The Effect of an Exogenous Fibrolytic Enzyme on Forage Digestibility Parameters

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree

Signature Date

Abstract

Title	:	The effect of an exogenous fibrolytic enzyme on forage digestibility parameters
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The ruminant has the ability to utilize forages more efficiently than any other production animal. The utilization of forage fibre is an important aspect of ruminant production systems, as this is the main source of energy available to the animal. The availability of high-fibre forage nutrients is, however, restricted by cell wall degradability, and since low quality forages contribute a great deal to ruminant production systems worldwide, the improvement of this degradation process is of major economic importance.

The use of exogenous fibre degrading enzymes has been proposed as a means of enhancing this process, with positive results being obtained from *in vitro* studies incorporating exogenous enzyme preparations. Positive *in vivo* results with regard to forage digestibility and other animal production parameters have consequently also been obtained following the addition of exogenous fibre-degrading enzyme preparations to the ruminant diet.

Two initial screening experiments were undertaken in order to identify fungal enzyme preparations that may have a positive effect on *in vitro* fibre degradability. The initial screening employed an *in vitro* organic matter digestibility technique, and was successful in identifying at least six enzyme preparations displaying enhanced digestibility results that were statistically significant. A second *in vitro* gas production procedure was used to confirm results obtained from organic matter

digestibility assays, as well as to increase screening capacity in order to evaluate new enzyme preparations more time-efficiently. Statistical analysis of results obtained from the secondary screening identified various enzyme candidates producing promising results. Only one of these, Abo 374, proved to be statistically superior to the control and other enzyme preparations.

A growth trial was subsequently conducted to assess the performance of this enzyme *in vivo*. The trial involved individual feeding of 32 Dohne Merino ram lambs grouped according to weights into four groups consisting of 8 lambs each. Each group represented a specific application level of enzyme to the wheat straw component of a high fibre diet, amounting to 10, 5, or 1 ml enzyme supernatant/kg straw. The enzyme was diluted with water at appropriate rates to obtain an application rate of 300ml/kg straw. The fourth (control) group was treated with water at the same application rate. The trial was conducted over a period of six weeks, during which feed intakes, weekly weight gains, as well as feed conversion efficiencies were recorded. Results suggested significant weight gains in the high (10ml/kg) and medium (5ml/kg) treatment groups, indicated by a *P*-value of *0.04*. Similarly, feed conversion efficiencies were improved for above-mentioned groups ($P=0.05$), while feed intakes did not differ significantly between the four experimental groups.

Uittreksel

Titel	:	Die effek van 'n eksogene veselverterende ensiem op ruvoer-verteerbaarheid parameters
	:	
Naam	:	Liezel Goosen
Promotor	:	Prof. C.W. Cruywagen
Instansie	:	Departement Veekundige Wetenskappe, Stellenbosch Universiteit
Graad	:	M. Sc. Agric

Die herkouer besit die vermoeë om ruvoere beter as enige ander produksiedier te kan benut. Die gebruik van ruvoervesel is 'n belangrike aspek van herkouer produksiesisteme, aangesien ruvoere die hoof bron van energie aan die herkouer verskaf. Die beskikbaarheid van hoë-vesel ruvoer nutriënte word egter beperk deur die degradeerbaarheid van die selwand, en aangesien lae kwaliteit ruvoere 'n groot bydrae tot wereldwye herkouer-produksiesisteme maak, is die moontlike verbetering van hierdie degraderingsproses van groot ekonomiese belang.

In 'n poging om hierdie verteringsproses te help bevoordeel, is die gebruik van eksogene veselverterende ensieme ondersoek, en positiewe resultate is verkry wanneer hierdie ensieme in *in vitro* studies gebruik is. Goeie verbeterings ten opsigte van ruvoer verteerbaarheid en ander diereproduksie parameters is ook verkry deur middel van *in vivo* studies waar eksogene ensieme by die ruvoer van herkouers gevoeg is.

Twee eksperimente is onderneem in 'n poging om ensiempreparate wat 'n moontlike positiewe effek op *in vitro* veselvertering mag hê, te identifiseer. Die eerste, 'n *in vitro* organiese materiaal verteerbaarheid tegniek, was suksesvol in die identifisering van minstens ses ensiem preparate wat statisties betekenisvolle verbeterings ten opsigte van verteringsresultate geproduseer het. 'n Tweede *in vitro* gasproduksie

prosedure is vervolgens gebruik om resultate verkry vanaf die eerste tegniek, te bevestig, asook om evalueringskapasiteit te vergroot en sodoende, nuwe ensiempreparate meer tydseffektief te evalueer. Statistiese evaluering van resultate verkry uit die tweede *in vitro* tegniek het 'n reeks ensieme met positiewe resultate opgelewer. Een van hierdie, Abo374, het statisties betekenisvolle resultate ten opsigte van die kontrole, sowel as ander ensieme getoon.

In 'n volgende eksperiment is 'n groeiproef gedoen om die effektiwiteit van hierdie ensiem *in vivo* te toets. In die proef is 32 Dohne Merino ramlammers op grond van hul gewig in vier groepe van agt skape elk verdeel, en individueel gevoer. Die groepe het verskillende toedieningsvlakke van die toetsensiem, toegedien tot die koringstrooi komponent van 'n hoë-vesel dieët, ontvang. Toedieningsvlakke was 10, 5, of 1ml ensiemkonsentraat/kg strooi. Elke groep se ensiemkonsentraat is verdun met die toepaslike hoeveelheid water om 'n toedieningsvlak van 300ml ensiemoplossing/kg koringstrooi te verkry. 'n Vierde groep is behandel slegs met water teen dieselfde toedieningsvlak, en het gedien as 'n kontrole. Die eksperiment is oor 'n periode van 6 weke uitgevoer. Tydens die proeftydperk is voerinnames, weeklikse gewigstoenames, sowel as voeromsetverhoudings, gedokumenteer. Resultate het betekenisvolle gewigstoenames in die hoë (10ml/kg) en medium (5ml/kg) groepe opgelewer, aangedui deur 'n *P*-waarde van *0.04*. Voeromsetverhoudinge het ook verbeteringe getoon vir bogenoemde twee groepe ($P=0.05$), terwyl voerinnames nie merkbaar tussen die vier groepe verskil het nie.

Contents

This work is dedicated to my Parents, without whom, none of this would be possible

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CHAPTER 1

A: Introduction

1. Forages and the Ruminant

Ruminants are well adapted to degrade plant cell walls, which enable them to convert plant fibres into products that can be utilized by man. The economic implications of this phenomenon are obvious. Additionally, the importance of fibre in the ruminant diet is underlined when conditions such as rumen acidosis, parakeratosis and abscessed livers, resulting from a lack of intake of fibrous material, are considered. Improvements in the ability of the animal to maintain the degradation process is therefore a major consideration, especially as this usually also leads to improved animal performance (Krause *et al.*, 2003). In developing countries, where ruminants sometimes form the only source of income for the small farmer, forages often form the major part of the diet for these animals, and in most cases, is their only source of nutrition. This is even the case in the developed world, where, in many instances, naturally occurring forages constitute the major part of the ruminant diet and can provide nutrients at low cost (Wilkins, 2000). Forages thus make a significant contribution to the overall nutritional economy of meat-, wool and milk-producing ruminants (Beever & Mould, 2000).

Fibre content of forages has a marked influence on the nutritive value thereof. Fibre makes up the bulk of the plant and provides an important source of energy to the microbes of the rumen (Yang *et al.*, 2002). Form of the fibre also plays a large role in this regard, as coarse fibre is needed to sustain rumen function. Rate of digestion, mainly influenced by the coarseness of the fibre, is important since this would influence the amount of energy available to the animal per unit of time (Van Soest, 1994).

These, and many more considerations, has made the efficiency of fibre digestion by the ruminant the topic of research for over 100 years (Krause *et al.*, 2003). Although great progress has been made in the development of products to improve protein and energy utilization, relatively little has been achieved in the forage area (Cruywagen, 1999).

Fibre digestion in the ruminant is relatively slow and incomplete (Yang *et al.*, 2002), as can be proven by the fact that fibre recovered from faeces is still fermentable (Krause *et al.*, 2003). Also supportive of this fact, is the improvement seen in the digestion of this feed component after mechanical and/or chemical pre-treatments, and more recently, the improvement obtained from the modification of plant lignin composition through genetic manipulation (Krause *et al.*, 2003). The efficiency of plant cell wall degradation depends on the interaction between the rumen microorganisms producing fibrolytic enzymes, and the anaerobic fermentation conditions in the fermentation chamber as provided by the host animal. This symbiosis between animal and microbe within the rumen is responsible for the formation of a highly effective system to the benefit of both parties involved. The rumen is host to a large community of microorganisms that digest feed particles. Some of these have the ability to hydrolyze and ferment plant carbohydrate polymers indigestible to most animals, hereby forming fatty acids to be utilized by the ruminant as an important metabolic fuel, as well as providing microbial cells which are absorbed in the lower digestive tract as a major source of protein and amino acids. One disadvantage, however, is that the rumen microbes also break down dietary protein, rendering it unavailable to host enzymes (Hungate, 1984).

A better understanding of these processes will allow the nutritionist to more accurately formulate feeds to meet the needs of the animal. Much progress has been made in this regard. For instance, the realization of the need and importance for nitrogen in the diet to allow for the degradation of fibre by fibrolytic microorganisms, resulting in the addition of urea to ruminant diets (Krause *et al.*, 2003), the improvement in digestibility of forages by mechanical and chemical treatments (Wilkins & Minson, 1970), and the construction of computer models to predict animal performance from feed ingredient characteristics (Fox *et al.*, 1995).

2. Forage Classification

Various systems are available for the classification and description of forages. Classification systems relating to forage quality are as yet insufficient, and therefore, the development of an accurate and acceptable classification system is an ongoing process (Van Soest, 1994).

The term 'forage' is defined by the Forage and Grazing Terminology Committee (1991) as 'edible parts of plants, other than separated grain, that can provide feed for grazing animals or that can be harvested for feeding' (as cited by Wilkins, 2000). A wide variety of feeds are considered within this term. These include feeds listed in Table 1. The narrower term 'forage crop' is also used, and refers to crops which are to be utilized by grazing or harvesting as a whole crop, e.g. sorghum and maize (Wilkins, 2000).

Table 1. Feed types included within the definition of forage (from Wilkins, 2000)

Herbage	Leaves, stems, roots of non-woody species, including sown and permanent grassland and crops that may be grazed or cut
Hay and silage	
Browse	Buds, leaves and twigs of woody species
Straw	

Although a wide range of feeds can be classified as forages, most within this broad definition contain substantial amounts of cell walls, and are therefore suitable as sources of feed fibre for herbivores naturally equipped for the efficient utilization of plant cell wall constituents through microbial digestion (Buxton & Mertens, 1995;

Wilkins, 2000). Grass is generally the principle source of forage, while maize and cereal straws such as barley and wheat are also widely used (Beever & Mould, 2000).

3. Forage Quality and Nutritive Value

The term 'nutritive value' refers to those aspects of forage composition that affect nutrition, but are independent of voluntary intake (Fisher *et al.*, 1995). 'Forage quality' is defined as the relative performance of an animal when fed herbage *ad libitum* (Sollenberger & Cherney, 1995), and therefore, include aspects of both nutritive value as well as voluntary intake (Fisher *et al.*, 1995). The quality of forages is dependent on nutrient concentration, rate of consumption, digestibility of consumed forage, and partitioning of metabolized products in the animal (Buxton & Mertens, 1995). Forages vary considerably, both within and between types, where composition and nutritive value are considered, as indicated in Table 2. This indicates that a specific type of forage will have a major effect on its contribution to a production system (Wilkins, 2000). This diversity presents both opportunities and challenges where these foods are considered for use in ruminant diets.

Table 2. Range in nutrient contents of different classes of forages (from Wilkins, 2000)

	Metabolizable energy (MJ kg^{-1} DM)	**Crude protein (g kg^{-1} DM)**
Temperate grasses, hays and silages	7.0-13.0	60-250
Tropical grasses	5.0-11.0	20-200
Maize silage	10.0-12.0	60-120
Cereal straw	5.0-8.0	20-40
Root crops	11.0-14.0	40-130
Kale and rape	9.0-12.0	140-220

In the view of many scientists, forage utilization has become the next nutritional barrier for dairy herds to improve nutritional efficiency, with forage quality and the prediction thereof being the main issue (Cruywagen, 1999). This statement is supported by Van Soest (1994), stating that forage quality may be the most important factor to currently influence the productivity of the ruminant. It is thus the responsibility of the ruminant nutritionist, for both economical and ecological purposes, to improve forage and fibre utilization of the ruminant, especially in developing parts of the world (Van Soest, 1994).

According to Sollenberger & Cherney (1995), the two main factors that affect forage quality include forage nutritive value and forage intake.

3.1 Factors affecting the nutritive value of forages

Nutritive value of forage refers to the chemical composition, digestibility and nature of digested products thereof (Sollenberger & Cherney, 1995). The quality of forages varies with plant species and the specific part of the plant under consideration. Principle factors affecting the nutritive value of forages include age and maturity, soil fertility, climatic circumstances and management conditions; with herbage maturity having the greatest effect on forage quality within species (Buxton & Mertens, 1995).

3.1.1 Plant growth stage

Stage of plant growth is the most important factor influencing the composition and thus nutritive value of pasture herbage (Buxton & Mertens, 1995). Generally, the nutritive value of forages decreases as the plant matures. The extent to which this occurs is dependent on the specific forage, with maturity and environment both playing a role in this regard. Within the development of any plant, varying stages of maturity within its structure are reached, and this contributes to the net whole forage composition (Van Soest, 1994).

As the plant grows, fibrous tissues increase, thus increasing the main structural carbohydrates (cellulose and hemicellulose) and lignin (McDonald *et al.*, 1995). Lignin is indigestible, and increased lignification as a result of increasing age of the plant leads to a decline in its nutritive value. Because rumination occurs in proportion to the cell wall content of the diet (Van Soest, 1994), this is however essential, as unlignified material would lead to insufficient rumination. As the plant matures, protein concentration proportionally decreases, and therefore a reciprocal relationship exists between protein and fibre in the growing and aging plant. This fact contributes to a decreased digestibility as the plant increases in maturity (McDonald *et al.*, 1995). A decreased proportion of leaves to stems observed with increasing maturity is generally also a contributing factor in this regard (Van Soest, 1994). This is due to the fact that stems generally have a higher concentration of cell walls than leaves, while additionally, in most plants, becoming less digestible at a more rapid rate than leaves as the plant matures (Buxton & Mertens, 1995).

3.1.2 Climatic and management conditions

The effects of climate and season on forage quality can be listed in order of importance as follows; temperature, light, water, fertilization and soil (Van Soest, 1994). Generally, environmental effects have a greater effect on forage yield than on quality, and this environmental influence is much smaller than the influence of forage maturity. Environmental changes influence forage characteristics such as growth rate, developmental rate, yield, and herbage quality (Buxton & Mertens, 1995).

In looking at climatic and management conditions, for example; where ensiled forages are considered, post-harvesting procedures such as procedures for field wilting, additive application and ensiling conditions all play a role. For hays, the weather, length of curing period and degree of mechanical handling influence quality. For artificially dried forages, drying temperature is important (Beever & Mould, 2000).

3.2 Factors affecting forage intake

Intake of forages is an important consideration in feeding the ruminant, as 65%-75% of variation in energy intake can be related to its effects (Buxton & Mertens, 1995). Forage intake is positively correlated to the accessibility and acceptability of the forage, and negatively correlated to the time spent in the digestive system of the ruminant (Sollenberger & Cherney, 1995). According to Mertens (1993) (as cited by Buxton & Mertens, 1995), cell wall concentration, extent of cell wall digestion, and passage rate (along with particle size reduction rate) are the factors most important in affecting intake. Although a great determining factor in the production of the ruminant, intake is both difficult to predict and to measure. Although there is generally a positive correlation between intake and digestibility, many of these comparisons have also proved to be low and inconsistent over plant species and plant parts. In addition, intakes vary with physiological production stage of the animal, thus complicating the prediction of forage intakes further. Physical factors, physiological status of the animal and external conditions are the main factors influencing forage intake in the ruminant (Buxton & Mertens, 1995). These are discussed in more detail in the following section.

3.2.1 Physical factors

Passage rate is a factor closely related to intake, and is determined by factors such as particle size, particle density, cell wall concentration and composition (Buxton & Mertens, 1995), degradability, and hydration properties of the feed (Fisher *et al.*, 1995). Gut fill occurs when the animal eats to an extent where the rumen and/or lower intestines reach a constant fill, and particle size of the feed is too large to pass from the rumen, thus physically limiting any further intake. Especially in ruminants with a high demand for energy, this is a limiting factor regarding intake, when the forage in question has a large NDF content, thus limiting the energy available to the animal. This physical fill limitation is therefore connected to cell wall concentration, which in turn is linked to bulk, rate of particle size reduction, and rate of

disappearance (flow rate) of the specific feed through the rumen (Buxton & Mertens, 1995).

When considering the feed itself, physical form and composition of the cell wall will also affect intake. The mechanical grinding and reduction of particle size of roughages accelerates ruminal breakdown through enhanced access of microbes to the cell walls, and therefore increases intake by partially destroying the structural organisation of cell (Fisher *et al.*, 1995). The rate and extent of this effect will therefore depend on the specific cell wall in question. Some effect is noted between leaves and stems in this regard, where leaf cell walls are generally broken down more easily, causing animals fed on leaves to consume about 40% more dry matter per day than those fed a higher percentage of stems (Mcdonald *et al*, 1995).

As mentioned previously, plant maturity has an influence on forage nutritional quality, with nutritional value generally declining with age. Maturity of forages also plays a role in regulating intake, as voluntary intake generally decreases with plant age. This situation is probably related to physiological fill, as plant cell wall concentrations increase with increased maturity. Similarly, leafy parts of the plant are generally consumed in greater quantities than stems because of a higher cell wall concentration in the latter (Buxton & Mertens, 1995).

3.2.2 Animal physiological status

The energy requirement of the animal varies with physiological stage, e.g., growth, maintenance, pregnancy or lactation etc. Physiological control of feed intake is a complex subject which, in part, refers to the situation that occurs when animals adjust their intake to satisfy their demand for energy. Intake would therefore be limited in a situation where an animal with a low energy requirement was fed a diet high in available energy and low in NDF concentration, therefore resulting in the animal, rather than the forage, regulating intake (Buxton & Mertens, 1995).

3.2.3 External factors

Intake can be influenced by external stimuli such as palatability, feed flavour, feed pH, social interactions, stress, diseases and management (Buxton & Mertens, 1995). Additionally, in grazing animals selection can, to an extent, affect the quality of the diet being consumed (Fisher *et al.*, 1995).

4. Dietary Fibre and the Plant

4.1 Definition of dietary fibre

Over the years, many definitions have been assigned to the term 'dietary fibre'. The term was originally shorthand for non-digestible constituents of the plant cell, being defined as "the skeletal remains of plant cells in the diet, which are resistant to hydrolysis by the digestive enzymes of man". This definition did not include polysaccharides such as food additives (e.g. plant gums and modified cellulose) added to the diet. Therefore, the definition was later revised to read, "all polysaccharides and lignin, which are not digested by the endogenous secretions of the human digestive tract" (Knudsen, 2001). This definition however included substances not of cell wall origin, which, in essence, should not be classified as fibrous. These substances are water-soluble, and therefore have a different action in the digestive tract than that of the insoluble neutral-detergent fibre. Pectin is an example of such a substance, as; despite being a cell wall constituent, it is easily separated from the cell wall structure and is readily fermentable (Van Soest, 1994). The term, 'dietary fibre complex' was used by Van Soest (1994) to describe all substances resistant to mammalian digestive enzymes, even though the mostly insoluble cell wall represents the only true fibre.

Researchers currently make use of either a physiological or a chemical definition, since, despite extensive research, no consensus as to a universal definition has been reached (Knudsen, 2001). Physiologically, dietary fibre is seen as "the dietary components resistant to degradation by mammalian enzymes", while chemically, it

refers to "the sum of non-starch polysaccharides and lignin" (Theander *et al.*, 1994). In most recent animal literature, dietary fibre is used to describe cell wall or storage non-starch polysaccharides (NSP) and lignin (Knudsen, 2001).

4.2 Chemistry and organisation of fibre in the plant cell

4.2.1 Plant carbohydrates

Dietary fibre is a complex mix of carbohydrate polymers associated with various other non-carbohydrate components (McDougall *et al.*, 1996). The non-structural (NSC) or non-fibre (NFC) carbohydrates, along with neutral detergent fibre (NDF), make up the two main classes of carbohydrates in ruminant diet formulation. Neutral detergent fibre is measured by chemical analysis, while the NSC/NFC value is calculated. The NFC fraction consists of many fractions that digest at different rates, produce different products of digestion and are difficult to separate analytically (Hall, 1998). Based upon location in the plant cell and individual nutritional characteristics, the variety of carbohydrates soluble in neutral detergent would better be referred to as neutral detergent-soluble carbohydrates (NDSC) rather than NSC or NFC.

Figure 1 shows the NDSC to include both structural and non-structural, as well as fibre and non-fibre carbohydrates. These include organic acids, sugars, oligosaccharides, starch, fructans, pectic substances, (1→3)(1→4) – ß – glucans plus other carbohydrates of similar solubility.

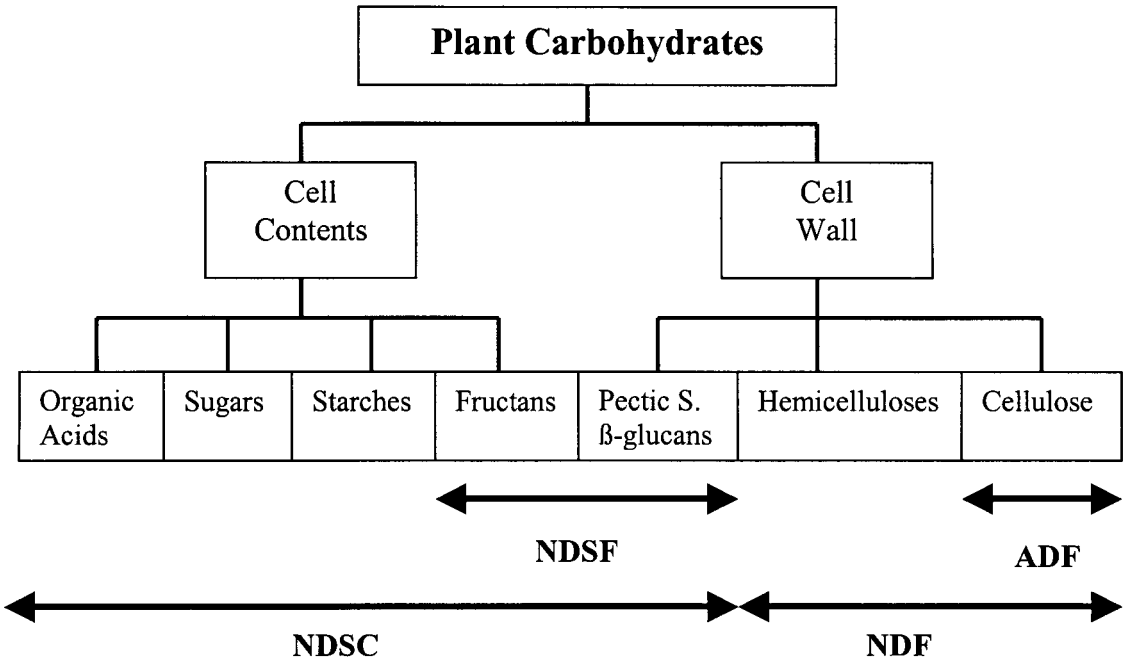

Figure 1. Plant Carbohydrate Fractions (Hall, 1998)

One way of classifying NDSC's is by dividing the fraction into that digested by the animal vs. that digested by ruminal microbes. The only carbohydrate linkages able to be hydrolized by mammalian enzymes are those contained in sucrose and starch. All other polymerised carbohydrates are digestible only by microbes.

NDSC is divided into fibre and non-fibre components. The latter group of carbohydrates consists of organic acids, sugars and starches, while the fibre fraction includes fructans, pectic substances, and (1→3)(1→4) – ß – glucans. Oligosaccharide classification depends on composition and linkages (Hall, 1998).

4.2.2 Cell wall polysaccharides

Forages can be divided into two main sections, namely the structural components of the cell wall, and the cell contents (Fisher *et al.*, 1995). Dietary fibre is predominantly found in the complex plant cell wall made up of a series of polysaccharides (Theander *et al.*, 1989; Knudsen, 2001), which consists of the phenolic polymer lignin, cellulose,

hemicellulose, pectin, protein, lignified nitrogenous substancces, waxes, cutin and mineral components (Van Soest, 1994).

Plant polysaccharides are broadly separated into two chemically defined types; the storage polysaccharide starch (α-glucan) and cell-wall polysaccharides (non-α-glucan). The building blocks of the cell wall polysaccharides are the pentoses (arabinose and xylose), the hexoses (glucose, galactose and mannose), the 6-deoxyhexoses (rhamnose and fucose), and the uronic acids (glucuronic and galacturonic acids). The polysaccharides mainly found in the plant cell wall include cellulose, arabinoxylans, mixed linked β(1-3)(1-4)-D-glucan (β-glucan), xyloglucans, rhamnogalacturonans and arabinogalactans (Theander *et al.*, 1989). Fibre composition varies according to the type of plant cell wall being considered (Van Soest, 1994).

The cell wall polysaccharides are referred to as NSP (non-starch polysaccharides) (Englyst, 1989). NSP comprises 700-900g kg^{-1} of the plant cell wall, the remaining being made up of lignin, protein, fatty acids, waxes etc. Plant cell wall NSP consists of a diverse group of molecules, displaying varying degrees of water solubility, size and structure (Knudsen, 2001). NSP is subdivided into soluble and insoluble fractions. The soluble fraction is soluble in water, and includes gums, pectins, mucilages and certain hemicelluloses. The insoluble fraction includes cellulose and the majority of the hemicelluloses (McDonald *et al.*, 1995).

NSP plus lignin are considered the major components of cell walls. Dietary fibre can be measured using one of two types of analyses, namely enzymatic-gravimetric and enzymatic-chemical methods. The enzymatic-gravimetric method attempts to isolate gravimetrically the part of the diet resistant to breakdown by digestive enzymes (Englyst, 1989), and gives no details as to polysaccharide type (McDonald *et al.*, 1995). The Association of Official Analytical Chemists (AOAC) method is based on this technique (Englyst, 1989). The enzymatic-chemical method removes starch enzymatically and measures dietary fibre in terms of its chemical constituents as NSP (Englyst, 1989), identifying individual carbohydrates in the diet (McDonald *et al.*, 1995). A difference between these two measurements of dietary fibre is that some

starch may be present in the AOAC procedure due to the fact that it is made resistant to hydrolysis by food processing with amylase. Dietary fibre measured as NSP by the enzymatic-chemical method contains no starch and is not affected by food processing, therefore making it possible to calculate the amount of fibre in processed foods and mixed diets from amounts of raw products in the food (Englyst, 1989).

It has become clear that starch included in the AOAC method only represents a small proportion of that escaping small intestinal digestion, leading to a proposal to classify starch based on its digestibility. Dietary fibre has always been seen as cell-wall polysaccharides, while the inclusion of starch therein was not considered. Defining dietary fibre as NSP provides a good index of the plant cell-wall polysaccharides, correlates to the original concept of dietary fibre, and is chemically precise (Englyst, 1989).

4.2.3 Lignin

Another major constituent of cell walls is lignin, which is described as very branched networks made up of phenylpropane units (Theander *et al.*, 1989). Lignin serves the dual purpose of anchoring the cellulose microfibrills and other matrix polysaccharides, as well as providing structure and rigidity to the walls to prevent physical and biochemical damage (Knudsen, 2001). It is seen as the most influential fibre component where nutrient availability of a feed is concerned (Van Soest, 1994), and is a major limiting factor regarding microbial degradation of the plant cell wall, especially regarding its association with structural polysaccharides (Nakashima *et al.*, 1988), where cross-linkages between lignin and cell wall polysaccharides could have a large effect on the digestibility of the specific polysaccharide (Buxton & Mertens, 1995). Statistically, the digestibility of a fibre fraction is dependent on its association with lignin. If this association is low, fibre content will be a poor predictor of digestibility (Van Soest, 1994). Lignin is seen as being virtually indigestible, and is found in forages in concentrations ranging from 3% to 12% of forage dry matter (Buxton & Mertens, 1995).

4.2.4 Implications

Cell wall composition varies between plants, as well as within plants, depending on the specific plant tissue under consideration. Maturity of the plant at harvest also has an effect in this regard (Knudsen, 2001, McDougall *et al.*, 1996). The physio-chemical properties of the cell wall polysaccharides are determined by their physical and chemical location within the cell wall. This influences their action in the gastrointestinal tract (Knudsen, 2001). Whereas plant cell-contents is almost completely available to livestock, the cell wall is rarely totally digestible. The cell wall contains a large proportion of the plant resistant to digestive enzymes of the mammalian intestine, and its availability is dependent on its composition and structure.

The two main factors used to determine the nutritive value and characteristics of feeds and forage when considering its biological components, are the proportion of plant cell wall and the degree of lignification. The cell wall can in some instances represent more than 50% of the organic matter in the forage, depending on its chemical composition and structure, indicating that cell wall concentration can have a large effect on the digestibility of a forage (Buxton & Mertens, 1995). It is found, however, that it is associations within the cell wall, rather than the proportion of individual components, which determine its digestibility. The physical properties of dietary fibre interact with its chemical properties, thereby determining physiological effects of the feed in question (Knudsen, 2001). Therefore, no single chemical analysis is sufficient in describing the biodegradability of the cell wall by microorganisms found within the rumen (Van Soest, 1994).

5. Chemical Analysis of Forage Fractions

Feed evaluation systems are designed to measure the capacity of the feed in question to sustain animal production and to supply in the nutritional demands of the specific animal production class (Beever & Mould, 2000). Forage quality is best evaluated

through animal performance. This is done by measuring characteristics such as intake, digestibility, and efficiency of utilisation. Of these, variation in intake accounts for 60-90% of variation in digestible energy available to the animal. Characteristics relating to intake and digestibility should therefore be measured routinely as indices of nutritional value (Cherney, 2000).

Chemical fractions associated with intake and digestibility include fibre, lignin and protein. As these are the main factors in determining nutrient supply, and thus animal performance (Mould, 2003), routine analysis of forages should therefore include determination of these, as well as an accurate dry matter (DM) determination. Determination of additional factors such as water-soluble carbohydrates, starch and soluble fibres etc. is dependent on the desired objectives of the specific research (Cherney, 2000).

Nutrient availability of a feed is determined by its chemical constitution. This refers to concentrations of present components, as well as to inhibitors and structures that may have an effect on their availability. Unlike *in vitro* methods, chemical analyses provide valuable information regarding the actual chemical constituents that influence digestion, thereby giving a better insight into the factors that may limit animal performance. They do not estimate nutritive value directly, but measure digestibility and intake through statistical association between the analysed components and feed quality (Van Soest, 1994). These statistical associations are being used increasingly, along with the use of models, to characterise forage fibre, lignin, protein as well as other chemical components and factors which may be limiting to the animal, in order to predict animal performance (Cherney, 2000). They provide a relatively simple and economical means of estimating animal responses to a specific feed source, as well as being a method of routine evaluation to control the quality of these feeds (Van Soest, 1994).

The realization that forage fibre content and composition plays a major part in forage quality and therefore could be used to predict intakes as well as nutritive value of a feed, sparked the need for a comprehensive system of forage fibre analysis in the laboratory. The development of such a system of analysis is complicated by the fact

that fibre is not nutritionally, chemically or physically uniform, and in addition, varies according to rumen size, intake, production level, and particle size of the fibre source (Van Soest *et al.*, 1991).

The use of acid- and neutral detergents by Van Soest & Wine (1967) provided a routine method of determining fibre in its individual fractions, namely acid detergent fibre (ADF) and neutral detergent fibre (NDF). Prior to this, the determination of feed fibre content extended to the estimation of crude fibre (CF) by the Weende system of feedstuff evaluation.

These fibre classification systems are discussed in the section that follows.

5.1 Proximate analysis

The proximate analysis procedure, or Weende classification system, has been in use for almost 150 years. It divides a food into six fractions; namely moisture, ash, crude protein, ether extract, crude fibre and nitrogen-free extractives. (Fisher *et al.*, 1995).

The carbohydrate of a food is contained in two fractions. These are crude fibre (CF) and nitrogen-free extractives (NFE). Crude fibre is determined by subjecting the residue from ether extraction to successive treatments with boiling acid and alkali of predetermined concentration. The organic residue represents the crude fibre. The sum of the amounts of moisture, ash, crude protein, ether extract and crude fibre (expressed in g/kg), subtracted from 1000, produces a difference representing the nitrogen-free extractives (NFE). The crude fibre fraction contains cellulose, lignin and hemicelluloses. A variable proportion of these can however also be found in the nitrogen-free extractives. The nitrogen-free extractives fraction is a heterogeneous mixture of all components not determined in the other fractions, and includes sugars, fructans, starch, pectins, organic acids and pigments, as well as the components mentioned above (Cherney, 2000).

The Weende procedure is simple, repeatable and relatively inexpensive, but several problems associated with the method caution against its use. It has been criticised as being imprecise, especially where crude fibre, ash and nitrogen-free extractives is concerned (McDonald *et al.*, 1995). For instance; ether extraction contains waxes and certain other compounds considered indigestible, while not recovering soaps in faeces, the main form of undigested excreted fatty acids (Van Soest, 1994). The crude protein determination assumes that all nitrogen (N) is contained in protein with an N content of 16%, which is inaccurate, since nitrogen from sources other than protein, such as free amino acids, amines, and nucleic acids is included in the determination (McDonald *et al.*, 1995; Cherney, 2000; Mould, 2003). Crude fibre also does not provide an accurate representation of the least digestible fibrous part of the feed in many cases (Sollenberger & Cherney, 1995), and similarly, nitrogen-free extractives (NFE) are not always a reasonable estimate of the highly digestible carbohydrate fraction, with crude-fibre digestibility exceeding nitrogen-free extract digestibility in about 30% of feedstuffs (Van Soest, 1994). The most serious error however, is the division of carbohydrates into crude fibre and NFE. In doing this, it is assumed that crude fibre contains all dietary cellulose, hemicellulose and lignin (Cherney, 2000), whereas varying amounts of lignin, hemicellulose and even cellulose may be solubilized and lost in the crude fibre preparation depending on the plant species in question (Van Soest, 1994). The division of carbohydrates into CF and NFE is inaccurate, and therefore is not, and should not be routinely used (Cherney, 2000; Van Soest, 1994).

5.2 Van Soest analysis

The proximate analysis procedure has therefore been replaced with alternative procedures developed by Van Soest, specifically for fibre rich feedstuffs. The method determines fibre fractions according to their degradability as those insoluble in neutral detergents (NDF: neutral detergent fibre) and those insoluble in acid detergents (ADF: acid detergent fibre), hereby creating the possibility of predicting intake and nutritive value of the test substrate (Mould, 2003). NDF measures hemicellulose, cellulose and

lignin, and ADF cellulose and lignin, so that hemicellulose is determined by difference (Knudsen, 2001).

The digestibility of a food depends on its chemical composition. The fibre fraction of a food has the greatest influence on digestibility, and both chemical composition as well as amount of fibre is important. In food analysis, the goal is to distinguish between cell wall fractions and cell contents. Cell contents are almost completely digested, while cell wall digestibility is much more variable and depends on the degree of lignification (lignin content of ADF), as well as on the structure of plant tissues (McDonald *et al.*, 1995).

5.2.1 Neutral detergent fibre (NDF)

In forages treated with boiling neutral detergent solutions of sodium lauryl sulphate and ethylenediaminetetraacetic acid (EDTA), cell contents dissolve, and a residue representing the plant cell wall remains (NDF). This is made up mainly of lignin, cellulose and hemicellulose, although minor cell wall components such as protein, bound nitrogen, minerals and cuticle can also be present. The cell wall component, pectin, is removed (Van Soest, 1994). This remaining cell wall fraction is subdivided into hemicellulose, which can be extracted using acid detergent solution, and cellulose plus lignin (ADF).

The NDF procedure was mainly developed for forages, but can be used for starch-containing foods if an amylase treatment is included (McDonald *et al.*, 1995). The inclusion of heat-stable α-amylase to assist in the removal of starch is recommended by Van Soest *et al.* (1991), as starch contamination of NDF, particularly in forages containing grain, can lead to an overestimate of the NDF value (Robertson & Van Soest, 1977; Beever & Mould, 2000). Cherney *et al.* (1989) reported that insufficient removal of starch from a sample often leads to difficulties with filtration, resulting in elevated NDF values. The extent of starch contamination is dependent on both the amount and type of starch found in the original feed sample (Beever & Mould, 2000).

Sodium sulphite was originally added to reduce nitrogenous contamination of the fibre. Insoluble indigestible animal proteins (or keratins), can be removed from the NDF fraction using sodium sulphite (Van Soest, 1994). The use thereof is however sometimes questioned because of the possibility to solubilize lignin (Van Soest *et al.*, 1991). The added use of sodium sulphite in the NDF procedure is still recommended for high protein forages, as this decreases nitrogen concentration in fibre and lignin values, and reduces within-sample variance.

The NDF fraction is a measure of cell wall content. This means that the energy available to the animal from a specific feed can be expressed as being inversely related to the proportion of NDF (or cell wall), found within the forage (Buxton & Mertens, 1995). NDF is also the chemical component of a feed that determines rate of digestion, with a negative relationship existing between NDF content and digestion rate. This causes feeds of equal digestibility, but differing in NDF content, to promote different intakes. Examples of these include grasses and legumes (McDonald *et al.*, 1995).

When all fibre fractions are considered, NDF relates most accurately to rumination, fill, passage and feed intake, and represents the total insoluble fibre matrix (Van Soest, 1991). It provides an estimation of the forage fraction which must first be degraded by microorganisms of the gastrointestinal tract in order to be metabolised by the ruminant (Fisher *et al.*, 1995). NDF is therefore the most important fibre fraction to take into consideration in ruminant feed analysis. The maximum NDF (or cell wall) content of a diet which will not negatively influence production in the ruminant is as high as 700-750 g NDF/kg DM for mature beef cows, 150-200 g NDF/kg for growing or fattening ruminants, and 270-290 g NDF/kg for high producing dairy cows in order to provide adequate energy while still containing enough fibre (Buxton & Mertens, 1995).

5.2.2 Acid detergent fibre (ADF)

The acid detergent fibre (ADF) analysis procedure is intended to be used as a preparative evaluation producing a residue which can be further used for the

determination of cellulose, lignin, Maillard products, silica, acid-insoluble ash and acid-detergent-insoluble N (ADIN) (Cherney, 2000). The ADF analysis divides the truly indigestible feed components, accumulated in the NDF procedure, into those fractions soluble and insoluble in 1 N acid (Van Soest, 1994). The ADF fraction represents the residue after refluxing with 0.5 M sulphuric acid and cetyltrimethyl-ammonium bromide and contains the crude lignin and cellulose fractions of plant material, while sometimes also including silica. It does not however include all cell wall constituents, as hemicellulose is soluble in the solution (Fisher *et al.*, 1995).

ADF determination is particularly useful for forages because of a good statistical correlation with digestibility (Van Soest, 1994; McDonald *et al.*, 1995), and is widely used to substitute for crude fibre as part of the proximate analysis procedure (Van Soest, 1994). Contamination with pectin can however inflate ADF values, especially in high pectin feeds such as citrus pulp and beet pulp. In these situations, sequential analysis of samples for NDF, followed by ADF, eliminates this problem (Hall, 1997).

5.2.3 Modified acid detergent fibre (MADF)

Even though statistical associations are noted in some instances, there is no valid theoretical basis to link ADF to digestibility (Van Soest *et al.*, 1991). The modified ADF (MADF) procedure of Clancy & Wilson (1966) (as cited by Van Soest, 1994) attempts to modify the ADF procedure in order to correlate better with forages of which the digestibility is known, so as to improve the predictability of the procedure. It differs from the Van Soest ADF procedure in that a slightly higher acid concentration (0.5 mol l^{-1}) and longer boiling time is used (Cherney, 2000). It has been shown that prolonged boiling with acid of higher concentration reduced bound nitrogen and improved the relationship between fibre and digestibility (Van Soest, 1994; Cherney, 2000). A preliminary step of drying the sample at 95°C however makes the sample unavailable for assaying for heat damage and unavailable protein, which is an important ADF application (Van Soest, 1994). Where heat-treated feeds are subjected to the ADF procedure, potential contamination from heat-damaged proteins, which have a low solubility in detergent solutions, should be considered so as not to overestimate the ADF fraction (Beever & Mould, 2000).

The Van Soest system of fibre analysis has become the primary standard for fibre evaluation of forages in the USA, with the resultant classification system for forage presented in Table 3.

Table 3. Classification of forage fractions using the detergent methods of Van Soest (Van Soest, 1967)

Fraction	***Components***
Cell contents (soluble in neutral detergent)	Lipids Sugars, organic acids and Water-soluble matter Pectin, starch Non-protein N Soluble protein
Cell wall constituents (fibre insoluble in neutral detergent)	
1. Soluble in acid detergent	Hemicelluloses Fibre-bound protein
2. Acid detergent fibre	Cellulose Lignin Lignified N Silica

The system separates carbohydrates into fractions based on nutritional availability (Sollenberger & Cherney, 1995). This results in a more reasonable estimate of the structural carbohydrates than does crude fibre (Figure 2), and allows for the prediction of other indices of forage quality, such as digestibility (Van Soest, 1994).

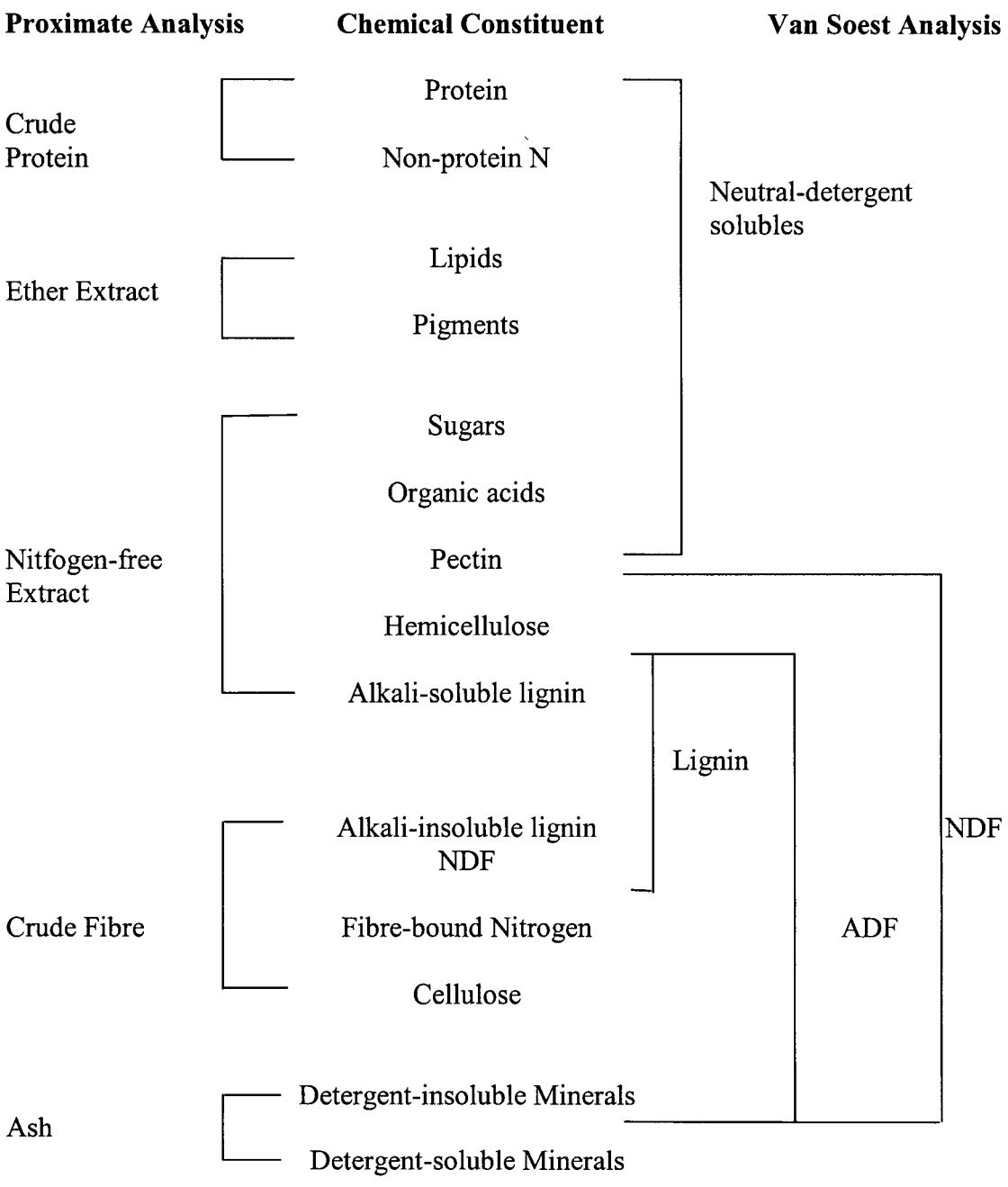

Figure 2. Contrast of Weende system and Van Soest system of carbohydrate analysis (Cherney, 2000).

The system does not divide feeds into chemically pure fractions. It is however, the nutritional uniformity of the fraction that is important (Cherney, 2000).

It is often found that chemical analyses do not correlate highly with digestibility results obtained from *in vivo* studies, and that microbial and enzymatic methods provide more comparable results (Van Soest, 1994). This is due to the fact that different forages, although chemically unique, may still have more or less the same *in vivo* digestibility; whereas samples identical in chemical composition (e.g. NDF) may differ significantly when fed to the animal (Cherney, 2000). This fact supports the continued use of *in vitro* and *in situ* methods (Van Soest, 1994). Two examples of these will be discussed in the following chapter.

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B: Fibrolytic enzymes in ruminant nutrition

1. Introduction

Dietary fibre is a major source of energy for the ruminant. However, its relatively slow and incomplete utilization in the rumen greatly limits animal production responses, and decreases productivity (Yang *et al.*, 2002). This is mainly due to a limited digestibility of forage plant cell walls, thus limiting the amount of available energy intake by the animal (Beauchemin *et al.*, 2004). With the plant cell wall comprising 40 to 70% of forage dry matter, and cell wall digestibility in the total digestive tract generally being less than 65%, even under ideal feeding conditions, the efficiency of forage utilization has become a major economic issue for ruminant production systems (Van Soest, 1994). This situation is accentuated under conditions sub-optimal for fibre digestion, such as when animals are fed high grain diets. Under these conditions, the total tract cell wall digestion could be as low as 50%, with ruminal digestion only contributing 35% to this total (Beauchemin *et al.*, 2001).

The use of fibrolytic enzymes in ruminant diets has sparked the interest of nutritionists for many years. Positive responses with regard to nutrient digestion and animal performance have been observed following exogenous enzyme additions (Beauchemin *et al.*, 1995, Lewis *et al.*, 1996, Krause *et al.*, 1998), with increases in dry matter and fibre digestion being obtained from *in situ* or *in vitro* trials (Feng *et al.*, 1996; Yang *et al.*, 1999; Columbatto *et al.*, 2003), as well as *in vivo* experiments (Feng *et al.*, 1996; Krause *et al.*, 1998; Rode *et al.*, 1999; Yang *et al.*, 1999).

Results following enzyme supplementation have however been inconsistent (Yang *et al.*, 1999; Nsereko *et al.*, 2000, Columbatto *et al.*, 2003c), with a combination of factors playing a role in creating this variability. These include factors such as diet composition, enzyme activity, diet component to which the enzyme is added (Krause *et al.*, 1998, McAllister *et al.*, 1999), type and level of enzyme applied, enzyme stability, method of enzyme application (Yang *et al.*, 2002), as well as physiological

status (Columbatto *et al.*, 2003b) and energy balance of the test animal (Beauchemin *et al.*, 2004b).

2. Enzyme Mode of Action

Theoretically, the use of exogenous enzymes could improve cell wall digestion in the ruminant by two possible means. Firstly, by the addition of products containing enzyme activities naturally limiting within the rumen; and secondly, by the use of enzyme products in situations where low rumen pH inhibits efficient fibre degradation through the depression of fibrolytic ruminal bacteria, such as when high-energy diets are fed (Morgavi *et al.*, 2000a; Beauchemin *et al.*, 2004).

More positive results are expected from high producing animals compared to those on maintenance energy level diets, as high energy levels cause a decrease in rumen pH while increasing intakes and passage rates. This provides an opportunity for exogenous enzymes to maintain high digestibility through improvements in rates of feed digestion, hereby overriding these factors (Beauchemin *et al.*, 2004a,b). Contrary to previous assumptions however (Lewis *et al.*, 1996), the generally lower optimal pH of exogenous enzymes as compared to endogenous ruminal enzymes does not override the negative effects on fibre digestion caused by a low rumen pH, such as when animals are fed high concentrate diets (Columbatto *et al.*, 2003a).

The mode of action of enzyme additives in the ruminant is as yet unknown (Morgavi *et al.*, 2000a; Yang *et al.*, 2002, Columbatto *et al.*, 2003a,b,c), but increases in total ruminal microbial population (Feng *et al.*, 1996; Nsereko *et al.*, 2002) as well as increased microbial protein synthesis (Rode *et al.*, 1999), have been reported. Hristov *et al.* (1998) postulated that an increase in fibre digestibility would enhance microbial protein synthesis. This would stimulate the total microbial population, thus leading to an increase in digestibility of the feed (Yang *et al.*, 1999).

Some research suggests that plant cell wall degrading enzymes stimulate fibre digestion in the rumen by enhancing enzymic activities. This effect was shown in *in vitro* trials conducted by Wallace *et al.* (2001). Additionally, trials have shown

increased degradation rate in the rumen (Yang *et al.*, 1999) while other enzymes have been shown to survive ruminal degradation, thus suggesting the possibility that exogenous fibrolytic enzymes could act both ruminally and postruminally (Gwayumba & Christensen, 1997; Hristov *et al.*, 1998).

Most commercial enzyme products consist of protein mixtures displaying several enzymic activities (Columbatto *et al.*, 2003c). Commercial preparations are, in addition, not clearly identified, thus complicating the identification of possible modes of action (Columbatto *et al.*, 2003b,c). Similarly, no standard method of enzyme application has as yet been identified to eliminate inconsistencies (Nsereko *et al.*, 2000). As forage is fed to ruminants in a variety of ways, from grazing forages to forage within a total mixed ration, the evaluation of various methods of enzyme delivery to the animal warrants some investigation (Lewis *et al.*, 1996).

Possible modes of action are discussed in the following section.

2.1 Pre-treatment effects

The treatment of feeds with fibrolytic enzymes prior to feeding has improved animal performance of dairy cattle (Rode *et al.*, 1999; Yang *et al.*, 1999; Kung *et al.*, 2002), beef cattle (Beauchemin *et al.*, 1995, McAllister *et al.*, 1999), and sheep (Pinos-Rodriguez *et al.*, 2002), and is seen by some nutritionists as a pre-requisite for any improvement in animal performance (Lewis *et al.*, 1996).

According to Beauchemin *et al.* (2004a), enzymes are most effective when added to the feed in liquid form prior to feeding. Spraying the enzyme on to the feed source may partially prevent the ruminal degradation of fibrolytic enzymes, as binding to the substrate could protect them from proteolytic inactivation in the rumen, thus making them more stable in the rumen environment (Fontes *et al.*, 1995; Beauchemin *et al.*, 2004a,b).

Conformational changes in the feed resulting from pre-feeding addition may have the same effect in preserving these enzymes (Fontes *et al.*, 1995). These structural feed

changes could also, in themselves, have a positive effect on degradation of the feed in question, as shown by Nsereko *et al.* (2000) in an experiment designed to eliminate all factors other than structural changes possibly influencing digestion characteristics. In this experiment, *in vitro* NDF degradation was improved following enzyme pre-treatment, with observed improvements being accentuated by longer pre-incubation times.

Exogenous enzyme addition prior to feeding is responsible for the release of reducing sugars and soluble carbohydrates (Beauchemin *et al.*, 2004a,b), with the degree of sugar release depending on type of feed and enzyme involved (Krause *et al.*, 2003). The partial solubilization of NDF and ADF has also been documented in some instances (Gwayumba & Christensen 1997; Hristov *et al.*, 1998). While this suggests an increase in the soluble feed fraction, as well as supporting evidence for improved rate of digestion and degradation from short-term *in situ* and *in vitro* incubations (Feng *et al.*, 1996; Beauchemin *et al.*, 2004a,), improvements regarding extent of *in situ* or *in vitro* degradation have not been observed in most instances (Yang *et al.*, 1999; Beauchemin *et al.*, 2004a). This suggests that only substrates which would normally be digested by rumen microbial enzymes are affected by the addition of exogenous enzymes to the diet (Krause *et al.*, 2003; Beauchemin *et al.*, 2004a).

2.2 Direct feeding effects

According to Beauchemin *et al.* (2004a), positive production responses observed from the addition of exogenous enzymes is most likely the result of post-ingestive effects.

Direct hydrolysis of feed within the rumen is a possible mode of action of exogenous feed enzymes (Morgavi *et al.*, 2000), providing evidence against the need for a pre-feeding incubation period of enzyme on a specific feed component (Beauchemin, 2004, Pinos-Rodriguez *et al.*, 2002), as well as supporting previous findings of improved fibre digestion when adding an exogenous fibrolytic enzyme to the concentrate component of the diet (Rode *et al.*, 1999; Yang *et al.*, 2000).

Direct feeding of fibrolytic enzymes have provided positive animal responses, and whereas it was previously thought that proteolytic activity in the rumen would inactivate direct-fed enzyme additives (Chesson, 1994), these positive results suggest a greater enzyme additive stability in the rumen than assumed in the past (Hristov *et al.*, 1998; Morgavi *et al.*, 2000b), hereby supporting the hypothesis surrounding increased hydrolytic activity within the rumen.

Enzyme stability in the rumen is reportedly linked to enzyme origin and type of enzyme activity (Morgavi *et al.*, 2001), while proteolytic activity of the specific donor animal also plays a role (Falconer & Wallace, 1998). Morgavi *et al.* (2001) reported greater levels of inactivation of polysaccharides when using rumen fluid of cows before, compared to after feeding. In addition, the increase in hydrolytic activity observed in the rumen when directly adding exogenous enzymes is shown to be dependent on the amount of enzyme added to the feed (Beauchemin *et al.*, 2004a).

The possibility also exists that exogenous enzymes stimulate the attachment of ruminal microbes to the fibre portion of the diet. This would explain the positive results on fibre digestion obtained from the addition of enzymes in small quantities. The mode of action by which exogenous enzymes stimulate attachment to plant cell walls also remains unknown, but the release of soluble sugars from the feed with enzyme application, as well as weakening of the feed surface to enhance attachment, may be contributing factors in this regard (Beauchemin *et al.*, 2004a,b).

2.3 Enzyme-ruminant synergy

Synergism between endogenous microbial enzymes of the rumen and exogenous enzymes fed to the animal refers to a net cooperative effect observed when feed enzymes are added, hereby creating an increased enzyme activity which exceeds the additive effects of each entity involved (Beauchemin *et al.*, 2004a).

Morgavi *et al.* (2000a) proposed a synergy between endogenous ruminal fibrolytic enzymes and exogenous fibrolytic enzymes to play a role in the positive animal

production responses associated with the feeding of enzymes. In his experiment, combined effects of endogenous and exogenous enzymes relating to hydrolytic activity within the rumen were much higher than estimations of individual activities, thus providing evidence of this synergism.

Since enzyme additives contribute very little to total enzyme activity within the rumen, and rumen microbiota inherently is well equipped for fibre digestion, this seems to suggest that enzyme additives should contain enzyme activities not normally found abundantly within the rumen (Krause *et al.*, 2003).

2.4 Proposed mode of action

More research is needed regarding the identification of the precise modes of action of exogenous fibrolytic enzymes in the ruminant (Morgavi *et al.*, 2000a). The mode of action on cell wall degradation of exogenous enzymes is complex, with various possible modes of action being proposed. A synergy or overlapping of some or all of these is also suggested (Beauchemin *et al.*, 2004a).

According to Beauchemin *et al.* (2004a), the end result of the addition of exogenous fibrolytic enzymes to the ruminant diet is an increase in total enzymic activity in the rumen by increasing its hydrolytic capacity through enhanced bacterial attachment, stimulation of microbial populations within the rumen, and synergistic effects; hereby enhancing the digestibility of the diet.

3. Commercial Enzymes

Products currently commercially available for use in animal diets represent mixtures of different enzymes displaying differing characteristics (Columbatto *et al.*, 2003b). The main structural polysaccharides in plant cell walls, namely cellulose and hemicellulose, are digested by cellulase and hemicellulase enzymes. Various different types of these, displaying variation in proportions and activities, are found in

commercial enzyme preparates. Additionally, commercial enzyme activities are determined at a temperature of ±60°C and pH of between 4 and 5. These enzyme optima do not correlate well with optimum conditions of 39°C and pH 6-7 found in the ruminant animal (Beauchemin *et al.*, 2004b), thus complicating the evaluation of such products. It is currently not known if any specific enzyme activity has a limiting effect on the rate and extent of digestion in the rumen; and if so, which enzymes are involved. The characterization of enzymes is thus of utmost importance in the selection process (Columbatto *et al.*, 2003b).

4. Enzyme Evaluation

4.1 Considerations

In selecting an enzyme candidate, it is important to remember that enzymes are substrate specific, and that enzyme activities vary according to the substrate offered. It is therefore clear that the optimum range of enzyme preparation activities will depend on the specific diet to which it is to be added. Enzymes should therefore be evaluated with this in mind, since plant cell wall structure and composition vary greatly between forage species (Beauchemin *et al.*, 2004b). Enzyme-feed specificity has, for instance, been documented by Columbatto *et al.* (2003b). It is also likely that enzyme activities limiting digestion are related to diet composition (Beauchemin *et al.*, 2004b).

Enzyme application level also plays a great role in observed animal production responses, and available results are variable. Beauchemin *et al.* (2004b) reported variable results over multiple trials using multiple enzyme application levels of different enzyme preparations. Responses tended to be non-linear, with diet composition playing a role in optimal inclusion levels. Over-, as well as under-supplementation is possible. It is however noted that high inclusion levels tend to be less effective than low levels of supplementation, suggesting possible competition between endogenous rumen microorganisms and exogenous enzymes.

Evaluation of possible candidates should also be conducted in the presence of rumen fluid to account for possible synergistic interactions between endogenous and exogenous fibrolytic enzymes, as postulated by Morgavi *et al.* (2000a). It has been shown that the effectiveness of exogenous fibrolytic enzymes cannot be predicted solely from enzyme activities.

4.2 Alternative methods

Since time, animal and financial constraints in most instances rule out the possibility of testing all possible enzyme candidates *in vivo*, reliable *in vitro* methods can be employed as screening systems to identify most promising enzymes. When employed under conditions (temperature, pH and feed substrate) similar to that of the animal test subject, they provide the advantage of being able to accommodate a wide range of feed samples, as well as allowing for the evaluation of various enzyme application levels. It must be noted, however, that the possible effect of enzyme delivery to the animal is not accounted for when using these evaluation techniques (Beauchemin *et al.*, 2004b).

In the present study, two *in vitro* screening systems, namely a gas production system and an *in vitro* organic matter digestibility (IVOMD) system were employed to identify enzyme candidates for possible future use in animal production systems.

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C: *In vitro* feed evaluation systems

1. Introduction

The aim of feedstuff evaluation systems is to provide insight into the ability of a feed to comply with the nutritional needs of the animal (Beever & Mould, 2000; Mould, 2003). The nutritive value of a feed is dependent on its chemical component concentrations and the rate and extent to which digestion takes place in the animal (Getachew *et al.*, 2004). Where forages are concerned, limitations regarding performance of the animal are most often a result of low voluntary intake and digestibility (Getachew *et al.*, 1998). Animal production responses obtained from *in vivo* feeding trials involving the specific animal and feed are the most accurate indication of nutritional value of a feedstuff. This approach, however effective, is most often time-consuming, impractical, laborious and costly, requires large amounts of feed, and limits to a great extent the number of feeds that can be screened at one time (Getachew *et al.*, 1998; Mauricio *et al.*, 1999; Beever & Mould, 2000). Chemical systems of analysis, on the other hand, while faster, having greater capacity, as well as providing absolute values, cannot take into consideration animal-feed interactions such as palatability, the effect of anti-nutritive factors and the extent to which diet composition influences digestibility (Mould, 2003). They therefore do not give an accurate reflection of the biological processes involved in the digestion processes within the animal (Van Soest, 1994).

2. Systems Currently in Use

Due to above-mentioned limitations, many attempts have been made to predict intake and digestibility through the use of alternative laboratory techniques. Biological methods that simulate the digestion process can be used to estimate digestibility of a feed (Getachew *et al.*, 1998). The four major alternative digestion techniques

(biological methods) currently available for the evaluation of nutritive value of ruminant feeds are (Theodorou *et al*, 1994; Getachew *et al*., 1998):

- digestion with rumen micro-organisms such as the Tilley & Terry (1963) procedure

- enzymatic methods such as cell-free fungal cellulase (Jones & Hayward, 1975, as cited by Beever & Mould, 2000)

- *in situ* incubation of samples in the rumen (Mehrez & Ørskov, 1977),

- the gas production method (Menke *et al*., 1979)

The method of Tilley & Terry (1963) represents the first routine *in vitro* system for the evaluation of animal feedstuffs. This method is widely used for large-scale feedstuff testing due to its convenience and extensive validation with *in vivo* values (Van Soest, 1994). The technique is described in a later section.

Since micro-organisms and enzymes are more sensitive to factors influencing the rate and extent of digestion than chemical methods, these biological methods are a more accurate estimation of ruminant feed value, and attempts to accurately predict digestibility from chemical composition of feeds using a regression equation has not yet proved successful (Van Soest, 1994).

This has led to the popularity of *in situ* feed evaluation systems (e.g. Ørskov *et al*., 1980). The nylon bag technique has been used for many years to provide useful estimates of rates of disappearance and potential ruminal degradability of feedstuffs (Mehrez and Ørskov, 1978). In this system, nylon bags containing substrate, are placed in the rumen of fistulated animals, and rates of fermentation calculated by removing bags at chosen time intervals and measuring dry matter disappearance (Blummel & Ørskov, 1993). The use of this method has however also been questioned, with animal welfare, costs of maintaining surgically modified animals and

restrictions on number of samples that can be tested being some of the concerns (Mauricio *et al.*, 1999). These considerations greatly limit the use of the method as a routine screening method for large numbers of samples (Getachew *et al.*, 1998). Much attention has consequently been given to the increased use of *in vitro* methods (Mauricio *et al.*, 1999).

Enzymatic digestibility assays, such as that developed by Jones & Hayward (1975) (as cited by Beever & Mould, 2000), use commercially produced enzymes instead of rumen micro-organisms as a source of microbial enzymes for the evaluation of feedstuffs, and therefore has the advantage of not requiring fistulated animals as inoculum donors, as well as eliminating variation introduced by the use of rumen inoculum. However, as this is also an end-point digestibility procedure, it presents disadvantages similar to that of Tilley & Terry (1963). In addition, results have not been validated with *in vivo* results to a great extent. The procedure may also be insensitive to factors affecting microbial degradation such as toxins and associative effects (Getachew *et al.*, 1998).

3. Considerations and Limitations Regarding Present Techniques

The process of microbial degradation of forages in the rumen is complex, involving interactions between microbes found in the rumen (bacteria, protozoa, fungi) as well as between these microbes and the host animal. When using alternative feed evaluation systems, in trying to simulate rumen conditions, consideration needs to be given to factors such as temperature, pH, buffering capacity, anaerobic conditions and source of nitrogen (Getachew *et al.*, 1998; Williams, 2000). In developing a suitable *in vitro* method, attention also needs to be given to factors such as repeatability and correlation of results with actual *in vivo* parameters (Getachew *et al.*,1998).

Current laboratory techniques measure degradation in the rumen, employing the rationale that a forage, consisting mainly of carbohydrate in the form of fibre, is digestible only by rumen micro-organisms. Effective degradability (digestibility

expected *in vivo*) however, also depends on the rate of degradation, potential degradability and fractional outflow rate from the rumen (Williams, 2000). While no *in vitro* technique is currently able to measure fractional outflow rate, existing techniques are capable of measuring potential degradability (Tilley & Terry, 1963), or both degradability and rate of digestion (*in situ*, cumulative gas production).

The two systems employed in the current study, namely an *in vitro* organic matter digestibility assay derived from that of Tilley & Terry (1963), as well as a gas production system based on the Reading Pressure Technique of Mauricio *et al.* (1999), are described in the following section.

4. Methods Used in Present Study

4.1 *In vitro* organic matter digestibility

Due to its high correlation with *in vivo* digestibility, *in vitro* organic matter digestibility (IVOMD) techniques have been widely used for the analysis of feeds, and a variety of IVOMD procedures are therefore currently available (Holden, 1999; Getachew *et al.*, 2004). All methods currently in use are based on the original *in vitro* method described by Tilley & Terry (1963). The original technique involves two stages resembling the rumen and lower digestive tract respectively, whereby forages are subjected to a 48 h fermentation in rumen fluid and a buffer solution, followed by digestion for 48 h with pepsin in a weak acid solution to remove undegraded plant cell matter and microbial protein (Mould, 2003). .

The method is simple, accommodates multiple samples at one time, is highly reproducible and provides an *in vitro* digestibility estimate which can be used to predict *in vivo* digestibility (Mould, 2003). The technique does, however, have a disadvantage in that only an end point measurement is provided, therefore giving no information as to the kinetics of forage digestion (Theodorou *et al.*, 1994). The sample is also destroyed in the course of residue determination, giving rise to the need for many replicates (Getachew *et al.*, 1998). The method was therefore modified by Goering & Van Soest (1970), by treating the residu after 48 h with neutral detergent

solution in order to estimate true dry matter digestibility (IVTD) (Beever & Mould, 2000).

While improvements regarding the accuracy of these procedures have been made through the modification of reagents used, little improvement has been made regarding labour efficiency or the accommodation of multiple samples at one time (Holden, 1999). Recently, the development of the DAISY^{II} *in vitro* fermentation system and fibre apparatus (ANKOM Technology Corp., Fairport, NY) has provided a means of analysing multiple feed samples simultaneously, thereby reducing variation as well as being more labour efficient (Holden, 1999; Getachew *et al.*, 2004). Two possible disadvantages of this system include, i) the movement of fine feed particles in and out of the filter bags used for incubation, thereby affecting residue weights and thus, digestibility and ii) the influence that samples may have on each other when incubated in the same vessel due to soluble matter released and having an effect on microbial activity in the rumen fluid (Wilman & Adesogan, 2000).

In an experiment conducted by Holden (1999), in which both a traditional method and the DAISY^{II} incubator (ANKOM Technology Corp., Fairport, NY) were used to determine dry matter digestibility of ten different feeds, the DAISY system provided good correlations with results obtained from traditional methods. This finding is supported by various researchers (Church & Peterson, 1960; Bezeau, 1965; Cherney *et al.*, 1993; Wilman & Adesogan, 2000). It was shown however, that the source of rumen fluid inoculum from test animals fed differing diets was shown to affect IVDMD estimations significantly. Church and Peterson (1960) also reported that, in addition, pH, amounts of rumen liquor and substrate used, as well as particle size of the substrate, could have marked effects on results from *in vitro* fermentations, while Cherney *et al.* (1993) also found that source of fibre in the donor animal diet could affect IVDMD values. Additionally, the 48h incubation period may not be an accurate estimation of the extent of digestion of various different feeds, as, in the case of fibrous feeds, retention time in the rumen in some instances greatly exceeds this time span, while the 48-h incubation allows for a greater extent of digestion of feeds low in fibre than is the case in the animal (Fisher *et al.*, 1995).

4.2 In vitro gas production

Rumen fermentation is associated with the formation of gas; either directly from microbial degradation of the feed, or indirectly from buffering of acids that are formed due to fermentation (Getachew *et al.*, 2004). In incubation of a feedstuff with buffered rumen fluid *in vitro*, carbohydrates are fermented to short chain fatty acids, gases (mainly CO_2 and CH_4) and microbial cells. On incubation, substrates are partially solubilized, making the soluble components easily fermentable. Gradually, fermentation of insoluble components starts to occur. These components need to be colonised by rumen micro-organisms before fermentation can commence, making fermentation rates of different components very dependent on initial concentration and composition of rumen microbes, as well as the ability of these to utilise the end products for growth. Different substrates resist these processes to differing extents, resulting in varying gas production profiles according to substrate used (Groot *et al.*, 1996). Getachew *et al.* (2004) reported distinct differences in fermentation patterns between corn silage, wheat silage, lucerne silage and lucerne hay. Generally, the gas produced in fermentation is proportional to net microbial metabolism, thus creating the possibility to use this value as an end point method for estimating digestibility (Van Soest, 1994). Another advantage of gas-based *in vitro* systems, is that it is non-destructive, thus making it possible to collect multiple measurements over a certain incubation period from a single fermentation vessel. A number of gas-based *in vitro* systems are currently in use, based on the assumption that the rate and extent of gas released upon fermentation resembles that which occurs in degradation in the animal (Mould, 2003).

Various gas production measurement techniques are available,:

- the Hohenheim gas method (Menke *et al.*, 1979)
- Liquid displacement system (Beuvink *et al.*, 1992, as cited by Getachew *et al.*, 1998)
- Manometric method (Waghorn & Stafford, 1993)

- Pressure transducer systems: manual (Theodorou *et al.*, 1994); computerised (Pell & Schofield, 1993); combination of pressure transducer and gas release (Cone *et al.*, 1996)

As early as 1943, Quin (as cited by Williams, 2000) measured gas production manometrically from incubation of feedstuffs in gastight flasks. The measurement of gas employing liquid displacement systems was suggested for the evaluation of ruminant feeds by Johnson (1963) and Trei *et al.* (1970) (as cited by Williams, 2000) and is still used today, while O'Hara *et al.* (1974) (as cited by Williams, 2000) observed differences in rates of gas production between substrates in a system measuring the release of gas by use of a syringe attached to a flask containing the substrate.

Menke and Ehrensvard (1974) proposed an indirect method for the evaluation of ruminant feeds *in vitro*, based on the use of syringes (Theodorou *et al.*, 1994). Menke *et al.*, in 1979 elaborated upon this method, and proposed a gas production system for the evaluation of ruminant feedstuffs based on the assumption that when feeds were incubated with rumen fluid and a buffered incubation medium *in vitro*, the amount of gas hereby released could accurately be related to digestibility, and therefore, to the energy value of the feed in question (Beever & Mould, 2000). In this system, syringes are incubated in a horizontally rotating rack at 39°C, and produced amounts of gas retained and measured manually by reading the position of the gas syringe plunger at different time intervals (Mauricio *et al.*, 1999). This so called Hohenheimer gas production test was modified by Blummel & Ørskov (1993) by incubating syringes vertically in a thermostatically controlled water bath, shaking them by hand and recording gas production at various time intervals over a 72 h period. The system is basically similar to that of Tilley & Terry (1963), but differs in that gas production, rather than organic matter lost, is used as parameter to describe the amount of substrate fermented (Blummel & Orskov, 1993).

In 1974, Wilkins developed an automated pressure transducer method for measuring the gas produced by micro-organisms. Here, a pressure transducer and pressure equaliser valve is attached to the metal cap of a test tube containing inoculum and a

culture medium, and gas pressure readings recorded on a strip-chart recorder. The method of Wilkins (1974) formed the basis for the future development of the pressure transducer method (Getachew *et al.*, 1998).

The system described by Pell & Schofield (1993) consisted of bottles containing sample and incubation medium. These are sealed with butyl rubber stoppers and crimp caps, and rumen fluid added by injection. A pressure sensor is inserted into each bottle and used to transmit data to a computer at hourly intervals for 48 hours, allowing for frequent cumulative gas production recordings. Sample sizes of 100mg showed significant sampling errors in gas production compared to larger sample size (200mg), indicating the need for the incubation of larger amounts of sample feed. These researchers also found reduced coefficients of variation among gas readings in stirred compared to unstirred samples (Pell & Schofield, 1993).

A system similar to that of Wilkins (1974) was proposed by Theodorou *et al.*, (1994) estimating rate and extent of feedstuff degradation from measured head space gases accumulated from microbial fermentation using a pressure transducer. Here, substrates are sealed in serum flasks, and accumulated headspace fermentation gases measured and released by a syringe/pressure transducer assembly. Despite the advantage of increased capacity over the system of Menke (1979), as well as reducing the need for surgically modified animals (Beever & Mould, 2000), the system poses some disadvantages. These include problems with syringe manipulation and the time taken to collect and enter readings manually. Cone *et al.* (1996) used a similar technique, using a pressure transducer, but incorporating an electric gas release valve. Hereby, gas is released when a pre-set upper pressure is reached, followed by the automatic closing of the valve when atmospheric pressure is again reached. The number of valve openings is recorded in a data logger. This allows for frequent gas production readings, offering an advantage over manual gas volume recordings.

4.2.1 Latest research

In 1999, Mauricio *et al.* proposed an *in vitro* feed evaluation system based on a semi-automated gas production technique similar to that of Theodorou *et al.* (1994).

Although similar, the technique differs in that accumulated head-space gas pressures are measured by a pressure transducer and directly fed into a computer spreadsheet. Simultaneous pressure and volume readings are then used to estimate generated gas volumes by the use of a quadratic function, hereby eliminating the need for a syringe in order to measure accumulated head space gases. This so called Reading Pressure Technique (RPT) improves upon the method of Theodorou *et al.* (1994) by increasing both the rate and accuracy at which readings are recorded, while greatly increasing analytical capacity (Mauricio *et al.*, 1999).

A brief summary of the development of gas production systems is given in Figure 2 (from Mould, 2003)

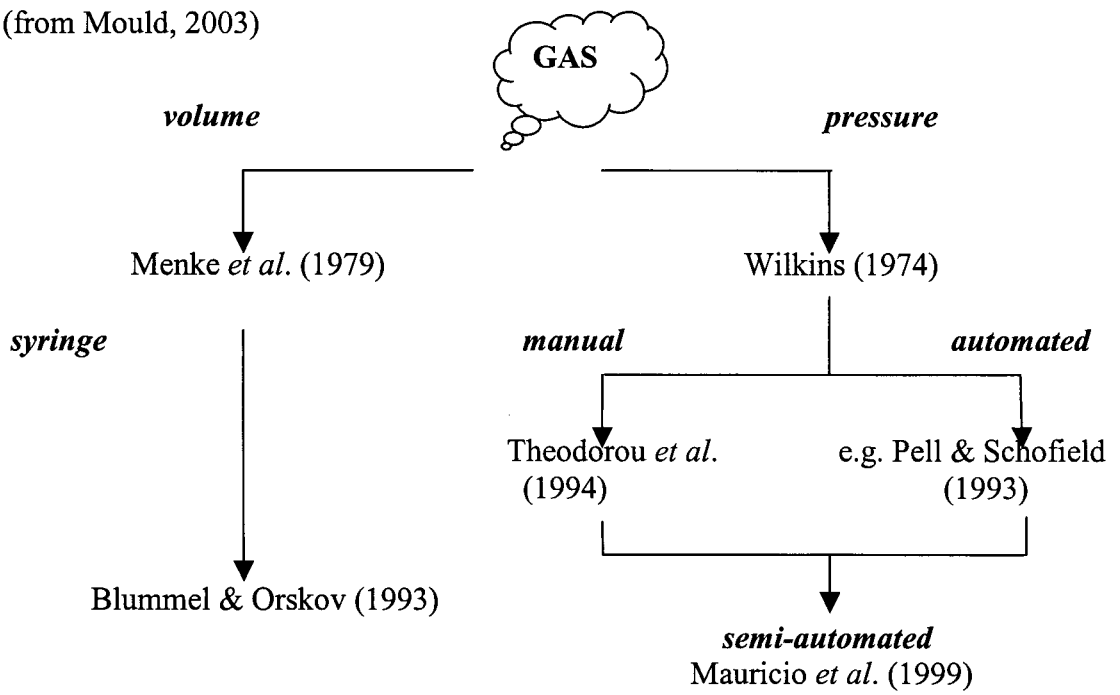

Figure 2. Development of gas-based feed evaluation systems. (adapted from Mould, 2003).

4.2.2 Advantages and limitations

In vitro methods, such as Tilley & Terry (1963), and the nylon bag technique make use of gravimetric measurements and measure disappearance of the substrate, all of which may not contribute to fermentation. In the gas measurement technique, focus is placed on the measurement of fermentation products, since products soluble but not fermentable do not contribute to gas production (Blummel & Orskov, 1993). The gas

production technique is a useful method for determining fermentation characteristics of both soluble and insoluble feedstuff fractions (Pell & Schofield, 1993; Getachew *et al.*, 1998). In addition, the gas production system is widely used because of the potential with regard to the accommodation of large numbers of samples. The technique is also less expensive and time consuming, and allows the researcher to maintain experimental conditions more accurately than do *in vivo* trials (Getachew *et al.*, 1998).

Blummel & Ørskov (1993) reported a good correlation between *in vitro* gas production profiles and the nylon bag technique with reference to feed intake and digestibility, with the nylon bag method appearing to be slightly more accurate in predicting performance of the animal. The close relationship between in *vitro* gas production parameters and voluntary feed intake was documented by Blummel & Becker (1997). Sileshi *et al.* (1996) also reported a significant relationship between DM digestibility determined from the residue remaining after gas production and that determined *in vitro* by the Tilley & Terry (1963) method. Getachew *et al.* (2004), however, reported a poor correlation between 24h gas production and *in vitro* true dry matter digestibility (IVTD), stating that this result could be due to feed constituents such as fat and protein contributing little or nothing to gas production, while still being degraded *in vitro*.

Correlation of *in vitro* gas production results with *in vivo* data has generally in the past provided mixed results. This is partly due to the fact that, since gas production only accounts for total VFA and gas production, no consideration is made for substrate used for microbial growth, since truly digested substrate is divided between VFA, gas and microbial biomass (Getachew *et al.*, 2004).

Menke *et al.*, (1979) concluded that despite high correlations between 24-h cumulative gas production and OM digestibility determined *in vivo*, feedstuffs showing low gas production rates may have higher *in vivo* digestibility than that suggested from gas production trials (Beever & Mould, 2000). Although gas production is closely related to degradation, no direct information is given regarding either extent of degradation or quantity of fermentation products (VFA's and

microbial protein) available to the animal (Mauricio *et al.*, 1999). The procedure therefore only provides a means of comparison, ideally suited for the purpose of screening large numbers of feedstuffs or treatments, providing information only on rates of fermentation.

To further validate results obtained from the procedure, examination of fermentation residues can be done to more accurately determine estimates of extent of degradation and fermentation efficiency (Blummel *et al.*, 1997). By incorporating this procedure, information would be gained on the amount of substrate used for the formation of all fermentation products, namely short-chain fatty acids, gases and microbial biomass.

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Trial Objectives

The inclusion of exogenous fibrolytic enzymes in the ruminant diet has in many cases been shown to have a positive effect on ruminant production parameters, as reported in the previous section. Due to the ever-growing need for more economic means with which to maintain and enhance the productive efficiency of these animals, and the utilization of fibrous feedstuffs being a major restrictive factor in this regard, such positive effects cannot be overlooked.

The present study was therefore conducted in order to identify possible enzyme candidates that may have the potential to be incorporated into a low quality, high-forage ruminant diet, and to assess the consequent effects such an enzyme may have on animal production parameters. Firstly, two *in vitro* screening methods were employed in order to identify enzymes that may have a positive impact on high-fibre forage digestibility. The most promising of these was consequently incorporated into a feeding trial to assess the *in vivo* effects of the fibrolytic enzyme preparation.

The research reported in the following chapters of this document were conducted on the hypothesis that enzymes identified in *in vitro* screening procedures as those having a positive effect on degradability parameters of wheat straw forage, would also have the ability to enhance animal performance parameters *in vivo*.

CHAPTER 2

General materials and methods

1. *In Vitro* Digestibility

Based on the original *in vitro* method of Tilley & Terry (1963), *in vitro* true digestibility (IVTD) of control and enzyme treated samples were determined using the DAISY^{II} *in vitro* fermentation system (ANKOM Technology Corp., Fairport, NY) according to the procedure described by the manufacturer. The prescribed buffer solution was, however, replaced with that of Goering & Van Soest (1970) and contained tryptose as a reducing agent. This alternative procedure was followed, as the former buffer solution appeared not to become reduced, thus influencing *in vitro* conditions and consequent results.

Feed samples were milled through a 2mm screen (Scientech RSA, Hammer Mill Ser. No 372, Cape Town, S.A.), and stored in sealed plastic bags at 4°C to ensure sample consistency. Milled feed samples were subsequently passed through a 124μm horizontal shaker for 5 minutes prior to use. This was done to eliminate the presence of dust and extremely fine material in the sample that may escape through the pores of the filter bags used for incubation, thus influencing results. According to procedures described by ANKOM, 0.25g (±0.01) of feed *(c)* was then weighed into Ankom F57 filter bags (ANKOM Technology Corp, Fairport, NY) and heat-sealed for incubation into the DAISY^{II} system. The filter bags were washed in acetone for 5 minutes and dried at 100°C overnight prior to use in order to determine bag dry weights *(a)* before addition of the feed sample. Percentage dry matter contents of the feed used as substrate *(d)* was determined gravimetrically. Dry matter (DM) values were then determined for each specific sample using the following equation:

$$\textbf{Sample DM} = d/100 \times c \qquad \ldots eq\ 1$$

where c = feed sample weight

d = DM percentage of wheat straw used as substrate

The DAISY^{II} incubation system consists of a chamber designed to accommodate four 4L glass jars as incubation vessels. Incubations are maintained at 39°C and incubation flasks are automatically rotated at a constant slow speed. Each incubation flask is able to accommodate 25 filter bags, therefore creating capacity for 100 samples per incubation (Holden, 1999). This included 2 empty heat sealed filter bags per jar for the calculation of a blank bag correction factor *(b),* which was calculated as the average of the two post-*in vitro* weights obtained from each empty bag.

Prepared filter bags containing the sample feeds were incubated with pre-prepared buffer solution into the daisy incubation system for 30mins to allow for the equilibration of temperature in the incubation jars before the introduction of rumen inoculum to the system. Contrary to manufacturer indications of 1600ml of buffer solution, 1400ml of buffer solution was added, and the remaining 200ml substituted with the appropriate enzyme preparation diluted with distilled water.

Equal amounts of rumen fluid inoculum were obtained from two ruminally canulated donor Merino rams that received a diet containing forages similar to those used as fermentation substrates. This was done in order to minimise between-trial error resulting from differences in rumen fluid inoculum. Although the concentration of micro-organisms in the rumen of pre-fed animals is lower than that found in animals 2-4 hours after feeding, less variation is generally observed between samples collected prior to feeding (Mauricio *et al.*, 1999). Donor animals were therefore fed each morning at 7.30, with feed being withheld on day of collection until after the collection had been completed. Rumen contents was removed by hand, squeezed through a layer of cheesecloth, and the rumen liquor, plus a small amount of collected solid rumen contents, transported to the laboratory in thermos flasks previously filled with hot tap water to pre-heat the flasks. Flasks were filled completely to eliminate the presence of oxygen while transporting the fluid. On arrival at the laboratory, all collected rumen fluid, including solid material, was pooled in an industrial blender, the container purged with CO_2, and the contents sealed and blended for 30 seconds. It

was then once again strained through a layer of cheesecloth before use to eliminate larger solid materials, and thus aid pipetting.

Following temperature adjustment of incubation jars, 400ml of prepared rumen inoculum was added to each incubation jar, the jar purged with CO_2 for 30 seconds, and then sealed and placed into the incubator for an incubation period of 24 hours. On completion of the incubation, the jars were removed, the contents emptied and the filter bags rinsed with cold tap water until the water became clear, taking care to inflict minimum mechanical agitation on filter bags. They were then air-dried for an hour before incubation in a 50°C oven for 24 hours to determine post-*in vitro* dry weights of the bags. Dried bags were subsequently weighed after 24 hours, and dry weights recorded.

Following dry weight determination, neutral detergent fibre (NDF) analyses were done sequentially on the same bags using the ANKOM$^{200/220}$ Fibre Analyzer (ANKOM Technology Corp., Fairport, NY) according to manufacturer specifications in order to determine *in vitro* true digestibility (IVTD) of the feed sample. As suggested by Van Soest (1991), the NDF analyses included the addition of heat stable α-amylase to assist in the removal of starch, as starch contamination of NDF could lead to an overestimate of this value. Sodium sulfite was also added to reduce nitrogenous contamination of the fibre (Van Soest, 1994). According to the specified procedure, samples were boiled in neutral detergent solution for 75 minutes, followed by three 5 minute washing procedures with boiling water containing 4ml of heat stable α-amylase in the first two. Bags were then removed and air-dried. This was followed by washing in acetone for 5 minutes, and overnight drying in a 100°C oven to determine post-NDF dry weights *(e)* of the bags. An average value of dry matter weights of the two blank bags after NDF was also recorded *(f)*.

Following oven-drying of the filter bags, sample bags containing the processed substrate were ashed in pre-weighed porcelain crucibles *(g)* at 500°C for 6 hours. Crucibles containing ash samples were subsequently weighed *(h)*, and dry ashing weights recorded. Again, an average value was calculated for the two blank bags

subjected to the ashing procedure *(j)*. These values were used to determine ash percentages, as well as OM percentages of each sample with the following equations:

$$\textbf{Ash \%} = ((h - g) - (a \times j) / (eq\ 1) \times 100 \qquad ...eq\ 2$$

where a = bag dry weight

eq 1 = sample DM

g = crucible dry weight

h = crucible plus ash dry weight post ashing

j = blank bags average ash weights

$$\textbf{OM proportion} = (100 - (eq\ 2)) / 100 \qquad ...eq\ 3$$

where *eq 2* = sample ash %

Percentage improvements in *in vitro* true digestibility (IVTD) was consequently determined using the following equation:

$$\textbf{IVTD degrad} = 100 - ((e - (a \times f/b) - (h - g)) / (eq\ 1) \times 100 \qquad ...eq\ 4$$

where a = bag dry weight

b = blank bags average dry weight

eq 1 = sample DM

e = sample + bag after NDF (DM)

f = blank bags average after NDF

g = crucible weight

h = post-ashing crucible weight

Resulting IVTD values for the enzymes tested were compared against IVTD values obtained from the control treatment in each trial run in order to compare results and to identify enzyme candidates providing improved IVTD values above that of the control treatment.

2. Gas Production

The gas production system described below is, unless otherwise stipulated, based on the Reading Pressure Technique (RPT) – an *in vitro* feed evaluation system based on a semi-automated gas production technique described by Mauricio *et al.* (1999). The system is similar to that of Theodorou *et al.* (1994), but differs in that gas volume is no longer measured with a syringe.

Glass vials of predetermined volume, similar to that used by both Theodorou *et al.* (1994) and Mauricio *et al.* (1999), ranging between 116,0-120,0ml, were used to conduct the incubations. Feed samples were milled through a 2mm screen (Scientech RSA, Hammer Mill Ser. No 372, Cape Town, S.A.), and subsequently passed through a 124um horizontal shaker for 5 minutes prior to use. In accordance with Mauricio *et al.* (1999), who suggested a quantity of no more than 1,0g degradable organic matter in order not to exceed the buffering capacity of the medium, an amount of 0,5±0,01g of substrate (as is basis) was accurately weighed into each vial.

Each enzyme tested using this procedure was represented by 9 incubation bottles. Six of these contained feed substrate, buffer, rumen fluid and enzyme treatment, and served as repetitions for the enzyme being tested. Three blank bottles, or 'enzyme blanks', excluding substrate, but containing buffer, rumen fluid and specific enzyme treatment, were also included for each trial enzyme. This procedure was followed in order to correct for gas production resulting directly from the inoculum or sources other than the substrate. A series of 9 incubation bottles, prepared as described above for enzyme treatments, but containing pure distilled water instead of enzyme treatment, was also included in each trial run. These 'reagent blanks' were included as a control treatment in order to compare results between trials.

For the purpose of this trial, various enzyme treatments and/or dilutions thereof were added at a rate of 1.0ml supernatant per bottle (i.e.,1,0ml supernatant per 0.5g of feed). To allow for a pre-incubation interaction time of enzyme on feed substrate, the bottles were then sealed with rubber stoppers, and incubated in an incubator oven at

39°C for 18 hours prior to the addition of buffer and rumen fluid to each bottle to initiate the incubation.

Rumen fluid was collected according to the procedure described for *in vitro* organic matter digestibility in section 1 of this Chapter. Following collection, the rumen fluid was placed in a glass container and continually stirred with the aid of a magnetic stirrer, while periodically purging the container with CO_2 during the course of the preparation to ensure that anaerobic conditions are maintained.

At this stage, the glass vials containing feed samples and enzyme or control solutions were removed from the incubator, the rubber stoppers removed, and 10ml of rumen fluid, 40ml of buffer solution, as well as a 20mm stirrer magnet, added to each vial. The buffer used was that described by Goering & Van Soest (1970), and contained tryptose as reducing agent to ensure the rapid initiation and progression of fermentation. Each bottle was purged with CO_2 before being re-sealed with a rubber stopper and aluminium crimp cap. This was followed by the release of pressure resulting from inserting the stoppers, by pushing a needle through the rubber stopper in order to start the incubation of each bottle at atmospheric pressure representing the zero value. The bottles were placed into the previously mentioned temperature controlled oven on magnetic stirrer plates that were connected to a time switch set to stir the contents of each bottle for 1 minute every 1.5 hours.

Individual readings were taken at pre-determined time intervals, dependent on the nature of the incubation, using a digital pressure gauge (SenSym ICT, Honeywell Inc., Morris N.J., USA) fitted with a 21gauge needle. The needle was inserted through the rubber stopper, and individual pressure readings recorded manually.

A calibration curve was set up to obtain a standard conversion factor in order to convert digital gas pressure readings, measured by a digital pressure gauge in psi-units, to gas volumes produced in milliliters. This was done by preparing a series of 34 glass incubation vials with buffer and rumen fluid according to the standard incubation procedure. Additionally, 1 ml of water (representing enzyme treatment volume), and a stirrer magnet, was added to each vial. Preparing the incubation vials

in this way ensured that the head space volume was similar to that allowed for in screening incubations. The vials were sealed with rubber stoppers and crimp caps. A needle was inserted through each rubber stopper to equilibrate each vial to atmospheric pressure before initiating the experiment. Carbon dioxide was consequently inserted into each vial in increasing volumes using a syringe and 21gauge needle. The amount of gas added ranged from 0ml to 70mls in increasing increments. The increments equalled 0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 50.0, 60.0, and 70.0ml of gas. The sequence was performed in duplicate.

The vials were incubated in an oven at 39°C for two hours. Thereafter, the gas pressure in each vial was recorded using the digital pressure gauge. As original amounts of gas were added at 25°C, the gas volume at the time of determining digital pressure readings at 39°C was estimated by the following equation:

$$\textbf{Gas at 39°C (ml)} = a \times ((39 + 273.15) / (25 + 273.15)) \qquad \ldots eq\ 5$$

where a = gas added at 25°C (ml)

This value was used to calculate volume fractions for each bottle as follows:

$$eq\ 5 / b \qquad \ldots eq\ 6$$

where b = head space of specific incubation vial

The average pressure reading obtained from the two vials incubated without adding gas, was subtracted from each of the pressure values obtained from vials where gas was added. These corrected pressure readings were plotted against volume fractions calculated for each vial, and a regression equation obtained.

It is clear from Figure 1 that a good correlation (R^2=0.9904) was found between the addition of pre-determined gas volumes and consequent pressure readings done with a

pressure gauge. It was therefore decided that the equation generated by the regression ($y = 0.0977x$) would be used as a standard equation to convert pressure readings obtained in screening experiments, to gas volumes.

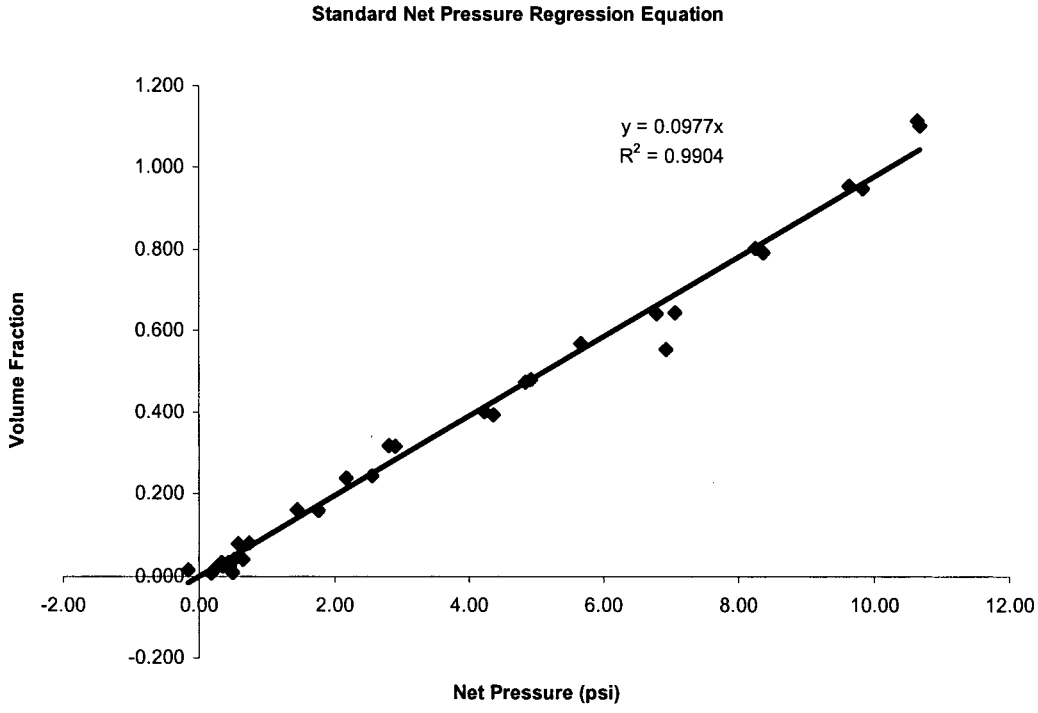

Figure 1. Regression of gas volumes added to incubation vials against psi pressures measured.

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CHAPTER 3

Screening of exogenous fibrolytic enzymes to determine fibre digestibility effects on wheat straw forage using two *in vitro* evaluation techniques

Abstract

The aim of the present study was to evaluate the effect on fibre digestibility of forages treated with various exogenous fibre-degrading enzyme preparations. This was done by the use of *in vitro* evaluation techniques in order to identify exogenous enzyme preparations that may have the potential to improve ruminant production through the enhancement of fibre digestibility in animals receiving high forage diets. Two *in vitro* screening methods, namely the DAISY^{II} *in vitro* organic matter digestibility technique (ANKOM Technology Corp., Fairport, NY), as well as an *in vitro* gas production method similar to the Reading Pressure Technique, were used in order to conduct the screening process. The former method identified various promising enzyme candidates following repeated evaluation. These were consequently evaluated in the gas production system as secondary screening, along with new enzyme candidates not previously evaluated in the initial screening process. Statistical evaluation of results obtained from gas production screenings produced seven enzyme candidates displaying promising results. Three of these were consistent with results obtained from initial screenings with the DAISY^{II} system, while the others had not been previously analysed. The experiment served to produce valuable information regarding the identification of possible enzyme candidates for future use in the ruminant feed industry, as well as suggesting a possible similarity between, and good repeatability of the two *in vitro* screening systems employed.

Introduction

Even though the ruminant is extremely well adapted to utilise high fibre forages, utilisation is restricted by plant cell wall digestibility (Van Soest, 1994; Beauchemin *et al.*, 2004), which seemingly is not optimal even under ideal feeding conditions

(Yang *et al.*, 2002). The efficient utilisation of naturally occurring forages is of major economic importance, as this provides a relatively inexpensive means of feeding ruminants, and in most instances make up the bulk, if not the total, of ruminant production systems all over the world (Wilkins, 2000; Beauchemin *et al.*, 2004).

The possibility of enhancing fibre digestibility, and thus increasing the amount of energy available to the animal through the addition of exogenous fibre digesting enzymes, has been researched for many years, and many positive responses have been obtained when feeding these enzyme products to specific production class animals (Feng *et al.*, 1996; Krause *et al.*, 1998).

Although *in vivo* trials provide the most accurate estimate of production responses expected from a specific experimental treatment, such as enzyme addition, conducting trials in this manner for the purpose of screening possible effective enzymes poses some difficulties and restrictions. These include time needed to conduct animal trials, costs related to feeding and care of animals, amount of animals needed in order to produce statistically significant results, as well as restrictions regarding the number of treatments such trials can accommodate at one time (Getachew *et al.*, 1998). These restrictions limit the use of *in vivo* trials as a screening system to identify effective enzyme products.

The use of biological laboratory methods that simulate the digestion process therefore provide a cost- and time-effective alternative to *in vivo* experimentation involving animals, and greatly increases the number of samples that can be evaluated at one time (Getachew *et al.*, 1998). The *in vitro* organic matter digestibility technique provides a procedure which is both repeatable, as well as being closely related to *in vivo* digestibility (Mould, 2003), while *in vitro* gas production can be used to determine fermentation characteristics of large numbers of samples at accurately maintainable experimental conditions (Getachew *et al.*, 1998).

Approximately three hundred fungal strains were cultivated by the Department of Microbiology of Stellenbosch University and the University of the North to obtain

enzyme-containing supernatants. Of these, 150 strains were selected for *in vitro* screening by the Department of Animal Sciences of Stellenbosch University. The current study was planned to screen these supernatants in order to identify the most promising fungal strains that might be used to produce a fibrolytic enzyme cocktail for potential use in the feed industry.

Materials and Methods

Experiment 1

Initial *in vitro* screening of enzymes was done using the DAISY^{II} *in vitro* fermentation system (ANKOM Technology Corp., Fairport, NY) in order to determine the rate and extent of OM and fibre degradation of wheat straw following treatment with specific enzyme preparations. Despite having limited screening capacity, the use of the *in vitro* fermentation system has been proven to be reliable, with the DAISY^{II} system being shown by researchers to provide repeatable and consistent results. It was therefore selected as an initial screening system in order to identify potential enzyme candidates for further testing.

Liquid preparations of enzyme supernatants were provided by the Department of Microbiology (Stellenbosch University), and incubations were initiated on the day of receiving each fresh enzyme. The wheat straw used as substrate was sourced in one bulk batch so as to ensure substrate uniformity throughout the screening process.

In vitro degradability assays were subsequently conducted according to the methodology described in Chapter 2 in order to determine OM degradability of the enzyme-treated feed samples. A neutral detergent fibre analysis procedure (NDF) was done sequentially on the same filter bags using the ANKOM$^{200/220}$ Fibre Analyser (ANKOM Technology Corp., Fairport, NY) according to manufacturer specifications in order to determine *in vitro* true digestibility.

As the DAISY^{II} *in vitro* fermentation system accommodates four incubation flasks, only three different enzyme preparations could be evaluated at one time because the fourth flask was used for a control treatment in each run. The enzyme-free control

was designated a negative control. A positive control, which was a *Trichoderma reesei* enzyme preparation, was also included in every run as a secondary control to compare results between assays. Therefore, only two experimental enzyme preparates could be evaluated per trial run.

Over the course of the initial screening period (*Experiment 1*), 24 different enzymes were evaluated, with 7 of these being repeated in multiple trial runs, amounting to 64 enzyme screenings in total, including the control treatments.

Experiment 2

The number of fungal enzyme preparations obtained from the Department of Microbiology for *in vitro* evaluation increased at such a rate that the requirement started to exceed the capacity. It was therefore decided to change to a gas production method of evaluation to increase capacity. The promising strains identified in *Experiment 1*, as well as all the new preparations received, were evaluated in *Experiment 2*.

The method used was based on the Reading Pressure Technique, as described in Chapter 2. The technique allows for the accommodation of multiple experimental enzyme samples as well as various sample repetitions within a specific enzyme, thereby greatly increasing evaluation capacity. In this way, promising enzyme candidates identified in *Experiment 1* could be compared under constant experimental conditions in the same trial run in order to minimise between-run variance. Thus, positive results obtained from *Experiment 1* were validated by the use of an *in vitro* system based on fermentation characteristics of the sample rather than organic matter disappearance.

The following general procedure was followed:

A volume of 1ml of diluted enzyme supernatant was added to 0.5g of feed substrate in incubation vials, as described in Chapter 2. These vials were sealed and allowed an

18h pre-incubation interaction time at 39°C before the addition of buffer and rumen fluid to initiate the incubation.

Each experimental run consisted of 9 incubation vials per enzyme, with the system able to accommodate 19 enzymes plus one control treatment (180 vials) per experimental run. The 9 vials representing each enzyme candidate included six fermentation vials containing wheat straw substrate, buffer and enzyme treatment, as well as 3 control vials ('control blanks') containing both enzyme treatment and buffer, but no substrate. This was done in order to correct for gas resulting from the enzyme and incubation medium in the absence of feed substrate. Each incubation run also included a series of 9 vials, as described above, treated with distilled water instead of an enzyme preparation. These 'reagent blanks' were used to correct readings from enzyme treated sample vials for gas production resulting from the incubation medium independent of enzyme treatment.

Important aspects that had to be investigated before final evaluations were documented included optimal and practical pressure reading times and enzyme dilution rates.

Optimal gas production reading times

In order to determine the optimal time to measure and compare gas productions following incubation, an experiment was conducted where cumulative gas productions of a control, Abo374, and *T.reesei*, were determined at 12, 18 and 24 hours respectively. These results are presented in Figure 1. . Enzyme treatment appeared to affect cumulative gas productions measured at different time intervals, and differences between enzyme treatments and the control appeared to become smaller after 24h incubation. Pressure readings at either 12h or 18h after incubation appeared to be optimal. Of these, a reading after 18h was the more practical option.

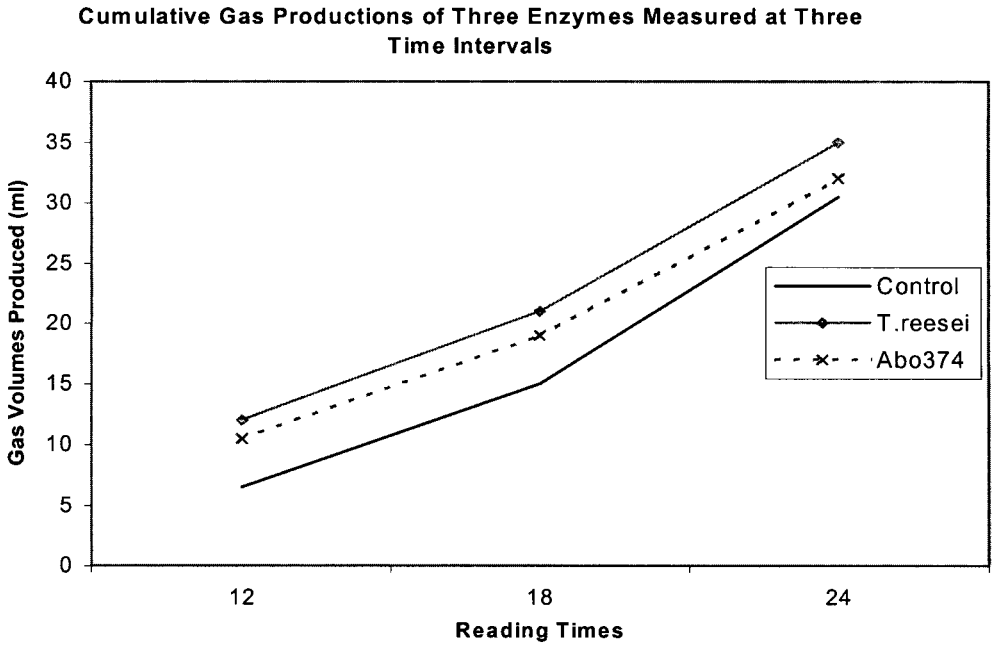

Figure 1. Effect of enzyme treatments on gas production measured at different time intervals.

It was consequently decided that gas production measurements were to be taken at 18 hours after initiating the incubation.

Enzyme dilution rates

Initial trials were conducted in order to determine the effects of enzyme concentration on gas production results, and to obtain the supernatant dilution level best suited to the gas production evaluation method in order to create a standard for future screening purposes. Two enzymes, namely Abo374 and *T.reesei*, were incubated at various dilution levels of enzyme supernatant to buffer along with a control treatment. The following dilution rates were used: 1:4, 1:8, 1:18, 1:28, 1:38. The trial was conducted according to the procedure already described, and therefore included an 18h pre-incubation interaction time of enzyme dilute on substrate, with subsequent addition of buffer and rumen fluid, and gas production readings recorded at 18h. Results are presented in Figure 2.

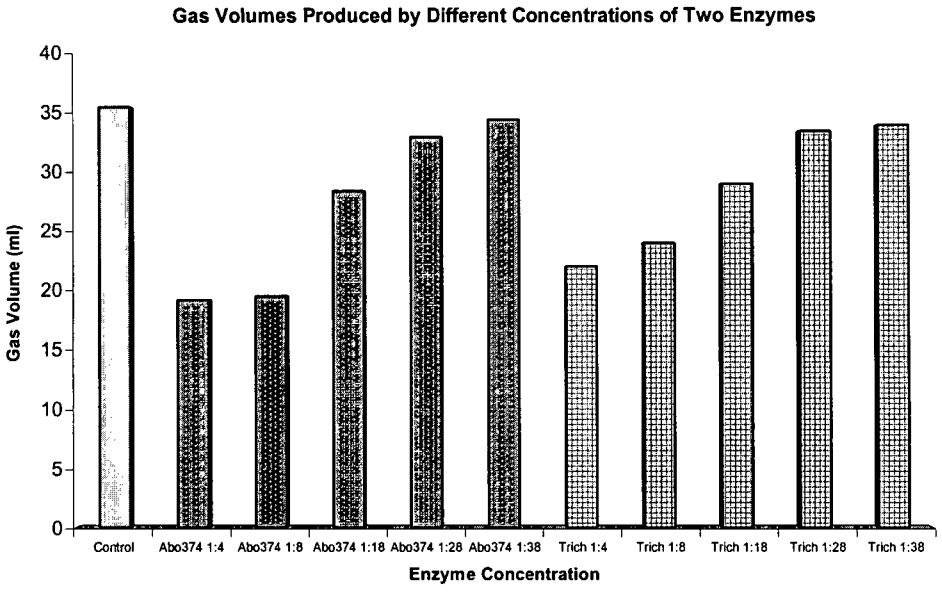

Figure 2. Effect of enzyme concentration of two enzyme preparations on gas production from wheat straw.

From Figure 2, it appeared as though high enzyme inclusion levels had a negative effect on *in vitro* gas production, and that optimal dilution levels regarding gas production on wheat straw may be even higher than those tested in the initial dilution experiment.

In addition to this, concern was also expressed regarding the concentration, or 'purity' of the enzyme supernatant used in previous experiments. Consequently, enzyme supernatants were filtered by the Department of Microbiology to produce the following fractions: A (unfiltered supernatant), B (concentrated fraction; >10K) and C (filtered fraction; <10K). The concentrated B fraction produced the highest gas production values in 7 out of 8 trials involving the different fractions. Following an experiment where the concentrated B fraction was diluted 1:10, 1:100, 1:1000 or 1:10 000 times, gas production results indicated improved gas production for dilution rates up to 1000 times. These however declined at the 1:10 000 fold dilution, and it was concluded that the optimal dilution rate of the concentrated fraction appears to be around 1:100 or 1:1000.

The filtration process resulted in the B fraction (>10K) being concentrated five fold. Due to time constraints regarding the filtration process, it was therefore suggested that the 1:1000 dilution of the B fraction should be comparable to a 1:200 fold dilution of the original enzyme supernatant (A fraction). A gas production trial was therefore conducted where both supernatants (1:200 diluted) as well as filtered fractions (1:1000 diluted) of the same sample batch were compared for four different enzymes. Results are presented in Figure 3, and it appears that fairly similar gas production volumes were obtained from the supernatant (A) compared to a concentrated filtered enzyme fraction (B) at their respective dilution rates of 1:200 and 1:1000. Based on these results, it was decided to use only the supernatant at a dilution rate of 1:200 fold for future enzyme screening experiments.

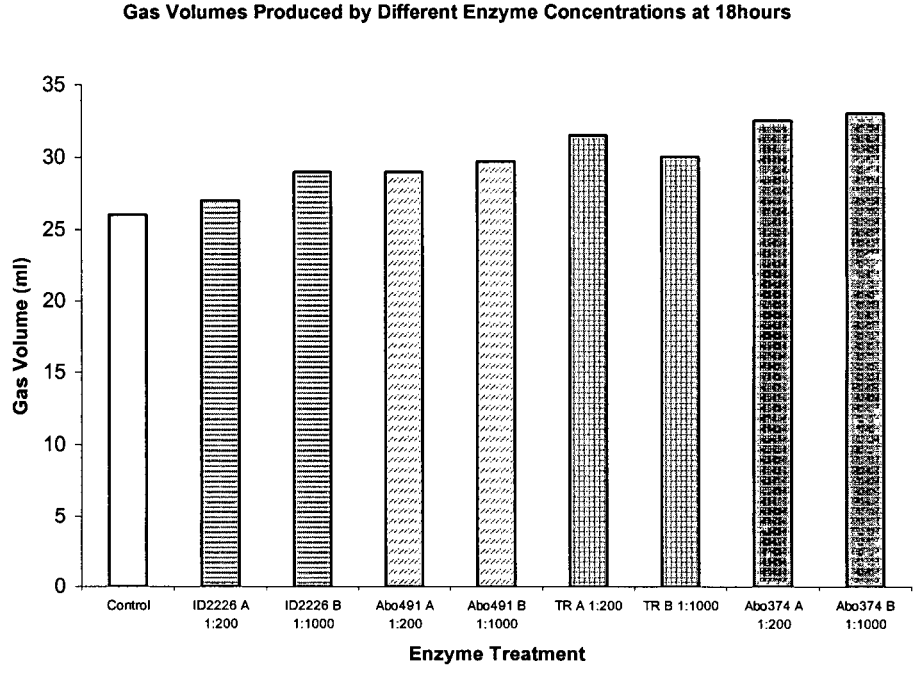

Figure 3. Effect of dilution rates (Supernatant = 1:200, Fraction B = 1:1000) on 18 hours gas production from wheat straw.

In order to facilitate statistical comparisons of enzymes tested across trials, cumulative gas volumes of the 'reagent blank' control treatments added in each trial run were normalised to a standard volume of 38.0 ml gas, representing the average gas produced by these control treatments across all conducted experiments. Gas

volumes from enzyme treatments were therefore adapted accordingly for each specific trial run control, thus eliminating between-run variation.

Statistical Analysis

Following repeated enzyme screenings using the gas production system, results from *in vitro* gas production were entered into the SAS statistical analysis program with the aid of Enterprise Guide. Enzymes were ranked according to normalised gas volumes produced against the normalised control using the One-way ANOVA procedure, employing Duncan's multiple range test to identify significant differences between individual means. Significance was declared at $P<0.0001$.

Results and Discussion

Experiment 1

Enzymes producing the most promising results following initial *in vitro* degradation screenings are presented in Table 1. Enzyme degradation results are presented in order of decreasing response, and therefore do not suggest a time-effect, but rather variability among repeated enzyme screenings. Variation in observed degradation results of repeat evaluations of enzyme candidates may be as a result of inconsistencies in enzyme activities between batches prepared on different evaluation days. Additionally, variation in rumen fluid (pH, activity) collected on different days is seen as representing the largest between-study error when conducting *in vitro* evaluations (Mauricio *et al.*, 1999), and was a likely contributing factor in variation observed in degradation results between experimental trial runs.

From these results, it appeared that *T.reesei*, Abo374, AZ7a2, FS2(2), Abo340 and Abo109 might have the potential to improve IVTD with >10%. These strains were further evaluated in *Experiment 2*.

Table 1. Percentage improvements in IVTD of most promising enzyme candidates following *in vitro* digestibility screening.

Enzyme	Repeat	Improvement in degradation (%)
Trichoderma reesei	1	40.0
	2	28.6
	3	22.4
	4	21.8
	5	10.0
	6	8.0
Abo374	1	29.3
	2	6.9
	3	5.7
AZ7a2	1	20.9
FS2(2)	1	13.0
Abo340	1	11.0
Abo109	1	10.4
Ab067	1	9.9
	2	5.1
G13	1	7.2
Abo87	1	5.7
Abo375	1	5.4

Experiment 2

Enzyme dilution rates

According to Figure 2, it appears that high concentrations of enzyme supernatant had an inhibitory effect on gas production. This would suggest a negative effect on rumen microbial fermentation. There is a possibility that high enzyme concentration results in most or all of the binding sites being occupied by exogenous enzymes, thereby preventing the attachment and subsequent action of rumen microbial enzymes on the substrate. This effect was also noted for certain specific enzyme activities on fibre

digestibility of treated alfalfa hay by Nsereko *et al.* (2000). Since it has been estimated that exogenous enzyme activities contribute less than 15% of total ruminal activity (Morgavi *et al.*, 2000; Beauchemin *et al.*, 2004), over-supplementation seems to be a very probable explanation.

Optimal gas production reading times

From the slopes of the lines displayed in Figure 1, it appears that enzyme treatment affected cumulative gas productions at the three measured time intervals and, as explained in the materials and methods section, it was decided to measure pressure readings after 18h incubation. Longer incubation periods (>24h) may also lead to gas pressure values beyond the specifications of the pressure gauge. This was shown in the methods of Theodorou *et al.* (1994) and Pell & Schofield (1993), where accumulated head-space gas pressure within the incubation bottle may negatively affect microbial fermentation, as well as potentially changing the solubility of gases within the system, therefore causing errors in gas measurements.

Validation of best enzyme candidates

The most promising strains identified in *Experiment 1*, as well as a large number of other strains, were evaluated in *Experiment 2*.

Statistical analysis of *in vitro* gas production results identified seven enzymes constantly producing gas volumes similar to, or above, that of the control treatment (Table 2). Since the gas volumes recorded for each enzyme was adapted (normalised) according to the control treatment for its specific trial run, it was assumed that variation between trials was accounted for, and that results across trials were therefore comparable. Of the seven candidates producing positive gas production results in *Experiment 2*, three of these were identified initially as producing promising degradation results from *Experiment 1*, with the *Trichoderma reesei* enzyme employed as a secondary control in initial degradation screenings being one of these. The others were not previously tested in the initial screening experiment. This suggested a possible consistency between results obtained from the two *in vitro* evaluation methods, as found by Blummel & Ørskov (1993). However, more experimental trial runs are needed in order to accurately assess this assumption.

Table 2. One-way Analysis of Variance ranking most promising enzyme candidates against the control according to Duncan's Multiple Range Test for normalised gas production (ml)

Means with the same letter are not significantly different			
Duncan Grouping	**Mean**	**N**	**Culture**
A	41.5059	60	Abo374
B	38.4746	48	*T.reesei*
B	38.0000	24	Control
B	37.7918	36	Abo212
B	37.5699	24	AZ22xyt
B	36.1282	48	Abo490
B	35.9666	36	Abo109
B	35.8963	24	EF09st

Strain Abo374 was the only enzyme candidate showing a significant improvement above that of the normalised control treatment, as well as over all other enzyme candidates. Looking at all the other promising candidates across all the different evaluation runs, the mean values were not statistically different to that of the control. However, there were individual evaluation runs where, apart from Abo374, strains AZ22xyt, Abo490, Abo109 and EF09st resulted in improvements of >10% over the control regarding both net gas production and DM disappearance.

Emphasis was placed on the results of the single best enzyme candidate, as the main objective of this experiment was to identify the single most effective enzyme for use in further tests involving *in vivo* experimentation. Consequently, this enzyme candidate (Abo374) produced results suggesting improvements in *in vitro* degradation on all six occasions of being tested in *Experiment 1*, while producing improved gas production results in most gas production trials conducted in *Experiment 2*. This provided positive information regarding the repeatability of both of these methods. It also provided positive information regarding the effectiveness of the enzyme preparation in question, suggesting that positive results may be expected when

incorporating this enzyme in an animal trial, as both of these evaluation methods aim to simulate conditions within the digestive system of the ruminant animal.

Conclusion

Due to the limitations associated with conducting animal trials, the need for a dependable and easily maintainable *in vitro* evaluation method which can accommodate a reasonable amount of samples, and at the same time incorporate the use of rumen fluid in order to simulate conditions within the ruminant, is essential to identifying products which may have a positive production effect on the animal *in vivo*.

The DAISY^{II} *in vitro* organic matter digestibility procedure is a classic example of such a system, with numerous scientific publications attesting to its accuracy, repeatability and correlation with *in vivo* parameters. Due to a restricted capacity, especially in situations such as in the present study where multiple enzyme candidates had to be evaluated and re-evaluated to confirm results, time limitations can become a restriction regarding rate of sample flow-through and thus, the generation of a substantial database.

For this reason, the gas production system can be employed as an alternative screening and validation method, as it is able to accommodate multiple samples and allow for various repetitions within a specific sample. Additionally, the procedure is less labour intensive than the original organic matter digestibility procedures.

Results obtained in the present study suggested consistency and repeatability of both of these *in vitro* procedures, as well as a possible consistency in results between the two methods. Consequently, these findings indicated that the use of either procedure can be recommended as initial screening method in order to identify treatments/products, etc. able to provoke a positive response regarding feed digestibility by estimating either organic matter digestibility (*Experiment 1*) or fermentation characteristics (*Experiment 2*) of the feed in question following specific

treatments or additions. The gas production method is especially useful when used as an initial screening system when many experimental samples need to be analysed.

Both of the *in vitro* procedures employed in present study were successful in identifying enzyme candidates producing positive responses. An overlap of enzymes identified by the two procedures also gave a good indication as to the repeatability regarding positive effects obtained from these enzymes, as improvements above that of the control were noted in repeated evaluation, especially in the secondary screening method. One of these enzymes, Abo 374, was shown by statistical analysis to be superior to the control and other enzymes, while other promising enzymes were shown not to provide results statistically different from that of the control treatment. This suggests that *in vitro* screening has aided in the identification of an exogenous fibrolytic enzyme that can be expected to provide positive animal responses when used to conduct further *in vivo* testing.

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CHAPTER 4

Effect of an exogenous fibrolytic enzyme on growth rate, feed intake and feed conversion efficiency in growing lambs

Abstract

This study was conducted to investigate the effects of an exogenous fibrolytic enzyme (Abo374) preparation on body weight gain, dry matter intake and feed conversion efficiency of growing rams. Thirty two Dohne Merino lambs with an initial mean (±SD) body weight of 30.3±2.5 kg were used in a randomised block design. Lambs were randomly assigned to one of four groups, A, B, C or D, corresponding to high (10ml/kg), medium (5ml/kg), low (1ml/kg) and control (no enzyme) treatments respectively. Wheat straw was pre-treated with different levels of the enzyme supernatant and mixed with lucerne hay and concentrate mix just before feeding. For each group, the enzyme supernatant was appropriately diluted with water and applied to the wheat straw component of the diet to correspond to a rate of 300ml/kg, with the control group, D, being treated with water at the same application rate. All groups were fed a uniform basal diet, with enzyme concentration being the only variable between groups. Following enzyme application, the wheat straw was stored in open plastic containers at room temperature for a pre-feeding incubation time of 18 hours before mixing with other feed components the following morning. Animals were fed individually at ad lib. plus 5%. Feed intakes and refusals were measured daily and animals weighed on a weekly basis to obtain cumulative group weights for a trial period of 6 weeks. Cumulative weight gains (kg) and feed conversion ratios (kg gain/kg dry matter intake) were 6.8 and 0.15, 7.1 and 0.16, 5.5 and 0.13, and 5.4 and 0.12 for the four groups respectively. Dry matter intakes were not affected. It was concluded that fibrolytic enzymes produced by Abo 374 are able to increase cell wall degradation and have potential to be used in the animal feed industry.

Introduction

Ruminants are well adapted to utilize plant cell walls, and the degradation thereof by the animal is of major economic importance. However; even under ideal feeding conditions, cell wall digestibility in the total digestive tract is still generally less than 65% (Van Soest, 1994). With plant cell walls typically contributing towards 40 to 70% of forage dry matter (Van Soest, 1994), improvement in the ability of the animal for this degradation process is therefore a major consideration. Although great progress has been made in the development of products to improve protein and energy utilization, relatively little has been achieved in the forage area.

Recent positive responses in feeding trials with regard to the use of fibrolytic enzymes as feed additives for ruminants resulted in the topic receiving much research interest in the past few years (Beauchemin *et al.*, 1995; Feng *et al.*, 1996; Yang *et al.*, 1999; Kung *et al.*, 2000). Diet composition (Beauchemin *et al.*, 1995), as well as diet component (McAllister *et al.*, 1999) to which the enzyme is added appear to influence its effectiveness. Pinos-Rodriques *et al.*(2002) found that the effect of an exogenous fibrolytic enzyme seems to be substrate-related. Possible pre-feeding effects resulting from incubation of enzyme to the feed prior to feeding also need to be further examined (Wallace *et al.*, 2001).

Although enzyme additives have in the past resulted in positive animal responses, the mode of action of exogenous enzymes in the ruminant is not fully understood (Columbatto *et al.*, 2003). The possibility exists that added enzymes may stimulate fibre digestion in the rumen (Wallace *et al.*, 2001) as well as increase total tract digestibility in ruminants (Yang *et al.*, 1999). Improvements in body weight gain and digestibility of high forage ruminant diets have been reported in studies where enzyme additives had been used (Beauchemin *et al.*, 1995; Feng *et al.*, 1996), with little effect on feed intake or feed conversion efficiency (Beauchemin *et al.*, 1995), while improvements in average daily gain, feed intakes and feed conversion efficiency have been found in others (McAllister *et al.*, 1999).

South Africa is faced with a shortage of high quality forages. Large quantities of grain crop by-products, such as wheat straw, are available after the harvesting season. In an attempt to improve the nutritive value of these low quality forages, a number of enzymes were cultivated on wheat straw at the Stellenbosch University. In the current study, one of the best enzyme candidates was chosen to determine the effect of enzyme pre-treatment of wheat straw on lamb growth, feed intake and feed conversion ratio.

Materials and Methods

Thirty-two Dohne Merino rams aged between 4 and 5 months with an initial mean (±SD) body weight of 30.3±2.5 kg were used in a six week growth trial. The lambs were housed individually in pens measuring 1,8m by 1,2m on wooden slatted floors in a semi-open metabolism building.

Lambs were weighed on two consecutive days one week prior to onset of the trial, and individual starting weights calculated as an average of these two measurements. Lambs were stratified according to starting weights and randomly allocated to one of four treatment blocks; A, B, C or D. Each group consisted of eight lambs and represented a specific treatment. Grouping was done according to weight to ensure equal size and weight distribution between treatments, hereby creating four uniform groups at onset of the trial. Lambs were consequently weighed weekly for the six weeks duration of the trial to obtain average weekly weights for each treatment.

All treatments received the same basal diet. The composition of the diet is as follows:

Table 1. Composition of basal experimental diet fed to lambs.

Component	Amount(%)
Physical Composition	
Wheat straw	39.2
Lucerne hay	27.6
Maize meal	19.7
Cottonseed oil cake	6.5
Molasses	5.7
Urea	1.1
Chemical composition	
Crude protein	13.0
Fibre	30.0
Ca	0.46
P	0.18

Lambs were fed individually with feed being offered at *ad lib* plus 5%. Treatment differences were established by the addition of varying concentrations of a locally produced enzyme preparation to the wheat straw component of the basal diet at an application rate of 300ml per kg wheat straw. The enzyme preparation (Abo 374) was prepared by the Department of Microbiology and received fresh on a daily basis. The enzyme supernatant was diluted with water to obtain a series of three different application rates corresponding to the four treatments, A, B, C and D.
The treatment applications were:

A: 10ml supernatant/kg straw (high application)
B: 5ml supernatant/kg straw (medium application)
C: 1ml supernatant/kg straw (low application)
D: Control treatment (no enzyme application)

The total amount of wheat straw required per group was weighed out daily and mixed with the appropriate diluted supernatant in an electric concrete mixer for 10 minutes. The control treatment (treatment D) was mixed with pure water at a similar application rate.

Individual amounts of treated wheat straw were weighed out for each lamb and stored overnight in open plastic containers at room temperature to ensure an incubation time of 18 hours prior to feeding. The treated wheat straw was mixed by hand with the other individually pre-weighed diet components at feeding the following day. Feed refusals were weighed back and dried daily to determine individual DM intakes.

Data was subjected to an ANOVA and treatment means were separated with a Bonferroni test to determine significant differences with the aid of Statistica 6.1 (2003). Significance was declared at $P<0.05$.

Results and Discussion

The effect of enzyme treatment of wheat straw on body weight gain, feed intake and efficiency of feed conversion is presented in Table 2.

The high and medium levels of enzyme treatment resulted in significantly higher weight gains by lambs, while the low level had no effect. Considering the relatively small number of animals (eight per treatment) and the variation usually observed in lamb growth rates, the effect is noteworthy. Increased weight gains of steers and growing heifers receiving enzyme treated forages have been reported by Beauchemin *et al.* (1995) and McAllister *et al.* (1999).

Table 2. Body weight gain, dry matter intake (DMI) and feed conversion efficiency (FCE) of Dohne Merino lambs receiving diets containing wheat straw treated with different levels of an enzyme supernatant (Abo 374) and control for a period of six weeks

Item	Treatment[1]				
	High (A)	Medium (B)	Low (C)	Control (D)	P-value
Cumulative weight gain (kg)	6.75[a]	7.13[a]	5.50[b]	5.41[b]	0.04
Total DMI (kg)	44.5	45.3	43.5	42.6	0.19
FCE (kg gain/kg DMI)	0.15[a]	0.16[a]	0.13[b]	0.12[b]	0.05

[1]Abo 374 supernatant application to wheat straw: High = 10mL/kg; Med = 5mL/kg; Low = 1mL/kg

[a,b]Means with common superscripts did not differ significantly ($P<0.05$)

Treatment did not affect feed intake, but feed conversion efficiencies were improved for the High and Med treatment groups, suggesting a possible improvement in the digestibility of the wheat straw component of the diet with certain levels of enzyme application. McAllister *et al.* (1999) observed an improvement in ADG, feed intake and efficiency of feed conversion in feedlot cattle receiving enzyme-treated silage. Yang *et al.* (2000) found an increase in total tract digestibility in lactating cows when high concentrate diets were supplemented with fibrolytic enzymes, but observed no response in sheep. In the current study, lambs received low concentrate diets and results suggested a possible positive effect of Abo 374 on digestibility based on the improved FCE. Although the enzyme was applied only to the wheat straw component of the basal diet, it is uncertain if its positive effect resulted specifically from this addition, combined with a pre-incubation interaction time; or whether other post-ingestive interactions of the enzyme within the rumen contributed to the observed improvement in feed conversion efficiencies, thus causing the improvements in weight gain observed within the High and Med application groups.

Weekly and cumulative weight gains are presented in Figure 1. The effect of the High and Med enzyme application levels are clearly observable at four and six weeks. It appears as if the effect started at an earlier stage, possibly already after two weeks, but that it did not become significant before four weeks. It appears as if an enzyme application rate of 5mL supernatant/kg wheat straw was as effective as 10mL/kg. The

level of application would obviously have an effect on the economical implications of the use of fibrolytic enzymes in the feed industry.

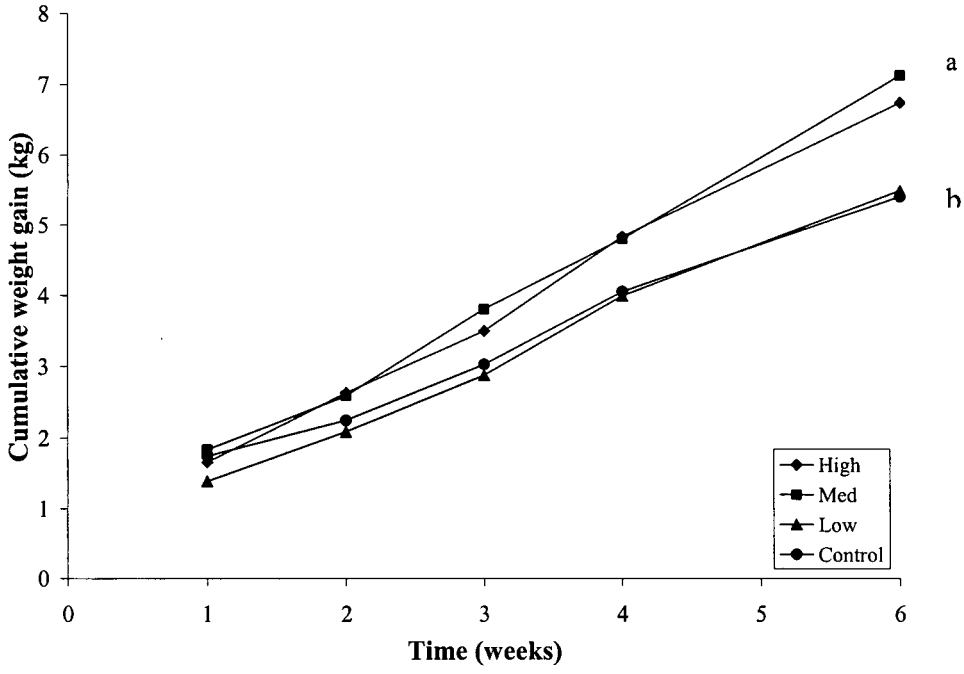

Figure 1. Weekly and cumulative weight gains of Dohne Merino lambs receiving diets containing wheat straw treated with different levels of an enzyme supernatant (Abo 374) and control. High, Med and Low refer to 10, 5, and 1 mL supernatant/kg wheat straw DM.
[a,b]Means with common superscripts did not differ significantly ($P<0.05$)

Conclusion

Pre-feeding treatment of wheat straw with Abo 374 supernatant at levels equivalent to 10 and 5mL supernatant/kg straw resulted in improved weight gains and feed conversion ratios compared to a low level treatment of 1mL/kg, or the Control with no enzyme treatment. This suggests that the fibrolytic enzymes produced by Abo374 have the potential to be effective in increasing cell wall digestibility and therefore have the potential to be used on a larger scale in the ruminant feed industry.

More information is needed with regard to optimal application levels of Abo 374, and the effect of pre-feeding incubation time of the enzyme on its effect post-feeding. Its influence on dry matter intakes over a prolonged test period, as well as *in vivo*

digestibility effects also need to be examined in order to validate the claim that Abo374 had a positive effect on the digestibility of the fibrous component of the diet. Additionally, its possible influence on other animal production parameters also needs to be further explored..

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General Conclusion

Forages make a significant contribution to the overall nutritional economy of meat-, wool and milk-producing ruminants worldwide, as they can provide essential nutrients at low cost. In South Africa, we are faced with a shortage of high quality forages, while low quality grain crop by-products, such as wheat straw, are readily available. Exogenous fibre degrading enzymes have the potential to improve animal production parameters by enhancing the degradability of these feedstuffs.

Results from *in vitro* digestibility and gas production screening experiments suggest that there are numerous enzyme strains that have the potential to enhance the digestibility of low quality forages. One of these enzyme preparations, Abo374, consistently provided enhanced degradability and fermentation results in the respective *in vitro* assays used. On the basis of these results, it was selected as the enzyme preparation most likely to have a positive response *in vivo*, and was consequently tested in a six week feeding trial. Medium and high treatment levels of 5 and 10ml enzyme supernatant per kg feed, applied to the wheat straw component of a forage-based basal diet, improved weight gains and feed conversion efficiencies of Dohne Merino lambs used in the trial.

It was concluded that the *in vitro* screening methods employed in this study proved to be both reliable and comparable in identifying enzymes that may have a positive animal production response. In addition, results from the *in vivo* study indicated positive animal production responses when feeding a high to moderate level of the exogenous fibrolytic enzyme preparation identified in laboratory screenings.

Results suggest that Abo374 has the potential to be used commercially to enhance fibre degradability of low quality forages fed to ruminants. More research is needed regarding optimal application levels of the enzyme, as well as the economic implications regarding its use in the ruminant feed industry.