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Digesta markers for evaluating the effect of exogenous enzymes on nutrient digestibility in beef feedlot cattle

By

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Submitted in a partial fulfilment of the requirements for the degree MSc (Agric):
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Declaration

I, Anna Maria van Wyk, declare that this dissertation, which I hereby submit for the degree MSc (Agric) Animal Science (Animal Nutrition) at the University of Pretoria is my own work and that it has not been previously submitted by me for a degree at this or any other tertiary institution.

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A.M. van Wyk

Pretoria

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Summary

Digesta markers for evaluating the effect of exogenous enzymes on nutrient digestibility in beef feedlot cattle

Supervisor: Prof. L.J. Erasmus

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Degree: MSc (Agric) Animal Science (Animal Nutrition)

The Food and Agriculture Organization (FAO) stated that it is vital to intensify animal and feed production in a sustainable manner. Producers are therefore increasing focus on methods to improve production efficiency. The use of exogenous enzymes in ruminant diets can improve production efficiency while also reducing waste products, thereby contributing to more sustainable food production. There are many combinations of enzymes that first have to be screened through *in vitro* methods, thereby selecting a number of enzyme combinations with the highest potential effectiveness. Ultimately, however, the best combinations need to be validated using *in vivo* digestibility studies. Ruminants, in general, have variable responses to supplementation with exogenous enzymes and the animal response to different enzymes can be influenced by the type of enzyme or combinations used as well as the experimental conditions. The *in vivo* validation of prototype enzymes by means of digestibility studies is therefore of utmost importance before commercialisation of a product.

To measure total tract digestibility the total faecal collection method is normally used. Total faecal collection is, however, labour intensive because all the faeces produced in a

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period of time must be collected and the animals must be confined to individual housing which may disturb the animals. Marker-based methods are less labour intensive are an attractive alternative to the total faecal collection technique. Before any marker can be used in digestibility studies, it should be validated to confirm the suitability of the specific marker in a specific diet.

The aim of this study was first to evaluate the effect of different enzyme prototypes on feedlot diet digestibility and secondly to validate different markers as an alternative to total faecal collection in digestibility studies using high maize feedlot diets.

Six ruminally cannulated steers were used to evaluate the apparent total tract nutrient digestibility using two different enzyme prototypes against a control diet. The steers were fed twice daily and received either the control diet or a control diet supplemented with either enzyme A or enzyme B. Three different markers, chromic (III) oxide (Cr_2O_3), acid insoluble ash (AIA) and acid detergent lignin (ADL) were validated against total faecal collection to determine which marker is most suitable in digestibility studies for feedlot diets containing high levels of maize (60%) and low levels of roughage (20%).

The mean apparent total tract digestibility (TTD) for dry matter (DM), starch and crude protein (CP) showed no difference ($P>0.05$) between the control diet and diets supplemented with enzyme A or enzyme B. The neutral detergent fibre (NDF) digestibility did show a difference ($P<0.05$) between the control diet and the diet supplemented with enzyme B. However there were no differences ($P>0.05$) between the control diet and the diet supplemented with enzyme A or between the two diets supplemented with the enzymes A and B. Acid insoluble ash and Cr_2O_3 showed no difference ($P>0.05$) between treatments in its ability to predict digestibility when compared to total faecal collection. Acid detergent lignin predictions differed ($P<0.05$)

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from total collection and is probably unsuitable to be used as a marker in high maize feedlot diets.

The use of enzymes containing xylanase, amylase and protease had no effect on nutrient total tract digestibility in our study; however, the enzyme-containing xylanase and β-glucanase had an effect on apparent total tract NDF digestibility. Both AIA and Cr_2O_3 can be used as markers to determine apparent total tract nutrient digestibilities in feedlot diets with a high maize content based on a comparison with the total faecal collection. Acid detergent lignin appears to be a poor marker in high concentrate feedlot diets due to the low ADL content of the diet.

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List of abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
ADL	Acid detergent lignin
AIA	Acid insoluble ash
ASS	Atomic absorption spectrophotometry
Ca	Calcium
Ca:P ratio	Calcium to phosphorus ratio
Cl	Chloride
cm	Centimetre
Co-EDTA	Cobalt ethylenediamine tetraacetic acid
CP	Crude protein
Cr_2O_3	Chromium (III) oxide
°C	Degree Celsius
DM	Dry matter
DMI	Dry matter intake
EE	Ether extract
FAO	Food and Agriculture Organization of the United Nations
FO	Faecal output
g	Gram
GIT	Gastro intestinal tract
h	Hour
HCl	Hydrochloric acid
HPC	High protein concentrate
H_2SO_4	Sulphuric acid
iADF	Indigestible acid detergent fibre

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ICP-ES	Inductively coupled plasma emission spectrometry
Kg	Kilogram
KL	Klason lignin
$KMnO_4$	Permanganate
ME	Metabolisable energy
mg	Milligram
MJ	Mega joule
ml	Millilitre
Na	Sodium
NDF	Neutral detergent fibre
NFE	Nitrogen free extract
nm	Nanometre
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
OM	Organic matter
P	Phosphorus
TC	Total collection
TMR	Total mixed ration
TTD	Total tract digestibility

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Chapter 1

Introduction

Livestock production is under the spotlight since consumers became aware of climate change and greenhouse gas emissions (Meissner *et al*., 2013). Consumers demand animal products that are produced in a sustainable manner (Capper, 2013) while producers are faced with the reality that the human population is growing significantly, increasing the demand for food production (Roughgarden, 1979). It is estimated that the human population would approach a total of 9.7 billion people by the year 2050 and producers are therefore under pressure to increase production while taking economic, environmental and social sustainability into consideration (Erasmus and Webb, 2013).

It is vital to intensify production in a sustainable manner in order to alleviate the environmental impact of livestock production (Steinfeld *et al*., 2006). Sustainability is a dynamic process (Capper, 2013) and the main objectives of food producers must be to meet the current demand for food without compromising the ability of future generations to produce food (Brundtland, 1987). Livestock producers, therefore, have to look at the efficiency of nutrient utilisation which will have the benefits of improved production and reduced environmental impact (Capper, 2013). The ruminant production (red meat) sector is especially criticised when compared to the monogastric (white meat) sector because of its lower efficiency and higher enteric methane emission. Du Toit *et al*. (2013) estimated that beef cattle contribute up to 89.4 kg methane per head per year. Feedlots specifically are under scrutiny to improve efficiency and reduce greenhouse gas emissions (Capper, 2013).

Production efficiency can be improved through various methods like increased intake, improving nutrient content of feed and by improving the digestibility of feed (Owens

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and Soderlund, 2006). The greatest improvement in production efficiency over the last few years was through the application of new technologies like feed additives to manipulate the above mentioned three factors.

Simmons (2009) stated that 70% of the increase in food production by 2050 should be from the use of efficiency-enhancing technologies. Examples of efficiency-enhancing technologies include the use of bovine somatotropin, processing methods to improve digestibility and availability of nutrients and the use of feed additives. Examples of feed additives are exogenous feed enzymes, yeast products, direct fed microbial products and phytonutrients. Another method used is genetic selection through the use of breeding values to improve feed conversion ratios and increased growth rates.

The adoption of some of these efficiency-enhancing technologies has been slow as the cost of the product outweigh that of other growth enhancing additives such as growth hormone implants (Meale *et al*., 2014). In addition, sustainability is an important global topic and natural feed additive alternatives are challenged to maintain current performance while also reducing the environmental footprint. Limited research exists on alternative feed additives such as enzymes in feedlot type diets and more research is needed on the effect of enzymes on beef production sustainability (Jennings and Wagner, 2014).

When using this efficiency-enhancing technologies or feed additives, it is necessary to quantify and validate the improved efficiency using in vivo methods that are reliable and repeatable. Measurements of digestibility by means of total faecal collection is still considered to be the golden standard to which other methods should be compared to (Marais, 2000), however, it is time-consuming and labour intensive. It is important to consider other methods as alternatives to total faecal collection to save time and labour.

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The use of external or internal marker-based methods has great potential, but the accuracy of the marker varies between diets (Cochran *et al*., 1986; Thonney *et al*., 1985). Limited information is available on which markers are most accurate for estimating digestibility in high concentrate diets for example feedlot diets with 20% or less roughage.

The aim of our study was to evaluate and validate different markers techniques against the total faecal collection technique to quantify apparent total tract nutrient digestibility of high concentrate feedlot diets that were supplemented enzyme combinations. It is envisaged that the outcome of this project would provide some guidelines on which marker to use in high maize feedlot diet digestibility experiments as an alternative to the labour intensive total faecal collection technique.

In the next chapter, a literature review on the use of markers in digestibility studies and enzyme combinations that was used in the study is presented. In the chapter thereafter the two enzyme combinations were compared to a control diet in terms of nutrient digestibility using total faecal collection. It is then be followed by a chapter where the different markers were validated against total faecal collection in digestibility studies with the two enzymes.

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Chapter 2

Literature Review

2.1 Enzymes: Mode of action, types and application

2.1.1 Introduction

The increasing demand for hormone and antibiotic free meat creates opportunities for alternative technology to improve growth and produce animal products more efficiently (Webb & Erasmus, 2013). Producers are trying to maximise productivity of feedlot cattle by focussing on more efficient feed digestion because feed accounts for approximately 70% of the total cost of production (Wang *et al*., 2003). Current methods to improve feed digestion focus on improving ruminal fermentation through alterations of the diet or the use of feed additives, alterations of animal behaviour or alterations of microbial populations that carry out fermentation (Nagaraja *et al*., 1997). Ionophores are most commonly used to alter the ruminal microbial population. This in conjunction with growth hormone implants is the most cost-effective ways to improve the average daily gain (ADG) and feed efficiency of feedlot cattle (Wang *et al*., 2003). Nutritionists started investigating the use of natural alternatives such as exogenous enzymes to replace ionophores antibiotics and hormone treatments. Since the supplementation of exogenous enzymes showed potential and positive effects to improve productivity in the poultry industry (Gearheart *et al*., 1996) as well as in the pig industry (Kiarie *et al*., 2012), a number of studies were initiated to investigate the potential of exogenous enzymes in ruminant diets.

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2.1.2 Production of exogenous enzymes

Exogenous enzymes are produced by a process called batch fermentation (Beauchemin *et al*., 2004). The process begins with a seed culture and growth media that typically contain carbohydrates, minerals, etc. When fermentation is complete (three to five days depending on the microorganism and conditions), the enzyme that is produced is separated from the fermentation residues as well as the source organism. The enzyme activity and characteristics can vary widely depending on the source organism used (Beauchemin *et al*., 2004). The source organisms can originate from bacteria or fungi were the most common bacterial strains used include *Bacillus subtillus*, *Lactobacillus acidophillus, Lactobacillus plantarium* or *Streptococcus faecium*. The most used fungal trains include *Aspergillus oryzae, Trichoderma reesei* and *Saccharomyces cerevisiae* (McAllister *et al*., 2001). Enzyme products do not contain live cells and are relatively purified and concentrated, thus having a very specific enzyme activity (Beauchemin *et al*., 2004).

2.1.3 Exogenous enzymes mode of action

Exogenous enzymes can improve the diet digestibility by acting pre-ingestive, ruminally or post-consumptive.

a) Pre-ingestive

Beauchemin *et al*. (2003a) reported that exogenous enzymes are most effective when applied prior to ingestion. Applying exogenous enzymes to feed prior to consumption may aid in releasing sugars from feedstuffs prior to consumption (Beauchemin & Rode, 1996). The released soluble sugars can provide additional available carbohydrates to microbes to stimulate microbial growth thus decreasing the lag time required for

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microbial colonisation (Forsberg *et al*., 2000) (Figure 1). However this quantity of soluble sugars released only represent a small portion of the total carbohydrates present in the diet, so it is unknown if the additional carbohydrates supplied to rumen microbes is significant (Beauchemin *et al*., 2003a).

Exogenous enzyme additives will only degrade substances that would naturally be digested by the endogenous enzymes of the rumen microbes (McAllister *et al*., 2001). The addition of exogenous enzymes prior to ingestion may act by starting digestion outside the animal. McAllister *et al*. (2001) showed that when applying a high concentration of an exogenous enzyme containing xylanase and cellulase to barley straw prior to ingestion; it clearly caused degradation of the barley straw (Figure 2). They did, however, not see the same effect on the barley straw when applying lower concentrations of the same exogenous enzyme. Pre-ingestive application of exogenous enzymes will help digestion as the limited colonisation of ruminal microbes on cell walls is a constraint to digestion and prior erosion to the cell wall may help to improve penetration of cellulolytic microbes. Prior application also allows the enzyme to bind to the feed to increase its resistance to proteolysis in the rumen (Beauchemin *et al*., 2003a).

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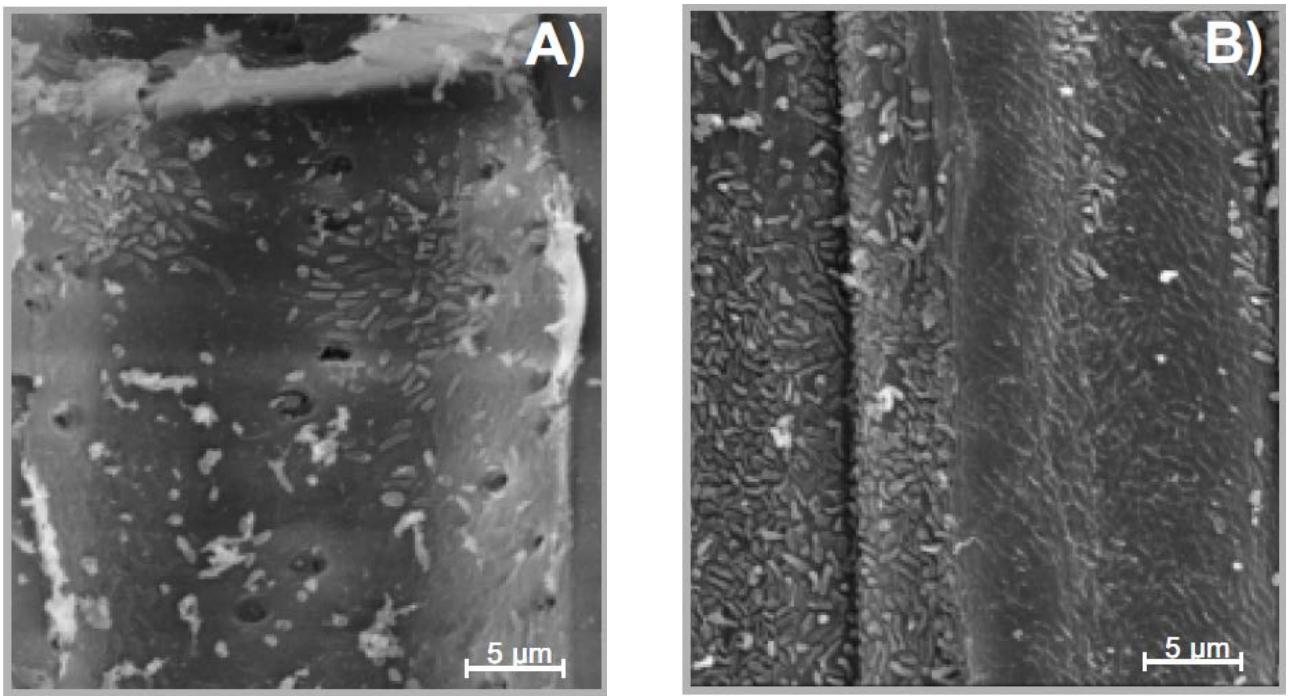

Figure 1: Electron micrograph scan taken 24 h after incubation on *Fibrobacter succinogenes* on maize fibre showing the difference between the control with no enzyme (A) and the treated fibre (B) (Adapted from Morgavi *et al.,* 2000a).

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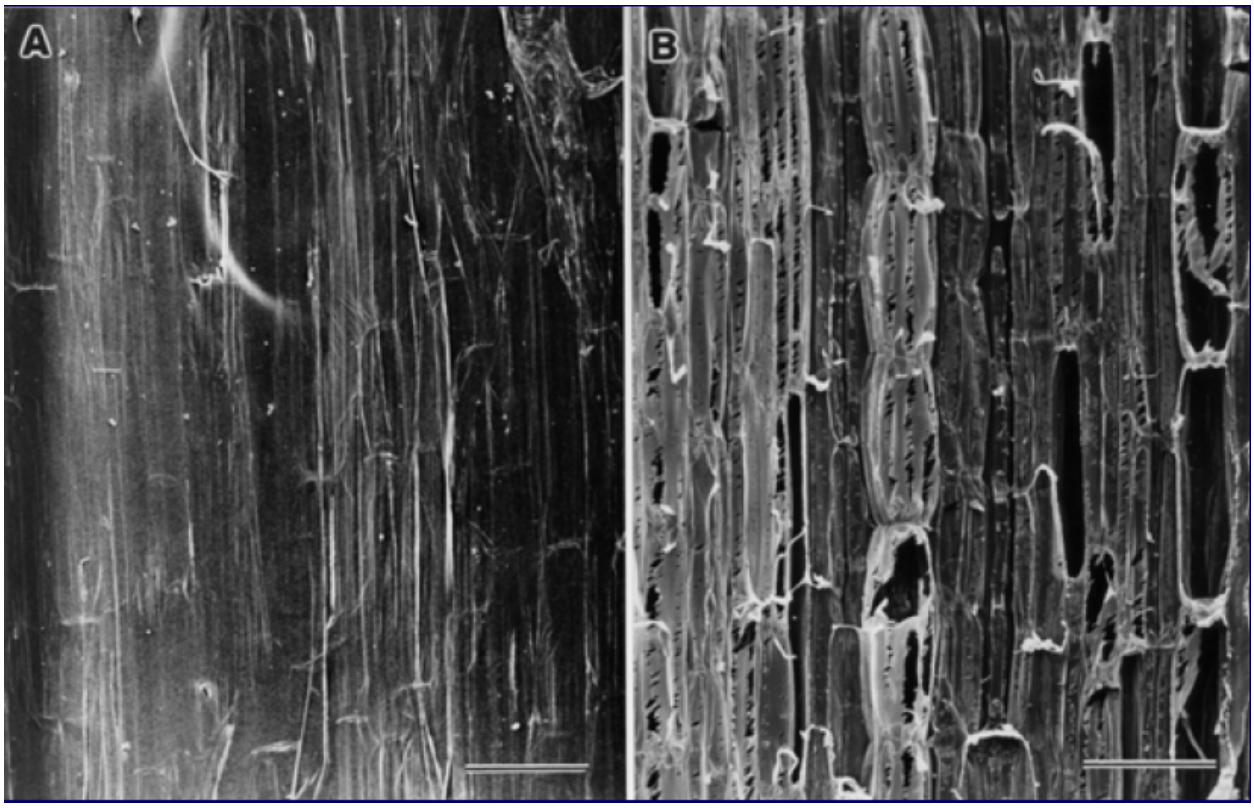

Figure 2: Electron micrograph scan of untreated barley straw (A) and barley straw treated with high concentrations of an exogenous enzyme containing xylanases and cellulases (B) (Adapted from McAllister *et al*., 2001).

b) Ruminal effects

Graham and Balnave (1995) reported that unprotected exogenous enzymes are rapidly inactivated in the rumen due to proteolytic activity. However, Hristov *et al*. (1998a) reported that studies showed that exogenous enzymes are more stable than previously thought. The stability of the exogenous enzymes depends on the source organism used to produce the enzyme, the enzyme activity and if the enzyme product is applied to the feed prior to ingestion (Beauchemin *et al*., 2003a). Fontes *et al*. (1995) reported that xylanase and cellulase are more stable in the rumen, probably due to glycosylation that protects them from inactivation by temperature and proteases. On the other hand, β-

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glucosidase and β-xylosidase are much more prone to inactivation and most of their activity is destroyed within an hour of incubation in rumen fluid (Beauchemin *et al*., 2003a).

Exogenous enzymes express itself in the rumen by means of five different effects, with each of the effects discussed in more detail below:

1) Direct hydrolysis

Exogenous enzymes enhanced the rate of *in situ* and *in vitro* digestion (Colombatto *et al*., 2002; Feng *et al*., 1996; Lewis *et al*., 1996). Although exogenous enzymes enhance the rate of *in situ* and *in vitro* digestion, exogenous enzymes did not improve the extent of *in situ* or *in vitro* digestion (Colombatto, 2000; Hristov *et al*., 1996; Nakashima *et al*., 1988). These studies suggest that exogenous enzymes improve the rate of digestion by improving the hydrolytic capacity in the rumen (Beauchemin *et al*., 2003a).

2) Synergism with ruminal microbes

The increase in ruminal hydrolytic capacity due to the use of exogenous enzymes may be underestimated when calculated using the microbial and exogenous enzyme activity due to synergy (Morgavi *et al*., 2000b). Synergy is the enhanced effect of the ruminal microbial enzymes and exogenous enzymes acting co-operatively. The result of this is increased enzyme activity that is greater than the additive effects of the ruminal microbes and exogenous enzymes (Beauchemin *et al*., 2003a). Bhat and Hazlewood (2001) reported synergy between cellulases and xylanases. In a study done by Wallace *et al*. (2001) the cellulase activity was reported to limit the rate of fermentation of maize and grass silage in the rumen. By increasing the enzymatic activities in the rumen by adding exogenous enzymes, the rate of the forage digestion increased, demonstrating that it is possible to achieve an enhanced enzyme activity due to synergism.

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3) Sub-optimal ruminal conditions for fibre digestion

When ruminants are intensively fed, the rumen pH might often be sub-optimal for fibrolytic bacteria to grow. Typically, when feeding dairy cows and feedlot cattle, the diets are highly fermentable causing the rumen pH to be below optimal at approximately 5.9 (Kung *et al*., 2000; Weimer, 1998). Some exogenous enzymes can be active at very low pH and when adding these to diets of ruminants with sub-optimal rumen pH, it might improve the fibre digestion. Yang *et al*. (2001) found an increase of 8% in the NDF digestibility due to exogenous enzyme supplementation at a rumen pH of 5.6 and also reported an increase of 18% when the rumen pH was 6.0. This shows that exogenous enzymes activities are reduced under low rumen pH conditions (Beauchemin *et al.,* 2003a).

4) Bacterial attachment and colonisation

There is evidence that exogenous enzymes stimulate the attachment of rumen microbes to plant fibres (Morgavi *et al*., 2000b). There are three possible mechanisms by which fibrolytic enzymes stimulate ruminal bacterial attachment to plant cell walls namely:

a) It is possible that applying exogenous enzymes to feed may cause the release of soluble sugars that increases the chemotactic attraction of ruminal fibrolytic bacteria to the plant surface (Beauchemin *et al*., 2003a).

b) It also may increase the “roughness” of the plant cell wall by prior digestion, making it more suitable for microbial colonisation (Morgavi *et al*., 2000b).

c) It may weaken the physical barriers for microbial attachment by prior digestion (Beauchemin *et al*., 2003a).

Previous studies (Colombatto *et al*., 2003a; Wang *et al*., 2001) showed that when supplementing exogenous enzymes, the microbial colonisation in ruminants increased and the feed particle associated enzyme activities improved. Morgavi *et al*. (2000a)

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showed that when supplementing low levels of *Trichoderma longibrachiatum* enzymes, the colonisation of *Fibrobacter succinogenes* increased on maize silage and alfalfa hay (Figure 1). However, Morgavi *et al*. (2000a) also reported that when supplementing high levels of exogenous enzymes, the exogenous enzymes may compete with the rumen microbes for binding sites on the feed, resulting in a lack of response.

5) Stimulation of rumen microbial population

Research shows that adding exogenous enzymes to the diet of a ruminant may increase the numbers of non-fibrolytic bacteria as well as fibrolytic bacteria in the rumen. A study (Nsereko *et al*., 2002) with an enzyme product from *Trichoderma longibrachiatum* showed that the total viable ruminal bacterial count increased with enzyme supplementation, however, as the dose of the exogenous enzymes increased; the increase in bacterial numbers disappeared, explaining the non-linear dose responses typically seen in vivo (Kung *et al*., 2000; Lewis *et al*., 1999; Beauchemin *et al*., 1995). The increased ruminal microbes may accelerate the digestibility of feed and this may amplify the synergy between ruminal enzymes and exogenous enzymes (Beauchemin *et al*., 2003a). The stimulation of ruminal microbe numbers may also result in increased microbial biomass as reported by Yang *et al*. (1999).

c) Post-ruminal effects

In non-ruminant animals, the primary mode of action for exogenous enzymes to improve animal performance is by decreasing the intestinal digesta viscosity (Choct, 2001). The non-starch polysaccharides in grains are viscous in nature and this causes prolonged intestinal transit time, modification of intestinal mucosa and even changes in the hormonal regulation due to the varied rate of nutrient absorption (Beauchemin *et al*., 2003a). When reducing the intestinal viscosity in the small intestine of cattle, the

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nutrient absorption can be improved but the intestinal viscosity of ruminants is generally one to two cPoise (Mirr *et al*., 2000) while intestinal viscosity in poultry may exceed 400 cPoise (Bedford, 1993).

The question if exogenous enzymes is still active in the small intestine was addressed by Hristov *et al*. (1998b) who found that when infusing exogenous enzymes abomasally, the cellulases and amylases did not survive the low pH and pepsin proteolysis (Morgavi *et al*., 2001), however xylanase activity in the duodenum increased by 12 - 30 fold. Despite this increased xylanase levels in the duodenum, the total tract digestibility did not improve. This suggests the possibility of synergism between exogenous enzymes and microbes, even in the large intestine, raising the possibility of exogenous enzymes aiding in an overall decreased manure output in cattle (Beauchemin *et al*., 2003a).

2.1.4 Variation in the response to supplementation to exogenous enzymes

Unfortunately, even if the mode of action is known, the outcome of supplementing any specific exogenous enzyme differs between studies. There are five possible causes of variation in animal production which include enzyme activity, enzyme inclusion level, feed-enzyme specificity, the method of enzyme application to the diet and the level of animal production (Beauchemin *et al*., 2003c). These aspects are discussed below:

a) Enzyme activity

The identification of enzyme activity is important to develop the most effective enzyme combination for ruminants (Beauchemin *et al*., 2003c). The assays used to determine enzyme activity is not always accurate and does not represent the rumen conditions (Beauchemin *et al*., 2004). Furthermore, the units of enzyme activity do not indicate the efficacy of the enzyme in the animal itself. The protein content of the enzyme also

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influences the activity, with an increased protein content the activity of the enzyme could also increase.

b) Enzyme inclusion level

Enzymes can be either over supplemented or under supplemented. Both will cause a reduction in efficacy of the enzyme as well as variability in the response (Beauchemin *et al*., 2003c).

c) Feed-enzyme specificity

Different enzyme combinations will be most effective for different types of diets e.g. diets that have a high or low roughage content or diets with high or low starch content. It is important to identify the enzymes that are the most effective for each type of diet (Beauchemin *et al*., 2003c).

d) Method of enzyme application to the diet

Some enzymes are most effective when applied in a liquid form 24 hours prior to consumption while others can be added to the diet immediately prior to the feed being fed. This can be due to some pre-ingestive action of the enzyme on the plant that will improve the resistance to proteolysis of the enzyme in the rumen as well as weaken the structure of the plant for ruminal microbial attack (Beauchemin *et al*., 2003c).

e) Level of animal productivity

The effect of enzymes will be most prominent in animals where fibre digestion is compromised due to a lower ruminal pH. Enzymes are mostly effective in animals fed for optimal production (Beauchemin *et al*., 2003c).

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2.1.5 Types of enzymes

In this study, two fibrolytic enzyme prototypes provided by a commercial company is investigated to evaluate the potential effects on diet nutrient digestibility. The effects of the enzymes were measured by calculating total tract digestibility using total faecal collection and alternatively by using markers to determine whether any marker is suitable as an alternative technique to total faecal collection in feedlot type diets. The one enzyme prototype used is a combination of xylanase and β-glucanase and the other enzyme prototype is a combination of xylanase, amylase and protease.

2.1.5.1 Xylanase

Beta–1,4-xylans are polysaccharides found in hemicelluloses (Guo *et al*., 2013). Xylanase is the primary enzyme which initiates the hydrolysis of the xylan backbone of plants and xylanases attack internal xylosidic linkages on the xylan backbone and release xylosyl residues (Wong *et al*., 1988). The primary end products that form when xylans are hydrolyzed are xylobiose, xylotriose and oligomers consisting of two to four xylosyl residues (Bajpai, 2009). The optimum pH for xylan hydrolysis is approximately five for most fungal xylanases but is normally stable between pH 2 - 9. Most produced xylanases are temperature stable and can stay intact up to a temperature of 90°C (Bajpai, 2009).

Different types of xylanases can be produced by fungi, bacteria, yeast, marine algae and protozoa. Fungi can produce several types of xylanolytic enzymes necessary for debranching substituted xylans. Typical fungal species that produce xylanase include *Aspergillus, Disporotrichum, Penicillium, Fusarium* and *Trichoderma* (Bajpai, 2009, Pariza and Cook, 2010). Xylanases need purification before the enzymes can be commercially applied and can be extracted by ultrafiltration in conjunction with solvent exchange

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through ion exchanges or by standard column chromatography techniques (Bajpai, 2009).

Supplementation of exogenous xylanases to animal diets showed promising results. Beauchemin *et al*. (1995) reported that adding an enzyme containing fibrolytic activities to a diet containing 91% forage (Timothy hay) and 9% concentrates, the live weight gain of cattle increased by 35% and feed conversion ratio increased by 10%. Beauchemin *et al*. (1997) also reported an 11% improvement in the feed conversion ratio and an increase of 6% ADG of feedlot steers fed a barley-based diet when supplemented with exogenous xylanases. Another study showed improved digestion of organic matter (OM) and NDF in the rumen and in the total digestive tract of dairy cows fed a concentrate diet supplemented with an enzyme mixture with high xylanase and endocellulase activity (Yang *et al*., 1999). McAllister *et al*. (1999) reported an improved ADG in growing feedlot steers without any alteration in the rumen pH when supplementing an exogenous xylanase.

The improved ruminal digestion associated with exogenous xylanase supplementation may result in improved microbial protein synthesis and a higher ruminal microbial colonisation of feed. Care should be taken not to overdose the enzyme since a higher concentration may compete with rumen bacteria for binding sites on the feed particles reducing overall endogenous bacterial activity (Beauchemin *et al*., 2000).

2.1.5.2 Alpha amylase

Starch is the nutrient with the highest concentration when feeding a high maize diet. Starch consists of amylose and amylopectin. Amylose is an insoluble linear polymer of glucose bound by α-1, 4 linkages while amylopectin is a highly branched polymer with α-1, 6 bonds at each branch point (Tricarico *et al*., 2008). Amylases are enzymes that

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cleave the O-glycosidic bonds in starch. Alpha-amylases (α-amylases) cleave the 1, 4-glycosidic bonds in amylose and amylopectin (Muralikrishna & Nirmala, 2005). Cleavage of amylose by α-amylase yields maltotriose and maltose and cleavage of amylopectin by α-amylose yields α-limit dextrins, maltose and glucose (Tricarico *et al*., 2008).

Amylases can be produced by fungi or bacteria and typical bacterial species that produce α-amylase include *Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus stearothermophilus and Bacillus subtillus.* A typical fungal species that produce α-amylase include *Aspergillus oryzae* (Hashemi *et al*., 2010).

The addition of exogenous α-amylase to ruminant diets is often neglected because the digestion of starch in the rumen is not a limiting factor and the rapid digestion of starch in the rumen can lead to digestive upsets such as ruminal acidosis and a decrease in intake (Owens *et al*., 1998). The most recent studies with supplemental α-amylase from *Aspergillus oryzae* which increased ruminal starch digestion in dairy cows also resulted in an increase in true ruminal digestibility of OM but had no effect on microbial nitrogen flow to the duodenum (Nozière *et al*., 2014). Previous research found that supplemental α-amylase decreases the proportion of acetate and butyrate and increases in the proportion of propionate in the rumen (Nozière *et al*., 2014; Tricarico *et al*., 2008; Hristov *et al*., 2000). Animal performance studies done on beef cattle fed a finishing diet with supplemental α-amylase showed an increase in live weight gain. The improvement in weight gain, however, was mediated by an increase in dry matter intake (DMI) of cattle (Tricarico *et al*., 2008).

It is proposed that supplemental α-amylase does not necessarily directly influence rumen starch digestion (Tricarico *et al*., 2008). The improved starch digestion is mediated through other effects such as the modification of the manner in which starch

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is digested. Research showed that in pure cultures of rumen bacteria, supplementing α-amylase improved the growth of bacteria that cannot grow, or grow slowly on starch such as *Butyrivibrio fibrosolvens* D1, *Selemononas ruminantium* GA192, *Megasphera elsdenii* T81 while bacteria that grow rapidly on starch such as *Streptococcus bovis* S1 or *Butyrivibrio fibrosolvens* 49 did not show any change from supplemental α-amylase. Supplementation with α-amylase also improved rumen epithelial growth in dairy calves (Tricarico *et al*., 2008).

2.1.5.3 Beta-glucanase

Beta-glucans (β-glucans) are produced when feed high in mixed-link (1-3)(1-4) β-glucans (like in barley based diets) are fed to animals (Ravindran *et al*., 2007). Beta-glucans may impair digestion and absorption of nutrients because of an increase in viscosity of the digesta (Hristov *et al*., 2000). Endogenous beta-glucanases (β-glucanases) hydrolyze cellulose chains randomly and produce cellulose oligomers while exogenous β-glucanases will produce cellobiose when the cellulose chain is hydrolyzed from the non-reducing end (Beauchemin *et al*., 2004).

Supplemented exogenous β-glucanases were found to be highly stable in the rumen environment (Morgavi *et al*., 2001) and certain strains are thermo-stable at high temperatures to withstand degradation during pelleting. Different types of β-glucanases can be produced by *Disporotrichum dimorhosporum, Penicillum emersonii, Aspergillus niger, Bacillus lentis, Bacillus subtillus* and *Trichoderma longibrachiatum*, although, the strain mostly used in animal feeds is from *Trichoderma reesei* (Beauchmemin *et al*., 2004; Coenen *et al*., 1995).

Limited studies have been conducted in ruminants to investigate the effect of β-glucanase on digestion. Hristov *et al*. (2000) evaluated the effect of ruminal dosing of β-

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glucanase and reported that exogenous β-glucanase supplementation decreases ruminal pH and increases total concentration of volatile fatty acids and acetate. Exogenous β-glucanase supplementation also increased the ruminal fibrolytic activity as well as the duodenal fibrolytic activity. This can be due to direct supplementation or from indirect increases in the rumen microbial activities. Supplementation of diets with exogenous β-glucanase had no effect on the dry matter intake (DMI) of animals but the digesta viscosity was reduced (Hristov *et al*., 2000).

Exogenous β-glucanase supplementation shows potential for use in ruminant diets but is most likely more effective when used in combination with other enzymes. More research is needed to confirm this.

2.1.5.4 Protease

Starch granules are surrounded by hydrophobic prolamin proteins which limit the accessibility to amylolytic rumen microbes. Proteolytic enzymes have been used as feed additives in silages to degrade these proteins in order to improve starch degradation in the rumen (Hoffman *et al*., 2011). The use of proteases as an exogenous enzyme has been limited because ruminal proteolysis has been considered excessive (Windle *et al*., 2014). Vera *et al*. (2012) found that proteolytic enzymes enhance fibre degradation by breaking down structural proteins that act as physical barriers to degradation.

The optimum pH at which exogenous proteases function differs greatly, depending from which strain of bacteria they are harvested from. Proteolytic enzymes from *Trichoderma* species and *Aspergillus* species have an optimal pH of 3 (Young *et al*., 2012) while others from certain strains of *Bacillus* species have an optimum pH of 10-11

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(Hadj-Ali *et al*., 2007). Various types of proteases can be produced by fungi and bacteria such as *Aspergillus, Trichoderma* and *Bacillus* species (Windle *et al*., 2014).

The use of exogenous protease enzymes in ruminant feed recently experienced renewed interest due to the realisation of other advantages when supplemented. Modern feeding practices aimed at improving production often cause a decrease in rumen pH, which in turn allow cellulolytic bacteria in the rumen to decrease and fibre degradation to decrease (Colombatto *et al*., 2007). The addition of exogenous fibrolytic enzymes can overcome this problem but supplementing exogenous proteolytic enzymes can also alleviate the problem since research found that the ruminal enzymatic activities of xylanase and endoglucanase increase with protease supplementation (Eun & Beauchemin, 2005). Research also revealed that *in vitro* gas production increased with the use of exogenous proteolytic enzymes (Vera *et al*., 2012; Eun *et al*., 2007; Eun & Beauchemin, 2005).

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Literature Review

2. 2 External and internal markers and methods of analysis

2.2.1 Introduction

There is a growing range of feed additives available for use in ruminant diets that offer the opportunity to improve dry matter intake, rumen fermentation efficiency, total tract nutrient digestibility, decreased methane production and improve overall animal productivity. Exogenous enzyme additives, in particular, have been reported to improve digestibility of diets that contain mainly forage (Feng *et al*., 1996; Lewis *et al*., 1996), but may also be effective in high concentrate diets such as feedlot diets by contributing to overcome the depression in fibre digestion that generally occurs in high concentrate diets (Krause *et al*., 2013; Beauchemin *et al*., 1997). *In vivo* digestibility studies are normally conducted as a first step in animal studies to evaluate the effectiveness of different enzymes or combinations thereof before large scale growth performance feedlot trials are conducted. The traditional method to perform digestibility studies involves total faecal collection, the use of metabolism crates, faecal collection bags, harnesses and catheters for female animals. This procedure is labour intensive and more importantly impacts the DMI of animals which then significantly affects nutrient digestibility (Galyean *et al*., 1986). This can mask the true potential of feed additives like enzymes to improve digestibility. To overcome this problem the use of external or internal markers can be considered as an alternative to the total faecal collection technique. Markers eliminate the use of metabolism crates and harnesses, allowing free movement of animals. It also eliminates the need for total faecal collection since with the marker technique only faecal grab sampling is needed (Galyean *et al*., 1986).

The ideal marker to be used in digestibility studies has specific defined properties. It should be indigestible and not absorbed nor affected by the gastrointestinal tract (GIT)

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or microbial population in the rumen. It must be physically similar to or in close association with the undigested nutrient that is being analysed. It should flow through the GIT at a similar rate as feed particles and should not separate from the feed. The ideal marker should furthermore be pharmacological inactive in the host animal and should be excreted without any change to its chemical composition and should be chemically unique to that it can be easily identified for analysis. In addition, there should be an accurate, sensitive and reliable analytical method available to analyse for the marker (Marais, 2000; Jagger *et al*., 1992; Kotb & Luckey, 1972). Markers can be classified in one of two categories: external and internal markers.

2.2.2 External markers

External markers are indigestible substances that can be added to the feed or digestive content. Examples of external markers include transition metals in oxide or salt forms like Cr_2O_3, cobalt ethylenediamine tetraacetic acid (Co-EDTA), titanium oxide, rare earth metals like ytterbium and other substances like even chain alkanes (Marais, 2000).

External markers can be used to predict faecal output through the use of grab sampling and knowing the marker concentration in the feed. The faecal output (FO) can be calculated using the following equation (equation 1):

$$\text{FO g/day (DM)} = \frac{marker\ \ dose\ rate\ \ (\frac{g}{day})}{Fecal\ \ marker\ \ \ concentration\ \ \ (\frac{g}{g}DM)} \qquad \text{(Equation 1, Galyean } et\ al.\text{, 1986)}$$

External markers can be administered through continuous dose or pulse dose methods (Galyean *et al*., 1986). The continuous dose method is when an external marker is continuously dosed or infused for a period of time until equilibrium is established before

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sampling is initiated. The faecal marker concentration is then analysed and used to estimate faecal output (equation 1). The length of the preliminary period and the frequency of dosing and sampling may affect the results. Most studies show optimal results if the preliminary period is five to seven days and the dosing frequency is twice daily (Galyean *et al*., 1986). External markers can also be administered through the pulse dose method where the marker is given once to estimate digesta flow or passage rate constants. One can also apply the pulse dose method to estimate faecal output via compartmental models. This method does, however, require frequent faecal grab samples to be collected in order to determine the passage rate and is, therefore, labour intensive (Galyean *et al*., 1986).

The most frequently used external marker to estimate faecal output is Cr_2O_3 (Marais, 2000; Mayes *et al*., 1995; Jagger *et al*., 1992). This marker is typically dosed for five to seven days until marker equilibrium is reached whereafter the faeces can be collected from the animal by faecal grab sampling (Ferret *et al*., 1999). The faecal output is then estimated from the marker concentration in the faeces using equation 1 (Forbes, 1995). Chromium oxide is insoluble in water and only slightly soluble in alkalis and acids. It can be administered through oral dosing, through the feed or intra-ruminally (Marais, 2000). Unfortunately, Cr_2O_3 has disadvantages such as possible carcinogenic properties (Delagarde *et al*., 2010; Peddie, 1982) and it can oxidise unsaturated fats (Steele & Clapperton, 1982). In some cases, it resulted in variable recovery rates, but it could possibly be due to incomplete recovery, due to physical loss of faeces, marker regurgitation, analytical errors or failure to establish marker equilibrium (Delagarde *et al*., 2010; Galyean *et al*., 1986). Dosing the marker twice daily seems to improve recovery rates and eliminate variation in excretion. Jagger *et al*. (1992) reported that when Cr_2O_3 is dosed at a rate of 5 g/kg feed, it caused an initial reduction in the DMI in pigs, but the effect was removed after the pigs acclimatised. In cannulated ruminants,

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this is not a problem as the marker is dosed intra-ruminally. Alternatively, internal markers can be used.

2.2.3 Internal markers

An internal marker is a substance that is naturally part of the forage or feed that is being consumed by the animal and it is not absorbed nor changed in the GIT (Marais, 2000; Huhtanen *et al*., 1994). Examples of internal markers include indigestible acid detergent fibre (iADF), indigestible lignin, AIA and odd-chain n-alkanes (Mayes *et al*., 1995).

The use of internal markers offer advantages such as that no preparation is needed because it is inherent to the feed, it is cost effective and convenient to use and when applying it as a marker, the animals does not have to be confined in order to administer the marker. Some disadvantages when using internal markers include the variable recovery results due to different analytical methods and the fact that some internal markers can be changed in the GIT resulting in the substance being tested in the feed may be chemically different from the marker in the faeces (Marais, 2000). Internal markers can be used to estimate apparent diet digestibility through the following equation (equation 2):

$$\text{Apparent total tract digestibility (\%)} = 1 - \left(\frac{marker\ \ concentration\ \ \ in\ feed}{marker\ \ concentration\ \ \ in\ feces}\right) \times 100$$

(Equation 2, Mayes *et al*., 1995).

When estimating the apparent total tract digestibility of a specific nutrient the following equation can be applied (equation 3):

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$$\text{Apparent TTD of nutrient (\%)} = 1 - \left(\frac{Marker\ \ concentration\ \ \ in\ feed}{Marker\ \ concentration\ \ \ in\ feces} \times \frac{Nutrient\ \ concentration\ \ \ in\ feed}{Nutrient\ \ concentration\ \ \ in\ feces}\right) \times 100$$

(Equation 3, Marais, 2000).

When using internal markers it is important to firstly determine if the chosen internal marker is suitable to be used on the specific type of diet chosen. Not all internal markers give accurate results on all types of diets and secondly one should make use of a standardised analytical method to quantify the internal marker in the feed and faeces (Huhtanen *et al*., 1994).

One internal marker that is used with great success is AIA. The use of this marker dates back to the 1950's where Kane *et al*. (1950) reported high recovery rates for acid insoluble ash. Shrivastava & Talapatra (1962) reported using an acid insoluble residue and found a recovery rate of 99.8%. Van Keulen & Young (1977) evaluated three different methods to analyse for acid insoluble ash and one of these three methods is still being used today (discussed in next section). McCarthy *et al*. (1974) reported that using AIA is even more accurate than using Cr_2O_3 in swine diets and Thonney *et al*. (1979) reported recovery rates of between 90% to 100% for AIA (depending on the cutting date of the hay used in the diet) and concluded that AIA is a preferred internal marker used to accurately predict TTD in sheep, cattle, pigs, poultry and horses (Bergero *et al*., 2004; Thonney *et al*., 1979; Van Keulen & Young, 1977; Vogtmann *et al*., 1975; McCarthy *et al*., 1974; Shrivastava & Talapatra, 1962).

Other internal markers that are used commonly include lignin or odd-chain alkanes. Lignin is used as it occurs in all forages and is theoretically indigestible, however, its complex structure can make it difficult to accurately quantify the marker (Galyean *et al*., 1986). Odd-chain alkanes are components of the cuticular wax layer covering all plants.

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Alkanes associate with the particulate matter in digesta and are stable through the GIT (Marais, 2000). Alkanes are mostly used in studies where herbage intake is estimated as well as where the specific species of herbage eaten is estimated as different herbages contain different chain length n-alkanes (Marais, 2000).

The use of markers in trials to estimate faecal output, TTD, passage rate, herbage species intake etc. Is becoming more popular in recent years and it offers great advantages above total faecal collection if one can accurately quantify the marker. In the following section, the analytical procedures used to quantify the markers used in this study will be discussed.

2.2.4 Laboratory methods to analyse for markers

The analytical methods used to analyse for markers in feed and faeces should be accurate and repeatable. The methods used to analyse for the markers used in this study (Cr_2O_3, AIA and ADL will be discussed below.

2.2.4.1 Chromic (III) oxide

In literature, several methods are described to analyse for Cr_2O_3. Some methods are more accurate than others due to several reasons that will be discussed below.

The analysis to determine Cr_2O_3 is based on two steps. The first is to use acid to digest any organic matter present. In this step the chromium valence is also changed from 3 + (sesquioxide) to 6 + (dichromate) which is quantifiable. The second step is to add an acid combination to quantify chromium (Souza *et al*., 2013). Several combinations can be used in the second step like nitric- and perchloric acids (Kimura & Miller, 1957),

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phosphoric acid solution (Williams *et al*., 1962) and sulphuric- and perchloric acids (Fenton & Fenton, 1979). To quantify chromium one can use atomic absorption spectrophotometry (AAS) (Williams *et al*., 1962), calorimetric method (Kimura and Miller, 1957) or by inductively coupled plasma emission spectrometry (ICP-ES) (Roofyael & Lyans, 1984). Yiakoulaki *et al*. (1997) determined that ICP-ES is more sensitive than AAS, but the equipment used is very expensive and not generally available in all labs. Scientists can thus use AAS or calorimetry. Souza *et al*. (2013) compared calorimetry to AAS as well as the influence of the three acids used on the results. They found that when using the mixtures of nitric- and perchloric acid of sulphuric- and perchloric acid, the recovery of chromium is more complete than when using phosphoric acid only. This can be due to possible influences on the formation of elemental chromium in the nitrous oxide flame of AAS when using phosphoric acid. In the study, it was also found that AAS based on nitric- and perchloric acid or sulphuric- and perchloric acid provided complete recovery of chromium while the calorimetric method showed lower recovery rates, regardless of the technique used (Souza *et al*., 2013).

Fenton & Fenton (1979) examined the effects of various wavelengths at which chromium can be quantified by examining 440 nanometres (nm), 375 nm and 350 nm. They concluded that as the wavelength decreased, the slopes of the curves used to correct for any deviation in the AAS value increased. They concluded that it was best to use the wavelength with the smallest slope for correction.

This method described by Fenton & Fenton (1979) is most commonly used by scientists all over the world as it is accurate and repeatable. This method also includes a step where the samples are ashed before acid digestion to remove all organic matter present. This improves the safety of the method as any present organic matter that is digested by perchloric acid has a high risk of explosion.

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2.2.4.2 Acid insoluble ash

Van Keulen & Young (1977) evaluated AIA as an internal marker using three different methods. They compared a new 2 N hydrochloric acid (HCl) based method to the concentrated HCl method of Shrivastava & Talapatra (1962) and to the 4 N HCl method described by Vogtmann *et al*., (1975). They found that there was no significant difference between the three methods for AIA recovery as well as predicting digestibility compared to total faecal collection. The use of the 2 N HCl method offer some advantages above the other methods such as being the most convenient method and the increased safety factor due to lower normality of the acid used. This method has an extra initial oven drying step prior to acid treatments in order to allow for DM determination. They also added an ashing step prior acid digestion in order to remove OM and reduce the strength of acid required to digest the sample. Prior ashing also allows for the avoidance of unpleasant odours that occur when feed or faeces are digested with acid (Van Keulen & Young, 1977).

Van Keulen & Young (1977) found no difference between the three AIA analytical methods ability to predict DM digestibility compared to total faecal collection. The use of the 2 N HCl method is advised as it has an added safety factor (lower normality of acid) and it is less time consuming when compared to the concentrated HCl and 4 N HCl methods (addition of prior drying step). This method is most commonly used in all animal science related studies and was utilised in this study as well.

2.2.4.3 Acid detergent lignin

Plant cell walls consist of a variety of structural polysaccharides cross-linked with proteins and phenolic components like lignin. Lignin is a noncarbohydrate phenolic

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polymer that is a multi-branched network consisting of phenyl propane units and can be partially linked to non-cellulosic polysaccharides (Hindrichsen *et al*., 2006). Lignin protects cell walls from degradation and physical damage due to the lignin-polysaccharide complexes that it forms. Lignin also anchors the cellulosic microfibrils and other matrix polysaccharides in cell walls (Slevendran, 1984).

Lignin can be used as an internal marker to determine digestibility and several assay methods have been approved by the AOAC (Hatfield & Fukushima, 2005). Lignin can be measured by using gravimetric methods, which is rapid and simple but may be unreliable (Muntifering, 1982). Lignin can be measured by an acid detergent method described by Goering & Van Soest (1970). The acid detergent method consists of hydrolytic and oxidative versions where the hydrolytic version is named acid detergent lignin using sulphuric acid (H_2SO_4) as digestive acid and the oxidative version is named permanganate lignin and use permanganate ($KMnO_4$) as digestive acid (Jung *et al*., 1997). Permanganate lignin measures true aromatic lignin but may include tannins, pigments or protein that is resistant to solubilization in acid detergent but are oxidised in permanganate solution (Van Soest 1987). The acid detergent lignin method described by Goering & Van Soest (1970) measures true lignin and cutin or mailard artefacts that are not digested during the digestion process and some true lignin may be destroyed by the acid step, leading to incomplete and or inaccurate results. Lignin can also be modified in the lower gut of the animal where lignin is converted to a soluble lignin-carbohydrate complex (Fahey & Jung, 1983). The soluble lignin-carbohydrate complexes measure gravimetrically as lignin leading to recoveries that are unreliable or inaccurate when using gravimetric methods to measure lignin (Fahey & Jung, 1983). Neilson & Richards (1978) suggested that up to 50% of the lignin of some forages may form soluble lignin-carbohydrate complexes that are measured in the acid detergent lignin fraction.

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Lignin can also be measured by the addition of a two-stage sulphuric acid hydrolysis to determine neutral sugar components of cell wall polysaccharides (Jung *et al*., 1997). This method is called Klason lignin (KL) and the method is described by Theander *et al* (1994). Klason lignin values are commonly higher that ADL but it can be due to the structural characteristics of lignin in grasses and legumes as well as possible protein contamination (Lai & Sarkanen, 1971; Van Soest, 1967). Hindrichsen *et al* (2006) showed a close positive correlation between KL and ADL and Jung *et al* (1997) concluded that ADL is slightly more consistently correlated with dry matter digestibility. Thus the use of ADL or KL is acceptable.

Another method of analysing lignin is by removing interfering phenolic compounds and protein by solubilizing it in 25% acetyl bromide prior to acetic acid solubilization and then determining its ultraviolet absorbance at 280 nm (Morrison, 1972). This method detects soluble lignin that is ordinarily destroyed or not detected by gravimetric analysis (Fahey *et al*., 1979). This method is more accurate when used to measure cell wall lignin and not whole plan lignin content. Lignin can also be measured using nuclear magnetic resonance spectroscopy (NMR) which characterises the composition and structural features of lignin when it is dissolved in a suitable solvent for solution-state NMR. Unfortunately, it is difficult to dissolve lignin (Hatfield & Fukushima, 2005).

Choosing a method to quantify lignin can be difficult as none of the methods is rapid, non-invasive and accurate as reported by Hatfield & Fukushima (2005). Consistency is important and all samples should be analysed using the same method. In our study, we, therefore, decided to use the method most commonly used in animal nutrition studies as well as the method used by our analytical lab as described by Goering & Van Soest

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(1970). This method is altered by the use of glass microfiber filters (934-AH) instead of asbestosis filters which are considered hazardous (Raffrenato & Van Amburg, 2011).

It is clear from above discussion that different methods for analysis for markers can significantly impact the results and therefore the method chosen for the analysis of a marker chosen for a specific type of diet should be carefully considered when performing digestibility studies.

In the next chapter, the experimental procedure and results will be discussed.

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Chapter 3

Effect of two enzyme prototypes on nutrient digestibility in feedlot steers using the total faecal collection technique

3.1 Introduction

In this study, the effect of two fibrolytic enzyme prototypes namely enzyme A and enzyme B were compared to a control diet without any enzyme supplementation by means of apparent total tract nutrient digestibility study using feedlot steers fed a total mixed ration. Apparent nutrient digestibility is calculated by subtracting the feacel nutrient excretion from the dietary nutrient intake. It does not account for endogenous losses via the faeces. The total faecal collection technique was used to calculate the apparent total tract nutrient digestibility. It should be noted that in the discussion it refers to apparent total tract digestibilities of the nutrients everywhere where total tract digestibilities are reported. Apparent total tract digestibility is calculated by subtracting the nutrients in the faeces from the dietary nutrient intake. It does not account for nutrients lost as methane or metabolic waste excretion.

3.2 Materials and methods

3.2.1 Animal ethics clearance

Ethical clearance was obtained from the Gauteng Department of Agriculture and a DECRA approval number was issued: EC003-14.

3.2.2 Location

This study was conducted at the Hatfield Experimental Farm situated in Pretoria in the Gauteng province of South Africa. This area is classified as a subtropical climate with

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hot rainy summers and cool dry winters. It has an average temperature of 24 °C and lies at an altitude of 1350 m above sea level.

3.2.3 Experimental design, animals and diets

Six ruminally cannulated (10 cm diameter, Bar Diamond, Parma, ID, USA) Nguni crossbred type steers with an average weight of 320 ± 20 kg at the onset of the trial were used in a replicated 3 x 3 Latin square design experiment. Nguni's are adapted to harsh African conditions and is very tolerant of extreme temperatures. Nguni's are medium frame type animals and a highly resistant to diseases (Bester *et al*., 2001). The steers were housed in individual stalls of 3 m x 5 m with concrete floors and a roof covering the feed and water troughs. Animals had *ad libitum* access to fresh water throughout the study and they were cared for according to animal husbandry practices on the research farm. Rectal temperatures were taken weekly to monitor animal health as described by Mitlöhner *et al*. (2001). The diet fed was a South African feedlot grower diet as described by Coetzer (2002a). The ingredient and chemical composition of the control diet are shown in Table 1.

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Table 1: Ingredient and chemical composition of the control diet fed to the experimental animals

Ingredient	% of diet "as is"
Dry rolled maize	60
Eragrostis curvula hay	20
Commercial concentrate (SB100)* containing a vitamin and mineral premix**	15
Commercial high protein concentrate (Procon 33)***	5

Nutrient Composition	g/100 g DM
DM	91.1
ME (MJ/kg DM)	11.1
NDF	26.3
ADF	13.2
Starch	43.9
CP	15.6
EE	2.2
Ash	5.3
Ca	0.7
P	0.3
Na	0.2
Cl	0.4
Monensin (mg/head/day)****	374
AIA	0.85
ADL	4.68

* SB 100 (Reg no V4498 (Act 36/1947)) is a molasses based feedlot concentrate. Its chemical composition consists of CP 30 g/ 100 g, Urea 8 g/100 g, CF 10 g/100 g, Moisture 16 g/100 g. It also contains ionophores and other vitamins and minerals. It is produced by Voermol (Hoofweg, Maidstone Village, Maidstone, 4380, South Africa).

** The vitamin and mineral premix used in the commercial concentrate contains manganese at 12 mg/100 g, copper at 3.9 mg/100 g, cobalt at 0.16 mg/100 g, iron at 12 mg/100 g, iodine at 0.18 mg/100 g, zinc at 20 mg/100 g selenium at 0.08 mg/100 g and vitamin A at 2 200 IU/100 g.

*** Procon 33 is a high protein product containing only natural proteins and no urea. It is high in rumen undegradable protein and suitable to use in diets for high producing animals. Its chemical composition is CP 33

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g/100 g, CF 15 g/100 g, Fat 6 g/100 g, Moisture 12 g/100 g. It is produced by Voermol (Hoofweg, Maidstone Village, Maidstone, 4380, South Africa).

**** Monensin intake is based on an average intake of 10 kg "as is" per animal per day.

DM – dry matter; CP – crude protein; EE – Ether extract; NDF – neutral detergent fibre; ADF – acid detergent fibre; Ca – calcium; P – phosphorus; Na – sodium; Cl – chloride; ME- metabolisable energy measured in mega joules (MJ)

The diet was supplied *ad libitum* throughout the study. Animals were adapted prior to the start of the study to the control diet for a 10 day period before the experimental phases began where the animals were randomly assigned to three groups of two animals to be part of the replicated 3 x 3 Latin square design. The treatments consisted of the control diet with no added enzyme, the control diet with 60 g added enzyme A and the control diet with 60 g added enzyme B. Enzyme A contained xylanase, amylase and protease while enzyme B contained only xylanase and beta-glucanase.

Each experimental period was 15 days with 11 days for adaptation to the experimental diet (Ametaj *et al*., 2009; Mathison *et al*., 1991) and four days for sample collection. Animals were fed twice daily at 8:00 am and 04:00 pm. The orts of each steer were collected daily just before the morning feeding during the sampling times in order to determine the daily feed intake of each steer. Before the faecal collection period proceeded, the concrete floor was cleaned with a high-pressure water hose. Total faecal output of each steer was collected immediately after spontaneous defecation from day 12 to 15 of each period and stored in a bucket. After each 24 hour collection period the faeces were weighed, blended and a subsample of 1 kg of each steer was stored in a refrigerator at 2°C. After each four day collection period during each experimental period, the representative sample of each day's faecal output was pooled into one faecal sample for each steer. Samples of feed and orts were also taken from day 12 - 15 and pooled within steer and period. The pooled 1 kg faecal and orts samples were

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stored at -20 °C until further analysis within steer and period. The total faecal collection procedure was used by Ponce *et al*., 2014 and Batista *et al*., 2016.

3.2.4 Laboratory analysis

Before analysis, the samples were thawed and dried at 60°C for 48 hours and ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a 1mm screen. Feed and faecal samples were analysed for DM (AOAC, 2000 procedure 934.01), NDF (AOAC, 2000 procedure 2002.04), starch (AOAC, 2000 procedure 996.11) and CP (AOAC, 2000 procedure 968.06). The orts samples were analysed only for NDF in order to determine whether feed selection occurred. The feed were also analysed for ADF (Goering & Van Soest, 1988), ether extract (AOAC, 2000 procedure 920.39), ash (AOAC, 2000 procedure 942.05), calcium (AOAC, 2000 procedure 965.17), sodium (AOAC, 2000 procedure 933.13), phosphorus (AOAC, 2000 procedure 965.17), chloride (AOAC, 2000 procedure 969.10) and metabolisable energy. The metabolisable energy was calculated by using the equation described below:

ME (kcal/kg DM) = (36*CP % + 81.8*EE % + 35*NFE %)

(Equation 3.1, Pauzenga, 1985)

Where kcal is kilocalories, EE is ether extract and NFE is the nitrogen-free extract. Kilocalories can be converted to megajoule by multiplying the amount of kcal by 0.004185. The NFE used is calculated using the following calculation:

NFE = 100% - (Moisture % - CP % - Cfat % - CF % - ash %)

(Equation 3.2, Van Soest *et al*., 1991)

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Chromic oxide was analysed according to the method described by Fenton and Fenton (1979), AIA according to the method described by Van Keulen and Young (1977) and ADL according to the method described by Goering and Van Soest (1973).

3.2.5 Statistical analysis

The data was analysed as a Latin square design using the GLM model (Statistical Analysis System, 2015) for mean effects over time. Repeated measures of analysis of variance with the GLM model were used for repeated period measures. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by the Fishers test (Samuels, 1989). A tendency for differences between treatments was declared at $P<0.10$. The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + P_j + A_k + e_{ijk}$$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

P_j = effect of the j^{th} period

A_k = effect of the k^{th} animal

e_{ijk} = error associated with each Y

3.3 Results and discussion

The TMR fed to the feedlot steers (Table 1) was formulated according to recommendations by Coetzer (2002a) based on the NRC (2000), with small adjustments

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based on the type of animal, feeding period in the feedlot and classification of the carcass according to the South African meat classification system. Meat is classified by age, fatness, confirmation, damage and sex (samic.co.za). The South African meat classification system is used to provide guidelines to meat traders by describing the requirements when purchasing a carcass and is also used to determine the selling price of the meat (samic.co.za).

The general South African feeding recommendations are based on steers entering the feedlot on ± 240 kg, a feeding period of ± 120 days and steers being slaughtered at ± 450 kg with a 14% fat content of the carcass (Henning, 2013). Ford (2012) described that the average weight of South African steers entering the feedlot is 235 kg and the average feeding period is 125 days. The general recommendation for monensin inclusion according to Elanco (Elanco Animal Nutrition, 2500 Innovation Way, Greenfield, IN, USA) is 5 - 40 g/tonne of feed based on a 90% DM basis. This should provide 50 – 480 mg/head/day. The TMR contained 374 mg monensin sodium per head per day based on a pre-treatment estimate of 10 kg intake per steer per day. According to Hutjens (2007), the acceptable levels of monensin supplementation is between 250 mg – 400 mg per head per day, therefore the ionophores levels in the trial TMR was within acceptable ranges.

In South African feedlots the recommended forage levels of feedlot finisher diets are generally 5 – 15%. The experimental TMR used was slightly higher at 20% to ensure rumen health as the animals were used in multiple trials due to the fact that they are cannulated. The experimental TMR contained a slightly higher CP level (15.6g/100 g DM) than the recommended range of 12 – 14 g/100 g on DM basis (personal communication with Dr Coetzer). This could be due to the usage of higher quality *Eragrostis curvula* hay than expected. The HPC used, Procon 33, consists of very high-

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quality bypass protein sources and no urea. The Ca:P ratio of the Control TMR was within recommended levels at 2:1 and the salt levels were also in the acceptable range with Cl at 0.4g/100 g and Na at 0.19g/100 g (Coetzer, 2002a,b). The effect of the different enzyme treatments on nutrient digestibility based on total faecal collection is shown in table 2.

Table 2: The effect of the different enzyme treatments at a dosage rate of 60 g on apparent nutrient digestibility (g/100 g) based on total faecal collection

	Treatment			
Nutrient	**Control**	**Enzyme A**	**Enzyme B**	**SEM**
DM	71.9	73.6	74.9	1.2
Starch	87.6	89.6	92.7	2.4
NDF	42.1[a]	46.5[ab]	48.5[b]	1.9
CP	64.8	65.4	63.4	1.4

DM-dry matter NDF- neutral detergent fibre CP-crude protein SEM- standard error mean
[a,b] Rows with different superscripts differ (P<0.05)

The mean total tract DM digestibility was 73.5 g/100 g with no difference (P>0.05) between treatments. The total tract DM digestibility was within the range (73.4 g/100 g) reported by Pinos-Rodrigues *et al.* (2008) where an exogenous fibrolytic enzyme was used in a diet with a forage: concentrate ratio of 40:60 and fed to growing lambs. Krause *et al*. (1998) conducted a study where fibrolytic enzymes were added to a feedlot diet based on rolled barley grain and found that the enzyme did not have any effect on the total tract DM digestibility. In our study, the total tract DM digestibility remained relatively stable between the different treatments (Table 2). This supports the statement by Burroughs *et al*. (1960) who reported that the addition of exogenous

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enzymes does not improve nutrient digestibility *per se*, but it does support an increase in the weight gain by making the nutrients more available to be used by rumen microbes. Applying exogenous enzymes prior to ingestion allow the enzymes to aid in sugar release by degrading some substances of the feed (Beauchemin *et al*. 2003a). This will make the nutrients more available to rumen microbes as the feed is degraded prior to ingestion (Figure 1 and Figure 2). In our study, the ADG was not measured since it was not one of the objectives of the study.

The mean starch total tract digestibility was 89.9g/100 g and it falls in the range of a previously reported study by Zinn *et al*. (1995) where a total tract starch digestibility of 89.8 g/100g was reported when feeding feedlot steers a diet with 65% dry rolled maize. Owens and Soderlund (2006) also reported a total tract digestibility 91.0 g/100 g when feeding dry rolled maize to feedlot cattle. Huntington (1997) found that the average starch total tract digestibility for ground maize was 93.5 g/100 g compared to dry rolled (92.2 g/100 g) and steam flaked maize (98.9 g/100g). The study did not indicate the size of the sieve of the ground maize. The mean total tract starch digestibility of steers fed the different treatments did not differ ($P>0.05$). This was expected as the two types of exogenous enzymes do not attack starch directly as it is classified as fibrolytic enzymes. It has been reported that the addition of exogenous fibrolytic and proteolytic enzymes to a high maize diet may lead to degradation of the pericarp and protein matrices of maize, which will improve the accessibility of the rumen microbes to the starch (Cowieson, 2005; Young *et al*., 2012). This was however not seen in this study.

The mean total tract NDF digestibility was 45.7 g/100 g. This is in the range of previously reported studies by Owens and Soderlund (2006) where a total tract NDF digestibility of 50.83 g/100 g was reported for dry rolled maize in feedlot steers. There are differences ($P< 0.05$) between the control diet and treatment with enzyme B.

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Enzyme B contains exogenous β-glucanase and its mode of action involves hydrolyzing cellulose chains randomly producing cellobiose (Beauchemin *et al*., 2004). Hristov *et al*. (2000) fed a barley-based diet to feedlot steers and reported that β-glucanase supplementation decrease ruminal pH and increase the total concentration of volatile fatty acids as well as increase the acetate concentration. They also found increased fibrolytic activity in the rumen as well as in the duodenal digesta (Hristov *et al*. 2000). Mendoza *et al*. (2014) reported that the inclusion of exogenous fibrolytic enzymes in feedlot finishing diets containing low amounts of forage should not have an effect on productivity; however the fact that the diets are usually high in starch causes the rumen pH to be low (<6) for the greatest part of the day. This affects the ability of the ruminal fibrolytic enzymes to digest fibre. This can explain why a difference in total tract NDF digestibility was noted in this study.

The mean total tract CP digestibility of the different treatments was 65.9 g/100 g. No differences (P>0.05) were observed between treatments. It is possible that the proteolytic enzyme supported the NDF degradation more than protein degradation as noted by Young *et al*. (2012). Eun & Beauchemin (2005) reported that exogenous proteolytic enzymes play a role in improving fibre digestion in alfalfa-corn diets *in vivo*. Colombatto *et al.* (2003b, c) reported an increase in NDF degradability of alfalfa hay and a TMR as a result of exogenous proteolytic enzyme supplementation. Vera *et al.* (2012) showed that supplementation of an exogenous proteolytic enzyme on a low forage diet (18.2% barley silage, 16% alfalfa hay and 65.8% concentrates on a DM basis) improved the total tract NDF digestibility by 26%. The suggested reason for this improved NDF digestibility when an exogenous proteolytic enzyme is supplemented is explained by Colombatto *et al.* (2003b, c). They suggest that the exogenous proteolytic enzyme may act on the nitrogen-containing cell wall components that act as a physical barrier to the rumen microbes to degrade fibre. By degrading the cell wall nitrogen components, the

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rumen microbes can have easier access to the fibre components (Vera *et al.* 2012). These results again support the statement that exogenous enzymes act in synergy with rumen microbes to support overall digestion and not necessarily specific nutrient digestion (Eun *et al*., 2007).

3.4 Conclusion

There were no treatment effects on total tract nutrient digestibility except for total tract NDF digestibility which was increased when enzyme B was fed compared to the control diet. Thus the use of enzymes containing xylanase, amylase and protease had no effect on any total tract nutrient digestibility in this study. However, the enzyme-containing xylanase and β-glucanase had an effect ($P<0.05$) on total tract NDF digestibility but no effect on the digestibility of the other nutrients measured.

Since South African feedlot diets contain relatively little fibre and are thus usually low in NDF, it would, therefore, most probably not be cost effective to attempt to increase NDF digestibility by means of enzyme supplementation. Results suggest that the two enzyme prototypes used were not effective in typical South African feedlot diets. More research is needed to determine if the enzyme has any effect on growth parameters and site of digestion of the nutrients even though no effect is seen on the total tract nutrient digestibility.

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Chapter 4

A comparison of the total faecal collection technique and marker-based techniques to estimate apparent nutrient digestibility in feedlot diets

4.1 Introduction

Although total faecal collection (TC) is the golden standard when *in vivo* total tract nutrient digestibility is determined, it is laborious, time-consuming and functionally impossible on large numbers of cattle (Marais 2000). When screening large numbers of feed additive prototypes such as enzymes or yeast strains that can potentially impact digestibility, total faecal collection is not practical. An alternative to total collection is to collect spot faecal samples and use markers to estimate the total faecal output. It is generally accepted that some markers are more suited to specific types of diets, for example, ADL is more suited when a high roughage diet is used than a low roughage diet. Each marker should be validated for the type of diet used (Jagger *et al*., 1992).

The two internal markers used in this study are AIA and ADL. Acid detergent lignin has good recovery rates in certain diets but it is difficult to quantify (Jagger *et al*., 1992), while AIA is easy to measure and accurate (Van Keulen and Young, 1977) but most studies conducted using AIA were based on diets high in barley grain and not maize (Huhtanen *et al*., 1994). There is, therefore, limited information on some markers when used in feedlot diets with high levels of ground maize.

The objective of this study was to compare the apparent total tract nutrient digestibility determined by total faecal collection with values estimated by using Cr_2O_3, AIA and ADL. It should be noted that in the discussion there is referred to apparent total tract digestibilities of the nutrients everywhere where total tract digestibilities are reported.

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The effect of the enzyme prototypes on the apparent total tract digestibility of the nutrients was only briefly discussed in this chapter as it is not one of the objectives of this study.

4.2 Materials and methods

4.2.1 Animal ethics clearance

Ethical clearance was obtained from the Gauteng Department of Agriculture and a DECRA approval number was issued: EC003-14.

4.2.2 Location

This study was conducted at the Hatfield Experimental Farm situated in Pretoria in the Gauteng province of South Africa. This area is classified as a subtropical climate with hot rainy summers and cool dry winters. This area mostly receives thunderstorms in the afternoon. It has an average temperature of 24 °C and lies at an altitude of 1350 m above sea level.

4.2.3 Experimental design, animals and diets

Six ruminally cannulated (10 cm diameter, Bar Diamond, Parma, ID, USA) Nguni crossbred type steers with an average weight of 320 ± 20 kg at the onset of the trial were used in a replicated 3 x 3 Latin square design experiment. Nguni's are adapted to harsh African conditions and tolerant to extreme temperatures. Nguni's are medium frame type animals and a highly resistant to diseases (Bester *et al*., 2001). The steers were housed in individual stalls of 3 m x 5 m with concrete floors and a roof covering the feed and water troughs. Animals had *ad libitum* access to fresh water throughout the study and they were cared for according to typical animal husbandry practices on

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the research farm. Rectal temperatures were taken weekly to monitor animal health as described by Mitlöhner *et al*. (2001). The diet fed was a typical South African feedlot grower diet as described by Coetzer (2002a). The ingredient and chemical composition of the control diet are shown in Table 1 (chapter 3, pg 33).

The diet was supplied *ad libitum* throughout the study. Animals were randomly assigned to three groups of two animals to be part of the replicated 3 x 3 Latin square design. The treatments consisted of the control diet with no added enzyme, the control diet with 30 g added enzyme A and the control diet with 30 g added enzyme B. Enzyme A contained xylanase, amylase and protease while enzyme B contained only xylanase and beta-glucanase. The marker treatments tested consisted out of total collection which was the control (treatment 1), treatment 2 was Cr_2O_3, treatment 3 was AIA and treatment 4 was ADL.

Each experimental period was 15 days with 11 days for adaptation to the experimental diet (Ametaj *et al*., 2009; Mathison *et al*., 1991) and four days for sample collection. Animals were fed twice daily at 8:00 am and 04:00 pm. The orts of each steer were collected daily just before the morning feeding during the sampling times in order to determine the daily feed intake of each steer. Before the faecal collection period proceeded, the concrete floor was cleaned with a high-pressure water hose. The total faecal output of each steer was collected immediately after spontaneous defecation from day 12 to 15 of each period and stored in a bucket. After each 24 hour collection period the faeces were weighed, blended and a subsample of 1 kg of each steer was stored in a refrigerator at 2°C. After each four day collection period during each experimental period, the representative sample of each day's faecal output was pooled into one faecal sample for each steer. Samples of feed and orts were also taken from day 12 - 15 and pooled within steer and period. The pooled 1 kg faecal and orts samples

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were stored at -20 °C until further analysis within steer and period. The total faecal collection procedures were used by Ponce *et al*. (2014) and Batista *et al*. (2016).

3.2.4 Laboratory analysis

Before analysis, the samples were thawed and dried at 60°C for 48 hours and ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a 1mm screen. Feed and faecal samples were analysed for DM (AOAC, 2000 procedure 934.01), NDF (AOAC, 2000 procedure 2002.04), starch (AOAC, 2000 procedure 996.11) and CP (AOAC, 2000 procedure 968.06). The orts samples were analysed only for NDF in order to determine whether feed selection occurred.

Chromic oxide was analysed according to the method described by Fenton and Fenton (1979), AIA according to the method described by Van Keulen and Young (1977) and ADL according to the method described by Goering and Van Soest (1973).

3.2.5 Statistical analysis

The data was analysed as a Latin square design using the GLM model (Statistical Analysis System, 2015) for mean effects over time. Repeated measures of analysis of variance with the GLM model were used for repeated period measures. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by the Fishers test (Samuels, 1989). A tendency for differences between treatments was declared at $P<0.10$. The linear model used is described by the following equation:

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$Y_{ij} = \mu + T_i + P_j + A_k + M_p + e_{ijkp}$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

P_j = effect of the j^{th} period

A_k = effect of the k^{th} animal

M_p = effect of the p^{th} marker

T_iM_p = effect of interaction of T and M

e_{ijkp} = error associated with each Y

4.3 Results and discussion

The internal marker content for the control diet and each dietary ingredient is shown in Table 3. The AIA content of the control diet was 0.9 g/100 g and lower compared to previous studies reported by Hristov *et al*. (2011; 2005; 2004). These researchers reported AIA contents of alfalfa and cereal silage based diets to be 1.4 g/100 g to 1.7 g/100 g (DM basis) which is higher than 0.5 g/100 g to 0.6 g/100 g (DM basis) reported for diets based on maize silage (Hristov *et al.,* 2004). Results of previous studies indicated that if the AIA content of diets were lower than 0.75 g/100 g, the nutrient digestibility predicted by using AIA may be biased (Thonney *et al*., 1985, Sunvold and Cochran, 1991). Thonney *et al*. (1985) stated that when a diet is high in grain, the low AIA content of the diet may cause variation in the samples collected which may cause biased results for AIA recovery. They also stated that when using AIA, the most suitable type of diets for using this marker is diets that are high in plant tissues like grass which is high in silica. Lee and Hristov (2013) reported that care should be taken when using AIA as a digestibility marker in TMR's as contamination from external sources like soil, which

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is high in silica, can cause biased results because soil will increase the ash content. Much of the AIA analysed is originating from silica (Thonney *et al*., 1985). The AIA content of maize in our study was 0.3 g/100 g which is higher as the value of 0.2 g/100 g as reported by Thonney *et al*. (1985). Results from this study did, however, show promising results for using AIA as a marker in feedlot diets. The recovery rates for the markers were determined by taking the amount of marker in the manure divided by the amount of marker in the feed (Marais, 2000). The AIA recovery rate in this study was 105.7% which is acceptable for predicting digestibilities and falls within the range of 90.1% to 105.9% previously reported (Van Keulen and Young, 1977, Thonney *et al*., 1979, Huhtanen *et al*., 1994).

Table 3: Internal marker content of the control diet and feed ingredients (DM basis)

Marker (%)	AIA	ADL
Basal diet	0.85	3.83
Maize	0.26	2.38
Eragrostis curvula hay	1.63	7.21
SB 100	2.41	4.23
Procon 33	0.38	6.65

The ADL recovery in this study was 126.7%. This is inconsistent with other research where recoveries of 82% were reported by Huhtanen *et al*. (1994) but Cochran *et al*. (1986) reported recoveries of between 52% and 116%. Differences in recovery rates depended on the nature of the diet as diets higher in ADL tend to show better recovery rates (Cochran *et al*. 1986). To accurately use ADL as a marker the diet should contain at least 6.0 g/100 g ADL (Van Soest, 1987). The dietary ADL content in this study was 3.8 g/ 100 g and is perhaps the reason for poor prediction of nutrient digestibility (Van Soest, 1987). Maize which makes up the bulk of the diet used in this study (60%) is low in ADL (2.4 g/100 g). Raffrenato *et al*. (2009) reported an average ADL content for various maize cultivars of 2.9 g/ 100 g and for the brown midrib cultivar an ADL content of 2.1

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g/100 g. The brown midrib cultivar is known for its lower lignin content and would thus have a higher digestibility than other cultivars (Raffrenato *et al*., 2009). The ADL content of a diet should thus be quantified before being considered as a digestibility marker. Acid detergent lignin should therefore not be used as a general marker but should be used in a typical high fibre diet with high ADL levels (Huhtanen *et al*., 1994).

The average recovery rate of Cr_2O_3 in this study was 100.5% and which is similar to other studies (Galyean *et al*., 1987, Jagger *et al*., 1992). Sampaio *et al*. (2011) reported recovery rates of between 94.4% - 104.7% when feeding diets containing grass silage, corn silage and concentrates at different levels. Delagarde *et al*. (2010) reported that Cr_2O_3 recovery is unaffected by the feeding level and it is able to predict faecal output accurately when compared to the total faecal collection technique.

The effect of the enzyme treatments on the nutrient digestibility is shown in Table 4 below. In this study at a dose rate of 30g of enzyme per day, the nutrient digestibilities between the control and treatment diets with Enzyme B did not differ for any of the nutrients ($P>0.05$). There was, however, an increase in the starch nutrient digestibility when Enzyme A was used. This can be explained by Cowieson (2005) who reported that the addition of exogenous fibrolytic and proteolytic enzymes to a high maize diet may lead to degradation of the pericarp and protein matrices of maize, which improves the accessibility of the rumen microbes to the starch (Cowieson, 2005; Young *et al*., 2012). It should, however, be interpreted with caution in this study as we did not see the same results in study one (chapter 3) where 60 g of the enzyme was dosed. Beauchemin & Rode (1996) reported that a higher application rate of enzymes does not necessarily increase animal performance with the effect depending on the substrate. The optimum enzyme level for any particular diet should be determined for individual enzyme preparations *in vivo* as the effects differ when being done *in vitro* (Romero *et al*., 2014).

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The same authors reported that when testing individual enzyme preparations *in vitro* the response of released reducing sugars to increased dosage rate is often linear, while this is not the case *in vivo*. More research is needed to assess the effect of the enzyme at different dosage rates. The effect of enzyme dosage level is not the focus of this study and will therefore not be elaborated on.

Table 4: The effect of the different enzyme treatments at a dosage rate of 30 g on apparent nutrient digestibility (g/100 g) based on total faecal collection

	Treatment			
Nutrient	**Control**	**Enzyme A**	**Enzyme B**	**SEM**
DM	70.5	73.6	71.3	2.1
Starch	93.6[a]	96.0[b]	93.5[a]	0.5
NDF	48.0	53.8	50.5	3.6
CP	65.3	69.2	67.1	2.4

DM-dry matter NDF- neutral detergent fibre CP-crude protein SEM- standard error mean
[a,b] Rows with different superscripts differ (P<0.05)

The total tract nutrient digestibilities as determined by total faecal collection and the external and internal markers are shown in Table 5:

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Table 5 Apparent total tract nutrient digestibilities (g/100 g) estimated by total faecal collection and external and internal digestibility markers*

	Marker				
Nutrient	**TC**	**Cr_2O_3**	**AIA**	**ADL**	**SEM**
DM	72.8[a]	72.3[ab]	76.3[a]	65.8[b]	2.4
Starch	94.7[a]	94.7[a]	95.3[a]	92.7[b]	0.5
NDF	51.7[ab]	51.0[ab]	58.4[b]	41.9[a]	4.1
CP	67.9[a]	67.4[ab]	72.3[a]	61.2[b]	2.7
Faecal output (kg DM/day)*	2.2[a]	2.3[ab]	1.9[a]	2.8[b]	0.2

TC- total collection, Cr_2O_3- chromium oxide, AIA- acid insoluble ash, ADL- acid detergent lignin
DM- dry matter, NDF- neutral detergent fibre, CP- crude protein, SEM- Standard error mean
[ab] Rows with different superscripts differ (P<0.05)
*Faecal output in kg DM/day was determined by total collection and predicted with calculations by using the external (Cr_2O_3) and internal markers (AIA and ADL).

The total tract nutrient digestibility between total faecal collection technique and Cr_2O_3 as a marker did not differ (P>0.05) for any of the nutrient (Table 5). This can be due to the recovery rate of 100.5%. These results suggest that Cr_2O_3 is suitable to be used as an external marker in digestibility studies with high maize diets such as typical in feedlot diets. The suitability of Cr_2O_3 as an external marker is supported by studies done by Galyean *et al*. (1987) and reviews published by Mayes *et al*. (1995) and Marais (2000). The previously mentioned authors reported that of Cr_2O_3 is easy to dose, and shows little diurnal variation when excreted. They also reported good recovery rates in most studies. It is also shown to be a suitable digestibility marker in other species like pigs (Jagger *et al*., 1992), dogs (Carciofi *et al*., 2007), goats (Yiakoulaki *et al*., 1997), dairy cattle (Ferret *et al*., 1999) and in horses (Vander Noot *et al*., 1967).

The total tract nutrient digestibility, when compared between total faecal collection and AIA, did not differ (P>0.05). For the estimation of total tract CP digestibility, the AIA

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estimation was numerically higher (72.3 g/100 g) when compared to TC (67.9 g/100 g). Similar results were observed with the DM (76.3 g/100 g) and NDF (58.4 g/100g) digestibilities. This can be due to the recovery rate that was 105.7% in this study. These results also suggest that AIA is suitable to be used as an internal marker in studies where high maize diets are fed like for feedlot cattle and feedlot lambs. Acid insoluble ash has successfully been used as internal marker in many studies and in different species like dairy cows (Lee and Hristov, 2013), beef steers (Thonney *et al*., 1985), horses (Bergero *et al*., 2004), sheep (Van Keulen and Young, 1977), pigs and poultry (McCarthy *et al*., 1974). Thonney *et al*. (1979) investigated whether AIA is suitable for estimating nutrient digestibility at different levels of concentrate feeding where concentrate levels ranged from 20% to 80%. They found that there was no significant difference between total faecal collection and AIA at different concentrate levels as long as the AIA content in the diet was above 0.75 g/100g. Thonney *et al*. (1985), however, reported that sampling error was a major cause of variation. They also reported that AIA is most suitable for diets that contain plant tissues like grasses as these tissues are high in silica that adding to AIA content (Thonney *et al*., 1985).

The total tract nutrient digestibility between total faecal collection and ADL differed ($P<0.05$) for all the nutrients analysed (Table 5) except for NDF digestibility. All total tract nutrient digestibilities were underestimated by using ADL as a marker compared to total faecal collection. The underestimation will probably be due to the high recovery rate (126.7%) of ADL. This indicates that ADL is not suitable for the use as an internal marker in studies where high maize diet feedlot diets are fed. This is in agreement with other studies (Sunvold and Cochran., 1991) where it was concluded that ADL delivers variable results and should be used with care. The lab results for the ADL analysed in the manure can be seen in appendix A. Note the high variation between samples of ADL analysed compared to for example the AIA analysed. Van Soest (1987) recommended

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that ADL should only be used if the diet contained at least 6 g/100g ADL on a DM basis. Results suggest that ADL is not a suitable marker to be used in any ruminant diet because of the large variation is seen in recoveries.

The basal diet used in this study only contained 3.5 g/100g ADL. The negative aspect when using ADL as a digestibility marker is inconsistent and variable recoveries as reported by Fahey and Jung (1983). Huhtanen *et al*. (1994) did, however, report that the use of different analytical methods to quantify lignin did not overcome the problems mentioned, namely incomplete and variable recoveries. However, Ferret *et al*. (1999) successfully used ADL in a study with dairy goats fed hay-based diets with little added concentrates. Those diets contained between 10-57 g/100 g ADL, confirming the recommendations by Van Soest 1987 that ADL can be considered as an internal marker in diets containing high levels of ADL such as roughage based diets. Huhtanen *et al*. (1994) also reported that ADL is suitable as an internal marker in silage based diets with different levels of concentrates added. Care should, however, be taken before this marker is used as a general internal marker for all types of diets. This is illustrated in Table 5 where the different total tract nutrient digestibility values predicted by all four methods namely total faecal collection, Cr_2O_3, AIA and ADL is shown. From this table, it is clearly demonstrated that ADL predicts different ($P>0.05$) values for all nutrients except NDF compared to total faecal collection. This has also been demonstrated in a study done by Kozloski *et al*. (2014) comparing ADL to n-alkanes and acid detergent fibre as well as Sunvold and Cochran (1991) who compared ADL to AIA, TC, acid detergent fibre and alkaline peroxide lignin.

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4.4 Conclusion

It can be concluded from this study that when compared to total faecal collection, Cr_2O_3 and AIA are suitable to be used as markers in high maize grain diets typically used in South African feedlot diets. The use of AIA is highly recommended since the analytical method to quantify this marker is easy to replicate and is accurate. It should, however, be noted that it is only recommended to use this marker when the marker content in the diet fed is above 0.75%. It is thus advisable to analyse the diet for AIA before potentially using the marker in digestibility studies.

Although there were no differences ($P<0.05$) in nutrient digestibilities when Cr_2O_3 were compared to total faecal collection, it is advised that Cr_2O_3 should be used with caution as Cr_2O_3 is a possible carcinogen. It is known that a chromium compound in the hexavalent state that is slightly soluble is one of the most potent carcinogens (Norset, 1981). Titanium oxide can be considered to be used as an alternative to Cr_2O_3 if the experiment dictates that an external marker should be used.

The results suggest that acid detergent lignin should not be used as an internal marker in high maize diets similar to South African feedlot diets. The recovery rate of ADL are highly variable and inconsistent and do not accurately predict the apparent total tract nutrient digestibilities. There is a need to find a more suitable analytical method to accurately quantify lignin in ruminant diets and more research is needed on this topic.

The use of specific internal and external markers, in general, is recommended as an alternative to the total faecal collection technique which is laborious and time consuming when performing apparent nutrient digestibility studies. Using a marker that

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has a relatively simple, safe and cost-effective laboratory analysis and yields accurate results can significantly reduce the cost when conducting *in vivo* digestibility studies.

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Appendix A.

Table A1 The ADL and AIA content (g/100 g) in the manure and feed as analysed by Nutrilab

	ADL %	AIA %
P1C1	13.5	5.0
P1C2	45.4	3.9
P1C3	49.9	4.0
P1C4	25.8	3.4
P1C5	14.7	3.6
P1C6	23.7	3.8
P2C1	21.6	3.2
P2C2	8.9	2.8
P2C3	10.9	3.4
P2C4	10.8	3.6
P2C5	25.1	3.6
P2C6	5.0	3.0
P3C1	5.9	3.5
P3C2	5.8	2.3
P3C3	6.3	3.9
P3C4	8.2	3.2
P3C5	7.8	3.6
P3C6	8.4	2.7
Feed batch 1	3.1	0.72
Feed batch 2	3.6	0.89

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