et al.*, 1999); improved cell wall digestibility *in vitro* (Colombatto *et al.*, 2003) or *in vivo* (Schingoethe *et al.*, 1999) for some exogenous fibrolytic enzyme preparations; while other studies indicated increases in milk production of lactating dairy cow (Tricarico *et al.*, 2008; Lewis *et al.*, 1999, Rode *et al.*, 1999; Schingoethe *et al.*, 1999; Kung *et al.*, 2000; Yang *et al.*, 2000) or improvements in the energy balance of transition cows (DeFrain *et al.*, 2005), increases in forage utilization, production efficiency and reduced nutrient excretion have also been reported (Beauchemin *et al.*, 2003). Increased feed intake was recorded in some cases and this was attributed to an increased palatability associated with the pre-ingestive release of

sugars during fibre hydrolysis and post-ingestive increases in digestion rate, extent of digestion and passage rate (Adesogan, 2005).

Table 1.1 Summary of the effect of exogenous fibrolytic enzymes.

Fibrolytic enzyme	Application	Result	Author
Cellulase and Xylanase	*In sacco* in steers on high fibre diet	Increased DM and CP in the a fraction, no effect on b or c fraction DM, increased CP of fraction c, no effect on NDF or ADF disappearance and production, and no effects were reported on the DMI, ADG or feed conversion	Alvarez *et al.* (2009)
Cellulase and Xylanase	*In vivo* in steers on wheat middlings and oat straw	increased ADF (wheat middlings), NDF and ADF (Oat straw) disappearance	Alvarez *et al.* (2009)
Cellulase and Xylanase	*In vivo* in lactating cross bred Beetle-Sannen goats where the enzyme was added to the concentrate supplement fed at 500g/d	Increased DM, OM, CP, NDF, ADF and total carbohydrates digestibility as well as increased microbial protein production and production effects such as improved milk yield, fat and SNF as well as increased feed intake and improved body weight	Bala *et al.* (2009)
Cellulase	*In vivo* in feedlot cattle with a high corn or roughage diet evaluated with exogenous fibrolytic enzymes applied to the protein concentrates	Higher ADG (7%), DMI (1%) and improved FCR (6%) and no effect reported for total tract digestibility	Burroughs *et al.* (1960)
Xylanase	*In vitro* in buffered rumen fluid from lactating Holstein cows on	Improved ruminal degradation and increased gas production as well as OMD	Eun *et al.* (2007)

	fresh low quality lucerne hay		
Xylanase	*In vivo* sheep on 70% grass hay: 30% concentrate	Increases in the ruminally insoluble potential degradable fraction of DM and fractional rate of degradation as well as increased propionate and decreased acetate: propionate	Giraldo *et al.* (2008)
Cellulase and Xylanase	*In vitro* incubated in the absence of rumen fluid or with buffered rumen fluid from non-lactating Holstein cows on Bahia grass hay	Increased DMD (absence of rumen fluid), decreased acetate concentrations, increased propionate and butyrate concentrations as well as decreased lag phase in the presence of rumen fluid.	Krueger *et al.* (2008)

DM (Dry matter); OM (Organic matter); CP (Crude protein); NDF (Neutral detergent fibre); ADF (Acid detergent fibre); DMI (Dry matter intake; ADG (Average daily gain); SNF (soluble non-fat); FCR (Feed conversion ratio); OMD (Organic matter digestibility); DMD (Dry matter digestibility)

It is likely that the majority of the positive effects of enzyme supplemented feeds on production responses are due to the ruminal effects. According to Adesogan (2005), supplementation increases the hydrolytic capacity of the rumen (indirectly reducing gut fill and increasing intake) by increasing bacterial attachment, stimulation of rumen microbial populations and synergistic effects with hydrolysis of ruminal micro-organisms with the net effect being increased enzyme activity in the rumen which in turn increases digestibility of the feed (Beauchemin *et al.*, 2003).

1.6. Exogenous fibrolytic enzymes in the nutrition of small ruminants

In the semi-arid and arid regions of the world, sheep and goats are increasingly produced due to their adaptation to these climates (Bala *et al.*, 2009). Studies using goats to ascertain the effect of exogenous fibrolytic enzymes have been limited and the responses poor due to the superior ability of goats to utilize fibre compared to that of large ruminants (Bala *et al.*, 2009). In addition, information on the effects of ruminal fermentation in small ruminants is scarce (Pinos-Rodriguez *et al.*, 2002). Yang *et al.* (2000) could not ascertain any effects of fibrolytic enzymes fed to goats while Bala *et al.* 2009 ascertain positive results on DM, OM, NDF, ADF, CP and total carbohydrate digestibility as well as increased milk yield in the third quarter of lactation along with a decrease in feed intake of up to 7%. Many researchers (Cruywagen *et al.*, 2004; Pinos-Rodriguez *et al.*, 2002; Giraldo *et al.*, 2008) observed positive results such as improved animal performance in sheep that gained significantly more body weight when fed the enzyme treated diet, and had improved feed conversion ratios (Cruywagen *et al.*, 2004 and

2008), on ruminal activity in sheep (Giraldo *et al.*, 2008) and DMI, OMI and CP intake were increased (Pinos-Rodriguez *et al.*, 2002).

Fibrolytic enzyme supplementation in small ruminants has received relatively little attention compared to that of large ruminants (Giraldo *et al.*, 2008). Only a limited number of small ruminants have been considered with regards to their responses to fibrolytic enzyme treatments (Marquardt *et al.*, 1997). Due to the importance of roughages in the diets of small ruminant under extensive conditions, during periods of high feed costs or poor feed quality it is important to explore the effect of adding exogenous enzymes to the diets of these small ruminant animals in order to improve the nutritive value of high roughage diets (El-Kady *et al.*, 2006).

1.7. Exogenous fibrolytic enzymes research techniques

The abundance of research on exogenous fibrolytic enzyme application appears to have been done using either *in vitro* or *in situ* studies. *In vitro* determination of the correct application dose that has the highest enzyme activity is very important. In order to do this there are a number of factors that one needs to pay attention, this will allow the direct comparisons among studies in which different enzyme products are used. The temperature and pH at which enzyme activities are assayed are most important when evaluating enzymes for specific purposes, for example direct fed or ensiling. In many of the published data the experimental conditions are not stated or they do not represent physiological conditions. The recommended conditions for conducting an assay are pH 6.0-6.5 at 39°C which reflects that of ruminal conditions. Because enzyme activity is expressed as an initial rate of reaction, it is important to ensure that the enzyme is at the maximum kinetic velocity for the duration of the assay. This can be done by making sure that the substrate concentration is well above that of the enzyme. Incubation time should not be too short or the number of samples that can be run at the same time should not be reduced. If the incubation time is too long the chances of obtaining misleading results are increased (Colombatto *et al.*, 2003).

Degradation kinetics of enzyme-treated feeds has mainly been described using the in situ techniques. However these methodologies are limited in terms of the number of samples that can be analysed at one time and they assume that all feed losses from the bags have been, or are degradable. There is therefore a need to develop suitable *in vitro* bioassays (eg. gas production) to provide an alternative to such in situ trails, especially when screening large numbers of samples or treatments (Colombatto *et al.*, 2003).

A very useful *in vitro* technique to measure effects of exogenous enzyme treatment of forages is the *in vitro* gas production technique, in which head space gas production can be measured throughout the incubation. Eun *et al.* (2007) evaluated 13 endoglucanases and 10 xylanases in this manner and were able to show increased gas production and organic matter digestibility (OMD) at 18h with many of these enzymes when applied to lucerne hay. Based on these initial screenings, two superior enzymes of each category were further evaluated, also in combination with each other. The authors found that the enzymes were effective in improving gas production and OMD, but that the combination of the two types of enzymes did not lead to any further improvement (Eun *et al.*, 2007). Gas production based techniques offer a potential as an alternative to in situ techniques as they account for both insoluble and soluble fractions. However, as gas production profiles are not necessarily directly related to substrate

degradation, the determinations of the undegraded residue have been recommended (Colombatto *et al.*, 2003).

1.8. Conclusion

The attempt to improve fibre digestion in ruminants is a continuous research area. The use of exogenous fibrolytic enzymes to enhance the quality and degradation of fibrous forages is on the verge of delivering practical benefits to the ruminant animal production systems. Several studies conducted with exogenous fibrolytic enzymes have mentioned an increase in the microbial activities in the rumen leading to increased digestion, which in turn is resulting in an increase in the animal performance traits, but results with exogenous fibrolytic enzymes in the ruminant systems are variable and somewhat inconsistent with some studies showing improvements (Lewis *et al.*, 1999, Rode *et al.*, 1999, Yang *et al.*, 1999, Nowak *et al.*, 2003, Cruywagen *et al.*, 2004, Bala *et al.*, 2009) while others are reporting either a negative effect or no effect at all (Vicini *et al.*, 2003, Bowman *et al.*, 2003, Baloyi., 2008), thus making their biological responses difficult to predict.

2. Objective

2.1. General objective

- The general objective of this study was to improve the utilization of a high roughage TMR for sheep through the optimal addition of a mixture of cellulase plus and xylanase plus exogenous fibrolytic enzymes.

2.2. Specific objective

- To investigate the effect of adding different proportions of cellulase and xylanase enzyme mixtures on *in vitro* gas and methane production characteristics, ruminal fermentation and *in vitro* digestibility.
- To investigate the effect of adding different proportions of cellulase and xylanase enzyme mixtures on *in vivo* digestibility and rumen fermentation parameter in sheep fed high roughage TMR.

3. Hypothesis

H1: Different proportions of cellulase plus and xylanase plus enzyme mixtures will improve the utilization of high roughage TMR by the sheep due to improvements in nutrient digestion, rumen fermentation parameters or fibre digestion or a combination of these. This in turn will reduce methane production per unit of digestible dry matter.

H0: The different proportion of cellulase plus and xylanase plus mixture has no influence on the utilization of high roughage TMR by the sheep. This means there will be no significant

difference between the different proportions of the enzyme mixture in terms of their effect on nutrient digestion, rumen fermentation and methane production.

CHAPTER 2
The effect of different proportions of cellulase and xylanase enzyme mixtures on nutrient degradation, total gas, methane and VFA production of Smutsfinger hay *in vitro*

ABSTRACT

This study investigated the effect of adding different proportions of cellulase and xylanase enzyme on nutrient degradation, total gas, methane and volatile fatty acid production. Cellulase and Xylanase enzymes have been reported by various authors to boost feed utilization efficiency. Cellulase and Xylanase enzyme mixtures were added to Smutsfinger hay to determine the gas and methane production at 0, 2, 4, 8, 12, 24 and 48 hours, including the fermentation parameters and nutrient degradation after the 48 hr incubation. Smutsfinger hay was pre-treated for 24 hr with the different enzyme solution (100% cellulase; 75% cellulase: 25% xylanase; 50% cellulose: 50% xylanase; 25% cellulase: 75% xylanase; 100% xylanase and a control with no enzyme). The samples were then incubated at 39°C in a shaking incubator under anaerobic conditions with buffer and ruminal fluid mixed in a 1:4 ratio. A semi-automated system was used to measure gas production and a gas chromatograph (GC) was used to measure methane production. Addition of different proportions of cellulase and xylanase plus enzyme mixtures to Smutsfinger hay, especially the 100% cellulase treatment, increased nutrient degradability, and total gas and methane production, lowered end fermentation pH and increased butyrate concentration as well as a tendency of higher acetate and total VFA proportions. Cellulase had a much greater effect than xylanase, and its effects decrease as its concentration in the enzyme mixture decreased below 75%.

1. Introduction

Forages play an important role in the animal industry worldwide. The structural fibre (cell wall) portion of forage contributes between 300 to 800 g/kg of forage dry matter and represents a major source of nutritional energy for ruminants. Unfortunately less than 50 % of the fibre fraction is readily digested and utilized by the animal (Hatfield *et al.*, 1999) thus any improvement in the digestibility of the forage cell wall can be of great benefit.

Increasing the digestibility of poor quality forages has been a topic of research for many years. Exogenous fibrolytic enzymes have been studied extensively in the last couple of decades as a viable means of improving the digestibility of forages typically used in ruminant nutrition. Preparations of enzymes that degrade cell walls such as cellulases and xylanases have the potential to hydrolyse forage fibre (Feng *et al.*, 1996). Many authors have reported on the successful use of this technology (Beauchemin *et al.*, 1995; 2003; Cruywagen *et al.*, 2004; Burroughs *et al.*, 1960; Rust *et al.*, 1965; Mohamed, 2013; Beauchemin, 1995; Feng, 1996). However, many inconsistent findings have also been reported and the variability in results is related to the types and activities of enzymes which are caused to a large extent by the organism from which they are produced, the substrate used for its growth and the culture conditions used (Considine *et al.*, 1989; Gashe, 1992). Other causes of variation or inconsistancies are the

composition of the substrate used, the method of enzyme application and the portion of the diet to which the enzymes are added (Beauchemin *et al.*, 1996; Hristov *et al.*, 1998).

Due to the importance of roughages in the diets of small ruminant under extensive conditions, it is important to explore the effect of adding exogenous enzymes to the diets of these small ruminant animals during periods of high feed costs or poor feed quality in order to improve the nutritive value of high roughage diets (El-Kady *et al.*, 2006).

The current *in vitro* study had the overall objective of investigating the total gas production and methane production characteristics of Smutsfinger hay treated with cellulase and xylanase enzyme and their combinations. This study also determined the VFA production, pH, DM, OM, NDF and ADF degradation of the Smutsfinger hay treated with the different proportions of cellulase and xylanase plus enzyme mixtures after a 48 hr incubation.

2. Material and methods

2.1. Ethical clearance for animal use

This study was approved by the Animal Use and Care Committee, project number EC113-13, University of Pretoria.

2.2. Laboratory analysis

2.2.1. Chemical analysis of Smutsfinger hay

Smutsfinger hay was analysed for DM, Ash, NDF, ADF and CP, OM was calculated. The detailed procedure is indicated below.

2.2.2. Dry Matter Determination

Dry matter was determined according to the official method of AOAC (2001), International method 934.01. A hay sample was weighed (2g) each into duplicate crucibles, which were oven dried at 105°C for 24 hr. The samples were removed from the oven, cooled and then weighed.

- $\%\ DM = \left(\frac{\text{Mass of sample after drying}}{\text{Mass of sample before drying}}\right) \times 100$

2.2.3. Ash Determination

Ash was determined according to AOAC (2001), International method 942.05. Samples of feed in the crucibles used for the dry matter determination were placed in the muffle furnace at 200°C for two hours, then at 600°C for four hours. The samples were left to cool overnight and the next morning they were taken out, cooled and weighed back.

- $\%\ Ash = \left(\frac{ASH}{DM}\right) \times 100$

2.2.4. Organic Matter Determination

Organic matter was calculated by deducting values of ash from the 100% DM.

- The % OM = 100% - ASH %

2.2.5. NDF and ADF determination

Neutral detergent fibre (NDF) was determined using the Filter Bag Technique (for A2000, A20001), ANKOM Technology (2016) Method 13. This method determines NDF as a residue remaining after digesting the feed sample in a neutral detergent solution. The remaining residues are predominantly hemicelluloses, cellulose, and lignin.

Acid detergent fibre was determined using the Filter Bag Technique (For A2000, A20001), ANKOM Technology (2016) Method 12. This method determines ADF as a residue remaining after digesting with acid detergent solution. The remaining residues are predominantly cellulose and lignin.

2.2.6. Crude Protein and Nitrogen Determination

The nitrogen (N) concentration of the test feed was determined by using the LecoTru Mac N, according to the Leco Instrumental manual procedure 968.06 AOAC (2000). Crude protein concentration was then determined by multiplying the N percentage by a factor of 6.25.

2.3. *In vitro* gas production measurements

2.3.1. Preparation of enzyme solution and substrate

Five treatments consisting of different mixtures of two exogenous fibrolytic enzymes, e.g. cellulase plus and xylanase plus at different proportions and a control were tested. The cellulase plus and xylanase plus enzymes used in the study were received from Dyadic International Inc., Florida, USA, and were concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8), respectively. They were produced by the fermentation of non-GMO *Trichoderma longibrachiatum* (formerly *Trichoderma reesei*). These enzymes are non-hazardous and food grade products. The level of addition was decided based on the outcome of a previous study at the University of Pretoria using the same enzymes (Gemeda *et al.*, 2014). About 20 *u*l of each enzyme treatment was added to a 100 ml of distilled water. The liquid enzyme solutions was stored in the refrigerator after its preparation. A control treatment was also prepared, which consisted of only distilled water. Smutsfinger hay was dried at 55ºC for 24hr and ground with a mill to pass through a 1 mm screen. About 0.5±0.01g of the milled sample was weighed into incubation vials that were rinsed for 3 minutes in acetone, dried and labelled with a permanent marker.

2.3.2. Treatment preparation

All enzyme treatments were applied 24 hours prior to incubation in order to allow for the enzyme-substrate interaction (Beauchemin *et al.*, 2003). This was to create a stable enzyme-feed complex to allow protection against proteolytic breakdown during fermentation and to start the alterations of the fibre structure thereafter. About 1ml of the appropriate enzyme treatment was then pipetted directly onto the substrate in the incubation vials 24 hr before the start of the *in vitro* incubation and left at room temperature

2.3.3. Rumen fluid collection and preparation

Rumen fluid was collected from mature rumen fistulated Merino sheep, and fed a lucerne based diet at the small stock section in the Experimental farm of the University of Pretoria. The animals where fed *ad libitum* with two feedings a day to ensure the constant availability of fresh feed. The rumen liquor was collected from a minimum of two animals and combined. It was collected in such a manner to try and ensure rumen content from three or four different sites within the rumen of the donor animals approximately an hour after feeding. The rumen

Table 2.1 Treatment groups for *In Vitro* study

Group	Treatment	Cellulase	Xylanase	Description
1	Treatment 1	100%	0%	TMR was treated with 0.4ml/kg hay cellulase plus and 0ml/kg hay xylanase plus.
2	Treatment 2	75%	25%	TMR was treated with 0.3ml/kg hay cellulase plus and 0.1ml/kg hay xylanase plus.
3	Treatment 3	50%	50%	TMR was treated with 0.2ml/kg hay cellulase plus and 0.2ml/kg hay xylanase plus.
4	Treatment 4	25%	75%	TMR was treated with 0.1ml/kg hay cellulase plus and 0.3ml/kg hay xylanase plus.
5	Treatment 5	0%	100%	TMR was treated with 0ml/kg hay cellulase plus and 0.4/kg hay xylanase plus.
6	Control treatment	0%	0%	No treatment with enzymes took place

content of each animal was mixed by hand before sampling. A small amount of rumen content was removed at a time and the rumen fluid was then strained through two layers of cheesecloth into a pre-warmed insulated flask. Any digesta other than the rumen liquor that was removed from the rumen was replaced.

Care was taken to keep the time of collection consistent throughout the trial to try and reduce the variation between the batches as little as possible. The flask was insulated and completely filled before being capped to keep the anaerobic milieu while they were transported to the laboratory as fast as possible (within one hour). This is to prevent the rumen fluid from cooling down.

At the laboratory the rumen fluid was strained again through a single layer of cheesecloth to ensure the removal of any large particles and transferred to a large glass beaker inside a 39°C water bath while being continuously purged with CO_2.

2.3.4. Preparation of incubation media

Preparation of the reagents was done in large quantities at least a day before *in vitro* incubation is done. All reagents were stored at 4°C.

Buffer solution

- Ammonium bicarbonate (NH_4HCO_3) 4 g
- Sodium bicarbonate ($NaHCO_3$) 35 g

This was dissolve in distilled water and brought up to 1 L in a volumetric flask.

Macro-mineral solution

- Sodium hydrogen phosphate, dibasic (Na_2HPO_4) 5.7 g
- Potassium phosphate, monobasic (KH_2PO_4) 6.0 g
- Magnesium sulfate, heptahydrate ($MgSO_4.7H_20$) 0.6 g

This was dissolve in distilled water and brought up to 1 L in a volumetric flask.

Micro-mineral solution

- Calcium chloride, dihydrate ($CaCl_2.2H_2O$) 13.2 g
- Manganese chloride, tetrahydrate ($MnCl_2.4H_2O$) 10.0 g
- Cobalt chloride, hexahydrate ($CoCl_2.6H_2O$) 1.0 g
- Ferric chloride, hexahydrate ($FeCl_2.6H_2O$) 8.0 g

This was dissolve in distilled water and brought up to 100 mL in a volumetric flask.

0.1% (wt/vol) Resazurin

- Resazurin 0.1 g
- Distilled water 100 mL

Dissolve and store in dark (amber coloured) bottle at 4ºC (in fridge).

The incubation media was done on the day the *in vitro* incubation was started**.** Approximately 2.5 g tryptone was dissolve completely in 500 mL water then 0.125 mL micro-mineral solution, 250 mL buffer solution, 250 mL macro-mineral solution and 1.25 mL 0.1% resazurin solution was added. The container with medium was then placed in water bath at 39ºC and continuously purged with CO_2 for 45 minutes. Approximately 0.313 g L-cysteine hydrochloride and 0.313 g sodium sulphide was then added and continuously purged with CO_2 for another 15 minutes or until solution turns grey to clear colour.
o A purple/pink colour indicates the presence of oxygen.
o A grey/clear colour indicates the solution is reduced.

The medium was then kept in the water bath and headspace saturated with CO_2 until the medium plus inoculum was transferred to incubation vials. One part of rumen fluid was added to four parts buffer solution and the required amount of this was then added to the incubation vials.

2.3.5. Sample incubation

Three independent runs with four replications of each treatment in each run were performed to ensure adequate replication for statistical purposes. The vials containing the enzyme-feed complex were placed in the incubation oven at 39°C to warm up the vials to prevent cold shock to the rumen fluid micro-organisms. Rumen fluid was then added to the medium in a ratio of 1:4 (rumen fluid: medium), this is an essential standard. Approximately 42 mL (84 mL/g substrate) of rumen fluid plus medium was then added to the enzyme-feed complex under a stream of CO_2 to each vial and the vials were then immediately closed with rubber stoppers and crimp sealed. The bottles were then placed back into the incubation oven. Once all the bottles were filled a needle was inserted through the rubber stopper of each vial

for about 5 seconds to release any amount of gas that might have built up and to create the starting point for the incubation. The rotary shakers were then turned on (~120 rpm).

2.3.6. Gas measurement and determination of methane production

At pre-determined time points, i.e. at 2 hrs, 4 hrs, 8 hrs, 12 hrs, 24 hrs and 48 hrs, measurement of the gas pressure and methane concentration was done. The time points were designed to avoid that pressure build up exceeds 7 psi because at pressures higher than 7 psi gasses will be re-absorbed into the liquid phase. In this study a semi-automated system consisted of a digital pressure gauge with a luer lock adapter and disposable needle was used for the gas pressure readings. The needle was inserted through the rubber stopper of each vial, the value was recorded and the next vial was sampled. Digital gas pressure readings were converted to gas volumes produced in millilitres using the following equation:

- Gas volume (mL) = 4.08 x gas pressure (psi)

A gas sample was taken during the measurement of gas pressure procedure using a gas tight syringe. The needle was inserted through the rubber stopper of each vial and about 3ml of gas is drawn up, the rest of the gas is then release before the incubation oven is closed and the rotary shaker turned on. The methane as a proportion of total gas is determined by gas chromatography (SIR 8610C gas chromatograph (GC) BTU Gas Analyzer GC System, Bad Honnef, Germany) equipped with a solenoid column packed with silica gel and a flame ionization detector. The sample drawn up is injected into the gas chromatograph which was already calibrated with standard CH_4 and CO_2. The measured methane concentration was related to the respective total gas volume measured in order to estimate its concentration (Travendale *et al.*, 2005)

2.4. Volatile Fatty Acids (VFA) production and pH measurement

At the end of the 48 hr fermentation the incubation vials are removed from the incubator and after gas pressure and gas samples are taken vials are placed on ice to stop the fermentation. The vials were opened as soon as possible to take the supernatant aliquots for VFA. The content of the vials was filtered through filter paper to separate the liquid from the fermentation residue. The pH of the fluid was then measured using a pH meter. For VFA determinations 4ml of 25% H_3PO_4 was added to 20ml filtered rumen sample. This was mixed well and stored in a freezer until it was required for analysis. The samples were analysed by defrosting and centrifuged at 4500rpm for 20 minutes and then filtered through Cameo 30 (0.45µm) filters. Approximately 1µl sample was then injected into the gas chromatograph. The preparation of the standard was done by cleaning a 100mL volumetric flask by adding approximately 50mL cooled distilled water and 2 ml ortho-phosphoric acid, then placing the flask on an accurate scale and adding, with a pipette, the fatty acids that was to be determined. An amount of 450 mg acetic acid, 200 mg propionic acid, 70 mg n-butyric acid, 25 mg iso-butyric acid and 25 mg n-valeric acid was added. The standard was repeatedly injected until consecutive results was comparable and the results calculate as follow:

- Peak area of sample/ Peak area of standard X standard concentration X dilution factors = mg/100 ml acid in the sample
- Divide by molecular mass of acid to obtain the concentration in mmol/100ml sample

2.5. Nutrient degradability

The fermentation residue that was left on the filter paper after the contents of the vials were filtered was then dried for 48h at 55°C. A chemical analysis was then done to determine the DM, Ash, OM, NDF and ADF. All the results were converted to a 100% DM basis and the degradability of nutrients calculated as follows.

- % Degradable Nutrient = $\frac{\text{(\% Nutrient in feed - \% Nutrient in residue)}}{\text{\% Nutrient in feed}} \times 100$

2.6. Calculations and statistical analysis

The experimental design used in this study was a completely randomized design. The data were statistically analysed using the ANOVA option of SAS (2004). Duncan test was done to determine the degree of significance between the means. Significant differences are declared at $p \leq 0.05$. Least square means and standard errors were calculated. The statistical design used was the Completely Randomised Design (CRD) and the model is:

- $Yij = \mu + Ti + \varepsilon ij$

Where: Yij = Observation for each variable measured
μ = Mean
Ti = Treatment effect
εij = Random error.

3. Results and Discussion

3.1. Nutrient composition of Smutsfinger hay

Smutsfinger hay was used solely as a substrate in the *in vitro* study. The nutrient composition of the Smutsfinger hay is summarised in Table 2.2. The hay was utilised to see if there was any difference in terms of the studied parameters for Smutsfinger hay treated with different mixtures of enzymes compared to the untreated hay. The enzyme treatment groups are summarised in Table 2.1.

Table 2.2 Nutrient composition of Smutsfinger hay (g/kg)

Chemical Components	As Is Basis	DM Basis
DM	881.00	1000.00
OM	891.57	876.93
ASH	108.43	123.07
CP	53.92	61.21
NDF	651.75	739.79
ADF	417.44	473.83

3.2. Gas measurement and determination of methane production

Table 2.3 shows the gas and methane production in mL/g DM of feed. Whereas Figure 2.1, 2.2 and 2.3 indicates the pattern of gas and methane production as well as methane per unit of gas produced over a 48 hr period. The trends as seen in Figure 2.1, 2.2 and 2.3 continues throughout the entire 48 hr incubation of Smutsfinger hay with different enzyme mixtures. However there is no significant difference between treatments in the 2 hr, 4 hr, 8 hr and 12 hr gas or methane production. The 24 hr gas and methane production showed a significant ($P<0.05$) difference between treatments, especially for enzyme mixtures with a higher concentration of cellulase resulting in higher gas and methane production as compared to that of the control. At 48 hr the difference between treatments in terms of gas and methane production becomes less substantial than gas and methane production at 24 hr but with 100% cellulase still having higher production of gas and methane than that of the control. This increase in gas production associated with the addition of cellulase is in agreement with Gemeda *et al.* (2014) who found higher gas production with the addition of cellulase enzyme at various time intervals. Similar to the findings of this study Kung *et al.* (2002) found higher gas production from forages treated with enzymes than that from untreated forage. The increased methane production associated with the cellulase addition also agrees with the findings of Chung *et al.* (2012) and Gemeda *et al.* (2015) who found an increased volume of methane gas with enzyme application for enzyme treated feed compared to that of the control. The findings of this study suggest that the 100% cellulase treatment would be the most effective in increasing the gas production but that a 50% mixture of the cellulase and xylanase enzymes would be the best additive to use when aiming for a higher gas production but still keeping the methane production as low as possible. This is due to its relative difference in terms of increasing gas production but lesser effect on increasing the methane production.

Table 2.3 Gas and methane production in mL/g of Smutsfinger hay treated with Cellulase and Xylanase enzymes

	Hours of incubation											
Treatments	2hr		4hr		8hr		12hr		24hr		48hr	
	GP	MP	GP	MP	GP	MP	GP	MP	GP	MP	GP	MP
100C:0X	2.4^{a}	0.028^{a}	7.8^{a}	0.259^{a}	20.8^{a}	1.639^{a}	36.3^{a}	5.306^{a}	103.4^{a}	26.092^{a}	179.8^{a}	69.252^{a}
75C:25X	2.2^{a}	0.030^{a}	7.3^{a}	0.263^{a}	19.9^{a}	1.639^{a}	35.8^{a}	5.380^{a}	99.8^{a}	25.512^{a}	172.8^{a}	67.377^{ab}
50C:50X	2.6^{a}	0.032^{a}	5.8^{a}	0.184^{a}	17.4^{a}	1.304^{a}	32.3^{a}	4.506^{a}	96.0^{a}	23.296^{ab}	170.4^{ab}	63.333^{ab}
25C:75X	4.2^{a}	0.052^{a}	10.1^{a}	0.351^{a}	16.9^{a}	1.398^{a}	30.8^{a}	4.620^{a}	80.9^{b}	20.552^{b}	153.1^{c}	60.272^{ab}
0C:100X	5.6^{a}	0.057^{a}	9.2^{a}	0.289^{a}	19.0^{a}	1.477^{a}	32.2^{a}	4.679^{a}	83.5^{b}	20.877^{b}	157.4^{bc}	59.675^{ab}
Control	4.4^{a}	0.050^{a}	7.8^{a}	0.252^{a}	18.6^{a}	1.422^{a}	32.4^{a}	4.574^{a}	82.7^{b}	20.295^{b}	155.2^{bc}	57.814^{b}
SEM	6.53	0.0007	14.68	0.0146	7.29	0.0374	6.08	0.1476	17.45	2.0653	29.50	12.1681

^{a,b,c} Means with different superscripts have a significant difference.
($p<0.05$ Duncan)
C (Cellulase plus); X (Xylanase plus); GP (Gas Production); MP (Methane Production); Control (No Enzyme)

Figure 2.1 Total gas production over a period of 48 hrs

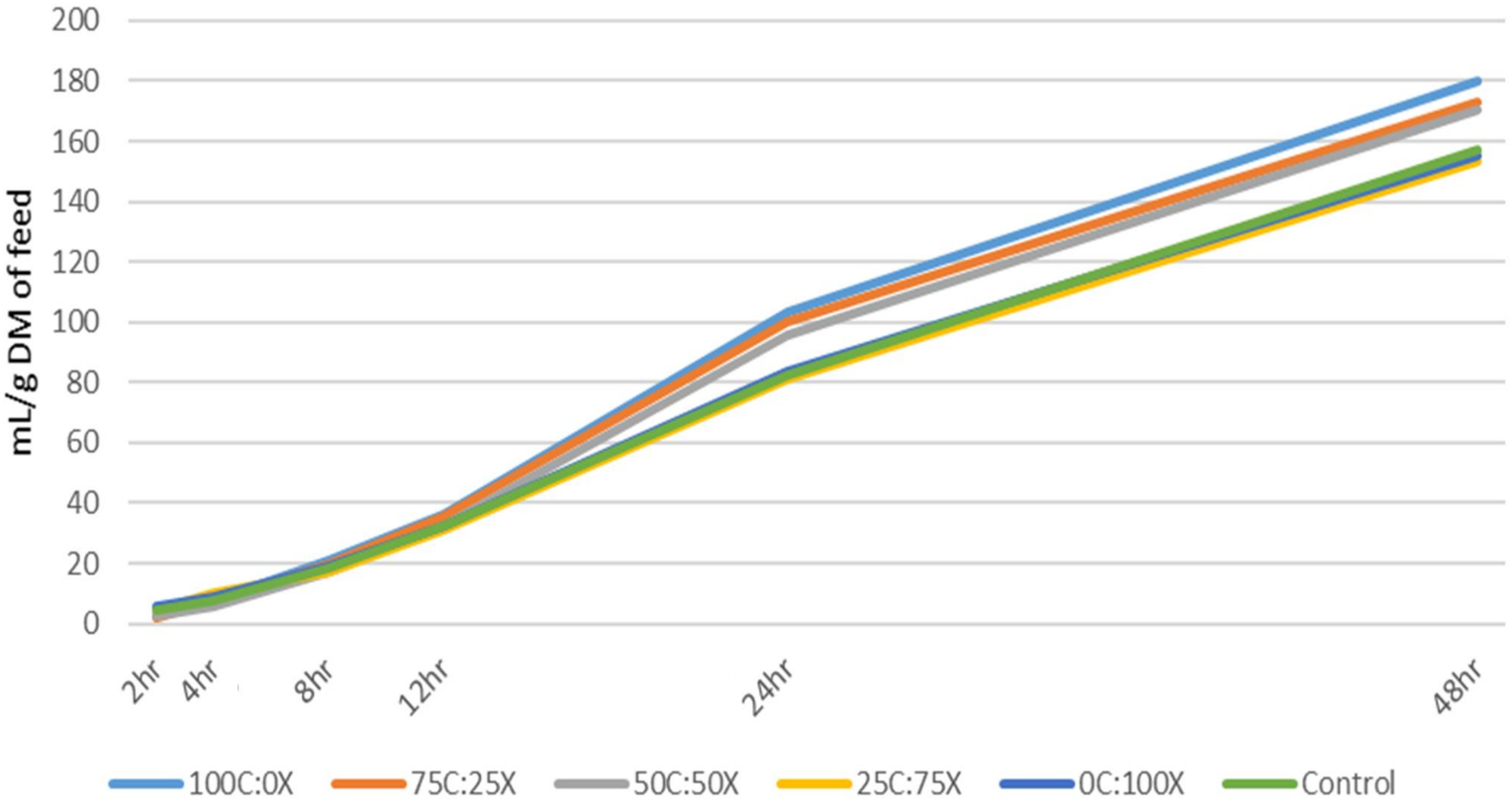


Figure 2.2 Methane production over a period of 48 hrs

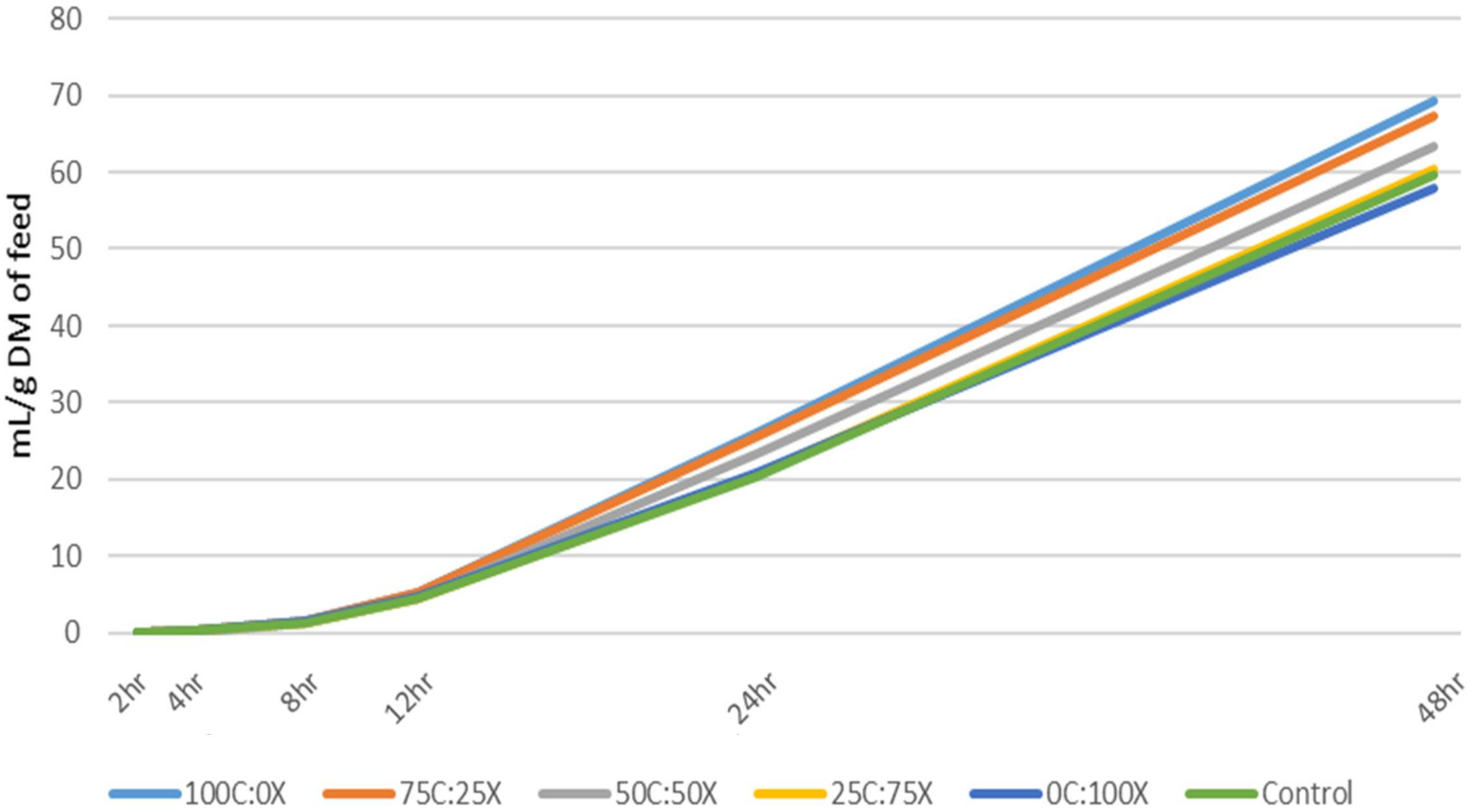
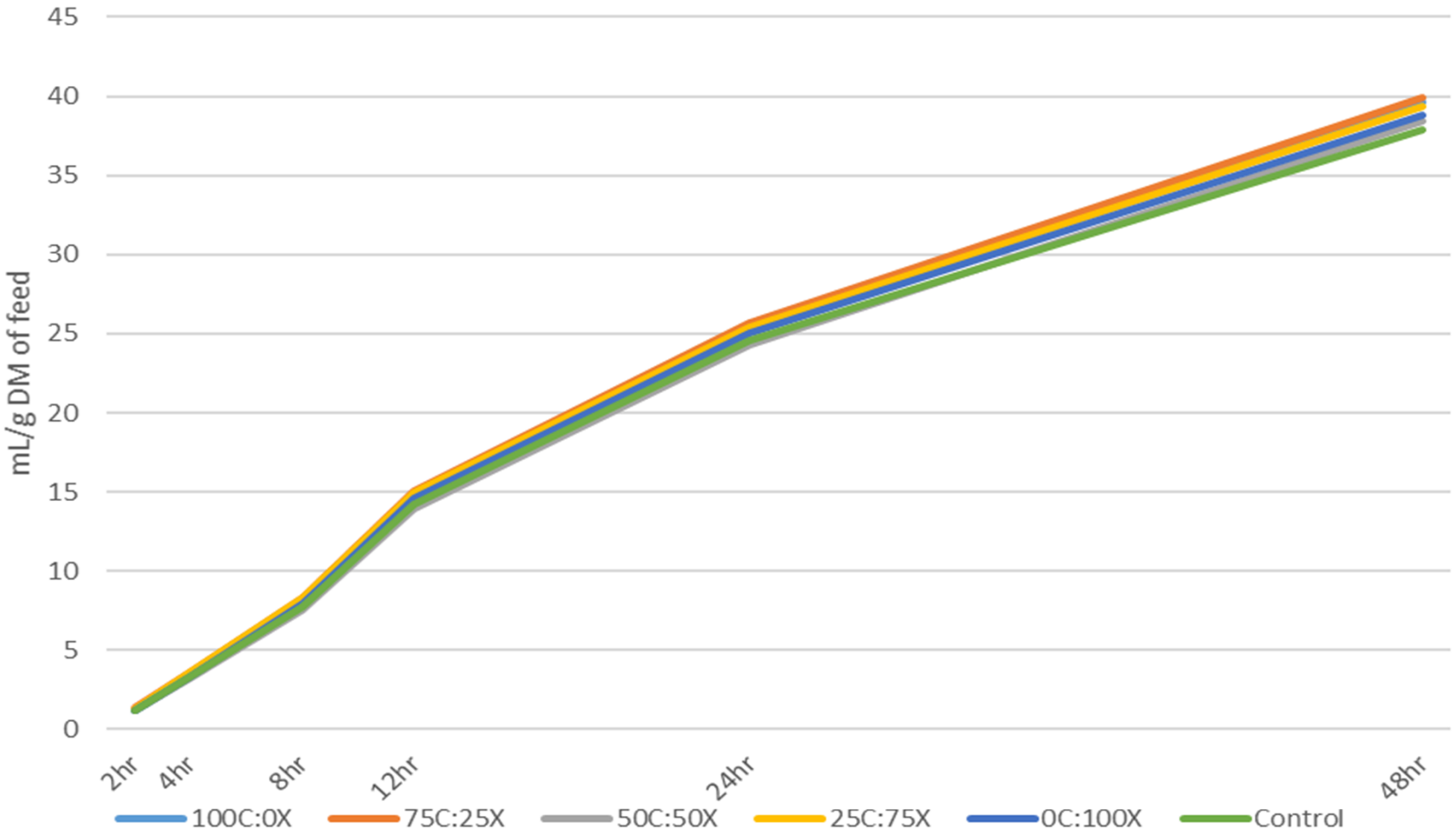


Figure 2.3 Methane produced per unit of gas produced over a period of 48 hrs



3.3. Volatile Fatty Acids (VFA) production and pH measurements

Table 2.4 shows the volatile fatty acid production in mM/L and end fermentation pH while figure 4 shows the pattern of butyric acid production for Smutsfinger hay with the different enzyme mixtures after a 48 hr incubation. The overall trend for volatile fatty acid production are the same for all volatile fatty acids as seen in Figure 2.4. There is no significant difference between the treatments in terms of acetate, propionate, iso-buterate, valerate, total VFA or the acetate: propionate ration. However, a significantly higher ($P<0.05$) butyrate production was observed for the 100% cellulase and xylanase treatments as compared to that of the control. This agrees with the study of Krueger *et al.* (2008) who also reported an increase in butyrate concentrations with the addition of cellulase and xylanase enzymes on Bahiagrass hay. In contrast with the findings of this study, Gemeda *et al.* (2014) recorded generally higher acetate and total VFA concentration for the cellulase and xylanase treated samples compared to the control samples. Kung *et al.* (2002) recorded that the volatile fatty acid production was not different among treatments. The results of this study also agree with those of Yang et al (2002) who found that enzyme addition did not significantly affect acetate and total VFA concentrations but did show increased molar proportions, with the 100 % cellulase and xylanase treatments in this study being the most effective compared to that of the control. The reason for the high butyrate concentration as well as a tendency of higher acetate and total VFA proportions in the 100% cellulase and xylanase treatmnets could be due to an increase in the reactivity of the enzyme after the 24 hr incubation that could have caused enhanced colonization and digestion of slowly degradable fibre fraction by ruminal micro-organisms.

There is no significant difference between the pH values of the control and that of the 50% and 75% xylanase treatment. However, there was a significant ($P<0.05$) difference between the other treatments and the control, indicating a reduction in the end fermentation pH with higher cellulase inclusion. The findings of this study agree with Lewis *et al.* (1996) who reported decreased ruminal pH in steers fed exogenous enzymes treated grass forage, with the 100 % cellulase and xylanase treatments being the most effective compared to that of the control.

3.4. Nutrient degradability

Table 2.5 shows the coefficient of degradability for DM, OM, NDF and ADF after 48 hr incubation of Smutsfinger hay with different enzyme mixtures. The results show that there was a significant ($P<0.05$) difference in degradability between the treatments and the untreated control. The general trend is the same for DM and OM digestibility while that of NDF and ADF responded the same. These finding correlate with those of Alvarez *et al.* (2009), Bala *et al.* (2009) and Lewis *et al.* (1996). The authors all reportedincreased nutrient degradability for test feed treated with cellulase and xylanase enzymes compared to that of the control. According to these results, it is shown that cellulase and xylanase can improve not only fibre degradation but also DM and OM, and that the best enzyme treatment for increased degradation would be a 100 % cellulase treatment. But, the mechanism for this improvement is not clearly known.

The 24 hr pre-incubation of feed sample with enzymes in our study might have shortened lag time and could be the reason for the observed results by a possible improvement in the attachment of micro-organisms to the plant cell wall components thus leading to increased

colonization (Nsereko *et al.*, 2000 and Wang *et al.*, 2001) and an alteration in the fibre structure due to the enzymes effects during the pre-incubation period giving microbes more access to the potentially fermentable fibre and improving fermentation of the feed (Sutton *et al.*, 2003; Elwakeel *et al.*, 2007; Giraldo *et al.*, 2008).

Table 2.4 VFA production in mM/L and end fermentation pH

Treatments	pH	Acetic	Propionic	Iso-Butyric	Butyric	Valeric	Total	A:P
100C:0X	6.53^{c}	43.33^{a}	14.84^{a}	1.40^{a}	4.87^{a}	1.89^{a}	66.32^{a}	2.91^{a}
75C:25X	6.53^{c}	42.67^{a}	14.59^{a}	1.38^{a}	4.71^{ab}	1.87^{a}	65.23^{a}	2.92^{a}
50C:50X	6.73^{a}	42.05^{a}	14.53^{a}	1.34^{a}	4.67^{ab}	1.83^{a}	64.42^{a}	2.89^{a}
25C:75X	6.63^{b}	41.12^{a}	14.37^{a}	1.34^{a}	4.64^{ab}	1.85^{a}	63.35^{a}	2.85^{a}
0C:100X	6.53^{c}	42.97^{a}	14.80^{a}	1.40^{a}	4.81^{a}	1.94^{a}	65.92^{a}	2.89^{a}
Control	6.68^{ab}	41.92^{a}	14.35^{a}	1.35^{a}	4.56^{b}	1.82^{a}	64.00^{a}	2.91^{a}
SEM	0.002	2.452	0.233	0.002	0.029	0.008	5.013	0.002

^{a,b,c} Means with different superscripts have a significant difference ($p<0.05$ Duncan)
C (Cellulase plus); X (Xylanase plus); A:P (Acetic:Propionic)

Figure 2.4 Burytic acid production in mM/L

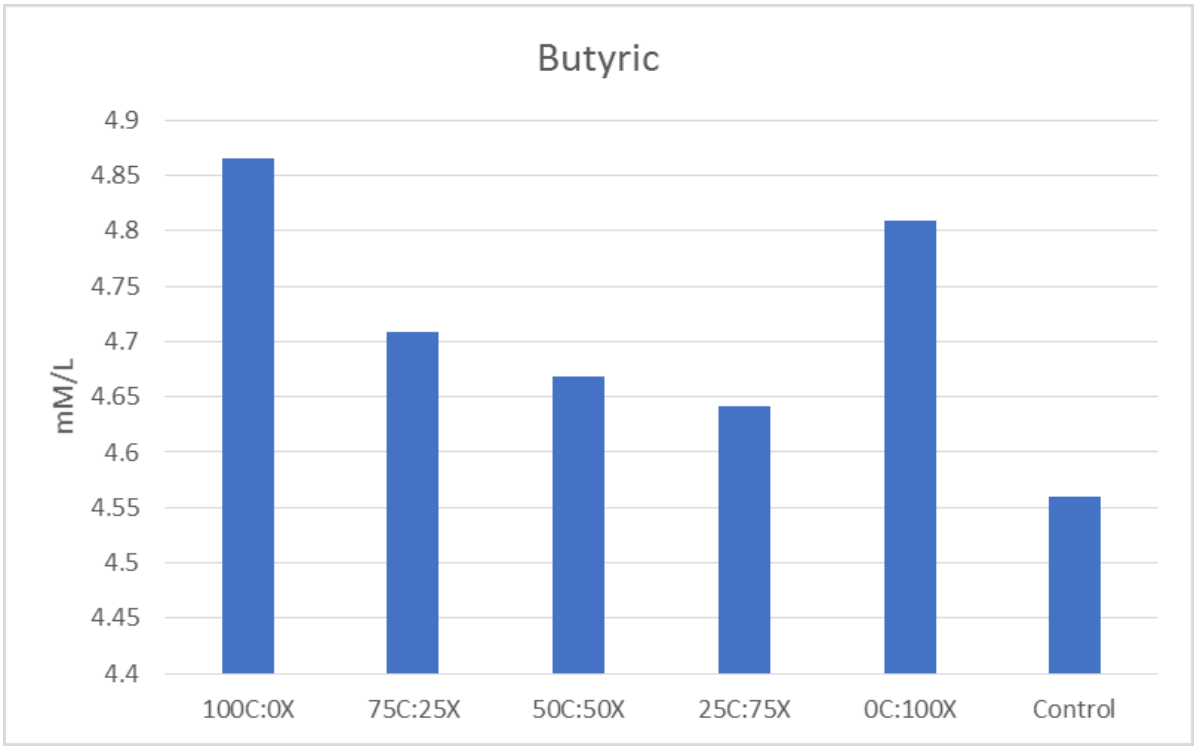


Table 2.5 Coefficient of degradability

Treatment	DM	OM	NDF	ADF
100C:0X	0.8305[a]	0.8236[a]	0.8371[a]	0.8366[a]
75C:25X	0.8285[a]	0.8226[a]	0.8314[ab]	0.8293[ab]
50C:50X	0.8261[ab]	0.8207[ab]	0.8289[ab]	0.8292[ab]
25C:75X	0.8289[a]	0.8235[a]	0.8255[b]	0.8265[ab]
0C:100X	0.8210[ab]	0.8160[ab]	0.8211[bc]	0.8194[bc]
Control	0.8164[b]	0.8097[b]	0.8125[c]	0.8126[c]
SEM	0.00003	0.00004	0.00003	0.00003

[a,b,c] Means with different superscripts have a significant difference.
($p<0.05$ Duncan)
C (Cellulase plus); X (Xylanase plus); DM (Dry Matter); OM (Organic Matter); NDF (Neutral Detergent Fibre; ADF (Acid detergent fibre)

4. Conclusion

Addition of different proportions of cellulase and xylanase plus enzyme mixtures, to Smutsfinger hay increased total gas and methane production, altered some VFA proportions and concentration, lowered end fermentation pH and enhanced the degradability of NDF and ADF of the test feed. Thus different proportions of cellulase plus and xylanase plus enzyme mixtures has improved the nutrient digestibility, rumen fermentation parameters, fibre digestion and gas production, but it does not reduce methane production per unit of digestible dry matter. For practical application the best enzyme treatment to use according to the results obtained in this study would be that of 100% cellulase treatment. Compared to the control, 100% cellulose treatment leads to higher gas production, methane production, butyrate concentration, decreased ruminal pH and improved DM, OM and fibre degradation. In the case where methane production is of importance, it would be best to apply the 75% mixture of the cellulase and xylanase enzymes treatment which leads to a higher gas production, butyrate concentration, decreased ruminal pH and improved DM, OM and fibre degradation but without a significant increase on the methane produced.

CHAPTER 3

Effect of different proportions of cellulase and xylanase enzyme mixtures on intake, nutrient digestibility and VFA production in Merino sheep fed a high forage diet.

ABSTRACT

Cellulase and Xylanase enzymes have been reported to be effective in increasing feed utilization. This study hereby investigated the effect of adding different proportions of cellulase and xylanase enzyme mixtures on intake, nutrient digestibility and volatile fatty acid (VFA) production in sheep fed high forage total mixed ration (TMR). TMR diets were prepared fresh every day starting with a 24h pre-incubation period of the Smutsfinger hay with the prepared enzyme solution. The treatments were 100% cellulose; 75% cellulase: 25% xylanase; 50% cellulase: 50% xylanase; 25% cellulase: 75% xylanase; 100% xylanase and an untreated TMR (control). The pre-incubated Smutsfinger hay was mixed with the appropriate amount of concentrate to form the TMR. A digestibility trial was conducted using a 6 x 6 latin square design and rumen fluid samples were collected and preserved for VFA analysis. Addition of the mixture of 50% cellulase and xylanase plus enzyme mixture, to Smutsfinger hay increased acetate and total VFA concentrations as well as enhanced the intake and the total tract digestibility of the NDF and ADF of the test feed. It appeared that the 50% treatment followed by 75 % cellulase tended to increase dry matter intake in g/head/unit metabolic weight. The 50% treatment increased digestibility of dry matter (DM), organic matter (OM) and crude protein (CP), but the increase in the digestibility was not significant, the treatment was however significantly higher than the control for the NDF and ADF digestibility, acetate production as well as total VFA production. There were no significant difference between the treatments for propionate and butyrate. Although the different proportions of cellulase and xylanase plus enzyme mixtures generally improved TMR intake, fibre digestion and alter the VFA concentration the best response in terms of improving these parameters were obtained with that a 50% cellulase and xylanase plus enzyme mixture.

1. Introduction

Roughages form the bulk in ruminant feeds (El-Kady *et al.*, 2006) and many production systems worldwide depend on roughage as the main nutritional component, leading to it playing an important role in the animal feed industry (Wilkins, 2000). Ruminants have adapted to utilize feed of very low quality to survive. But only a small percentage (10-35%) of the gross energy intake of these poor-quality roughages are retained as nett energy in the end, leading to poor production efficiency because the animal may not be able to digest 20-70% of the cellulose (Marquardt *et al.*, 1997).

The challenge in successful farming with ruminants, be it dairy cattle, beef cattle or sheep and goats lies in the effective utilization of feed resources, as the feeding costs present the largest component of production costs. Of the typically utilized feeds, forage comprises the biggest part of the feeding cost and hence presents a logical area of research for the improvement of its utilization. Exogenous fibrolytic enzymes present one way of improving fibre digestibility (Johnston, 2000). Due to the importance of roughages in the diets of small ruminant under extensive conditions, during periods of high feed costs or poor feed quality it

is important to explore the effect of adding exogenous enzymes to the diets of these small ruminant animals in order to improve the nutritive value of high roughage diets (El-Kady *et al.* 2006).

Exogenous fibrolytic enzymes as additive in ruminant nutrition is a growing interest as a way of improving digestibility of fibrous feeds and the use of this biotechnology to enhance quality and digestibility of fibrous forage is on the verge of delivering practical benefits to ruminant production systems. Its addition in small ruminants has received relatively little attention compared to that of large ruminants (Giraldo *et al.* 2008), only a limited number of small ruminants have been considered with regards to their responses to fibrolytic enzyme treatments (Marquardt *et al.* 1996), and only a limited amount of different roughages have been used. With many positive effects on utilization, ruminal fermentation and digestibility for enzyme addition to ruminant diets being reported.

The current *in vivo* study had the overall objective of investigating the effect of supplementing different proportions of cellulase and xylanase enzyme mixtures on the DM, OM, NDF and ADF digestibility in sheep fed high roughage TMR, as well as the effect of that supplementation on VFA production in the sheep. These general methodologies will be discussed in length at the following sections.

2. Material and methods

2.1. Ethical clearance for animal use

This study was completed with the ethical clearance from the Animal Use and Care Committee, project number EC113-13, University of Pretoria.

2.2. Location and the composition of the total mixed ration (TMR)

This part of the experiment was conducted at the Experimental farm of the University of Pretoria at the small ruminant stock section. Mature sheep were fed a TMR formulated at maintenance level. The TMR had the following ingredients as summarised in Table 3.1, with the Smutsfinger hay purchased from a local farmer and the concentrate portion manufactured by and purchased from Epol PTA West. Table 3.2 and 3.3 shows the nutrient composition of both the Smutsfinger hay and the TMR.

Table 3.1 The total mixed ration comprised of the following feed ingredients

Ingredients	**%**
Yellow Maize (Local)	33.85%
Wheat Middling	6.2%
Molasses	8%
Limestone Powder-Hand Add	0.47%
Salt Fine	1.22%
Urea	0.08%
Vit/Min Sheep	0.15%
Smuts finger hay	50.03%

Table 3.2 Nutrient composition of Smutsfinger hay (g/kg)

Chemical Components	As Is Basis	DM Basis
DM	881.00	1000.00
OM	891.57	876.93
ASH	108.43	123.07
CP	53.92	61.21
NDF	651.75	739.79
ADF	417.44	473.83

Table 3.3 Nutrient composition of TMR (g/kg)

Chemical Components	As Is Basis	DM Basis
DM	927.46	1000.00
OM	841.12	906.90
ASH	86.35	93.10
CP	71.59	77.19
NDF	373.05	402.23
ADF	216.73	233.68

2.3. Experimental design and treatments

A 6 x 6 latin square design was used where six sheep were allocated to one of the six treatments at six different periods. Table 3.4 shows the different treatments groups. The first adaptation and experimental period had a duration of 24 days of which 14 days were an adaptation period and the other 10 days were used for data collection on feed intake, faecal output, rumen digestibility and rumen sampling. For the second to last experimental period the adaptation period was 10 days with a data collection period of 10 days. The sheep were fed a TMR formulated for maintenance level during the entire experimental period. Feeding was *ad lib* twice a day, at 6:00 and 18:00, during the experimental period. Fresh clean water was available at all times. Feed offered and refused was recorded daily and the body weights were recorded at the beginning of each experimental period and at the end of the entire trial.

2.4. Measurement of feed intake and digestibility

The TMR was prepared fresh for every day. Smutsfinger hay was pre-incubated for 24 hrs with the prepared enzyme solution for each treatment separately. On the morning of the feeding the pre-incubated Smutsfinger hay was mixed with the appropriate amount of concentrate to form the TMR. The animals were housed in single metabolic cages with a sheep assigned to each experimental treatment. The sheep were fed on *ad lib basis and* were fitted with a faecal collection bag during the last three days of the adaptation to allow them to adapt

to the faecal bags before the data collection period of 14 days during which the faecal bags stayed on. The amount of feed fed and refusal during each feeding every day was weighed, recorded and sub-sampled. The faeces were also weighed, recorded and sub-samples taken for freezing on a daily basis. The animals were allowed to adapt to the untreated TMR for 14 days before the adaptation period started for the first experimental period. Fresh clean water was available at all times. Each sheep was weighed and weights recorded before the start of each adaptation period and at the end of the entire trial. The sheep were kept at ambient temperatures and natural day length. The feed, refusal and faecal samples that were frozen during collection were then dried, ground and analysed at the end of the experimental trial in the lab at the University of Pretoria to determine the DM, ash, OM, CP, NDF and ADF.

Table 3.4 The treatment groups.

Group	Treatment	Cellulase	Xylanase	Description
1	TMR treatment 1	100%	0%	TMR was treated with 0.4ml/kg Hay cellulose plus and 0ml/kg Hay xylanase plus.
2	TMR treatment 2	75%	25%	TMR was treated with 0.3ml/kg Hay cellulose plus and 0.1ml/kg Hay xylanase plus.
3	TMR treatment 3	50%	50%	TMR was treated with 0.2ml/kg Hay cellulose plus and 0.2ml/kg Hay xylanase plus.
4	TMR treatment 4	25%	75%	TMR was treated with 0.1ml/kg Hay cellulose plus and 0.3ml/kg Hay xylanase plus.
5	TMR treatment 5	0%	100%	TMR was treated with 0ml/kg Hay cellulose plus and 0.4/kg Hay xylanase plus.
6	Control treatment	0%	0%	No treatment with enzymes took place

2.5. Determination of volatile fatty acids (VFA)

A modified technique of Webb (1994) was used to determine the VFA concentrations as discussed in chapter 2 section 5. Rumen liquor had been collected twice a day for 4 consecutive days by extending the collection time by three hours in the subsequent days. Rumen content was squeezed through two layers of cheese cloth into containers and a small amount of inoculum was added.

The rumen fluid was prepared for the Gas Chromatographic method for VFA determinations, 4ml of 25% H_3PO_4 was added to 20ml filtered rumen sample. This was mixed well and stored in a freezer until it was required for analysis.

2.6. Laboratory analysis

The feed, refusal and faeces samples collected during the trial were analysed for DM, ASH, NDF, ADF and CP, and the OM was calculated.

2.6.1. Dry Matter Determination

Dry matter was determined according to the official method of AOAC (2001), International method 934.01. The hay samples were weighed 2g each into duplicate crucibles, which were oven dried at 105°C overnight. In the morning the samples were removed from the oven, cooled and then weighed back.

- % DM = (Mass of sample after drying) x 100
  Mass of sample before drying

2.6.2. Ash Determination

Ash was determined according to AOAC (2001), International method 942.05. Still in the crucibles dry matter analysed samples were placed in the muffle furnace at 200°C for two hours, then at 600°C for four hours. The samples were left to cool overnight and the next morning they were taken out, cooled and weighed back.

- % Ash =(ASH) x 100
  DM

2.6.3. Organic Matter Determination

Organic matter was calculated from the DM and Ash percentages.

- The % OM = 100% - ASH %

2.6.4. NDF and ADF determination

Neutral detergent fibre (NDF) was determined according to the Filter Bag Technique (for A2000, A20001), ANKOM Technology Method 9. This method determines NDF, which is the residue remaining after digesting in detergent solution. The remaining residues are predominantly hemicelluloses, cellulose, and lignin.

Acid detergent fibre was determined according to the Filter Bag Technique (For A2000, A20001), ANKOM Technology Method 8. This method determines ADF, which is the residue remaining after digesting with H2SO4 and CTAB. The fibre residues are predominantly cellulose and lignin.

2.6.5. Crude Protein Determination

The N concentration of the test feed was determined by using the LecoTru Mac N, according to the Leco Instrumental manual procedure 968.06 AOAC (2000). Crude protein concentration was then determined by multiplying the N percentage by a factor of 6.25.

2.7. Calculations and statistical analysis

The experimental design used in this study was a completely randomized design. The data were statistically analysed using the ANOVA option of SAS (2004). Duncan test was done to determine the degree of significance between the means. Significant differences are declared at $p \leq 0.05$. Least square means and standard errors were calculated. The statistical design used was the Completely Randomised Design (CRD) and the model is:

- $Yij = \mu + Ti + \varepsilon ij$

Where: Yij = Observation for each variable measured
μ = Mean
Ti = Treatment effect
εij = Random error

3. Results and Discussion

3.1. Intake and Digestibility

Table 3.5 shows the DM intake in g/head/kg W $^{0.75}$. There was a significant (P<0.05) difference between the treatments in terms DM intake per unit metabolic body weight (g DM/head/kg W 0.75) where addition of cellulose and xylanase in the range of 25-75 % in the enzyme mixtures improved DM intake in g /head/kg W 0.75 compared to the control treatment. The results of this study agree with those of Chen *et al.* (1992), Fredeen *et al.* (1993) and Bala *et al.* (2009) who all found a significant increase in DM intake with the addition of cellulase and xylanase enzyme mixtures. In contrast to other findings, Lewis *et al.* (1996) and Rode *et al.* (1999) showed that exogenous fibrolytic enzymes directly fed to the animal, or added to the feed did not affect the DM intake of the cattle it was fed to. The lack of response in terms of intake improvement may be because enzymes were being fed directly into the rumen and were not pre-treated for 24 hrs before feeding as we have done in this experiment, which gave the enzymes ample time to pre-act on the feed.

However, other studies found different responses to enzyme addition for instance Rodriguez *et al.* (2002) reported that addition of cellulose and xylanase increased the DM intake while Feng *et al.* (1996) reported that DM intake was increased by fibrolytic enzymes when added to dry forages, but not when fresh forages were used. This study shows that a 50 % cellulase and xylanase mixture improved dry matter intake the most, and this finding agrees with the findings of Bala *et al.* (2009) where the addition of a 50% mixture of cellulase and xylanase enzymes prior to feeding was more effective in small ruminants than feeding either of the enzymes separately or not at all. According to Decruyenaere *et al.* (2009) the gut fill capacity, in relation to forages characteristics, can be considered as a main factor of regulation of voluntary intake. Intake appears limited by the maximal volume that the digestive tract can reach (Allison, 1985; Allen, 1996), if the transit rate of digesta can be increased when the quality of forage is poor, intake can increase (Johnson *et al.*, 1991; 1992; Van Soest, 1994 cited by Schettini *et al.*, 1999). The increase in intake thus appears to be due to increased total tract digestibility of the fibre proportion of the feed, allowing the animal to empty its gut better making more space for additional intake.

Table 3.6 shows the coefficient of the total track digestibility of different nutrients in sheep. The fibrolytic enzymes did not significantly affect the digestibility of DM, OM and CP component though there is a tendency for higher values in the enzyme included treatments than the control. This agrees with the findings of Burroughs *et al.* (1960), Theurer *et al.* (1963) and Perry *et al.* (1966) who all reported no effect of fibrolytic enzymes on the digestibility of DM, OM and CP. In this study, however, the digestibility of the fibre component (NDF and ADF) was significantly (P<0.05) increased for the 50% cellulase and xylanase mixture as compared to that of the control. These findings agrees with the findings of other researchers who found an increase in *in vivo* digestibility of different quality forages (Bala *et al.*, 2009; Feng *et al.*,

1996; Geraldo *et al.*, 2008). The mechanism responsible for this increase in digestibility might be attributed to the synergistic action of the cellulase and xylanase enzymes. A lot of the synergistic action between cellulases and xylanase is believed to come from the ability of xylanase to expose the cellulose microfibril core, either by removing the hemicellulose or the hemicellulosic side chains (Selig *et al.*, 2008).

Table 3.5 Intake per unit metabolic weight (g DM/ head/kg W $^{0.75}$)

Treatments	Intake g DM/head/kg W $^{0.75}$
100C:0X	47.59^{d}
75C:25X	62.3^{bc}
50C:50X	78.2^{a}
25C:75X	58.4^{bc}
0C:100X	64.7^{b}
Control	60.9^{bc}
SEM	13.23

p<0.05 Duncan)
C (Cellulase plus); X (Xylanase plus); DM (Dry Matter)

Table 3.6 Coefficients of digestibility

Treatments	DM	OM	CP	NDF	ADF
100C:0X	0.61^{a}	0.62^{a}	0.46^{a}	0.49^{ab}	0.40^{ab}
75C:25X	0.63^{a}	0.64^{a}	0.51^{a}	0.49^{ab}	0.44^{ab}
50C:50X	0.68^{a}	0.69^{a}	0.58^{a}	0.58^{a}	0.52^{a}
25C:75X	0.60^{a}	0.61^{a}	0.48^{a}	0.43^{b}	0.35^{ab}
0C:100X	0.66^{a}	0.67^{a}	0.54^{a}	0.52^{ab}	0.43^{ab}
Control	0.60^{a}	0.62^{a}	0.45^{a}	0.43^{b}	0.24^{b}
SEM	0.006	0.005	0.014	0.010	0.036

^{a,b,c} Means with different superscripts have a significant difference.
(p<0.05 Duncan)
C (Cellulase plus); X (Xylanase plus); DM (Dry Matter); OM (Organic Matter); CP (Crude Protein); NDF (Neutral Detergent Fibre; ADF (Acid detergent fibre)

3.2. Volatile Fatty Acids (VFA) production

Acetate is a product from fermentation of fibre and feeds high in rapidly fermentable carbohydrates lead to microbial populations that produce relatively more propionate and butyrate than acetate (Moran, 2005). Table 3.7 shows the volatile fatty acid production in mM/L. There was a significant difference ($p<0.05$) between the control and the 50:50 cellulase and xylanase mixture in terms of acetate and total VFA production. These results agree with the *in vitro* results reported in chapter 2 and that of Gemeda *et al.* (2014) who recorded that, acetate and total VFA concentration generally tends to be higher for the enzyme treated samples compared to the control samples. The mechanism responsible for the increase in acetate production might be attributed to the synergistic action of the cellulase and xylanase enzymes in the 50% mixture treatment, which resulted in more NDF and ADF digestibility than the control treatment (Table 3.6).

Table 3.7 VFA production in mM/L

Treatments	Acetic	Propionic	Iso-Butyric	Butyric	Valeric	Total	A:P
100C:0X	36.88^{b}	11.65^{a}	1.24^{bc}	2.27^{a}	0.65^{a}	52.70^{b}	3.34^{a}
75C:25X	36.14^{b}	12.24^{a}	1.09^{c}	2.26^{a}	0.614^{a}	52.34^{b}	3.08^{a}
50C:50X	42.10^{a}	12.95^{a}	1.59^{a}	2.59^{a}	0.71^{a}	59.94^{a}	3.38^{a}
25C:75X	36.37^{b}	11.79^{a}	1.13^{bc}	2.36^{a}	0.66^{a}	52.31^{b}	3.20^{a}
0C:100X	40.00^{b}	13.61^{a}	1.46^{ab}	2.24^{a}	0.67^{a}	57.99^{ab}	3.15^{a}
Control	39.58^{b}	11.99^{a}	1.28^{abc}	2.42^{a}	0.66^{a}	55.92^{b}	3.51^{a}
SEM	15.456	3.189	0.065	0.106	0.011	30.56	0.171

^{a,b,c} Means with different superscripts have a significant difference ($p<0.05$ Duncan)
C (Cellulase plus); X (Xylanase plus); A:P (Acetic:Propionic)

4. Conclusion

Addition of the mixture of 50% cellulase and xylanase plus enzyme mixture, to Smutsfinger hay increased acetate and total VFA concentrations as well as enhanced the intake and the total tract digestibility of the NDF and ADF of the test feed. This suggests that a 50% cellulase and xylanase plus enzyme mixture is the optimal concentration that can be used for practical application in order to improve nutrient digestibility, rumen fermentation parameters and fibre digestion *in vivo*.

CHAPTER 4

1. General Conclusion and Recommendations

Ruminant production systems all across the world are mostly based on available natural pastures and harvested crop residues. These forages can be of extremely poor nutritive value as they consist of high fiber content comprising of cellulose and hemicellulose. The utilization of these forages are limited by its low quality (high fiber and low energy contents) and lack of a constant supply from these sources. Increasing the efficiency with which forages are digested by the micro-organisms in the rumen, has been the subject of extensive investigations for over a century. A number of techniques have been studied, these including genetic-, physical-, chemical- and biological technology such as breeding, processing, thermal/pressure treatment, NaOH and CaOH, ammonia, urea, urine, inoculants, ionophores and enzymes. In the past two decades, the application of exogenous fibrolytic enzymes as additives in ruminant feeds has become the topic of research worldwide and these enzymes are included in animal diets to improve feed utilization. Many positive effects have been reported and the enzymes have been shown to have the potential to increase forage utilization by increasing digestibility of feeds and the nutrients from rumen microbes, improving production efficiency and reducing nutrient excretion. These improvements in feed utilization have been reported in dairy cows especially as well as feedlot cattle and some results in small ruminants such as sheep and goats have been reported. The literature available, reporting on such positive effects, serve as evidence that exogenous fibrolytic enzymes can indeed be included in ruminant diets to improve feed digestibility. However, the data available do point out varied responses of ruminants to exogenous fibrolytic enzymes treatment of their diets, focus only on a limited number of feedstuffs and a small proportion focusing on the small ruminants. They also do not state what proportions of these enzymes give the best results.

During this study, six different treatments consisting of different enzyme mixtures were used. Pure cellulase and xylanase, 75% cellulase: 25% xylanase, 50% cellulase: 50% xylanase, 25% cellulase: 75% xylanase at the dosage of 1g/kg roughage as well as the control treatment were compared. With the *in vitro* study the addition of different proportions of cellulase and xylanase plus enzyme mixtures, to Smutsfinger hay increased total gas and methane production, increased butyrate concentration, lowered end fermentation pH and enhanced the degradability of NDF and ADF of the test feed. Thus different proportions of cellulase plus and xylanase plus enzyme mixtures will improve the nutrient digestibility, rumen fermentation parameters, fiber digestion and gas production, but it did not reduce methane production per unit of digestible dry matter. Based on the results from the *in vitro* study the best enzyme treatment would thus be the 100% cellulase treatment because, compared to the control, the 100% cellulose treatment leads to higher gas production, methane production, butyrate concentration, decreased ruminal pH and improved DM, OM and fibre degradation. In the case where methane production is of importance it would be best to use the 50% mixture of the cellulase and xylanase enzymes treatment which will still have a higher gas production, butyrate concentration, decreased ruminal pH and improved DM, OM and fiber degradation but without significantly increasing the methane produced. The result of the *in vivo* study differed from those of the *in vitro*, both revealed the general positive effect expected from the enzyme treatment. However the optimal enzyme concentration differed between the two studies. With the *in vivo* study the addition of the 50% cellulase and xylanase plus enzyme mixture to Smutsfinger hay increased acetate and total VFA concentrations as well as enhanced the intake and the total tract digestibility of NDF and ADF for the test feed. Thus revealing that the 50% cellulase and xylanase plus enzyme mixture is the optimum level that can be used for practical application in order to improve nutrient digestibility, rumen fermentation parameters

and fibre digestion *in vivo*. This difference in response between the two *in vitro* and *in vivo* studies can be ascribed to animal effect and the effect of the concentrate in the diet of the *in vivo* study.

Throughout the research that has been conducted, it is apparent that application of exogenous fibrolytic enzymes has given varied results. Research was also conducted using different methodologies and it appears that the use of exogenous fibrolytic enzymes is variable concerning the combinations, concentrations and the substrate it is applied to and this limits its use in ruminant diets. Further research to compile a comprehensive catalogue for farmers on the use of these enzymes is definitely necessary allowing for an easy guide as to which enzymes in what combination, and what concentration is optimal for their specific substrate in use before this enzyme should be made available to commercial ruminant farmers.

In summary, our study demonstrated that the use of exogenous fibrolytic enzymes definitely has merit for improving the digestibility and intake of roughage. This effect of this treatment is expected to happen during the early stages of digestion, decreasing gut fill via increased passage of digesta due to increased fibre digestion. This increased fibre digestion is likely due to the modification of the plant cell walls causing more exposure of components and allowing micro-organisms quicker access to the plan cell content. For these effects to be observed the correct enzyme combinations, concentration and application needs to be applied to the specific substrate in use.

2. Critical Evaluation

While conducting the *in vitro* study to obtain the gas and methane production values the rubber stoppers used on the vials were punctured for every reading, this could have led to some gas leaking from the vials and contributing to the higher SEM values that were obtained. This can be overcome by use of a technique – if found – that does not puncturing the rubber for every reading.

Throughout this study, six different treatments consisting of different enzymes mixtures were used. Pure cellulase and xylanase plus, 75% cellulase: 25% xylanase, 50% cellulase: 50% xylanase, 25% cellulase: 75% xylanase at the dosage of 1g/kg roughage dry matter as well as the control treatment were compared. The enzymes application at 24 hrs prior to feeding for pre-incubation effects involved a tiresome process of using buckets to sprinkle water on the roughage and hand mixing the roughage as well as again hand mixing in the concentrate proportion just before feeding. This was a very labor-intensive process taking up a lot of time. In addition, the hand mixing may not 100% guarantee even distribution of enzymes through all the roughage. It can be suggested that a more effective and faster method of enzyme mixing be employed, when an experiment of this nature is undertaken again, perhaps a small concrete mixer could be used or any other specialized equipment for mixing feed. During the 24 hour incubation, storing the roughage which had been mixed with water and enzymes could encourage mycotoxin development. This problem can be rectified by keeping enzyme-mixed feed under cooler temperatures, to prevent any mycotoxin development while allowing enzyme action until the next feeding.

During the *in vivo* study, a TMR of Smutsfinger hay and a concentrate proportion were used. Though only the roughage was treated with the enzymes, it makes it very difficult to conclude with certainty that the results of the *in vivo* study were purely those of the work of the enzyme on the roughage. In addition, that there was no interaction of the enzyme with the concentrate proportion from the time it was mixed into the diet, to the feeding and then digestion. Although it was assumed that, the enzymes only worked on the roughage proportion, it cannot be concluded. To be certain it is suggested that a pure diet of just Smutsfinger hay be used when a research trail of this nature is undertaken again.

The animals used in this *in vitro* study did not have ideal body weights with a great variation between individuals. It is thus suggested that animals with more uniform body weights be obtained for more reliable results, especially with regards to the intake. In addition to this it is also suggested that the animals should be given a period for adaptation to their environment after being transported to a new facility and before being cannulated. This will reduce the stress of the animals and increase chances of recovery after the operation. A proper recovery period is also suggested for animals to become accustomed to their new state (fistulation) after the operation, to human presence and handling. Seeing to these factors will allow for a more health, stress free animal that will perform better.

Also using cannulated animals is not at all flawless and it is not uncommon to experience difficulty with regards to the functioning of the rumen fistula. High pressure build up in the rumen in some instances lead to the plug being pushed out, causing fluid and digesta losses as well as altering the anaerobic environment. All of these factors will interfere with the normal functioning of the rumen and negatively influencing results obtained during the research trial.

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