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The use of exogenous enzymes and essential oils to enhance ruminal feed degradation and reduce methane production *in vitro*

by

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List of abbreviations

ADF	acid detergent fibre
ADL	acid detergent lignin
ANOVA	analysis of variance
Cellulase	cellulase PLUS
CO_2	carbon dioxide
CP	crude protein
DM	dry matter
EFEs	exogenous fibrolytic enzymes
EOs	essential oils
*iv*GP	*in vitro* gas production
ivCH_4	*in vitro* methane production
*iv*OMD	*in vitro* organic matter digestibility
mg	milligrams
Mixture	1 : 1 wt wt^{-1} mixture of cellulase and xylanase
ml	millilitres
SE	standard error
NDF	neutral detergent fibre
NFC	non fibre carbohydrates
OM	organic matter
Xylanase	xylanase PLUS

Declaration

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Signature_____

Date _____

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Poster Presentation

Effect of exogenous fibrolytic and protease enzyme on *in vitro* ruminal digestion, gas and methane production of *Eragrostis curvula* hay. 6th Greenhouse Gas and Animal Agriculture Conference, February 2016, Melbourne, Australia.

Abstract

The use of exogenous enzyme and essential oil to enhance ruminal feed degradation and reduce methane production *in vitro*

by

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Improving production efficiency and reducing the environmental impact of ruminant production systems would enhance the sustainability and global competitiveness of the livestock sector. Therefore, increasingly more emphasis is being put on climate warming threats and the intensification of livestock production systems in modern livestock practices. Greater awareness is placed on improving the utilization of conventional and non-conventional feedstuffs. In addition, the potential improvements that pure and mixtures of exogenous enzymes alone and in combination with essential oils (EOs) might have on rumen fermentation characteristics and reduction of enteric methane need to be studied in ruminants. In this regard identifying effective supplementation programmes for specific livestock rations is a step in the right direction. This study assessed several feed additives in order to produce the most promising ratio between methane emissions and degradable organic matter. The first experiment in this study aimed to determine the effect of treating three substrates with graded levels of cellulase PLUS (cellulase), xylanase PLUS (xylanase) and a mixture (1 : 1 wt wt^{-1} mixture of cellulase and xylanase) on *in vitro* gas production (*iv*GP). The substrates were pre-treated for 24 hours with these three exogenous fibrolytic enzymes (EFEs)

at five levels of application, namely 0, 0.5, 1, 1.5 and 2 ml g^{-1} DM, in order to identify the optimum level of application that improves ruminal degradation of these substrates. In the first experiment, the addition of EFEs showed a tendency towards significantly improved *iv*GP throughout the incubation period and across all three substrates ($P < 0.05$). The optimal dose was determined as 1.5 ml g^{-1} DM and this was used in the subsequent *in vitro* experiments. This experiment was followed by evaluating the influence of exogenous enzyme blends (fibrolytic and proteolytic) on *iv*GP, *in vitro* organic matter degradability (*iv*OMD) and *in vitro* methane production (*iv*CH_4). A factorial treatment combination of fibrolytic enzymes, namely control; xylanase at 1.5 ml g^{-1} DM (xylanase); cellulase at 1.5 ml g^{-1} DM (cellulase); and a 1 : 1 cellulase-xylanase mixture at 1.5 ml g^{-1} DM (mixture) and protease enzyme (0 and 0.5 ml g^{-1} DM substrate (protease)) were assessed using two substrates, that is, *Eragrostis curvula* and sunflower oilcake meal. The second experiment complemented the findings of the first experiment in that fibrolytic enzymes may generally increase *iv*GP. However, the addition of protease improved only *iv*GP of sunflower oilcake meal ($P < 0.05$). Fibrolytic enzymes improved *iv*OMD of sunflower oilcake meal, whilst the addition of protease improved the *iv*OMD of *Eragrostis curvula* ($P < 0.05$). The addition of fibrolytic enzymes increased *iv*CH_4, but not throughout the incubation period ($P < 0.05$). The addition of protease reduced *iv*CH_4 production of *Eragrostis curvula* at some incubation intervals ($P < 0.05$*)*. In contrast, the addition of fibrolytic enzyme increased *iv*CH_4 *iv*GP^{-1} and *iv*CH_4 *iv*OMD^{-1} for *Eragrostis curvula* substrate *(*$P < 0.05$*)*. The final experiment, namely the influence of various EOs in combination with fibrolytic enzymes on *iv*GP, *iv*CH_4 and *iv*OMD after 48 h of incubation of *Eragrostis curvula,* was investigated. The treatments included control with no enzyme and no EOs (T1); a 1 : 1 cellulase-xylanase mixture at 1.5 ml g^{-1} DM (enzyme) (T2); and a combination of this enzyme treatment and the EOs (Next Enhance®) (a commercial product that has 43 : 3.85 wt wt^{-1} of cinnamaldehyde and diallyldisulphide + diallyltrisulphide) (T3); cinnamon (T4); garlic (T5); and a blend of cinnamon and garlic oil (T6). These treatments were assessed in an *iv*GP study using *Eragrostis curvula* hay as a substrate. The third experiment also indicated that the addition of fibrolytic enzyme could increase *iv*GP and *iv*CH_4 and to some extent showed a tendency to increase the *iv*OMD ($P <$ 0.05) of the *Eragrostis curvula* substrate. Among the EOs evaluated, garlic and a combination of CinnGar with enzyme reduced *iv*GP compared with the enzyme treatment alone ($P < 0.05$). Furthermore, all of the EOs in combination with the enzyme treatment showed a tendency to reduce enteric methane production during the early stages of the incubation period compared with the enzyme treatment alone ($P < 0.08$). CinnGar in combination with enzyme reduced *iv*CH_4 throughout the incubation period ($P < 0.05$*).* Furthermore, a clear tendency was noted for the reduction in *iv*CH_4 and *iv*GP *iv*OMD^{-1} for the garlic and CinnGar treatments ($P < 0.095$). Based on the results of this study, *in vivo* evaluation of the mixture

enzyme treatment in combination with the CinnGar treatment is recommended to verify the repeatability of the results. Further studies on the use of these exogenous enzyme and EOs combinations under different conditions (rumen fluid donors, ruminal pH, substrate, dose levels, etc.) should also be conducted in order to attain a more informed recommendation on their efficacy. Following successful screenings, the best additives should be validated in *in vivo* trials.

General introduction

The production of meat and milk products from ruminants for human consumption is increasingly seen as one of the main contributing factors to harmful greenhouse gases (De Vries and De Boer, 2010). On the other hand, the global population is projected to reach more than 9.7 billion people by 2050 (FAO, 2016a) with a concomitant 60% increase in demand for food and livestock products (Alexandratos and Bruinsma, 2012). As a consequence of this rising demand, livestock production is expected to double by 2050 (Garnett, 2009). This in turn requires more research on efficient utilization of feed resources in combination with mitigation strategies aimed at reducing the ever-increasing harmful greenhouse gases. Sustainable animal production, guided by increased efficiency of resource utilization and reduced environmental impact, is seen as the solution going forward. Two mechanisms of such a solution are to utilize feed ingredients better through improvement in digestibility and to create an improved digestive environment (Binder, 2016). More recent studies have indicated that without modern feed additives sustainable animal nutrition would not be possible (Alexandratos and Bruinsma, 2012; Garnett, 2009). Feed additives such as enzymes and essential oils (EOs) have the potential to lower the amount of resources (land, feed, water and energy) used in livestock production, thereby leading to the production of healthy nutritious food for a growing world population (De Vries and De Boer, 2010). Methane, along with carbon dioxide (CO_2) and nitrous oxide, is an important greenhouse gas, which contributes to the global warming effect (Steinfeld *et al*., 2006). In livestock production, methane is produced in anaerobic conditions in the rumen by enteric methanogenic bacteria that use CO_2 and hydrogen as their building blocks (Leng, 2008). Various factors influence the production of methane in the rumen. The effects of diet and substrate degradability on the mitigation of ruminal methane are some of the factors that could be altered (Singh *et al*., 2005; Smith *et al*., 2005). The production of methane is desirable from a fermentative standpoint. However, it requires a significant amount of feed energy to produce methane. Cattle spend between 2% and 12% of their gross energy in the synthesis of this greenhouse gas (Beauchemin *et al*., 2009).

The use of exogenous fibrolytic enzymes (EFEs) has shown positive effects in terms of modulation of rumen fermentation and improvement of fibre degradation (Beauchemin *et al*., 2003; Adesogan *et al*., 2007; Krueger *et al*., 2008). Cellulase and xylanase in particular, which were introduced in ruminant diets, exhibited positive results in terms of enhanced dry matter (DM) intake (Beauchemin *et al*., 2000), increased digestibility (Krueger *et al*., 2008), increased live weight gain of beef cattle (Beauchemin *et al*.,

1999), and increased milk production of dairy cows (Yang *et al*., 1999; Adesogan *et al*., 2007). Further improvement was achieved through the use of proteolytic enzymes in combination with fibrolytic enzymes (Gado *et al*., 2009), although the results need to be verified for substrates with different characteristics and enzymes from various sources. The intended benefit of proteolytic addition is that this enzyme would degrade the cell wall protein and thereby improve access of ruminal microbiota to more digestible substrates (Colombatto and Beauchemin, 2009). Elghandour *et al*. (2013) reported improvements in degradability and fermentation parameters *in vitro* with an enzyme cocktail containing endoglucanase, alpha-amylase, xylanase and protease. Gado *et al*. (2009) confirmed these findings after conducting *in vivo* studies using the same enzyme cocktail.

Essential oils are organic compounds that are derived from plant secondary metabolism. Some EOs have antimicrobial activity. Calsamiglia *et al*. (2007) and Benchaar *et al*. (2007) conducted detailed reviews of EOs and their potential as additives to ruminant nutrition. Following the ban on the use of antibiotic growth promoters in Europe, renewed interest was ignited in EOs as rumen microbial modifiers. The majority of studies reported a positive effect in terms of an increase in the ratio of propionate to acetate, and a decrease in methane and ammonia nitrogen production, without decreasing the total production of volatile fatty acids (Busquet *et al*., 2005; Cardozo *et al*., 2005; Castillejos *et al*., 2006).

Cinnamaldehyde is the primary active compound of cinnamon oil and has antimicrobial activity against gram-positive and -negative bacteria. Busquet *et al*. (2005) tested cinnamaldehyde in a continuous flow study and reported reduced acetate production and increased propionate and butyrate production. The fermentation profile of cinnamaldehyde is similar to that of known antimethanogenic compounds such as carbon monoxide (Hino and Russel, 1985), suggesting that cinnamaldehyde has antimethanogenic properties, but its consistency over a range of feeds needs to be confirmed with further studies. Garlic oil, which is also EOs, is a combination of plant molecules derived from steam distillation (Lawson, 1996). Garlic oil has broad spectrum activity against gram-positive and gram-negative bacteria (Reuter *et al*., 1996). Busquet *et al*. (2006) showed that garlic oil reduces acetate and branched chain volatile fatty acids and increases propionate and butyrate. This fermentation profile is similar to that of antimethanogenic compounds such as carbon monoxide and amicloral that are applied to the rumen (Lamera *et al*., 2017).

There is somewhat limited and contrasting information in the literature about these feed additives and their associated effects on methane production. Nutritional interventions that contribute to the improvement of the efficiency of nutrient utilization, reduction of energy losses, and mitigation of enteric methane production are generally regarded as favourable (Monteny and Chadwick, 2006; Beauchemin *et*

al., 2008). However, it is crucial to introduce economical and low-risk mitigation strategies in a responsible manner to ensure that these strategies are adopted by livestock producers and that the integrity of the food supply chain remains intact. This research has an overall objective of improving the efficiency of utilization of commonly used feed resources in South Africa through the use of relatively safer additives to improve livestock production under intensive and extensive systems of production while reducing the carbon footprint of such systems.

Specific objectives

1. To determine the optimal level of application of three exogenous fibrolytic enzyme that maximize the *in vitro* gas production of three feed ingredients that are commonly used in ruminant nutrition in South Africa
2. To investigate the effect of adding exogenous protease in combination with exogenous fibrolytic enzyme on *in vitro* gas production, *in vitro* methane production and *in vitro* organic matter degradability of two substrates
3. To determine the effect of adding essential oil in combination with exogenous fibrolytic enzyme on ruminal fermentation and methane production in *Eragrostis curvula* hay used as a substrate

Chapter 1

Literature review

1.1. Sustainable animal production

Producing sufficient quantities of animal protein to feed an ever-expanding world population has become one of the primary objectives and concerns of our time. The challenge to produce enough animal protein is complicated, because it has to be done in such a manner as to sustain our planet. To achieve this goal, global production must curtail greenhouse gas emissions and deforestation. Therefore, we need to function within the critical confines of the planet's biosphere. This requires continuous use of innovative and research-based solutions that reduce the amount of resources – land, water, energy and feed – necessary to produce the required animal products to feed the world population. In recent times, nations all over the world have increased their efforts towards combatting climate change. At the United Nations Conference on Climate Change hosted in Paris in 2015, the vast majority of nations agreed on concrete measures to be taken to mitigate global warming (Paris Agreement, 2016). A 2015 Nielsen survey found that consumers generally want to be more environmentally friendly and do their own research about the products they purchase (Insights, 2016).

The United Nations estimates that global population will increase from 7.3 billion in 2015 to 9.7 billion by 2050. This estimation was made by its medium-variant projection (United Nations, 2015). This figure is stated with some uncertainty as factors such as economic growth, educational progress, national health and family planning can alter population growth. Even minor differences in fertility rates can have a major impact on world population growth (United Nations, 2015). In addition, over the last couple of decades, per capita income in the majority of world nations has increased, which has resulted in shifts in consumption patterns towards the use of high-quality protein from domestic and game animals. Therefore, the demand for animal protein such as meat, eggs, fish and milk has increased. In some more affluent nations, overconsumption of protein has resulted in imbalanced diets. Consumption of excessive amounts of red meat and processed meat may result in health risks such as obesity, diabetes, cardiovascular disease and even cancer (Worldwatch, 2016). The global middle class is projected to increase to an estimated 5 billion by 2030. This is a significant expansion from 1.8 billion in 2009, and most of this growth is projected to occur in Asia (Kharas, 2010). The Food and Agriculture Organization of the

United Nations studied the correlation between protein intake and increasing affluence. They found that per capita protein intake increases in a linear fashion when annual income ranges below 10 000 US dollars (United Nations, 2009). Furthermore, an ever-increasing proportion of the global population is moving from rural to urban areas. Urban growth exhibits positive consequences, as a higher proportion of the population have access to clean water, electricity, medical treatment and job opportunities. However, this increase is accompanied by greater strain on public institutions such as the electrical grid, infrastructure, health and education, as they attempt to meet the growing demand (Forbes, 2016). Urbanized populations tend to consume a greater range of dietary components compared with rural populations, which are defined by higher intake of meat, eggs, milk and fish (WHO, 2016). The Food and Agriculture Organization reported that in excess of 1 billion people suffered from chronic hunger in the 1990s. This figure declined to 793 million in 2015. Although these people consume enough energy to survive, harmful deficiencies of iodine, iron, vitamins and protein prevail (FAO, 2016). Hence, the goal should not be confined to producing adequate food for the ever-growing world population, but extended to provide a balanced diet with high-quality protein for everyone.

1.2. Ruminal digestion and methane production

Feed ingredients are digested in the rumen by symbiosis between the host animal and the microbes in the rumen (McDonald *et al*., 2011). Some microorganisms such as ciliated protozoans play a critical role in fibre fermentation by improving the attachment of other microorganisms to feed particles. They do this themselves by engulfing feed particles that enter the anaerobic rumen, while others do this by digesting rumen-degradable particles through secretions called fibrolytic enzymes (Krause *et al*., 2003). The rumen is a complicated environment consisting of various complementary and opposing relationships among microorganisms. Ruminal digestion is further complicated by the diversity of rumen microorganisms, which consist of a wide spectrum of bacteria, protozoa, fungi and methanogenic microorganisms (Kamra, 2005; Cieslak *et al*., 2013). The presence of fibre-fermenting bacteria that produce hydrogen is related directly to methanogens because of hydrogen transfer between these microbes (Morgavi *et al*., 2010). The animal in return has the primary products of microbial fermentation available for utilization (Weimer, 1998; Krause *et al*., 2003). These include volatile fatty acids, energy, microbial protein, CO_2 and methane (Marzorati *et al*., 2017). The production of methane takes place through the exchange of metabolites between hydrogen-synthesizing microorganisms and hydrogen-utilizing microorganisms (Kobayashi,

2010). This continuous exchange of hydrogen enables constant fibre fermentation in the rumen (Sakurai *et al*., 2012).

Methane production accounts for 7–17% of the loss of gross energy ingested, depending on the type of ingested material (Bayat *et al*., 2012; Hristov *et al*., 2013). However, a certain degree of methane production is required to maintain a healthy rumen environment as it restricts hydrogen build-up, which prevents the inhibition of dehydrogenase required in the oxidation of reduced co-factors (Martin *et al*., 2009). Following the reduction of cofactors such as NADH, NADPH and FADA, methanogens can utilize the hydrogen and in turn reduce CO_2, which results in the production of methane. The associated loss of energy from this process can be exacerbated when low-quality diets are fed that lack sufficient essential nutrients for healthy ruminal microbial growth (Charmley et al. 2016).

1.3. Constraints to fermentation of fibrous feed utilization

The outer layer of plant cell walls consists of pectin, cuticle and epicuticular waxes, which enable the plant to defend itself against dehydration and the invasion of phytopathogens. However, these protective layers of forages, grains and legumes may act as obstructions to feed utilization by the ruminal microbiota in the rumen (Selinger *et al*., 1996). These obstructions restrict microbial attachment to plant cell walls, thereby limiting ruminal fermentation. The infiltration of plant cell walls by the microbiota usually occurs through mechanical breakdown, namely chewing, cutting, grinding and pelleting, or at the stomata and lenticels. The extent of microbial establishment and the mechanisms of attachment differ among microbial species in the rumen (Varga and Kolver, 1997). The attachment is critical to effective fibrous feed fermentation and requires a well-balanced and adapted rumen microbial environment (McAllister *et al*., 1994; Min *et al*., 2014). The microbial population bind to the cell walls in various ways, including specialized binding proteins and receptors and physico-chemical forces such as Van der Waals forces (McAllister *et al*, 1994).

Breakdown of plant cell walls in the rumen is due mostly to the presence of fibrolytic bacteria such as *F. succinogenes, R. flavefaciens* and *R. albus,* which secrete fibrolytic enzymes with specific activities (Weimer, 1996; Varga and Kolver 1997). During this process the excreted cellulolytic enzymes adhere to the substrate, preventing other microorganisms and their enzymes from having access to the site of hydrolysis. Therefore, rumen cellulolytic bacteria can utilize the products of cellulolytic hydrolysis first (McAllister *et al*., 1994). Less is known about the exact role of fungi and protozoa compared with bacteria.

Fungi infiltrate the cell wall of lignified tissue. Fungal enzymes possess a broad range of activities, making fungal cellulases and xylanases the most active fibrolytic enzymes (Selinger *et al*., 1996). Similarly, protozoa contain all of the primary enzyme activities and therefore have great capacity to digest plant cell walls (Selinger *et al*., 1996). McDonald *et al*. (2011) concluded that the ruminal microbial population works in a complementary manner in the digestion of fibrous components by secreting a broad range of enzymes with multiple activities.

Various factors can lead to the restriction of fibrous feed degradation in the rumen. These include:

- The physical and chemical arrangement of plant components that prevent microbial colonization (McAllister *et al*., 1994)
- The balance among species of microorganisms in the rumen, the interactions between these species, the type of fibrolytic enzymes secreted and their relative concentrations as well as the manner of colonization of each species (Varga and Kolver, 1997)
- Secreted microbial fibrolytic enzymes, which affect colonization and hydrolysis of other microbial species (McAllister *et al*., 1994)
- Host animal and feed processing factors that determine the nutrient supplies in the rumen, such as mastication, salivation, grinding and pelleting (McDonald *et al*., 2002)
- The particle size of the substrate, which determines the area available for initial microbial colonization (Gimeno *et al*., 2015; Kazemi-Bonchenari *et al*., 2017)
- The degree of lignin, because it acts as a physical barrier to microbial colonization, leading to many substrates that can be digested only from the interior (Jung *et al*., 2012)
- The pH of the rumen, which affects the rumen microbial environment (Plaizier *et al*., 2009)

A depression of ruminal pH can be expected when feeding more grains and less roughage. The production of less sodium bicarbonate occurs because the need for mastication and rumination is lowered. This shift inevitably results in a reduction of ruminal pH, which can lead to a decline in cellulolytic microorganisms and ultimately fibre digestion. It is critical that the diet should contain sufficient fibre and the correct particle size to encourage rumination and combat low ruminal pH (Grant, 1997). Differences observed in the mastication actions of animals may affect rumination and contractions in the digestive tract, which can influence the utilization of fibrous substrates (Firkins *et al*., 1998).

1.4. Exogenous enzymes in ruminant nutrition

Enzymes are proteins that catalyse chemical reactions in biological systems. Digestive enzymes are of particular importance to nutritionists as they play a critical role in the breakdown of nutrients in the gastrointestinal tract of animals (McDonald *et al*., 2011). In animal nutrition, enzymes may be further divided into two groups, namely endogenous (originating from the animal) and exogenous (originating from microorganisms). These enzymes increase the rate of degradation of feed substrates that can be absorbed by the animal itself or the microbial population within the animal (Krueger *et al*., 2008).

Suitable exogenous enzymes can be added to feed substrates in an attempt to increase their nutritive value by improving the efficiency of digestion and absorption (Valdes *et al*., 2015). Various types of exogenous enzyme are available with different modes of actions, such as fibrolytic enzymes, proteolytic enzymes, amylolytic enzymes and phytases, which act on the fibre, protein, starch and unavailable phosphorous fraction of feedstuffs, respectively (Bedford and Partridge 2011). In this study the discussion focuses on fibrolytic and proteolytic enzymes.

1.4.1. Exogenous fibrolytic enzymes in ruminant nutrition

Investigation into the use of exogenous fibrolytic enzymes (EFEs) in ruminants began in the early 1950s. This was based on their potential to convert lignocellulose to glucose and other soluble sugars. Lignocellulose is the most abundant and renewable source of energy on earth, but is slowly degradable. Following advances in fermentation technology and biotechnology, the industrial production of EFEs was initiated in the 1980s (Bhat, 2000). In animal nutrition, the use of feed enzymes showed the potential to enhance the efficiency with which animals utilize raw materials (Beauchemin *et al*., 2003). Ruminal digestion and absorption of plant cell walls are inefficient. According to Van Soest (1994), less than 65% of the potential nutritive value of plant cell walls is degraded in the rumen. Overcoming this inefficiency can lower feed costs and reduce the environmental pressure of ruminant-based production systems (Sheppy, 2001).

Commercial enzymes, commonly referred to as cellulases or xylanases, rarely consist of pure enzyme products, and usually contain secondary enzyme activities such as amylases, proteases, esterases, and

pectinases (McAllister *et al*., 2001). This is advantageous as a wide spectrum of enzymes is required to degrade the structural carbohydrates in plant cell walls (Morgavi *et al*., 2000a). However, identifying pure enzymes that are responsible for any positive interaction with feed digestion becomes a challenge. Common practice is to include enzymes that are suitable for a range of feed types (Beauchemin *et al*., 2003). This always leads to highly variable results when enzymes are included in ruminant formulations. Investigating the effects of pure enzyme products on specific feedstuffs can enable more precise formulation. The addition of exogenous enzymes to suitable and commonly used feedstuffs would ultimately result in more reliable performance. Fibrolytic enzymes could result in increased nutrient digestibility. These nutrients can then be utilized by the microbial population in the rumen, facilitating more rapid microbial growth and increasing microbial colonization of feed substrate particles (Alvarez *et al*., 2009). Giraldo *et al*. (2008) observed a shift in the fibre structure due to the enzymatic effects. This, together with increased feed colonization, constitutes the lag time before the start of microbial digestion (Yang *et al*., 1999; Elwakeel *et al*., 2007). There is a synergistic effect between certain EFEs and microbial enzymes produced in the rumen. Hence hydrolytic activity in the rumen can be increased (Morgavi *et al*., 2000a).

1.4.2. Exogenous proteolytic enzymes in ruminant nutrition

Proteolytic enzymes are involved in the utilization of proteins and therefore have a direct contribution to the growth of an animal. Some studies have been conducted on the addition of proteolytic enzymes in combination with fibrolytic enzymes. The hypothesis is that proteolytic enzymes may degrade the cell wall protein, thereby improving the availability of more digestible nutrients to rumen microorganisms (Colombatto and Beauchemin 2009). Enzyme cocktails containing endoglucanase, xylanase, alpha-amylase and protease have shown increased digestibility and ruminal fermentation during *in vitro* (Elghandour *et al*., 2013) and *in vivo* (Gado *et al*., 2009) evaluation studies. The addition of protease to whole-corn plants after harvest increased the proteolytic stages of the ensiling process, resulting in increased ruminal *in vitro* starch degradation (Young *et al*., 2012). The addition of exogenous enzyme to low-quality roughage may increase digestibility and reduce methane per organic matter digested (Beauchemin *et al*., 2008; Grainger and Beauchemin 2011), but the observed results varied substantially. An *in vivo* screening conducted by McGinn *et al*. (2004) did not reduce methane per organic matter digested. On the other hand, Giraldo *et al*. (2007) reported increased *iv*CH_4 per organic matter digested

for grass hay treated with exogenous enzymes during the early stages of the incubation period. It is evident from the varying results that more research into the appropriate application of protease in various types of feedstuffs in ruminant diets needs to be conducted.

1.5. Factors affecting enzyme efficacy in ruminants

The effectiveness of exogenous enzymes is measured by their ability to improve the digestibility of feed and thereby unlock feed nutrients to an extent that they can be incorporated by the animal or via the animal's microbial population. Thus exogenous enzymes should supplement the enzymatic activities that are limiting digestion (Morgavi *et al*., 2000b). Various factors contribute to the efficacy of exogenous enzymes.

1.5.1. Enzyme–substrate preparation and interaction period prior to feeding

High moisture content of the feed substrate can increase the hydrolysis of carbohydrates from complex polymers (Morgavi *et al*., 2000a; Nsereko *et al*., 2000; Wallace *et al*., 2001). The subsequent solubilization of acid detergent fibre (ADF) and neutral detergent fibre (NDF) can cause the rapid spread of microorganisms and reduce the lag time needed for microbial establishment (Morgavi *et al*., 2000b; Devillard *et al*., 2004). Therefore, applying liquid enzyme treatment to a dry substrate is recommended. Furthermore, applying a pre-incubation time of enzymes and substrate together enables absorption and binding of the enzyme to the substrate. Enzymes that had interaction with the substrate prior to consumption appear to have higher efficacy *in vivo*. This is probably due to its higher resistance to proteolytic inactivation in the rumen resulting from a protective enzyme-substrate complex that has formed (Fontes *et al*., 1995; Kung *et al*., 2000). Colombatto *et al*. (2007) recommended an enzyme-substrate interaction time of 12–36 hours before consumption. In addition, increasing the proportion of substrate to which the enzyme is added improves its viability in the rumen. It was found that adding enzymes to the macro raw materials that make up 45% of the ration was more effective compared with including them in the premix, which comprised 0.2% of the diet (Bowman *et al*., 2002).

1.5.2. Enzyme dose rate

The optimal dose rate is of utmost importance if exogenous enzymes are to alter digestion efficiently (Eun *et al*., 2007; Jalilvand *et al*., 2008). The optimal dose rate is influenced by the type of feed and its chemical composition (Pinos-Rodriquez *et al*., 2002; White *et al*., 1993), DM content and pH (Colombatto *et al*., 2007). It is critical to establish the optimal dose of enzymes for the substrate in question to attain the highest efficacy.

1.5.3. Identification of primary enzymatic activities

Enzymes that are utilized in the livestock sector were often developed for other purposes, because primary enzymatic activities might differ from those needed to improve ruminal fermentation. It is therefore a critical step to establish the primary enzymatic activities when evaluating potential enzyme supplements in ruminants (Eun and Beauchemin, 2007). Because of the vast array of enzymes that are available to the livestock industry, it is crucial to match the substrate with the best-suited enzyme treatment (Hristov *et al*., 1998; Beauchemin *et al*., 2003).

1.5.4. Substrate composition

The effects of exogenous enzyme supplementation are substrate specific because the efficacy of enzymes that improve nutrient degradation relies on their activities, which in turn is related to the chemical composition of the substrate (Pinos-Rodriguez *et al*., 2002).

1.6. Enteric methane mitigation strategies

Various factors contribute to enteric methane production, namely the availability and composition of ingested feed (Beauchemin *et al*., 2008), the ratio of acetate to propionate in the rumen (McAllister *et al*., 1996), the types of carbohydrates after fermentation (Santoso *et al*., 2003), NDF and ADF concentrations (Hindrichsen *et al*., 2003), and the characteristics of the forage that is consumed (Arthington and Brown, 2005). One or more of these scenarios must hold true to see a reduction in possible enteric methane production:

- Decreased ruminal hydrogen production without impeding digestibility
- Increased hydrogen usage in metabolic pathways producing metabolites other than methane, for example butyrate production
- Inhibitory effects on methanogenic microorganisms without negatively affecting the rumen environment

Alterations to the ration by lowering the proportion of fibre in exchange for concentrate should reduce methane production per unit DM consumed (Lovett *et al*., 2003). In a study conducted by Agle *et al* (2010), a dairy cow ration with 50% concentrate produced 44% higher methane compared with a diet with 70% concentrate. Ruminants consuming feed with a higher proportion of grains or starch generally emitted lower methane. However, because of the cost implications and the lack of concentrates, multiple livestock production systems cannot use concentrates as a methane mitigation strategy. Many researchers have concluded that increasing the quality of forage fermentation could result in the reduction of enteric methane emissions (Moss, 2000; Boland *et al*., 2009; McGeough *et al*., 2010). Some of the strategies that show promise in ruminants consuming high roughage diets are described in the subsequent sections.

1.7. Essential oils in ruminant nutrition

The need to optimize energy and protein utilization in the rumen has been discussed extensively (Nagaraja *et al*., 1997; Busquet *et al*., 2006; Cardozo *et al*., 2006). Alteration of the microbial population is a viable option to achieve this goal. The supplementation of antibiotic ionophores has been successful in this regard, but their use in the livestock industry faces serious social acceptance issues. Therefore, the use of

natural product alternatives to optimize the microbial population has been investigated (McIntosh *et al*., 2003). Some EOs have been shown to have antimicrobial activities that can reduce peptidolysis, methanogenesis and deamination in the rumen (Nagaraja *et al*., 1997; Cardozo *et al*., 2006). Increasing public concern over global warming and greater emphasis on rumen-derived methanogenesis has directed considerable research efforts towards suppressing ruminal protozoa because of their close association with methanogens (Williams and Coleman, 1992).

Essential oils (EOs) are blends of volatile aromatic secondary metabolites that are extracted from plants by steam distillation (Greathead, 2003). In addition to various health effects (Harborne and Williams, 2000; Reddy *et al*., 2003), EOs have antimicrobial activity against gram-positive and gram-negative bacteria, protozoa and fungi (McIntosh *et al*., 2003; Burt, 2004). The antimicrobial activity of EOs is probably related to their disruption of microbial cell membranes, leading to impaired electron transport, ion gradients and translocation (Griffin *et al*., 1999). The investigation of EOs in livestock production commenced in the 1960s (Borchers, 1965; Oh *et al*., 1967; Nagy and Tengerdy, 1968). Most of the research efforts into EOs were abandoned because of the use of antibiotics in animal feed in the 1970s. However, following the ban on antimicrobial growth promoters in Europe, renewed efforts have been conducted in the study of EOs as rumen fermentation modifiers. Numerous studies have been published since then. The positive results obtained in these studies included an increase in propionate production and reductions in acetate, methane and ammonia nitrogen production. Pure culture studies revealed that EOs affected mostly ruminal hyperammonemia-producing bacteria *(Peptostreptococcus anaerobius, Clostridium aminophilum, Clostridium sticklandii)* and fungi (McIntosh *et al*., 2003). Consequently, the rate of deamination of amino acids was significantly reduced in this study. Other *in vitro* studies using batch or continuous cultures reported variable effects of EOs on deamination of amino acids (Newbold *et al*., 2004; Busquet *et al*., 2006; Castillejos *et al*., 2006). When a combination of cinnamaldehyde (0.6 g day^{-1}) and eugenol (0.3 g day^{-1}) was fed *in vivo* (Cardozo *et al*., 2006), acetate and ammonia-nitrogen concentrations decreased and propionate concentration increased, which led to an accumulation of peptides and amino acids in ruminal fluid. Even though the author did not measure methane production in this study, the change in the volatile fatty acid profile is consistent with decreased methane production. However, if methanogenesis is inhibited by EOs, and the electrons are not used in the formation of methane or if these electrons are not efficiently relocated into other sinks, the re-oxidation of cofactors and interspecies hydrogen transfer could be impaired (Nagaraja *et al*., 1997). Deamination discharges one pair of reducing

equivalents per mole of ammonia released. The decrease in the redox potential caused by the inhibition of methanogenesis could then inhibit deamination (Russell and Martin, 1984).

1.7.1. Cinnamaldehyde in ruminant nutrition

Cinnamaldehyde is a phenylpropanoid with a broad range of antimicrobial actions against gram-positive and -negative bacteria (Calsamiglia *et al*., 2007). Cinnamaldehyde is the primary active compound of cinnamon oil *(Cinnamomum cassia)* (Calsamiglia *et al*., 2007). After conducting a continuous culture experiment, Cardozo *et al*. (2004) found that cinnamon oil altered the nitrogen metabolism of the ruminal microbial population by impeding peptidolysis. Cinnamon and cinnamaldehyde reduced total volatile fatty acid and ammonia-nitrogen concentrations, and cinnamaldehyde showed stronger effects when compared with cinnamon oil (Busquet *et al*., 2006). These studies indicate that the additional component in cinnamon oil may interact with cinnamaldehyde, and that pure cinnamaldehyde delivers more favourable effects on ruminal fermentation. Busquet *et al*. (2005) reported lower molar concentration of acetate and increased molar concentration of propionate and butyrate following the addition of cinnamaldehyde. This is a fermentation profile that is comparable with those seen in the application of other antimethanogenic compounds, indicating that the mode of action of cinnamaldehyde may inhibit methanogenesis (Hino and Russel, 1985; Yang *et al*, 2010), although this needs to be confirmed by further research.

1.7.2. Garlic oil in ruminant nutrition

Garlic oil consists of a mixture of molecules that are formed within the plant or after oil extraction and processing (Lawson, 1996). Garlic oil is known for its antiparasitic, anti-oxidant, insecticidal, anti-inflammatory, hypoglycaemic and anti-cancer properties, but its antimicrobial activity against a broad range of gram-positive and negative bacteria is of particular interest in the livestock sector (Reuter *et al*., 1996). The addition of garlic oil reduced acetate and branched chain volatile fatty acids while increasing propionate and butyrate (Busquet *et al*., 2005; Busquet *et al*., 2006). These effects are consistent with alterations observed on the addition of methane inhibitors to the rumen environment (Lamera *et al*., 2017). Busquet *et al*. (2005) demonstrated that garlic lowered the methane to volatile fatty acid (µmol)

ratio from 0.20 to 0.05. Because methane is the primary hydrogen sink of rumen fermentation, a reduction in methane production leads to increased levels of reducing equivalents that are utilized in the formation of additional propionate and butyrate (Van Nevel and Demeyer, 1988). Diallyl disulfide and allyl mercaptan are the primary components in garlic oil, which are responsible for the observed reduction in methane production (Busquet *et al*., 2005). Kamel *et al*. (2007) confirmed these results. The modes of action of garlic oil and its primary constituents differ from other EOs (Busquet *et al*., 2005). Gebhardt and Beck (1996) found that the addition of garlic oil resulted in the inhibition of *Archaea* microbes in rumen via the deactivation of HMG-CoA reductase, leading to reduced levels of the isoprenoid unit required for cell membrane rigidity. Busquet *et al*. (2003) noted increased milk production in dairy cattle after the addition of garlic oil. However, no alteration in milk production was found following the addition of powdered garlic in dairy cows. These results may be different when applying garlic oil, as the primary active compounds are identified and synthesized from oil extraction (Lawson, 1996; Yang *et al*., 2006). Few studies have been conducted on the effects of garlic oil *in vivo,* therefore more research should be done in order to compare the results obtained from *in vitro* studies with those of *in vivo* trials.

1.8. Conclusion

The goal of the livestock industry should not be confined to producing adequate food for an ever-growing world population, but should be extended to providing a balanced diet that contains high-quality protein for everyone in a sustainable manner. The production of enteric methane represents a significant loss of gross energy ingested. It is critical that the diet provided to ruminants should promote a well-balanced rumen microbial environment to promote efficient feed fermentation. Suitable exogenous enzymes can be supplemented to feed substrates to increase their nutritive value by improving the efficiency of fermentation. However, identifying pure enzymes responsible for any positive interaction with feed digestion is a challenge. It is therefore necessary to determine the effect of pure enzyme products on certain feed ingredients that could ultimately lead to more precise formulations. The addition of fibrolytic enzymes could result in increased nutrient digestibility, resulting in more rapid microbial growth and increased microbial colonization of feed substrate particles. It has been found that proteolytic enzymes in combination with fibrolytic enzymes can degrade cell wall protein, thereby improving the availability of more digestible nutrients to rumen microorganisms. This could lead to increased digestibility and efficiency of ruminal fermentation. Some of the most important factors that influence the efficacy of

exogenous enzymes include the enzyme-substrate interaction period prior to ingestion**,** and application of the most suitable dose rate of enzymatic activities on specific substrates. Research suggests that effective reduction of enteric methane emissions could be achieved by improving the process of ruminal fermentation. The addition of certain EOs supports these findings. EOs have shown the capacity to increase the ratio of propionate to acetate and reduce enteric methane and ammonia production. In particular, cinnamaldehyde and garlic oil have been shown to have antimethanogenic characteristics, although there is a general lack of studies on the effect of EOs on methanogenesis.

1.9. Hypotheses of the experiments

To address the specific objectives of this study, a series of experiments were designed in order to test the following hypotheses:

- The addition of various levels of fibrolytic enzymes to *Eragrostis curvula*, sunflower oilcake meal and hominy chop does not affect the *in vitro* gas production of these substrates.
- The addition of exogenous protease enzyme in combination with fibrolytic enzyme on *Eragrostis curvula* and sunflower oilcake meal does not affect the *in vitro* gas production, *in vitro* methane production or the *in vitro* organic matter degradability of these substrates.
- The addition of various essential oil in combination with fibrolytic enzyme to *Eragrostis curvula* substrate does not affect its *in vitro* gas production, *in vitro* methane production or the *in vitro* organic matter degradability.

Thus the following three experiments were conducted and the findings are reported as Chapters 2, 3 and 4 of this dissertation.

- Experiment 1: Effects of various levels of fibrolytic enzyme addition on *in vitro* gas production of *Eragrostis curvula*, sunflower oilcake meal and hominy chop (Chapter 2)
- Experiment 2: Effects of fibrolytic enzyme with or without the addition of protease enzyme on *in vitro* digestibility, gas and methane production (Chapter 3)
- Experiment 3: Use of essential oil in combination with fibrolytic enzyme to reduce *in vitro* ruminal methane production (Chapter 4)

Chapter 2

Effects of various levels of the addition of fibrolytic enzymes on *in vitro* gas production of *Eragrostis curvula*, sunflower oilcake meal and hominy chop

2.1. Abstract

Investigating the interaction between exogenous enzymes and individual substrates is a crucial step towards identifying the best enzymatic supplementation for a specific livestock ration. The aim of this study was to determine the effect of treating three substrates with graded levels of cellulase PLUS (cellulase), xylanase PLUS (xylanase) and their mixture (1 : 1 wt wt^{-1} mixture of cellulase and xylanase) on *in vitro* gas production (*iv*GP). The substrates were pre-treated for 24 hours with the three exogenous fibrolytic enzymes (EFEs) at five levels of application: 0, 0.5, 1, 1.5 and 2 ml g^{-1} DM. Rumen fluid was collected from six cannulated sheep fed a lucerne hay-based diet was used as a source of inoculum. Substrates used in the *in vitro* batch culture were *Eragrostis curvula*, sunflower oilcake meal and hominy chop. *In vitro* gas production was measured in 125 ml glass vials using a pressure transducer connected with a digital gauge, following anaerobic fermentation in a water bath set at 39 °C. Gas pressure readings were taken after 2, 4, 8, 12, 18, 24, 36 and 48 hours of incubation. The addition of exogenous enzymes had shown a tendency towards significantly improved *iv*GP throughout the incubation period and across all three substrates ($P < 0.05$). Enzyme addition resulted in increased cumulative *iv*GP following the 48-hour incubation period of 7.2 – 14%, 7.66 – 21% and 3 – 11% for cellulase, xylanase and mixture treatments, respectively. Considering the economic implications and the relative increase in *iv*GP per unit of additional enzyme revealed across the three treatments and substrates, an application rate of 1.5 ml g^{-1} DM was identified as the optimal dose to be used in subsequent *in vitro* and *in vivo* studies.

2.2. Introduction

The use of exogenous enzymes as feed additives in ruminant feeds has produced encouraging effects in terms of improved nutrient utilization of raw materials, increased production efficiency, and lowered nutrient excretion (Beauchemin *et al*., 2003). These positive results are not always replicated, partly due to the intricate nature of the ruminant digestive system and partly to enzyme-substrate interaction. This has resulted in variability in the efficacy of exogenous enzyme products across substrates (Colombatto *et al*., 2003a). In addition, the complexity of the rumen requires that exogenous and endogenous enzyme activities of rumen microbes should complement each other (Morgavi *et al*., 2000b). Additional information is required on enzymatic activities, optimal dose rates, and *in vitro* fermentation characteristics to establish the desired enzymatic effects on specific feed ingredients used locally in commercial ruminant rations (Eun and Beauchemin, 2007; Jalilvand *et al*. 2008). The type and optimum inclusion rate of exogenous enzymes are thus dependent on the diet under consideration. There is a need to identify suitable types and optimal dose rates of exogenous enzyme treatments for locally important substrates to utilize these additives more efficiently. This study assessed the enzymatic treatments of cellulase, xylanase and a 1 : 1 mixture at five levels (0, 0.5, 1, 1.5, 2 ml g^{-1} DM) on the *iv*GP characteristics of three substrates, namely *Eragrostis curvula*, sunflower oilcake meal and hominy chop, which are commonly used in the diets of ruminants in South Africa.

2.3. Materials and methods

The following subsections describes the methodology followed in this chapter.

2.3.1. Description of study area

The activities of two registered Dyadic enzyme products (cellulase and xylanase) on three commercial feed ingredients were determined using the *iv*GP method. The *in vitro* experiments were conducted at the Nutrition Laboratory of the Department of Animal and Wildlife Sciences, University of Pretoria. Cellulase and xylanase enzymes were obtained from Dyadic International Inc. Florida, USA, as

concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8) respectively, as well as a 1 : 1 wt wt^{-1} combination of these enzymes. Cellulase and xylanase enzymes were produced by the fermentation of non-genetically modified *Trichoderma longibrachiatum*. *Eragrostis curvula*, sunflower oilcake meal and hominy chop were used as the test substrates. The substrates were treated with 0, 0.5, 1, 1.5 and 2 ml g^{-1} DM of the three enzymes, namely cellulase, xylanase and a mixture of the two at the same inclusion levels.

2.3.2. Feed sample collection and preparation

Eragrostis curvula, sunflower oilcake meal and hominy chop were collected from a commercial feed mill in Gauteng. The feed samples were dried at 55 °C for 48 hours in a forced air oven. For all *in vitro* studies and chemical composition analyses, the samples were milled through a 1-mm sieve in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) to decrease the variations in particle size in a particular sample (Tilley and Terry, 1963). The samples were stored in resealable plastic bags at 4 °C to preserve the chemical composition of the feed samples.

2.3.3. Chemical analysis of substrates

The substrates were analysed for dry matter (DM), ash, organic matter (OM), ether extract (EE), crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). Dry matter was determined according to the method of AOAC International (method 934.01 AOAC, 2000). The samples were analysed for OM and ash by combustion in a muffle furnace at 250 °C for 2 hours followed by an additional 4 hours at 600 °C (method 942.05) (AOAC, 2000). The EE fraction was analysed in the Tecator Soxtec (HT6) system (method 920.39) (AOAC, 2000). The CP fraction was measured on a Leco FP-428 nitrogen and protein analyser (Leo Corporation, St. Joseph, MI, USA) according to the combustion method 968.06 (AOAC, 2000). NDF, ADF and ADL fractions were analysed using an ANKOM analyser (ANKOM Technology, Fairport, NY) according to the methods described by Van Soest *et al*. (1991).

2.3.4. Enzyme assay

Enzyme activities were determined with single polysaccharides as a substrate in triplicate with the inclusion of a blank. Xylanase activity was assayed with 1% (w v^{-1}) birchwood xylan as a substrate according to the procedure described by Bailey *et al*. (1992). Endoglucanase and exoglucanase enzyme activities were assayed according to the method described by Wood and Bhat (1988). Enzyme activity was studied at a range of pH levels (4, 5, 6 and 7). One unit of activity was defined as the amount of enzyme required to release 1 µmol equivalent of glucose or xylose min^{-1} g^{-1} of enzyme under the conditions of the assay.

2.3.5. Substrate preparation and enzyme dilution

Using identical glass vials of 125 ml capacity, 400 mg ± 0.05 mg of each substrate was weighed into each incubation vial. The required dose rates were diluted to deliver the specific concentration of enzymes in 1 ml aliquots. The density of the enzyme and carrier mixture was different from that of water and was corrected for. A fresh batch of dilutions were prepared for every run using the same method to ensure that the efficacy of the enzyme dilutions remained the same. The amount of 1 ml of enzyme treatment was added to each vial 24 hours prior to the start of the incubation. The control treatment received 1 ml of distilled water to standardize the volume for the vials.

2.3.6. Buffer medium preparation

The buffer, macro mineral and micro mineral solutions were prepared according to the methods of Goering and Van Soest (1970). The micro mineral solution was stored in dark brown glass bottles at 4 °C for no longer than a month to guarantee its stability.

The buffer medium was prepared before rumen fluid collection. The buffer medium consisted of distilled water, buffer, macro mineral and micro mineral solution mixed with tripticase. Thereafter, appropriate amounts of 0.1% (wt $volume^{-1}$) resazurin, L-cysteine hydrochloride and sodium-sulphide were weighed and added to the solution after all the components had been completely dissolved. The solution was then placed on the heating and stirring plate at 39 °C with a constant stream of CO_2 running through the

solution. Rumen fluid were collected when the solution was fully reduced, which was indicated by the clear colour.

2.3.7. Collection of rumen fluid from donor sheep and *in vitro* gas production measurement

An adaptation period to the basal diet (lucerne hay) of fourteen days was given prior to the collection of rumen fluid for *in vitro* evaluations. The six Merino wethers were fed *ad libitum*. The feed was offered to the animals in two fractions, in the morning at 7:30 and again at 15:30. Rumen fluid collections were done at 08:30 a.m. throughout the trial, after the morning feeding. Approximately 250 ml of ruminal fluid was collected from each of the six donor animals. Collections were done from at least three sites in the rumen. Rumen contents were filtered through four layers of cheesecloth into a pre-heated thermos flask. The flask was filled with rumen fluid to maintain anaerobic conditions. In the laboratory, the rumen fluid was filtered once more through two layers of cheesecloth with continuous streaming with CO_2. Appropriate amounts of rumen fluid were added to a 5 L beaker containing the reduced buffer solution already maintained at 39 °C. To maintain 39 °C, the beaker was placed on a hotplate with temperature regulator and stirrer. A 20 mm magnetic stirrer was spun to distribute the mixture evenly while being flushed with CO_2 (Grant and Mertens, 1992).

The vials were filled with 33 ml (82.5 mL g^{-1} substrate) of reduced buffer medium and rumen fluid under a constant stream of CO_2. At least 80 ml of headspace was kept to avoid pressure build-up higher than 7.0 psi during the incubation period. Following inoculation, blue stoppers equipped with 1.5 inch 22-gauge needles and three-way stopcocks were used to seal each vial. Crimp seals were not required for these stoppers. Following inoculation, the stopcock of every vial was opened for five seconds to release the small amount of gas that might have built up and create a starting point for the incubation run. The vials were returned to the water bath at 39°C and the sliding tray was turned on at 50 rpm. Four bottles with only rumen liquor and reduced buffer solution was added to each experimental run as blanks to correct the gas produced from the bottle treated with various treatments.

An incubation period of 48 hours was used in this experiment. The gas pressure was recorded by a pressure transducer (PX4200-015GI from Omega Engineering, Inc., Laval, QC, Canada) with a three-way stopcock compatible connection on the tip, as described by Theodorou *et al*. (1994). Gas pressure was

measured and released at 0, 2, 4, 8, 12, 18, 24, 36 and 48 hours of incubation. Gas measurements at each interval were recorded in psi units and later converted to volume (ml of gas produced) using a calibration curve with pressure on the one axis and volume on the other. After 48 hours of incubation, fermentation was terminated by placing all the bottles on ice. In this study, four replicate bottles were used for every sample and its corresponding treatments in a run. Three independent runs were conducted as a replicate in different batches.

2.3.8. Calculations and statistical analysis

A completely randomized experimental design was used in this study. The data were statistically analysed using the GLM option of SAS (version 9.4, 2013). Separate analysis was undertaken for each of the three enzymes in order to test the effects of enzyme levels, substrate type and interaction between enzyme level and substrate type. Where the F-test showed significant differences, the differences between means were determined using Tukey's test.

2.4. Results

2.4.1. Chemical composition

The proximate analysis of the three substrates used in this study is presented in Table 2.1. These three substrates are readily included in ruminant diets. The chemical compositions of these substrates differ significantly. Hominy chop had the lowest ash content (23.9 g kg^{-1} DM). The highest CP content was recorded for sunflower oilcake meal (390 g kg^{-1} DM). *Eragrostis curvula* had the highest acid ADF (475 g kg^{-1} DM), NDF (770 g kg^{-1} DM) and ADL (42 g kg^{-1} DM) content, followed by sunflower oilcake meal ADF (278 g kg^{-1} DM), NDF (350 g kg^{-1} DM) and ADL (20 kg^{-1} DM) and finally hominy chop ADF (13 g kg^{-1} DM), NDF (25 g kg^{-1} DM) and ADL (7 kg^{-1} DM).

The EE content (95 g kg^{-1} DM) of hominy chop was much higher than that of sunflower oilcake meal (24 g kg^{-1} DM) and *Eragrostis curvula* (21 g kg^{-1} DM). Hominy chop was the only substrate containing starch (498 g kg^{-1} DM).

Table 2.1. Chemical composition of sunflower oilcake meal, *Eragrostis curvula* and hominy chop

Chemical components	Substrate composition g kg^{-1} DM		
	Sunflower oilcake meal	*Eragrostis curvula*	Hominy chop
DM	949	953	938
Ash	58.6	57.8	23.9
OM	890	895	914
EE	24	21	95
CP	390	87	98
NDF	350	770	25
ADF	278	475	13
ADL	20	42	7
Starch	-	-	498

DM: dry matter; OM: organic matter; EE: ether extract; CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; ADL: acid detergent lignin

2.4.2. Enzyme activity

The enzyme activity profile was determined at a temperature of 39 °C and various pH levels. The results are shown in Table 2.2. The highest endoglucanase activity was observed at a pH of 6, 4 and 4 for cellulase, xylanase and mixture, respectively. However, no significant exoglucanase activity was observed for any of the enzymes. The highest hemi-cellulase activity was observed at a pH of 5, 4 and 4 for cellulase, xylanase and mixture, respectively.

Table 2.2 Enzyme activities of cellulase, xylanase and mixture and the amounts of released sugar equivalents at four pH levels

Enzyme product *(n=12)*	*pH*	Cellulase activity		Hemi-cellulase activity
		Endo-glucanase activity *(µmol min^{-1} mL^{-1})*	*Exoglucanase activity* *(µmol min^{-1} mL^{-1})*	*Xylanase activity* *(µmol min^{-1} mL^{-1})*
Cellulase PLUS (cellulase)	4	5347	24	1128
	5	5973	25	1385
	6	6044	25	1234
	7	4219	24	1209
Xylanase PLUS (xylanase)	4	1000	25	6413
	5	801	25	6147
	6	802	24	4466
	7	775	23	3907
Cellulase : Xylanase PLUS (mixture)	4	1075	24	5698
	5	921	24	5469
	6	901	24	4935
	7	818	24	2475

2.4.3. *In vitro* gas production

The effects of various levels of cellulase, xylanase and a mixture of cellulase and xylanase enzyme treatments on the cumulative *iv*GP of the three test substrates are shown in Tables 2.3, 2.4 and 2.5.

There was no significant interaction effect between substrate and the level of cellulase enzyme treatment. At the 2-hour and 4-hour incubation intervals only the 2 ml g^{-1} DM level produced significantly ($P < 0.05$) higher levels of *iv*GP compared with the control treatment. The 1.5 and 2 ml g^{-1} DM levels produced significantly higher levels of *iv*GP during the 8-hour and 12-hour intervals. Thereafter no significantly different ($P < 0.05$) *iv*GP occurred between enzyme levels and the control treatment, although a strong tendency was observed that showed an increase of *iv*GP with an increase in enzyme treatment level (Table 2.3).

Similarly, there was no significant interaction effect between substrate and the level of xylanase enzyme treatment. The effect of xylanase enzyme level is depicted in Table 2.4. At the 2-hour incubation interval level, 1.5 and 2 ml g^{-1} DM resulted in significantly higher *iv*GP compared with the control treatment. Generally, gas production rose with an increase in xylanase enzyme level. This trend continued from the 4-hour interval up to and including the 24-hour interval, with 1, 1.5 and 2 ml g^{-1} DM levels producing significantly higher *iv*GP compared with the control treatment. The 36-hour and 48-hour intervals reverted to the initial pattern in which the 1.5 and 2 ml g^{-1} DM levels produced significantly higher *iv*GP compared with the control treatment. Over the incubation period, a clear trend was observed that indicated a rise in *iv*GP with an increasing level of xylanase treatment (Table 2.4).

There was no significant interaction effect in terms of gas production between substrate and the level of cellulase-xylanase-PLUS enzyme treatment. The effect of enzyme application rate for the mixture is depicted in Table 2.5. Application rate of the mixture at 2 ml g^{-1} DM resulted in significantly higher *iv*GP throughout the incubation period compared with the control. Treatment level 1.5 ml g^{-1} DM recorded significantly higher *iv*GP compared with the control at 4-, 8- and 12-hour incubation intervals. However, a clear trend was observed throughout the incubation period, which indicated that an increase in application rate of the mixture resulted in an increase in *iv*GP (Table 2.5). Enzyme addition resulted in increased cumulative *iv*GP following the 48-hour incubation period of 7 – 14%, 8 – 21% and 3 – 11% for cellulase, xylanase and mixture treatments, respectively.

The substrate effect as depicted in Tables 2.3, 2.4 and 2.5 showed the same pattern, regardless of enzyme treatment. *In vitro* gas production between the substrates was significantly different in terms of gas production trends. *Eragrostis curvula* produced the lowest amount of *iv*GP throughout the incubation period. Sunflower oilcake meal had the highest *iv*GP during the first 12 hours. Following the 12-hour incubation interval, hominy chop continued to produce gas at a significantly higher rate than the other substrates and resulted in the highest cumulative *iv*GP after the 48-hour incubation period.

Table 2.3 *In vitro* gas production of substrates treated with cellulase PLUS (cellulase) enzyme product at four dose levels

	Gas production (ml g^{-1} DM) at various time intervals (hours of incubation)							
Treatments (n=12)	2	4	8	12	18	24	36	48
Cellulase level								
0	12.18^{b}	17.94^{b}	30.70^{b}	44.35^{b}	64.22^{a}	95.16^{a}	126.29^{a}	141.88^{a}
0.5	13.18^{ab}	20.34^{ab}	34.71^{ab}	52.81^{ab}	72.43^{a}	105.75^{a}	135.89^{a}	152.13^{a}
1	13.37^{ab}	20.98^{ab}	35.53^{ab}	52.92^{ab}	72.90^{a}	106.73^{a}	139.40^{a}	155.23^{a}
1.5	13.85^{ab}	21.73^{ab}	37.29^{a}	55.09^{a}	76.70^{a}	108.73^{a}	140.59^{a}	156.35^{a}
2	14.85^{a}	23.61^{a}	39.79^{a}	57.55^{a}	73.40^{a}	112.31^{a}	144.65^{a}	161.95^{a}
SEM	*0.44*	*0.93*	*1.50*	*2.22*	*2.07*	*4.34*	*3.10*	*3.31*
Substrate (n=12)								
Eragrostis curvula	6.99^{c}	10.43^{c}	17.59^{c}	24.78^{b}	36.08^{c}	59.14^{c}	95.60^{c}	112.41^{b}
Sunflower oilcake meal	18.01^{a}	29.59^{a}	49.78^{a}	67.23^{a}	80.28^{b}	100.71^{b}	114.09^{b}	122.03^{b}
Hominy chop	15.45^{b}	22.73^{b}	39.44^{b}	65.62^{a}	99.44^{a}	157.36^{a}	202.40^{a}	226.06^{a}
SEM	*3.33*	*5.60*	*9.49*	*13.89*	*18.76*	*28.46*	*32.95*	*36.39*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

Table 2.4 *In vitro* gas production of substrates treated with a xylanase PLUS (xylanase) enzyme product at four dose levels

	Gas production (ml g^{-1} DM) at various time intervals (hours of incubation)							
Treatments (n=12)	2	4	8	12	18	24	36	48
Xylanase level								
0	13.41^{c}	22.57^{d}	38.45^{d}	61.46^{c}	86.06^{c}	108.00^{c}	132.29^{c}	145.89^{c}
0.5	13.91^{c}	23.86^{cd}	40.96^{cd}	65.20^{bc}	92.5^{bc}	115.56^{bc}	139.13^{bc}	153.55^{bc}
1	14.61^{bc}	25.26^{bc}	43.35^{bc}	69.85^{b}	96.92^{b}	120.32^{b}	143.00^{bc}	157.68^{bc}
1.5	15.42^{b}	26.66^{b}	44.74^{b}	70.47^{ab}	98.85^{ab}	123.61^{ab}	150.22^{ab}	167.12^{ab}
2	16.96^{a}	29.15^{a}	48.60^{a}	76.30^{a}	106.23^{a}	131.82^{a}	159.39^{a}	176.48^{a}
SEM	*0.62*	*1.14*	*1.72*	*2.52*	*3.35*	*3.98*	*4.66*	*5.33*
Substrate (n=12)								
Eragrostis curvula	8.15^{b}	14.31^{c}	24.08^{c}	37.79^{c}	53.72^{c}	73.62^{c}	102.45^{c}	125.58^{b}
Sunflower oilcake meal	18.24^{a}	32.20^{a}	55.49^{a}	80.20^{b}	97.39^{b}	111.30^{b}	124.38^{b}	129.36^{b}
Hominy chop	18.20^{b}	29.98^{b}	50.09^{b}	87.98^{a}	137.02^{a}	175.67^{a}	207.59^{a}	225.50^{a}
SEM	*3.36*	*5.63*	*9.70*	*15.60*	*24.06*	*29.79*	*32.02*	*32.69*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

Table 2.5 *In vitro* gas production of substrates treated with 50 : 50 cellulase-xylanase PLUS mixture (mixture) at four dose levels

	Gas production (ml g^{-1} DM) at various time intervals (hours of incubation)							
Treatments (n=12)	2	4	8	12	18	24	36	48
Mixture level								
0	17.93^{b}	31.33^{c}	58.19^{c}	89.63^{c}	124.18^{b}	143.92^{b}	168.04^{b}	183.5^{b}
0.5	18.7^{ab}	32.74^{bc}	60.65^{bc}	93.02^{bc}	128.91^{ab}	149.79^{ab}	173.45^{ab}	188.87^{ab}
1	19.29^{ab}	33.36^{abc}	62.3^{abc}	95.41^{bc}	131.45^{ab}	152.55^{ab}	176.2^{ab}	191.71^{ab}
1.5	20.3^{ab}	35.15^{ab}	64.69^{ab}	98.05^{ab}	134.4^{ab}	155.73^{ab}	180.02^{ab}	195.9^{ab}
2	21.03^{a}	36.07^{a}	66.69^{a}	102.18^{a}	140.31^{a}	162.77^{a}	185.54^{a}	203.63^{a}
SEM	*0.55*	*0.85*	*1.49*	*2.14*	*2.70*	*3.13*	*2.96*	*3.39*
Substrate (n=12)								
Eragrostis curvula	8.19^{c}	13.89^{c}	25.83^{c}	41.97^{c}	69.49^{c}	92.58^{c}	126.89^{c}	149.76^{c}
Sunflower oilcake meal	28.49^{a}	51.05^{a}	84.6^{a}	109.99^{b}	134.91^{b}	147.61^{b}	158.19^{b}	164.68^{b}
Hominy chop	21.67^{b}	36.26^{b}	77.09^{b}	135.01^{a}	191.15^{a}	218.66^{a}	246.07^{a}	263.74^{a}
SEM	*5.96*	*10.80*	*18.47*	*27.80*	*35.15*	*36.49*	*35.67*	*35.77*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

2.5. Discussion

2.5.1. Description and chemical composition

Eragrostis curvula is a tufted perennial grass that is used mainly for pasture or hay. It is a common raw material in ruminant feed and forage in southern Africa. Sunflower oilcake meal is the remainder after solvent extraction of oil from sunflower seeds *(Helianthus annuus).* South Africa is a major producer of sunflower oil, and thus sunflower oilcake meal is readily available for use in the livestock sector. Hominy chop is a by-product of corn milling. It consists mainly of fractions of the endosperm of the corn kernel *(Zea mays).* The slower rate of *iv*GP recorded for *Eragrostis curvula* could be due to lack of readily fermentable material in the substrate, as evidenced by low starch and high fibre content reported for this study (table 2.1). Fermentable organic matter and fibre content have been reported to have major effects on the rate and extent of *iv*GP (Blummel and Becker, 1997). Sunflower oilcake meal has lower fibre content than *Eragrostis curvula,* as well as some readily fermentable sugar, which indicates a faster rate of *iv*GP compared with *Eragrostis curvula* hay. Hominy chop contains relatively low levels of fibre and high levels of readily fermentable material such as EE, starch and sugar. These characteristics are partly responsible for the faster rate of *iv*GP at early incubation times and high total *iv*GP potential.

2.5.2. Enzyme activity

Endoglucanase and exoglucanase tests were used to measure cellulase activity, while the hemi-cellulase test indicated the xylanase activity. The activities for the cellulase and xylanase enzymes tested in this study were relatively higher than other reported values (Colombatto *et al*., 2003; Eun and Beauchemin, 2007). It is not always possible to compare these results one on one as the enzyme sources and characteristics may differ, coupled with contrasting analytical methods between laboratories. The cellulase, xylanase and mixture showed optimum performance at a pH of 6, 4 and 4, respectively, which agrees with the manufacture's recommendations of 4.8. In this study, the xylanase and cellulase activities of the pure enzyme products were similar to those obtained by Belete *et al.* (2014) after analysing the same enzyme products. However, these activities were higher than other values that have been reported

(Colombatto *et al*., 2003; Eun and Beauchemin, 2007). In this study the enzyme activity of the mixture was analysed as well. In this case the endoglucanase activity was significantly reduced, while most of the hemi-cellulase activity remained constant compared with the activity of the pure xylanase enzyme products. This analysis indicates that the enzyme activity of pure enzymes can be altered when combined with other enzymes. Thus, this explains why the two pure enzyme products, namely cellulase and xylanase, do not necessarily show additive effects when combined. From the results of this study, the mixture of xylanase and cellulase might not be the ideal treatment to enhance endoglucanase activity. However, these enzymes might perform differently in the rumen environment owing to endogenous enzyme interactions and differences in substrates, temperature and pH (Eun and Beauchemin, 2007). The wide discrepancy between the ideal conditions of an enzyme assay and the rumen environment justifies assays being conducted on a broader range of conditions as well as standardizing these methods to determine exogenous enzyme activities in order to mimic the rumen environment more closely.

2.5.3. *In vitro* gas production

In vitro gas production is an indication of feed fermentation, mainly the carbohydrate component. The degradation of substrate in the rumen is directly and positively correlated with *iv*GP (Menke *et al*., 1997). Results in this study show that *iv*GP rose with an increase in dose rate. This effect was most prominent in the initial stages of incubation and at higher dose rates, and was applicable to cellulase, xylanase and their mixture (Tables 2.3, 2.4 and 2.5). In agreement with these results were those of Eun and Beauchemin (2007), who used alfalfa hay under *in vitro* conditions, of Giraldo *et al*. (2008), who experimented with a hay-concentrate (70 : 30) mixture for sheep, and of Pinos-Rodriguez *et al*. (2007), who reported on various forage-to-concentrate ratios fed to lambs. All authors reported improvements in the rate of fibre component degradation when substrates were treated with EFEs. Furthermore, previous studies reported that cellulase, xylanase and other EFEs could increase cumulative *iv*GP and the rates of *in vitro* fermentation of grass and maize silage (Wallace *et al*., 2000). In contrast, other researchers reported that the *iv*GP rate was not improved by fibrolytic enzyme supplementation. According to these researchers, incorrect enzyme dosage and enzyme-substrate combinations were the primary factors for the lack of improvement (Yang *et al*., 2000; Kung *et al*., 2002).

In this study, the increase in *iv*GP was less prominent with the mixture treatment. This could be due to the reduced endoglucanase activity of the mixture, as seen in the enzyme activity assay. Although multiple

studies have shown that exogenous enzyme treatments could improve fibre fermentation and the rate of fermentation of various substrates, the mechanism of this improvement is not fully understood. Possible reasons for the positive effect in terms of rate of *iv*GP could be explained by improved microbial colonization of the substrate cell wall (Wang *et al*., 2001), resulting in an increased rate of fermentation. Alteration of the substrate cell wall structure improved ruminal fermentation because of the enzymatic effects, which resulted in a more favourable surface for microbial fermentation and increased access to potentially fermentable components (Giraldo *et al*., 2008, Elwakeel *et al*., 2007). The 24-hour pre-treatment of the substrate with the exogenous enzymes in this study might have improved the substrate–enzyme complex, resulting in higher efficacy of the enzyme in the incubation media (Alvarez *et al*., 2009). But this effect was less prominent in the later stages of the incubation period. This could be explained by the disruption of the enzyme-substrate complex by ruminal enzymes and its proteolytic effects, thereby reducing the duration of the efficacy of the exogenous enzymes within the rumen (Kung *et al*., 2000). Another possible explanation could be that as the incubation period progressed, the potential substrate on which exogenous enzymes could act declined, slowing down the rate of *iv*GP (Colombatto *et al*., 2003).

The *iv*GP profiles of the three substrates were significantly different, regardless of enzyme treatment or dose rate. This could be owing to a higher amount of readily fermentable material in hominy chop and sunflower oilcake meal compared with *Eragrostis curvula* (Table 2.1). After 18 hours of incubation, hominy chop showed a much higher capacity of total *iv*GP compared with the other substrates owing to the presence of significantly more fermentable material in the form of starch and lower NDF and ADF content. The large variation in *iv*GP patterns between these substrates emphasizes the necessity to determine the interaction between enzymes and specific substrates.

2.6. Conclusion

The enzyme activity profile in this study indicated that most of the activity remained higher at pH 4–6. Enzyme products such as EFEs should be capable of tolerating conditions closer to those of the rumen environment to be used effectively as additives in ruminant nutrition. Cellulase and xylanase used in this study were effective at 0.5 ml g^{-1} DM of substrate. However, at 0.5 ml g^{-1} DM their effect was relatively lower compared with higher application rates (1, 1.5, 2 ml g^{-1} DM). This is understandable as EFEs are not always produced to be used in the feeding system of ruminants. The *iv*GP in this study indicated that all

of the enzyme treatments had a measurable effect on total *iv*GP, especially during the earlier incubation intervals of the tested substrates. The cumulative effect increased with increasing level of application, reaching the highest level at 2 ml g^{-1} DM for cellulase and the mixture. In addition, the associated costs related to higher enzyme application rate need to be considered to justify the return that will be obtained per unit increase in enzyme application level. Thus taking into consideration the efficiency of application, the pre-treatment of *Eragrostis curvula*, sunflower oilcake meal and hominy chop with cellulase, xylanase and a mixture at an application rate of 1.5 ml g^{-1} DM resulted in more economical *in vitro* ruminal fermentation throughout the incubation period. Cellulase, however, enhanced gas production only during the first 12 hours of the incubation period, and this was obtained at an application rate of 2 ml g^{-1} DM.

Chapter 3

Effect of addition of fibrolytic enzyme with or without protease on *in vitro organic matter* degradability, total gas and methane production

3.1. Abstract

The potential improvements that exogenous protease enzymes in addition to exogenous fibrolytic enzymes (EFEs) might have on ruminal fermentation need to be studied to enhance the utilization of high forage diets by ruminants. This study evaluated the influence of exogenous enzymes on *in vitro* gas production (*iv*GP), *in vitro* organic matter degradability (*iv*OMD) and *in vitro* methane production (*iv*CH_4). A factorial treatment combination of fibrolytic enzymes (control, xylanase PLUS at 1.5ml g^{-1} DM (xylanase), cellulase at 1.5ml g^{-1} DM (cellulase) and a 1 : 1 cellulase-xylanase PLUS mixture at 1.5ml g^{-1} DM (mixture) and protease enzyme (at 0 and 0.5ml g^{-1} DM substrate (protease)) was evaluated using two substrates (*Eragrostis curvula* and sunflower oilcake meal). Rumen fluid was collected from six cannulated sheep fed a Lucerne hay-based diet and used as a source of inoculum. *In vitro* gas production and *iv*CH_4 were measured using a pressure transducer after 3, 6, 10, 14, 24, 36 and 48 hours of incubation. Gas samples was taken from every vial at each of these intervals. Thereafter, gas samples were analysed for methane concentration using a SRI gas chromatograph. In addition, *in vitro* organic matter digestibility of the feed was measured after 48 hours of incubation. The results of this study indicated that fibrolytic enzymes may generally increase *iv*GP. However, the addition of protease had no effect on *Eragrostis curvula,* while it improved the *iv*GP of sunflower oilcake meal ($P < 0.05$) when used alone. Fibrolytic enzymes in the form of cellulase and xylanase alone improved the *iv*OMD of sunflower oilcake meal, while only the addition of protease improved the *iv*OMD of *Eragrostis curvula* ($P < 0.05$). It appears that the addition of fibrolytic enzymes increased *iv*CH_4 for both substrates, but not throughout the incubation period *($P < 0.05$)*. The addition of protease reduced *iv*CH_4 of *Eragrostis curvula* at 10–14 hour intervals *($P < 0.05$)*. Similarly, the addition of fibrolytic enzyme alone increased *iv*CH_4 *iv*GP^{-1} and *iv*CH_4 *iv*OMD^{-1} for *Eragrostis curvula* ($P < 0.05$), but not for sunflower oilcake meal. The results obtained in this study highlight the variation in the effects of exogenous enzymes on different substrates. Therefore, conducting further studies on the

effects of protease addition in combination with fibrolytic enzymes and by itself on a range of substrates would be justified.

3.2. Introduction

Improving production efficiency and reducing the environmental footprint of ruminant production under an extensive system would enhance the sustainability of the system and global competitiveness of livestock sector. Hence, progressively more emphasis is being put on climate warming risks and the intensification of livestock production systems in modern livestock practices. Greater awareness is placed on improving the utilization of traditional and non-traditional feedstuffs. As a consequence, various feed additives have been evaluated in order to produce the most favourable ratio between methane emissions and degradable organic matter. The application of exogenous enzymes has led to better utilization of nutrients by contributing to the degradation of cellulose, hemicellulose and protein in forages commonly fed to ruminants (Pinos-Rodríguez *et al*, 2002). Interest in the use of exogenous enzymes has grown in recent times. This is due mainly to an emphasis being placed on the reduction of feed costs and easier access to good-quality enzymes (Adesogan *et al*, 2007; He *et al*. 2014). The application of protease in combination with fibrolytic enzymes is believed to have positive effects on nutrient digestibility by degrading protein molecules situated on the cell wall, which increases nutrient exposure to microbial degradation (Colombatto and Beauchemin, 2009). Enzyme cocktails of endoglucanase, alpha-amylase, xylanase and protease activity have resulted in increased nutrient digestibility and improved ruminal fermentation profiles in both *in vitro* (Elghandour *et al*, 2013) and *in vivo* (Gado *et al*, 2009) trials. Ruminal fermentation contributes to greenhouse gasses by the production of methane (Kim *et al*, 2016; Perme *et al,* 2016). Reduction in enteric methane would not only reduce greenhouse gas production, but also improve the energy efficiency of substrates (Johnson and Johnson, 1995). Previous studies have shown that exogenous enzymes can improve digestibility and in some cases reduce methane production (Grainer and Beauchemin *et al*, 2011), but this response is not consistent across studies. The observed response to exogenous enzymes depends on the type of enzyme, mode of application, dose level and substrate. The current study was undertaken to investigate the effect of addition of three fibrolytic enzyme treatments with or without protease enzyme on *iv*GP, *iv*CH_4 and *iv*OMD of *Eragrostis curvula* and sunflower oilcake meal.

3.3. Materials and methods

The following subsections describes the methodology followed in this chapter.

3.3.1. Description of study area

This study evaluated the effect of three EFEs treatments in combination with or without protease enzyme on *iv*GP, *iv*CH_4 and *iv*OMD. The *in vitro* experiments were conducted at the Nutrition Laboratory of the Department of Animal and Wildlife Sciences, University of Pretoria. The fibrolytic enzymes used in this study consisted of cellulase and xylanase (Dyadic International Inc., Florida, USA), which is a concentrated liquid of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8), and the mixture, which is a 1 : 1 wt wt^{-1} combination of these enzymes. The fibrolytic enzymes were produced by the fermentation of *Trichderma longibrachiatum* (non-genetically modified organism). The protease enzyme was dried *Bacillus licheniformis* PWD-1 fermentation solubles mixed with ground limestone and natural flavouring. The protease enzyme used is commercially available under the trade name Cibenza® DP 100. *Eragrostis curvula* and sunflower oilcake meal were used as the test substrates. These substrates were treated with a fibrolytic enzyme, namely control, cellulase, xylanase and their mixture in combination with or without protease enzyme. In each run, control samples (substrates without enzyme treatment), blanks for treatments (incubation medium and each enzyme treatment) and rumen fluid (rumen fluid and buffer medium) were incubated with the treatments. The dose rate of the fibrolytic enzymes determined earlier in Chapter 2 was used, whereas the dose rate of the protease enzyme was adapted from previous studies conducted by Colombatto and Beauchemin (2009) and Cantet *et al*. (2015). Each treatment was replicated with four vials per run and three independent runs were carried out.

3.3.2. Feed sample collection and preparation

The *Eragrostis curvula* and sunflower oilcake meal described in Section 2.3.2 were used as substrates in this study.

3.3.3. Chemical analysis of substrates

The chemical analysis of the substrates was similar to those described in Section 2.3.3 of this study.

3.3.4. Enzyme assay

The enzyme assay described in Section 2.3.4 was applicable to the fibrolytic enzymes used in this study. However, protease enzyme activity was determined by the manufacturer and reported on a certificate of analysis as 6000,000 U g^{-1} minimum protease activity.

3.3.5. Substrate preparation and enzyme dilution

The same procedures that were described in Section 2.3.5 were followed for *Eragrostis curvula* and sunflower oilcake meal and the fibrolytic enzymes and protease enzyme.

3.3.6. Buffer medium preparation

The method described in Section 2.3.6 was followed in this study.

3.3.7. Collection of rumen fluid from donor sheep and *in vitro* measurements

The procedure described in Section 2.3.7 was followed in this study with these amendments: the vials were placed in an *in vitro* incubation oven at 39°C with a sliding tray at 120 rpm instead of the water bath described in Section 2.3.7. To correct for gas production of the rumen fluid component, four blanks, which comprised rumen fluid and the specific treatment, were included in every run. Gas pressure was measured and released at 3, 6, 10, 14, 24, 36 and 48 hours of incubation. During each interval, a gas sample was

taken from every vial. The gas samples were kept in gastight vials and analysed for concentration of methane using a SRI gas chromatograph shortly after collection. Finally, following the 48-hour incubation period, the vials were placed on ice to stop incubation. *In vitro* organic matter degradebility was determined after the 48-hour incubation period following procedures described by Van Soest *et al*. (1966).

3.3.8. Calculation and statistical analysis

A completely randomized block experimental design was used in this study. The run was used as a blocking factor. The data were analysed statistically using the GLM option of SAS (version 9.4, 2013). The statistical model included the effect of fibrolytic enzyme, protease and interaction effect between fibrolytic enzyme and protease. Where the F-test showed significant effect, differences between the means were determined using Tukey's test.

3.4. Results

3.4.1. *In vitro* gas production

A significant interaction effect between fibrolytic enzymes and protease was observed for sunflower oilcake meal throughout the incubation period (Table 3.1). The addition of fibrolytic enzymes alone resulted in significantly higher *iv*GP throughout the incubation period compared with the control treatment. The same effect was observed with the addition of protease compared with the control treatment. However, no significant increase in *iv*GP was observed due to the addition of fibrolytic enzymes in the presence of protease (Table 3.1). Similarly, no significant increase in *iv*GP was observed due to the addition of protease in the presence of fibrolytic enzymes.

Table 3.1 *In vitro* gas production of sunflower oilcake meal treated with various enzyme products over 48 hours

		In vitro gas production (ml g^{-1} DM)			
Interval (hours of incubation)	*Enzyme treatment (n=12)*	Control	Xylanase	Cellulase	Mixture
3	Control	25.4_b^2	30.1_a^1	31.2_a^1	32.1_a^1
	Protease	29.9_a^1	32.0_a^1	32.6_a^1	31.3_a^1
	SEM	*0.83*	*0.83*	*0.83*	*0.83*
6	Control	44.1_b^2	51.5_a^1	52.5_a^1	53.7_a^1
	Protease	52.1_a^1	53.3_a^1	54.9_a^1	52.9_a^1
	SEM	*0.99*	*0.99*	*0.99*	*0.99*
10	Control	64.5_b^2	73.4_a^1	73.6_a^1	75.5_a^1
	Protease	75.8_a^1	75.1_a^1	77.1_a^1	74.6_a^1
	SEM	*1.17*	*1.17*	*1.17*	*1.17*
14	Control	80.5_b^2	90.9_a^1	90.7_a^1	92.9_a^1
	Protease	93.9_a^1	92.3_a^1	94.5_a^1	91.6_a^1
	SEM	*1.36*	*1.36*	*1.36*	*1.36*
24	Control	103.8_b^2	116.3_a^1	115.3_a^1	116.8_a^1
	Protease	119.3_a^1	116.7_a^1	120.0_a^1	116.2_a^1
	SEM	*1.55*	*1.55*	*1.55*	*1.55*
36	Control	114.6_b^2	129.1_a^1	128.1_a^1	129.4_a^1
	Protease	132.1_a^1	129.2_a^1	132.7_a^1	128.5_a^1
	SEM	*1.77*	*1.77*	*1.77*	*1.77*
48	Control	121.2_b^2	137.7_a^1	136.6_a^1	138.1_a^1
	Protease	140.7_a^1	138.0_a^1	141.1_a^1	137.0_a^1
	SEM	*1.93*	*1.93*	*1.93*	*1.93*

Means with different numbers (superscripts) within a column and letters (subscripts) within a row differ significantly in a section at indicated P value, $P < 0.05$

The interaction effect between fibrolytic enzymes and protease was significant only at the 24-hour interval in the 48-hour incubation period of *Eragrostis curvula*. The addition of fibrolytic enzymes resulted in significantly higher *iv*GP compared with the control throughout the rest of the incubation period. The addition of protease did not alter the *iv*GP profile throughout the incubation period (Table 3.2).

Table 3.2 *In vitro* gas production of *Eragrostis curvula* treated with various enzyme products over 48 hours

	In vitro gas production (ml g^{-1} DM) at various time intervals (hours of incubation)					
Treatments (n=12)	3	6	10	14	36	48
Fibrolytic enzymes						
Control	4.39[b]	7.55[b]	14.33[b]	24.81[b]	92.76[b]	116.45[b]
Xylanase	5.43[a]	9.25[a]	17.09[a]	28.47[a]	96.75[a]	119.76[a]
Cellulase	6.11[a]	9.86[a]	17.31[a]	28.25[a]	96.78[a]	120.14[a]
Mixture	6.80[a]	10.76[a]	18.64[a]	29.54[a]	97.65[a]	120.63[a]
SEM	*0.56*	*0.56*	*0.63*	*0.67*	*0.95*	*1.05*
Protease						
0 ml	5.74[a]	9.39[a]	17.02[a]	28.10[a]	96.09[a]	119.23[a]
40 ml	5.62[a]	9.32[a]	16.66[a]	27.44[a]	95.88[a]	119.25[a]
SEM	*0.4*	*0.4*	*0.44*	*0.47*	*0.67*	*0.74*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

With the exception of the 24-hour incubation period, the addition of fibrolytic enzymes resulted in significantly higher *iv*GP compared with the control in the absence of protease. The addition of protease in combination with fibrolytic enzymes did not result in higher *iv*GP compared with the control. In fact, the combination of protease and xylanase produced significantly lower *iv*GP compared with xylanase alone (Figure 3.1).

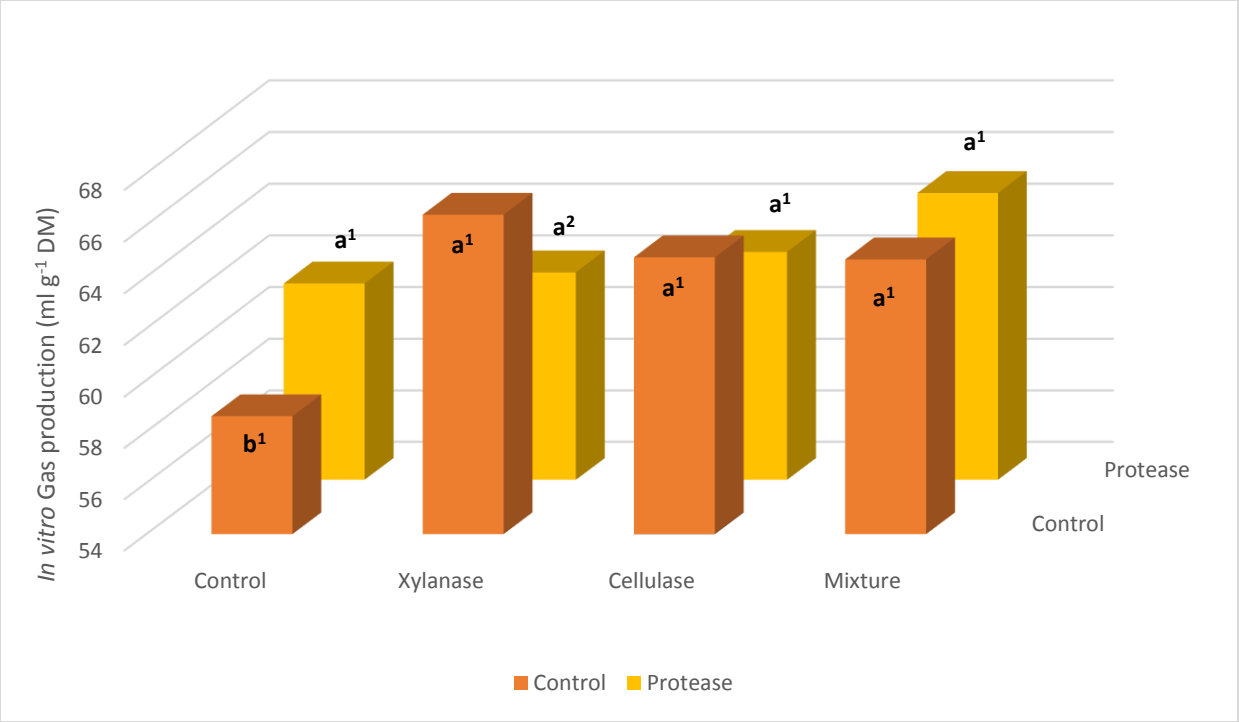

Figure 3.1. Cumulative *in vitro* gas production of *Eragrostis curvula* treated with various enzyme products at the 24-hour interval

Means with different letters (subscripts) within an x-axis and different numbers (superscripts) within a z-axis differ significantly at indicated P value $P < 0.05$

3.4.2. *In vitro* methane production

During the incubation of sunflower oilcake meal, an interaction effect between fibrolytic enzyme and protease was observed only at the 48-hour incubation interval (Table 3.3 and Figure 3.2). The addition of fibrolytic enzymes increased cumulative *iv*CH_4 at the 3-, 6-, 24- and 36-hour intervals, although this was not consistent across the various enzyme types and at the 10- and 14-hour intervals. The addition of protease did not affect cumulative *iv*CH_4 production during the first 36 hours of incubation.

Table 3.3 Cumulative *in vitro* methane production from sunflower oilcake at various time intervals

	Methane production (ml g^{-1} DM) at various time intervals (hours of incubation)					
Treatments (n=12)	3	6	10	14	24	36
Fibrolytic enzymes						
Control	0.51^{b}	1.15^{b}	2.27^{a}	3.38^{ab}	5.53^{b}	6.69^{b}
Cellulase	0.61^{a}	1.31^{ab}	2.31^{a}	3.32^{b}	5.65^{ab}	6.91^{ab}
Mixture	0.61^{a}	1.29^{ab}	2.35^{a}	3.46^{ab}	5.70^{ab}	6.91^{ab}
Xylanase	0.58^{ab}	1.35^{a}	2.49^{a}	3.69^{a}	5.99^{a}	7.27^{a}
SEM	*0.03*	*0.06*	*0.09*	*0.11*	*0.15*	*0.17*
Protease						
0 ml	0.58^{a}	1.26^{a}	2.35^{a}	3.52^{a}	5.83^{a}	7.06^{a}
40 ml	0.58^{a}	1.29^{a}	2.36^{a}	3.40^{a}	5.61^{a}	6.83^{a}
SEM	*0.02*	*0.04*	*0.07*	*0.08*	*0.1*	*0.12*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

In the absence of protease, the addition of fibrolytic enzymes resulted in significantly higher *iv*CH_4 when compared with the control treatment. The addition of protease in combination with fibrolytic enzymes did not affect *iv*CH_4 when compared with the control treatment. In the presence of protease, the addition of cellulase and mixture produced significantly lower *iv*CH_4 compared with xylanase used in combination with protease (Figure 3.2).

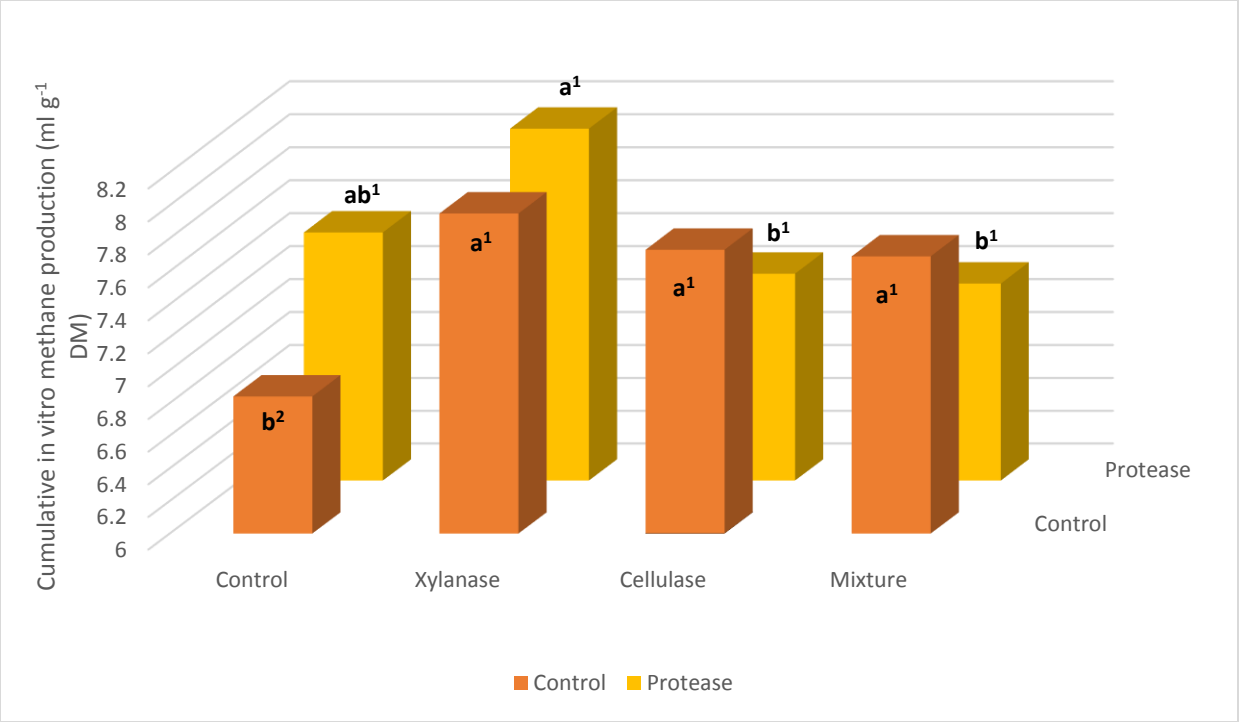

Figure 3.2. Cumulative *in vitro* methane production of sunflower oilcake meal treated with various enzyme products at the 48-hour interval

Means with different letters (subscripts) within an x-axis and different numbers (superscripts) within a z-axis differ significantly at indicated P value, $P < 0.05$

For *Eragrostis curvula,* a significant interaction effect between fibrolytic enzyme and protease in terms of cumulative *iv*CH_4 was observed at the 48-hour incubation interval only (Figure 3.3). The addition of a mixture resulted in significantly higher *iv*CH_4 during the first 14 hours of the incubation period compared with the control treatment. Xylanase and cellulase showed a tendency of increasing *iv*CH_4 at 3- and 6-hour intervals. Compared to the control, xylanase increased *iv*CH_4 production at the 10- and 14-hour intervals, while cellulase did not affect *iv*CH_4 at these intervals. At the 24- and 36-hour intervals, fibrolytic enzyme addition did not affect *iv*CH_4 when compared with the control treatment. The addition of protease generally reduced *iv*CH_4 at the 10- and 14-hour intervals compared with the treatments that did not contain protease. However, protease did not affect *iv*CH_4 at 24- to 36-hour intervals when compared with the control (Table 3.4).

Table 3.4 Cumulative *in vitro* methane production from *Eragrostis curvula* at various time intervals

	In vitro methane production (ml g^{-1} DM) at various time intervals (hours of incubation)					
Treatments (n=12)	3	6	10	14	24	36
Fibrolytic enzymes						
Control	0.02^{b}	0.05^{b}	0.15^{b}	0.37^{b}	1.96^{a}	4.11^{a}
Xylanase	0.03^{ab}	0.07^{ab}	0.22^{a}	0.48^{a}	2.14^{a}	4.52^{a}
Cellulase	0.03^{ab}	0.07^{ab}	0.16^{b}	0.35^{b}	1.99^{a}	4.24^{a}
Mixture	0.048^{a}	0.10^{a}	0.23^{a}	0.49^{a}	2.15^{a}	4.43^{a}
SEM	*0.01*	*0.01*	*0.02*	*0.02*	*0.13*	*0.15*
Protease						
0 ml	0.04^{a}	0.08^{a}	0.21^{a}	0.46^{a}	2.06^{a}	4.40^{a}
40 ml	0.03^{a}	0.06^{a}	0.17^{b}	0.38^{b}	2.06^{a}	4.25^{a}
SEM	*0.01*	*0.01*	*0.01*	*0.02*	*0.09*	*0.1*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

At the 48-hour incubation interval, the addition of xylanase alone resulted in significantly higher *iv*CH_4 compared with the control as well as the cellulase and xylanase mixture, while in the presence of protease, xylanase resulted in higher *iv*CH_4 than the mixture enzyme. The addition of protease alone resulted in significantly higher cumulative *iv*CH_4 compared with the control treatment, whereas the addition of protease to xylanase and cellulase resulted in significantly lower *iv*CH_4 compared with the substrate treated with these fibrolytic enzymes alone (Figure 3.3).

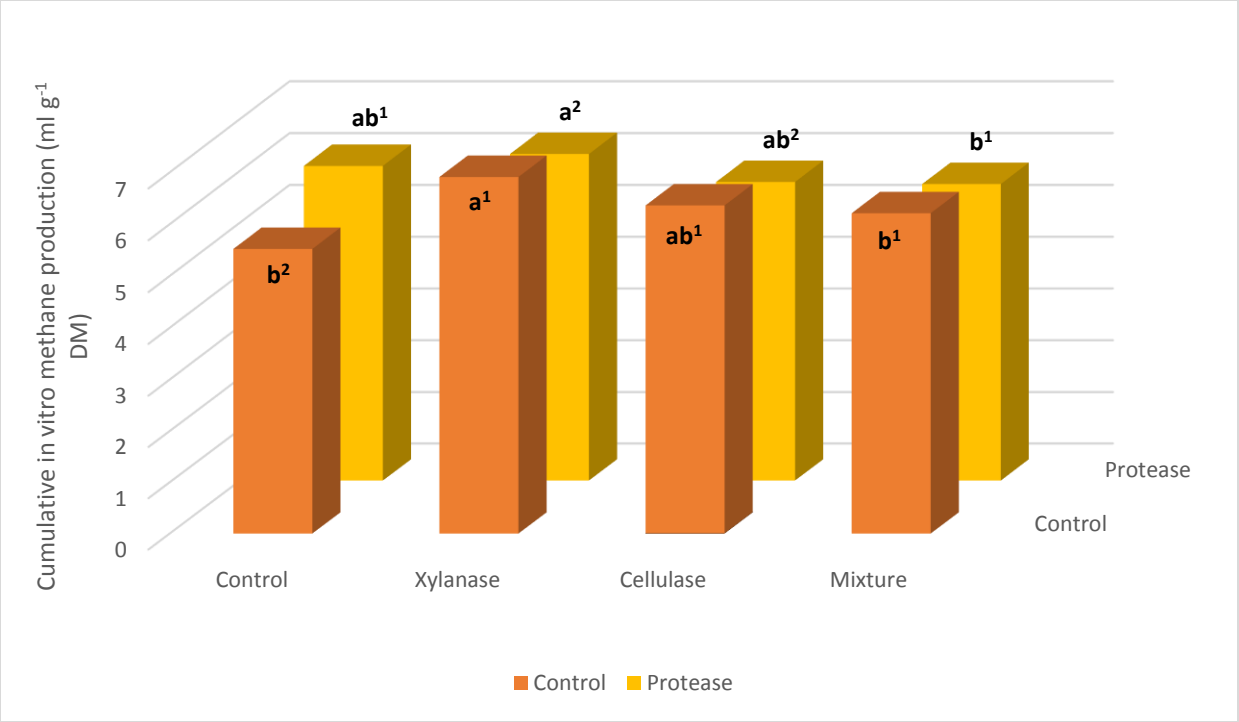

Figure 3.3. Cumulative *in vitro* methane production of *Eragrostis curvula* treated with various enzyme products at the 48-hour interval

Means with different letters (subscripts) within an x-axis and different numbers (superscripts) within a z-axis differ significantly at indicated P value, $P < 0.05$

3.4.3. *In vitro* organic matter degradation

For sunflower oilcake meal, the addition of xylanase and cellulase increased *iv*OMD compared with the control treatment. The addition of the mixture showed a tendency to increase *iv*OMD, but this increase did not statistically differ ($P > 0.05$) from the control. The addition of protease to sunflower oilcake meal did not affect *iv*OMD after the 48-hour incubation period (Table 3.5).

Table 3.5 Percentage *in vitro* organic matter degradability of sunflower oilcake meal following 48-hour *in vitro* incubation

Treatments (n=12)	*In vitro* organic matter degradability (%)
Fibrolytic enzymes	
Control	73.52[b]
Xylanase	77.05[a]
Cellulase	76.71[a]
Mixture	75.01[ab]
SEM	*0.8*
Protease	
0 ml	75.25[a]
40 ml	75.89[a]
SEM	*0.57*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

The addition of fibrolytic enzymes to *Eragrostis curvula* did not affect *iv*OMD following the 48-hour incubation period when compared with the control. However, the addition of protease resulted in significantly higher *iv*OMD compared with control the treatments without protease (Table 3.6).

Table 3.6 Percentage *in vitro* organic matter degradability of *Eragrostis curvula* following 48-hour *in vitro* incubation

Treatments (n=12)	*In vitro* organic matter degradability (%)
Fibrolytic enzymes	
Control	52.62[a]
Xylanase	52.54[a]
Cellulase	52.35[a]
Mixture	51.95[a]
SEM	*0.63*
Protease	
0 ml	51.35[b]
40 ml	53.38[a]
SEM	*0.44*

Means with different letters (superscripts) within a column differ significantly in a section at indicated P value, $P < 0.05$

3.4.4. *In vitro* methane production per unit of gas produced

In the absence of protease, the addition of fibrolytic enzymes did not alter the *iv*CH_4 *iv*GP^{-1} of sunflower oilcake meal when compared with the control treatment. Similarly, the addition of protease alone did not alter *iv*CH_4 *iv*GP^{-1} when compared with the control treatment without protease. The addition of protease in combination with cellulase reduced *iv*CH_4 *iv*GP^{-1} compared with the substrate treated with cellulase alone. Furthermore, xylanase in combination with protease produced significantly higher *iv*CH_4 *iv*GP^{-1} when compared with the control or other treatments that contained protease (Table 3.7).

Table 3.7 *In vitro* methane production per unit of gas produced after 48-hour incubation of sunflower oilcake meal

	ivCH_4 *(*ml g^{-1} DM*) iv*GP^{-1} *(*ml g^{-1} DM*)*	
Fibrolytic enzyme (n=12)	No protease	Protease
Control	0.057_a[1]	0.054_b[1]
Xylanase	0.058_a[1]	0.059_a[1]
Cellulase	0.057_a[1]	0.052_b[2]
Mixture	0.056_a[1]	0.053_b[1]
SEM	*0.001*	*0.001*

Means with different numbers (superscripts) within a row and letters (subscripts) within a column differ significantly at indicated P value, $P < 0.05$

ivCH_4: *in vitro* methane production; *iv*GP: *in vitro* gas production

A significant interaction effect between the fibrolytic enzyme and protease enzyme treatment was observed at 48 hours in terms of *iv*CH_4 per unit of *iv*GP of *Eragrostis curvula*. The addition of fibrolytic enzymes alone resulted in significantly higher *iv*CH_4 *iv*GP^{-1} compared with the control treatment, while the addition of protease alone did not affect *iv*CH_4 *iv*GP^{-1} when compared with the control treatment. However, when protease was added in combination with the mixture and the cellulase treatment, a reduction in *iv*CH_4 *iv*GP^{-1} was observed compared with *Eragrostis curvula* treated with these fibrolytic enzymes only. Furthermore, in the presence of protease the fibrolytic enzyme mixture resulted in significantly lower *iv*CH_4 *iv*GP^{-1} when compared with the control treatment with protease (Table 3.8).

Table 3.8 *In vitro* methane production per unit of gas produced after 48-hour incubation of *Eragrostis curvula*

	*ivCH₄ (*ml g^{-1} DM*) ivGP^{-1} (*ml g^{-1} DM*)*	
Fibrolytic enzyme (n=12)	No protease	Protease
Control	0.048_{c}^{1}	0.051_{ab}^{1}
Xylanase	0.056_{a}^{1}	0.053_{a}^{1}
Cellulase	0.053_{ab}^{1}	0.048_{bc}^{2}
Mixture	0.051_{b}^{1}	0.047_{c}^{2}
SEM	*0.001*	*0.001*

Means with different numbers (superscripts) within a row and letters (subscripts) within a column differ significantly at indicated P value, $P < 0.05$

ivCH_4: in vitro methane production; *iv*GP: *in vitro* gas production

3.4.5. *In vitro* methane production per unit of *in vitro* organic matter degraded

In terms of the 48-hour *iv*CH_4 *iv*OMD^{-1} for sunflower oilcake meal, an interaction effect was observed between the fibrolytic enzyme and protease enzyme treatments. When compared with the control, the addition of fibrolytic enzymes alone showed no difference in terms of *iv*CH_4 *iv*OMD^{-1}. Similarly, the addition of protease alone did not affect *iv*CH_4 *iv*OMD^{-1} when compared with the control. However, in the presence of protease the mixture and cellulase resulted in reduced *iv*CH_4 *iv*OMD^{-1} compared with the control treatment with protease. In the presence of fibrolytic enzymes the addition of protease did not alter *iv*CH_4 *iv*OMD^{-1} when compared with the control with fibrolytic enzymes (Table 3.9).

Table 3.9 *In vitro* methane production per unit of *in vitro* organic matter degraded following 48-hour incubation of sunflower oilcake meal

	ivCH$_4$ (ml g^{-1} DM) *ivOMD^{-1} (%)*	
Fibrolytic enzyme (n=12)	No protease	Protease
Control	9.59_a[1]	9.90_{ab}[1]
Xylanase	10.21_a[1]	10.67_a[1]
Cellulase	10.05_a[1]	9.52_b[1]
Mixture	10.26_a[1]	9.58_b[1]
SEM	*0.334*	*0.334*

Means with different numbers (superscripts) within a row and letters (subscripts) within a column differ significantly at indicated P value, $P < 0.05$

*iv*CH$_4$: in vitro methane production; *iv*OMD: *in vitro* organic matter degradability

No interaction effect between the fibrolytic enzyme and protease enzyme applied to *Eragrostis curvula* was observed following the 48-hour incubation period. In the absence of protease, the addition of xylanase resulted in higher *iv*CH$_4$ *iv*OMD^{-1}, while the mixture and cellulase had shown a tendency to increase *iv*CH$_4$ *iv*OMD^{-1} compared with the control treatment. Addition of protease alone did not affect *iv*CH$_4$ *iv*OMD^{-1} compared with the control treatment. However, the addition of protease in combination with cellulase reduced *iv*CH$_4$ *iv*OMD^{-1} when compared with the control treatment without protease applied in combination with cellulase (Table 3.10).

Table 3.10 *In vitro* methane production per unit of *in vitro* organic matter degraded following 48- hour incubation of *Eragrostis curvula*

	*ivCH$_4$ (*ml g^{-1} DM*) ivOMD^{-1} (%)*	
Fibrolytic enzyme (n=12)	No protease	Protease
Control	10.95_{b}^{1}	11.01_{a}^{1}
Xylanase	13.35_{a}^{1}	11.92_{a}^{1}
Cellulase	12.38_{ab}^{1}	10.91_{a}^{2}
Mixture	12.05_{ab}^{1}	11.01_{a}^{1}
SEM	*0.473*	*0.473*

Means with different numbers (superscripts) within a row and letters (subscripts) within a column differ significantly at indicated P value, $P < 0.05$

ivCH_4: *in vitro* methane production; *ivOMD*: *in vitro* organic matter degradability

3.5. Discussion

In vitro gas production is an effective parameter that can be used to identify the efficacy of exogenous enzymes in some of the less degradable components of cell walls (Cantet *et al*., 2015). Generally, an increase in *iv*GP during the initial incubation period indicates a positive response to the treatments ($P < 0.05$). In this study *iv*GP was increased significantly during the early hours of incubation when the fibrolytic enzymes were applied to each of the substrates alone. However, the addition of protease improved only the *iv*GP of sunflower oilcake meal. These results are in agreement with those of previous researchers, who reported higher gas production during the early incubation intervals of lucerne leaves treated with exogenous enzymes (Colombatto *et al*. 2003), in roughage (Elghandour *et al*. 2013), and in grass hay/concentrate (70:30) fed to sheep (Giraldo *et al*., 2008) *in vitro*. However, Jalilvand *et al*. (2008) did not find any differences in 24-hour *iv*GP with the addition of an exogenous enzyme mixture (cellulase, xylanase, alpha-glucanase, protease and amylase activity) to lucerne hay, wheat straw and maize silage. In contrast to the findings of this study the mixture often contained suboptimal enzyme activity, which could explain the results reported by these findings.

Colombatto *et al*. (2003b) concluded that exogenous enzyme treatments increased cellulose and xylan fermentation *in vitro,* owing primarily to greater hydrolytic activity. Improvements in organic matter degradability, NDF and CP degradability following the addition of enzymes have been recorded in dairy

cow diets (Rode *et al*. 1999; Elghandour *et al*. 2013). This study supports these findings because the addition of xylanase and cellulase resulted in significantly higher *iv*OMD of sunflower oilcake meal, while protease alone had no effect on *iv*OMD of sunflower oilcake meal. In contrast, during the incubation of *Eragrostis curvula*, fibrolytic enzyme had no effect on *iv*OMD, while the addition of protease resulted in significant improvements of *iv*OMD. This indicates that different responses are possible when using the same exogenous enzymes, depending on the substrate and thus determine the efficacy of enzymes used in a specific area.

The use of proteolytic enzymes in ruminant feed was not seen as a viable option owing to arguments that suggest that the addition of proteolytic enzymes leads to increased dietary and endogenous protein degradation, resulting in higher N wastage (Eun and Beauchemin 2008). This argument is supported by Colombatto *et al*. (2003) and Cantet *et al*. (2015), who found that protease lowered the *iv*OMD of silages and forages treated with the same exogenous protease enzyme. However, this was not always supported by the results obtained in this study, because protease addition increased *iv*GP of sunflower oilcake meal and *iv*OMD of *Eragrostis curvula*. Similarly, Eun and Beauchemin (2005) reported improved organic matter, nitrogen and hemicellulose digestion in dairy cattle fed diets with high and low forage inclusion rates and treated with protease. In a meta-analysis by Eun and Beauchemin (2008) on protease enzymes, the authors found improvements in *iv*OMD due to protease addition, but also noticed that high inclusion of protease could have detrimental effects on ruminal forage degradation. These collective results highlight the importance of using correct dose levels and identification of appropriate enzyme mixture for specific ruminant feed in question. It is critical to develop effective methodologies to determine the efficacy of enzymes in ruminant nutrition, because multiple factors (substrate, dose rate, rumen pH, etc.) may play a significant role in affecting the anticipated response.

In previous studies, methane emissions were mitigated by the action of exogenous enzymes on structural carbohydrates and protein in the substrates to which they were applied (Beauchemin *et al*. 2008). Currently, few studies have investigated the correlation between the addition of exogenous enzymes and ruminal methane production and their reported results are inconsistent. Treatment of total mixed ration fed to dairy cows with fibrolytic enzymes resulted in increased degradation of DM, NDF and acid detergent fibre (ADF), but did not affect methane production (Giraldo *et al*. 2007). Similarly, McGinn *et al*. (2004) reported no change in methane production or NDF and ADF digestibility following an *in vivo* study with steers fed barley silage supplemented with protease enzymes. In this study, the addition of fibrolytic enzymes to *Eragrostis curvula* substrate resulted in significant increases of *iv*CH_4, but not throughout the

incubation period. Sunflower oilcake meal treated with fibrolytic enzymes seems to have produced higher levels of *iv*CH_4 at all intervals except for the 10- and 14-hour intervals, whereas *Eragrostis curvula* treated with mixture produced higher *iv*CH_4 for the first 14 hours of the incubation period, while the addition of xylanase resulted in increased *iv*CH_4 at the 10- and 14-hour incubation intervals. In contrast, the addition of protease did not have an effect on the *iv*CH_4 production of sunflower oilcake meal, but reduced the cumulative *iv*CH_4 production of *Eragrostis curvula* at the 10- and 14-hour incubation intervals.

According to Cantet *et al*. (2015), fermentation parameters such as *iv*GP and *iv*CH_4 per gram of organic matter digested gives a more accurate indication of ruminal fermentation because methane is produced exclusively from the rumen fermentable carbohydrate fraction. The expression of *iv*GP and *iv*CH_4 per unit of fermented feed should thus be preferred when comparing various substrates. By expressing the results of the current study in this way it was found that the addition of fibrolytic enzyme increased *iv*CH_4 *iv*GP^{-1} and *iv*CH_4 *iv*OMD^{-1} for *Eragrostis curvula* only. Protease had no effect on these parameters for either of the substrates. The addition of xylanase in combination with protease increased *iv*CH_4 *iv*GP^{-1} for sunflower oilcake meal, while the addition cellulase and mixture in combination with protease reduced *iv*CH_4 *iv*OMD^{-1} compared with the protease treatment alone. Furthermore, the addition of cellulase increased *iv*CH_4 *iv*GP^{-1} for sunflower oilcake meal compared with cellulase addition alone. For *Eragrostis curvula*, the mixture and protease combination reduced *iv*CH_4 *iv*GP^{-1} compared with the protease treatment alone, while the cellulase and protease combination reduced both *iv*CH_4 *iv*GP^{-1} and *iv*CH_4 *iv*OMD^{-1} and the protease and mixture combination reduced *iv*CH_4 *iv*GP^{-1} only compared with protease addition alone. This study showed variable results depending on the substrate and enzyme treatment that were used. These results warrant a more in-depth study into the use of protease and into the interaction between fibrolytic and protease on various substrates in ruminant nutrition.

3.6. Conclusion

The aim of this study was to determine the effect of protease addition alone and in combination with fibrolytic enzymes on *iv*GP, *iv*OMD and *iv*CH_4 production of two feed substrates commonly used in the livestock industry. Fibrolytic enzymes alone increased the *iv*GP of both *Eragrostis curvula* and sunflower oilcake meal throughout the incubation period and resulted in an increase of *iv*CH_4 throughout the bulk of the incubation period. Protease addition to sunflower oilcake meal showed some potential in increasing

*iv*GP and reducing *iv*CH_4 *iv*OMD^{-1} when used in combination with exogenous cellulase and mixture. The addition of protease in the presence of fibrolytic enzymes to *Eragrostis curvula* showed potential to reduce *iv*CH_4, reduce *iv*CH_4 *iv*GP^{-1} when used in combination with cellulase and mixture and reduce *iv*CH_4 *iv*OMD^{-1} when used in combination with cellulase. The results obtained in this study compared with published data on this topic highlight the erratic effects of exogenous enzymes on various substrates. Therefore, further investigation on the use of protease alone and in combination with fibrolytic enzymes on specific substrates used in ruminant nutrition is recommended.

Chapter 4

The use of essential oil in combination with fibrolytic enzymes to reduce *in vitro* ruminal methane production

4.1. Abstract

Fibrolytic enzymes have been used successfully to improve rumen fermentation of high forage diets. However, there is an associated increase in methane production due to higher proportion of acetate in the fermentation product. The potential improvements that exogenous fibrolytic enzymes (EFEs) might have on ruminal fermentation need to be studied further with essential oils (EOs) to reduce methane emission associated with the use of these enzymes. This study evaluated the influence of various EOs in combination with fibrolytic enzymes on *iv*GP, *iv*OMD and *iv*CH_4 after 48 hours of incubation of *Eragrostis curvula*. The treatments included control with no enzyme and no EOs (T1); a 1 : 1 cellulase- xylanase mixture at 1.5 ml g^{-1} DM (enzyme) (T2); and a combination of this enzyme treatment and the EOs (Next Enhance®, which is a commercial product) at 43:3.85 wt wt^{-1} of cinnamaldehyde and diallyl disulfide + diallyl trisulphide) (T3); cinnamon (T4); garlic (T5); and cinnamon and garlic oils (T6)). These treatments were assessed in an *in vitro* gas production study using *Eragrostis curvula* hay substrates. Rumen fluid, collected from six cannulated sheep fed a lucerne hay-based diet, was used as inoculum. The parameters *iv*GP and *iv*CH_4 were measured using a pressure transducer with a digital gauge at 39 °C after 3, 6, 10, 14, 24, 36 and 48 hours of incubation. Gas samples was taken from every vial at each interval. The gas samples were analysed for methane concentration using a SRI gas chromatograph. *In vitro* organic matter digestibility of the feed was measured after 48 hours of incubation. The results indicated that the addition of enzymes appears to have increased *iv*GP and *iv*CH_4 and showed a tendency to increase *iv*OMD ($P < 0.05$). The addition of EOs, garlic and CinnGar in combination with enzyme reduced *iv*GP compared with the enzyme treatment alone ($P < 0.05$). Furthermore, all of the EOs in combination with the enzyme treatment showed a tendency to reduce methane production during the early stages of the incubation period compared with the enzyme treatment alone ($P = 0.08$). CinnGar in combination with enzyme was the only treatment that reduced *iv*CH_4 throughout the incubation period ($P < 0.05$). A reduction in *iv*CH_4 and *iv*GP per unit *iv*OMD was revealed for the garlic and CinnGar treatments ($P < 0.095$). Further studies

on these EOs combinations under different conditions (ruminal pH, EOs sources, substrate, etc.) and dose level should be evaluated to make a more informed recommendation on their efficacy. After successful screenings, these additives should be validated in *in vivo* trials.

4.2. Introduction

Essential oils are volatile plant secondary metabolites that are gaining popularity as ruminant feed additives, serving as alternatives to traditional antibiotic growth promoters (Wallace, 2004). The current situation has led to increased research into plant-derived compounds and their effects on ruminal fermentation (Calsamiglia *et al*., 2007). EOs have been shown to have promising effects through selective anti-bacterial activity, inhibition of ruminal methane emissions, enhancement of ruminal propionate proportion and bypass protein to the intestine (Benchaar *et al*., 2007; Calsamiglia *et al*., 2007). Of particular interest to this study is the ability of EOs to mitigate enteric methane production. McIntosh *et al*. (2003) found that EOs inhibit the energy metabolism of rumen microorganisms such as *Methanobrevibacter smithii*, a rumen *archae* that contributes to methane production. EOs have been studied for their effect on methane mitigation using both *in vivo* and *in vitro* studies, but the reported responses in terms of methane production have been variable, which may be related to the large diversity in the nature of these compounds (Calsamiglia *et al*., 2007). *In vitro* screening has proved an effective procedure to identify plant compounds with potentially positive effects (Beauchemin *et al*., 2008). However, this routine screening needs to be complemented by evaluating multiple EOs compounds and combinations at different dose rates. Moreover, owing to the ever-increasing risk of global warming and the contribution of rumen methane in it, there is continuous effort to screen further EOs and combination options. The aim of this study was to test the effects of EOs such as cinnamaldehyde, garlic oil and their mixture in the presence of fibrolytic enzymes on *iv*GP, *iv*CH_4 and *iv*OMD.

4.3. Materials and methods

The following subsections describes the methodology followed in this chapter.

4.3.1. Description of study area

This study included five additives as experimental treatments and a control without an enzyme or EOs. The first treatment was a control without enzyme or addition of EOs (T1). T2 was a 1 : 1 wt wt^{-1} mixture of two pure enzyme products, consisting of concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8) at 1.5 ml g^{-1} DM (mixture) dose rate. The dose used in this study was determined in previous studies and was described in more detail in Section 2.3.1. The remaining four treatments included an enzyme mixture in combination with a commercial EOs product (Next Enhance®) at 43:3.85 wt wt^{-1} of cinnamaldehyde and diallyl disulfide + diallyl trisulfide (T3); cinnamaldehyde (T4); garlic oil blend (T5); and a 1 : 1 wt wt^{-1} combination of cinnamaldehyde and the garlic oil blend (T6). The parameters included *iv*GP, *iv*CH_4 and *iv*OMD. *Eragrostis curvula* hay was used as the test substrate. The *in vitro* experiments were conducted at the Nutrition Laboratory of the Department of Animal and Wildlife Sciences, University of Pretoria.

4.3.2. Feed sample collection and preparation

Eragrostis curvula described in Section 2.3.2 was used as substrate in this study.

4.3.3. Chemical analysis of substrates

Chemical analysis described in Section 2.3.3 was used in this study.

4.3.4. Enzyme assay

The enzyme assay described in Section 2.3.4 was used for the fibrolytic enzyme mixture in this study.

4.3.5. Substrate and treatment preparation

The procedure described in Section 2.3.5 was followed for *Eragrostis curvula* and the enzyme mixture. Cinnamaldehyde and garlic oil were bought through Sigma-Aldrich©. Average concentrations of garlic oil and cinnamaldehyde were selected based on previous *in vitro* research (Busquet *et al*., 2005). The concentrations were dissolved in ethanol in a ratio of 1 : 10. All additives were stored at 5 °C in a smoked glass flask (Busquet *et al*., 2004).

4.3.6. Buffer medium preparation

The method described in Section 2.3.6 was followed to prepare buffer medium for this study.

4.3.7. Collection of rumen fluid from donor sheep and *in vitro* measurements

The procedure described in Section 3.3.7 was followed to collect rumen fluid from donor sheep and undertake *in vitro* gas production measurements with the modifications that gas pressure was measured and released at 2, 4, 8, 12, 24, 36 and 48 hours of incubation.

4.3.8. Calculations and statistical analysis

A completely randomized block experimental design was used in this study. The data were analysed statistically using ANOVA (analysis of variance) procedure of SAS (version 9.4, 2013). The statistical model included the effect of treatments and block, which is an independent run that was used in this study. Where the F-test showed significance, difference between treatments, means were separated using Tukey's test.

4.4. Results

4.4.1. *In vitro* gas production

Generally, *Eragrostis curvula* treated with fibrolytic enzyme had a higher *iv*GP throughout the incubation period compared with the control. Inclusion of garlic significantly reduced *iv*GP at the 8-, 12-, 24-, 36- and 48-hour incubation intervals, whereas CinnGar significantly reduced cumulative *iv*GP at 4-, 8-, 12-, 24-, 36- and 48-hour incubation intervals compared with the enzyme treatment (Table 4.1). When compared with the enzyme treatment, the inclusion of commercial product or cinnamon oil did not affect the *iv*GP at the 4-, 8-, 24-, 36- and 48-hour incubation intervals.

Table 4.1 *In vitro* gas production of *Eragrostis curvula* treated with various enzyme and essential oil combinations over 48 hours

	Gas production (ml g^{-1} DM) at various time intervals (hours of incubation)						
Treatments (n=12)	2	4	8	12	24	36	48
Control	2.44^{a}	4.90^{ab}	11.73^{bc}	24.45^{b}	60.83^{b}	93.33^{b}	118.27^{b}
Enzymes	4.51^{a}	7.88^{a}	15.57^{a}	29.12^{a}	67.01^{a}	100.05^{a}	124.95^{a}
Commercial	3.46^{a}	6.31^{ab}	13.94^{ab}	27.34^{a}	64.12^{ab}	96.82^{ab}	121.04^{ab}
Cinnamon	5.00^{a}	8.07^{a}	14.81^{ab}	27.08^{a}	63.55^{ab}	96.00^{ab}	121.00^{ab}
Garlic	2.42^{a}	5.14^{ab}	11.77^{bc}	24.15^{b}	60.53^{bc}	93.13^{b}	116.91^{b}
CinnGar	2.19^{a}	4.08^{b}	10.58^{c}	22.72^{b}	59.84^{c}	92.13^{b}	115.97^{b}
SEM	*0.84*	*0.76*	*0.64*	*0.48*	*0.76*	*1.07*	*1.09*
p-value	*0.1598*	*0.0187*	*0.0014*	*<0.0001*	*0.0004*	*0.0033*	*0.0016*

Means with different letters (superscripts) within a column differ significantly at indicated P value, P < 0.05

4.4.3. *In vitro* methane production

Fibrolytic enzyme increased cumulative *iv*CH_4 production at the 2-, 4- and 8-hour incubation intervals compared with the control treatment, but did not affect *iv*CH_4 production after the 12-hour incubation interval. All EOs in combination with the enzyme treatment reduced methane production at the 2-, 4- and 8-hour incubation intervals when compared with the enzyme treatment alone. Cinnamon, garlic and CinnGar continued to reduce *iv*CH_4 at the 12-hour interval. Only CinnGar reduced *iv*CH_4 throughout the incubation period, while garlic reduced *iv*CH_4 at the 48-hour incubation interval again (Table 4.2)

Table 4.2 Cumulative *in vitro* methane production from *Eragrostis curvula* treated with various enzyme and essential oil combinations over 48 hours

	Methane production (ml g^{-1} DM) at various time intervals (hours of incubation)						
Treatments (n=12)	2	4	8	12	24	36	48
Control	0.000^{b}	0.010^{b}	0.060^{b}	0.237^{ab}	1.427^{ab}	2.890^{ab}	4.193^{ab}
Enzymes	0.017^{a}	0.033^{a}	0.120^{a}	0.337^{a}	1.677^{a}	3.247^{a}	4.593^{a}
Commercial	0.003^{b}	0.010^{b}	0.063^{b}	0.253^{ab}	1.417^{ab}	2.883^{ab}	4.150^{ab}
Cinnamon	0.003^{b}	0.013^{b}	0.070^{b}	0.227^{b}	1.443^{ab}	2.907^{ab}	4.260^{ab}
Garlic	0.003^{b}	0.010^{b}	0.060^{b}	0.223^{b}	1.343^{ab}	2.700^{ab}	3.903^{b}
CinnGar	0.000^{b}	0.003^{b}	0.047^{b}	0.197^{b}	1.263^{b}	2.593^{b}	3.890^{b}
SEM	*0.00*	*0.00*	*0.01*	*0.02*	*0.08*	*0.13*	*0.14*
p-value	*0.0015*	*0.0021*	*0.0023*	*0.0164*	*0.0725*	*0.0614*	*0.0458*

Means with different letters (superscripts) within a column differ significantly at indicated P value, $P < 0.05$

4.4.4. *In vitro* organic matter degradation

No difference was observed between treatments in terms of *iv*OMD, *iv*CH_4 *iv*OMD^{-1}, *iv*CH_4 *iv*GP^{-1}, and *iv*GP *iv*OMD^{-1} following the 48-hour incubation period (Table 4.3). However, a clear tendency was observed for garlic and CinnGar to reduce *iv*GP *iv*OMD^{-1} (ml g kg^{-1}) and *iv*CH_4 *iv*OMD^{-1} (ml g kg^{-1} DM) when compared with the enzyme alone treatments. Inclusion of enzyme showed a tendency ($P < 0.085$) to increase *iv*OMD

(g kg^{-1} DM) when compared with the control.

Table 4.3 *In vitro* organic matter degradability and the relationship between methane, gas production and organic matter digestibility *in vitro* after 48 hours of incubating *Eragrostis curvula* with essential oils and fibrolytic enzymes

Treatments *(n=12)*	*iv*OMD (g kg^{-1} DM)	*iv*CH_4 *iv*OMD^{-1} (ml g kg^{-1} DM)	*iv*CH_4 *iv*GP^{-1} (%)	*iv*GP *iv*OMD^{-1} (ml g kg^{-1})
Control	488.3[a]	8.53[a]	3.52[a]	241[a]
Enzymes	506.5[a]	8.97[a]	3.64[a]	246[a]
Commercial	494.8[a]	8.32[a]	3.41[a]	245[a]
Cinnamon	493.8[a]	8.56[a]	3.49[a]	245[a]
Garlic	492.0[a]	7.88[a]	3.32[a]	237[a]
CinnGar	492.9[a]	7.82[a]	3.31[a]	235[a]
SEM	*0.38*	*0.26*	*0.10*	*2.82*
p-value	*0.0855*	*0.0704*	*0.2592*	*0.0944*

Means with different letters (superscripts) within a column differ significantly at indicated P value, $P < 0.05$

*iv*OMD: *in vitro* organic matter degradability; *iv*CH_4: in vitro methane production; *iv*GP: in vitro gas production

4.5. Discussion

Eragrostis curvula treated with enzyme had a higher *iv*GP throughout the incubation period and increased cumulative *iv*CH_4 production in the initial stages of the incubation period compared with the control. This is in agreement with the results obtained in the preliminary studies (Section 3.5) and was discussed extensively.

Numerous studies on the use of EOs in ruminant nutrition have been conducted with variable results (Busquet *et al*., 2005; Klevenhusen et al., 2011; McGinn et al., 2006). Arguments against the comparison of results between studies can be made as there is a lack of studies using identical EOs compositions (Castro-Montoya *et al*., 2015). Therefore, differences as reported could be due to variations in the EOs products and the efficacy of their active compound in the rumen environment. In addition to the EOs source, the ration fed has a major effect, and varies considerably among studies. The ration fed has a major impact on ruminal pH which in turn affects the efficacy of EOs. When the pH of the rumen is decreased, acids tend to become undissociated and more hydrophobic. In this state, interaction with cell membranes occurs more frequently. The antimicrobial action of EOs is more prominent under these conditions. As a result, the microbial population is more susceptible to the effects of EOs at low pH (Skandamis and Nychas, 2000). In this study, the rumen fluid was obtained from donor sheep under maintenance conditions. It was expected that the rumen fluid would have a relatively high pH compared with production animals such as dairy cows or feedlot steers on high concentrate rations. These conditions might differ from those in another study. The pH in turn can affect the efficacy of both the enzyme and EOs treatments. Furthermore, the methodology, EOs variations, and substrate might explain differences between studies.

More importantly, variations among results are concerning. Beauchemin and McGinn (2006) found no effect on methane emissions when supplementing a blend of EOs and spice extract, although Mohammed *et al*. (2004) reported a significant decrease in methane emissions relative to dry matter (DM) intake when supplementing dairy heifers with horseradish oil. Klevenhusen *et al*. (2011) supplemented garlic oil in sheep rations, and did not find any effect on methane emissions, whereas Wang *et al*. (2009) found that garlic oil supplementation reduced methane emissions by 12% compared with the control. Castro-Montoya *et al*. (2015) observed that dairy cattle fed total mixed ration supplemented with a blend of EOs reduced daily methane emissions. In the same study, beef cattle emitted lower daily methane emissions relative to bodyweight after EOs supplementation. Interestingly, the *in vitro* experiments conducted by these researchers were anticipated to replicate the effects observed *in vivo*. However, no decrease in *iv*CH_4 production was observed in 24-hour incubations. Even with extended incubation (96 hours) and a consistent supply of substrate and additive, *iv*CH_4 was not inhibited. Castro-Montoya *et al*. (2015) proposed that these results could be explained by the differences in the modes of action of EOs *in vitro* and *in vivo*, hence more research is required to verify this theory. In this study, the addition of garlic and CinnGar in combination with enzyme significantly reduced *iv*GP and *iv*CH_4 when compared with the enzyme treatment alone, whereas the commercial product or cinnamon addition had no effect on *iv*GP

and *iv*CH_4 production when compared with the enzyme treatment alone treatment. Furthermore, all of the EOs in combination with the enzyme treatment appeared to have reduced *iv*CH_4 during the early stages of the incubation period compared with the enzyme treatment alone. Cinnamon, garlic and CinnGar continued to reduce *iv*CH_4 at the 12-hour interval, but only CinnGar reduced *iv*CH_4 throughout the incubation period. No difference was observed between treatments in terms of *iv*OMD, *iv*CH_4 *iv*OMD^{-1}, *iv*CH_4 *iv*GP^{-1} and *iv*GP *iv*OMD^{-1} following the 48-hour incubation period because of the addition of any of the EOs treatments. However, a clear tendency was noted for reduction in *iv*CH_4 and *iv*GP *iv*OMD^{-1} for garlic and CinnGar treatments. A possible explanation for these observations is that the source and dose of the EOs treatments were not suitable to influence these parameters. Therefore, that authors recommend adding parameters such as rumen VFA profile and rumen ammonia-nitrogen concentration in future studies.

4.6. Conclusion

The addition of an enzyme increased *iv*GP and *iv*CH_4 and showed a tendency to increase *iv*OMD. Garlic and CinnGar in combination with the enzyme reduced *iv*GP compared with the enzyme treatment alone. All of the EOs in combination with the enzyme treatment showed a tendency to reduce methane production during the early stages of the incubation period compared with the enzyme treatment alone. Of all the treatments, however, CinnGar in combination with enzyme was the only one that reduced *iv*CH_4 throughout the incubation period. Furthermore, a clear tendency was noted for reduction in *iv*CH_4 and *iv*GP per unit of *iv*OMD for garlic and CinnGar treatments. Further studies on these EOs combinations under different conditions (ruminal pH, EOs sources, substrate, etc.) and dose levels should be evaluated to make more informed recommendations on their efficacy. Following successful screenings, the use of these additives should be validated in an *in vivo* trials.

Chapter 5

5.1. General conclusion and recommendations

In the ruminant production sector of southern Africa, extensive production systems are characterized by a shortage of feed during the dry season. The bulk of available feed is characterized as poor in quality with low digestibility, low efficiency of nutrient utilization, and relatively high methane emissions compared with the degree of production (growth, reproduction, milk yield, etc.) that is achieved. This is particularly true among small-scale farmers. Supplementing such rations with concentrate and good-quality roughage could improve production performance, but is not always economically feasible. Another strategy is to improve the utilization of poor-quality feed while minimizing the loss of energy through enteric methane production. Feed additives can contribute to this much required improvement, but it is critical to determine the most appropriate feed additives and optimum inclusion levels to be applied with the specific feed substrate.

The use of exogenous enzymes as additives in ruminant feeds is a safe intervention. Its application has had positive effects in terms of improved nutrient utilization of raw materials, increased production efficiency, and lowered nutrient excretion. The complex nature of the rumen demands that exogenous and endogenous enzyme activities of rumen microbes should complement each other. Therefore, additional information is required to better comprehend enzymatic activities, to determine optimal dose rates, and to improve *in vitro* fermentation characteristics, and thereby enhance the utilization of specific feed ingredients in commercial ruminant rations. This study determined the optimal dose rate of application of three EFEs (exogenous fibrolytic enzymes) on three substrates in terms of improving *iv*GP (*in vitro* gas production). It was found that all of the enzyme treatments had a significant positive effect on total *iv*GP, especially during the earlier incubation intervals of the tested substrates. The cumulative effect increased with higher level of application, reaching the highest gas production level at an application rate of 2 ml g^{-1} DM. However, the associated cost related to a higher application rate of enzyme may not necessarily be justified by the observed level of net return. Thus taking into consideration the cost/benefit ratio of the application rate, the pre-treatment of *Eragrostis curvula*, sunflower oilcake meal and hominy chop with cellulase, xylanase and their mixture at 1.5 ml g^{-1} DM is recommended to improve *in vitro* ruminal fermentation at an economical level. The study thus confirmed that EFEs could have a positive effect on *iv*GP, which is related to improved organic matter and fibre degradation of the

substrates. Furthermore, the concentration of EFEs is positively correlated with increased *iv*GP. However, the cost of EFEs should be balanced with its potential benefit to establish the most economically viable dose rate of EFEs on the substrate in question. By supplementing EFEs to *Eragrostis curvula*, it could be possible to improve the nutrient utilization of various feedstuffs commonly used in ruminant nutrition.

Improving production efficiency and reducing the environmental footprint of ruminant production systems would enhance sustainability and the global competitiveness of the livestock sector. Therefore, greater awareness is placed on improving the utilization of traditional and non-traditional feedstuffs. The application of exogenous enzymes has led to better utilization of nutrients from forages commonly fed to ruminants. Protease addition to *Eragrostis curvula* and sunflower oilcake meal showed potential to increase *iv*GP and reduce *iv*CH_4, *iv*CH_4 *iv*OMD^{-1} and *iv*CH_4 *iv*GP^{-1} when used in combination with EFEs. With the appropriate application of exogenous enzymes in common feed substrates used in livestock production systems, it is possible to improve the utilization of these feed resources in animal production and decrease the environmental impact in the process. The supplementation of EFES alone is expected to increase *iv*CH_4 production. If the results observed in this study could be replicated *in vivo,* one may suggest that a combination of EFES and protease could be a viable supplementation option. However, these results were not conclusive and thus did not improve the understanding of the interactions between exogenous enzymes, various substrates and the rumen environment under the circumstances of this study. Further research is required to achieve better understanding of the use of protease alone and in combination with fibrolytic enzymes and other feed additives on feed substrates used in ruminant nutrition. By conducting such studies, it is possible to improve digestibility while reducing enteric methane production of feed substrates.

An ever-increasing focus is being placed on food security and more efficient and environmentally sustainable livestock production systems. Researchers have the responsibility to evaluate every viable avenue that could contribute towards achieving these overarching goals. One of these avenues could be to establish the potential additive or complementary effects of various supplements on various feed substrates used in the livestock industry. Increasing the knowledge of how best to supplement livestock rations in a focused and precise manner would make significant progress towards achieving these critical goals. Essential oils (EOs) are volatile plant secondary metabolites that are gaining popularity as ruminant feed additives, due mainly to sanctions against the use of antibiotic growth promoters in the European stock feed industry. These restrictions are likely to be adopted by more nations. Preparing for this likelihood would assist ongoing efforts towards achieving food security. In addition to their potential antimicrobial properties, EOs could mitigate enteric methane production. Based on these results, EOs and

exogenous enzyme could act as effective supplements, if used fittingly. In this study, all of the EOs and exogenous enzyme combinations induced a tendency to reduce methane production in the early stages of the incubation period compared with the enzyme treatment alone. However, of all the EOs, CinnGar in combination with enzyme was the only combination that reduced *iv*CH_4 throughout the incubation period. Furthermore, a clear tendency was noted for reduction in *iv*CH_4 and *iv*GP *iv*OMD^{-1} for garlic and CinnGar treatments.

Further studies on these EOs (garlic and CinnGar) and exogenous enzyme combinations under different conditions (ruminal pH, EOs sources, substrate, etc.) and dose levels should be evaluated to make more informed recommendations on their efficacy. The *in vitro* screening and evaluation of feed additives offer an economical and efficient means of identifying multiple potential additives to specific feed substrates. These *in vitro* screenings could be used for evaluation of wider options with an aim of identifying the best additives or a combination of additives that can be included in an *in vivo* digestibility study in order to verify the repeatability of the *in vitro* result by determining the *in vivo* responses. However, the final recommendation regarding the true value of feed additives for ruminants in terms of improving feed utilization could be confirmed only through the use of animal evaluation and production trials.

5.2. Critical evaluation

This section highlights some important points or ideas that were not covered during the implementation of this study, but that would have probably improved the findings and conclusion of this work.

1. When evaluating large numbers of treatments at a time using an incubation oven instead of a shaking incubation water bath (see Chapter 2) would have produced data with lower experimental error during the early incubation hours. The proposed reason for the difference in the effectiveness of the two systems is that the shaking water bath used in this study was big and thus did not adequately regulate the temperature of the environment as evenly as the shaking incubation oven. Furthermore, the sliding tray is not as effective at simulating the rumen environment. Because of the mechanism, 50 revolutions per minute were possible for the shaking water bath, whereas the shaking incubation oven could easily achieve speeds of 120 revolutions per minute, as recommended in most literature. Thus the shaking incubator was used in Chapter

3 and Chapter 4, which allowed to give clear improvements in the quality of the data. It would be recommended that a shaking incubation oven should be used where possible instead of an incubation water bath in future studies.

2. It is accepted that an enzyme assay could better indicate the potential of digesting feed by measuring its protein density. In this study protein density was not performed.
3. Inclusion of additional treatment in the form of higher enzyme dose levels would enable researchers to plot the plateau of a dose-response curve that would have better supported the optimal dose level that was chosen (see Chapter 2).
4. Although the *in vitro* parameters tested for in this study have good correlations with rumen fermentation, generating additional parameters that complement the gas production data such as *in vitro* volatile fatty acid production would have improved the interpretation of the gas production data and potentially strengthened the conclusions.
5. Fibrolytic and protease enzymes and EOs produced variable effects on *in vitro* fermentation. In this study, the main focus was on alterations to *in vitro* fermentation characteristics and *in vitro* methane production. The effects of these additives on the *in vitro* microbial population (protozoa, bacteria, fungi and methanogenic archaea) changes were not monitored. A better understanding of the pattern and extent of changes in methanogens and other microorganisms *in vitro* due to the addition of these additives could better improve our understanding and illustrate the biochemical process and mechanism involved in the altered *in vitro* fermentation characteristics. Therefore, the addition of these parameters to similar studies in the future is recommended.
6. In this study the pH of the rumen fluid collected from fully grown wethers should have been recorded during the collection time. The pH of the rumen fluid collected from animals fed higher forage diets is expected to be significantly higher than the pH of rumen fluid collected from high producing dairy cows or feedlot steers fed high concentrate rations. It is known that certain enzymes and EOs are less effective in such environments characterised with high pH.
7. An *in vivo* evaluation trial using methane chambers should be considered to verify the results. This would provide more accurate data that help us to illustrate the effects of exogenous enzymes and EOs oils on nutrient digestibility and enteric methane production.
8. In order analyse the data further one should consider to evaluate the effect of the exogenous fibrolytic enzymes on the rate of fermentation of each substrate using the France's Model (France et al. J Theor. Biol. 1993. 163:99-111).

References

Adesogan, A.T., Ma, Z.X., Romero, J.J. and Arriola, K.G., 2007. Improving cell wall digestion and animal performance with fibrolytic enzymes. Journal of Animal Science 92: 1317-1330.

Agle, M., Hristov, A.N., Zaman, S., Schneider, C., Ndegwa, P. and Vadella, V.K., 2010. Effects of ruminally degradable protein on rumen fermentation and ammonia losses from manure in dairy cows. Journal of Dairy Science 93: 1625-1637.

Alexandratos, N. and J. Bruinsma., 2012. World agriculture towards 2030/2050: The 2012 revision. ESA Working Paper 12-03. FAO, Rome.

Alvarez, G., Pinos-Rodriguez, J.M., Herrera, J.G., Garcia, J.C., Gonzalez, S.S. and Barcena, R., 2009. Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fibre rations. Livestock Science 121: 150-154.

AOAC, 2000. Official method of analysis 920.39 (17th edition). Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 934.01 (17th edition). Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 942.05 (17th edition). Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 968.06 (17th edition). Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

Arthington, J.D. and Brown, W.F., 2005. Estimation of feeding value of four tropical forage species at two stages of maturity. Journal of Animal Science 83: 1726-1731.

Bailey, M.J., Biely, P. and Poutanen K., 1992. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23: 257-270.

Bayat, A. and Shingfield, K.J., 2012. Overview of nutritional strategies to lower enteric methane emission in ruminants. Agricultural Science Day. 1-7.

Beauchemin, K.A. and McGinn, S.M., 2006. Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. Journal of Animal Science 84: 1489–1496.

Beauchemin, K.A., McAllister, T.A. and McGinn, S.M., 2009. Dietary mitigation of enteric methane from cattle. Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 4: 1-18.

Beauchemin, K.A., Yang, W.Z. and Rode, L.M., 1999. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. Journal of Dairy Sciences 82: 378-390.

Beauchemin, K. A., Colombatto, Morgavi, D.P. and Yang, W.Z., 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. Journal of Animal Science 81: 37–47.

Beauchemin, K.A., Kreuzer, M., O'Mara, F. and McAllister, T.A., 2008. Nutritional management for enteric methane abatement: A review. Australian Journal of Experimental Agriculture 48: 21-27.

Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi, D.P. and Kampen, R., 2000. Evaluation of a non starch polysaccharidase feed enzyme in dairy cow diets. Journal of Dairy Sciences 83: 543-553.

Bedford, M.R. and Partidge, G.G., 2011. Enzymes in farm animal nutrition. 2nd edition. 12 – 54. MPG Books Group, Bodmin, United Kngdom.

Belete, S.G., Hassen A. and Morgavi, D., 2014. *In vitro* fermentation, digestibility and methane production of two roughages and a total mixed ration as influenced by cellulase and xylanase enzyme application levels. Journal of Animal Science 85: 28-160.

Benchaar, C., Petit, H.V., Berthiaume, R., Ouellet, D.R., Chiqeutte, J. and Chouinard, P.Y., 2007. Effect of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. Journal of Dairy Science 90: 886-897.

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnology Advances 18: 355-383.

Binder, M., 2016. Life cycle thinking in animal production. AMINONews. Special edition (2016): Sustainability.

Blummel, M. and Becker, K., 1997. The degradability characteristics of fifty-four roughages and roughage detergent fibre as described by *in vitro* gas production and their relationship to voluntary feed intake. British Journal of Nutrition 77: 757-768.

Boland, T.M., Hart, K.J., Pierce, K.M., Lynch, B.M., McDonnel, L.R., Murphy, D., Kelly, A.K. and Kenny, D.A., 2009. The effect of pre-grazing herbage mass on growth rate and methane emissions of grazing beef cattle. Journal of Dairy Science 92: 343-339.

Borchers, R., 1965. Proteolytic activity of rumen fluid *in vitro*. Journal of Animal Science 24: 1033-1038.

Bowman, G. R., Beauchemin, K.A. and Shelford, J.A., 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. Journal of Dairy Science 85: 3420–3429.

Burt, S., 2004. Essential oils: Their antibacterial properties and potential applications in foods – A review. International Journal of Food and Microbiology 94: 223-253.

Busquet, M., Calsamiglia, S., Ferret, A. and Kamel, C., 2004. Effects of different doses of plant extracts on rumen microbial fermentation. Journal of Dairy Science 87: 213.

Busquet, M., Calsamiglia, S., Ferret, A. and Kamel, C., 2006. Plant extracts affect *in vitro* rumen microbial fermentation. Journal of Dairy Science 89: 761-771.

Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C. and Burt, S., 2005. Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous culture. Animal Feed Science and Technology 124: 597-613.

Busquet, M., Greathead, H., Calsamiglia, S., Ferret, A. and Kamel, C., 2003. Effect of garlic extract and cinnamaldehyde on milk production, composition and residues in high production cows. ITEA 24: 756-758.

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L. and Ferret, A., 2007. Essential oils as modifiers of rumen microbial fermentation: A review. Journal of Dairy Science 90: 2580-2595.

Cantet, J.M., Colombatto, D. and Jaurena, G., 2015. Methane production and *in vitro* digestibility of low quality forages treated with a protease or a cellulase. Animal Production Science. http://dx.doi.org/10.1071/AN14988

Cardozo, P.W., Calsamiglia, S., Ferret, A. and Kamel, C., 2004. Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. Journal of Animal Science 82: 3230-3236.

Cardozo, P.W., Calsamiglia, S., Ferret, A. and Kamel, C., 2005. Screening for the effects of natural plant extracts at different pH on *in vitro* rumen microbial fermentation of a high-concentrate diet for beef cattle. Journal of Animal Science 83:2572-2579.

Cardoso, P.W., Calsamiglia, S., Ferret, A. and Kamel, C., 2006. Effects of alfalfa extract, anise, capsicum and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high concentrate diet. Journal of Animal Science 84: 2801-2808.

Castro-Montoya, J., Peiren, N., Cone, J.W., Zweifel, B., Fievez, V. and Camoeneere, S., 2015. *In vivo* and *in vitro* effects of a blend of essential oils on rumen methane mitigation. Livestock Science 180: 134-142.

Castillejos, L., Calsamiglia, S. 7 Ferret, A., 2006. Effect of essential oils active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. Journal of Dairy Science 89: 2649-2658.

Charmley, E., Williams, S.R.O., Moate, P.J., Hegarty, R.S., Herd, R.M., Oddy, V.E., Reyenga, P., Staunton, K.M., Anderson, A. and Hannah, M.C., 2016. A universal equation to predict methane production of forage-fed cattle inAustraliad. Animal Production Science 56: 169–180.

Cieslak, A., Szumacher-Strabel, M., Stochmal, A. and Oleszek, W., 2013. Plant components with specific activities against rumen methanogens. Animal Science 7: 253-265.

Colombatto, D. and Beauchemin, K.A., 2009. A protease additive increases fermentation of alfalfa diets by mixed ruminal microorganisms *in vitro*. Journal of Animal Science 87: 1097-1105.

Colombatto, D., Hervas, G., Yang, W.Z. and Beauchemin, K.A., 2003. Effects of enzyme supplementation of a total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. Journal of Animal Science 81: 2617-2627.

Colombatto D., Morgavi D.P., Furtado A.F. and Beauchemin K.A., 2003a. Screening of exogenous enzymes for ruminant diets: relationship between biochemical characteristics and *in vitro* ruminal degradation. Journal of Animal Science 81: 2628-2638.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E., 2003b. Use of fibrolytic enzymes to improve the nutritive value of ruminal diets: a biochemical and *in vitro* rumen degradation assessment. Animal Feed Science and Technology 107: 201-209.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E., 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. Animal Feed Science and Technology 137: 150-162.

De Vries, M. and De Boer, I.J.M., 2010. Comparing environmental impacts for livestock products: A review of life cycle assessments. Journal of Livestock Science 128: 1–11.

Devillard, E., Goodheart, D.B., Karnati, S.K.R., Bayer, E.A., Lamed, R., Miron, J., Nelson, K.E. and Morrison, M., 2004. Ruminococcus albus 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture. Journal of Bacteriology 186: 136–145.

Elghandour, M.M.Y., Salem, A.Z.M., Ganzalez-Ronquillo, M., Bórquez, J.L., Gado, H.M., Odongo, N.E. and Peñuelas, C.G., 2013. Effects of exogenous enzymes on *in vitro* gas production kinetics and ruminal fermentation of four fibrous feeds. Animal Feed Science and Technology 179: 46-53.

Elwakeel, E.A., Titgemeyer, E.C., Johnson, B.J., Armendariz, C.K. and Shirley, J.E., 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. Journal of Dairy Science 90: 5226-5236.

Eun, J.S. and Beauchemin, K.A., 2005. Effects of proteolytic feed enzyme on intake, digestion, ruminal fermentation and milk production. Journal of Dairy Science 88: 2140-2153.

Eun, J.S. and Beauchemin, K.A., 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. Animal Feed Science and Technology 132: 298–315.

Eun, J.S. and Beauchemin, K.A., 2008. Relationship between enzymatic activities and *in vitro* degradation of alfalfa hay and corn silage. Animal Feed Science and Technology 145: 53-67.

Eun, J.S., Beauchemin, K.A. and Schulze, H., 2007. Use of fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. Journal of Dairy Science 90: 1440-1451.

Firkins, J. L., Allen, M.S., Oldick, B.S. and St-Pierre, N.R., 1998. Modelling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. Journal of Dairy Science 81: 3350-3369.

Flachowsky, G. and Lebzien, P., 2012. Effects of phytogenic substances on rumen fermentation and methane emissions: A proposal for a research process. Animal Feed Science and Technology 176: 70–77.

FAO. 2016. FOASTAT online database. http://www.foa.org/hunger/en/ FAO

Fontes, C. M., Hall, G.A., Hirst, J., Hazlewood, B.A. and Gilbert, H.J., 1995. The resistance of cellulases and xylanases to proteolytic inactivation. Applied Microbiology and Biotechnology 43: 52–57.

Forbes, 2016. http://www.forbes.com/sites/danielrunde/2015/02/24/urbanization-development-opportunity/#797904d62779.

Gado, H.M., Salem, A.Z.M., Robinson, P.H. and Hassan, M., 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. Animal Feed Science and Technology 154: 36–46.

Garnett, T., 2009. Livestock-related greenhouse gas emissions: Impacts and options for policy makers. Journal of Environmental Science and Policy 12: 491–503.

Gebhardt, R. and Beck, H., 1996. Differential inhibitory effects of garlic-derived organosulfur compounds on cholesterol biosynthesis in primary rat hepatocyte cultures. Lipids 31: 1269-1276.

Gimeno, A., Alami, A., Abecia, L., de Vega, A., Fondevila, M. and Castrillo, C., 2015. Effect of type (barley vs. maize) and processing (grinding vs. dry rolling) of cereal on ruminal fermentation and microbiota of beef calves during the early fattening period. Animal Feed Science and Technology 199: 113–126.

Giraldo, L.A., Carro, M.D., Ranilla, M.J. and Tejido, M.L., 2007. Influence of fibrolytic enzymes on *in vitro* methane production and rumen fermentation of a substrate containing 60% grass hay. Livestock Research for Rural Development 19: 185-187.

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

Goering, H.K. and Van Soest, P.J., 1970. Forages fiber analysis, apparatus, reagents, procedures, and some applications. USDA Agricultural Handbook 379: 1–20.

Grainger, D. and Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Animal Feed Science and Technology 166-167: 308-320.

Grant, R.J. 1997. Interactions among forages and non forage fibre sources. Journal of Dairy Science 80: 1438-1446.

Grant, R.J. and Mertens, D.R., 1992. Impact of *in vitro* fermentation techniques upon kinetics of fiber digestion. Journal of Dairy Science 75: 1263-1272.

Greathead, H., 2003. Plant and plant extract for improving animal productivity. Proceedings of Nutrition Society 62: 279-290.

Griffin, S.G., Wyllie, S.G., Markham, J.l. and Leach, D.N., 1999. The role of structure and molecular properties of trepenoids in determining their antimicrobial activity. Flavour Fragrance Journal 14: 322-332.

Harborne, J. B. and C. A. Williams., 2000. Advances in flavonoid research since 1992. Phytochemistry 55: 481-504.

He, Z.X., He, M.L., Walker, N.D., McAllister, T.A. and Yang, W.Z., 2014. Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains with solubles: ruminal fermentation, digestibility, and growth performance of feedlot steers. Journal of Animal Science 92: 3978-3987.

Hindrichsen, I.K., Kreuzer, M., Machmuller, A., Bach Knudsen, K.E., Madsen, J. and Wettstein, H.R., 2003. Methane release and energy expenditure of dairy cows fed concentrates characterized by different carbohydrates. In Souffrant, W.M., Metges, C.C. (Eds.), Proceedings of the EAAP-Symposium on Energy and Protein Metabolism and Nutrition. Pp. 413-416.

Hino, T. and Russel, J.B., 1985. Effects of reducing-equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen organisms and their cell extracts. Applied and Environmental Microbiology 50: 1368-1374.

Hristov, A.N., McAllister, T.A. and Cheng, K.J., 1998. Effect of dietary or abomasal supplementation of exogenous polysaccharide- degrading enzymes on rumen fermentation and nutrient digestibility. Journal of Animal Science 76: 3146-3156.

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson, B. and Tricarico, J.M., 2013. Mitigation of methane and nitrous oxide emissions from animal operations: II. A review of manure management mitigation options. Journal of Animal Science 91: 5070-5094.

Insights, 2016. The sustainability imperative. http://www.nielsen.com/ca/en/insights/reports/2015/the-sustainability-imperative.html.

Jalilvand, G., Odongo, N.E., López, S., Naserian, A., Valizadeh, R., Sharodi, F.E., Kebreab, E. and France, J., 2008. Effects of different levels of an enzyme mixture on *in vitro* gas production parameters of contrasting forages. Animal Feed Science and Technology 146: 289-301.

Johnson, K.A. and Johnson, D.E., 1995. Methane emissions from cattle. Journal of Animal Science 73: 2482-2492.

Jung, H.G., Samac, D.A. and Sarath, G., 2012. Modifying crops to increase cell wall digestibility. Plant Science 186: 65–77.

Kamel, C., Greathead, H.M.R., Tejido, M.L., Ranilla, M.J. and Carro, M.D., 2007. Effects of allicin and diallyl disulfide on *in vitro* rumen fermentation of a mixed diet. Animal Feed Science and Technoglogy 84: 1203-1232.

Kamra, D.N., 2005. Rumen microbial ecosystem. Current Science 89: 124-135.

Kazemi-Bonchenari, M., Salem, A.Z.M. and López, S., 2017. Influence of barley grain particle size and treatment with citric acid on digestibility, ruminal fermentation and microbial protein synthesis in Holstein calves. Animal 11: 1295–1302.

Kharas., H.J., 2010. The emerging middle class in developing countries. OECD Development Centre, Paris.

Kim, E.T., Hwang, H.S. and Lee, S.M., 2016. Effects of medicinal herb extracts on in vitro ruminal methanogenesis, microbe diversity and fermentation system. Asian-Australas Journal of Animal Science 29: 1280-1286.

Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M. and Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Animal Feed Science and Technology 166: 356–363.

Kobayashi, Y., 2010. Abatement of Methane Production from Ruminant: Trends in the manipulation of Rumen Fermentation. Asian-Australian Journal of Animal Science 23: 410-416.

Krause, D.O., Denman, S.T., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T. and McSweeney, C.S., 2003. Opportunities to improve fibre degradation in the rumen: Microbiology, ecology, and genomics. FEMS Microbiology Reviews 27: 663-693.

Krueger, N.A., Adesogan, A.T., Staples, C.R., Krueger, W.K., Kim, S.C. and Litell, R.C., 2008. Effects of method of applying fibrolytic enzymes or ammonia to Bermuda grass hay on feed intake, digestion and growth of beef steers. Journal of Animal Science 86: 882-889.

Kung, J.R., Treacher, R.J., Nauman, G.A., Smagala, A.M., Endres, K.M. and Cohen, M.A., 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. Journal of Dairy Science 83: 115-122.

Kung, L., Cohen, M.A., Rode, L.M. and Treacher, R.J., 2002. The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. Journal of Dairy Science 85: 2396-2402.

Lamera, E., Bouacida, S., Marc, L.B., Bouazizc, Z. and Bouraiou, A., 2017. Sequential MCR/Fisher indolization strategy for the construction of polycyclic carbazole derivatives. Journal of Tetrahedron Letters. 58: 1305–1307.

Lawson, L., 1996. The composition and chemistry of garlic cloves and processed garlic. Pages 37-107 in Garlic. The science and therapeutic application of *Allium sativum* L. and Related Spieces. H.P. Koch and L.D. Lawson (eds). Williams and Wilkins, Baltimore, MD.

Leng, R.A., 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A report to the Department of Climate Change Commonwealth Government of Australia, Canberra, ACT, Australia. Available at: www.penambulbooks.com.

Lovett, D., Lovel, L.S., Stack, L., Callan, J., Finlay, M., Conolly, J. and O'Mara, F.P., 2003. Effect of forage concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livestock Production Science 84: 135-146.

Martin, C., Morgavi, D.P. and Doreau, M., 2009. Methane mitigation in ruminants: From microbe to farm scale. The Animal Consortium 4: 351-365.

Marzorati, M., Vilchez-Vargas, R., Bussche, J.V., Truchado, P., Jauregui, R., El Hage, R. A. and Van de Wiele, T., 2017. High-fiber and high-protein diets shape different gut microbial communities, which ecologically behave similarly under stress conditions, as shown in a gastrointestinal simulator. Molecular Nutrition and Food Research 61: 150-160.

McAllister, T. A., Bae, H.D., Jones, G.A. and Cheng, K.J., 1994. Microbial attachment and feed digestion in the rumen. Journal of Animal Science 72: 3004-3018.

McAllister, T.A., Okine, E.K., Mathison, G.W. and Cheng, K.J., 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. Canadian Journal of Animal Science 76: 231-243.

McAllister, T. A., Hristov, A.N., Beauchemin, K.A., Rode, L.M. and Cheng, K.J., 2001. Enzymes in ruminant diets. In: M. R. Bedford and G. G. Partridge (eds). Enzymes in farm animal nutrition. CAB International, Wiltshire, UK.

McDonald, P., Edwards, R., Greenhalgh, J.F.D., Morgan, C.A., Sinclair, L.A. and Wilkinson, R.G., 2011. Animal nutrition. 7th edition. Harlow, Pearson Education, Prentice Hall, England.

McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.A. and Newbold, C.J., 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Applied Environmental Microbiology 69: 5011–5014.

McGeough, E.J., O'ki, E.L.Y.P., Hart, K.J., Moloney, A.P., Boland, T.M. and Kenny, D.A., 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. Journal of Animal Science 88: 2703-2716.

McGinn, S.M., Beauchemin, K.A., Coates, T. and Colombatto, D., 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes. Yeast, and fumaric acid. Journal of Animal Science 82: 3346-3356.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D. and Scneider, W., 1997. The estimation of digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. Journal of Agricultural Science 93: 217-222.

Min, B.R., Solaiman, S., Shange, R. and Eun, J.S. 2014. Gastrointestinal bacterial and methanogenic archaea diversity dynamics associated with condensed tannin-containing pine bark diet in goats using 16S rDNA amplicon pyrosequencing. International Journal of Microbiology 11.

Mohammed, N., Ajisaka, N., Lila, Z.A., Hara, K., Mikuni, K., Kanda, S. and Itabashi, H., 2004. Effect of Japanese horseradish oil on methane production and ruminal fermentation *in vitro* and in steers. Journal of Animal Science 82: 1839–1846.

Monteny, G.J. and Chadwick, D., 2006. Greenhouse gas abatement strategies for animal husbandry. Agriculture Ecosystems and Environment 112: 163-170.

Morgavi, D. P., Newbold, C.J., Beever, D.J. and Wallace, R.J., 2000b. Stability and stabilization of potential feed additive enzymes in rumen fluid. Enzyme Microbiology and Technology 26: 171–177.

Morgavi, D.P., Forano, E., Martin, C. and Newbold, C.J., 2010. Microbial ecosystems and methanogenesis in ruminants. Animal Science 4: 1024-1036.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., Iwaasa, A.D., Yang, W.Z., McAllister, T.A. and Wang, Y., 2000a. Synergy between ruminal fibrolytic enzymes and enzymes from *Trichoderma Longibrachiatum*. Journal of Dairy Science 83: 1310–1321.

Moss, A.R., 2000. Methane production by ruminants: Its contribution to global warming. Annales of the Zootechnie 49: 231-253.

Nagaraja, T.G., Newbold, C.J., Van Nevel, C.J. and Demeyer, D.I., 1997. Manipulation of ruminal fermentation. Pp.523-632 in The Rumen Microbial Ecosystem, 2nd edition. P.N. Hobson and C.S. Stewart (eds).Blackie Academic and Professional, London.

Nagy, J.G. and Tengerdy, R.P., 1968. Antibacterial action of essential oils of Artemisia as an ecological factor. II. Antibacterial action of volatile oils of Artemisia tridentate (Big Sagebrush) on bacteria from the rumen of mule deer. Applied Microbiology 16: 441-444.

Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R. and Wallace, J., 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 114: 105-112.

Nsereko, V. L., Morgavi, D.P., Beauchemin, K.A. and Rode, L.M., 2000. Inhibition of ruminant feed enzyme polysaccharidase activities by extracts from silages. Canadian Journal of Animal Science 80: 523–526.

Oh, H.K., Sakai, T., Jones, M.B. and Longhurst, W.M., 1967. Effect of various essential oils isolated from Douglas Fir Needles upon sheep and deer rumen microbial activity. Applied Microbiology 15: 777-784.

Paris Agreement, 2016. http://ec.europa.eu/clim/policies/international/negotians/paris/index_en.htm.

Perme, B., Chaudhary, L.C., Agarwal, N. and Kamra, D.N., 2016. Effect of phyto additives and Saccharomyces cerevisiae on rumen fermentation and microbial profile in buffaloes. Indian Journal of Animal Science 86: 197-202.

Pinos-Rodriquez, J.M., Gonzalez, S.S., Mendoza, G.D., Barcena, R., Cobos, M.A., Hernandez, A. and Ortega, M.E., 2002. Effects of exogenous fribrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. Journal of Animal Science 80: 3016-3020.

Pinos-Rodriquez, J.M., Moreno, R., Gonzalez, S.S., Robinson, P.H., Mendoza, G. and Alvarez, G., 2007. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. Animal Feed Science and Technology 166-167: 113-121.

Plaizier, J.C., Krause, D.O., Gozho, G.N. and McBride, B.W., 2009. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. Journal of Veterinary Science 176: 21-31.

Reddy, L., Odhav, B. and Bhoola, K.D., 2003. Natural products for cancer prevention: a global perspective. Pharmacology and Therapeutics 99: 1-13.

Reuter, H.D., Koch, J.P. and Lawson., 1996. Therapeutic effects and applications of garlic and its preparations. The Science and Therapeutic Application of Allium sativum L. and Related Species. Williams and Wilkins, Baltimore, MD. 135-212.

Russell, J. B., and Martin, S.A., 1984. Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms *in vitro*. Journal of Animal Science 59: 1329–1338.

Sakurai, K., Arai, H., Ishii, M. and Igarashi, Y., 2012. Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. Journal of Bioscience and Bioengineering 113: 343–348.

Santoso, B., Kume, S., Nonaka, K., Kimura, K., Mizokoshi, H., Gamo, Y. and Takahashi, J., 2003. Methane emission, nutrient digestibility, energy metabolism and blood metabolites in dairy cows fed silages with and without galacto-oligosaccharides supplementation. Asian-Australian Journal of Animal Science 16: 534-540.

Selinger, L.B., Forsberg, C.W. and Cheng, K.J., 1996. The rumen: A unique source of enzymes for enhancing livestock production. Anaerobe 2: 263-284.

Sheppy, C., 2001. The current feed enzyme market and likely trends. In: Enzymes in farm animal nutrition. M.R. Bedford and G.G. Partridge (eds). CAB, London, UK. pp 1-9.

Singh, B., Sahoo, A., Sharma, R. and Bhat, T.K., 2005. Effect of polyethylene glycol on gas production parameters and nitrogen disappearance of some tree forages. Animal Feed Science and Technology 123-124: 351-364.

Skandamis, P.N. and Nychas, G.J., 2000. Development and evaluation of a model predicting the survival of Escherichia coli O157:H17 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentration. Applied Environmental Microbiology 66: 1646-1653.

Smith, A.H., Zoetendal, E.G. and Mackie, M., 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microbial Ecology 50: 197-205.

Steinfeld, H., Gerber, P., Castel, V., Rosales, M. and de Haan, C., 2006. Livestock's long shadow. FAO, Rome, Italy.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllen, A.B. and France, J., 1994. A simple gas production method using pressure transducers to determine the fermentation kinetics of ruminant feed. Animal Feed Science and Technology 48: 185-197.

Tilley, J.M.A. and Terry, R.A., 1963. A two-stage technique for the *in vitro* digestion of forage crops. Journal of the British Grassland Society 18: 104-111.

United Nations, 2009. The state of food and agriculture. Food and Agriculture Organization of the United Nations, Rome.

United Nations, 2015. World population prospects: The 2015 Revision. United Nations, New York.

Valdes, K.I., Salem, A.Z.M., Lopez, S., Alonso, M.U., Rivero, N., Elghandour, M.M.Y., Dominguez, I.A., Ronquillo, M.G. and Kholif, A.E. 2015. Influence of exogenous enzymes in presence of Salix babylonica extract on digestibility, microbial protein synthesis and performance of lambs fed maize silage. Journal of Agricultural Science 153: 732-742.

Van Nevel, C.J. and Demeyer, D.I., 1988. Manipulation of rumen fermentation. In: P.N. Hobson (ed). The rumen microbial ecosystem. Elsevier Applied Science, NY, 387-443

Van Soest, P.J., 1994. Nutritional ecology of the ruminant. 2nd edition. Cornell University Press, Ithaca, NY, USA.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A., 1991. Symposium: Carbohydrate methodology, metabolism and nutritional implications in dairy cattle. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74: 3583-3597.

Van Soest P.J., Wine R.H. and Moore L.A., 1966. Estimation of the true digestibility of forages by the *in vitro* digestion of cell walls. In: Proceedings of the 10th International Grasslands Congress. Pp. 438-441.

Varga, G.A. and E.S. Kolver, 1997. Microbial and animal limitations to fibre digestion and utilization. Journal of Nutrition 127: 819S-823S.

Wallace, R.J. 2004. Antimicrobial properties of plant secondary metabolites. Proceedings of the Nutrition Society 63: 621-629.

Wallace, R. J., Wallace, S.J., McKain, N., Nsereko, V.L. and Hartnell, G.F., 2000. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms *in vitro*. Journal of Animal Science 79: 1905–1916.

Wang, C.J., Wang, S.P. and Zhou, H., 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Animal Feed Science and Technology 148: 157–166.

Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A.D. and Yang, W., 2001. Effect of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique (rusitec). British Journal Nutrition 85: 325-332.

Weimer, P.J., 1996. Why don't ruminal bacteria digest cellulose faster? Journal of Dairy Science 79: 1496-1502.

Weimer, P. J., 1998. Manipulating ruminal fermentation: A microbial ecological perspective. Journal of Animal Science 76: 3114-3122.

White, B.A., Mackie, R.I. and Doerner, K.C., 1993. Enzymatic hydrolysis of forage cell walls. In Forage cell wall structure and digestibility. American society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, 455-484.

WHO, 2016. Global and regional food consumption patterns and trends. http://www.who.int/nutrition/topics/3_foodconsumption/en/index4.html.

Williams, A.G. and Coleman, G.S., 1992. The rumen protozoa. Springer-Verlag, New York.

Wood, T.M. and Bhat, M.K., 1988. Methods for measuring cellulose activities. In W.A. Wood and S.t. Kellogg (eds). Methods in enzymology. Aceademic Press London, UK, 87-112.

Worldwatch, 2016. Global meat production and consumption continue to rise. http://www/worldwatch.org/global-meat-production-and consumption-continue-rise-1.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M., 1999. Effects of enzyme feed additives on extent of digestion and milk production of lactating dairy cows. Journal of Dairy Science 82: 391-403.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M., 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. Journal of Dairy Science 83: 2512-2520.

Yang, W.Z., Chaves, A.V., He, M.L., Benchaar, C. and McAllister, T.A., 2006. Effects of monensin and essential oil on feed intake, milk yield and composition of lactating dairy cows. Journal of Animal Science 86: 598-609.

Yang, W.Z., Ametaj, B.N., He, M.L., Benchaar, C. and Beauchemin, K.A., 2010. Cinnamaldehyde in feedlot cattle diets: intake, growth performance, carcass characteristics, and blood metabolites. Journal of Animal Science 88: 1082-1092.

Young, K.M., Lim, J.M., Der Bedrosian, M.C. and Kung, L., 2012. Effects of exogenous protease enzymes on the fermentation and nutritive value of corn silage. Journal of Dairy Science 95: 6687-6694.