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# Effect of exogenous fibrolytic enzyme supplementation on rumen fermentation, *in sacco* NDF disappearance and total tract digestibility of feedlot steers fed hominy chop/maize based diets.

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**Submitted in partial fulfillment of the requirements for the degree**
**M.Sc. (Agric.): Animal Science: Animal Nutrition**

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**Declaration:**

**I, Gino Renzo Galetti, declare that this dissertation/thesis for the degree M.Sc. (Agric.) Animal Science: Animal Nutrition at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.**

**Signature:……**

**Date:……**

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## List of Abbreviations

| | |
|---|---|
| **ADF** | **Acid detergent fibre** |
| **AIA** | **Acid insoluble ash** |
| **aNDF$_{om}$** | **Neutral detergent fibre on organic matter basis analysed using Alpha-amylase enzyme** |
| **Ca** | **Calcium** |
| **CP** | **Crude protein** |
| **DM** | **Dry matter** |
| **DMI** | **Dry matter intake** |
| **EE** | **Ether extract** |
| **EFE** | **Exogenous fibrolytic enzyme** |
| **GE** | **Gross energy** |
| **HC** | **Hominy chop** |
| **IVOMD** | ***In vitro* organic matter digestibility** |
| **ME** | **Metabolizable energy** |
| **MJ** | **Mega joule** |
| **NFC** | **Non -fibre carbohydrates** |
| **P** | **Phosphorus** |
| **SARA** | **Subclinical rumen acidosis** |
| **SSF** | **Solid state fermentation** |
| **TMR** | **Total mixed ration** |
| **IU** | **International unit** |
| **VFA** | **Volatile fatty acid** |

# Summary


## Effect of exogenous fibrolytic enzyme supplementation on rumen fermentation, *in sacco* NDF disappearance and total tract digestibility of feedlot steers fed hominy feed/maize based diets.

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The focus of this study was to determine the effect of exogenous enzyme supplementation on rumen fermentation parameters, since a healthy or improved rumen environment favors more efficient growth performance and contributes to higher total tract digestibility, the focus being on the South African feedlot industry. The effects on total tract digestibility, *in sacco* digestion and dry matter disappearance of Neutral detergent fibre (NDF) as well as rumen fermentation patterns were studied using three differing levels of formulated fibre. The primary energy component were different combinations of maize and hominy chop, leading to differing dietary fibre levels. Hominy chop is a by-product of the South African maize milling industry and often a raw material used extensively in feedlots around the country due to the ease of supply and possible cheaper acquisition price than maize.

The experimental trial was designed as a 6x6 latin square, repeated in time, within a factorial arrangement of treatments (3x2). The treatments included non-enzyme and enzyme treatment of three diets differing in starch and fibre content, the primary constituents being maize and hominy chop. During each feeding cycle, enough time for adaption to the new diet was allowed as well as enough time for enzyme washout to avoid carry-over effects from the previous treatment. Digestibility was measured using an internal marker, *in sacco* incubation was performed on the different experimental diets as well as hominy chop on its own, and lastly, rumen fluid samples were analysed for pH, volatile fatty acid (VFA) and ammonia-nitrogen levels.

Total tract crude protein and starch digestibility did not differ significantly between treatments ($P<0.05$), but fibre digestibility was affected by enzyme treatment. For both the 75HC:25M and 25HC:75M diets, the enzyme supplementation increased ADF digestibility ($P<0.05$) from 43.79% to 54.12% and 46.11% to 54.49% respectively. No significant effects were seen between the same the diets on the different treatments for NDF digestibility, while there were significant effects across all the diets for both ADF and NDF digestibility ($P<0.05$). The mean NDF digestibility ($P<0.05$) improved from 54.73% to

60.10%, while the mean ADF digestibility ($P<0.05$) improved from 46.11% to 54.49% for the non-enzyme vs enzyme treatment respectively.

The enzyme supplementation did not affect the *in sacco* DM and NDF disappearance for either the hominy chop or the TMR when comparing results within experimental treatments. There were also no overall effects of enzyme vs non-enzyme supplementation on *in sacco* hominy chop and TMR DM and $NDF_{om}$ ruminal digestibility ($P < 0.05$).

Within experimental diets as well as overall, enzyme supplementation did not affect any of the rumen fermentation parameters measured. Results suggest that enzyme supplementation can play an important role in improving apparent total tract fibre digestion when feeding diets where part of the maize component is replaced with hominy chop, as is many times the case in Southern African feedlots.

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# Chapter 1
## Introduction and overview of the South African feedlot industry

There are two primary beef cattle production systems commonly practiced around the world. Extensive rearing of cattle that graze natural vegetation, or the intensive, controlled feeding of cattle to a specific body composition or body weight. The intensive feeding of cattle is generally referred to as feedlotting. Although differing systems exist, the common goal is to improve the efficiency of animal growth while minimizing the cost of that growth. The main aim of a feedlot is to transform an otherwise unmarketable calf via intensive feeding, into a high-quality source of beef product. It has been proven that the most efficient manner of achieving this is by feeding high concentrate diets in feedlots. Concentrate diets are more energy dense and digestible than forage based diets that are consumed by cattle under extensive production systems, therefore supplying more potential nutrient substrate to the animal for growth.

In the past, a copious amount of research has been done to equip the feedlot industry and feedlot operators with tools to improve beef production efficiency and profitability. Feedlots face an ever growing list of challenges, ranging from increased and inconsistent feed prices, quality and availability of feed and public scrutiny concerning feedlot pollution and animal welfare (Meeker, 1999). The use of antimicrobials in feedlot cattle has raised public concern. According to McEwen & Fedorka-Cray (2002), these concerns are based on increased antimicrobial resistance of certain zoonotic enteropathogens. These bacteria could be transferred through the food chain to humans or the resistant genes of commensal bacteria could be transferred to the zoonotic enteropathogens. According to Duff & Galyean (2007), antimicrobial resistance needs to be closely monitored and managed.

Research efforts therefore have been directed towards the development of natural alternatives such as direct fed microbials, organic acids, essential oils and exogenous enzymes, with the goal being to maintain or increase feedlot productivity through alternatives that will not risk public safety. This is of crucial importance, as beef consumption worldwide is predicted to increase due to global population growth (Brandebourg *et al.*, 2013).

Recent positive responses in feeding trials with regard to the use of fibrolytic enzymes for ruminants have resulted in the topic receiving much research interest (Cruywagen & Goosen, 2004; Atrian & Shahryar, 2012). Several studies using enzyme additives in ruminant diets containing mainly forage have resulted in improvements in digestibility of diets and feedstuffs for ruminants (Beauchemin *et al.*, 2000; Pinos-Rodríguez *et al.*, 2008). Enzyme mixtures may also be beneficial in high concentrate feedlot diets (Beauchemin *et al.*, 1997; Krause *et al.*, 1998; Zinn & Salinas, 1999).

Enzyme application is complicated. Ruminal microbes already produce similar enzymes, but fibre digesting ruminal bacteria can be inhibited when ruminal pH is below the range of pH 6.2 - 6.8. This pH

range is optimal for fibre digesting bacteria (Colombatto *et al.*, 2007). In cattle fed typical feedlot diets, the rumen pH can range from 5.6 - 6.5, with the average pH typically around 5.8 - 6.2 (Nagaraja & Lechtenberg, 2007). Many of the commercial enzymes, however, have a low pH optimum and the greatest benefits have been observed with grain-based rations (Russell, 2002).

Unlike the US, feedlot diets in South Africa utilize hominy chop alone or in combination with maize as the primary ingredient. Maize contains a neutral detergent fibre content (NDF) of 9% compared to NDF values of 35% and up to 55% for hominy chop (Macgregor, 2000), depending on the fat and crude protein (CP) content. Replacing most of the maize in feedlot diets with hominy chop would therefore significantly increase NDF content of the diet. Profit margins in feedlots are low and increased ruminal or total tract digestibility through fibrolytic enzyme supplementation can potentially improve feed efficiency and profitability. The similarities of many meat products mean that they have to compete on a cost-of-production bases (Meeker, 1999). Therefore, improving the efficiency of animal growth via ruminal or total tract digestibility by using exogenous enzymes, makes sense. We are not aware of any studies where the effect of fibrolytic enzymes were evaluated in hominy chop/maize based diets under South African feedlot conditions.

1.1) An Overview of the South African feedlot industry

The South African beef industry provides meat produce for 50 million consumers, with an individual average per capita consumption of 15.50 kg/year. Currently, 625,000 tons is produced domestically, while 50,000 tons is imported per year. One hundred commercial feedlots supply mainly the domestic market, with the largest concentration of feedlots in the Free State province and the largest concentration of cattle per feedlot in the Gauteng province. These feedlots have a capacity of 650,000 cattle with an annual throughput of 1,800,000 per annum (Ford, 2017).

The largest portion of consumers prefer A grade meat (95%), while the other 5% prefer AB grade (1-2 permanent incisors). Cattle are fed on average for 135 days, generally from an average weight of 253 kg to 465 kg and slaughtered with an average dressing % of 58.5 (Ford, 2017). While the South African feedlot industry has changed significantly since the 1970's, embracing many new strategies and technologies to improve feedlot efficiency and profitability along the way, there still is room for improvement.

Feedlot operators have begun to look at meat ageing strategies and muscle profiling in terms of product quality. The efficiency of calf procurement is on the increase, and together with preprocessing and back-grounding of newly acquired stock, has helped to greatly improve efficiency of production. A larger emphasis has been put on being mindful of the environmental effects feedlotting has. This includes the water and carbon footprint of feedlotting, waste water management via aquaculture and the increased utilization of waste products in feed (Ford, 2017). Feedlots in South Africa have shown an ability to embrace and adapt to new challenges, and can hopefully keep on doing so in future.

The majority of grain produced in South Africa is maize, due to being the major feed grain as well as the major staple feed of the population. After sugar cane, maize is the second largest crop produced in South Africa. About 60% of the maize produced is white maize, which is used for human consumption, while the other 40%, being yellow maize, is utilized for animal feed production (broiler and layer operations).

The South African feedlot industry will typically use yellow maize to some degree in its diet formulations, but the largest proportion of ingredients will be maize by-products such as hominy chop, due to it being in abundance and cheaper than yellow maize. Hominy feed or chop is produced as a by-product of the dry-maize milling process and consists of the pericarp, germ, soft endosperm and maize sweepings (Leeson *et al.*, 1988). Hominy chop usually presents as a finely ground combination of these portions of maize (Larson *et al.*, 1993), and can vary greatly in its composition due to varying maize quality and the nature of the milling process (Wall *et al.*, 1971).

In terms of nutritive value in comparison to maize, hominy chop has less starch but is higher in crude protein (CP), neutral detergent fibre (NDF) and fat content (Larson *et al.*, 1993). The National Research Council (NRC, 1984) suggests that hominy chop has a higher (6%) energy value than maize, primarily due to the higher fat content. Presently, this is not be the case due to more oil extraction from maize. Wall *et al.* (1971) reported that high temperatures during expeller processing of maize to remove oils could influence the protein availability of hominy chop as high temperatures are known decrease the digestibility of certain amino acids. In a study performed by Larson *et al.* (1993) comparing hominy feed for finishing ruminants, it was found that increased total tract digestibility was due to the smaller particle size and therefore higher passage rate of hominy chop compared to maize. The NDF content and digestibility was found to increase as hominy chop content increased, indicating that the fibre portion was more digestible than that of roughages such as maize silage. In conclusion, Larson *et al.* (1993) states that hominy chop can be substituted for up to 40% of the maize content in finishing rations for cattle.

The objective of this study is to examine the effect of an exogenous enzyme mixture (Fibrozyme, Alltech Inc, Nicholasville, KY) on rumen fermentation patterns, *in situ* NDF disappearance and total tract digestibility in feedlot steers fed diets differing in non-forage NDF content (maize: hominy chop ratio).

# Chapter 2

# Literature review - Forage digestibility in ruminants and the role of exogenous enzymes

## 2.1) Ruminant classification and feeding behavior

Ruminants are an animal type of great importance to man, purely due to the fact that they convert carbohydrates and proteins that are otherwise indigestible and chemically inaccessible to man, into useful and nutritious products. Hofmann (1989) classified ruminants into three well defined categories; concentrate-selectors (40%), intermediate feeders (35%) and grass and roughage eaters (25%). The digestive systems of these animals are different, but similar. Many factors have contributed to the differentiation between these groups, namely behavior, climate, habitat pressure and ecological opportunity (Hofmann, 1989).

Concentrate selectors are those ruminant species that are not suited to utilizing plant fibre, and rather consume high quality forage which is easily digestible. These animals feed frequently, alternating with short periods of rumination (Hofmann, 1989). Examples are bushbuck (*Tragelaphus scriptus*), the northern giraffe (*Giraffa camelopardalis*) and greater kudu (*Tragelaphus strepsiceros*). There are no domesticated examples in this category.

Intermediate feeders are those that choose a mixed diet and show a marked degree of forage selectivity. They can adapt in a short period of time to what forage is available (seasonal anatomical adaptations), increase their intake to meet requirements and adjust productive activities (lactation, energy deposition etc.) according to periods of optimal forage production and availability (Kay, 1987; Hofmann, 1989). When the available forage lignifies, these species can switch to fruit and seeds. Examples include Impala (*Aepyceros melampus*), Thompson's gazelle (*Eudorcas thomsonii*) and Eland (*Taurotragus oryx*). A domesticated example is the goat (*Capra hircus*), descendant from the bezoar (*Capra aegagrus*) (Pfister & Malechek, 1986).

Finally, grass and roughage eaters are those that are most adapted to digesting the fibrous portion of plants. They feed for longer periods at a time, followed by long periods of rumination (Hofmann, 1989). Rumination is an important function to these species, as it allows the chewing of cud which in turn increases the degree to which fibrous fibres are broken up to allow greater accessibility for microbial enzymes. This category contains the most domesticated species and includes cattle (*Bos tuarus, Bos indicus*), sheep (*Ovis aries*), the African buffalo (*Syncerus caffer*) and waterbuck (*Kobus ellipsiprymnus*) to name a few (Hofmann, 1989). In ruminant nutrition, the largest advantage with the use of exogenous enzymes would be seen in this category, as this is where the most fibrous feeds are consumed and where the utilization of those feeds are the least efficient.

An overview of exogenous enzymes in ruminant diets will follow, with emphasis on the limitations in forage digestibility, the mode of action of exogenous enzymes and the utilization of exogenous enzymes in the feedlot industry.

## 2.2) Limitations in forage digestibility

### *2.2.1) Plant anatomy and digestibility*

As explained above, ruminants have developed a mutualistic relationship with the microorganisms in their gut, allowing a larger degree of utilization of plant material. Inherently, ruminants do not produce the enzymes needed to breakdown fibrous components, the predominant component of the plant cell wall.

The quality of plants can be influenced by the arrangement and the amount of certain tissues (Akin, 1986). Plant species follow specific photosynthetic pathways depending on the environment they are adapted to (Ehleringer & Monson, 1993). Species of grass following the $C_3$ photosynthetic pathway grow in more temperate environments and have a higher ratio of mesophyll to vascular tissue compared to grass species adapted to more tropical environments. These grass species follow the $C_4$ photosynthetic pathway. According to Akin (1986), the mesophyll tissue is the most easily degraded by rumen microorganisms.

The degree to which the parenchyma bundle sheath that occurs in leaf blades is surrounded and protected by the plant cell wall can affect the digestibility of plants. The parenchyma in $C_3$ species seems to be more easily degraded because of the higher degradability of the cell wall protecting the bundle sheath. The parenchyma bundle sheath in $C_4$ plants is partially degraded by rumen microbes due to being a more rigid, thick-walled structure (Akin, 1986).

It is found that lignified tissues are the least digestible by rumen microbes (Akin, 1989). The stems of plants thus follow the same pattern of digestibility due to the higher degree of lignification. The epidermis, sclerenchyma ring and vascular tissue in stems all contain lignin to some degree, making stems more fibrous compared to other plant fractions. Furthermore, stem tissue such as the parenchyma cells become more lignified with maturity and therefore less digestible (Jung & Allen, 1995).

Grass species are found to be less digestible than legumes. According to Jung (1989) and Buxton & Russell (1988), the higher rate of cell wall degradation in legumes is due more localized lignin deposition and more core lignin than non-core lignin. When lignin is localized to a higher degree, the physical restriction of cell wall digestion is minimized. Legume species follow the same maturation process as grass species, where lignification of the legume leaf with maturity decreases the digestibility. This being said, Sharma *et al.* (1988) reports that the effect is not as large as in grass species. The illustration below gives an indication of the process of cell maturation in plants.

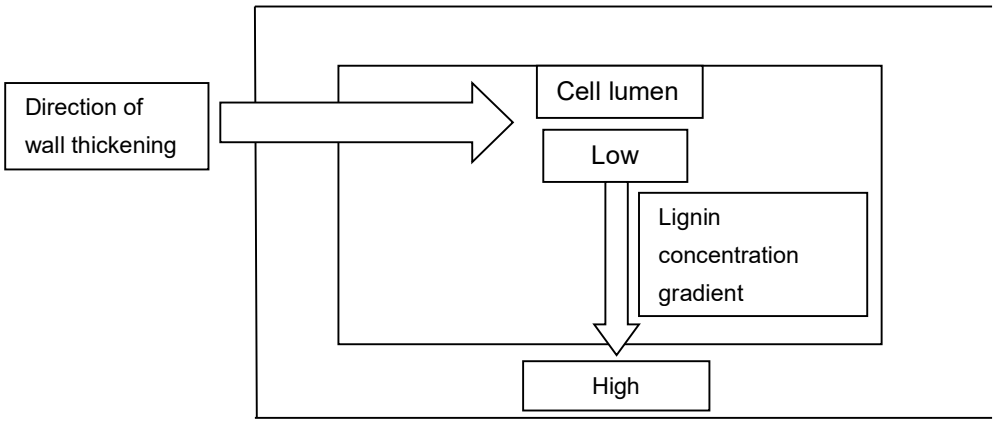


Figure 1: Proposed process of plant cell maturation illustrating the process of cell wall thickening and maturation (Adapted from Jung & Allen (1995).

*2.2.2) Cellular components of the plant cell wall*

The plant cell wall is composed of many different components. The plant cell wall functions to provide mechanical strength, maintain cell shape, regulate transport, provide protection and serve as a storage unit for food reserves to the plant (Brett & Hillman, 1985). The structural plant polysaccharides occurring in the plant cell wall, namely cellulose, hemicellulose and pectin, are the main components which ruminants have evolved to digest in order to obtain energy (Meale *et al.*, 2014). Waxes, phenolic acids and proteins occur with these carbohydrates, influencing the availability to microbial degradation.

Cellulose is composed of covalently linked cellobiose units which are held together by hydrogen bonds. Cellulose occupies 9 – 25% of the cell wall (Salisbury & Ross, 1992). These cellobiose units in repetition form chains which in turn form microfibrils of various widths (Akin, 1986). Cellulose is reported to be almost completely hydrolyzed by mixed populations of rumen bacteria (Dehority & Scott, 1967; Russell & Wilson, 1996; Weimer, 1996; Beever & Mould, 2000). According to Akin (1986), other factors are involved in the inhibition of complete cellulose digestion by rumen microbes. Had it been otherwise, cellulose would be completely available to rumen microbes.

Hemicellulose occupies 25 – 50% of the cell wall (Evert, 2006). Thompson & Soltes (1983) suggested that the structure of hemicellulose consists of linear or branched polymers occurring in

association with other carbohydrates and uronic acids. According to Akin (1986), hemicellulose is formed by a linear glucan chain together with xylan side chains attached to side units of arabinose and uronic acids. Furthermore, hemicelluloses exists as a matrix polysaccharide in conjunction with phenolic substances surrounding cellulose fibrils (Thompson & Soltes, 1983). According to Brice & Morrison (1982) and Lindberg *et al.* (1973), microbial hemicellulases found in the rumen are able to hydrolyze the glycosidic bond between xylose residues which leads to the removal of arabinose side units from the hemicellulose structure. This in turn leads to increased degradation of hemicellulose because of the increased accessibility to the hemicellulose structure by microbial enzymes. Therefore it can be concluded that resistance to microbial degradation is not due to structural differences between carbohydrate polymers, but rather due to physical protection by lignin as well as covalent bonding of phenolic acids to cell wall polysaccharides (Kerley *et al.*, 1988).

Lignin, together with ferulic acid and ρ-coumaric acid, is known as phenolic compounds. Van Soest & McQueen (1973) describes lignin as having plastic properties, allowing wood to have a rigid structure. Lignin and carbohydrates in the plant cell wall are closely associated. Lignin and hemicellulose are covalently linked by at least three bonds. The carbohydrate residues xylose, glucose and arabinose are involved in this linkage. According to Morrison (1980), there is no evidence of covalent linkage between cellulose and hemicellulose. Therefore lignin is the most likely cause of limited availability of structural plant polysaccharides to microbial enzymatic degradation (Van Soest & McQueen, 1973; Hartley *et al.*, 1974).

The fibrous portion of plant forage will always have a place in the ruminant diet. Fibre acts to stimulate the scratch receptors in the rumen wall and thereby stimulate rumen function (Van Soest *et al.*, 1991). This is measured and termed as neutral detergent fibre (NDF) during analyses. Neutral detergent fibre includes cellulose, hemicellulose and lignin (Van Soest *et al.*, 1991; Miller *et al.*, 2008), and therefore gives an estimation of the amount of fibre in feed.

## 2.3) The process of microbial digestion in the rumen

The rumen microbial population consists mainly of bacteria, fungi and protozoa. According to McAllister *et al.* (1994a), ruminal microorganisms adhere to the rumen wall, but the majority (75%) are found to be associated with partially digested feed particles. Several authors agree that a prerequisite for the digestion of feed is the attachment of rumen microorganisms to substrate entering the rumen (Miron *et al.*, 1990; Miron, 1991; McAllister *et al.*, 1994b). The period of time before microbial attachment occurs, known as the lag-time, differs between bacteria, protozoa and fungi. Bacteria have an estimated lag-time of 5 minutes (Cheng *et al.*, 1977), protozoa have a lag-time of 5 – 15 minutes (Amos & Akin, 1978; Bauchop, 1979a), while fungi have a lag-time of within 2 hours (Orpin, 1977). The degree of colonization and the mode of attack is specific to the microbial species in question (McAllister *et al.*, 1994a).

Bacteria have been recognized as the main microorganism in the rumen involved in fibre digestion (Akin, 1986). Originally it was thought the main species of bacteria responsible for hydrolytic activity in the rumen were the gram positive cocci *Ruminococcus albus* and *Ruminococcus flavefaciens*, and the gram negative rod species *Bacteroides succinogenes* (Cheng *et al.*, 1977). But, according to several authors, these species only make up a mere 1 – 3% of the total amount of bacteria in the rumen, therefore contributing a small part to the hydrolytic capacity (Stevenson & Weimer, 2007; Mosoni *et al.*, 2011). Research indicates that the abovementioned bacteria are able to produce cellulases, cellobiase, xylanase and pectinase (Pettipher & Latham, 1979; Groleau & Forsberg, 1981). These bacteria all follow the same means of colonization by attaching to plant substrate via a glycocalyx (Demeyer, 1981; McAllister *et al.*, 1994a; Varga & Kolver, 1997). The glycocalyx has negatively charged polysaccharide fibres which adhere to the positively charged plant polysaccharides of the plant cell wall via a polar bond (Costerton *et al.*, 1978). Glycocalyx bonds have been observed between adjacent bacterial cells, indicating that "physiologically complimentary" bacteria species together aid in plant polysaccharide degradation (Costerton *et al.*, 1978). The parenchyma bundle sheath of $C_4$ grass species as well as the epidermis of lignified schlerenchyma of leaf blades need the attachment of bacterial cells in order to be degraded (Akin, 1986), whereas the mesophyll and other cell wall tissue in $C_3$ grasses seem to be degraded without the need of bacterial attachment (Akin, 1986).

The physiological conditions within the rumen are important for the adequate growth and functioning of bacteria. It is known that the cellulolytic activity of rumen bacteria can be inhibited if the rumen pH falls below pH 6.2 – 6.8 (Matte & Forsberg, 1992; Colombatto *et al.*, 2007). In many instances this is the case, where high grain rations results in decreased rumen pH levels due to rapid fermentation of the grain (Krause *et al.*, 1998). Enzymes produced by fibrolytic bacteria cannot survive at the lower pH levels and thus fibre digestion is implicated (Colombatto *et al.*, 2007). In this regard, aiding the microbial enzymes through supplying exogenous enzymes that have a lower pH optima would be beneficial.

Fungi play a vital role in forage digestion due to being able to physically attach to lignified tissue (Bauchop, 1979a). Bauchop (1979a) further states that the degree of attachment, colonization and growth of fungi on fibrous particles indicates an important role in plant digestion. Motile zoospores of chytrid-like fungi have the ability to swim to sites of attachment and colonization. Species identified are *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis* (Bauchop, 1979b). According to Orpin & Letcher (1979), fungi have a full complement of enzymes capable of degrading the structural carbohydrates of the plant wall. Fungi are more effective in degrading lignified tissues then bacteria and will solubilize but not metabolize phenolic acids (Akin & Borneman, 1990).

A number of authors report that protozoa produce the necessary enzymes such as cellulases and hemicellulases in order to digest plant cell walls (Clarke & Bauchop, 1977; Williams, 1986). Williams (1986) further reports that although hemicellulytic activity was similar, protozoal glucanase activity is higher than that of bacteria. Protozoa furthermore have the ability to freely attach to and dissociate from feed particles (Williams, 1986), suggesting that ruminal protozoa could play an important role in exposing

the more fragile plant tissues and increasing available surface area for bacterial digestion. Williams (1986) however states that in the absence of protozoa, efficient bacterial activity and fermentation continues. Amos & Akin (1978) report that in the absence of bacterial activity, protozoa only contributed 28% towards the fibre digestion of the total rumen population. In conclusion, the role protozoa play in fibre digestion is small when compared to that of bacteria.

## 2.4) Exogenous enzymes

### *2.4.1) An introduction to exogenous enzymes*

An enzyme is defined as a naturally occurring biocatalyst produced by cells in the body and functions to bring about biochemical reactions (McAllister *et al.*, 2001). Exogenous enzymes are those that are produced via a batch fermentation process, utilizing the enzyme producing capability of microorganisms (Beauchemin *et al.*, 2004b). Exogenous enzymes are used in many industrial applications. These applications range from food and wine, textile and laundry, pulp and paper, bio-fuel production, agriculture and as feed additives to improve animal feed utilization (Bhat, 2000; Beg *et al.*, 2001; Van de Vyver, 2011).

Exogenous enzymes, in the context of feed additives, are used to aid in the catalyzation of degradation reactions of feed components into their chemical constituents. Exogenous enzymes are marketed on the bases of their digesting capacity and are therefore referred to as cellulases or xylanases. In most cases, these enzyme preparations do not consist of a singular enzyme, but rather a mixture of enzymes such as amylases, pectinases and proteases (McAllister *et al.*, 2001).

Over many years, the ruminant digestive system has evolved to be able to digest the fibrous nature of forage through a relationship of mutualism with microorganisms in the rumen (Sudweeks *et al.*, 1981; Hofmann, 1989; Morgavi *et al.*, 2013). These microorganisms are responsible for producing the fibre digesting enzymes that the ruminant animal otherwise would lack, therefore aiding in digestion, while the rumen of the ruminant provides an environment adequate for the growth and proliferation of microorganisms. Hatfield *et al.* (1999) unfortunately reports that less than 50% of the fibre portion of forage dry matter is digested and utilized by the animal. Fibrous feed will always have a place in formulated ruminant diets (Meale *et al.*, 2014). Ruminants need fibre to maintain a healthy rumen environment. Concentrates alone cannot be fed due to the risk of acidosis. From a cost point of view, fibrous feeds are more economical than concentrates. The development and use of exogenous enzymes that have a synergistic relationship with rumen microbiota could aid in increasing digestion and utilization of the fibrous potion (Meale *et al.*, 2014).

The experimentation with exogenous enzymes can be dated back to the 1960's, with the focus mainly on the proteolytic and amylolytic kind (Burroughs *et al.*, 1960; McCarthy *et al.*, 2013). Specifically, in ruminant nutrition, the idea was to increase the efficiency by which ruminants obtain energy from structural plant polysaccharides in order to produce high quality products for human consumption. This is

crucial due to the ever-growing human population and the consequent demand for food. The adoption of this technology has been slow due to the cost of enzymes outweighing the cost of other feed additives such as ionophores, antibiotics and hormone implants (Beauchemin *et al.*, 2004b).

Variable results obtained during enzyme trials and insufficient understanding of the mode of action of exogenous enzymes hindered further development and progress (Sujani & Seresinhe, 2015). Recent advances in biotechnology leading to reduced production costs and well defined commercial enzymes have encouraged increased research into the use of exogenous enzymes (Chen *et al.*, 1995; Beauchemin *et al.*, 1997). According to Krause *et al.* (2003) exogenous enzymes are increasingly been seen as a cost-effective means of improving feed efficiency.

*2.4.2) How are exogenous enzymes produced?*

Enzymes used as animal feed additives are produced via a batch fermentation process utilizing the enzyme producing capabilities of microorganisms in a growth media. This growth media usually contains nitrogen, minerals, carbohydrates and surface active agents (Beauchemin *et al.*, 2004b). When produced commercially and thus on large scale, the production process stretches for 5 to 7 days, depending on the microorganism used. The strain of microorganism as well as the growth conditions affect the type and activity of enzyme produced (Lee *et al.*, 1998). Enzyme products are concentrated and purified and therefore have specific enzymatic activity. However, many enzymes are required to be affective against the complex structural arrangement of the plant cell walls (Chesson & Forsberg, 1997; Krause *et al.*, 2003; Morgavi *et al.*, 2013). Commonly, in the case of fibrolytic enzymes products, commercial preparations are labeled as having cellulolytic or xylanitic activity, but in actual fact invariably have pectinases, proteases, esterases and amylases present as secondary enzyme activities to make said enzyme products effective against forage material (McAllister *et al.*, 2001).

According to McAllister *et al.* (2001), enzyme products are primarily derived from four main bacterial species namely *Bacillus subtilis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Streptococcus faecium* , three fungal species (*Aspergillus oryzae*, *Trichoderma reesei* and *Saccharomyces cerevisiae*). Within a single microorganism species, it is possible that the types and activity of enzyme produced can vary due to the strain of microorganism used as well as the selected growth substrate and culture conditions. This is advantageous as it creates the possibility of a wide variety of enzyme products that allow many combinations for the use as exogenous enzyme products, but at same time introduces variability when comparing the efficacy of enzymes because results cannot be (Behera & Ray, 2016) compared to a specified standard.

Recent advancements in exogenous enzyme production leans towards the use of solid state fermentation, or SSF. Popularity of this technique has risen due to several advantages over the more traditional technique of submerged fermentation, in which reactive biomolecules (e.g. bacterial species) are submerged in a liquid such as a nutrient broth. Behera & Ray (2016) define SSF as “Fermentation in the absence or near-absence of free water and intimately provides the natural habitat for the growth of

the microorganism on surface of solid material or substrate". The majority of microorganisms used in SSF are fungi and bacteria. Advantages of this method over submerged fermentation include higher production of crude enzymes, as well as a more complete combination of enzymes needed to degrade lignocellulose structures (Behera & Ray, 2016). Cadirci *et al.* (2016) reports that specifically with *Aspergillus niger*, catabolite repression, which limits enzyme production, seems to be lower. Furthermore, a single reactor can be used for the processes of hydrolysis and fermentation, reducing the incidence of contamination and making the production process more cost effective (Couto & Sanromán, 2006).

2.5) The mode of action of exogenous enzymes used in animal nutrition

Understanding the mode of action of exogenous enzymes is the only way the use and application of enzymes can be improved upon. Once understood, possible advantageous and disadvantageous effects of exogenous enzymes can be explained and improved on. It is important to remember that all the following possible modes of action form a complex enzyme interaction and are not separate form one another (McAllister *et al.*, 2001).

Pre-consumptive effects have been noted by several authors. Evidence indicates that applying exogenous enzyme prior to consumption leads to the release of reducing sugars from feedstuffs (Beauchemin *et al.*, 1996; Hristov *et al.*, 2008). Some authors have described the phenomenon as a "pre-ingestive attack" of the enzymes on the plant fibre (Beauchemin *et al.*, 2004a). The release of these sugars partially arises from the solubilization of NDF and acid detergent fibre (ADF) (Hristov & Broderick, 1996; Gwayumba & Christensen, 1997; Krause *et al.*, 1998). Pell & Schofield (1993) postulated that enzyme absorption onto the substrate is a prerequisite for the hydrolysis and release of sugars, while Forsberg *et al.* (2000) reasons that the sugar release acts as a chemo-attractant for the rumen microbes and a readily available source of carbohydrates. A notable effect of this is the shortened lag-time required for microbial colonization due to the increased supply of carbohydrates leading to encouraged microbial proliferation (Forsberg *et al.*, 2000).

In many cases, the biggest constraint to digestion is the limited colonization and penetration by microbes and their enzymes. Beauchemin *et al.* (2004a) states that the application of exogenous enzymes prior to consumption alters the feed structure, thereby increasing the accessibility of microbes and their enzymes to the feed particles. According to Fontes *et al.* (1995), applying enzymes to feed prior to consumption increases the binding of the enzymes to the feed and thereby reduces the susceptibility of the enzyme to proteolytic inactivation in the rumen. In short, the increased release of soluble sugars, decreased lag-time, alteration of feed structure and enhanced enzyme binding is consistent with the observed increased soluble fraction and rate of *in situ* digestion observed by several authors (Hristov *et al.*, 1996; Krause *et al.*, 1998; Forsberg *et al.*, 2000)

Many ruminal effects are present as well. Exogenous enzymes essentially are a protein structure, and therefore one would assume that introduction into the rumen would result in proteolysis by inherent stomach enzymes and inactivation due to the temperature in the rumen (McAllister *et al.*, 2001). Work done by Hristov *et al.* (1998) and Morgavi *et al.* (2001) prove that the cellulase and xylanase activity of

exogenous enzyme products are more stable than expected in the rumen. This could be due to their glycosylation which protects them from inactivation. Hristov *et al.* (1998) further elucidates that exogenous enzymes increase xylanase and cellulase activity in the rumen, with evidence of eventual declining activity in the rumen fluid due to enzyme outflow with the rumen fluid phase and inactivation of the enzymes over time, as shown in table 1.

Table 1: Mean substrate degrading activities of ruminal fluid and duodenal digesta following administration of Enzyme 1 and Enzyme 2 into the rumen, indicating eventual decline of enzyme activity due to enzyme outflow from the rumen and enzyme inactivation, adapted from Hristov *et al.* (1998).

| | **Site** | | | | | |
|---|---|---|---|---|---|---|
| | Rumen | | | Duodenum | | |
| Activity/treatment[1] | h | Mean[2] | SEM[3] | 0 h | Mean | SEM |
| *CMC-ase* | | | | | | |
| Control[4] | 1.9 | 17.9[a] | 1.04 | 1.31 | 0.85[a] | 0.14 |
| Enzyme 1 | 5.6 | 48.1[b] | 7.29 | 0.83 | 3.82[b] | 0.59 |
| Enzyme 2 | 5.3 | 53.4[b] | 3.34 | 1.58 | 3.77[b] | 0.86 |
| *Xylanase* | | | | | | |
| Control | 3.3 | 70.9a [a] | 2.93 | 0.66 | 0.84[a] | 0.11 |
| Enzyme 1 | 1.9 | 105.9[b] | 12.00 | 1.32 | 2.82[b] | 0.36 |
| Enzyme 2 | 2.4 | 336.6[c] | 8.55 | 1.03 | 23.3[c] | 5.20 |
| *B-Glucanase* | | | | | | |
| Control | 06.7 | 79.1[a] | 5.04 | 0.34 | 0.53[a] | 0.08 |
| Enzyme 1 | 04.9 | 127.2[b] | 12.00 | 0.34 | 2.41[b] | 0.64 |
| Enzyme 2 | 04.7 | 163.9[c] | 5.46 | 0.61 | 3.75[b] | 0.33 |
| *Amylase* | | | | | | |
| Control | 2.3 | 44.8[a,b] | 4.97 | 0.60 | 0.54[a] | 0.06 |
| Enzyme 1 | 1.4 | 39.4[a] | 6.25 | 0.88 | 0.51[a] | 0.18 |
| Enzyme 2 | 7.8 | 59.2[b] | 8.10 | 1.02 | 1.32[b] | 0.28 |
| *Reducing sugars*[5] | | | | | | |
| Control | 0.5 | 3.4[a] | 0.36 | ND[6] | - | - |
| Enzyme 1 | 0.2 | 5.6[b] | 0.30 | ND | - | - |
| Enzyme 2 | 0.8 | 6.1[b] | 0.65 | ND | - | - |

[1] Activity is expressed as mmol of reducing sugar per ml rumen fluid or duodenal digesta per 1 min.

[2] The 0 h figures were omitted from the treatment means.

[3] Standard error of the mean ($n$=18 for Control; $n$=12 for enzyme treatments).

[4] Control: no enzyme addition; Enzyme 1 and 2: enzymes added directly to rumen at 135g day$^{-1}$.

[5] Reducing sugars in ruminal fluid expressed as µmol ml$^{-1}$.

[6] ND, not detected (<0.1 µmol ml$^{-1}$).

[a,b,c] Means within the same column and activity having different superscripts differ at $P$<0.05.

[A,B] Means within the same column and activity having different superscripts differ at $P$<0.01.

Due to the stability of exogenous enzymes in the rumen, the possibility of increased digestion through direct hydrolysis of feed exists. Although this effect has been found to occur in some *in vitro* studies (Forwood *et al.*, 1990; Varel *et al.*, 1993; Feng *et al.*, 1996), this effect was lacking in other *in vitro* and *in situ* studies (Firkins *et al.*, 1990; Varel & Kreikemeier, 1994; Kung *et al.*, 2000), or the response was present due to the amount of enzyme applied rather than the fact that an exogenous enzyme was supplemented (Yang *et al.*, 2000). According to Beauchemin *et al.* (2004a), these results indicate that direct hydrolysis only affects feed components which would naturally be degraded by enzymes produced by rumen microbes. The addition of exogenous enzymes would contribute a minor part to increasing the total hydrolytic capacity of the rumen, because of the levels typically used (Feng *et al.*, 1996; Yang *et al.*, 2000).

A synergistic effect has been found to occur between exogenous enzymes and ruminal microorganisms (McAllister *et al.*, 1994a; Morgavi *et al.*, 2000a; Beauchemin *et al.*, 2004a). This leads to an enhanced effect and therefore a net increase in the enzymatic activity in the rumen that surpasses the additive effects of the individual exogenous enzymes and endogenous microbial enzymes.

Insufficient types and quantities of enzymes, unfavourable rumen conditions and poor interaction of microbial enzymes with target substrates result in limited digestion of plant cell wall material (McAllister *et al.*, 2001). Cross-linking between *p*-coumaryl and feruloyl groups to arabinoxylans has been shown by Hatfield *et al.* (1999) to be a limiting factor in plant cell wall digestion. Tenkanen *et al.* (1991) has shown that *Apergillus oryzae* produces an esterase capable of digesting the ester bridges between ferulic acid, ρ-coumaric acid and arabinoxylan. In this way *Aspergillus oryzae* synergistically acts with rumen microbial enzymes to aid in digestion.

In another range of experiments conducted by Morgavi *et al.* (2000a), a clear synergistic effect was found between an exogenous enzyme produced by the fungi *Trichoderma longibrachiatum* and endogenous enzymes produced by ruminal bacteria in feedlot steers. *Trichoderma longibrachiatum* produces enzymes capable of degrading cellulose and xylanose. These researchers also reported that a substantial cooperative effect on the digestion of xylan as well as the degredation of carboxymethyl cellulose at low pH levels. The synergistic effect was determined by measuring the control monosaccharide level and the resulting monosaccharide level when the exogenous enzymes were added to the diet. Morgavi *et al.* (2000a) therefore concluded that the synergistic effect accounts for the increase in fibre digestion.

At present it is not known whether exogenous enzymes block or expose additional microbial adhesion sites on the surface of feeds (McAllister *et al.*, 2001). Cellulose and hemicellulose can be degraded by free enzymes or by a combination of many enzymes in an organized enzymatic complex known as a cellulosome (Teeri, 1997). These enzymes act in a synergistic manner to catalyze the digestion of cellulose (Bayer *et al.*, 1998). In the case of microbial adhesion, cellulosomes may aid in the adhesion of microbes to the surface of cellulosic material of the plant cell wall (Pell & Schofield, 1993;

Beguin & Alzari, 1998). According to Beguin & Alzari (1998), anaerobic fungi and Clostridium species in the rumen degrade fibre via cellulosomes. Furthermore, ruminococci may rely on cellulosomal structures to degrade fibre as well (McAllister *et al.*, 2001). It is well known that adequate cellular adhesion is needed for effective digestion of forages (McAllister *et al.*, 1994b; McAllister *et al.*, 2001). According to Beauchemin *et al.* (2004a), the release of soluble sugars due to applying enzymes prior to feeding could increase the chemotactic attraction of fibrolytic rumen bacteria to the surface of plant material. Morgavi *et al.* (2000b) proposed that increased roughness on the plant surface due to the exogenous enzymes applied may aid in increased microbial colonization activity.

Low rumen pH conditions are a physiological barrier to the adequate functioning of rumen microbial enzymes. Most fibrolytic microbial enzymes function optimally at pH above 6.2 (Matte & Forsberg, 1992; Colombatto *et al.*, 2007). Exogenous enzymes produced by aerobic fungi have the ability to function at lower rumen pH levels, when compared to the pH optima of microbial fibrolytic enzymes (McAllister *et al.*, 2001). According to Gashe (1992) fibrolytic enzymes produced by aerobic fungi have a pH optima of between 4.0 to 6.0. It is well known that unfavourable rumen pH conditions lead to inhibited bacterial growth and therefore compromised fibre digestion. This often is the case in feedlot and dairy cattle, where high-grain rations result in rumen pH levels being below 6 for large portions of time (Nocek, 1997; Krause *et al.*, 1998). This occurs due to rapid fermentation of feed carbohydrates (Morgavi *et al.*, 2000a). In this regard, exogenous fibrolytic enzymes would aid in fibre digestion and aid in circumstances of poor rumen conditions due to high-grain rations (Beauchemin *et al.*, 1997).

2.6) Stimulation of rumen microbial populations

The stimulation of fibrolytic and non-fibrolytic bacteria populations has occurred when exogenous enzymes have been used as feed additives. In most cases, bacteria utilizing cellobiose or glucose as energy source have been those in which increased numbers have been observed (Beauchemin *et al.*, 2004b). Nsereko *et al.* (2002) supplemented dairy cows with incremented amounts of exogenous fibrolytic enzymes from *Trichoderma longibrachiatum*. They found that the total amount of viable bacteria increased in a quadratic manner. Mostly it was found that the cellobiose-utilizing, xylanolytic- and amylolytic bacteria numbers increased, but there was no effect on the cellulose-utilizing bacteria. Similarly, Giraldo *et al.* (2008) found stimulated growth of cellolytic bacteria 4 hours after feeding in the rumens of cannulated sheep when an exogenous enzyme mixture was deposited directly into the rumen, thus avoiding any pre-feeding effects. The enzyme used contained a mixture of endogluconase and xylanase activity. No effect was seen on endogluconase and amylase activity.

When higher supplemented enzyme levels were used, the increase in bacterial numbers was diminished. According to Kung *et al.* (2000) and Beauchemin *et al.* (1995), the quadratic response seen may explain why non-linear dose responses are seen *in vivo*. Nsereko *et al.* (2002) speculated that excessive exogenous enzyme from *Thrichoderma longibrachiatum* that attached to feed reduced the “space” available for endogenous microbial attachment and therefore did not result in increased bacterial numbers.

Stimulation of microbial numbers by exogenous enzymes could increase the synergistic effect and therefore accelerate digestion due to the larger microbial population (Beauchemin *et al.*, 2004a).Yang *et al.* (1999) reports that this could explain why an increased microbial protein flow to the duodenum has been observed.

Post-ruminally, the greatest effect has been seen with xylanase activity (McAllister *et al.*, 2001; Beauchemin *et al.*, 2004a). Hristov *et al.* (1998) reports that xylanase activity in the duodenum increased by 30% with the use of exogenous enzymes. Cellulose and amylase activity in the small intestine was not significant because of the susceptibility to pepsin and low intestinal pH levels (Morgavi *et al.*, 2001). It was found that with the increase in xylanase activity, intestinal viscosity of feed decreased (Hristov *et al.*, 1998). Reduced intestinal viscosity seen with increased xylanase activity is advantageous to feedlot and dairy cattle that experience increased intestinal viscosity due to the high level of grain they consume. Nutrient absorption could be improved in situations where grain based diets are fed (Mir *et al.*, 1998).

Beauchemin *et al.* (1998) found improvement in total tract digestion of barley grain based diets fed to dairy cattle mainly due to improved fibre and starch digestion in the lower digestive tract. This is advantageous as it allows for the absorption of released sugars and therefore energy and nitrogen benefits which would not occur if feed remains undigested in the rumen or is used as nutritive source for the rumen microbes.

## 2.7) Production responses when using fibrolytic enzymes in ruminant diets

### *2.7.1) Exogenous fibrolytic enzyme effects in dairy cattle*

The use of exogenous fibrolytic enzymes (EFE) in commercial dairy settings has been met with mixed emotions. Many *in vivo* studies have shown a large standard deviation in responses to exogenous fibrolytic enzymes, and therefore the use of these enzymes have not been widely adopted (Beauchemin *et al.*, 2003). In a study conducted by Rode *et al.* (1999), early lactation Holstein cows were supplemented with an exogenous enzyme mixture of xylanase and cellulase. No effect on DMI was seen, however there was an increase in total digestible nutrients (NDF, ADF and CP) measured via Chromium oxide marker, and therefore and increase in milk yield (35.9 vs. 39.5 kg/d). This seems to agree with a study performed by Holtshausen *et al.* (2011), reporting milk-and milk components yield as well as increase DMI. After enzyme supplementation, it was concluded that improved rumen fermentation attributed to an increase in fibre degradation and therefore improved volatile fatty acid profiles (particularly acetate) and increased microbial protein production.

In another more recent meta-analysis by Arriola *et al.* (2017), 15 studies (17 experiments and 36 comparisons), all from peer-reviewed journals and all using continuous experimental designs, were included in the meta-analysis. Authors emphasized the use of early lactation cows for EFE studies. This

could be because the increases in DM and NDF digestibility are critically required during the early stages of lactation. Arriola *et al.* (2017) concluded that across all the studies included, EFE supplementation did not affect DMI, but did improve NDF and DM digestibility as well as milk yield. Although cellulase-xylanase enzymes increased milk yield the most, statistically it could not be proven that this enzyme type was superior to all enzyme types. Therefore, more studies using cellulase-xylanase enzymes are needed.

Furthermore, it was shown that increasing enzyme application rate did not improve the measured parameters but increasing the duration of study showed positive responses in terms of yield and content of milk protein. This suggests that studies longer in duration are needed (Arriola *et al.*, 2017). Lastly, the type of EFE used made little difference to the variation in milk yield across the different studies. This highlights the fact that EFE labeling and characterization is not adequate enough.

The abovementioned could explain why some studies have yielded inconclusive results. More work is warranted specifically with regards to application rate, interactions with rumen microbes, dietary interactions and actual enzyme composition (Masey O'Neill *et al.*, 2014).

*2.7.1) Exogenous enzymes effects in beef cattle*

Similar to the dairy industry, the adoption of exogenous enzyme technology in the beef industry has been equally slow. This can be attributed to more cost-effective additives such as ionophores, antibiotics and hormone implants, and the proven benefits of these additives (Beauchemin *et al.*, 2006). One would expect better responses to EFE in dairy rations which contain more forage than the grain-based feedlot rations, but some positive effects have been shown (Masey O'Neill *et al.*, 2014). The addition of EFE to rolled barley grains in feedlot finishing diets reported by Beauchemin *et al.* (1999), resulted in increased live-weight gain (1.40 vs. 1.53kg/day) and a 10% increase in feed to gain. These responses could be due to EFE aiding fibre digestion in low rumen pH conditions, which are not adequate for optimal functioning of fibre digesting bacteria (Beauchemin *et al.*, 2003). Meale *et al.* (2014) stresses that the variable responses seen in beef cattle are dependable on the dosage of EFE used, the diet the enzyme is applied to as well as the time applied in relation to feeding.

He *et al.* (2015) evaluated the effect of EFE supplementation on *in situ* feed digestibility at two different levels to cannulated animals fed a backgrounding diet (He *et al.*, 2014) as well as an intensive finishing diet in a feedlot setting. The backgrounding diet was a high forage diet containing 50% barley silage, 25% barley grain, 10% grass hay and 15% dried distillers grains with solubles (DDGS). It was found that during this phase, the high level EFE supplementation increased the NDF disappearance of the barley silage linearly from 14.9% to 18.9%, while a linear increase in CP digestibility was seen as well. This indicates and improvement in total tract NDF and CP digestibility through improved FE.

During the feedlot phase, in which the diet contained 60% rolled barley grain, 30% wheat DDGS and 10% barley silage, it was found that starch digestibility increased as the enzyme application increased, while there was a decline in the incidence of liver abscesses ($P = 0.03$). There were no other differences in nutrient digestibility, ruminal pH or VFA production (He *et al.*, 2014). This indicates the

potential health benefits EFE could have during the feedlot phase as well as potential increased starch digestibility.

From the above discussion it can be concluded that there are benefits to supplementing EFE's, but as Masey O'Neill *et al.* (2014) concludes, more research needs to be done to understand the variable responses seen in dairy and feedlot cattle.

## 2.8) Advances in microbial research

A subject which has received a marked degree of interest over the last few years is the use of DNA sequencing and bioinformatics as tools for our understanding of certain complex areas applicable to ruminants. Of those areas, advances have been made in the genomic study of the ruminant digestive tract and its microbiota (Morgavi *et al.*, 2013). These genomic studies have allowed for the study of microbial diversity under different dietary and production conditions. Furthermore, the sequencing of microbial genomes through metagenomics allows for detailed understanding of microbial physiology, with the focus on understanding the enzymatic means by which microbes digest plant polysaccharides (Morgavi *et al.*, 2013). The ruminant animal can be defined as a superorganism due to the fact that the ruminant has evolved over time to have an intimate association with it symbiotic microbiota (Morgavi *et al.*, 2013). The ruminant relies on a complex and interrelated relationship for many things. The rumen microoganisms offer protective, immunological, developmental and nutritional functions and can be considered an organ on their own, housed by the ruminant in conditions suitable to the growth and proliferation.

Handelsman (2004) defines metagenomics as the genomic analyses of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms. Through metagenomics, the study of the ecology and physiology of microorganisms is possible, and therefore will facilitate the design of improved culturing strategies. Furthermore, genomic analyses and pure culture studies can be linked due to the use of metagenomics (Handelsman, 2004).

Metagenomics can be divided into functional metagenomics and sequence based metagenomics. According to Morgavi *et al.* (2013), functional metagenomics has been used to identify hydrolytic enzymes in the rumen, with the greatest interest in those hydrolytic enzymes applicable to the degradation of plant polysaccharides. Pioneering work in this field was initiated by Ferrer *et al.* (2005), who identified a number of enzymes from a dairy cow metagenomic library, including esterases, endoglucanases and a cyclodextrinase. Following this, more work has been done by various authors. Novel lipases (Liu *et al.*, 2009; Bayer *et al.*, 2010), polyphenol oxidases (Beloqui *et al.*, 2006) and cysteine phytases (Huang *et al.*, 2011) have been screened using functional metagenomics. In addition to this, an enzyme capable of degrading a product of the organophosporus insecticide chlorpyrifos (Math *et al.*, 2010), has been identified. Functional metagenomics has been able to identify new enzymes in the rumen and therefore is a useful tool if we are to produce exogenous enzymes needed to aid and improve

digestion in the ruminant animal (Morgavi *et al.*, 2013). Table 2, adapted from Morgavi *et al.* (2013) indicates various enzymes identified through metagenomics studies

Table 2: Plant polysaccharide-degrading enzymes identified from rumen metagenomics libraries, indicating enzymes identified through metagenomics studies. (Adapted from Morgavi *et al.* (2013).

| CAZy family) | Activity (number) | Animal |
|---|---|---|
| GH3 | b-glucosidase | Cow |
| GH3 | b-xylosidase | Cow |
| GH3 | Unspecified (15) | Cow |
| GH5 | Endo-glucanase (7) | Cow |
| GH5 | Cellodextrinase (2) | Buffalo |
| GH5 | Endo-glucanase (12) | Buffalo |
| GH5 | Endo-b-1,4-glucanase | Buffalo |
| GH5 | Endo-b-1,4-glucanase | Cow |
| GH5 | Endo-b-1,4-glucanase (2) | Cow |
| GH5 | Endo-xyloglucanase | Cow |
| GH5 | Exo-xyloglucanase | Cow |
| GH5 | Unspecified (27) | Cow |
| GH5 | Endo-glucanase/xylanase | Yak |
| GH5/GH26 | Glucanase/mannanase/xylanae | Cow |
| GH5/GH26 | Unspecified (1) | Cow |
| GH8 | Unspecified (2) | Cow |
| GH9 | Unspecified (20) | Cow |
| GH10 | Unspecified (21) | Cow |
| GH13 | Cyclomaltodextrinase | Cow |
| GH26 | Endo-glucanase (2) | Cow |
| GH26 | Mannanase (2) | Buffalo |
| GH26 | Unspecified (1) | Cow |
| GH43 | Exo-a-1,5-L-arabinase | Cow |
| GH43 | Endo-a-1,5-L-arabinase | Cow |
| GH43 | Arabinosidase/xylosidase (3) | Cow |
| GH43 | Endo-xylanase | Cow |
| GH48 | Unspecified | Cow |
| GH57 | α-amylase | Cow |
| CE6 | Acetyl-xylan esterase | Cow |
| - | Unspecified esterase (11) | Cow |
| - | Unspecified esterase (2) | Cow |

The motivation for sequence based metagenomics in animal studies comes from the large sequencing projects on human gut microbial communities backed by the European Union (Morgavi *et al.*, 2013). Studies in this manner have been done on the specific microbial community attached to switchgrass incubated in the cow rumen (Hess *et al.*, 2011) and anaerobic fungi and protozoa in the musk oxen rumen (Qi *et al.*, 2011). According to (Morgavi *et al.*, 2013), it would be helpful if this metagenomic data could be placed in a taxonomic context, meaning the use of a reference set of rumen microbial genome sequences to aid in explaining the data.

Through these genomic and metagenomic studies, the microbial organ can be considered with regards to nutrition and other production aspects. It has been shown that mammalian microbiomes have a large set of functions in common (Muegge *et al.*, 2011). This makes it possible to extrapolate data obtained from human and other animal genome studies to ruminants. Gut microbes are in some cases largely shared between species, making it possible that some ideas can be enforced universally across humans and livestock species (Ley *et al.*, 2008). It seems that the focus will be on using the techniques of metagenomics to study rumen carbohydrase complexes from a range of different backgrounds. In the last few years, a study group from New Zealand has been collecting rumen samples from across the world with the aim of identifying carbohydrases that are critical in plant polysaccharide digestion in a variety of different species (Meale *et al.*, 2014). This could potentially aid in identifying essential carbohydrases that are lacking in ruminants, possibly leading to the formulation and development needed to supplement these deficiencies via exogenous enzymes.

# Chapter 3
# Materials and methods

## 3.1) Ethical clearance for the use of experimental animals

Ethical clearance for this study was granted by the Research Ethics Committee of the University of Pretoria as project number EC060-15, attached in addendum 1.

## 3.2) Location

The study was conducted on the University of Pretoria, Hatfield Experimental farm, located in the Gauteng province, Pretoria, South Africa. The experimental farm, at an elevation of 1308m, is equipped with a small-scale feedlot, specifically designed to house a small number of cattle for the purpose of experimental trials. The climatic conditions are typical to the Highveld conditions of South Africa, where summer rainfall occurs between October and March, followed by a dry winter season. Average daily maximum temperatures range from 19°C in July to 28°C in December. The coldest time of the year is during the month of July when the average minimum temperature falls to 3°C. The study was conducted between the months of April and August.

## 3.3) Housing

The feedlot located at the University of Pretoria Experimental farm is constructed of 20 outdoor pens in total, 10 on each side. The pens are each 3m x 5m in proportion, each with its own feeding trough and an ad lib water source. The feedlot runs from east to west in length, with the southern side of the feedlot used for this study. A well-equipped processing crush and corridor is located in close proximity to the feedlot, allowing for sampling without excessive handling of animals. The neck clamp was used minimally, as the experimental animals got used to the sampling techniques and routine. All excess vegetation and grass was kept short to avoid grazing during movement towards the crutch.

## 3.4) Experimental design, animals and treatments

The experimental design consisted of a 6x6 Latin square within a factorial arrangement (3x2) of treatments. Treatments were randomly allocated to 6 ruminally cannulated Pin$^2$zyl steers, each housed in their own pen. Each animal remained in the same pen for the duration of the trial, while the treatments were randomized between pens. All animals were fitted with rumen cannulae from Bar Diamond Industries (Bar Diamond, Inc., Parma, ID, USA), prior to the onset of the trial. All animals were individually weighed, tagged and vaccinated by the experimental farm staff. All animals were 15 months of age at the onset of the trial.

The treatments consisted of three total mixed ration (TMR) diets, with or without the exogenous fibrolytic enzyme (EFE) added. The major difference between the experimental diets was the hominy chop: maize ratio. These treatments are shown below:

- Treatment one (T1): 75% hominy chop (HC); 25% maize (M)
- Treatment two (T2): 75% hominy chop (HC); 25% maize (M) with enzyme (E)
- Treatment three (T3): 50% hominy chop (HC): 50% maize (M)
- Treatment four (T4): 50% hominy chop (HC): 50% maize (M) with enzyme (E)
- Treatment five (T5): 25% hominy chop (HC): 75% maize (M)
- Treatment six (T6): 25% hominy chop (HC): 75% maize (M) with enzyme (E)

Animals were fed their respective treatments at 08h00, 14h00 and 20h00. The seventh back-up animal was maintained on treatment 3 (T3) for the duration of the trial, following the same feeding schedule. Each of the six experimental periods was 23 days in length and structured as follows:

- One experimental period was structured as follows:
- 23 days in total.
- 14 days for adaption.
- 4 days digestibility trial.
- 3 days *in sacco* trial starting on day 19.
- 3 days rumen fermentation study starting on day 21.

### 3.5) Exogenous enzyme description

The experimental exogenous enzyme used was a product of Alltech Inc (Alltech Inc, Kentucky, USA) called Fibrozyme®. This exogenous enzyme is in a powder form and contains fermentation products from *Aspergillus niger* and *Trichoderma viride.* The exogenous enzyme thus is a fibrolytic enzyme used to aid cellulose and hemicellulose digestion, the NDF portion of the plant cell wall. According to Pinos-Rodríguez *et al.* (2008), the xylanase activity was guaranteed at 100 xylose units/g with one xylose unit defined as the amount of enzyme that liberates 1 µmol of xylose per minute under assay conditions. Álvarez *et al.* (2009) defined Fibrozyme (Alltech Inc) as having xylanitic and cellulolytic activity of 43.4 UI and 31.0 UI respectively.

### 3.6) Ingredients and chemicals composition of experimental diets

In total six diets were used for the duration of the trial. The supplemental enzyme (Fibrozyme, Alltech Inc) was carefully premixed into the diets to make sure of even distribution. Preparation and mixing of the diets was performed at a commercial plant using a batch mixing system. As per recommendation by the manufacturer, each animal received 15g DM enzyme per day.

The experimental diets were similar to those typically seen in South African feedlots, with a dietary composition consisting of hominy chop and maize and as the primary ingredients, formulated based on the Nutrient Requirements of Beef Cattle (NRC, 2000). The grain component made up 75% of the ration. *Eragrostis curvula* hay, milled to a shorter length, was used as a roughage source, as well as all necessary by products needed to formulate a well-balanced diet. A mold inhibitor (MycoCURB® liquid, Kemin Industies Inc, Iowa, USA) was added to each experimental diet to prevent mold growth. The product is a blend of organic acids designed to maintain the freshness of grains used in formulation. The vitamin and mineral premix used was a standard feedlot premix supplied by Pennville Animal Nutrient Solutions (Pennville (Pty) Ltd, Pretoria West, Gauteng, RSA). The diets were fed with or without the exogenous enzyme.

Days 1 to 14 serve as a period for adaptation to the new diet. As mentioned, diets were randomly allocated to each animal, with or without the treatment enzyme. The adaptation period is critical to allow the digestive system of the animal, particularly the microbes, to adapt to the new diet (Fernando *et al.*, 2010). Disregarding the adaptation period would result in decreased animal performance and digestive upsets such as acidosis (Nagaraja & Titgemeyer, 2007).

Another function of the adaptation period was to serve as an enzyme washout period. Fourteen days was chosen as optimum, with several authors in agreement (Chen *et al.*, 1995; Kincaid *et al.*, 2005; Hristov *et al.*, 2008; Miller *et al.*, 2008; Phakachoed *et al.*, 2012), while 21 days became too expensive. The washout period is critical to avoid enzyme carry-over effects from the previous treatment. The adaptation period furthermore served to determine the feed intake of each experimental animal, allowing the calculation of the required feeding level of each animal for the following experimental periods.

Table 3: Ingredient composition of the high NDF (75HC:25M), medium NDF (50HC:50M) and low NDF (25HC:75M) diets evaluated with or without supplemental enzyme.

| | Diets | | |
|---|---|---|---|
| Ingredients (% DM) | 75HC:25M | 50HC:50M | 25HC:75M |
| *Eragrostis curvula* hay | 9 | 9 | 9 |
| Hominy chop | 56.3 | 37.5 | 18.8 |
| Ground maize | 18.8 | 37.5 | 56.3 |
| Whole cottonseed | 5.0 | 5.0 | 5.0 |
| Molasses | 8.0 | 8.0 | 8.0 |
| Limestone | 1.4 | 1.4 | 1.4 |
| Urea | 1.0 | 1.0 | 1.0 |
| Salt | 0.4 | 0.4 | 0.4 |
| Vitamin-Mineral Premix | 0.1 | 0.1 | 0.1 |

Table 4: Chemical composition (%DM) of the experimental diets.

| | Diets[1] | | |
|---|---|---|---|
| | 75HC:25M | 50HC:50M | 25HC:75M |
| ME (MJ/kg DM) | 12.9 | 13.1 | 13.3 |
| NFC[2] | 49.2 | 54.6 | 60.0 |
| CP | 12.9 | 12.6 | 12.3 |
| Sol CP (%CP) | 39.8 | 40.5 | 41.2 |
| NDF | 27.1 | 23.1 | 19.1 |
| ADF | 12.0 | 11.1 | 10.3 |
| EE | 7.1 | 6.2 | 5.4 |
| Starch | 39.0 | 44.7 | 50.4 |
| Calcium | 0.8 | 0.8 | 0.8 |
| Phosphorus | 0.5 | 0.5 | 0.5 |

[1] HC:M – Hominy chop:maize ratio

[2] NFC – Non-fibre carbohydrates (%) = 100 – ((%)crude protein + (%)NDF + (%)fat (ether extract) + (%)ash) (Polakova *et al.*, 2010).

3.7) Apparent digestibility study

The apparent digestibility study commenced on day 15 of each cycle and ended on day 18, with the aim of determining apparent total tract digestibility. During this period, 200g/kg feacal grab samples were taken directly from the rectum of each animal twice daily at 08h00 and 16h00. The test animals were herded into the crutch together and individually restrained using the neck clamp for the samples to be taken. The holding area was cleared of any plant material that could be consumed during the short waiting period. Each feacal sample was clearly marked using permanent marker before being composited within steer and period frozen until analyses. During analyses, DM and ash content of each pooled faeces sample was determined, as well as Ammonia-nitrogen, $aNDF_{om}$, CP, starch. In addition to the feacal samples taken, samples of feed and orts (200g/kg) will be taken and frozen before analysis of DM (method 930.15), N (method 990.0.) and ash (method 985.01) of AOAC (1995) and aNDF (Van Soest *et al.*, 1991).

Acid insoluble ash was used as an internal marker to determine total tract digestibility, as well as calculate feacal output for the determination of apparent specific nutrient digestibility. The formulas used were as follows:

$$Apparent\ total\ tract\ digestibility\ (\%) = 1 - \left(\frac{Feed\ marker\ concentration\ (\%)}{Fecal\ marker\ concentration\ (\%)}\right) * 100.$$ (McDonald *et al.*, 2011)

$$Fecal\ output\ (FO)\left(\frac{Kg}{day}\right) = DMI(kg) * \left(\frac{Feed\ marker\ concentration\ (\%)}{Fecal\ marker\ concentration\ (\%)}\right).$$ (McDonald *et al.*, 2011)

$$Apparent\ nutrient\ digestibility\ (\%) = \left[\frac{(DMI * Nutrient\ in\ feed) - (FO * nutrient\ in\ feces)}{(DMI * Nutrient\ in\ feed)}\right] * 100.$$ (McDonald *et al.*, 2011)

3.8) *In sacco* study

The *in sacco* study commenced on day 19 to 21 of each cycle. The ruminal disappearance of hominy chop and TMR was determined using the nylon bag technique as described by Ørskov *et al.* (1980). Twelve bags were incubated per animal, 6 containing hominy chop and 6 containing the respective TMR. Samples were milled using a 2mm screen and 5.000g of feed was placed in polyester bags (10x20 cm, 52µm pore size) provided by Bar Diamond Industries (Bar Diamond, Inc., Parma, ID, USA). New bags were used for each animal and each cycle. Incubation times were 2, 4, 6, 12, 24 and 48 hours from the onset time of incubation (06h00 am). According to Ørskov *et al.* (1980), concentrates need 12 – 36 hours of incubation, while roughages need from 24 to 72 hours of incubation.

The process of sequential removal as described by Cruywagen (2006), was used. This process involves the use of a stocking as the housing for the incubation bags while inside the rumen of the animal. A 'catcher' stocking is attached to the cannula plug. The 'catcher' holds the receptacle stocking in place. The 'receptacle' contains the incubation bags, each of which is separated from the next by a knot tied into the 'receptacle' stocking. A large glass marble is placed at the end of the 'receptacle' stocking to act as a weight while in the rumen. In order to retrieve an incubation bag, the 'receptacle' is freed from the 'catcher', allowing the removal of the incubation bags at proceeding time points without the exposure of the remaining bags to the atmosphere (Cruywagen, 2006).

Figure 2: Rumen cannula plug used during experimental trial illustrating the ring used to anchor the *in sacco* incubation bags in the rumen during the *in sacco* study of dry matter and NDF disappearance.

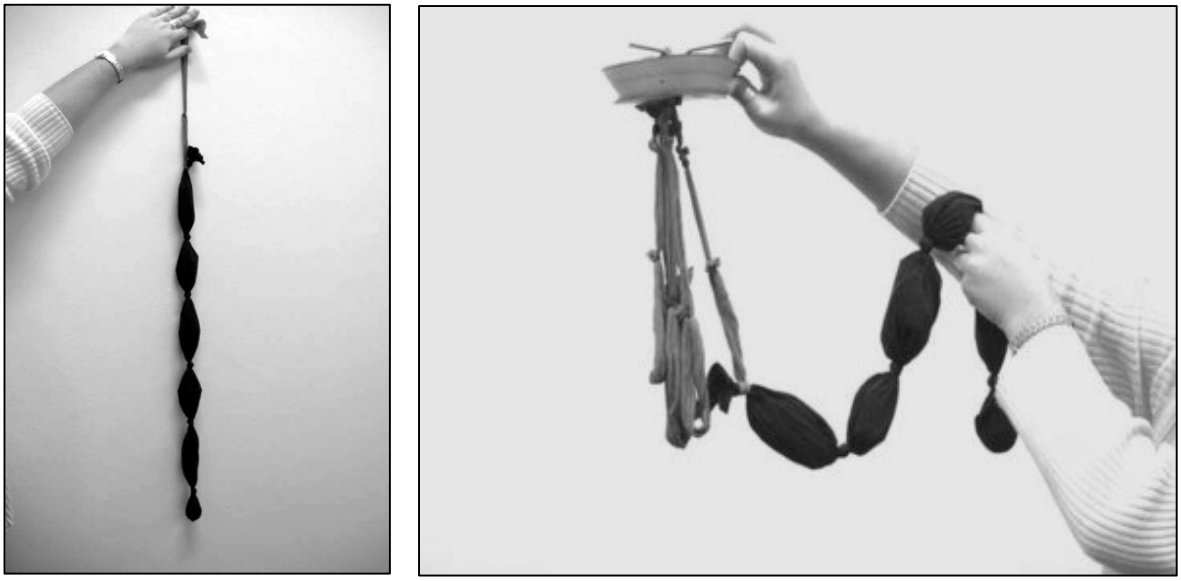

Figure 3: The use of stockings as housing for *in sacco* incubation bags, anchored to cannula plug as a technique for sequential removal as illustrated by Cruywagen (2006).

Once a bag was removed from an animal, it was immediately put on ice to terminate the fermentation process. Once all the bags were removed, cold water was used to hand wash the bags lightly in a circular motion. Bags from different treatments were not mixed and bags from each animal were kept separate. Proceeding the light wash, bags were inserted into a freezer as soon as possible. This occurred less than half an hour after removal from the animal. The *in sacco* bags remained frozen until completion of the trial.

The washing procedure used involved hand washing of the *in sacco* bags. Bags were individually washed in a gentle manner under running cold water until the water passing through the bag was clear. The bag material was cleaned by rubbing the bag between the finger and thumb. This was done by myself only and each bag was washed as uniformly as possible. A timer was used to aid in this regard. This washing method coincides with recommendations on *in sacco* bag washing by Ørskov *et al.* (1980).

Control bags (zero-hour interval) were prepared for each animal for each cycle for the respective TMR and hominy chop incubations. These bags were not incubated, but handled and washed in exactly the same manner as the incubated bags in order to account for loss of very fine bag material during the washing process.

The bags were then dried at 55°C for 24 hours, after which dry matter loss was determined. Following dry matter loss determination, all bags were analysed for $aNDF_{om}$ (Ankom, 2017b), using an Ankom A200 Fibre Analyzer and F57 fibre filter bags from Ankom Tecnologies. The ashing procedure for determination of organic matter followed the recommendations by Ankom tecnologies (2 hours ashing in a muffle furnace at 600°C).

3.9) Ruminal fermentation study

The ruminal fermentation study started on day 21 of each cycle. The experimental animals were maintained on their respective randomly assigned diets. Starting at 06h00 on the 21$^{st}$ day, 300mL ruminal fluid samples would be taken at intervals of 12 hours. Therefore 06h00 and 18h00, 09h00 and 21h00 (day 22), 12h00 and 00h00 (day 23). pH was measured immediately using a digital pH meter .Forty five mL of rumen fluid will be added to 5mL 50% $H_2SO_4$ solution (sulfuric acid) for determination of ammonia-nitrogen (Broderick & Kang, 1980) using an autoanalyzer.

For VFA determination by gas chromatography, 5mL of a 10% NaOH solution will be added to 45mL rumen fluid (Webb, 1994). For analyses, approximately 10 mL of rumen fluid / NaOH mixture is added to 1.1 mL 50% Orthophosphoric acid in a centrifuge tube for centrifugation in a cooled chamber (< 10°C) at 4500 rpm for 20 minutes. Following this, the clear supernatant and precisely 1 mL of the internal standard are pipetted into clean bottles and stored in a refrigerator until required for analyses.

3.10) Sampling and analysis

Samples of feed and orts were taken during the digestibility trial period. These samples were kept in a fridge until further analysis. Pooling of feed and orts per treatment was done at the end of the trial period. Feed, orts and feacal sample analyses were done at Nutrilab, Department of Animal and Wildlife Sciences, University of Pretoria. All *in sacco* samples where immediately stored in a freezer until after the trial and only then analysed for DM loss and $aNDF_{om}$. Rumen fluid samples were analysed for ammonia-nitrogen (Broderick & Kang, 1980) at Nutrilab, while volatile fatty acid analyses was performed at the University of the Freestate, Bloemfontein, South Africa.

Table 5: Total sampling and analysis procedures performed during the experimental trial.

| Sample type and amount | | Analyses | Reference |
|---|---|---|---|
| **Feed** 6 pooled samples | | DM | Procedure 934.01 (AOAC, 2000) |
| | | ADF | Ankom (2017a) |
| | | $aNDF_{om}$ | Ankom (2017b) |
| | | EE | Procedure 920.39 (AOAC, 2000) |
| | | Ash | Procedure 942.05 (AOAC, 2000) |
| | | IVOMD | Tilley & Terry (1963) |
| | | CP | Procedure 968.06 (AOAC, 2000) |
| | | AIA | Procedure Ba 5b-68 (AOAC, 2000) |
| | | Starch | Procedure 996.11 (AOAC, 2000), Megazyme kit (Megazyme U.C., Wicklow, Ireland) |
| | | GE | CAL2K-1 (Digital Data Systems Pty Ltd, Randburg, Gauteng, South Africa) |
| **Orts** 6 pooled samples | | DM | Procedure 934.01 (AOAC, 2000) |
| | | $aNDF_{om}$ | Ankom (2017b) |
| | | Starch | Procedure 996.11 (AOAC, 2000), Megazyme kit (Megazyme U.C., Wicklow, Ireland) |
| **Faeces** 36 pooled samples | | DM | Procedure 934.01 (AOAC, 2000) |
| | | $aNDF_{om}$ | Ankom (2017b) |
| | | ADF | Ankom (2017a) |
| | | EE | Procedure 920.39 (AOAC, 2000) |
| | | Ash | Procedure 942.05 (AOAC, 2000) |
| | | CP | Procedure 968.06 (AOAC, 2000) |
| | | AIA | Procedure Ba 5b-68 (AOAC, 2000) |
| | | Starch | Procedure 996.11 (AOAC, 2000), Megazyme kit (Megazyme U.C., Wicklow, Ireland) |
| | | GE | CAL2K-1 (Digital Data Systems Pty Ltd, Randburg, Gauteng, South Africa) |
| **Rumen fluid** 72 pooled samples | | VFA's (36) | Webb (1994) |
| | | $NH_3$-N (36) | Broderick & Kang (1980) |
| ***In sacco* study** | 432 *in sacco* bags and 72 control *in sacco* bags | $aNDF_{om}$ | Ankom (2017b) |

3.11) Statistical analysis

Data was analysed as a 6x6 latin square, repeated in time, within a factorial arrangement of treatments (3x2). Main effects and interaction will be analysed using the GLM option of SAS and LSMEANS of SAS will be used to compare means (SAS, 2016).

Ruminal *in sacco* DM and $aNDF_{om}$ kinetics will be estimated using the equation of Ørskov & McDonald (1979):

$$p = a + b (1 - e^{-ct'}).$$

The terms of this equation are explained as follows:

p = rumen degradability of nutrients at a time "t" (DM and $NDF_{om}$ disappearance)

a = the intercept representing the degradation at time zero

b = insoluble but potentially degradable fraction by microorganisms in the rumen.

c = describes the degradation rate of "b" per hour (the rate constant for the degradation of "b")

t = time of incubation in hours

Non-linear parameter, a,b,c will be estimated by an interactive least square procedure (NLIN) (SAS, 2016). Significant differences will be declined if $p < 0.05$ and trends at $p < 0.10$.

3.12) Feed intake discussion

Deciding on the amount of feed to be consumed by each test animal was a challenge due to none of the animals being similar in body weight at the onset of the experimental trial. It is important to remember that this study was not for the purpose of measuring growth, typically seen during feedlot trials, but rather to measure the effects of exogenous enzyme treatment on rumen parameters. For this reason, to ensure that the recommended amount of enzyme was consumed by each animal with the minimal amount of orts, it was decided that the animals would be fed at 90% of average individual *ad lib* intake. The average minimum feed intake would be established during the 14-day adaption period during each feed change over. This technique would best mimic what happens in practice and ensure the correct amount of enzyme intake every day.

It was considered to top dress the enzyme or administer the enzyme via the rumen cannula. Top-dressing, however is not recommended, since enzyme intake in practice occurs throughout the day as the animals feed, which agrees with what is recommended by the supplier. Administering the enzyme through the rumen cannula in effect is the same as topdressing the enzyme, and poses the risk of negatively affecting the rumen environment by introducing oxygen to an anaerobic environment (Santra & Karim, 2003). Opening and closing the rumen cannula was there for kept to a minimum as far as possible.

Below in table 6, the mean intakes of the individual test animals are shown for each of the 6 cycles during the experimental trial:

Table 6: Mean DMI for each cycle for each animal in the experimental trial.

| Animal | C1 | C2 | C3 | C4 | C5 | C6 | Mean intake |
|---|---|---|---|---|---|---|---|
| **1** | 8.7 | 9.42 | 10.2 | 12.12 | 15.94 | 17.11 | 12.25 |
| **2** | 8.92 | 9.56 | 10.23 | 14.78 | 16.71 | 13.72 | 12.32 |
| **3** | 8.64 | 9.74 | 10.67 | 12.05 | 14.98 | 17.08 | 12.19 |
| **4** | 8.9 | 9.54 | 10.2 | 13.95 | 16.21 | 15.33 | 12.36 |
| **5** | 8.89 | 9.84 | 11.01 | 13.12 | 13.6 | 14.65 | 11.85 |
| **6** | 8.92 | 9.41 | 10.56 | 13.01 | 14.39 | 17.12 | 12.24 |

Once the mean minimum feed intake level was established, 90% of this feeding level would be maintained for the duration of feeding cycle.

# Chapter 4
# Results and discussion

4.1) The effect of exogenous fibrolytic enzyme on apparent total tract nutrient digestibility.

Acid insoluble ash (AIA) was used as internal marker for the estimation of apparent total tract digestibility. During the course of the trial, all animals consumed the different diets with minimal orts left behind. Feeding level per animal was such that the recommended level of 15g/day of exogenous enzyme was consumed by each animal. The largest amount of orts recorded during the digestibility trial occurred on a single occasion and was a mere 4% of the total daily amount fed, specifically to animal 4. The main effects were seen in the fibrous fractions, namely the NDF and ADF fractions. The mean apparent nutrient digestibility and impact of enzyme supplementation on $NDF_{om}$ is shown in table 7:

Table 7: Mean non-enzyme vs. enzyme comparison for apparent $NDF_{om}$ digestibility for each diet tested during the experimental trial.

| Treatment | Non-enzyme | Enzyme | Mean |
|---|---|---|---|
| 75HC:25M | $53.46^{ab}$ | 60.77 | 57.11 |
| 50HC:50M | $60.41^{a}$ | 60.42 | 60.41 |
| 25HC:75M | $50.34^{b}$ | 59.11 | 54.72 |
| Ave | $54.73^{1}$ | $60.10^{2}$ | |
| Se | | | 3.13 |

$^{a,b}$ Column means with different superscripts differ ($P < 0.05$)

$^{1,2}$ Row means with the different superscripts differ ($P < 0.05$)

Table 7 indicates that within experimental treatments, there were no differences between enzyme supplemented and non-enzyme supplemented animals ($p < 0.05$). In general, enzyme addition to each individual diet contributed towards a numerical increase in $NDF_{om}$ digestibility. More importantly, when comparing non-enzyme vs. enzyme treated animals, regardless of treatment, the enzyme supplemented animals had a higher $NDF_{om}$ digestibility compared to the non-enzyme treated animals (54.71% vs. 60.10%) ($p < 0.05$). A significant effect was shown between the untreated 50HC:50M and 25HC:75M diets. An overall comparison for each diet (the mean for the enzyme and non-enzyme treatment), did not yield a significant effect, with the largest numerical NDF digestibility seen for the 50HC:50M diet (60.41%).

Considering the ADF fraction, which consists of lignin and cellulose, significant differences were shown between the enzyme and non-enzyme treatment of the high fibre diet (75HC:25M) as well as between the enzyme and non-enzyme treatment of the high starch diet (25HC:75M). These results are represented in table 8 below:

Table 8: Mean apparent nutrient digestibility values for ADF for enzyme and non-enzyme treatments using AIA as internal marker.

| Treatment | Non-enzyme | Enzyme | Mean |
|---|---|---|---|
| 75HC:25M | 43.79[1] | 54.12[2] | 48.96 |
| 50HC:50M | 50.29 | 54.35 | 52.32 |
| 25HC:75M | 44.25[1] | 54.99[2] | 49.62 |
| Ave | 46.11[1] | 54.49[2] | |
| Se | | | 3.29 |

[a,b] Column means with different superscripts differ (P < 0.05)

[1,2] Row means with different superscripts differ (P < 0.05)

There were no differences seen for crude protein and starch digestibility for any of the diets tested under enzyme and non-enzyme conditions (P < 0.05).

As seen with the NDF digestibility, ADF digestibility followed the same trend for total enzyme vs. non-enzyme effect across all the diets, with enzyme addition improving digestibility (46.11% vs. 54.49%). The same trends seen for the differing diets for NDF digestibility, were seen for ADF digestibility.

The main digestibility effects occur on the fibrous portion of the feed, as can be expected when supplementing an exogenous fibrolytic enzyme. The use of exogenous fibrolytic enzymes in ruminant diets can be justified due to the low inherent digestibility characteristics of these diets, as well as in attempt to reduce the amount of more expensive grain ingredients used in formulated rations, while maintaining animal growth and performance. Exogenous fibrolytic enzymes aid the rumen by predigestive attack on the fibrous fraction of the plant cell wall, as well as working together with inherent microbial enzymes to degrade nutrients in the feed (McAllister *et al.*, 1994b).

Considering the NDF fraction, on average, a significant difference was seen between enzyme and non-enzyme treatments across all diets for NDF digestibility (54.73% vs. 60.10%), an indication that the use of the enzyme shows a trend for improvement of the utilization of the NDF fraction in feed, although it might not be significant. This has more than not been the case in literature, where improvement of NDF digestibility has not been significant. Álvarez *et al.* (2009) demonstrated in a study on beef steers fed high fibre rations where two exogenous fibrolytic enzymes were applied separately (both enzymes consist of a xylanase/cellulase mixture), that NDF disappearance was not improved. The diets tested were much higher in NDF content than in this study. A meta-analysis on the use of fibrolytic exogenous enzymes in ruminant diets compiled by Tirado-González *et al.* (2018), concluded that although DMI was improved in beef cattle, NDF digestibility was not improved. Records of 74 journal articles were used to compile the study.

While there was an improvement in the NDF digestibility for the high fibre diet, an equal improvement in the digestibility of the low fibre, high starch diet containing 75% maize, was measured.

As stated by Beauchemin *et al.* (2003), more consistent results have been recorded for exogenous fibrolytic enzymes supplemented to high grain diets compared to high fibre diets. In a series of experiments conducted by various authors, fibrolytic enzymes were investigated on barley based diets. Beauchemin *et al.* (1997) reported an increase in feed efficiency with the use of a xylanase enzyme when applied to a 95% barley based diet. The diets tested had an average starch content of 48%, similar to the starch content of the 25HC:75M diet tested (50%). This increase in feed efficiency was attributed to the improvement in diet digestibility seen when using the enzyme. Eivemark (1997) repeated the experiment under *in vitro* conditions and found consistent increases in *in vitro* gas production as well as increases volatile fatty acid (VFA) responses to the enzyme treatments compared to the control (no enzyme supplementation). The conclusion by these authors was that the use of exogenous fibrolytic enzyme in barley based diets improved increased feed efficiency due to improved diet digestibility.

Considering the ADF fraction, more significant differences were seen compared to the NDF fraction. These significant differences were seen for the high fibre and the low fibre diets, as well as a significant average enzyme effect for across all the diets tested. It has often been the case were improvements in ADF digestibility were seen compared to NDF digestibility. Krause *et al.* (1998) tested a xylanase enzyme on barley based diets and reported a 14% increase in ADF digestibility, while numerical but not significant increases in NDF digestibility were seen.

Many factors could contribute to the difference in digestibility of the ADF and NDF fractions seen. The first reason, and probably the most likely explanation, could be the difference in composition of the ADF and NDF fractions. By definition, the ADF fraction consists of the lignified portion of the plant cell wall as well as cellulose (Van Soest, 1967), while the NDF fraction consist of lignified tissue, hemicellulose and cellulose (Van Soest *et al.*, 1991; Miller *et al.*, 2008).

Cellulose has the highest degree of digestibility of the fibre fractions, mainly due to being the most digestible by a mixture of ruminal microbial enzymes when compared to hemicellulose and lignin (Dehority & Scott, 1967; Russell & Wilson, 1996; Weimer, 1996; Beever & Mould, 2000) and having a negative correlation with the amount of lignin present, therefore, as lignin concentration increases, the digestibility of cellulose decreases. Lignin is the least digestible of the fibre fractions (Van Soest & McQueen, 1973) and because lignin is closely associated with the hemicellulose and cellulose fractions, it restricts the availability of cellulose to digestive enzymes (Van Soest & McQueen, 1973; Hartley *et al.*, 1974). Hemicellulose and lignin are covalently linked by at least three bonds, while there is no evidence of this linkage between hemicellulose and cellulose (Morrison, 1980). Therefore, when hemicellulose is measured, as is the case with the NDF fraction, a lower degree of digestibly is seen in the NDF fraction when compared the ADF fraction.

It is possible that the composition of the exogenous enzyme is not suited towards the solubilization of lignin, which makes up part of the NDF fraction and is the least digestible of the fibre fractions (Van Soest *et al.*, 1991; Miller *et al.*, 2008) therefor lowering the digestibility measurement of NDF and indicating more efficacy in the ADF fraction of which lignin is not a part of. During the digestion of NDF, xylans must be removed to gain access to the protected cellulose and therefore efficacy could be reduced to such an extent that adequate exposure of lignin to the exogenous enzyme does not occur (Grabber *et al.*, 2002). It is known as well that exogenous enzymes lose efficacy as times passes due to eventual enzyme breakdown and outflow from the rumen (Hristov *et al.*, 1998).

Another reason for the improved digestion of the ADF fraction seen in comparison to the NDF fraction, could be the higher availability of starch and therefor energy for microbial digestion, particularly one the low fibre, high starch diet (25HC:75M). Ferraretto *et al.* (2013) reported in a study of cereal grain types and digestion, that as much as a 0.61% decrease in NDF digestibility and a 0.48% decrease in total tract digestibility can be seen in ruminant animals per percentage-unit increase in dietary starch content.

As concluded by Tirado-González *et al.* (2018), improvements in dietary digestibility using exogenous fibrolytic enzymes is possible when the dietary composition is taken into consideration and the correct balance of xylanase to cellulase ratio is selected in the enzyme used.

4.2.1) The effect of exogenous fibrolytic enzyme on *in sacco* $NDF_{om}$ and DM digestibility of hominy chop.

During the *in sacco* study, the three TMR diets as well as hominy chop were incubated separately in incubation bags over several time points. This was done under each treatment condition. In order to compare the digestion curves statistically, the equation of Ørskov & McDonald (1979) was used to determine DM and $aNDF_{om}$ kinetics for the Hominy chop samples incubated under the different treatment conditions. The calculated "p- values" (rumen degradability of the nutrient at time "t") were compared and analysed using least square procedure (NLIN) (SAS, 2016). These results are represented in table 9, which indicates the degree of $NDF_{om}$ and DM ruminal degradation between the enzyme and non-enzyme treatments of hominy chop regardless of the dietary treatments, and is visually represented in figure 4:

Table 9: The effect of fibrolytic enzyme and non-enzyme treatment on hominy chop $NDF_{om}$ and DM disappearance over time during the *in sacco* study.

| | Hominy chop $NDF_{om}$ | | Hominy chop DM | |
|---|---|---|---|---|
| Time (h) | Non-enzyme | Enzyme | Non-enzyme | Enzyme |
| 0 | 24.8 | 25.2 | 25.5 | 25.3 |
| 2 | 30.6 | 31 | 37.9 | 38.6 |
| 4 | 31.5 | 32 | 40.8 | 40.7 |
| 6 | 32.7 | 32.4 | 43.4 | 42.6 |
| 12 | 34.1 | 34.2 | 46.1 | 47.6 |
| 24 | 37.5 | 38.8 | 51.5 | 53.9 |
| 48 | 48.9 | 46.8 | 62.9 | 63.2 |

[a,b] Row means with different superscripts differ ($P < 0.05$).

As can be seen, following statistical analyses, no significant differences were seen between the digestibility measured at the different timepoints. It follows that the same result was seen when comparing the sigmoidal curves produced using the Ørskov & McDonald (1979) equation, as can be seen in figure 4 below:

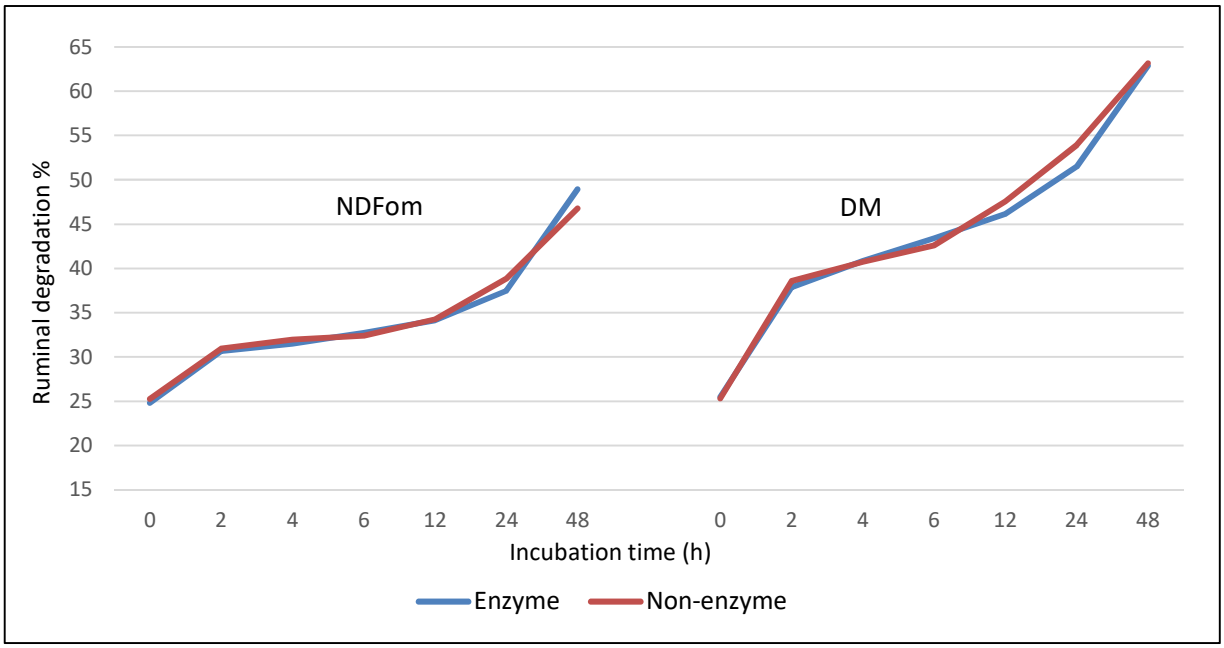


Figure 4: Overall enzyme vs. non-enzyme comparison of *in sacco* hominy chop $NDF_{om}$ and DM disappearance for the enzyme and non-enzyme treatments.

As can be seen from table 9, no significant differences were seen between hominy chop $NDF_{om}$ and DM ruminal degradation under the differing treatments. From figure 4, indications for both nutrients show that there were minimal differences in ruminal degradation between treatments, with resulting similar endpoints of degradation as well as similar rates of degradation.

Figure 5 illustrates the dry matter ruminal degradation for the incubation of hominy chop. The best representation of these results is expressed by means of comparisons of treatment level (enzyme or non-enzyme) over time. The 75HC:25M (high NDF) diet showed the largest degree of difference between the treatments, specifically at the 24h incubation timepoint, where a more defined lag-phase was seen. The lag phase is defined as the period during which no degradation, or a reduced level of degradation occurs and can be influenced by many factors such as the time it takes for rumen microflora to attach to feed, the composition of the feed consumed and the alteration of that feed for degradation (Mertens, 1977; Van Milgen *et al.*, 1991). Once again, the non-enzyme treatment had a higher endpoint of dry matter degradation. When comparing the 50M:50HC and low NDF diet (25HC:75M), no differences between treatments were shown.

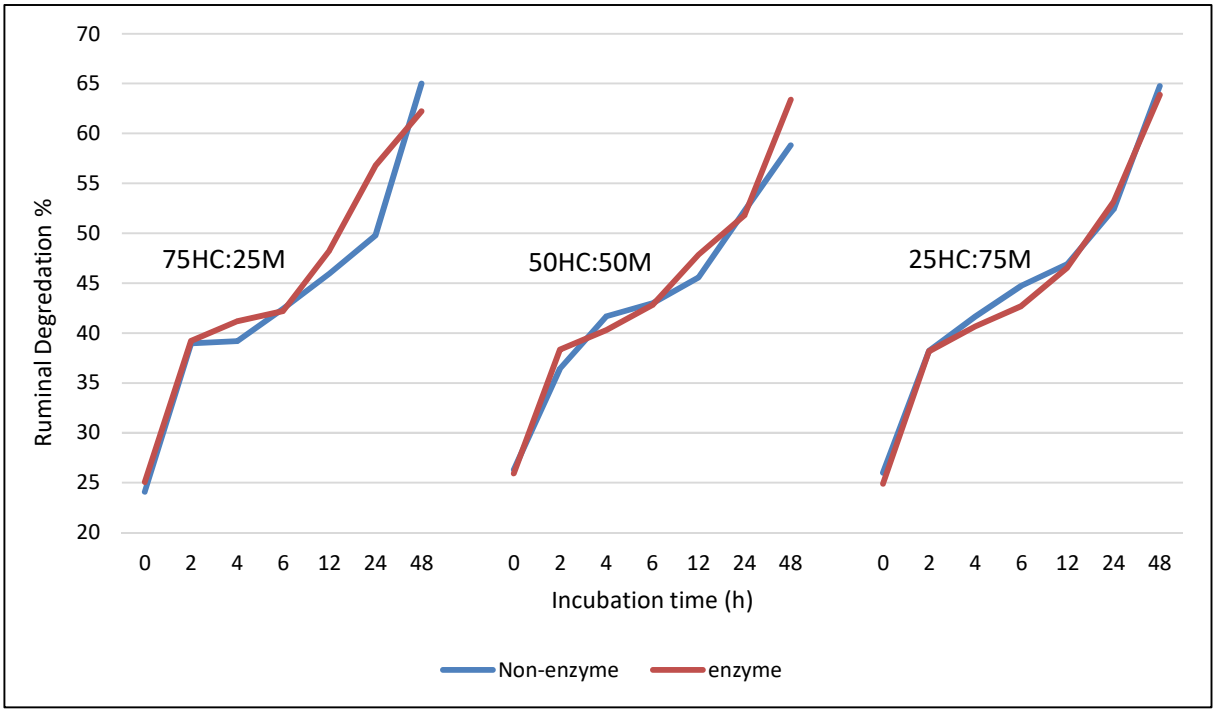


Figure 5: Enzyme vs. non-enzyme comparison of *in sacco* hominy chop DM ruminal degradation in diets differing in HC:M ratio.

It is possible that the steeper degradation curve shown for the enzyme treatment of the high fibre diet (75HC:25M) could be due to the following: Exogenous enzyme addition to the feed could aid in shortening the lag time via pre-ingestive attack of the fibrous fractions in the feed. This has been postulated by several authors who agree that this phenomenon aids degradation by release of soluble sugars through solubilization of NDF and ADF in the feed (Beauchemin *et al.*, 1996; Hristov *et al.*, 2008). The release of these sugars provides the energy as well as chemo-attractant needed by the rumen microflora for attachment once the feed is in the rumen (Hristov & Broderick, 1996; Gwayumba & Christensen, 1997; Krause *et al.*, 1998), increasing microbial colonization and proliferation, leading to reduced lag-time (Forsberg *et al.*, 2000).

Another possible reason could be the synergistic effect seen between exogenous enzymes and ruminal microorganisms (McAllister *et al.*, 1994a; Morgavi *et al.*, 2000a; Beauchemin *et al.*, 2004a). This synergism leads to an enhanced effect and therefore a net increase in the enzymatic activity in the rumen. The increased enzymatic capacity aids in increasing degradation rate, therefore aiding in reducing microbial lag-time. All these effects together increase the DM degradation of the feed.

Considering $NDF_{om}$ ruminal degradation of hominy chop, figure 6 illustrates the $NDF_{om}$ ruminal degradation of hominy chop under the enzyme and non-enzyme treatments:

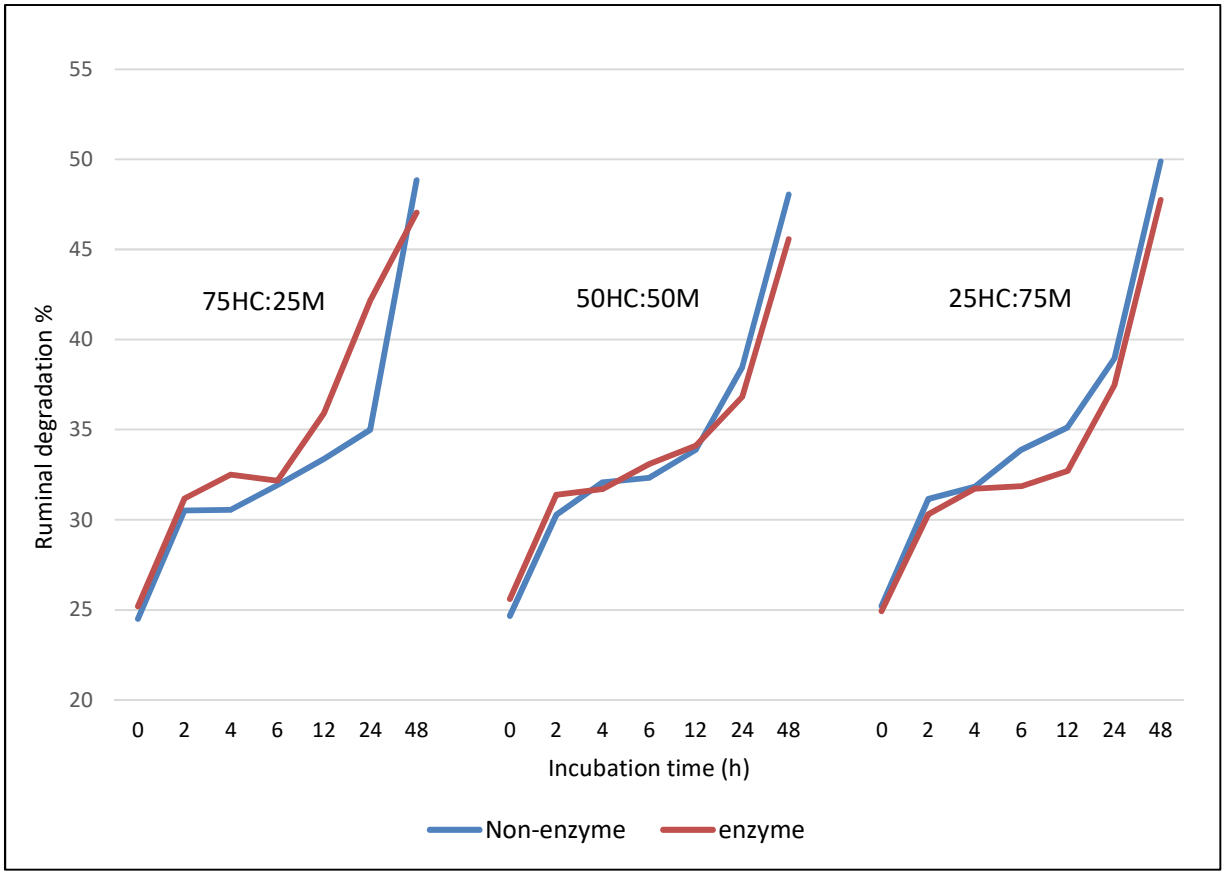


Figure 6: Enzyme vs. non-enzyme comparison for *in sacco* Hominy chop $NDF_{om}$ ruminal degradation in diets differing in HC:M ratio.

Figure 6 shows that under the high NDF diet (75HC:25M), a higher, more constant numerical rate of hominy chop $NDF_{om}$ digestion was observed between the 6 and 48-hour time points when treated with the exogenous fibrolytic enzyme, compared to the non-enzyme treatment, which resulted in a more defined lag-phase. Statistically, a significant difference was seen at the 24h time point of incubation. Interestingly, the endpoint of digestion was higher under non-enzyme conditions, and the rate of digestion for both treatments was equal for the first 2 hours after the onset of incubation.

Considering the *in sacco* ruminal degradation of hominy chop when incubated in the 50HC:50M diet, most aspects of the degradation curves for both treatments are similar, with a slightly higher endpoint of $NDF_{om}$ digestion for the non-enzyme treatment. No differences were observed for any of the time points when enzyme vs non-enzyme treatments were compared ($p>0.05$). The low NDF diet (25HC:75M) interestingly showed a larger degree of $NDF_{om}$ digestibility for hominy chop when not treated

with the fibrolytic enzyme. In addition to this, a higher rate of digestion was seen for the non-enzyme treatment, followed by a higher endpoint in $NDF_{om}$ digestion. Results suggest that there was a well-defined lag-phase between the 4 and 12 hour intervals for the enzyme treatment.

The same principles explained under the DM degradation of the incubated hominy chop, can be applied to explain the improved $NDF_{om}$ seen under the enzyme treatment conditions. A rumen environment that was beneficial toward increased $NDF_{om}$ digestion of hominy chop vs. the non-enzyme conditions was seen. It could be said that the increased digestibility of the high fibre diet due to fibrolytic enzyme addition (significant difference seen for apparent ADF total tract digestibility) lent toward beneficial conditions for a more constant rate of hominy chop digestion due to the ready supply of soluble sugars. Thus, the energy needed by the rumen microbes was readily available, aiding in enhancing digestion. The conditions created in the rumen furthermore shifted the rumen microbial population towards fibrolytic bacteria, rather than amylolytic bacteria.

This shift in rumen microbial population improves the chances for synergism between exogenous enzymes and rumen microbes, which as well creates a net increase in the enzymatic capacity of the rumen (McAllister *et al.*, 1994b; Morgavi *et al.*, 2000a; Beauchemin *et al.*, 2004a). Due to fibrolytic enzyme use and the available sugars acting as chemo-attractant, faster and more consistent attachment of microbes to the hominy chop substrate could have occurred (Cheng *et al.*, 1977; Forsberg *et al.*, 2000). It is, however, unclear why there were no differences at the earlier time points of incubation.

A clear phenomenon that can be seen from figure 6 is the decreased efficacy of the enzyme treatment when applied to the diet it is least suited to improving nutrient degradation, the low fibre diet (25HC:75M). The use of exogenous fibrolytic enzyme on maize based diets has been shown to not be effective in improving nutrient degradation. Beauchemin *et al.* (1997) tested the effects of an exogenous fibrolytic enzyme mixture on barley- and maize based diets in feedlot steers. He found no improvement in dietary degradation of the maize based diets.

4.2.2) The effect of exogenous fibrolytic enzyme on *in sacco* $NDF_{om}$ and DM digestibility of the TMR diets.

Once again, the equation of Ørskov & McDonald (1979) was used to determine DM and $aNDF_{om}$ kinetics for the TMR samples incubated under the different treatment conditions. The calculated "p-values" (rumen degradability of the nutrient at time "t") were compared and analysed using least square procedure (NLIN) (SAS, 2016). Overall, the addition of the fibrolytic enzyme did not have any effect on either the $NDF_{om}$ or DM ruminal degradation of the different TMR treatments. No significant differences for *in sacco* ruminal degradation was seen between the enzyme and non-enzyme treatments at the specific incubation time points, as indicated in table 10 and visually represented in figure7:

Table 10: *In sacco* $NDF_{om}$ and DM disappearance for the different TMR diets evaluated under enzyme and non-enzyme treatment conditions during the *in sacco* study.

| | TMR $NDF_{om}$ | | TMR DM | |
|---|---|---|---|---|
| Time (h) | Non-enzyme | Enzyme | Non-enzyme | Enzyme |
| 0 | 33.49 | 33.52 | 18.73 | 18.18 |
| 2 | 39.57 | 40.69 | 37.30 | 36.48 |
| 4 | 41.09 | 40.35 | 39.44 | 37.89 |
| 6 | 40.36 | 40.42 | 39.25 | 39.98 |
| 12 | 40.50 | 43.08 | 42.74 | 44.20 |
| 24 | 43.71 | 46.19 | 46.37 | 50.85 |
| 48 | 51.73 | 52.97 | 57.61 | 58.84 |

[a,b] Row means with the different superscripts differ ($P < 0.05$)

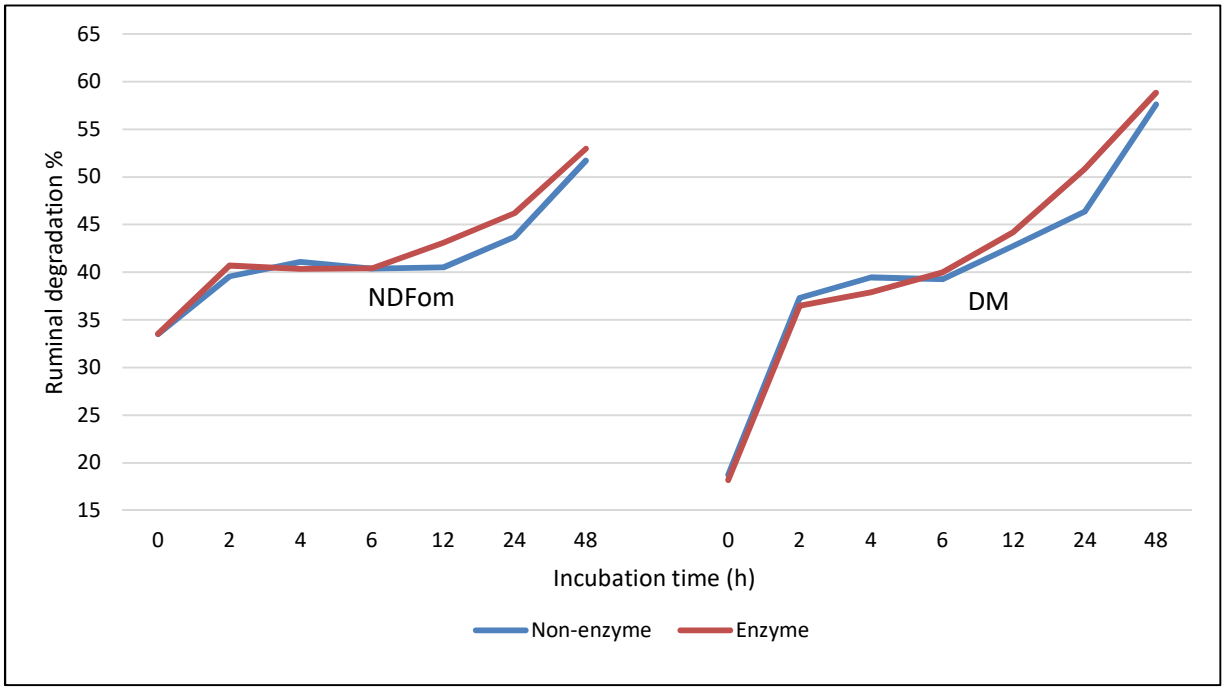


Figure 7: Overall enzyme vs. non-enzyme comparison of *in sacco* TMR $NDF_{om}$ and DM disappearance for the enzyme and non-enzyme treatments.

Larger differences were seen for the $NDF_{om}$ and DM digestion of the TMR incubated feed than the hominy chop incubated feed. When considering the effect of fibrolytic enzyme treatment on the DM ruminal degradation of the different TMR feeds incubated as indicated in figure 8, the following can be seen:

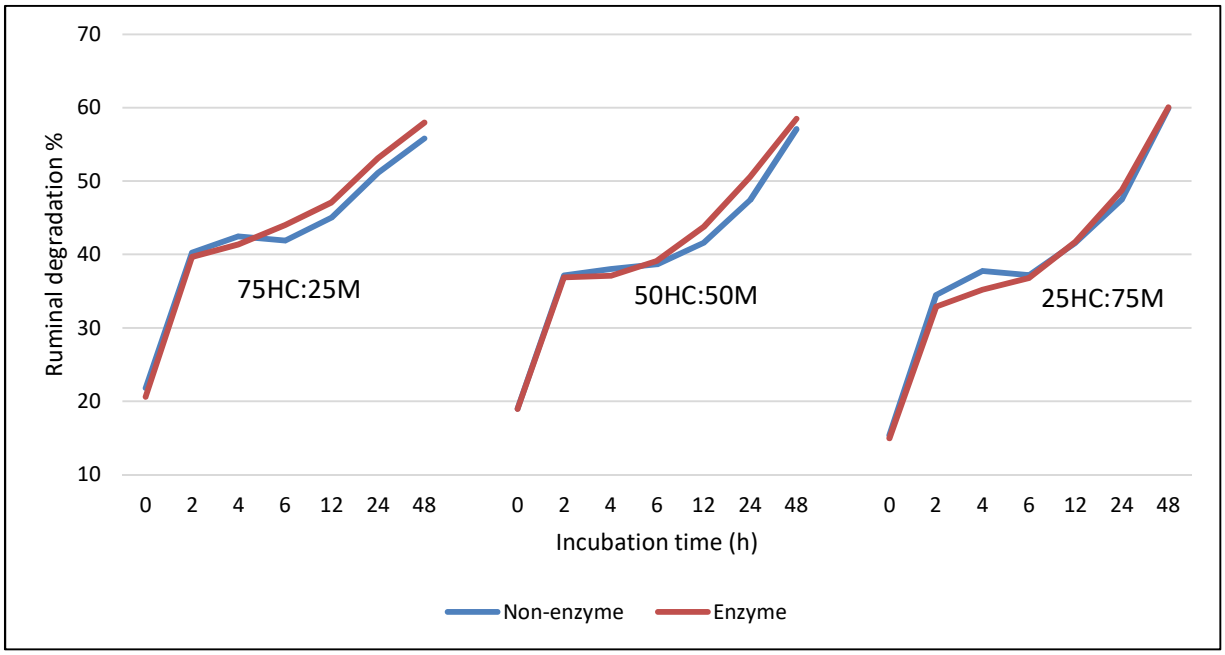


Figure 8: Enzyme vs. non-enzyme comparison *in sacco* TMR DM ruminal degradation for the diets differing in HC:M ratio

Considering the ruminal DM degradation of the TMR feeds as seen in figure 8 above, the degradation curves display as one would expect diets differing in digestibility would, with the high fibre diet (75HC:25M) showing the lowest degree of degradability and the high starch diet (25HC:75M) showing the highest degree of degradability. Noticeable is the differing starting points of digestion and the differing endpoints of digestion. The inherent digestibility of a diet is influenced by the fibre (lignin, NDF and ADF) content of that feed, where a higher fibre content lowers digestibility as well as passage rate of the feed (Jung & Allen, 1995).

Each diet follows the same shape of degradation curve, resulting in similar amounts of DM degradation at 48 hours of incubation. Comparing the diets on their own, the most promising trend could be seen for the high fibre diet (75HC:25M), with the enzyme treatment outperforming the non-enzyme treatment. The 50HC:50M diet initially showed a steeper degradation curve, after which the non-enzyme treatment caught up towards the 48-hour mark. No differences are shown for the low fibre (25HC:75M) diet. A noticeable similarity is the sharp rate of dry matter disappearance seen between 0 and 2 hours of incubation.

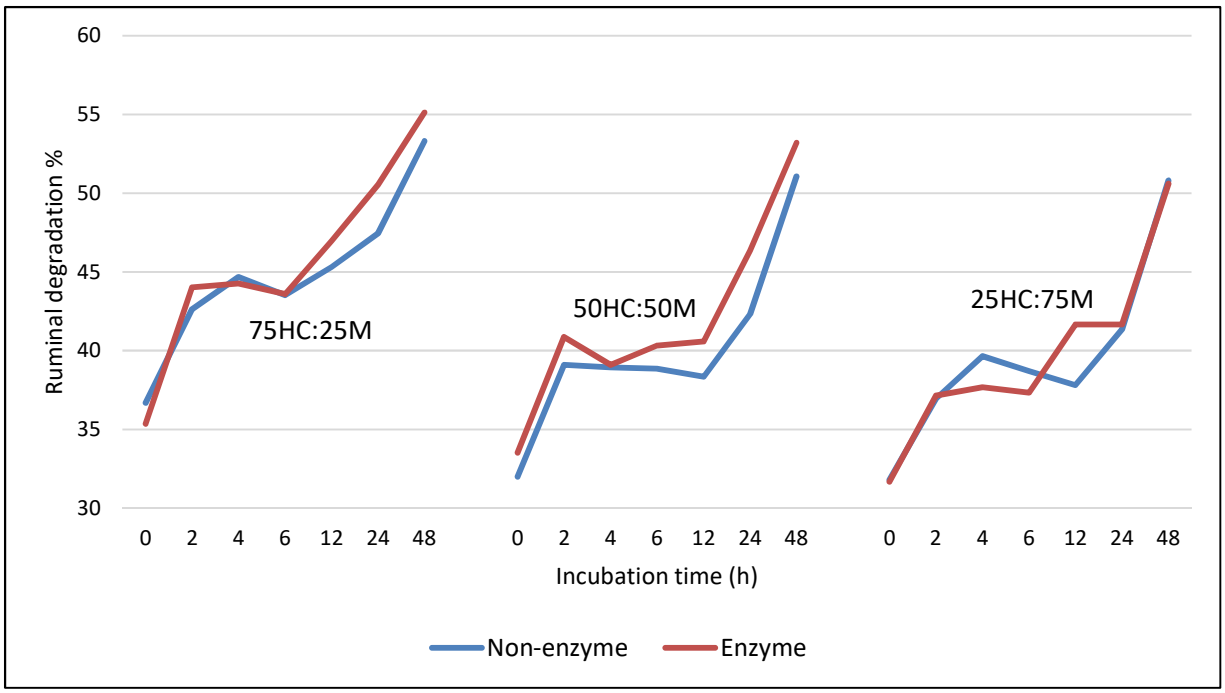


Figure 9: Enzyme vs. non-enzyme comparison of *in sacco* TMR $NDF_{om}$ degradation of diets differing in HC:M ratio.

Figure 9 indicates a larger degree of *in sacco* digestibility for the high NDF diet (75HC:25M), both under enzyme and non-enzyme conditions. A larger degree of NDF digestibility would be expected with the high NDF diet, as was seen in the digestibility study. The high NDF diets shows the same length of lag-phase between the enzyme and non-enzyme treatment (2 – 6-hour incubation time points), followed by a more prominent increase in $NDF_{om}$ digestibility under the enzyme conditions. This was not a significant effect, but a positive trend in improved $NDF_{om}$ digestibility due to enzyme supplementation. The explanations for this could be the synergistic effect seen when exogenous fibrolytic enzymes are supplemented, assisting and aiding the inherent ruminal enzymes in digestion (McAllister *et al.*, 1994b; Morgavi *et al.*, 2000a; Beauchemin *et al.*, 2004a).

Considering the low NDF diet (25HC:75M), no difference could be seen in terms of endpoint of digestion, as would be expected. The addition of exogenous fibrolytic enzyme did however result in a shortening of the lag time in comparison to the non-enzyme treatment. This was not significant, but showed a positive trend. This could relate to pre-ingestive attack by the exogenous enzymes as explained by Beauchemin *et al.* (2004a). In summary, it can be said that higher end points of digestion can be seen with the enzyme supplementation, with the effect decreasing as the diets decrease in NDF content and increase in starch content.

In a real-time Polymerase chain reaction study performed by Tajima *et al.* (2001), it was proven that fibrolytic bacteria species such as *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* are prominent when exposed to high fibre diets and will decline in numbers with a decrease in dietary fibre. Leedle *et al.* (1982) explains that xylan-xylose and pectin bacterial subgroups respectively comprise half and a third of the total bacterial population under high-forage conditions. These groups, together with cellulolytic bacteria, declined under high-concentrate dietary conditions, while soluble carbohydrate bacteria were present regardless of diet type.

The fact that these soluble carbohydrate bacteria are present, could as well indicate why the shortened lag time was seen under the enzyme conditions of the low fibre diet (25HC:75M). The bacteria already are present and able to use the soluble sugars released during the pre-ingestive attack phase brought on by the addition of the exogenous enzymes (Hristov & Broderick, 1996; Gwayumba & Christensen, 1997; Krause *et al.*, 1998), and therefore result in the shortened lag time.

4.3) The effect on rumen fermentation patterns

During ruminal fermentation, the end goal is the adequate degradation of plant polysaccharides to sources of energy and peptides that can be used by the animal for growth and production. These sources of energy predominantly reside in the volatile fatty acid pools produced during ruminal fermentation by the ruminal microbes. When comparing the overall effect of enzyme vs non-enzyme treatment supplementation, irrespective of dietary TMR, no differences were seen between treatments for the pH, Ammonia-nitrogen and volatile fatty acids measured in the rumen fluid samples taken, as can be seen in table 11:

Table 11: Overall enzyme vs non-enzyme comparison for rumen parameters measured *in vivo*.

| Parameters | Enzyme | Non-enzyme | SE |
|---|---|---|---|
| · pH | 6.03 | 6.09 | 0.028 |
| · Propionate (mmol/L) | 25.31 | 24.55 | 0.002 |
| · Acetate (mmol/L) | 50.65 | 54.12 | 0.003 |
| · Butyrate (mmol/L) | 4.73 | 5.02 | 0.0003 |
| · NH3-N (mg/dL) | 8.79 | 8.91 | 0.32 |

[ab] Row means with different superscripts differ ($P < 0.05$).

These results reiterate the fact that exogenous enzyme supplementation will not necessarily be beneficial in terms of improving feed degradation or increased nutritional contributions by the diets fed. The enzyme used must carefully be considered together with the composition of the diet as well as the desired outcomes (Tirado-González *et al.*, 2018). For this reason, it was decided to compare parameter results between the enzyme and non-enzyme treatments of the different TMR diets tested, as indicated in table 12 below.

Table 12: Effect of enzyme supplementation on rumen fermentation parameters irrespective of TMR ratio of HC:M.

| Parameter | Enzyme | Non-enzyme | SE |
|---|---|---|---|
| | 75HC:25M | | |
| · pH | 6.08 | 6.08 | 0.046* |
| · Propionate (mmol/L) | 19.19[1] | 23.78 | 0.003* |
| · Acetate (mmol/L) | 44.35 | 56.04 | 0.005* |
| · Butyrate (mmol/L) | 4.27 | 5.43 | 0.0006* |
| · Total VFA production (mmol/L) | 67.81 | 85.25 | |
| · NH3-N (mg/dL) | 8.02[1] | 7.87[1] | 0.55* |
| | 50HC:50M | | |
| · pH | 6.03 | 6.12 | |
| · Propionate (mmol/L) | 26.53[1,2] | 22.36 | |
| · Acetate (mmol/L) | 54.59 | 49.99 | |
| · Butyrate (mmol/L) | 5.32 | 4.75 | |
| · Total VFA production (mmol/L) | 86.44 | 77.1 | |
| · NH3-N (mg/dL) | 7.60[1] | 9.09[1,2] | |
| | 25HC:75M | | |
| · pH | 5.97 | 6.06 | |
| · Propionate (mmol/L) | 30.22[2] | 27.49 | |
| · Acetate (mmol/L) | 53.01 | 56.34 | |
| · Butyrate (mmol/L) | 4.6 | 4.88 | |
| · Total VFA production (mmol/L) | 87.83 | 88.71 | |
| · NH3-N (mg/dL) | 10.76[2] | 9.80[2] | |

[a, b] Row means with different superscripts differ ($P < 0.05$).
[1, 2] Column means for the same parameter between dietary conditions with different superscripts differ ($P < 0.05$).
* Applies to all parameters measured in the table.

No differences were observed for pH between treatments for the individual experimental TMR's tested, with lower numerical pH ranges were seen under the enzyme treatment compared to the non-enzyme treatment, while the lowest pH average was observed for the 25HC:75M diet for the enzyme treatment (5.97). There were no effects on total VFA production or differences between individual VFA's measured for the same TMR diet. Significant differences were seen between the different diets on the enzyme treatment, where the 75HC:25M diets differed significantly from the 25HC:75M diet. The 50HC:50M diet did not differ from the 75HC:25M or 25HC:75M diets.

If we concentrate on the high fibre diet (75HC:25M), the expected outcomes can be seen in terms of volatile fatty acid production, with a larger proportion of acetic acid being produced in comparison to propionic acid. The opposite is seen with the high starch diet (25HC:75M), where a larger proportion of propionic acid was produced in relation to acetic acid. This is in agreement with Ferraretto (2017), who states in a paper discussing dietary starch content and the impact on diet digestibility, that concentrate diets, or those that are higher in starch content, produce a higher degree of propionic acid, while fibrous diets produce a higher degree of acetic acid. Furthermore, a decrease in NDF digestibility is expected when the starch content of a diet increases, due to a decrease in rumen pH because of more rapid fermentation of starch. Ferraretto *et al.* (2013) reported in a study of cereal grain types and digestion, that as much as a 0.61% decrease in NDF digestibility can be seen in ruminant animals per percentage-unit increase in dietary starch content.

A good indicator of the conditions within the rumen could be rumen pH (Nagaraja & Titgemeyer, 2007). Overall, the pH levels under the enzyme treatment conditions were seen as lower than the non-enzyme treatments (6.03 vs. 6.09), but these differences were not significant (P>0.05) (table 12). Several authors agree that optimal rumen function in terms of digestive enzymes occurs above a pH of 6.2 (Matte & Forsberg, 1992; Colombatto *et al.*, 2007), although exogenous enzymes are supposed to aid in digestion when rumen pH is not optimal (Russell, 2002).

Below follows a chart indicating the measured pH levels for each dietary condition and treatment at specific time intervals. As seen from the chart, it was clear that the rumen pH declined below the optimal level for ruminal digestion from as early as 1 hour after the first feeding time at 08h00.

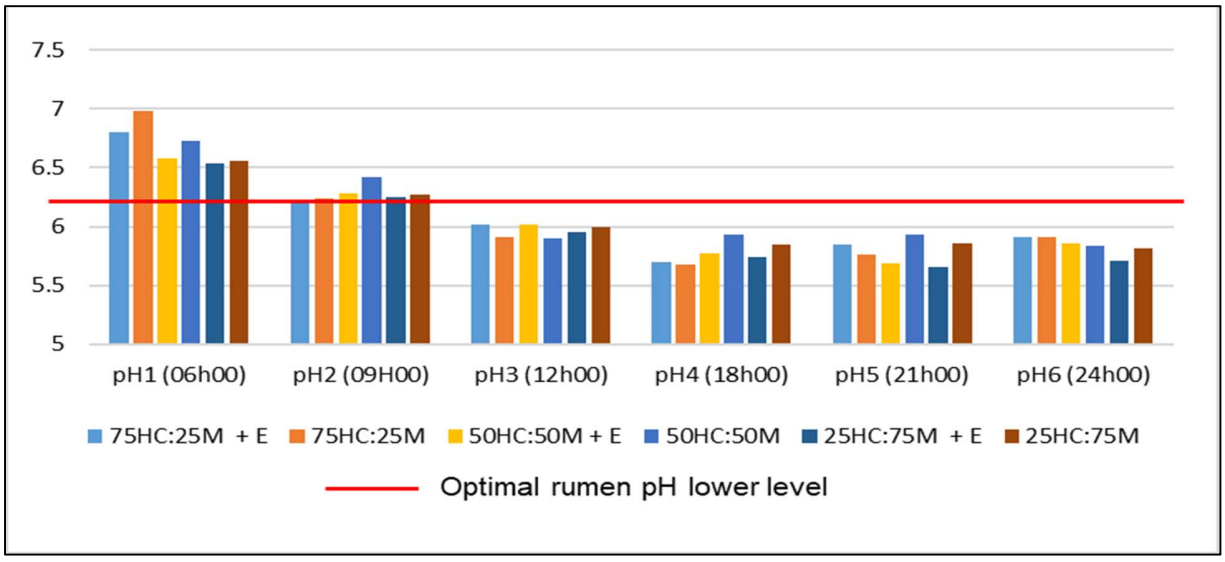


Figure 10: Rumen pH levels measured from rumen fluid sampes taken at specific timepoints during the rumen fermentation study.

As seen from the chart, the rumen pH declines below the optimal level for ruminal digestion from as early as 1 hour after the first feeding time at 08h00. Therefore, we can agree that conditions were not suited towards adequate rumen function and that instances where increased rumen volatile fatty acid production was seen under exogenous enzyme treatment, proves that exogenous enzymes can aid in digestion in cases where rumen pH is not optimal for digestion (Russell, 2002).

It seems that overall, the exogenous enzyme treatment of the high starch diet (25HC:75M), resulted in the best outcome of VFA production and rumen ammonia-nitrogen production between all the diets tested. The lowest rumen pH was seen on this treatment. A possible explanation for the lower rumen pH, could be the more rapid production of VFA's, which being acids, reduce the rumen pH until adequate absorption in the rumen occurs. Lewis *et al.* (1996) demonstrated in an exogenous fibrolytic enzyme trial, that lower rumen pH levels were seen together with higher VFA levels after feeding. In another exogenous enzyme trial performed by Hristov *et al.* (2000), supplementing increasing levels of exogenous polysaccharide degrading enzyme, rumen pH was seen to decrease with positive effects on nutrient digestibility and rumen ammonia-nitrogen. It is as well possible the higher level of volatile fatty acids seen under the enzyme treatment of the high starch diet could be due to the VFA's not being absorbed as rapidly from the rumen through the rumen epithelium. Dijkstra (1994) reports that the fractional absorption rates of volatile fatty acids are reduced with an increase in rumen pH, and that the assumption that volatile fatty acid absorption is directly proportional to concentration in rumen fluid, is false. Therefore, larger amounts of volatile fatty acids where present when the samples were taken at the comparable time points to the other treatments.

There has been very little research done where exogenous fibrolytic enzymes have been tested on high starch dairy diets and even less on high starch feedlot diets. This could be due to a number of reasons, one being that the expected increase in dietary digestibility is not expected as would be with exogenous amylolytic enzymes.

Ammonia-nitrogen measured during the trial, showed significant differences between diets on the same treatment, but not between the same TMR diet treated under enzyme and non-enzyme conditions. Considering the enzyme treatment, the 25HC:75M diet differed significantly from the 75HC:25M and the 50HC:50M diets (10.76 mg/dL vs. 8.02 mg/dL and 7.60 mg/dL respectively). The non-enzyme treatment of the differing diets showed significant differences between the 75HC:25M and the 25HC:75M diet. The 50HC:50M diet did not differ significantly from the others for the non-enzyme treatment.

For the production of microbial protein in the rumen, ammonia is seen as a key metabolite and literature reports that 60 – 80% of rumen ammonia is incorporated into microbial protein (Pisulewski *et al.*, 1981). Rumen microbial protein production is optimal when the pH conditions are above 6.2 and there is enough energy available for use by the rumen microbes for protein synthesis, mainly from the fermentation of available dietary starch (Ferraretto, 2017) and a tendency for amylolytic bacteria to be more proteolytic in comparison to cellulolytic bacteria (Siddons & Paradine, 1981; Wallace *et al.*, 1997).

Many plant proteins are trapped within the fibre matrix that makes up the plant cell wall and therefor it has been found that NDF-bound proteins are only degraded by proteolytic bacteria once cellulolytic bacteria have depolymerized cellulose. We can see from the results indicated in table 12 that the enzyme treatment showed a trend in improving the ammonia-nitrogen production for the high fibre and high starch diets. Considering the rumen parameters measured in a trial performed by Álvarez *et al.* (2009) studying the effects of exogenous fibrolytic enzymes in beef steers, higher rumen pH values were seen together with improved crude protein solubility values (higher ammonium-nitrogen values).

## Conclusion

The study of exogenous enzyme effects is a broad and varied field, even more so when specifically looking at rumen parameters.

Regarding improvement in digestibility, significant differences were seen overall for ADF and NDF under the enzyme conditions, although these effects were not replicated for the *in sacco* study focused on dry matter and NDF disappearance. An improved rate of dry matter and NDF disappearance was apparent for both the TMR and hominy chop samples incubated during the *in sacco* study, resulting increased efficiency of feed utilization. Synergism between rumen microbial enzymes and compounding enzyme effects could be the result due to a stable enzyme-substrate complex formed. This could be an indication of an advantage using and exogenous enzyme vs. using none, as improved feed efficiency definitely is an attribute sought after in feedlot rearing.

No significant differences were noted between the enzyme and non-enzyme treatment regarding the rumen parameters measured during the rumen fermentation study. It could be noted that significant differences were seen between the same parameters measured on the enzyme treatment, specifically for propionate and ammonia-nitrogen. Propionate production was the highest on the high starch diet (25HC:75M), while ammonia-nitrogen production followed suit and was the highest on the high starch diet (25HC:75M). Both these results are beneficial in terms of energy for growth and substrate for microbial protein production in the rumen of the animal. Therefor it can be concluded that the application of an exogenous fibrolytic enzyme for the purpose of improving feed utilization, may it be ‘n ration higher in fibre due to the substitution of maize for hominy chop or higher in starch due to the inclusion of maize, could be beneficial.

It is important to note that animal responses are greatest when fibre digestion is compromised and when energy is the first-limiting nutrient in the diet. A number of reasons can be brought forward to explain the variable responses seen in exogenous enzyme trials as seen across the many trials performed. Experimental conditions resulting in energy not being the limiting nutrient, the differing enzyme activities and characteristics, incorrect supplementation of exogenous enzyme product (over- or under supplementation) and the incorrect method of enzyme application to animal or substrate could all be factors contributing to conflicting results between enzyme studies (Beauchemin *et al.*, 2003).

## Critical evaluation

A few points would have to be addressed and done differently if this research trial would be recreated. The main concern was the fact that the experimental animals were not of the same body weight at the onset of the trial. It would have been more suitable to rear a new batch of steers to the same body weight, freshly cannulate them and then proceed with the trial from there. This would have aided in determining the feeding level used per animal throughout the treatments easier, to ensure that the prescribed amount of enzyme was administered. This was a challenge due to financial and time constraints.

A second point worth mentioning would be the use of a different marker for the total tract digestion study. During the trial, complications in the dosing of the planned chromium oxide were encountered and it was decided to use an internal marker, calculated via the acid insoluble ash procedure, instead. Had the experimental animals been new animals, the incidence of bloating due to acidosis would have been less, making the dosing of the chromium oxide via the rumen cannula easier. The animals simply had been exposed to too many incidences of acidosis prior to this experimental trial. In some instances, chromium oxide was lost due to rumen content spilling out of the cannula when opening the rumen. I believe the adaptation period was adequate, as several studies suggested and agreed 14 days was enough.

Other than that, the experimental trial proceeded well and was a worthwhile experience. it was a privilege to be able to work with the animals, knowing I could possibly make a worthwhile contribution.

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Addendum 1 – Ethical clearance for use of research animals:

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

# Animal Ethics Committee

| | |
|---|---|
| PROJECT TITLE | **Effect of exogenous fibrolytic enzyme supplementation on rumen fermentation, in sacco NDF disappearance and total tract digestibility of feedlot steers fed hominy feed/maize based diets** |
| PROJECT NUMBER | **EC060-15** |
| RESEARCHER/PRINCIPAL INVESTIGATOR | **GR Galetti** |

| | |
|---|---|
| STUDENT NUMBER (where applicable) | **UP_ 11022002** |
| DISSERTATION/THESIS SUBMITTED FOR | **MSc** |

| | | |
|---|---|---|
| ANIMAL SPECIES | **Bovine** | |
| NUMBER OF ANIMALS | **6** | |
| Approval period to use animals for research/testing purposes | | **1 August 2015 – 1 August 2016** |
| SUPERVISOR | **Prof. LJ Erasmus** | |

*KINDLY NOTE:*

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

| | |
|---|---|
| **APPROVED** | Date 27 July 2015 |
| CHAIRMAN: UP Animal Ethics Committee | Signature |

*S4285-15*