The effect of grinding size and amylase enzyme supplementation on potential ruminal and total tract starch digestion of maize in dairy cows

By

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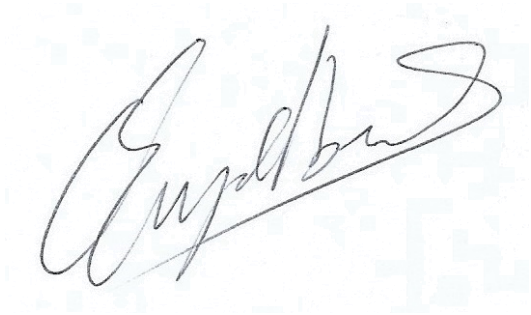
DECLARATION

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



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SUMMARY

The effect of particle size and amylase enzyme supplementation on ruminal and total tract starch digestion of maize in dairy cows

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Although the market price of maize depends on environmental and political factors, thus deeming it variable, South Africa uses maize as the primary energy concentrate in ruminant diets, providing the energy for high-performance animals. Producers recently experienced the severity of the impact of these environmental factors, with maize prices reaching a record high during 2016 due to droughts from 2014 to 2016, decreasing the milk-to-feed price ratio in South Africa to a critical level. Since international milk prices still have not recovered after the 2014 global price crash, it is critical to formulate diets accurately and purposefully for optimal production. One factor that can significantly affect this goal is finding ways to reduce the wastage of nutrients in the digestive tract.

Many factors, including genetics, cultivar, variety, geographical location, year, climatic conditions, and agronomic practices, directly influence the nutrient value and digestibility of grains. In addition to these production parameters, the ratio of amylose to amylopectin, which makes up the type of starch contained in the endosperm of grains, and the particle size of milled grains have a substantial effect on the digestibility of the grain component in feed. The encapsulation of maize starch particles in a bed of hydrophobic proteins called prolamin primarily influences the digestibility of the starch in maize. The type of starch is the second factor that affects starch digestibility since amylose is less digestible than amylopectin because

of its molecular structure. It is commonly accepted that finer ground maize leads to less starch in the manure, thus indicating improved starch digestion.

The digestibility of grains is directly altered by the amylose-to-amylopectin ratio of grains. *In-vitro* rumen digestibility increased as the amylose content of grains decreased. Amylose has tighter intermolecular bonding between starch molecules than amylopectin. Commercial feed enzyme development is a radical innovation in dairy cow nutrition. Ronozyme®. Rumistar is an α-amylase enzyme specifically developed to improve starch digestion in the rumen of ruminants. This study aimed to determine the efficiency and the interaction between maize processing and the addition of exogenous enzymes and to provide additional insights into these two most important factors that affect ruminal starch disappearance and the post-ruminal digestibility of starch in ruminants.

This trial was conducted in two phases, assessing total tract starch digestion in the first phase and ruminal digestion in the second. The first phase of the trial was conducted on a commercial dairy farm. One diet was fed throughout the trial period, with the maize component ground into three different milling sizes (coarse maize with a mean particle size of >3 mm, fine maize with a mean particle size of <3 mm, and micro-milled maize with a particle size of <1 mm). The cows were randomly allocated to two treatment groups. The feed of one group was supplemented with an additional exogenous amylase enzyme, while the other group served as the control. This study reported the impact of different particle sizes in the maize fraction of the feed and the addition of an amylase enzyme on milk yield, milk composition, and the manure nutrient profile. Phase Two of the trial was conducted as a cross-over design with four rumen-cannulated cows at the University of Pretoria's experimental farm to determine the rate of starch disappearance over time. Each cow served as a repetition of the trial, and the study was executed in two periods, with one sampling day per period. Before the start of the study, two of the cows were adapted to the enzyme treatment by adding the enzyme directly into the rumen via a cannula twice a day for 21 days before the 24-hour *in-sacco* trial to allow the cows to adjust to the diet and conditions. Ruminal pH and temperature were measured and recorded at every enzyme insertion. The treatments were inverted for the second round. A seven-hour, *in-vitro* starch digestibility assay was run with an adapted rumen fluid mixture for the different treatments. Ruminal volatile fatty acid production was measured for the different treatment runs.

In the first phase, which focused on total tract starch digestibility, no significant differences were found in milk yield when the diets with different maize particle sizes supplemented with amylase enzyme were compared. Milk fat was not affected by either the supplementation of the amylase enzyme or the particle size of the maize component in the feed. However, a significant interaction was found between treatment and particle size ($P<0.05$). The feeding of coarse maize resulted in higher milk fat production in the control group that received the base diet with no supplemental enzyme, but the effect was suppressed when the amylase enzyme was added. Particle size significantly affected milk protein, with a smaller particle size resulting in an increased milk protein percentage ($P<0.05$).

The particle size of the maize component in the diet significantly affected the faecal starch content, with less starch in the faeces of the cows eating the finer ground particles ($P<0.05$). Manure neutral detergent fibre was significantly affected by the maize particle size and enzyme interaction. Overprocessing effects seemed to reduce the neutral detergent fibre content of micro-milled maize in conjunction with the supplemental enzyme. Across the particle sizes, the mean protein content of the manure was significantly lower for the enzyme treatment, suggesting reduced hindgut fermentation as a result of improved rumen fermentation.

In the second phase, ruminal starch degradability was measured using a seven-hour *in-vitro* digestibility assay. Analysed digestibility of coarse maize was half that of fine maize, with the digestibility of micro-milled maize being an additional 25% higher than fine maize.

As expected, in the *in-sacco* digestibility assay, digestibility curves showed that the maize particle size had a significant impact on the soluble fraction A, which increased with the degree of processing ($P<0.005$). The particle size did not affect the insoluble fraction B. The fast-digestible fraction was lower with the supplemental enzyme, but total tract digestibility was improved ($P<0.05$). This corresponded with the volatile fatty acid results, showing increased volatile fatty acid production with the supplemental enzyme across all maize particle sizes. Propionic acid production was raised, and the acetate-to-propionate ratio was reduced.

There is no doubt that the use of exogenous enzymes with amylolytic and proteolytic activity in diets with a high starch amylase content for ruminants is already under way although the precise mode of action and the limitations of the metabolic system in grains are not yet well understood. More research is necessary to understand all the factors that are influencing and

being influenced by these enzymes in order to utilise them for the benefit of the animal and the producer.

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
CM	Coarsely ground maize (>3 mm)
CP	Crude protein
CSD	Calculated starch digestibility
DM	Dry matter
DMI	Dry matter intake
ED	Effective degradability
FM	Finely ground maize (<3 mm)
IF	Insoluble fraction
IVTD	*In vitro* true digestibility
$IVTD_{DM}$	*In vitro* true dry matter digestibility
kd	Fractional degradation rate
KNU	Kilo Novo Units
ME	Metabolisable energy
MJ	Megajoules
MMM	Micro-milled maize (<1 mm)
MP	Metabolisable protein
mRDC	Modelled rapidly degradable carbohydrates
mTDC	Modelled total degradable carbohydrates
MUN	Milk urea nitrogen
N	Nitrogen
NDF	Neutral detergent fibre
NIR	Near-infrared technology
NRC	National Research Council
NSC	Non-structural carbohydrates
OM	Organic Material
P	Phosphorus
PSD	Percentage starch disappearance
RDP	Ruminal digestible protein
REML	Restricted maximum likelihood

SARA	Sub-acute ruminal acidosis
SD	Standard deviation
SER	Standard error of the mean
TMR	Total mixed ration
TTSD	Total tract starch digestibility
uCT	X-ray microcomputed tomography
VFA	Volatile fatty acid

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Chapter 1

1. General Introduction

The maize price is affected by environmental and political factors, causing it to be highly volatile. It is, however, still the primary energy source in ruminant diets, providing energy in the form of starch to high-producing animals. Producers felt the severity of this monetary impact when maize prices reached a record high during 2016 because of the droughts between 2014 and 2019, decreasing the milk income to feed price ratio in South Africa to a critical level of sustainable income for dairy farmers (Bureau for Food and Agricultural Policy, 2022). International milk prices have still not recovered after the 2014–2016 global price crash (Charles, 2016), and the input costs involved in maintaining the high production propensity of the modern dairy cow while ensuring high reproduction rates and health status are increasing. It is, therefore, increasingly important when formulating diets to control the balance of all nutrients accurately and to optimise them precisely for optimum efficiency. One way that this could be achieved is by finding ways to reduce the wastage of nutrients in the digestive tract through increased digestibility, thereby improving the efficiency of nutrient utilisation. Genetics, cultivar, variety, geographical locations, year, climatic conditions, and agronomic practices all have a direct impact on the nutrient value and digestibility of grains (Huntington, 1997; Offner *et al*., 2003).

In maize, the encapsulation of the starch particles in a bed of hydrophobic proteins called prolamin is the primary factor that affects the digestibility of starch (Larson & Hoffman, 2008; Hoffman & Shaver, 2009). Current practices are based on the fact that when maize is ground into a finer particle size, the prolamin encapsulation is damaged, giving microbes better access to the starch molecule (Owens *et al*., 1986; Beauchemin *et al*., 1994). The analysed starch content of the manure indicates maize digestion (Fredin *et al*., 2014). Feeding finer ground maize leads to manure with a lower starch content and improved digestion (Knowlton *et al*., 1998; Rémond *et al*., 2004; Owens and Soderlund, 2006; Fredin *et al*., 2015).

In addition to production parameters, the type of starch (as defined by the ratio of amylose to amylopectin contained in the endosperm of grains) has a major impact on the digestibility of the grain component of the feed (Huntington *et al*., 2006). Amylose has tighter intermolecular bonds between the starch molecules than amylopectin (Buléon *et al*., 1998) and is much harder

to digest than amylopectin because of its molecular structure (Huntington *et al*., 2006). The digestibility of grains is thus directly altered by the amylose-to-amylopectin ratio of grains (Sajilata *et al*., 2006). *In-vitro* rumen digestibility of maize increases as the amylose content of grains decreases (Stevnebø *et al*., 2006).

Recently, there has been a renewed interest in using nutrient feed additives to improve the efficiency of nutrient use in ruminants. Feed additives such as yeast products, phytonutrients, and calcified marine algae have shown the potential to replace antibiotic ionophores (Jouany & Morgavi, 2007; Tassoul & Shaver, 2009; Khiaosa-Ard & Zebeli, 2013). Moreover, in recent studies, significantly improved general health, reproduction, and production and feed efficiency in dairy cattle were observed (Krehbiel *et al*., 2003; Kung, 2004; AlIbrahim *et al*., 2010; Cruywagen *et al*., 2015; Hutjens, 2016; Julien *et al*., 2017; Oh *et al*., 2018).

The use of feed enzymes is a radical innovation developed over the past 10 years that improves the digestion of nutrients fed to ruminants, resulting in increased performance in ruminants with high nutritional requirements (Jouany & Morgavi, 2007). While most of the research on enzymes focuses on fibre digestion with fibrolytic enzymes, the primary component in rations for high-producing animals is starch, which suggests that finding a way to manipulate starch digestion with enzymes may lead to improved production efficiency (Tricarico *et al*., 2008). Through the years, increased milk production and feed efficiency, improvement of starch digested in the rumen, and enhanced energy balance were found by supplementing with exogenous feed enzymes (Tricarico *et al*., 2005; DeFrain *et al*., 2005; Klingerman *et al*., 2009; Gencoglu *et al*., 2010; Nozière *et al*., 2014; Andreazzi *et al*., 2018). The varying production responses and the cost of the enzymes limit the commercial application of starch enzymes. However, new factors such as the resistance to antibiotics and other growth stimulants, as well as the increasing maize price, necessitates another look at the value and understanding of the specific advantage of the physiological mechanism of enzymes for dairy cows.

Ronozyme® Rumistar is a new addition to the several, already commercialised enzymes. It is, however, a pure amylase selected for ruminal conditions to maximise maize starch utilisation and fibre digestion in early lactation (Nozière *et al*., 2014). This enzyme catalyses the hydrolysis of maize starch to oligosaccharides in the rumen without compromising the rumen pH (Bach, 2011). The produced oligosaccharides can then be used as an energy source by the fibre-degrading microbes (Flint *et al*., 2012); this is also known as ‘cross-feeding’. The

additional energy that is available through the stimulation and action of the fibrolytic microbes reduces the digesting time for fibre, thus increasing the degradation of organic matter in the rumen (Varga & Kolver, 1997; Krause *et al*., 2003)) . Hence, an improvement in the total tract digestibility of the diet is expected, resulting in improved utilisation of the diet's energy potential and leading to better animal production and performance efficiency (Tricarico *et al*., 2007). Faecal starch concentration accurately predicts total tract starch digestibility (TTSD) (Fredin *et al*., 2014).

The two principal sources of indigestibility, the prolamin structure embedding the starch molecules and the amylose-to-amylopectin ratio, affect each other to varying degrees. Results from studies comparing the effect of different ratios of amylose-to-amylopectin on the starch digestibility of maize are affected by the particle size of the grains that are fed (Doorenbos *et al*., 2017). For example, for dry-rolled maize, the combination of fine particles of grain with a floury endosperm, a thin or loose pericarp, and a low amylose-to-amylopectin ratio will maximise starch digestion (Owens & Soderlund, 2006).

If grinding to a smaller particle size improves the primary indigestibility and adding exogenous amylose enzyme improves the secondary indigestibility, combining these two processing measures could potentially have an added positive effect on the starch digestibility of maize. The optimal combination of particle size and amylose enzyme addition should be researched further.

This trial aimed to provide additional insights into two of the most important factors affecting rumen starch disappearance and post-ruminal digestibility of starch in ruminants, the amylose-to-amylopectin ratio and the prolamin structure. This was achieved by assessing ruminal starch degradation values and total tract starch disappearance and monitoring the effects on milk production and milk composition.

The goal of this study was to firstly investigate the effect of the particle size of maize in combination with the addition of a pure exogenous amylose enzyme on the ruminal and total tract digestibility of maize in dairy cows. The second goal was to investigate the effect of the enzyme supplementation on ruminal NDF degradation by considering the following objectives:

1. Production and milk parameters.

2. A faecal analysis as an indicator of TTSD.
3. Starch digestion for 3 different particle size maize and NDF digestion of a finely ground lucerne sample in a seven-hour *in-vitro* study.
4. Ruminal starch degradation curves for 3 different particle size maize and NDF digestion curve of a finely ground lucerne sample using the *in-sacco* method.
5. Rumen fermentation parameters (VFA) for enzyme vs non enzyme treated rumens.

The study was conducted in two phases. In the first phase, an on-farm trial was conducted to investigate the effect of maize particle size and the addition of an exogenous amylase enzyme on milk production parameters and total tract starch digestion. In the second phase, *in-vitro* and *in-sacco* studies were conducted to investigate the effect of treatment on the ruminal parameters of pH change, volatile fatty acid (VFA) production, seven-hour *in-vitro* degradation, and *in-sacco* ruminal starch and neutral detergent fibre (NDF) disappearance over a 24-hour period.

Chapter 2

2. Literature Review

2.1 Introduction

Maize (*Zea mays*) is grown commercially in more than 25 counties. Globally it is the most widely grown cereal crop with an annual production of over a billion tons worldwide, making up nearly 40% of the total grain production of 2.6 billion tons (United States Department of Agriculture [USDA], 2019). The highest South African maize production was in 2017, with 17.5 million tons being recorded (SAGL, 2021). Maize is used mainly for human consumption, animal feed, and ethanol production (Ranum *et al*., 2014). According to the *Biofuels Industrial Strategy of the Republic of South Africa* published by the Department of Minerals and Energy (DME) in December 2007, maize for ethanol production in South Africa will become increasingly critical in the future (DME, 2007).

To satisfy the ever-growing need for food by the exponentially growing global population, the amount of genetic research has led to the development of highly productive modern maize cultivars with much-improved yields. Over the last decade, maize cultivars have been genetically modified to be resistant to drought, diseases, and pests, and these cultivars are used extensively in South Africa (SAASTA, 2014). Genetic modification of maize has increased the production potential of the plants (Pellegrino *et al*., 2018), but according to Owens (2005), the impact on the kernel and specifically on the endosperm morphology needs to be determined to ensure the impact off the modification on digestibility is properly described in nutritional predictions.

Cereal grains are an indispensable component of ruminant feed because they are responsible for the major energy requirements of dairy cows. Starch is the main component of cereal grains that the rumen microbes will break down and ferment into VFA. Rumen microbes have the first opportunity to break down and utilise the starch from the ingesta. The shape, character, and processing of the endosperm are the most critical factors that contribute to the variation in the nutrient availability of maize (Van Zyl, 2017). The ruminal and total tract digestibility of different sources of maize fluctuates extensively depending on the type of maize endosperm,

particle size, gelatinisation by heat processing, conservation method, and other factors (Owens *et al*., 1997).

Intricate interrelations between several factors determine the rate and extent of starch digestion in the rumen. These include the source of the dietary starch, grain type, the structure of the grain kernels, the genotype of the cereal, diet composition, mechanical and chemical processing, degree of adaptation of ruminal microbiota to the diet (Huntington, 1997), and the availability of starch for enzymatic attack. Both animal and grain characteristics influence the fermentation of grain in the rumen and the digestion of starch in the small intestine (Zinn *et al*., 2002; Owens & Soderlund, 2006).

According to the National Research Council (NRC,2001), maize contains 730 g of starch per kg of dry matter (DM), and this supplies 75% of the metabolisable energy (Ranum *et al.*, 2014) of maize, ranging between 12.5 MJ ME/kg DM and 13.5 MJ ME/kg DM. The remaining amounts of nutrients contained in maize is relatively low, with fat ranging between 35 g/kg DM and 40 g/kg DM and protein ranging from 73 g/kg DM to 95 g/kg DM (NRC, 2001). Structural carbohydrates measured by acid detergent fibre (ADF) are as low as 30 g/kg DM to 35 g/kg DM while NDF, ranges between 90 g/kg DM and 95 g/kg DM. Although these values are well established and published, the supply of degradable substrates in the rumen, the rumen function, and the profiles of the microbial protein and fatty acids that are available for metabolism after absorption determine the utilisation of these nutrients by ruminants (Dijkstra *et al*., 2012; Huhtanen & Nousiainen, 2012). Most nutrient requirement models use metabolisable energy (ME), NE_l, and metabolisable protein (MP) values calculated by considering digestibility and estimating rumen fermentation and intestinal total tract digestibility to determine the nutrients that are truly supplied (Tylutki *et al*., 2008; Volden, 2011; CVB, 2012).

2.2 The Structure of Maize Kernels

The understanding of starch digestion starts with an understanding of the structure of cereal grains. Cereal grains consist of a central, tiny embryo or germ surrounded by a thick endosperm layer and a thin pericarp layer. Different morphological structures are clearly distinguished by Van Kempen *et al*. (2003) in nutritional studies with piglets when they compared the percentages of NDF and phosphorus (P) in the various layers of the maize kernels and the

impact on digestibility thereof. The maize endosperm contains <4% NDF and 0.09% P compared with the germ that contains 17% NDF and 0.97% P while the pericarp contains 33% NDF and 0.29% P.

2.2.1 Maize Germ

The germ is the reproductive centre containing the living tissue for the next generation plant and consists of the embryonic axis including the plumule and radicle, the precursors for roots, stem, leaves and grain. (Evers *et al*., 1999). These are surrounded by the nutrition storage and protection layer called the scutellum, which constitutes 15% of the maize kernel (Evers *et al.*, 1999). This layer comprises mainly of, simple soluble sugars, and hormones consisting of protein (Serna-Saldivar, 2010). The germ is the reason for the existence of the maize, and the rest of the seed functions as a protective mechanism for the germ to enhance its chances of germination into the next generation plant (Evers *et al*., 1999). Proper embryo protection from environmental factors (moisture, temperature, seed coverage, and light) is essential until the correct ecological conditions initiate the conditions for annual reproduction. Water absorption starts the germination process by causing the renewal of a particular enzymatic activity that leads to cell division until the embryo breaks through the pericarp (Hoffman & Shaver, 2010). The germ of modern, high-yielding maize cultivars is proportionally more extensive and contains more oil. Owens & Zinn (2005) speculate on whether the additional oil would have a negative effect on the microbial health of the rumen.

2.2.2 Maize Endosperm

The middle layer of the seed, the endosperm, comprises approximately 75%–80% of the total weight of the maize kernel. The endosperm is the most significant component of the kernel. It consists of starch and protein particularly in the peripheral and corneous endosperm (Evers *et al.*, 1999) and supplies nutrients for the growth of the embryonic axis at the subsequent germination of the plant (Evers *et al.,* 1999). Ranum *et al.* (2014) describe cereal grains as the fruits of cultivated grasses that store nutrients for the germinating seed of the next generation of plants.

About 80% of the endosperm consists of starch in the form of microscopic granules (Giuberti *et al*., 2014). The profile of the starch content and the structure is specific to the species. The second-most prominent component in the endosperm is proteins. Proteins in maize kernels are

mainly storage proteins and enzymes that have an impact on grain quality (Sylvester-Bradley & Folkes, 1976; Hoffman & Shaver, 2011). In the natural reproduction cycle of maize, the nutrients in the endosperm are of the correct composition to mobilise nutrition in order to support plant growth after germination (Evers *et al.*, 1999). The biological function of the middle layer or endosperm is to provide the primary source of nutrients for the embryo until the seedling can start producing nutrients for itself. Four explicit layers can be distinguished in the endosperm: the aleurone layer, the peripheral layer, the corneous, and the floury endosperm (Kotarski et al., 1992). The aleurone layer contains enzymes and inhibitors but no starch granules (Huntington, 1997).

Hydrophobic proteins called prolamins protect the starch in the endosperm. Pure starch is highly hydrophilic, and premature hydration of the endosperm would hinder germination (Hoffman *et al.*, 2011). Therefore, starch cannot be efficiently stored in the maize endosperm as pure starch.

Although the endosperm in maize contains almost no structural carbohydrates and has no ADF or NDF as protection, it is still challenging for rumen microbes to attack the kernel for starch fermentation because of the abundant storage proteins in the form of albumins, globulins, prolamins, and glutelins that are found as a protein matrix surrounding the starch granules (Kotarski *et al.*, 1992; McAllister *et al.*, 1993). Once the pericarp layer is breached, the rate of access to the starch granules is governed by the protein matrix and the endosperm cell walls (Kotarski *et al.*, 1992; Corona *et al*, 2006).

Many bacteria that are capable of digesting starch lack β-glucanases and are thus incapable of degrading the endosperm cell wall (Morgavi *et al.*, 2013). These bacteria depend on cellulolytic organisms to penetrate and enable access to the starch granules (Hua *et.al.*, 2022). Lipids, including phospholipids, free fatty acids (Baldwin, 2001; Svihus *et al.*, 2005), and some proteins on the surface of the starch granules play a role in reducing the contact between enzyme and substrate and reducing the extent of granule swelling due to increasing hydrophobicity, thus interfering with its digestion (Guyton, 2002).

The zein content of the vitreous outside region of the maize endosperm is much higher than the opaque, softer inside area (Holding, 2014). Tsai *et al*. (1978) noted how environmental conditions play a role in determining the formation of the vitreous endosperm when they found

that reduced zein synthesis was caused by nitrogen (N) depletion, resulting in soft and starchy kernels throughout.

The final protection that the endosperm provides consists of a complicated structure of polysaccharides, including cellulose, arabinoxylans and β-glucans, proteins, and esterified phenolic acids. According to Hoffman & Shaver (2010), this cannot truly be described as fibre since the term is confusing, being method or function dependent. The fibre fraction of maize comprises the cellulose in the endosperm cell wall, the lignified cell walls of the outer protective pericarp, and the testa contents and cuticles.

2.2.3 Maize Pericarp

The ultimate protective outer layer, the pericarp, is high in pentosans, cellulose, hemicellulose, and other insoluble and indigestible polymers. This layer is essentially the seed coat and primarily protects the seed from pathogens (Hoffmann & Shaver, 2010). The fibre in the pericarp plays a protective, structural, and metabolic role in the cereal kernel. The embryo needs protection from moisture, temperature, insects, and other environmental conditions until the ideal prerequisites for germination exist. When the pericarp of the whole grain is intact, rumen microbes cannot attach to the grain kernel, making it entirely resistant to ruminal microbial fermentation (Eastridge *et al*., 2011).

The energy source is stored in the grain kernels and used as nutrition of the seed embryos. The pericarp is the unpalatable, impenetrable, outer covering that protects this energy source from outside infestations and predation by wild populations (Evers *et al.*, 1999).

2.3 Maize Cultivars

The endosperm quantity and quality, the composition profile of the endosperm, and the physical characteristics of the kernels are used to classify maize into five categories: dent, flint, waxy/floury, popcorn, and sweet (Fox & Manley, 2009). Dent, flint, and waxy maize are used in ruminant nutrition (Van Zyl, 2017).

The kernels of dent or field maize (*Zea mays indentata*) contain hard and soft starch with a vitreous to floury endosperm ratio of 2:1, which causes it to become indented as it matures (Dickerson, 2003). This variety is primarily used commercially for maize starch manufacturing

in addition to food, animal feed, and industrial products. The kernels of flint maize (*Zea mays indurate*) are hard, horny, rounded, short, and flat, with the soft and starchy endosperm entirely enclosed by a hard outer layer (Corona *et al*., 2006; Fox & Manley, 2009).

Waxy maize contains only branched-chain starch (more than 99% amylopectin) compared with the traditional maize varieties that contain 72%–76% amylopectin and 24%–28% amylose. The floury endosperm contains the most amount of starch. However, these starch granules are not bound to a protein matrix (Pérez & Bertoft, 2010), which causes the floury endosperm to be more susceptible to processing effects (Kotarski *et al.*, 1992; Zinn *et al*., 2002). Moreover, not only the genotype and cultivar affect the size of the starch granules and their characteristics but also, environmental factors (e.g. different temperatures, locations, and rainfall) greatly affect both (Panozzo & Eagles, 1998).

2.4 Maize Starch Categories

The starch in maize consists of one of two polymers, amylose or amylopectin; both are made up of α-1-4 linked glucose residues arranged in different patterns (Huntington *et al.*, 2006). Amylose is a single or a double-helical polymer chain that consists of α-D-glucose molecule units (Deckardt *et al*., 2013) that are linearly bound to each other through α-1-4 glycosidic bonds. Amylose chains have a molecular mass of about 105 g/mol (Chen *et al*., 1997) and a degree of polymerisation up to 6 680 d.p. (Hizukuri & Takagi, 1984). There are two known types of amylose, Type A and Type B, and they vary with the placement of the double helices and the water units per cell (Bertoft, 2017).

Amylopectin is a branched polymer with its α-(1→4) linked glucose chains linked together by an α-(1→6) bond at every 20 to 25 glucose residues. Raw amylopectin is more digestible in the rumen than amylose (Iqbal *et al.*, 2009). When the processing of grains for animal feed is limited to dry rolling (Mohd & Wootton, 1984), maize hybrids with proportionally higher amylopectin positively affect feeding.

The strong linear helix structure of amylose causes it to be less favourable for microbial penetration and, therefore, less fermentable than the leaf-like, branched structure of amylopectin (Huntington *et al.*, 2006). The intermolecular bindings between amylose starch molecules are stronger than that of amylopectin, making amylopectin easier to ferment and

amylose more resistant to microbial fermentation in the rumen (Corona *et al.*, 2006). According to Owens & Zinn (2005), the specific amylose content in maize varies from 24% to 30% but can be as low as 2% in particular cultivars. The ratio of amylose to amylopectin content varies between grain cultivars, and according to Kotarski *et al.* (1992), with less than 2% amylose in waxy cultivars and up to 34% amylose in non-waxy cultivars. The genetic makeup of waxy maize cultivars results in the production of amylopectin only (Dickerson, 2003).

2.5 Maize Kernel Protein

Seed storage proteins called prolamins are present in the endosperm of cereal grains such as wheat, barley, rye, sorghum, oats, and maize and supply amino acids for germination and seedling development. The name prolamin is derived from the amino acids proline and glutamine, which are found in high concentrations in the storage proteins of grains (Fox & Manley, 2009). Proline is exceptionally hydrophobic and capable of intricate pleating. Therefore, proteins containing high proline levels develop profoundly hydrophobic tertiary networks by forming a tight cross-linked matrix that encapsulates the starch granules into a hydrophobic capsule. These networks are only soluble in aqueous alcohol solutions (Momany *et al.*, 2006). Prolamin is degraded mainly by fermentation with lactic and acetic acid. The prolamin proteins are associated with starch and have specific scientific and historical names depending on the cereal grain (Hoffman & Shaver, 2010).

Table 2.1: Grain type with the associated prolamin protein and level of encapsulation.

Cereal grain	Scientific name	Level
Wheat	Gliadin	Medium–Low level
Barley	Hordein	Low Level
Rye	Secalin	Medium–Low level
Maize	Zein	High Level
Sorghum	Kafirin	High–Very High Level
Oats	Avenin	Low Level

Source: Hoffman & Shaver (2010)

According to Corona *et al.* (2006), small grains have a lower prolamin content than maize (zeins) or sorghum (kafirin). Zein protein bodies are divided into four groups: alpha, beta,

gamma, and delta (Lending & Larkins, 1989). Temporal and spatial regulation of the zein gene expression, transcription level, and interactions between the different types of zein proteins influence the protein formation in maize (Woo *et al*., 2001; Kim *et al*., 2002). Both zein and kafirin proteins are inherently resistant to digestion by the disulphide cross-linked nature of the γ-prolamins, packing them into protein bodies and forming a shell with a relatively low surface area considering the amount of prolamin that is packaged (Holding, 2014). The surface outside the starch molecules contains the zein proteins. As the grains mature, the different zein types (β-zein and γ-zein) form a cross-linked network with the α-zein and δ-zein, encapsulating the starch molecules (Mu-Forster & Wasserman, 1998). The degree of cross-linking determines the vitreousness of the endosperm, which affects starch degradability (Ngonyamo-Majee *et al*., 2008). The protein (prolamin, zein) matrix in maize resists proteolytic attack. It restricts access to bacterial amylases to encompass starch granules, as opposed to barley whose rapid digestion is facilitated by a diversity of proteolytic bacteria that readily penetrate the protein matrix. The barley endosperm is homogeneous throughout, and starch granules are more loosely associated with the protein matrix (McAllister *et al*., 2006). More than 70% of the endosperm of maize consists of zein, which is classified as a low-quality protein that is severely lacking the essential amino acids, lysine and tryptophan (Mertz *et al*., 1964).

The proteins in sorghum (kafirins) are equally deficient in these essential amino acids but are even less digestible because of the high level of disulphide cross-linking (Aboubacar *et al*., 2001). An excessively tough protein starch matrix is formed by attaching the zein protein bodies to the maize starch granules to develop vitreous maize (Hoffman, 2009). Starch granules also link together when amorphous, non-crystalline amylopectin molecules on the surface interact to fill the spaces between them. The chemical protein bonds in lieu of the starch-protein bonds strengthen the matrix. The starch granules embedded in the zein-protein matrix in vitreous endosperm are much stronger than the starch granules of floury maize endosperm (Gibbon *et al*., 2003). The level of zein present influences the ratio of vitreous to floury endosperm or the vitreousness of maize and affects the hardness of the maize kernel (Delcour & Hoseney, 2010).

The inherent genetic code of a specific cultivar of maize, the environmental conditions, and the maturity level determine the vitreousness of maize kernels (Erasmus, 2003). The vitreousness of maize fundamentally affects rumen digestibility, fermentation, and TTSD (Ngonyamo-Majee *et al.*, 2008).

2.6 Digestibility of Starch in Maize Kernels

Animal species and breed, production and health status, the grain cultivar, gene expression, growth and storage environments, usage in the diet, and processing of the grain kernels all influence the rate and degree of ruminal starch fermentation and the utilisation of the end products of fermentation (Firkins *et al*., 2001; Hoffman & Shaver, 2011). Improvement of ruminal starch fermentation will increase the propionate-to-acetate ratio (Deckardt *et al.*, 2013). However, it can also reduce the rumen pH to the detriment of ruminal fermentation (Rowe *et al*., 1999).

2.6.1 Effect of Animal Species and Breeds

There is a vast difference between the capability and the efficiency of carbohydrate digestion within species and between different species, ranging from poultry with the highest digestion capability of maize at 100% to horses in which even finely ground maize (FM, <3 mm) is poorly digested at 30% (Rowe *et al.*, 1999). According to Black (2008), the available energy in maize depends on the genetic code of the cultivar and the environmental effects; these influence the type and the composition of the endosperm and the maturity level. This cannot be directly correlated with the starch content of the maize. The division of energy required for maintenance and production is a function of the metabolisable energy and all the factors that influence digestibility (Black, 2008). Although sheep and cattle are ruminants, sheep are better digesters of maize in terms of ruminal and total tract digestion because of the anatomical differences in digestive capability and the different sizes of the gastrointestinal tracts. Additionally, sheep can chew grain more effectively and select maize kernels for ruminating, which changes particle flow dynamics through the total tract (Rowe *et al*., 1999) and increases total rumen digestion.

2.6.2 Effect of Genotype

In barley-based diets, the starch digestion in the rumen is more than 30% higher than in maize-based diets (Philippeau *et al*., 1999), which confirms the earlier work of McCarthy *et al.* (1989). Improved ruminal digestion results in improved milk yield in dairy cows. Spicer *et al.* (1986) compared the ruminal starch digestion of sorghum-based diets with maize-based and barley-based diets in beef steers. They found sorghum-based diets to be significantly less

digestible (74%), followed by maize-based diets (84%). Barley-based diets (88%) had the highest digestibility when compared with maize- and sorghum-based diets. The authors concluded that the same factors that increase starch digestibility also influence the digestion of feed protein and microbial protein production. Herrera-Saldana & Huber (1990) compared five cereal grains using *in-vitro* and *in-situ* methods for analysing ruminal starch digestibility to demonstrate the differences between rumen degradation. They listed the cereal grains according to its digestion rates, with oats being the highest, followed by wheat, barley, and maize and lastly, milo. Philippeau *et al.* (1999) found a significant difference in starch digestion between the dent genotypes (with 60.8% digestibility) and the flint genotypes (with 34.8% digestibility). The authors also reported different sites of digestion in ruminants for the different genotypes (Philippeau *et al.*, 1999).

2.6.3 Effect of Vitreousness

The accumulation and packaging of prolamins into the endoplasmic reticulum protein bodies cause vitreous endosperm to form during kernel development. Gibbon *et al.* (2003) scanned amylopectin from different grain cultivars using electron microscopy. They compared the linking pattern of the starch granules and determined how the granules are physically embedded into the prolamin-protein matrix (Figure 2.1). Their results clearly show starch granules heavily embedded in the prolamin-protein matrix in the vitreous endosperm compared with the starch granules in opaque maize endosperm with little and loose embedding by prolamin-protein. Vitreousness can be determined by manual dissection of whole grain kernels and indexed on-farm by laying the matured, halved maize kernels on a background lightbox to compare the translucidity (Hoffman & Shaver, 2011). However, performing this as a regular commercial analysis at a laboratory is not practical. It is also not measurable in ground maize or high-moisture maize. A compounding issue is that the prolamine content is unstable in grains. As the kernel matures, the starch content and the prolamin-protein protection increase (Sniffen & Ward, 2011).

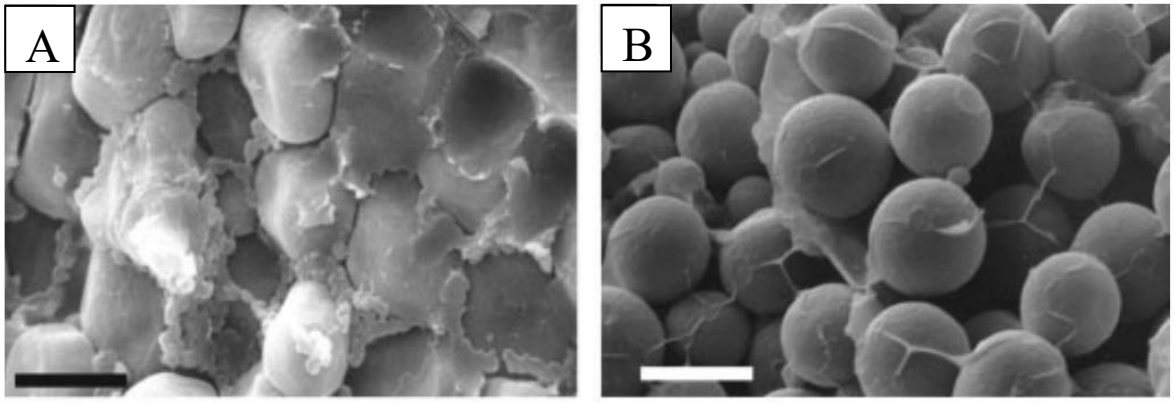

Figure 2.1: Electron microscopy of mature maize kernels to showing the difference between vitreous (A) and opaque (B) maize cultivars in the surface structure of the starch granules and the density of the prolamin in which it is embedded. Source: Gibbon *et al.* (2003).

Ruminal starch degradation and fermentation potential are negatively correlated with the vitreousness of the maize endosperm (Ngonyamo-Majee *et al.*, 2008) because of the encapsulation of starch granules by a strong zein protein-starch matrix that limits the access of rumen microbes to the starch molecules (Gibbon *et al.*, 2003). Ruminal starch and total tract starch disappearance also decrease as maize endosperm vitreousness increases with a decrease in moisture level and progressed maturity (Correa *et al*., 2002; Szasz *et al.*, 2007). Holding (2014) reasons that vitreous endosperm is primarily not an animal food source, but its original function is to supply nutrients for embryonic growth, controlling the growth rate of the embryo during germination and seed development by nourishing and protecting the maize embryo against insect and fungal attack and drying before harvest.

2.6.3.1 Factors Affecting Vitreousness

The Vitality of the Plant

The N fertility status of the maize crop during the growing season influences the prolamin content of the grain. A lack of moisture and excessive rainfall during pollination affects the prolamin content of maize (Hoffman & Shaver, 2011). Poor N status leads to reduced yield, whether from N leaching or denitrification. According to Hoffman & Shaver (2011), maize kernels from standard hybrids grown under poor environmental conditions such as excessive

rainfall and thus growing in an N-deficient environment would be more opaque and contain only half of the expected average prolamin concentration.

Preservation of Maize Kernels

Improved starch digestion in high-moisture maize or maize silage depends on the degradation of prolamins through fermentation (Hoffman & Shaver, 2011). The longer the maize is subjected to ensiling conditions and the higher the intensity of the fermentation, the more prolamin degradation will occur, leading to improved starch digestion. Soluble protein and ammonia concentrations are markers of prolamin degradation in high-moisture maize (Hoffman, 2009).

Processing Effects

Processing methods include all modifications, whether physical processes such as rolling, breaking, cracking, and grinding of grains or chemical processes involving water, heat, and pressure (Nocek & Tamminga, 1991). Even when there are no genetic or chemical differences between the grains, starch digestion can differ because the fermentation potential of grain is related to the mean particle size of the grain (Hoffman *et al*., 2012). According to Blasel *et al*. (2006), for every particle size enlargement of 100 µm in milled grains, starch access by α-amylase will decline by 26.8 g/kg of starch. McAllister *et al.* (1993) found that grinding maize and barley from an average particle size of 3 mm to a particle size of 2 mm increased starch digestion in the rumen for both grain types. Yang *et al*. (2001) reported an increase in ruminal starch digestion of 12.3% when they fed eight lactating dairy cows fine-rolled barley instead of coarse grain barley. Callison *et al*. (2001) compared the ruminal digestibility of starch for maize ground into three different particle sizes: coarse at 4.9 mm, medium at 2.6 mm, and finely ground at 1.2 mm. They reported a positive quadratic effect from 49.8% to 87% on true ruminal degradability as the particle size decreased.

Steam flaking allows the prolamin-protein matrix to degrade through the application of heat, moisture, and mechanical action, making the starch more available for ruminal fermentation. Huntington (1997) compared dry-rolled and steam-processed maize and sorghum diets. The author clearly showed that digestibility increased from 75% to 85% with steam-flaked maize and from 52% to 78% with steam-flaked sorghum. In addition, Zinn *et al.* (2002) demonstrated that processing steam-flaked grains further to achieve a smaller flake thickness or density improved the performance of feedlot cattle by 18% (much higher than the NRC tabulated

values). The prolamin-protein matrix embedding the starch limits starch digestion, and if the flaking process disrupts this protein matrix, it devalues itself.

Quality control factors such as steam-chest temperature, steaming time, roll groove, roll gap, and roll tension are critical during the flaking process to ensure sufficient flaking is achieved to improve starch digestion (Sindt *et al*., 2006). Other quality standards for steam-flaked maize include the rate of flaking and the distribution of kernels across the rolls (Gutierrez *et al*., 2017). Flake thickness, flake density, starch solubility, and enzyme reactivity must be measured and controlled (Zinn *et al*., 2002).

2.7 Digestion of Maize

2.7.1 Ruminal Effects

Carbohydrates and protein are the main components that are degraded and fermented in the rumen. The carbohydrate fraction consists of sugar, starch, pectin, and NDF, each with different features, distinct ruminal degradation patterns, and fermentation characteristics. Total VFA production depends on the intake of rumen fermentable organic matter (Dieho *et al*, 2016)However, each fraction will result in a different VFA profile being produced by the rumen microbes (Nozière *et al*., 2011). The VFA ratio of acetate, propionate, and butyrate produced by rumen microbes affects milk production and composition. These are influenced by diet composition or through the interconversions between the VFAs (Sutton *et al*., 2003).

The effects on milk production and composition are evident for every carbohydrate fraction, even when the energy supply is constant. Any change in the starch source alter the VFA production ratio, intestinal digestibility, and other metabolic responses, although this does not necessarily result in a direct milk production response (Lechartier & Peyraud, 2011; Ferraretto *et al*., 2013; Piccioli-Cappelli *et al*., 2014). For lactating dairy cows, the energy supply is determined by how much the starch can be fermented and used by the rumen microbes to maximise microbial protein (Hall & Herejk, 2001; Andreazzi *et al*., 2018).

Ruminal pH is critical in enhancing the microbial population and in improving ruminal digestion. (Castillo-González *et al*., 2014; Li & Hanigan, 2020). It is controlled primarily by the buffering effects of saliva production, absorption of the VFAs produced by the rumen microbes, and the evacuation of digesta to the lower tract (Dieho *et al*., 2016; Castillo- López

et al., 2020). Increased carbohydrate supply to the rumen microbes will increase fermentation and VFA production. (Dijkstra *et al*., 2012). The additional VFAs produced in the rumen must be compensated for by additional buffering and increased VFA absorption or they will result in a variable or reduced ruminal pH, leading to an unhealthy rumen environment (Deckardt *et al*., 2013; Dieho *et al*., 2016) This will reduce ruminal microbe numbers, which leads to excessive carbohydrates, causing even more VFA production and the onset of sub-acute ruminal acidosis (SARA). Therefore, a reduction of ruminal pH will decrease milk production (Dijkstra *et al*., 2012).

Fractional passage and degradation rate are just as crucial for healthy ruminal degradation as the supply of degradable carbohydrates (Van Duinkerken *et al*., 2011). The fractional degradation rate (kd) refers to the fraction of the substrate degraded per unit of time. According to Volden (2011), using this value directly in linear programming for feed formulation is impossible because passage and degradation rate also depends on processing and cow and management factors. In a study by Doorenbos *et al*. (2017), degradation rates and other characteristics of the carbohydrate components were assigned to feedstuffs and called modelled rapidly degradable carbohydrates (mRDC) and modelled total degradable carbohydrates (mTDC). These nutrients effectively consider the total carbohydrate supply and the degradation rate while still formulating a diet with linear programming. Changes in mRDC resulted in responses in milk production, while fat-protein-corrected milk and butterfat were influenced quadratically. Milk protein increased linearly by increasing the MrdC in formulation (Doorenbos *et al.*, 2017).

2.7.2 Volatile Fatty Acid Production

Bird *et al*. (1999) tested the relationship between particle size and grain type in barley as measured by the fermentation rate. With an increased particle size from 0.5 mm to 4 mm, the total acid production decreased from 79 mmol to 41 mmol, the lactic acid production declined from 44 mmol to 3 mmol, and starch digestion was reduced from 79% to 46% (Bird *et al*., 1999). These effects were less pronounced in sorghum and oats. Lactic acid was not produced when sorghum was fed, and only a moderate decline in lactic acid production was found for oats (Bird *et al*., 1999). It follows that the rate of starch digestion of barley in the rumen can be controlled with particle size, but particle size has a negligible effect on the digestion of sorghum and oat starch (Bird *et al*., 1999).

Another categorisation of starch is rumen-resistant and non-rumen-resistant starch. Rumen-resistant starch is not fermented by rumen microbes but digested by amylase enzymes secreted by the pancreas in the small intestine or degraded in the caecum (Deckardt *et al.*, 2013). Products produced in the caecum are mostly lost to the animal and will often lead to hindgut acidosis (Huntington *et al.*, 2006; Gressley *et al.*, 2011). Rumen microbes ferment non-rumen-resistant starch by producing VFAs (Deckardt *et al.*, 2013). Proportional ratios of the VFAs change according to diet composition (Brandao & Faciola, 2019).

Starch digestion depends on ruminal amylolytic enzymes to break through the surface area, hydrolysing the starch molecules (Gibbens, 2014). Starch granules typically have too high a molecular weight for bacteria to ingest (Hua *et al*., 2022). Therefore, bacteria need to generate enzymes to cleave the α-1,4 bonds of amylose or α-1,6 bonds of amylopectin (Hua *et al*., 2022). Amylases are categorised into three groups according to their hydrolytic activity. Endoamylases split the α-1,4 bond randomly in the interior of the starch-producing oligosaccharides. Exoamylases cleave the α-1,4 bond at the starch molecule's non-reducing end, forming dextrins. Glucoamylases, Isoamylases and Pullulanases are categorised in the debranching enzyme group. (Hua *et al*., 2022). The two major enzymes involved in the breakdown of starch are α-amylase and β-amylase. Alpha-amylase detaches molecules from amylose and amylopectin, whereas β-amylase only cleaves molecules from the ends of the chains. Therefore, the functioning of the enzymes is limited to the surface of amylopectin (Cerrilla & Martínez, 2003).

Rumen microbes are specific in the substrate that they ferment and the type of VFA that they produce. Many rumen microbes, including *Bacteroides amylophilus, Bacteroides ruminocola, Butyrivibrio fibrisolvens, Selenomona lactylitica, Prevotella ruminocola, Streptococcus bovis, Eubacterium ruminantium, Ruminococcus bromii, Ruminobacter amylophilus, Succinimonas amylolytica,* and *Lactobacillus spp,* produce propiolytic enzymes, but *Ruminobacter amylophilus* and *Streptococcus bovis* produce amylolytic enzymes. These bacteria are the primary starch fermenters, while some fungi and protozoa are also involved. Increasing the non-rumen-resistant starch inflates propionate production relative to acetate and butyrate production and reduces ruminal pH when these products are not absorbed as produced (Fredin *et al*., 2015).

Numerous methods to determine the influence of endosperm characteristics on the nutrient value were evaluated. Fox & Manley (2009) compared particle size indexes with near-infrared technology (NIR), and Guelpa *et al*. (2015) describe a calibration procedure using X-ray microcomputed tomography (uCT) for differentiating vitreousness, hardness, and density for a range of maize cultivars and could classify them accordingly, with 88% accuracy.

2.7.3 Rumen Metabolic Health

Ruminants fed high-concentrate diets with a reduced particle size of the grain through fine grinding can develop metabolic disorders (Owens, 2005). To increase the extent of starch digestion of grains fed to ruminants, the author recommends other processes such as steamrolling, steam flaking, and fermentation (high moisture storage) rather than feeding FM. Furthermore, Owens (2005) suggests that grain characteristics should dictate the processing method. Steam flaking to a thinner density will increase starch digestibility in the rumen. In a study by Zinn *et al.* (2002), when the density of the steam-flaked fell below 0.31 kg/l, the dry matter intake (DMI) of feedlot steers was reduced without increasing starch digestion, which increased the variability of weight gain among animals within a pen and predisposed the cattle to acidosis and bloat. The authors recommended optimising the steam-flaking process based on faecal starch analysis (Zinn *et al.*, 2002). According to Owens & Soderlund (2006), ruminal starch digestion in lactating cows is maximised by thinner flaked maize because of the shorter ruminal retention time and TTSD in lactating cows compared with finishing cattle.

Owens & Soderlund (2006) indicate that a combination of very fine grain with a floury endosperm, a thin or loose pericarp, and a low amylose-to-amylopectin ratio is needed to maximise starch digestion for whole and dry-rolled maize. These processing methods will increase starch digestion in the rumen or the small intestine or in both locations.

2.7.4 Disruption of the Metabolic System

According to Owens *et al*. (1998), ingesting a large amount of readily fermentable carbohydrates leads to the accumulation of lactic acid in the rumen. Some bacteria will change their fermentation products from acetate to lactate in the presence of these readily available sources of carbohydrates as the rate of glycolysis increases (Russell & Diez-Gonzalez, 1998). An *in-vitro* measurement of the lactic acid content, which correlates positively with total VFA

production, could supply a sensitive index of fermentation rate. A positive correlation was found when this was applied to barley, wheat, oats, and lupins (Bird *et al*., 1999)

2.7.5 Digestion in the Small Intestine

Microbes cannot attach to the pericarp of whole maize kernels, thus making them resistant to ruminal fermentation (Eastridge *et al.*, 2011). Increasing the rumen-resistant starch in the diet of animals causes an upsurge of starch presence in the lower digestion sites of the GIT (Owens & Soderlund, 2006). The abomasum of a ruminant is subjected to a continual influx and outflow of digesta, and hydrochloric acid is produced to maintain a constant pH of 2.1 to 2.2 (Constable *et al*., 2006). In addition to producing digestive enzymes such as pepsin, and depending on the abomasal outflow rate, the abomasum also receives pancreatic lipase and amylase secreted by the pancreas. Starch in the abomasum and duodenum are hydrolysed by α-amylase and β-amylase, both of which are secreted by the pancreas, resulting in maltotriose composed of (4-8) glucose moieties and still containing the α-(1-6)-linkages. These linkages are impossible for amylases to hydrolyse since debranching enzymes are required to break the bonds (Cerilla & Martínez, 2003).

The duodenum and the small intestine operate as active nutrient absorption sites (Deckardt *et al*., 2013). As digesta travels down the GIT from the abomasum, the pH is raised from 2.2 to 7.5 by pancreatic and liver secretions in the duodenum, activating duodenal enzymes to function. Bile secretions from the gall bladder also improve digestion. More energy is lost digesting starch in the rumen due to methane and heat loss than digestion in the small intestine, making digestion in the small intestine more efficient (Owens & Soderlund, 2006). However, the amount of starch digested in the small intestine is limited. According to Owens & Soderlund (2006), when all processing methods are averaged, starch digestibility in the small intestine declines when the quantity of starch that enters the small intestine increases. However, when considering different processing methods, starch digestion remains proportional to the entry rate (Giuberti *et al*., 2014).

The movement towards bypassing nutrients by the rumen to save on energy costs was challenged in a literature review by Cabrita *et al*. (2006), showing more rumen degradable starch availability and increased microbial N supply. Dairy cows can improve milk production through increased metabolisable nutrient supply, including microbial protein synthesis in the

rumen when the available ruminal energy content of the diet increases (Gozho & Mutsvangwa, 2007). Cerilla & Martínez (2003) state that although intestinal starch digestion is similar in ruminant and monogastric animals, there is still inconsistency in the effectiveness of intestinal starch utilisation. The effectiveness of starch utilisation is controlled through the reduction of amylase secretion and activity by GIT hormones, depending on the starch hydrolysate in the duodenum (Kreikemeier *et al*., 1990). Another controlling factor that Fushiki & Iwai (1989) investigated is that protein in the duodenum stimulates the pancreas to secrete protease-sensitive cholecystokinin-releasing peptide, which acts as a monitor peptide. Thus, because of this negative feedback control, protein in the diet may also control starch digestion in the duodenum.

2.7.6 The Effect of Ruminal Conditions on Maize Starch Digestion

The primary starch-digesting bacteria in the rumen are *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola, Butyrivibro fibrisolvens, Succinomonas amylolytica,* and *Selenomonas ruminantium*. These bacteria can all digest starch, but none can produce the total number of enzymes needed to digest the complete grain kernel (McAllister *et al*., 2001). Bacterial species must form a complementary team to complete starch digestion in the correct sequence. This sequence starts the attraction and attachment of amylolytic bacteria to the grain kernel surface (McAllister *et al*., 1994). These bacteria then multiply to produce enough digestive enzymes to activate nutrients and create digestive sinkholes in the surface area of the starch granules. This attracts secondary colonisers, which populate the area. According to McAllister *et al.* (2001), grain processing and particle size can change this sequential development, thereby influencing the rate and the extent of digestion in the rumen. Simerlar to an increase in the rumen population increases starch digestibility, an increase in available starch changes the rumen environment. An increased starch fermentation rate, due to processing, the grain type, or the concentration of the starch fraction, will result in a decline in the rumen pH (Zinn *et al*., 1995; Philippeau *et al.*, 1999; Yang *et al.*, 2001).

When steam-flaked maize and sorghum diets were compared with dry-rolled grain diets for Holstein cows in studies by Plascencia & Zinn (1996), Joy *et al*., (1997), and Crocker *et al.* (1998), a higher ruminal propionate concentration and a lower acetate-to-propionate ratio were consistently found with steam-flaked diets. The study of Dhiman *et al*. (2002) confirmed this result when the rumen microbial production in Holstein cows fed a diet with steam-flaked

maize decreased the acetate-to-propionate ratio compared with the diet with CM (coarsely ground maize, >3 mm) or FM.

2.7.7 Other Microorganisms

Although rumen bacterial populations are well documented, the rest of the rumen microbial ecosystem is much less characterised. The rumen contains a complex mixture of archaea, bacteria, protozoa, and fungi to break down plant material efficiently and contribute to bacterial fermentation, converting roughage into metabolites and nutrients for the ruminant (Hagen *et al*., 2021). Jouany & Ushida (1999) state that holotrich and ectodiniomorphid protozoa can degrade starch. It is presumed that protozoa account for up to 50% of the starch digestion in the rumen. The engulfment rate for protozoa is inversely related to the size of the engulfed particle, resulting in a higher digestion rate of small starch granules than large maize particles (Fondevila & Dehority, 2001). The primary function of protozoa is that they are predators of amylolytic bacteria, engulfing and digesting starch-digesting bacteria at a rate of 130 – 21 200 bacteria/protozoa/hr at bacterial densities of 10^9 cells/ml rumen fluid (Russell & Hespell, 1981), thereby reducing the amylolytic bacteria population in the rumen and modulating the rumen pH to a higher level (Ushida *et al*., 1991). The diet dictates the numbers of protozoa in the rumen, with the numbers increasing when the grain is introduced into a forage-based diet (Hristov *et al*., 2001) but decreasing when very high grain levels are included in the diet (Mendoza *et al*., 1999). The proliferation of Entodiniomorphid protozoa will prevent a pH reduction in the rumen by actively metabolising lactic acid (Ushida *et al*., 1991). The reduction of protozoa numbers exacerbates a low rumen pH, increasing the risk of acidosis (Brossard et al., 2004). Certain fungi such as *Neocallimastix frontalis* not only produce amylolytic enzymes but also possess additional hyphae with which they exert a physical force to penetrate fibrous plant structures such as the grain pericarp, enhancing bacterial penetration and therefore improving starch degradation. (McAllister *et al*., 2001).

2.7.8 Availability of Starch for Enzymatic Attack

The processing of grain disrupts the prolamin protein in which the starch granules of the grain are embedded, allowing these starch granules to be available for rumen fermentation and digestion (Kotarski *et al*., 1992; McAllister *et al*., 1993; Owens *et al*., 1986). Tóthi (2003), determined that dairy cows fed on a diet with steam-flaked maize are inclined towards a smaller rumen starch pool size than cows fed on a diet in which the maize fraction is dry ground because

of a lower DMI, and discussed the inconsistent effects of steam flaking on the DMI reported by researchers. Chen *et al*. (1994) found steam-flaking increased DMI, but Oliviera *et al*. (1993) recorded a reduced DMI. Knowlton *et al*. (1996) also found significantly lower rumen DM content, starch, and NDF pool sizes in cows that were fed on diets containing ground maize than in cows fed diets containing only cracked maize. An increase in the passage rate can cause the digesta from FM diets and the steam-flaked diets to escape rumen fermentation quicker than the digesta from comparable diets (Knowlton *et al*., 1996). According to McKinney (2006), the advantage achieved by processing should be offset by the equipment and running costs, including labour costs.

2.7.9 Rumen pH and Microbial Products

The processing methods of the grains fed to the ruminants determine the rumen characteristics, and these affect the composition of rumen microbes and VFA production (Ren *et al*. 2019). Ruminal concentrations of acetate decline, and the acetate-to-propionate ratio is reduced for cows fed on steam-flaked maize compared with cows fed on diets with dry ground maize. (Corona *et al*., 2005). However, recent research by Malekkhahi *et al*. in 2020 found the opposite. In their study, an increase in the acetate-to-propionate ratio with steam-flaked versus dry-rolled maize was found, and the ratio declined when super-conditioned maize was fed to cows. Ahmadi *et al*. (2020) reported reduced propionate production and total VFA concentration when the grain source was changed from ground maize to steam-flaked maize. They found no acetate and butyrate concentration changes or acetate-to-propionate ratio fluctuation. The form of the starch source did not affect the ruminal concentration of isobutyrate, isovalerate, valerate, or total VFA (Ahmadi *et al*., 2020). In other studies, steam-flaked maize consistently increased rumen propionate production and lowered the acetate-to-propionate ratio in Holstein cows (Oliveira *et al*., 1993; Plascencia & Zinn, 1996; Joy *et al*., 1997). Crocker *et al*. (1998) also reported elevated butyrate levels in the steam-flake diets. None of these studies reported any significant effects on rumen pH, suggesting that the buffering capacity of steam-flaked diets is sufficient.

2.7.10 Caecal Fermentation

While the rate and extent of fermentation and the production of VFA in the rumen and caecum are identical, the caecum should not be liable for more than 5%–10% of the total tract digestion

(Gressley *et al*, 2011). Caecal epithelium absorbs the VFAs produced by caecal microbes (Gressley, 2011).

Hindgut acidosis results from an increased production of short-chain fatty acids, including lactic acid. It occurs when the absorption capacity is exceeded by an exaggerated flow from the small intestine due to animal stress, health considerations (including SARA), and environmental or dietary factors (Shabi *et al*., 1999). Usually, high-producing animals are more susceptible to hindgut acidosis caused by the feeding of denser, high-grain diets rather than low-density, high-roughage diets (Plaizier *et al*., 2008). These high-density, high-grain diets often lead to SARA, which accelerates the post-ruminal flow of fermentable carbohydrates (Plaizier *et al*., 2008). Additionally, decreased intestinal pH causes damage to the epithelium, which can clearly be seen by mucin casts in the faeces of dairy cows (Gressley *et al*., 2011). This damage to the epithelium layer generates a break in the barrier protecting the animal from external elements in the digesta, which can lead to the occurrence of liver abscesses and laminitis (Meissner *et al*., 2017).

2.7.11 Dry Matter Intake

Elevated starch digestion in the rumen leads to a decrease in DMI in ruminants (Brake & Swanson, 2018). The fermentation process produces VFA and lactate, reducing the pH to the detriment of the cellulolytic microbes and lowering fibre digestion and DMI restriction, leading to decreased fibre digestibility and DMI (Tóthi, 2003). This is the ideal breeding ground for the metabolic disorders of acute ruminal acidosis (ARA) and SARA, rumen inflammation leading to leaky gut, laminitis, liver abscesses, and polyencephalomalacia (Plaizier *et al*., 2009). Ruminal acidosis increases as the starch level and digestion rate increase (Callison *et al*., 2001). Compared with steers fed on less digestible, dry-rolled maize, depressed DMI was reported in steers fed on steam-flaked maize (Owens *et al*., 1997; Barajas & Zinn, 1998). However, Knowlton *et al*. (1998) saw an increase of 1.2 kg in DMI (from 21.3 kg to 22.5 kg per cow per day) with both dry and high-moisture maize fed in ground form rather than rolled form. They also found an improvement in total tract starch digestion when the maize component was ground into a smaller particle size (Knowlton *et al*., 1998).

Increasing the ruminant feed intake has been reported to decrease ruminal starch digestion. Russell & Hespell (1981) found that increasing the maize intake of steers by 1.9 kg from 8.7 kg

to 10.6 kg decreased ruminal starch digestion. However, this was contradicted by other studies, which demonstrated that restricting feed intake did not alter starch digestibility in sheep (Hart & Glimp, 1991; Hatfield *et al*., 1993) and in steers (Murphy *et al*., 1994; Zinn *et al.*, 1995).

2.7.12 Exogenous Amylases for Dairy Cows

Commercial enzymes utilized in the livestock feed industry are typically derived from microbial fermentation. The manufacturing process involves a batch fermentation method, starting with the inoculation of a seed culture into a growth medium (Beauchemin *et al*., 2003). When the fermentation is complete, the enzyme protein is separated from the source organism as well as from the fermentation residues. The fermentation extract is then concentrated and purified, to produce commercial enzyme products with precise control over their enzymatic activities. Enzyme supplements designed for ruminant diets are predominantly sourced from ligninolytic fungi, particularly *T. longibrachiatum*, *A. niger*, and *A. oryzae*, as well as bacteria, mainly belonging to the *Bacillus* genus (Carrillo-Díaz *et al*., 2022) to increase the availability of cell wall carbohydrates. This starts by stripping away lignin and/or hemicellulose, which allows cellulose to be accessible to cellulolytic enzymes that enables the breakdown of the polysaccharide fraction of NDF into fermentable sugars, particularly glucose, through hydrolysis. Types and activities of enzymes vary widely, based on the strain selected, growth substrate, and culture conditions even when the source organisms may be similar (Beauchemin *et al*., 2003). The impact, however, of incorporating cellulases and xylanases on ruminant performance varies significantly due to numerous external factors. Despite the abundant literature on the utilization and mechanisms of exogenous fibrolytic enzymes in ruminants (Beauchemin *et al*., 2003), current research suggests variations in their effects on different components of the diet.

Weiss *et al*., 2011 examined whether exogenous enzymes could offer a potential substitute for the extensive mechanical processing of maize. The inclusion of exogenous enzymes with fibrolytic properties into dairy cow diets has been shown to improve the digestibility of DM, NDF, and CP (Bowman *et al.*, 2002). Weiss *et al*. (2011) listed studies where TTSD was increased by the addition of a number of exogenous amylases to ruminant diets (Klingerman *et al*., 2009; Gencoglu *et al.*, 2010) as well as studies where TTSD was not affected (Hristov *et al*., 2008; Klingerman *et al*., 2009). In their study, Weiss *et al*. (2011) concluded that supplementing a concentrate feed with the particle size of the maize fraction ground coarsely,

with amylase did not alter milk production or the digestibility of energy or starch. However, in line with previous research, NDF digestibility was improved. According to Noziere *et al*. (2014), improvement of the OM digestibility is sometimes linked with improved NDF digestibility, without any notable impact on starch digestibility (Gado *et al*., 2009; Klingerman *et al*., 2009; Gencoglu *et al*., 2010) or true ruminal OM digestibility (Hristov *et al*., 2008).

Klingerman *et al*. (2009) pointed out that while ruminal starch digestion is typically not considered a limiting factor, there is both *in-vitro* and *in-vivo* evidence indicating that animal performance and efficiency might be improved by addition of amylase enzymes. An increase in the digestibility of starch in the rumen can be achieved by supplementing an exogenous amylase, providing stability in the rumen fluid (Klingerman *et al.*, 2009; Nozière *et al.*, 2014). In an *in-vitro* study, Rojo-Rubio *et al*. (2001) found that an amylase derived from *Bacillus licheniformis*, increased ruminal digestion of starch from sorghum and maize. Similarly, Mora-Jaimes *et al*. (2002) observed improved ruminal starch digestion in steers with α-amylase from *B. licheniformis* treated sorghum, achieving 82.85% digestion, as well as with glucoamylase from *A. niger* treated sorghum, achieving 87.23% digestion, compared to 75.13% digestion with untreated feed. The consequent higher availability of starch in the rumen elevates the ruminal microbial yield. This results in improved feed efficiency through intake regulation induced by the increased liver oxidation of propionate (Allen *et al*., 2009).

Despite this, the impact of supplemental exogenous amylase on DMI, milk yield, milk solids, and production efficiency has been inconsistent (Engstrom, 2013; Nozière *et al*., 2014; Tóth & Tóthi, 2016; Andreazzi *et al.*, 2018). Hristov *et al.* (2008) concluded that some exogenous enzymes resist ruminal degradation, thus maintaining their advantage in the lower GIT. Klingerman *et al*. (2009) fed exogenous enzymes in diets with standard starch content and observed a higher milk yield and positive effects on digestibility. When exogenous enzymes were fed to animals on a low-starch diet, increased total tract digestibility, lower DMI, and improved fat-corrected, solids-corrected, and energy-corrected milk feed conversions (kg/kg of DMI) were reported (Gencoglu *et al*., 2010). Improving feed conversion on lower starch diets for dairy cows has real potential for economic viability (Ferraretto *et al*., 2011). However, increased starch degradation in the rumen, can inadvertently trigger ruminal acidosis, resulting in decreased rumen microbe production which will reduce the secretion of milk solids and milk production (Oba and Allen, 2003). The type and processing of starch in the diet will impact the effect of enzymatic treatment on ruminal starch digestibility. Oba and Allen (2003) noted that

the improvement in ruminal starch digestibility was 24.2 and 12.6 percentage units when high-moisture maize replaced finely ground mature maize in diets with high (32%) and low (21%) starch content, respectively, showing a limited capacity of the rumen to digest more resistant starch sources. The addition of amylase to flint maize increased VFA accumulation after 6 hours of ruminal *in vitro* fermentation, while it had no effect on floury maize (Klingerman *et al*., 2009). Moreover, exogenous amylase supplementation has been found to enhance total-tract digestibility of NDF (Gencoglu *et al*., 2010; Weiss *et al*., 2011), likely due to increased availability of starch hydrolysis products to fiber-digesting bacteria in the rumen (Tricarico *et al*., 2008).

Andreazzi *et al.* (2018) propose a hypothetical mode of action for amylase that suggests that the production of oligosaccharides from amylose and amylopectin from low amylase levels stimulates the cross-feeding mechanisms of some rumen bacteria. The authors did not detect a change in the VFA profile in the rumen of enzyme-supplemented cows. Their studies showed that *Selenomonas ruminantium, Megasphaera elsdenii*, and *Butyrivibrio fibrisolvens* grew poorly on starch but grew rapidly when an amylase was added to the starch-containing medium (Andreazzi *et al*., 2018). The release of maltodextrins (a breakdown product of native starch) by the enzyme was used for growth of these bacteria (Klingerman *et al.*, 2009). Nozière *et al*. (2014) also observed a change in the VFA profile to a decreased acetate in relation with increased propionate, isovalerate, valerate, and caproate, when diets with higher than 30% starch levels were fed. In contrast, high enzyme doses extensively digest starch to disaccharides and monosaccharides, not participating in similar cross-feeding mechanisms. Some enzyme preparations contain additional substances such as *S. cerevisiae* fermentation solubles, which may contribute to the responses (Klingerman *et al*., 2009).

Ronozyme® Rumistar 600 (CT) (hereafter referred to as Rumistar) is a new, commercially available enzyme that is unique in its mode of action, which stimulates the development and absorptive capacity of the rumen epithelium by increasing the molar proportion of butyrate in the rumen (Tricarico *et al*., 2005). Rumistar catalyses the hydrolysis of maize starch to oligosaccharides in the rumen without compromising the pH. Oligosaccharides can be used as an energy source by the fibre-degrading microbes; this is also known as ‘cross-feeding’ (Engstrom, 2013). When the degradation of organic matter in the rumen, is increased, an overall improvement of the total tract digestibility of the ration would be achieved (Nozière *et*

al., 2014). Higher total tract OM digestibility exploits the energy potential of the diet and leads to better animal performance (Klingerman *et al.*, 2009).

The primary determinants of starch digestibility, in order of importance, are (1) particle size, (2) the virtuousness (prolamin content) of the dry grain kernel, and (3) moisture and length of ensiling time for fermented maize (Oba & Kammes-Main, 2022). The following chapter describes the materials and methods used in the study.

Chapter 3

3. Materials and Methods

3.1 Introduction

The study was conducted in two phases. The purpose of the first phase was to investigate the effect of the particle size of the maize component in the diet of dairy cows in combination with the addition of an exogenous enzyme (Rumistar) on TTSD, milk production, and milk composition. To achieve this, an on-farm trial was carried out over a period of three months, commencing in April 2017 and with the first adaptation period ending in June 2017.

The second phase of the trial was comprised of *in-sacco* and *in-vitro* trials that were conducted over a three-month period commencing in August 2017 and ending at the end of October 2017. During the second phase, ruminal pH changes were investigated in addition to seven-hour *in-vitro* digestion, *in-sacco* ruminal starch, NDF disappearance over a 24-hour period, and the VFA of rumens with and without an addition of anan exogenous enzyme.

3.2 Experimental Phase One

Phase One was conducted on the commercial dairy farm of Job Legemaat (Rayton, Pretoria). The farm is situated on a plateau of extensive grasslands at an altitude of 1 423 m above sea level, and the herd averages 35 L of milk per cow per day. This experiment was divided into three periods, and the diet for each period contained maize that had been ground to a different particle size, FM, CM and MMM (micro-milled maize, <1 mm). The cows were divided into a control group and a treatment group for each of the three periods. The diets of both groups were the same except an exogenous amylase enzyme was added to the diet of the treatment group.

The following parameters were measured:

- Daily milk production per cow.
- Milk-composition parameters for individual cows on a weekly basis.
- Weekly NASCO sieve analysis on a constituted manure sample for each treatment group.
- Individual manure starch and NDF content.

Because of facility constraints and cow numbers, it was not possible to adopt a standardised experimental design such as a randomised complete block design. A meta-analysis approach was followed to address the effect of time, synthesising a single data group from the results of the experiments conducted in the three separate studies (Barker *et al*., 2021).

3.2.1 Experimental Animals

Two groups of cows were used, each comprising of 20 early-lactation, high-producing, multiparous Holstein cows that were milked three times daily in a single-row, rapid-exit parlour. According to NRC standards, the 70 lactating cows were fed a typical total mixed ration (TMR) formulated for a milk production of 40 L per cow per day (NRC, 2001). The TMR was fed for *ad-lib* consumption, and water was freely available. The cows were randomly divided into two groups to form a control group and a treatment group, each comprising 20 cows. The cows for each treatment group were blocked by lactation number, 305-day mature milk production, and days in milk.

3.2.2 Experimental Diets and Treatments

A granular amylase formulation (Rumistar) with an amylase activity of 600 Kilo Novo Units (KNU) per gram was provided by DSM Nutritional Products (Basel, Switzerland) and Novozymes (Bagsvaerd, Denmark). Rumistar is stable in concentrate, grain mixes, and premixes. It shows good stability in pelleted concentrate (up to 85°C pelleting temperature). The recommended dose is 300 KNU Rumistar/kg DMI.

When feeding a TMR, the amylase should be mixed into the concentrate portion, grain mix, mineral mix, or premix before mixing with forage in order to allow homogenous distribution in the TMR. Rumistar can also be fed in a concentrate via an automatic feeder (transponder feeding).

During all three rounds, the control groups received a commercial 18% protein-concentrated dairy feed. This was mixed on-farm to a TMR, as shown in Table 3.1 and Table 3.2. The treatment groups received the same diet supplemented with Rumistar at 12.5 g per cow per day as a part of a specifically manufactured premix. The chemical composition of the commercial

18% protein-concentrated dairy feed and the TMR mixed on the farm listed in Table 3.2 was calculated and verified weekly, with quality control NIR analysis.

Table 3.1: Ingredient composition (g/kg DM) of the dairy feed and the TMR mixed on the farm for the control and treatment groups.

Ingredient	Dairy Meal (kg/ton)	TMR (kg per cow per day)
Yellow Maize	416	5.62
Hominy Chop	120	1.62
Soya Hulls	25	0.34
Molasses Liquid	50	0.68
Cotton Oilcake 33%	20	0.27
Extruded Full-Fat Soya	65	0.88
Soya Oilcake 47%	110	1.48
Sunflower Oilcake 36%	20	0.27
Local Fishmeal 65%	50	0.68
Limestone	10	0.13
Magnesium Oxide	3	0.04
Ammonium Sulphate	2	0.03
Urea	8	0.11
Monocalcium Phosphate	1	0.01
Salt	10	0.13
Sodium Bicarbonate	5	0.07
Megalac	80	1.10
Vitamin & Micromineral Premix [a]	1	0.01
Acid Buff	4	0.05
Brewers Grain (Wet DM = 22%)[b]	-	16.00
Maize Silage (Wet DM – 36.4%) [b]	-	16.00
Eragrostis Hay [b]	-	2.5
Lucerne Hay 18% [b]	-	6.5

[a] Premix added to the treatment group diet contained Ronozyme® Rumistar 600 (CT). [b] Mixed together with the commercial concentrated feed in a mixer wagon on the farm.

Table 3.2: Nutrient profile of the TMR fed (g/kg) of the dairy feed and the TMR on the farm for all the control and treatment groups.

Nutrient (g/kg DM)	Dairy Meal (g/kg)	TMR (kg DM per cow per day)
Dry Matter (g/kg as fed)	877.75	31.05
ME $_{Ruminant}$ (MJ/kg DM)	11.85	315.65
NSC	454.59	12.84
Fat	73.68	1.28
Crude Protein	181.51	4.395
Lysine	7.05	0.17
ADF	69.20	8.34
NDF	149.52	12.95
Calcium	10.29	0.27
Phosphorus	4.25	0.12
Magnesium	3.60	0.06
Potassium	8.60	0.35
Sodium	2.88	0.06

ME: Metabolisable energy; NSC: Non-structural carbohydrates; ADF: Acid detergent fibre; NDF: Neutral detergent fibre.

In the first period, the particle size of the maize component of the 18% protein dairy concentrate was milled to a standardised size of <3 mm and called FM. The control group received the FM diet, and the treatment group received the FM diet + Rumistar. An adaptation period of three weeks commenced, followed by one week of sample collection.

The adaptation period and the sampling and resting cycle were repeated for another two experimental periods. The control and treatment groups were kept similar but the particle size of the maize in the feed for both the control and treatment groups in period two was changed the same dairy concentrate manufactured with the maize component ground to a coarser particle size (>3mm) and termed as CM instead of FM. In period three, the particle size was changed to concentrate with MMM; this was wet-milled to achieve a constant particle size of

<1 mm. While altering the particle size of the maize component of the feed, the premix used in the dairy concentrates of the treatment groups contained Rumistar, and the premix used in the dairy concentrates of the control groups did not include any supplemental enzymes (Table 3.3).

Table 3.3: Dairy premix composition

Ingredient (Composition per unit of premix)	**Units**	**Dairy feed (No Rumistar)**	**Dairy feed (with Rumistar)**
		Rumistar Control PX	**Rumistar Treatment PX**
Vitamin A	IU	5 600 000	5 600 000
Vitamin D3	IU	600 000	600 000
Vitamin E	Mg	50 000	50 000
Vitamin B1	Mg	50	50
Vitamin B12	Mg	30	30
Niacin	Mg	6 000	6 000
Manganese	Mg	80 000	80 000
Iron	Mg	10 000	10 000
Zinc Oxide 75%	Mg	160 000	160 000
Copper	Mg	30 000	30 000
Cobalt	Mg	1 500	1 500
Iodine	Mg	2 300	2 300
Selenium	Mg	250	250
Organic Zinc	Mg	30 000	30 000
Organic Selenium	Mg	250	250
Levucell	Mg	100 000	100 000
Monensin	Mg	18 000	18 000
Flavomycin 8%	Mg	100 000	100 000
Ronozyme® Rumistar	Mg	-	926 000
Unit Size	Kg	3.00	3.00

3.2.3 Sampling and Analyses

The experimental phase was divided into three periods, and for each period, there were three weeks of adaptation followed by a week of sample collection. Sample days were Monday, Wednesday, and Friday. Milk samples and individual faecal samples were collected.

During the fourth week of each experimental period, fifteen individual manure samples (±500 g) were collected rectally from each treatment group on the sampling days after the evening milking into pre-labelled Ziplock plastic bags. According to Fredin *et al.* (2014), the starch content of manure is not affected by the day of the week that it is sampled but definitely by the time of day that it is collected. Manure sampling, therefore, was done three times during the week at the same time each day. Selected animals for both the control and the treatment groups were restrained in a cattle crush as they left the parlour after the evening milking. A gloved, lubricated hand was passed gently through the anus, and any faecal material present was withdrawn. A rectal evacuation was stimulated by gently massaging the rectal wall. Approximately 500 g of manure was collected from each individual animal and dropped into a pre-labelled Ziplock plastic bag. The air was squeezed out, and the sample was sealed and frozen on dry ice. The following day, samples were labelled and dried in a foil container in a forced-air oven at 55°C for 48 hours at the Nutrilab facility at the University of Pretoria.
Samples were then sent to the Central Analytical Laboratories (CAL) (5 Cartwright St, Stormill, Roodepoort, 1709) for NIR analysis. of the following parameters: DM, Starch, Protein, and NDF. According to Reeves & van Kessel, (2000) and Fredin *et al.* (2015), NIR analysis can successfully be used for the measurement of the starch content of manure (compared with quick tests: $R^2 = 0.95$, $P<0.001$) and (compared with wet chemistry: $R^2 = 0.88$, $P<0.001$) respectively and can be used as a predictor of TTSD.

Individual milk samples were collected for each group on days 1, 3, and 5 during the sampling week and preserved before submission to Mérieux NutriSciences Laboratories (Stellenryk Building, Constantia Square Office Park, 526 16th Street, Randjespark, Midrand, 1685, South Africa) for total solids, milk fat, and milk protein content. Daily milk production data were recorded throughout the trial period. Because the treatments with the different sizes of maize particles were done consecutively, the data were statistically tested with a meta-analysis approach model to remove the effect of time.

Feed samples of the dairy concentrate, lucerne, and maize silage were analysed by Central Analytical Laboratories (CAL) (5 Cartwright St, Stormill, Roodepoort, 1709) for verification of nutrient content of the following parameters: DM (Moisture Loss on Drying: AOAC 930.15 / AOAC 945.15 / AOAC 935.29), Starch (Total Starch = Megazyme: AOAC Method 996.11 / AACC Method 76-13.01), Crude Protein (Dumas: Gafta Method 4.2 / AOCS Ba4e-93 / AOCS Ba4F-00 / AOAC 990.03 / AOAC 992.23 / ISO 16634-2), and NDF (Ankom Method 15) and all batches were verified with NIR (Perten DA 7250) analysis through the standard quality control system at the ALZU Feeds quality control laboratory. The TMR intake of each group of cows was recorded by weighing the feed given, and the orts left on a daily basis. Adaptations to the daily ration were made to ensure *ad-lib* intake of TMR.

Additional manure samples were also collected for analysis with the NASCO Digestibility Analyser on the scheduled sample collection dates. One constituted faecal sample per group was collected for NASCO analysis on a NASCO Digestion Analyser (eNasco Product Number: C26728N, NWF Agriculture, Wardle, Nantwich, Cheshire, CW5 6AQ).

Fifteen individual manure samples of approximately 200 g were collected from each treatment group pen into a 20-litre bucket to make up one composite sample per treatment group. These manure samples were mixed thoroughly to ensure a representative subsample was obtained. From the composite sample, a 1 kg representative sample was weighed and washed through the sieves of the NASCO Digestibility Analyser using clean water. The residue on each sieve was weighed, photographed, and stored in a pre-labelled Ziplock plastic bag for drying at the Nutrilab facility at the University of Pretoria. The variability of the manure was noted according to the 5% rule. The manure from a group of cows consuming the same diet should be similar. It is considered normal if 5% or less cows demonstrate different manure from the rest of the group.

The NASCO separator comprised three tiers of metal screens, top 4.76 mm, middle 2.38 mm, and bottom 1.59 mm, and these were used to divide the manure into size fractions. Less than 10% of manure residue on the top screen is perceived as ideal (Mertens, 1997). According to the author, more than 10% of the manure sample left on the top screen indicates some metabolic issue with the diet. Reasons for these metabolic issues include inadequate effective fibre in the daily ration, a sudden ration change to which the cows are not adapted, rumen-degradable protein available for rumen microbes too low for effective fibre digestion, inadequate sugar

and starch available for microbial production, or excess unsaturated fat in the diet, inhibiting the microbial population (Deckardt *et al*., 2013).

The presence of long particles or undigested material in the manure can indicate that cows are not ruminating properly, are sorting feed, and have an excessive grain-feeding rate that is causing the rumen pH to decrease and the digesta flow rate to increase. It can indicate that the diet is lacking effective/long fibre. In addition, when inadequately processed grain is fed into the diet, the attachment capability of rumen microbes is limited, and digestibility is decreased. Rumen acidosis, an imbalance of the non-fibre carbohydrate to ruminal digestible protein ratio (NFC: RDP) in the diet, or a disproportionate amount of moisture (<40% DM in the TMR) can all lead to an increase in digesta flow rate, reducing effective digestion (Nousiainen *et al*., 2009)

Less than 20% manure residue left on the middle screen is perceived as ideal. A residue on the middle screen comprising more than 30% of the total sample can indicate an imbalance of NFC: RDP, a poor balance of starch and protein degradability in the daily diet, inadequate grinding of grains, or an excessive grain feed rate. The ideal manure residue on the bottom screen should be more than 50% of the original sample. There should be little, if any, recognisable feed present.

3.2.4 Statistical Analysis

Initially, the control and treatment groups (n=20) were analysed with the t-test for two independent samples to find differences between effects per maize milling size. To compare the three milling sizes, a meta-analysis approach was used (Payne *et al*., 2015) A combined analysis of the three milling sizes with enzyme addition was performed using the restricted maximum likelihood (REML) procedure (Payne *et al*., 2015). Unlike the 2 x 3 factorial analysis of variance (ANOVA), this method does not pool the variances over the full nine-week trial but applies the variance from each period separately, which ensures more efficient estimates. The number of days after calving, which is not influenced by the milling size or enzyme treatments, was used as a covariate in the analysis to adjust the mean estimates of the response variables that were to be analysed (Payne *et al*., 2015).

3.3 Experimental Phase Two

The second phase of the trial was conducted at the dairy section of the Hatfield experimental farm of the University of Pretoria using cows with a mean milk production of 24 L per day. The experimental design was a cross-over design, using two cows per treatment and was conducted over two experimental periods. During each period, maize ground to three different particle sizes was analysed in the cows receiving an exogenous enzyme treatment and in the control cows not receiving the enzyme. The diets of both groups were the same except for an exogenous enzyme added directly into the rumen of the cows in the treatment group. The following parameters were measured:

- Rumen pH twice daily at milking time.
- Weekly VFA throughout the adaptation and sampling periods of the rumens of cows with and without the exogenous enzyme added.
- Seven-hour *in-vitro* digestibility assay for starch on maize ground to three particle sizes and NDF on finely ground lucerne.
- 24-hour *in-sacco* digestibility for starch on maize ground to three particle sizes and NDF on finely ground lucerne.

3.3.1 Experimental Animals

Four multiparous, rumen cannulated, high-producing Holstein cows with mean body weights of 652 kg ±35.0 kg, milk yield of 24 L ±4.0 L, days in milk 100 days ±20 days, parity 4.5 ±1.29 (mean ±SD) and housed in semi-enclosed stalls on the Hatfield experimental farm of the University of Pretoria were used in the trial. For the duration of this trial, the cows remained in their regular production group and were treated exactly the same as the rest of the lactating cows. The only interference that the trial had on their routine was adding the enzyme into the rumen twice daily after milking. These cows were milked first in their group, and the enzyme was added in the crush passage as they left the parlour. Thereafter, they joined their group before returning to the camp.

3.3.2 Experimental Diets and Treatments

All cows were fed *ad lib.* A standard TMR formulated for high-producing dairy cows (NRC, 2001) and consisting of a commercial dairy meal containing maize meal, soybean oilcake,

hominy chop, molasses, urea, rumen inert fat, and a vitamin/mineral premix was mixed on-farm with chopped lucerne hay and eragrostis hay. The TMR contained 165 g/kg crude protein, 317 g/kg NDF, and 225 g/kg starch on a DM basis. Feed samples of the dairy concentrate, lucerne, and maize silage were analysed by Central Analytical Laboratories (CAL) (5 Cartwright St, Stormill, Roodepoort, 1709) for verification of nutrient content of the following parameters: DM (Moisture Loss on Drying: AOAC 930.15 / AOAC 945.15 / AOAC 935.29), Starch (Total Starch = Megazyme: AOAC Method 996.11 / AACC Method 76-13.01), Crude Protein (Dumas: Gafta Method 4.2 / AOCS Ba4e-93 / AOCS Ba4F-00 / AOAC 990.03 / AOAC 992.23 / ISO 16634-2), and NDF (Ankom Method 15) and all batches were verified with NIR (Perten DA 7250) analysis through the standard quality control system at the quality control laboratory, to confirm the composition (Table 3.4). Cows had unlimited access to clean water.

Table 3.4: Nutrient analysis of the commercial dairy feed and the TMR on the farm for the control and treatment groups.

Nutrient (g/kg DM)	Dairy Meal (g/kg)	TMR (g/kg)
Dry Matter (g/kg as fed)	877.75	794.15
ME $_{\text{Ruminant}}$ (MJ/kg)	11.81	11.27
NSC	454.59	358.56
Fat	73.68	45.68
Crude Protein	181.51	164.95
Lysine	7.05	6.11
ADF	69.20	297.82
NDF	149.52	316.51
Calcium	10.29	1.12
Phosphorus	4.25	5.2
Magnesium	3.60	2.12
Potassium	8.60	12.5
Sodium	2.88	2.25

ME: Metabolisable energy; NSC: Non-structural carbohydrates; ADF: Acid detergent fibre; NDF: Neutral detergent fibre.

Maize ground to three different particle sizes was tested with and without the addition of an exogenous amylase enzyme that was administered directly into the rumens of the treatment cows, resulting in six dietary treatments. The particle sizes were the same as in phase one

(i.e. CM: >3 mm, FM: <3 mm, and MMM: <1 mm). The MMM was wet-milled to achieve a constant particle size of <1 mm.

A cross-over design with four cows was used to determine the rate of starch disappearance over a 24-hour time period. Each cow served as a repetition of the trial. The study was executed in two periods of 21 days adaptation, followed by one sampling day when 24-hour *in-sacco* studies were conducted. Cows were adapted to the enzyme treatment by adding the enzyme directly into the rumen via a cannula twice a day. The cows were given a three-day resting period before the second trial period commenced. This followed the same pattern of 21 days adaptation followed by a 24-hour *in-sacco* study.

During each period, two of the four cows were administered an exogenous enzyme directly into the rumen. The other two cows served as controls (i.e. no enzyme treatment). In the second period, the cows in the control and treatment groups were switched, and the trial was repeated. Treatments were repeated on the same cows after a recovery and rest period of three days and an adaptation period of 21 days, interchanging control and treatment cows before the second interchange period for a total of two rounds. This allowed the cows to adjust to the diet and the conditions in addition to stabilising and adapting the rumen microflora to the additional enzyme. Ruminal pH was measured and recorded twice daily at every enzyme insertion throughout the trial period for all trial cows. The enzyme was mixed with 50 ml of artificial saliva and introduced into the rumen. The control cows received 50 ml of artificial saliva only, at the same time.

The cows remained in one group in the general herd environment throughout the total trial period. Insertion of the enzyme occurred twice daily, directly after the morning milking at 6h00 and after the evening milking at 19h00. Trial cows were milked first in their group, and the insertion was done while they waited in the holding pen for the rest of the group to exit the parlour. They re-joined the group before leaving the holding pens.

3.3.3 Sampling and Analyses

Ruminal pH and temperature were measured by inserting the portable digital pH meter (Extech PH110 Waterproof ExStik pH Meter, Tequipment, Interworld Highway, LLC 205 Westwood Avenue, Long Branch, NJ 07740) into the central rumen through the cannula at a 90^{o} angle

into the liquid phase of the rumen and not exceeding the midline of the cow (Duffield *et al.*, 2004). The measurements were recorded twice daily after milking at every enzyme insertion. A seven-hour, *in-vitro* degradation analysis was performed with rumen fluid from the control and the treatment cows. Rumen fluid was taken every week for a VFA production analysis of the cows with and without exogenous amylase enzyme. The sampling day entailed an *in-sacco* digestion assay over a 24-hour period.

Every week at the morning and evening handling of the cows during the adaptation and testing period, rumen fluid samples were taken for VFA production analysis. Samples containing 5 ml rumen fluid mixed with 1 ml preservation solution (5% glycerol) were frozen and analysed after the trial was completed. The analysis was conducted in the microbiology laboratory of the University of the Free State using gas chromatography on a gas chromatograph (Agilent Technologies 7890B [FID] split injector) fitted with a capillary column treated with polyethylene glycol terephthalic acid (Rosmalina *et al*., 2020).

After the 21-day adaptation period of the two cows to the enzyme treatment, a seven-hour *in-vitro* digestibility assay was done using the DAISY^{II} Incubator and two different sets of rumen fluid (control and test) on all six treatments consisting of maize ground to three different particle sizes (MMM, FM, and CM) in the cows receiving additional enzyme and the control cows. Additionally, the lucerne was analysed for NDF disappearance in the cows receiving additional exogenous enzymes and the control cows. The 21 days of adaptation were followed by the sampling day when a 24-hour *in-sacco* method of degradation analysis was conducted as described by **Ø**rskov *et al*. (1980) and adapted by Cruywagen (2006).

The lucerne samples were milled with a 2 mm screen using a Retch Ultra Centrifugal Mill (ZM200, Rheinische Strobe 36, Germany) and sieved through a 120 µm screen. After removing the dust and extremely fine particles, the *in-sacco* lucerne samples from the residue on top of the screen were weighed. The particle sizes of the maize samples were standardised for this trial, coarse >3 mm, fine <3 mm, and micro-milled <1 mm.

Before the onset of the *in-sacco* period, 6 g of the substrate was weighed into dacron bags (F57, ANKOM Technology®, Macedon, NY, USA) with empty dimensions of 10 cm by 20 cm and which ANKOM had tested to retain particles measuring >53 µm. The feed sample amount to the net surface area of the bag ratio, excluding areas of the bag prohibited from being in contact

with the feed and the closed end, was 13.75 mg/cm^2 (total surface area of a bag = 10 cm x 20 cm x 2 sides = 400 cm^2, hence 6 g x 92% DM = 5 500 mg. Thus, 5 500 mg DM weighed / 400 cm^2 = 13.75 mg/cm^2), which was well within the prescribed range of 10–15 mg/cm^2 (Nocek, 1988).

Since rapidly digestible FM was inserted into the rumen, it was expected that most of the maize would be digested after incubation in the rumen for more than eight hours. Duplicate samples were weighed for all treatments and for periods longer than eight hours to ensure that enough residue would be available for analysis. The duplicate bags were dried separately and weighed for the residual DM but pooled for the NDF and starch analysis.

The bags were closed with a colour-coded cable tie and weighed again. Figure 3.1 shows the 48 bags, representing the different incubation periods of 15 min, 30 min, 60 min, 90 min, and 2, 4, 8, 12, 16, and 24 hours together with the duplicates for the 12-, 16-, and 24-hour periods.

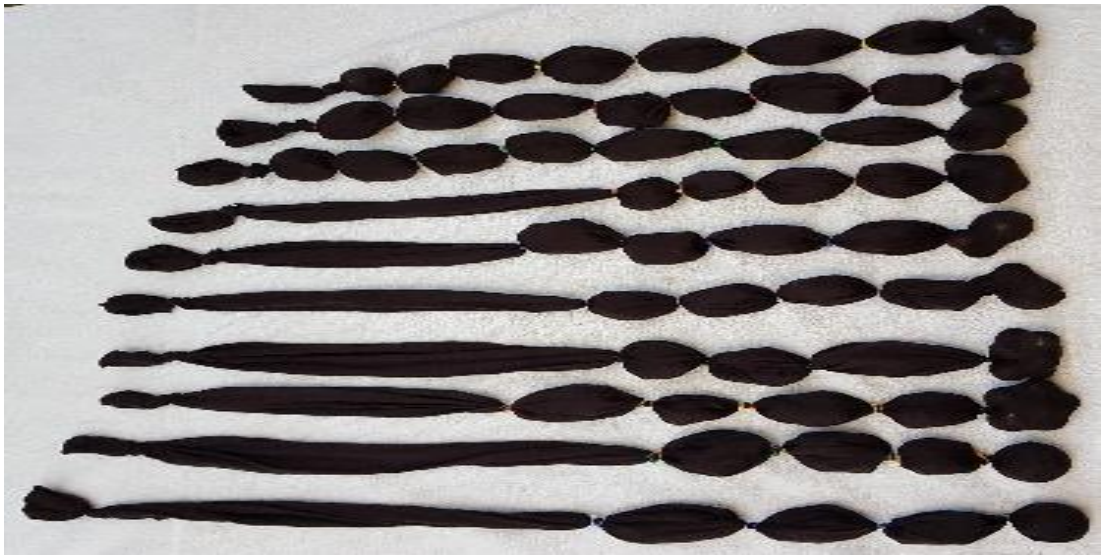

Figure 3.1: Sample bags prepared for the different time periods of the *in-sacco* analysis.

Following the method described by Cruywagen *et al*. (2011), the bags were placed in tandem into weighted opaque stockings and tied to the lid of the rumen cannula via a heavily weighted central line consisting of a stocking with 750 g marbles as the bottom weight. The weight of the central line tied to the cannula lid ensured that the bags remained submerged in the rumen fluid during incubation (Figure 3.2). The strings of the tandem samples in the stockings were

looped onto the main line before pushing them well into the rumen, allowing the bags to move freely within the ruminal contents.

The bags were inserted in reverse time order of removal (i.e. the bags incubated for 24 hours were inserted into the rumen first at 14:00, directly after the noon milking). This was followed by the rest of the bags according to the incubation schedule in reverse.

Figure 3.2: Sample bags on the central line simulating how they were inserted into the rumen.

The bags were removed simultaneously (Vanzant *et al*., 1998) at 14:00 on the next day and immediately inserted into a bucket of iced water to stop microbial activity. The bags were then rinsed properly for at least 20 minutes under running water until the water ran clear. Control bags were prepared and soaked in water for one hour, then washed and dried under similar conditions to the incubated samples as a control for particle loss and as the initial starch value of the sample (Cruywagen *et al*., 2011).

Thereafter, the bags were oven dried at 55°C for 24 hours in a forced-air oven, cooled in a desiccator, and weighed to determine the DM left in each bag. The contents of each bag were removed and sent to Nvirotek Laboratories in Brits for DM analysis (Association of Official Agricultural Chemists [AOAC], 2000, procedure 934.01) and starch analysis (AOAC, 1984, procedure 996.11). The NDF content was determined with the ANKOM 200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, New York, USA). Percentage disappearance of DM, starch, and NDF at each incubation time was calculated from the proportion remaining after incubation in the rumen by analysing the residues at each incubation time period. The DM, starch, and NDF disappearance values were fitted to a non-linear model using the equation of Ørskov & McDonald (1979).

3.3.4 Statistical Analysis

Given the non-independence of the sample in the proposed cross-over design, the use of a mixed modelling approach for the data analyses was recommended. Digestion curves for each data set were evaluated to examine the *in-sacco* degradation kinetics of dietary starch and NDF in each of the treatments in the rumen. Dry matter and crude protein (CP) disappearances were expressed as percentages of the incubated samples. To determine DM and CP degradability parameters, an iterative least-square procedure was used to fit the data to the following one-compartment model according to Ørskov & McDonald (1979) and as described by López *et al*. (1999):

$$p = a + b\,(1 - e^{-ct})$$

where p = degradation at time t

A least-square procedure estimates non-linear parameters:

a = rapidly soluble fraction.

b = the potentially degradable fraction that will degrade over time.

c = the rate of degradation of the b-fraction.

From the *in-sacco* data, an IF (insoluble fraction) can be calculated by the equation (Ørskov *et al*., 1980):

$$IF = 100 - (A+B)$$

A = Soluble fraction A
B = Insoluble degradable fraction B

Because ruminal retention time affects the extent of degradation, a fractional outflow rate of undegraded nutrients from the rumen (kp) was considered López *et al*. (1999). Effective degradability (ED) was calculated from the kinetic parameters obtained from exponential adjustment, assuming fractional passage rates (k_p) of 0.02, 0.05, and 0.08 h^{-1}:

$$ED=A+B\times(k/(k+k_p))$$

The different turnover rates (k_p) used were based on feeding high-production dairy cows (Chaves *et al*., 2006). This study also compared actual disappearance values obtained after 16 hours of incubation. The non-linear parameters a, b, and c and the ED values were submitted to a one-way ANOVA with the aid of SAS PROG ANOVA (SAS, 2000). Significance was declared at $P\leq0.05$.

Chapter 4

4. Results and Discussion

4.1 Analysis Parameters

The current study was conducted to determine the effect of maize particle size and exogenous amylase enzyme supplementation on performance, potential starch utilisation, and rumen fermentation in Holstein cows. The study was carried out in two phases. During Phase One, the effect of particle size of the maize component in the diet in combination with the addition of an exogenous amylase enzyme treatment on milk production and manure assessments was investigated. Phase Two entailed a ruminal digestion study analysing *in-vitro* and *in-sacco* digestion, ruminal pH fluctuations, and VFA production of rumens with and without an exogenous enzyme added.

4.2 Phase one: Effect of Amylase Supplementation and Maize Particle Size on Performance, Milk Composition, and Manure Assessment.

4.2.1 Introduction

Along with water, energy is a critical nutrient requirement of the dairy cow. Energy cannot be chemically analysed with chemicals in a laboratory. Since energy comes from the digestion of carbohydrates, protein, and fat, researchers have attempted to predict an energy value for feeds based on the amount of each nutrient and the assumed or measured digestibility and availability to the cow (Weiss & Tebbe, 2018; Owens & Hicks, 2019). Starch is the most influential nutrient supplying energy to the high-producing cow (Nocek & Tamminga, 1991), and any significant improvement in the digestibility of starch can contribute substantially to the efficiency of nutrient utilisation in dairy cows (Moharrery et al., 2014).

Furthermore, a significant energy-to-protein interaction has an impact on milk production, protein content, and yield. Although milk production may be affected by numerous dietary nutrients, energy and protein are the most influential. The response corresponds with the two more-limiting factors, energy and protein (Brun-Laffleur *et al.*, 2010). The effects of energy and protein in the diet on milk production and composition can, however, not be separated and were used by Brun-Lafleur *et al*. (2010) to develop a model that predicts the responses of milk

yield, protein yield, and protein content of milk. This model predicts the impact that changes in the diet composition have on fluctuations in milk yield and milk composition relative to the genetic potential of the cows. It shows that with additional energy and protein supply in the diet, an upsurge in milk production, milk protein, and milk fat yield can be expected, but these two factors are not additive. Friggens *et al*. (1995) agree that an increase in energy supply in the diet increases the milk production of dairy cows. Moreover, according to Broderick (2003), the milk protein content can be augmented by increasing the energy supply in the diet of dairy cows. However, Friggens *et al.* (1995) report that the milk fat content is not affected by this increase in energy supply but is instead, slightly decreased.

The understanding of the dietary effect on milk composition response should be divided into intake and metabolic effects (Piccioli-Cappelli *et al*., 2014). Furthermore, the parity and genotype of the cows will also affect milk production and composition. These characteristics may also interact with the dietary supply of nutrients. Therefore, cows of different genotypes may show different milk yield and composition responses (Horan *et al*., 2005; Fulkerson *et al*., 2008).

4.2.2 Milk Production

As shown in the results for the milk production responses in Table 4.1, there were no differences between particle size and enzymatic treatment. Additionally, no interactions could be detected (P = 0.79) between the enzyme treatments and the maize particle size. The mean milk production for enzyme and control treatments was 37.1 L (control group) and 36.4 L (enzyme group). In contrast, the mean milk production for the cows fed different maize particle sizes was 36.4 L (CM), 37.5 L (FM), and 36.4 L (MMM).

Table 4.1: Effect of exogenous amylase enzyme supplementation and maize particle size on mean milk production of dairy cows.

	Mean Milk Production (litres/day)		
Maize Particle Size	**Control**	**Enzyme**	**Mean for Milling**
Coarse Maize (>3mm)	36.3 ± 1.5	36.5 ± 1.5	36.4 ± 1.0
Fine Maize (<3 mm)	37.6 ± 1.9	37.3 ± 1.9	37.5 ± 1.3
Micro-Milled Maize (<1mm)	37.3 ± 1.5	35.5 ± 1.6	36.4 ± 1.1
Mean for Treatment	37.1 ± 0.9	36.4 ± 1.0	-

Milk production means and standard errors are shown. There were no differences detected between treatments (p>0.05).

Based on published literature (Fredin *et al.*, 2015; Doorenbos *et al.*, 2017), it was expected that a finer particle size would have had a positive effect on overall milk production. Finer ground maize provides a larger surface area and hence, more starch is exposed for bacterial attachment, thus breaking down starch more effectively and releasing more energy for milk production. However, the results of the current study are in contrast with the findings of Fredin *et al.* (2015) and Doorenbos *et al*. (2017). Starch is broken down by enzymatic hydrolysis. Therefore, depending on the size of the surface available, increasing maize particle size would be inversely related to the digestion of starch. (Rémond *et al*., 2004; Giuberti *et al*., 2014). However, according to Gallo *et al*. (2016), starch digestion also depends on the influence of fermentation end products and the availability of post-ruminal carbohydrate sources, which in turn, is affected by the starch level and intake of the diet and the particle size of the grain component. High-producing cows consuming high intakes of a diet formulated with high starch levels were used in the present study. Moreover, the particle size of maize elicits a modification of the ruminal starch degradability, which affects both the extent of digestion and the site where digestion occurs. The digestion site influences the generation of the different nutrient ratios (VFA and glucose) that are used with varied efficiencies for energy production (Ramos *et al*., 2009; Gallo *et al.*, 2016).

The milk production of the cows that were fed the diet containing the three particle sizes showed no response to the addition of the enzyme to the diet. This disagrees with results reported by Harrison & Tricario (2007), Tricarico *et al.* (2008), and Klingerman *et al.* (2009), who found a quadratic rise in milk production with the supplementation of amylase enzyme in the diet of dairy cows. Tóth & Tóthi (2016) theorise that the reason for improved milk production in cows fed supplemental exogenous enzymes is the more effective metabolism of nutrients that is seen in altered rumen fermentation and plasma metabolites. Contrary to these findings, some studies found no effect on milk production with the supplementation of exogenous amylase (Ferraretto *et al.*, 2011; Weiss *et al.*, 2011; Vargas-Rodriguez *et al*., 2014), while McCarthy *et al*. (2013) reported a negative effect of supplemental exogenous amylase on milk production.

The speed, rigour, and completeness of fermentation of feed ingredients are determined not only by their chemical composition but also by their physical form in the diet. When grains of identical composition are processed to a higher level, it will lead to more rapid and complete

fermentation in the rumen, causing the pH level to drop faster and more steeply and increasing the risk of SARA (Oetzel, 2003). Owens & Soderlund (2006) warned that increasing the concentrate levels of the diet with a finer ground grain can result in metabolic disorders that decrease milk production. Because of the higher and rapid starch availability of MMM with the additional amylase availability from the enzyme treatment, it is suspected that stressors were induced in the rumen bacteria, causing periods of sub-clinical acidosis in the animal (Elmhadi *et al*., 2022).

Both Gencoglu et al. (2010) and Ferraretto et al. (2011) reported improved milk production efficiency through either producing a higher milk yield with no changes in DMI or by maintaining milk yield with a lower DMI. These results were achieved with a low dietary starch content of 21%. Fredin et al. (2015) and Doorenbos et al. (2017) described the rationale of finer ground maize supplying a larger surface area and more exposed starch and thus positively influencing overall milk production. Neither Nozière et al. (2014) nor Weiss et al. (2011) found improvements in DMI, milk yield, and efficiency when including amylaze enzymes in low starch (20% and 26%) diets or in diets with coarsly ground maize. Results regarding milk production and DMI of dairy cows with the addition of exogenous amylase enzyme are variable and seem dependent on the level and type of starch used in the diet. However, they can also be attributed to other intrinsic animal and extrinsic environmental factors (Andreazzi et al., 2018).

4.2.3 Milk Composition

Energy availability has a direct impact on milk composition. Additional energy will increase milk protein until no free milk urea nitrogen (MUN) is available (Broderick, 2003). Propionate production is increased by more readily digestible feed energy, a high hydrogen concentration in the rumen after feeding, or a faster passage rate of feed from the rumen, raising the propionate-to-acetate ratio (Ren *et al*., 2019; Wang *et al*., 2023). Acetate is the precursor for the short-chain fatty acids that are synthesised in the mammary gland, and therefore, acetate is responsible for milk fat production. An increased propionate-to-acetate ratio caused by an increase in rapidly digestible carbohydrate/starch energy in the feed will negatively affect subsequent milk fat production (Lechartier & Peyraud, 2011). Although protein and milk fat percentages are measured and reported, the actual economic value of the response would be in total protein and fat yield per cow per day.

4.2.3.1 Milk Fat

Milk fat is a gauge of fibre digestion and a healthy rumen, and together with milk protein, it can be an indicator of stressors in the rumen such as SARA. Crocker *et al*. (1998) found a linear decrease in milk fat production that reflected the changes in the VFA pattern and concentrations as changes in starch and fibre digestion were observed. Reynolds (2006) describes the interaction of the type and particle size of forage with the type and processing of the grain fraction in diets. When the NDF digestibility is compromised in any way, milk fat production is negatively affected. Moreover, the site of digestion has an impact on milk fat production. When starch is directly infused into the duodenum, depressed DMI is observed, which is closely associated with a decrease in milk fat percentage (Huntington, 1997; Reynolds *et al*., 2001). According to Oetzel (2007), ruminal acidosis is one of the three main factors causing milk fat depression, not by reducing propionate absorption but by inhibiting the bacteria responsible for fatty acid biohydrogenation, allowing more trans fatty acids to be absorbed. An increase in rumen-degraded starch leads to a decrease in DMI (Bradford & Allen, 2007), which depresses milk fat production. A negative relationship exists between the total amount of starch digested in the rumen and milk fat production (Firkins *et al*., 2001; Ferraretto *et al*., 2013).

The mean milk fat percentage was 3.84% CM, 3.85% FM, and 4.08% MMM. There was no effect of maize particle size on the mean milk fat content ($P>0.05$), but a significant interaction was found between maize particle size and enzyme treatment ($F_{2,68.2} = 10.6$, $P = 0.008$). This result suggests that the effect of the enzyme is more pronounced when lower starch degradability is expected with an increase in maize particle size as there is more substrate for the enzyme to make a difference.

Regarding the absence of the enzyme, higher milk fat percentages were observed in the CM treatment (4.28%) compared to the FM treatment (3.69%; $p < 0.05$) (Figure 4.1). It was assumed that the lower starch availability caused by the larger maize particles led to a reduced propionate-to-acetate ratio agreeing with Crocker *et al*. (1998) who stated that an increase in the degradability of ruminal starch reduces milk fat production following a change in the molar percentage of propionate production over acetate production. A linear decrease in milk fat percentage was found as lower digestible dry-rolled maize was replaced by higher digestible

steam-flaked maize following the change in the molar percentages of acetate and propionate (Crocker *et al.*, 1998).

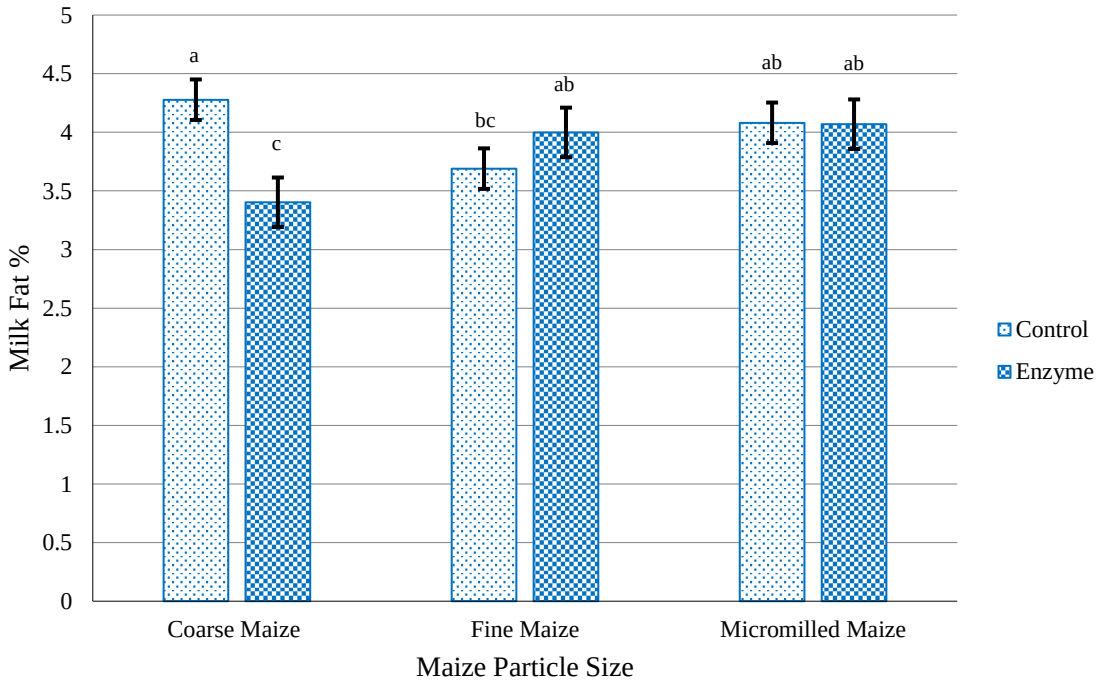

Figure 4.1: Average milk fat percentage of milk produced by cows fed a diet containing coarse maize (>3mm), fine maize (<3mm), and micro-milled maize (<1mm) with and without enzyme treatment. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. [abc] Treatment means with different superscripts differ significantly (P<0.05).

Krause & Combs (2003) found decreased milk fat production relating to lower DMI when rumen-degraded starch was increased. Diluting rumen-degraded starch by increasing the NDF fraction of the diet results in a higher DMI and milk fat percentage (Beckman & Weiss, 2005). Lower hydrogen is available in the rumen when coarse, less digestible maize is fed to cows. According to Wang *et al.* (2023), this affects the thermo-dynamic, hydrogen-consuming fermentation pathways and promotes the production of acetate and butyrate over propionate production.

In the enzyme group, however, lower milk fat percentages were detected in the CM group (3.40%) than in the groups fed the diets containing finer ground maize (FM 3.99% and MMM 4.07%) (Figure 4.1). This result contradicts the prediction of Ferraretto *et al.* (2013) in their

meta-analysis study that DMI and milk fat would be reduced when starch digestibility is increased. The result also deviates from the results of Gencoglu *et al*. (2010), Ferraretto *et al*. (2011), Weiss *et al*. (2011), Nozière *et al*. (2014), and Andreazzi *et al*. (2018) who reported no impact of amylase supplementation on milk fat. By increasing the intake of higher fermentable starch, a decrease in the acetate-to-propionate ratio from 2.7 to 2.1 was reported by Lechartier & Peyraud (2011). In the current study, the milk fat produced by the cows fed diets containing all the finer ground maize supplemented with an amylase enzyme was similar to the milk fat produced by the fine maize fraction fed to cows in the control group with no added enzyme (Figure 4.1). This indicates that the amylase enzyme contributed to more propionate production than acetate production, increasing the propionate-to-acetate ratio when the amylase enzyme was included in the diet containing lower digestible maize (CM), similar to the improvement in milk fat induced by the smaller particle size in the control group.

However, as seen in Figure 4.1 and contrary to what has been reported, in the enzyme group, the milk fat production of the cows fed the diet containing finer ground maize was positively affected by the finer particle size. It is postulated that this result could have been due to a cross-feeding effect. The catalytic hydrolysis of starch to oligosaccharides by the amylase enzyme and the increased starch availability due to the smaller particle size led to more time being available for fibrolytic bacteria to exert the effect of improved fibre digestion (cross-feeding). According to Salfer *et al*. (2021), the abundance of individual rumen microbe populations cannot be directly derived from the intake of growth substances since an increase in fibre digesters after feeding high starch diets was seen. Salfer *et al.* (2021) state that more complex dynamics exist between rumen microbial populations in relation to cross-feeding.

4.2.3.2 Milk Protein

Increasing the energy supply in the diet will improve the protein content of the milk that is produced, especially if there is an oversupply of protein and an undersupply of energy (Broderick, 2003). Milk protein, therefore, can be used as a barometer of the energy that is available to the rumen bacteria for milk production.

Data showed no effect of the enzyme treatment on milk protein, although maize particle size significantly affected the protein content of milk produced ($P<0.05$) (Figure 4.2.) The mean milk protein percentage was 2.93% for CM, 2.93% for FM, and 3.12% for MMM, showing

that a diet consisting of MMM resulted in the production of milk with a higher milk protein content than in the diets containing the coarser-ground particle sizes ($P<0.05$). Grinding the maize component in the diet to a particle size finer than 1 mm positively affected the milk protein percentage, indicating that more energy was available for milk production (Figure 4.2).

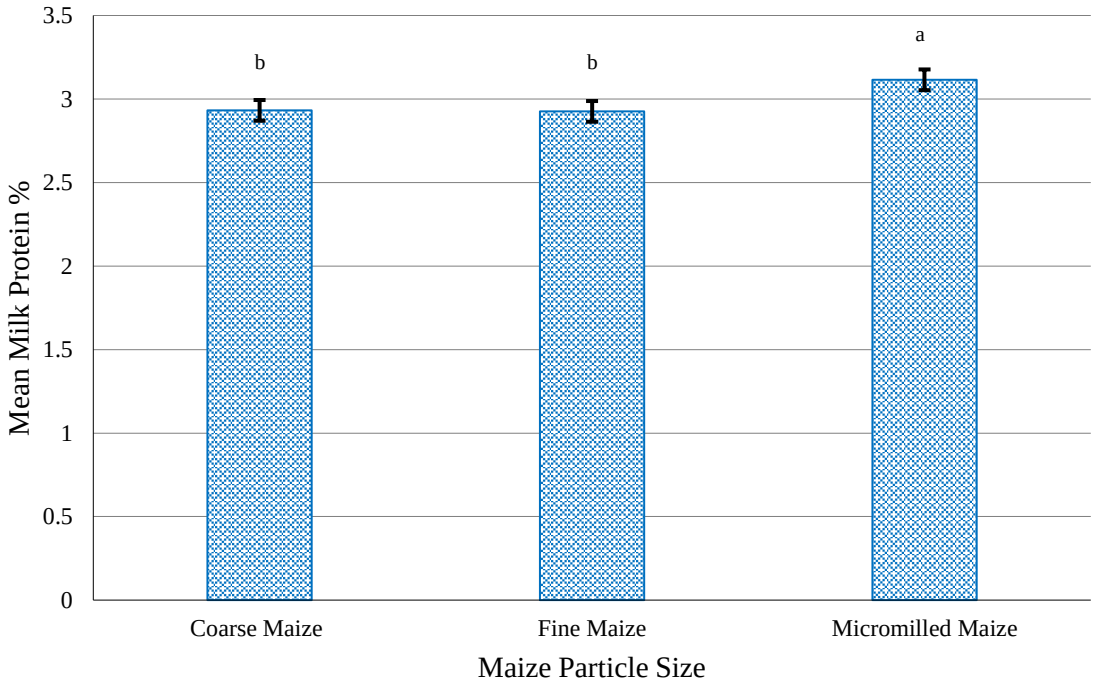

Figure 4.2: Mean milk protein produced by cows fed a diet containing coarse maize (>3mm), fine maize (<3mm), or micro-milled maize (<1mm) across both control and enzymatic treatment. [ab] Treatment means with different superscripts differ significantly (P<0.05). Means were calculated from the treatment diets (with added enzyme) and the control diets (with no added enzyme). Data are presented as means ± SEM.

The results agree with Firkins (2008) who theorised that an increased starch degradability in the rumen would promote microbial protein synthesis. However, Firkins (2008) cautioned that this comes at the expense of NDF digestibility in the rumen, which negatively influences ruminal digestion.

The mean milk protein percentage was 3.02% for the control group and 2.3% for the enzyme treatment group. No significant effects of the enzyme treatment or the interaction of milling size and treatment on the mean milk protein content of the milk were found (Treatment: $F_{2,70.2}=2.41$, $P=0.123$; Interaction: $F_{2,70.2}=0.31$, $P=0.734$). This agrees with

Andreazzi *et al.* (2018) who reported that secretions of milk fat and protein were not changed by exogenous supplementation of amylase. In trials on supplemental exogenous enzymes added to reduced starch diets, Gencoglu *et al*. (2010) found a positive response in milk protein and a reduction in MUN content with an added enzyme. This aligns with the study of Oba & Allen (2003) that reported increased microbial protein production due to improved ruminal starch digestibility.

However, Hristov *et al*. (2008) found that additional starch supplied in the rumen by intraruminal dosing as opposed to the addition of NDF increased ruminal ammonia concentrations and ruminal microbial production, thus increasing milk protein production. In the study of Ferraretto *et al.* (2011), no change in the percentage of milk protein was noticed when exogenous amylase was added to a reduced starch diet of lactating cows.

Klingerman *et al.* (2009) tested different exogenous amylase enzymes at low and high dosages and found no effect on milk protein. Her study showed however, that the DMI was reduced in low-starch diets when exogenous amylase was added, indicating improved feed-to-milk conversion. Hence, improving feed efficiency by adding an exogenous starch enzyme, results in a direct economic benefit. Tóth & Tóthi (2016) found no response in milk protein production when adding exogenous enzymes to the cows' diets. Nevertheless, they reported a significant reduction in the lactose content of the milk, which indicates a lower DMI. However, contradictory results were reported by Nozière *et al.* (2014), who found an increased lactose content in milk with the feeding of the exogenous enzyme but no effects on starch digestion.

A meta-analysis by Ortiz-Rodea *et al*. (2013) on a range of exogenous enzymes fed in various dosages reported a poor relationship between the addition of an exogenous enzyme and milk composition. In our study, no significant differences were detected in the protein content when the exogenous enzyme was added to the diet of lactating cows.

4.2.4 Manure NIR Results for Starch and NDF

The distinctive traits of a specific plant cell wall comprise one of the intrinsic characteristics of feed that can affect the digestibility of a diet (Jung & Allen, 1995). Intrinsic digestibility is the potential, extent, and rate of digestion under ideal conditions when only the substrate traits limit digestion (i.e. digestibility determined at maintenance) (Huhtanen *et al*., 2006). When

intakes of dairy cows increase, different extrinsic factors (i.e. concentrate to roughage ratio, time of feeding, environmental impact) will influence the digestion and passage rates of the digesta, which in turn, will affect the digestibility of feedstuffs. Increased intakes will increase the passage rate, depressing digestibility (De Sousa *et al*., 2018). Milk production controls DMI until the physical fill and ruminal metabolic health dominate the influence (Allen, 2000). According to Cavallini *et al*. (2023), NDF in the faeces is an indicator of NDF intake and degradation, gut fill, and the potential of the dietary fibre to stimulate chewing and rumination. When high starch intake replaces the fibre content, microbial populations shift away from the fibre digesters, leading to more fibre in the manure. A positive correlation exists between NDF measured in the faeces and total tract DM digestibility, but a negative correlation is seen between faeces NDF and total tract NDF (Lacey *et al*., 2020). The negative correlation indicates that when cows digest NDF better, they have less NDF in their faeces. Higher faecal indigestible NDF contributes to an increased level of NDF in the faeces and, therefore, potentially degradable NDF can be calculated by subtracting indigestible faecal NDF from faecal NDF. Correlations between potentially degradable NDF and total tract DM digestibility (-0.31) and between potentially degradable NDF and total tract NDF digestibility (-0.97) were found, confirming that cows with higher digestion efficiency have less potentially digestible NDF in their faeces (Lacey *et al*., 2020).

Firkens *et al*. (2001) and Ferraretto *et al*. (2013) reported that 70% to 100% of starch is digested in the total gastrointestinal tract of dairy cows. A myriad of factors such as particle size, grain processing, storage method, harvest maturity, moisture content, duration of silo fermentation, and maize endosperm type can have an impact on starch digestibility. Firkens *et al.* (2001), Taylor & Allen (2005), Allen *et al*. (2008), Lopes *et al.* (2009), Hoffman *et al*. (2011), Ferraretto *et al*. (2013), and Fredin *et al.* (2014) note that starch analyses of manure could be used to indicate excessive starch content in manure (>5%), suggesting opportunities to improve production and nutrient utilisation by ration adjustments.

4.2.4.1 Faecal Starch

The starch content of the faeces samples was analysed to examine the effect of starch digestibility. Starch digestibility can be affected by various factors, which include the following:

- Particle size: Poorly ground or processed grain will have a negative impact on starch digestibility (Knowlton *et al*., 1998; Firkins *et al*., 2001; Rémond *et al*., 2004; Ferraretto *et al*., 2013).
- Maize Silage Processing: Poorly processed kernels in maize silage can result in lower digestibility (Hoffman *et al*., 2011).
- Moisture content: Maize silage with >35% DM tends to be less digestible (Ferraretto & Shaver, 2012).
- Fermentation: Starch digestibility is enhanced in fermented maize, unlike unfermented maize. Digestibility continues to increase in storage for 4–6 months after ensiling (Hoffman *et al*., 2011).

Table 4.2 provides a guideline for interpreting faecal starch (Fredin *et al.*, 2015). Grain particles will always be visible in the manure of high-producing dairy cows because a high feed intake and a high-energy diet are needed to meet the energy needs, resulting in an accelerated digesta passage rate. This often results in more starch being supplied that can be fermented in the rumen.

Table 4.2: Guidelines for the interpretation of faecal starch.

Faecal Starch (%DM)	Guidelines
<3%	Starch digestion is good, and there is no need to investigate starch sources.
3%–5%	Total tract starch digestibility (TTSD) is 93% or better; may have some opportunity to adjust rations or management practices.
>5%	Starch digestibility can be improved; individual sources of starch should be investigated.

Source: Fredin *et al.* (2014)

Although undigested starch leaving the rumen can be digested more effectively in the small intestine, the benefit of ruminal starch digestion is the increased production of microbial protein (DeFrain *et al*., 2005). Starch digestibility in the small intestine is limited by the site's digesta passage rate and the effectiveness of glucose utilisation (Oba & Allen, 2003; Larson *et al*., 2009). The challenge is to determine the optimum amount of grain and the extent of grain processing in order to optimise digestion; this varies between cows and production systems.

Since the different maize particle sizes were fed consecutively, all data were statistically tested with a meta-analysis model to remove the variability of the time period. The feeding of a ration containing MMM led to less starch in the manure (2.26%) than in the diets containing FM (3.42%) and CM (4.08%) ($P<0.05$) (Figure 4.3).

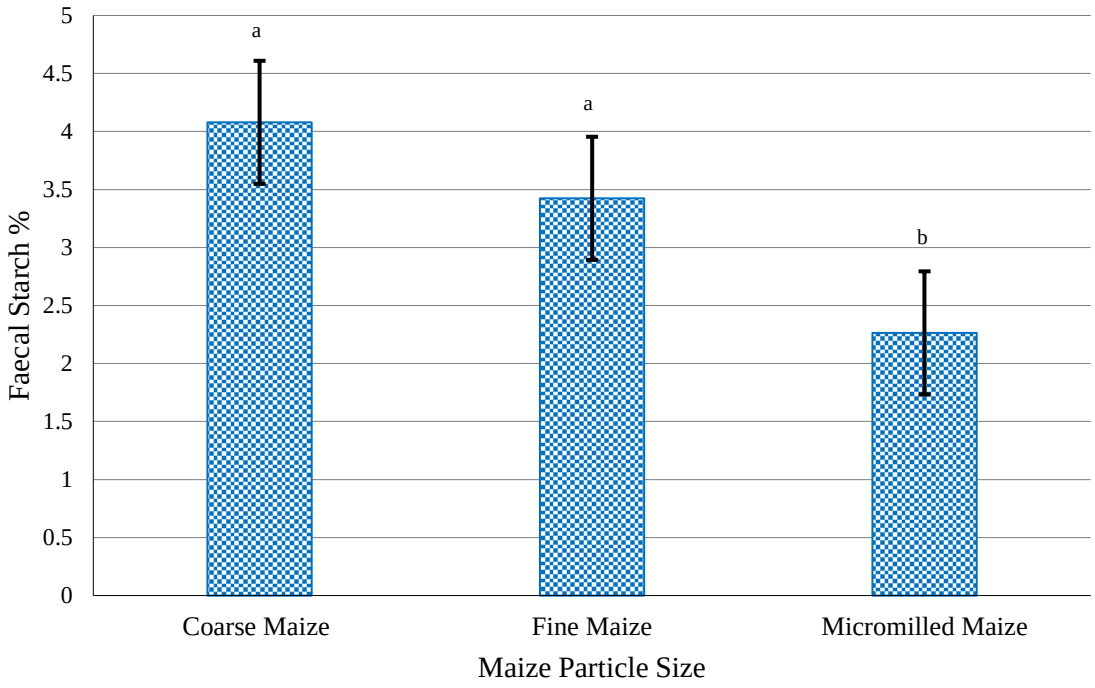

Figure 4.3: Average starch percentage in manure excreted by cows fed a diet containing coarse maize (>3mm), fine maize (<3mm), and micro-milled maize (<1mm) with and without enzyme treatment. Data are presented as means ± SEM. [ab] Treatment means with different superscripts differ significantly (P<0.05).

The results of the present study agree with Fredin *et al.* (2014) that total starch digestibility correlates with faecal starch concentration. They found a strong linear relationship between TTSD and faecal starch content, which is represented in the equation: TTSD = 100% - (1.25 x Faecal Starch percentage) ($R^2 = 0.94$). When the maize fraction is more processed, less faecal starch is found, indicating higher TTSD. Similar close relationships were found by Owens & Zinn (2005), Corona *et al.* (2006), and Zinn *et al.* (2007). Bird *et al.* (1999) also found reduced total tract starch digestion with increased grain particle size. The results of the current study highlight the impact of maize particle size on the amount of starch flowing through the digestive tract into the manure and demonstrate that a significant degradation of the prolamin

structure of starch will improve access to the starch molecules by the enzyme, leading to enhanced starch digestion for diets with highly processed maize (MMM).

No differences in the starch content of the manure were found between the control group (3.27%) and the enzyme-treated group (3.24%) (P>0.05). This is in agreement with Hristov *et al.* (2008) who reported no changes in ruminal and total tract starch digestion for lactating dairy cows fed a balanced diet containing supplemental amylase enzymes. Tricarico *et al.* (2005) also did not find any changes in starch digestibility with the addition of exogenous polysaccharide-degrading enzymes to diets of steers and lactating cows. However, earlier studies reported improved digestion (López -Soto *et al.*, 2000; Murillo *et al.*, 2000; Bowman *et al.*, 2002; Beauchemin *et al.*, 2003; DeFrain *et al.*, 2005; Tricarico *et al.*, 2007). The abovementioned authors concluded that the mode of application, the interactions of different feed ingredients, and the inhibiting effects of specific feeds affect the response of the exogenous enzyme on nutrient digestibility.

The addition of exogenous amylase enzymes increases the degradability of nutrients in the rumen by up to 4% but not necessarily the total tract digestibility of organic matter and starch. Nozière *et al.* (2014) argue that the reason for this could be that the deficit of ruminal digestibility is compensated post-ruminally when no exogenous enzyme is supplemented.

4.2.4.2 Faecal NDF

According to Mgbeahuruike (2007), manure characteristics can be used as an indicator of digestive health and are affected by feed moisture, NDF content, and the mean retention time of the feed in the animal's digestive tract. Manure can range from normal, a medium porridge-like consistency falling into a pile measuring 2.5 cm to 5.0 cm high (Varga, 2003), to distorted, runny, foamy, or stiff and clay-like. Manure is scored according to an adapted Ireland-Perry & Stallings' (1993) scale ranging from liquid (1) to hard (4). Abnormality is caused by many factors, including metabolic stress, digestion disturbances, protein-to-energy imbalances, heat stress, poisoning, bacterial and parasitic infections, and too little fibre in the diet. Low NDF values of manure indicate good rumen fermentation, while high NDF values signal improper fermentation of the fibre in the diet (Nørgaard *et al.*, 2004). The effect of the particle size of the maize fraction of the diet and the enzyme treatment on the faecal NDF is shown in Table 4.3.

Table 4.3: Effect of maize particle size and enzyme supplementation on the mean NDF percentage in the faeces of cows.

Maize Particle Size	Mean Manure NDF Content (%)		
	Control	Enzyme	Mean for Milling (P<0.05)
Coarse Maize (>3mm)	42.96 ± 1.0	43.98 ± 1.0	43.47^{d}± 0.7
Fine Maize (<3 mm)	44.95^{b} ± 0.8	47.37^{a} ± 0.8	46.16^{c} ± 0.5
Micro-Milled Maize (<1mm)	41.49 ± 0.9	39.60 ± 0.9	40.53^{e} ± 0.6
Mean for Treatment	43.13 ± 0.5	43.64 ± 0.5	-

Mean percentages and standard errors are shown. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. ^{ab} Treatment means with different superscripts differ significantly within rows, while ^{cde} mean for milling with different superscripts differ significantly within column (P<0.05).

On average when the enzyme treatments are not taken into account shown in the mean for milling column, the particle size of the maize in the feed had a significant effect on the NDF fraction in the manure. The highest NDF was found in the faeces of the cows consuming FM diets. The diet formulated with MMM indicated the lowest NDF in the faeces. The mean faeces NDF per milling size was 43.47% for CM, 46.16% for FM, and 40.53% for MMM (P<0.05). These results are supported by other studies (Hall, 2002; Varga, 2003; Mgbeahuruike, 2007), who observed an increase in the number of long fibre particles in the cows' manure when the starch content of the diet was increased. An acidic rumen environment decreases fibre digestion due to the defaunating effects of VFAs and leads to a diminished number of fibre-degrading microbes (Nozière *et al*., 2011). The reduced fermentation of the fibre particles was ascribed to a lower residence time of digesta in the rumen and a reduced ruminal pH. Both the limited time spent in the rumen by the digesta and the reduced fibre-degrading microbes in the rumen could have caused an increase in the fibre content of the manure of dairy cows fed high-concentrate rations. Ireland-Perry & Stallings (1993) described the increased faecal NDF in cows fed higher concentrated diets and devised a scale to evaluate manure. Huhtanen *et al*. (2021) report an increased faecal NDF output with increased feeding level, concentration, and digestion of dietary starch.

The reasons for the lower NDF content of the MMM versus the other particle sizes are unclear, but it can be considered that, the micro-milling of the maize leads to higher digestible starch in

the rumens of cows consuming the diet with MMM validated by the significant improvement in milk protein production of cows in this group. This affects the rumen microbial population even more, resulting in passing undigested feed particles to the large intestine and caecum, increasing hindgut fermentation and reducing the NDF content of the manure of the cows in this group. Higher starch content and the digestibility of the grain component dictated by the processing rate and extent leads to increased post-ruminal starch digestion (Kreikemeier *et al*., 1990). Sanz-Fernandez *et al*. (2020) demonstrated the exponential effect of the starch supply on duodenal starch flow and showed how the starch digested in the rumen decreases as dietary starch increases. Although this shift could be beneficial as digestion in the small intestine is perceived to be more efficient than ruminal digestion (Owens *et al*., 1986; Knowlton *et al*., 1998; Callison *et al*., 2001), duodenal digestion also decreases with the increased influx of starch (Huntington, 1997). As the starch content and digestibility of the diet increase, disproportionately high starch levels reach the caecum (Sanz-Fernandez *et al*., 2020).

No significant difference was observed between the starch excreted by the control group and the starch excreted by the treatment group, where the mean manure NDF content was 43.13% for the control group and 43.64% for the treatment group (P>005). Nozière *et al*. (2014) reported similar results when they did not observe any effects on the ruminal and total tract digestibility of NDF, ADF duodenal N flow, or improvements in microbial synthesis with the inclusion of the same enzyme. In contrast with the enzyme not affecting the faecal NDF in the coarse maize diet, faecal NDF increased when added to the fine maize treatment (P<0.05). The faecal NDF content of the control FM group was lower than in the treatment cows (44.95% and 47.37%, respectively), as shown in Table 4.3. Adding the amylase enzyme to the fine maize diet increased the provision of starch in the rumen, increasing the diet concentrate load and the propionic acid producers. This can result in less acetic-acid-producing bacteria, affecting the ruminal pH and could lead to a decline in NDF digestion.

In the MMM diet with added enzyme, the expected higher starch availability in the rumen (due to the finer particle size and added enzyme) could have resulted in adverse effects of increased starch such as early stages of acidosis and hindgut fermentation. However, there was no significant difference in faecal NDF between the micro-milled diet with added enzyme and the control. It was speculated that the cross-feeding mechanism of the enzyme was activated in this circumstance.

4.2.4.3 Faecal Protein

High-producing dairy cows require a highly concentrated ration with high levels of rapidly fermentable carbohydrates in order to maximise the energy supply and achieve their production potential. Such a ration is commonly lacking in sufficient and effective fibre, with negative consequences on cows' general health and reproduction (NRC, 2001; Krause & Oetzel, 2006; Oetzel, 2007; Crnkic & Hodzic, 2012; Abdela, 2016).

When rumen fermentation is diminished, undigested fibre reaching the hindgut could lead to secondary fermentation (Hall, 2002). Extensive hindgut fermentation is a well-known result of overfeeding highly digestible carbohydrates or feeding diets lacking in effective fibre, often leading to problems in cow health and production (Hall, 2002). Limited nutrient absorption in the hindgut is of little value to the animal and is detrimental to general animal health. Amino acids are not effectively absorbed in the hindgut of cows, causing the loss of microbial protein (Darragh & Hodgkinson, 2000; Varga, 2003, Lapierre *et al*., 2006). When excessive nutrients are fermented in the hindgut, the gas and acid produced here will present in the faeces, changing the consistency and appearance (Gressley *et al*., 2011; Dijkstra *et al*., 2012). The evaluation of manure in relation to diet and production environment indicates rumen function, rate, and site of digestion. The microbial protein produced in the hindgut will exit with the manure (Hall, 2002). Microbes produced via hindgut fermentation will be excreted in the manure, leading to higher manure protein values. Lukas *et al*. (2005) reported a positive interaction between faecal protein and diet OM digestibility. As diet organic matter digestibility increases, faecal organic matter decreases and undigested microbial protein increases. These authors suggest that this interaction could be used indirectly to estimate diet organic matter digestibility from faecal protein analysis (Lukas *et al*., 2005).

An excessive amount of mucus in the faeces indicates inflammation of the intestinal epithelial cells caused by low pH in the hindgut due to excessive fermentation. Mucin casts are observed on the manure of cows fed diets that are excessively concentrated and showing early signs of SARA (Hall, 2002). Mucin could be a source of endogenous protein in the faeces indicating extensive hindgut fermentation. Hence, manure protein values can be used to indicate hindgut fermentation. The mean manure protein values are shown in Table 4.4.

Table 4.4: The effect of maize particle size and enzyme supplementation on the protein percentage of the faeces of cows.

Maize Particle Size	Mean Manure Protein Content (%)		
	Control	Enzyme	Mean for Milling (P<0.05)
Coarse Maize (>3mm)	19.06± 0.3	18.94 ± 0.3	19.00 ± 0.2
Fine Maize (<3 mm)	19.66 ± 0.2	18.77 ± 0.2	19.22 ± 0.1
Micro-Milled Maize (<1mm)	19.83 ± 0.3	19.43 ± 0.3	19.63 ± 0.2
Mean for Treatment	19.52[a] ± 0.2	19.04[b] ± 0.2	-

Means and standard errors are shown. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. [ab] Treatment means with different superscripts differ significantly within rows (P<0.05).

Faecal protein content was expected to increase as the particle size of the maize in the diet decreased. Improved starch digestibility was expected to lead to an increased digesta flow rate and increased hindgut fermentation caused by the initial stages of ruminal acidosis. The mean manure protein content per milling size was 19.00% for CM, 19.22% for FM, and 19.63% for MMM (P<0.1). The protein content of manure of cows, fed any of the three different particle size did not differ.

A difference was observed between the control group and the treatment group (P<0.05), where the mean manure protein content was 19.52% for the control group and 19.04% for the enzyme treatment group (P<0.05). This lower value indicates that the amylase treatment could have positively affected rumen fermentation, which would have had a reduced effect on hindgut fermentation. However, the biological significance of this is questionable.

4.3 Phase Two: Effect of Amylase Supplementation and Maize Particle Size on Rumen Fermentation Parameters and *In-Sacco* Digestibility

4.3.1 Introduction

High levels of maize are used as energy sources for high-producing dairy cows (Giuberti *et al.*, 2014). One of the main factors affecting ruminal digestibility is the resistance of the maize kernels to degradation by the rumen microbes. The structure of the endosperm together with the surrounding pericarp of the individual grain kernels also affects ruminal starch degradation (Huntington, 1997; Dehghan-Banadky *et al.*, 2007). The processing of maize kernels improves

the access of microbes to the starch fraction (Offner *et al.*, 2003; Hoffman *et al.*, 2012), improving starch fermentation. However, the overprocessing of maize kernels is just as detrimental to the animal because it can lead to excessive VFA production and lactic acid production, resulting in a significant increase in the total acid load in the rumen. This reduces rumen pH, mobility, and function, and can cause other rumen disorders (Rémond *et al.*, 2004; Hindle *et al.*, 2005; Gozho & Mutsvangwa, 2007).

In this phase, an investigation into which combination of amylase and particle size would optimise energy availability for the high-producing dairy cow was carried out. The aim was to describe the effects of mean particle size as a component of nutrients and determine the parameter coefficients to improve the modelled energy availability from the starch fraction by describing the nature of the available energy substrates and VFAs (Firkins *et al.*, 2001; Offner & Suavant, 2004; Larsen *et al.*, 2009).

4.3.2 Rumen pH

During the second phase of the trial, cows were adapted to the different treatments for a period of 25 days at the dairy section of the experimental farm of the University of Pretoria in Hatfield. Two of the treatment cows received the enzyme in a buffer solution directly into the rumen, while the other two animals received the buffer solution only. A seven-hour *in-vitro* digestibility study and a 24-hour *in-sacco* degradability study was conducted during this time. The health status and adaptation capabilities of the trial animals were monitored by taking pH measurements twice daily at 7h00 and 19h00 after milking and while introducing the control and treatment solutions into the rumen. Although rumen cannulation is, according to Nocek (1997), the recommended method to obtain representative rumen fluid samples for research purposes, Tajik & Nazifi (2011) warned that the cows' rumen environment and digesta could be disturbed.

As shown in Figure 4.4, rumen pH measurements were taken throughout both trial periods from day zero, during adaptation to the different treatments and until the 24-hour sampling period of the *in-sacco* trial commenced. This was repeated during the cross-over treatment. As shown in Figure 4.4, significant differences in the pH means were found in the weekly morning and afternoon measurements. The average morning pH values during the first week of adaptation were 6.4, 6.7 for the next eight days, and 7.2 for the last week before sampling,

excluding the pH of the sampling day (P<0.05). The average afternoon pH values during the first week of adaptation were 5.8 and 6.0 for the next eight days, and 6.4 for the last week before sampling (P<0.05), excluding the pH of the sampling day.

The pH measurement of the rumen fluid at a single time point could be used to monitor the prevalence of rumen acidosis, which is known to contribute to a range of metabolic disorders (Plaizier *et al*., 2008, Abdela, 2016). Thresholds for SARA indication are set by measuring ruminal pH two to five hours after feeding for a set sample of cows through all production groups. The accepted classification is ≤5.5 = abnormal; 5.6–5.8 = marginal; >5.8 = normal. A herd is classified as having a problem with SARA if one or more groups have two or more animals with a pH of ≤5.5 (Jonsson *et al.*, 2018).

It is clearly shown in Figure 4.4 that the pH levels of the rumens during the adaptation and the trial show, on average, a definite increase for the two sampling periods. This indicates that all trial animals adapted to the daily handling and ruminal pH measurements.

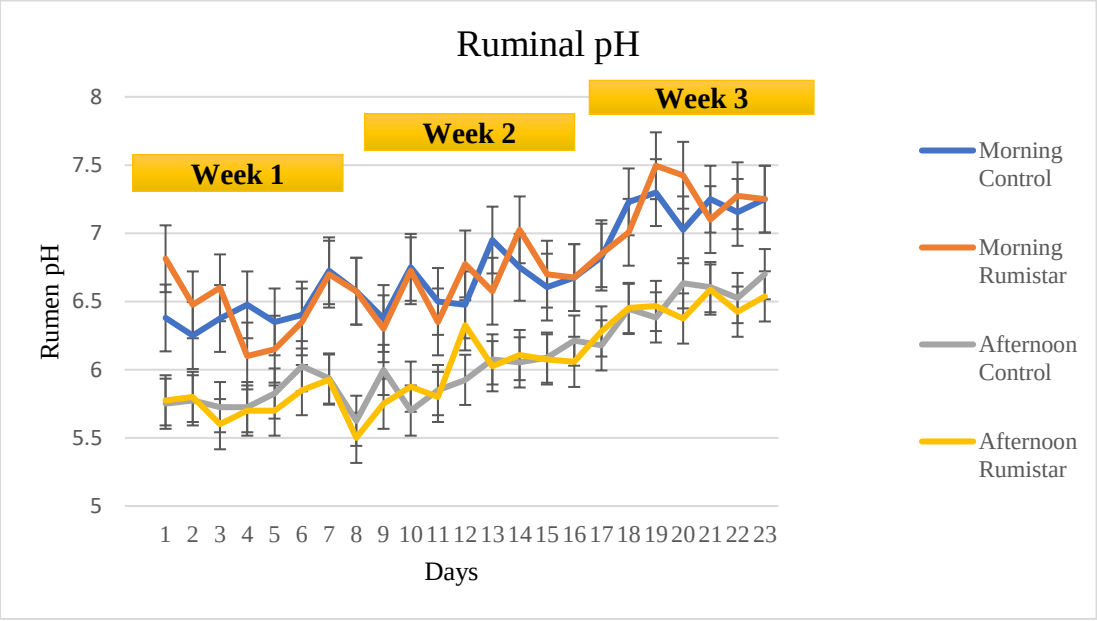

Figure 4.4: Rumen pH throughout a 25-day trial period depicting twice-daily pH results from cows receiving either a control buffer solution or an enzyme treatment with buffer solution.

Although measurements were collected twice daily at the same time, the rumen pH was higher in the morning than in the afternoon over the trial period for all treatments, as shown in Figure

4.4. None of the observed results was below the pH threshold of 5.6. This does not reflect the hourly rumen pH fluctuation, which would better reflect ruminal health.

4.3.3 Volatile Fatty Acids

Rumen microbes produce VFAs with methane and CO_2 gas, and microbial cells use digested digestible carbohydrates as their energy source. It follows that the production of VFAs can be used as a parameter in measuring the efficiency of energy supply to the microbes (Klingerman *et al*., 2009). The DM intake level of cows, especially the level, character, and degradation rate of the carbohydrates, determines the makeup of the microbial population (McCann *et al*., 2016), which establishes the composition of the VFA profile in the rumen (Zhang *et al*., 2020). Acetate production increases as the population of fibrolytic microbes expands, and propionate production rises as the population of amylolytic microbes grows with higher concentrate feeding (Nozière *et al*., 2010). Moreover, diets with high levels of water-soluble concentrates expand the protozoa population, resulting in higher butyrate production than propionate production (Brossard *et al*., 2004). The proportion of ruminal VFA in cows fed a high-starch diet changed to reduced acetate, and in the study conducted by Nozière *et al.* (2014), this was balanced with correspondingly increased levels of propionate, isovalerate, valerate, and caproate. The profile of the produced VFAs can be used to project starch digestion compared with fibre digestion in the rumen. Sutton *et al*. (2003) found a clear shift in the rumen fermentation pattern from high acetate and butyric acid production on a high-fibre diet. The results of the acetic acid, propionic acid, and the acetic to propionic ratio are reported in Table 4.5. In the current study, the total VFA produced did not differ significantly (156.5 mmol/L for the control cows versus 187.2 mmol/L for the treatment cows) (Figure 4.5). This also agrees with the results of the study done by Tricarico *et al.* (2005). Klingerman *et al.* (2009) reported an interaction between the effect of the enzyme on VFA production and the type of maize, with the highest impact being on flint versus floury and dent maize. Tricarico *et al.* (2005) found similar values for total ruminal VFA; ranging between 150 mmol/L and 165 mmol/L at different levels of an α-amylase enzyme derived from *Aspergillus oryzae* that was added to the feed.

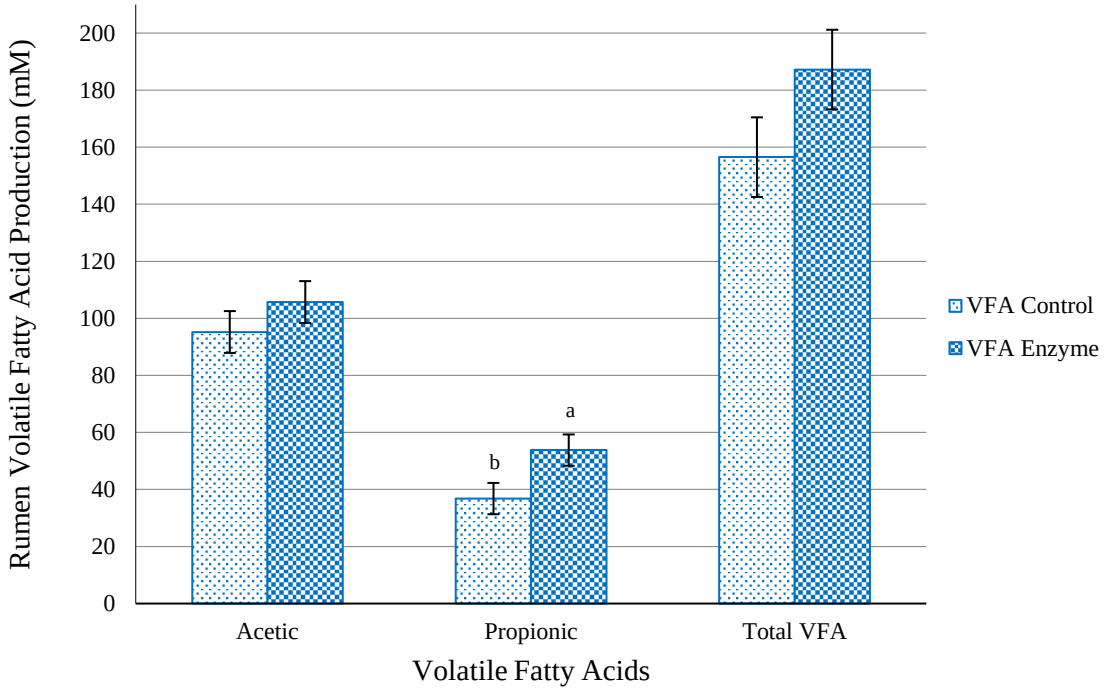

Figure 4.5: Total volatile fatty acid production, acetic acid production, and propionic acid production in the rumens of cows treated with supplemental enzyme versus control cows. Data are presented as means ± SEM. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. [ab] Treatment means with different superscripts differ significantly (P<0.05). Where no superscript is indicated, there is no significant difference within the VFA.

No significant differences between the acetic acid production in the rumens of cows supplemented with an amylase enzyme and the control group were found (P>0.05), with the acetic acid production of the control group at 95.2 mmol/L and the enzyme-treated group higher at 105.7 mmol/L (Table 4.5). The rumen microbes of cows treated with a supplemental enzyme produced significantly higher levels of propionic acid at 53.8 mmol/L than the control group at 36.8mmol/L (P<0.05). This agrees in part with the conclusion of Nozière *et al.* (2014) that supplementation of an amylase has the propensity to increase the total VFA and the molar proportions of propionate and valerate to the detriment of acetate and butyrate levels. Andreazzi *et al.* (2018) observed no difference in total-tract starch and NDF digestibility nor any changes in the VFA production and proportions of acetate, propionate, and butyrate through amylase supplementation, which is in agreement to this study.

The acetate-propionate ratio showed a significant decline in the enzyme-supplemented cows from 2.75 mmol/L (P<0.05) for the control cows to 2.33 mmol/L (P<0.05) for the treatment cows (Table 4.5). Klingerman *et al.* (2009) used VFA productions to evaluate the influence of amylase enzymes because they are the direct end products of rumen microbial fermentation. A dose-response linear increase in VFA production was found by Klingerman *et al.* (2009) with the supplementation of enzymes for flint and dent maize. In our study only propionic acid production was increased with the addition of the enzyme which also altered the VFA production ratio.

Table 4.5: Volatile fatty acid content (mmol/L) and profiles of rumen fluid of cows adapted to supplemental enzyme treatment and control cows.

	Volatile Fatty Acid of rumen fluid mmol/L (P<0.05)	
Testing Parameter	Control	Enzyme
Acetic (mmol/L)	95.2±7.33	105.7±7.33
Propionic (mmol/L)	36.8^{a}±5.49	53.8^{b}±5.49
Total VFA (mmol/L)	156.5±13.96	187.2±13.96
Acetic: Propionic	2.75^{a}±0.09	2.33^{b}±0.09

Means and standard errors are shown. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. [ab] Treatment means with different superscripts differ significantly within rows (P<0.05).

Table 4.5 compares the effect of additional enzyme treatment on the rumen production of VFA with a control. The present study found no significant response in total VFA and acetic acid. Although these results do not contradict the observations of Klingerman *et al.* (2009) regarding enzyme mixtures using primarily α-amylase activity, only propionic acid production and the propionate-acetate ratio increased significantly, indicating a change in the VFA profile rather than a linear improvement of total VFA production. This disagrees with Bach (2011) who reports no effects on total VFA production but shows a tendency for supplemental amylase enzyme to increase acetate production.

Tricarico *et al.* (2008) speculated that supplemental amylase enzyme elevates butyrate while reducing propionate in the rumen and hence, no increase in rumen starch digestion should be attributed to the amylase enzyme. Butyrate is the VFA mainly used for maintenance and, therefore, additional butyrate available in the body will supply the energy required to increase

rumen wall breadth and improve papillae length and capillary development (Weigand *et al*., 1975).

4.3.4 Seven-Hour In-Vitro *Starch Digestibility*

An *in-vitro* starch digestibility test can be used to measure the potential starch digestion of a product (Gallo *et al*., 2016). The test prescribes an incubation time of seven hours as it considers the time spent in the rumen and indicates the time of feeding when ruminal acidosis usually occurs (Krause & Combs, 2003; Allen, 2012).

The current study used the rumen fluid of the dairy cows that had already been adapted to the treatments (enzyme and control) in Phase Two to conduct a seven-hour *in-vitro* study for the starch digestion analysis of the three milling sizes of maize (CM, FM, and MMM) and the degradation analysis of finely ground lucerne (NDF). The results are shown in Table 4.6.

Table 4.6: Seven-hour maize (starch) and lucerne (NDF) degradability for both the control and enzyme treatment groups.

	Mean Seven-Hour Degradability of Starch (Maize) and NDF (Lucerne) (Starch/NDF Degraded [% DM Basis])		
Maize Particle Size (Starch Analysis)	**Control**	**Enzyme**	**Mean for Milling (P<0.05)**
Coarse Maize (>3mm)	16.72 ± 0.73	13.75 ± 0.73	15.24^{e} ± 0.51
Fine Maize (<3mm)	36.22 ± 0.54	34.93 ± 0.54	35.58^{d} ± 0.38
Micro-Milled Maize (<1mm)	40.43 ± 0.92	37.86 ± 0.92	39.14^{c} ± 0.65
Mean for Treatment	31.12^{a} ± 0.43	28.85^{b} ± 0.43	-
Lucerne (NDF Analysis)	**Control**	**Enzyme**	
Finely Milled Lucerne (NDF)	30.94^{a} ± 0.746	24.57^{b} ± 0.746	-

Means and standard errors are shown. Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. ^{ab} Treatment means with different superscripts differ significantly within rows (P<0.05). ^{cde} Treatment means with different superscripts differ significantly within columns (P<0.05).

As expected, significant differences among the milling sizes were observed (P>0.05). The digestibility ranged from the lowest level of 15.24% for coarse maize to 35.58% for fine maize and up to 39.14% for MMM. Gallo *et al*. (2016) concluded that fermentation kinetic parameters

and *in-vitro* starch digestibility of grains are mainly influenced by the particle size of the grain. The results of the present study confirmed these findings. Although carbohydrates can be described as fast or slow fermented, properties such as mean particle size can change the fermentation rate within ingredients. Hoffman *et al.* (2012) use mean particle size, ammonia-nitrogen, and prolamin content of maize to predict maize digestibility.

There were no differences in the seven-hour starch digestibility between the enzyme treatment groups and the control groups within the three different particle size groups ($P>0.05$). The seven-hour digestibility was 13.7% for coarse maize with enzyme versus the control at 16.72%, 34.93% for fine maize with enzyme versus the control at 36.22%, and 37.86% for MMM with enzyme versus the control at 40.43%.

The mean seven-hour starch digestibility across all maize milling sizes was lower for the enzyme-treated cows at 28.85% than the control at 31.12% ($P<0.05$). The seven-hour NDF digestibility of the lucerne sample followed the same pattern, with the enzyme group being significantly lower at 24.57% than the control group at 30.94% ($P<0.05$). It is unclear why these unexpected and low digestibility results were obtained for the enzyme-treated group since they are contrary to the results of studies by Rojo-Rubio *et al.* (2001), Mora-Jaimes *et al.* (2002), and Klingerman *et al.* (2009), and it is speculated that because more starch is available in the rumen, increasing propionate production, lead to a reduction of cellulolytic bacteria degrading NDF.

4.3.5 In-Sacco *Digestibility Study in a 24-hour Period*

The importance of determining ruminal digestion of starch versus total tract starch digestion has been indicated by Sauvant (1997). He stated that although slower starch degradation reduced the amount of microbial growth and fermentable organic matter, the efficiency of the microbial growth was unaltered. The author also noticed changes in the proportions of acetate to propionate and a higher rumen pH (Sauvant, 1997). A slower starch degradation rate increases DMI, but milk yield remains unchanged (Lechartier & Peyraud, 2011). However, milk fat and protein fluctuate with slow versus rapidly degradable starch feeding changes (Cabrita *et al.*, 2007).

Ruminal digestion per se can be used to limit the risk of rumen acidosis. The starch digestion site determines the end products (nutrients) of that digestion (Ramos *et al*. 2009). For starch degradation, the end product could be either VFAs or glucose. Volatile fatty acids are produced through microbial starch digestion in the rumen, but glucose is produced by enzymatic digestion in the small intestine and is utilised at a higher efficiency. Undegraded starch escaping the rumen varies from 5%–65% depending on the level in the diet, the DMI of the cow, the maize characteristics (e.g. vitreousness) (Rémond *et al*. 2004), and the processing of the grain (Huntington *et al*. 2006). The undegraded fraction escapes the rumen and is subjected to enzymatic digestion in the lower digestive system (Van Gastelen *et al*. 2020).

This study focused on maize and lucerne residues that were collected through rumen incubation at different times (15 min, 30 min, 60 min, 90 min, and 2, 4, 8, 12, 16, and 24 hours). Using the data, degradation curves assuming three different fractional passage rates (KpB) from the rumen of $0.02h^{-1}$, $0.05h^{-1}$ and $0.08h^{-1}$, as shown in Figure 4.6 (ED05) were drawn for each treatment as shown in Fig 4.6.

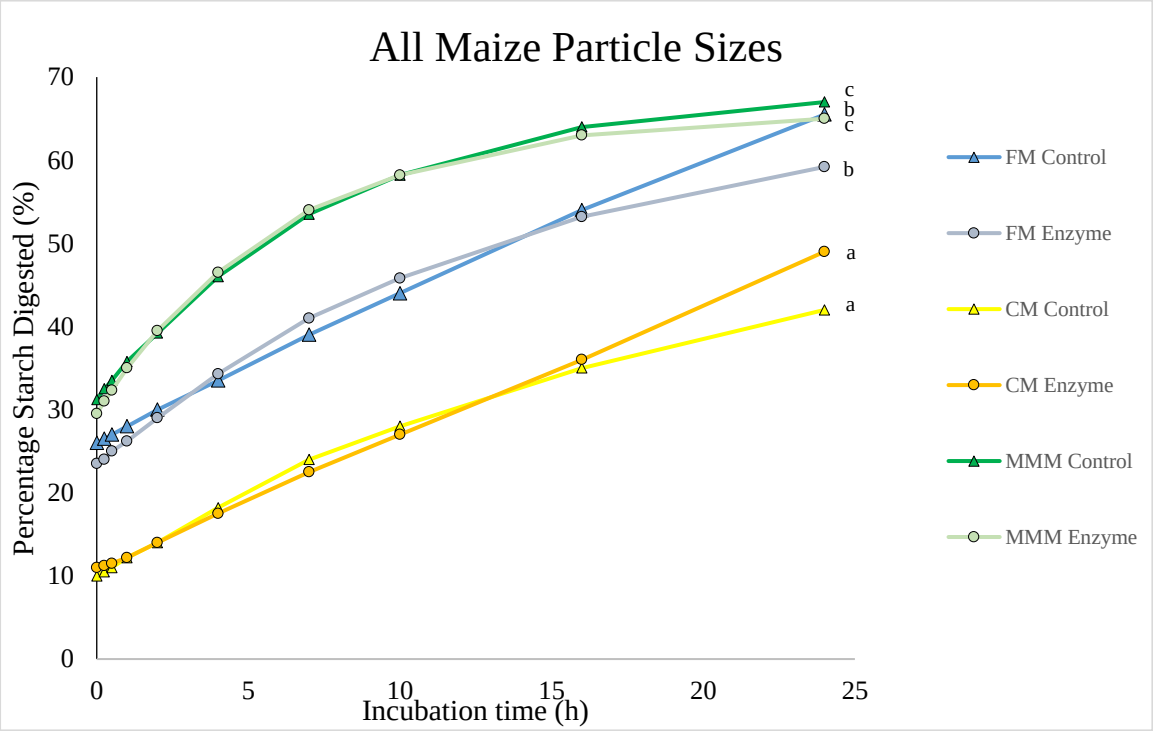

Figure 4.6: Degradation curves for maize at different particle sizes (coarsely ground, finely ground, and micro-milled). Data are presented as means ± SEM. ^{abc} Treatment means with different superscripts differ significantly (P<0.05).

The ratios between the quantities of starch that disappeared for each time period after placement of the sample into the rumen to the start-up amount of washed starch without insertion into the rumen were used as blanks and were calculated and plotted onto an Ørskov ruminal degradability graph to predict effective degradability at different digesta flow rates (Sveinbjörnsson *et al.*, 2007). No lag phase was detected for any of the digestion patterns.

The percentage *in-sacco* starch disappearance of CM over a 24-hour period shows the adjusted R^2 = 94.2% with the standard error of the mean (SER) = 0.0263. Comparing the curves for the enzyme treatment versus the control for CM shows the initial digestion to be equivalent. The degradation of maize in the enzyme-treated animals lagged behind the control group for approximately 10 hours. However, after a more extended retention period, the CM digested by the enzyme-treated animals showed continual improvement, up to 50% disappearance. In contrast, maize degradation in the control group tapered at approximately 40% disappearance. No differences were found in the fractional degradability of tested digesta flow rates ED02, ED05, or ED08 ($P>0.05$) between the control group and the enzyme-treated group for CM.

The percentage starch disappearance of FM over a 24-hour period shows the adjusted R^2 = 93.1 with SER = 0.0342. The degradation curve for the enzyme-treated group lagged behind the control group during the initial stages. After two hours, an improvement in degradation was observed in the enzyme-treated group. This tapered at approximately 15 hours with a 58% disappearance, while the starch disappearance in the control group was sustained beyond 60%. No differences were found in the fractional degradability of tested digesta flow rates ED02, ED05, or ED08 ($P>0.05$) between the control group and the enzyme-treated group for FM.

The percentage starch disappearance of MMM over a 24-hour period shows the adjusted R^2 = 77.7% with SER = 0.0684. The starch disappearance for both the control and the enzyme-treated groups with MMM followed the same degradation curve for up to 12 hours. Thereafter, the starch disappearance in the control group was higher than in the enzyme-treated group, with both tapering down at 65%. No differences were found in the fractional degradability of the tested digesta flow rates ED02, ED05, or ED08 ($P>0.05$) between the control group and the enzyme-treated group for MMM.

The percentage of NDF disappearance of lucerne over 24 hours shows the R^2 = 93.1% with SER = 0.029 (Figure 4.7). The lucerne degradation curve is similar to the curve for MMM

where both the enzyme-treated groups and the control groups follow the same trend, tapering at approximately 12 hours and 58% and with the enzyme-treated group being lower than the control group. No difference was found between the digestion pattern of the control group and the treatment group for NDF in lucerne (P>0.05).

These results support the results of Hristov (2008) who found no effects on NDF degradability, caused by supplementation with xylanase and amylase enzymes. However, these results are in agreement with the results of Zilio *et al*. (2019) who hypothesised that exogenous enzymes would increase NDF degradability and VFA production and would improve milk production and composition. On the contrary, Tricarico *et al*. (2005) and Andreazzi *et al*. (2018) found a positive effect on the performance of mid-lactation cows with the addition of exogenous amylaze enzymes.

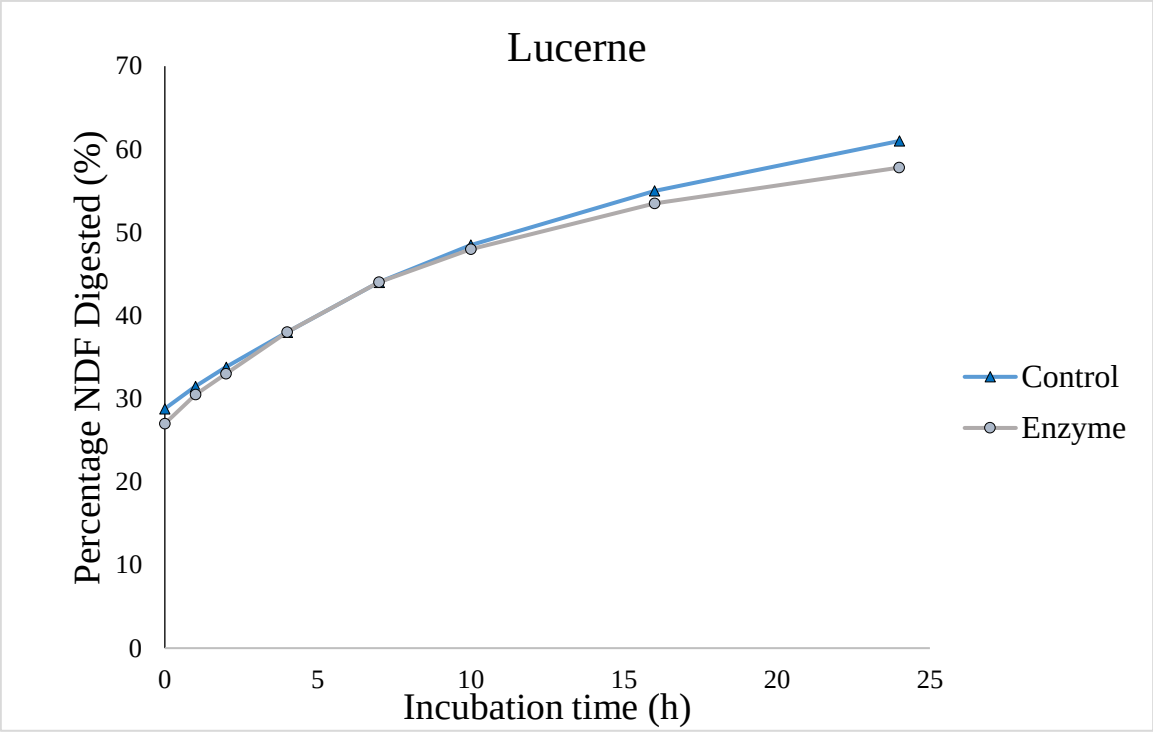

Figure 4.7: Digestion curve for lucerne treatment and control. Data are presented as means ± SEM. There is no significant difference between the control and enzyme treatment.

The starch digestibility, reported by the seven-hour *in-vitro* analysis and the seven-hour degradability, calculated from the 24-hour *in-sacco* assay, was compared using the degradation curves to calculate starch digestion at the seven-hour time period (Table 4.7). Increasing

particle size has a direct and adverse effect on the digestibility of starch. Contrary to the lower starch digestibility of the enzyme-treatment results obtained in the seven-hour *in-vitro* digestibility assay, no differences between the enzyme-treated groups and the control groups in the *in-sacco* degradability at seven hours were shown.

The analysed seven-hour *in-vitro* results and the calculated seven-hour *in-sacco* results showed the effect of maize particle size on starch digestibility. Degradability results for CM, FM, and MMM analysed by the seven-hour *in-vitro* analysis were 15.24%, 35.58%, and 39.14%, respectively (P<0.05) and calculated on seven hours in the 24-hour *in-sacco* trial were 15.17%, 25.92%, and 34.77%, respectively (P<005).

Table 4.7: Comparing the seven-hour *in-vitro* starch degradability analysis results with a calculated degradability at the seven-hour time point in the 24-hour *in-sacco* trial.

Maize Particle Size	Seven-Hour *In-Vitro*			*In-Sacco* Starch Degradability				
	Control	Treatment	Mean for Milling	Control		Treatment		Mean for Milling
				PSD	CSD	PSD	CSD	
CM	16.72	13.75	15.24^{e}	25	15.81	23	14.54	15.17^{e}
FM	36.72	32.93	35.58^{d}	39	24.66	41	25.92	25.29^{d}
MMM	40.43	37.86	39.14^{c}	55	34.77	55	34.77	34.77^{c}
Mean for Treatment	31.12^{a}	28.85^{b}	-		25.08		25.08	

PSD: Percentage of starch disappearance; CSD: Calculated starch digestibility; CM: Coarse Maize (>3mm); FM: Fine Maize (<3mm); MMM: Micro-Milled Maize (<1mm). Control: No enzyme added. Enzyme: Exogenous amylase enzyme added. ^{ab} Treatment means with different superscripts differ significantly within rows (P<0.05). ^{cde} Treatment means with different superscripts differ significantly within columns (P<0.05).

In-sacco starch digestibility was calculated at seven hours in the 24-hour run time and compared with the seven-hour *in-vitro* digestibility study. Chemical analyses determined the starch at time 0 = 63.22% and NDF at time 0 = 55.71%. The significance of each study (*in-vitro and in-sacco*) is confined within the analysis.

The results of both trials agree with Rémond *et al.* (2004) who found a 70% digestibility of starch in finely ground maize compared with a 54% digestibility of starch in coarse maize. Callison *et al*. (2001) noted that decreasing the maize particle size to 4.8 mm, 2.6 mm, and 1.2 mm affected the ruminal degradability of non-structural carbohydrates to 49.8%, 46.5%, and 87.0%, respectively. However, there was less improvement in TTSD because of post-ruminal digestion compensating for the lower digestion of coarse maize. The degree of starch access based on starch recovery by enzymatic hydrolysis was used by Blasel *et al.* (2006) to test the impact of maize particle size on starch degradability, and they found that for every 0.1 mm reduction in maize particle size, the degree of starch access increased by 26.8 g/kg starch. Fredin *et al*. (2015) confirmed increased degradability through processing maize to a finer particle size and showed increased propionate production and lower pH in the rumens of cows fed diets with a finer maize particle size. Gallo *et al.* (2016) calculated a ratio of 6.3% increase of *in-vitro* rumen starch degradability for every 1 mm reduction in maize particle size.

The data obtained by the *in-sacco* digestibility study over a 24-hour period are summarised in Table 4.8. No differences were found between the control groups and the supplemental enzyme-treated groups regarding the digestion kinetics (P>0.05). These included the soluble fraction A, the insoluble fraction B, and the calculated fractional degradability at all the tested digesta flow rates for the three maize particle sizes that were analysed. No interactions between treatment and maize particle size were detected (P>0.05).

Table 4.8: Starch degradability kinetics of coarse maize (>3mm), fine maize (<3mm), micro-milled maize (<1mm), and NDF degradability of lucerne with and without enzyme treatment.

Maize Particle Size	Fractions (%)	Control	Treatment
Coarse Maize (>3mm)	Soluble Fraction A	10.11 ± 0.89	10.51 ± 0.44
	Insoluble Degradable Fraction B	49.44 ± 14.62	61.82 ± 12.57
	Fractional Degradability ED02	41.81± 4.69	48.2 ± 4.20
	Fractional Degradability ED08	27.1 ± 1.09	28.4 ± 0.77
Fine Maize (<3mm)	Soluble Fraction A	24.92 ± 0.56	23.17 ± 0.57
	Insoluble Degradable Fraction B	47.78 ± 9.9	55.92 ± 11.52
	Fractional Degradability ED02	58.65 ± 4.46	61.13 ± 4.53
	Fractional Degradability ED08	44.6 ± 0.96	44.078 ± 0.61
Micro-Milled Maize (<1mm)	Soluble Fraction A	31.84 ± 0.56	28.58 ± 1.33
	Insoluble Degradable Fraction B	45.16 ± 12.449	38.25 ± 4.51
	Fractional Degradability ED02	66.03 ± 5.51	61.75 ± 3.20
	Fractional Degradability ED08	53.16 ± 1.19	53.16 ± 0.78
Means for Treatment across all particle sizes	Soluble Fraction A	22.29^{a} ± 0.45	20.75^{b} ± 0.45
	Insoluble Degradable Fraction B	47.60 ± 6.41	52.00 ± 6.41
	Fractional Degradability ED02	55.50 ± 2.56	57.00 ± 2.56
	Fractional Degradability ED08	41.52 ± 0.53	41.86 ± 0.53
Lucerne	Soluble Fraction A	29.02 ± 0.54	28.08 ± 0.67
	Insoluble Degradable Fraction B	44.2 ± 2.795	42.86 ± 9.65
	Fractional Degradability ED02	61.08 ± 0.75	58.34 ± 3.36
	Fractional Degradability ED08	47.09 ± 0.97	45.93 ± 0.52

Means and standard errors are shown. Fraction A: Highly degradable water-soluble fraction; Fraction B: Insoluble, but potentially slowly degradable fraction; Fractional Degradability ED02: Degradable fraction at a particle passage rate of 2% per hour; Fractional Degradability ED08: Degradable fraction at a particle passage rate of 8% per hour.

[ab] Treatment means with different superscripts differ significantly within rows (P<0.05).

The soluble fraction A for the mean of all maize particle sizes was lower for the enzyme-treated groups (20.75%) than for the control groups (22.29%) ($P<0.05$), showing that the enzyme treatment resulted in a less soluble starch fraction across the particle sizes ; however, this is of little significance. For the mean insoluble degradable fraction B (47.60% and 52.00%) or for any of the fractional degradability digesta flow rates (means for ED02 of 55.50% and 57.00% and means for ED08 of 41.52% and 41.86%), no differences were detected between the control groups and the enzyme groups ($P>0.05$). The amylase enzyme appeared to slow the degradation rate down in the very early stages of digestion (Figure 4.6). This may be the result of the cross-feeding effect caused by amylose degraded to oligosaccharides rather than maltose (Tricarico *et al*., 2007; Salfer *et al.* 2021). Theoretically, it also may indicate a short-term reduction in rumen pH below the 5.6 threshold level directly after feeding, as increased VFAs are produced when more starch is available (Cone, 1990). This is in agreement with Zinn *et al.* (1995), Philippeau *et al*., (1999), Yang *et al.* (2001), and Rowe *et al.* (1999) who demonstrated that increased starch fermentation rates due to processing could result in a decline in rumen pH.

The ED calculated at three digesta flow rates (ED02, ED05, and ED08) showed no significant differences when the supplemental enzyme was added (Table 4.8). The effective NDF digestibility of lucerne was similar in the supplemental enzyme-treated group and the control group ($P>0.05$).

The results of the current study contradict the findings of Gencoglu *et al*. (2010) and Engstrom (2013). However, the current study agrees with Tricarico *et al*. (2005) and Hristov *et al*. (2008) who found no difference in starch degradation in the rumen when supplementing the diets with amylase. Klingerman *et al*. (2009) report that adding exogenous amylase enzyme to the diet of dairy cows increases starch degradability, probably reducing rumen pH and acetate production while increasing propionate production, which would result in reduced milk fat production (Allen, 1997). Tricarico *et al*. (2005) report that adding amylase enzyme to an average starch diet does not affect ruminal digestibility. Tricarico *et al*. (2007) found that adding exogenous amylase enzyme to the diet of dairy cows increased ruminal acetate and butyrate and decreased propionate proportionally. The authors suggest that a cross-feeding mechanism is initiated by the amylase enzyme, causing the hydrolysis of starch to maltodextrins that are used by both amylolytic and non-amylolytic bacteria, and thus modifying the VFA production pattern

(Tricarico *et al*., 2007). Gencoglu *et al.* (2010) thought that this might be why adding exogenous enzymes to a reduced starch diet does not affect milk fat.

Although no differences between the control groups and the treatment groups were found in terms of starch degradability, an apparent effect of the maize particle size on the fractional degradability was observed in the 24-hour *in-sacco* digestion assay (Table 4.9).

Table 4.9: The means of milling for starch degradability kinetics of maize with different particle sizes with and without enzyme treatment.

	Starch Degradation Kinetics: Means for Milling				
Maize Particle Size	**Soluble Fraction A (%)**	**Insoluble Degradable Fraction B (%)**	**Fractional Degradability ED02 (%)**	**Fractional Degradability ED05 (%)**	**Fractional Degradability ED08 (%)**
Coarse Maize (>3mm)	$10.31^{c} \pm 0.56$	55.90 ± 7.85	$45.00^{b} \pm 3.14$	$33.50^{c} \pm 1.23$	$27.75^{c} \pm 0.65$
Fine Maize (<3 mm)	$24.04^{b} \pm 0.56$	51.80 ± 7.85	$55.99^{b} \pm 3.14$	$49.32^{b} \pm 1.23$	$43.81^{b} \pm 0.65$
Micro-Milled Maize (<1mm)	$30.21^{a} \pm 0.56$	41.70 ± 7.85	$63.90^{a} \pm 3.14$	$57.46^{a} \pm 1.23$	$53.50^{a} \pm 0.65$

Mean percentages and standard errors are shown. ^{abc} Treatment means with different superscripts differ significantly within columns ($P<0.05$).

The milling size of the maize particles significantly influenced the soluble fraction of the maize particles ($P<0.05$). The mean soluble fraction A was 10.31% for CM, 24.04% for FM, and 30.21% for MMM. No significant differences were found for the influence of milling size on the insoluble fraction B (55.90% for CM, 51.80% for FM, and 41.70% for MMM) ($P>0.05$). The fractional degradability among the means of the different particle sizes clearly shows the impact of particle size on starch digestibility in the rumen. Coarser particles degrade slower and less than finer particles.

At the slowest assumed passage rate of $0.002h^{-1}$, the fractional degradability of MMM (ED02 = 63.90%) was higher than for CM and FM (ED02 = 45.00% and 55.99%, respectively) ($P<0.05$). At faster assumed digesta flow rates, the difference becomes more pronounced, with the fractional degradability of all three particle sizes differing significantly. The highest fractional degradability (ED08) for MMM was 53.50%, for FM was 43.81%, and for CM was

27.85%. This shows that at a slower passage rate, the particle size of the maize has a negligible influence on degradability. At a faster passage rate, normally observed in high-producing cows (Allen, 2000), milling the grain component to a smaller particle size improves the degradability. This confirms statements from Huntington (1997), McAllister *et al.* (2001), and Owens *et al*. (2009) on the influence of kernel processing on maize digestibility.

The data of the present study reflect an insoluble fraction (Table 4.9) for CM of 33.8%, an insoluble fraction for FM of 24.16%, and an insoluble fraction for MMM of 28.09%, as calculated using the equation above. The insoluble fraction for CM is expected to be higher than for FM. The higher insoluble fraction for MMM could be the result of grain overprocessing. A number of studies described the effects of overprocessing grain, resulting in a torrent of VFAs and causing the rumen pH to drop to below the threshold of 5.5, which causes hypertonicity of the rumen, increased lactate production, propionate flooding of the liver, and other metabolic distress (Hindle *et al*. 2005; Rémond *et al*. 2004; Gozho & Mutsvangwa, 2007; Gallo *et al*., 2016).

4.4 General discussion

It will be economically and highly beneficial to manipulate and improve the efficiency of starch digestion in the rumen because it constitutes one of the significant fractions of the diets that are fed to high-producing cattle (Tricarico *et al.*, 2008). Hristov *et al*. (2008) demonstrated that using feed additives such as enzymes could have considerable potential in achieving this.

No significant differences were found in milk yield with the enzyme treatment or the different particle sizes of the maize component in the feed (Table 4.1). Of the milk composition components, milk fat was not significantly influenced by either the supplementation of the amylase enzyme or the particle size of the maize component in the feed (Figure 4.1). However, a significant interaction was found between enzyme treatment and particle size. The feeding of coarse maize to the cows resulted in higher milk fat production in the control group, but the effect was suppressed when the amylase enzyme was added (Figure 4.1). Milk protein was analysed as an indicator of changes in energy supply with the different treatments. As expected, only particle size significantly affected the milk protein, and finer particle size resulted in an increased protein percentage in the milk (Figure 4.2). Based on published literature, more energy for milk production is released when finer ground maize is fed because of the larger

surface area and a more exposed starch structure, causing rumen microbes to break down and use the starch more effectively (Doorenbos *et al*., 2017).

Only the particle size of the maize component in the diet significantly affected manure starch content, (Figure 4.3) showing less starch throughflow with finer ground particles. Manure NDF was significantly affected by the particle size and interaction with the enzyme (Table 4.3). Overprocessing effects seemed to reduce the manure NDF content of MMM in conjunction with the supplemental enzyme. A reduction in manure protein with the addition of the amylase enzyme to the diet might indicate a reduction in hindgut fermentation (Table 4.4). Enzyme treatment did not affect total VFA production (Figure 4.5), but the propionic acid and the ratio of acetic acid to propionic acid were altered. Propionic acid production increased nearly twofold with the supplemental enzyme treatment, which led to the ratio of acetate to propionate decreasing significantly (Table 4.5).

Both the statements that particle size mainly influences starch digestibility for flint and dent maize (Gallo *et al.,* 2016) and the speculation that maize degradability, can be predicted by particle size, (Hoffman *et al.*, 2012) were confirmed by the seven-hour *in-vitro* digestibility trial (Table 4.6), showing a doubling of degradability from 15.25% for CM to 35.58% for FM. Furthermore, MMM had an even higher degradability of 39.14%. The seven-hour *in-vitro* digestibility assay of the maize with supplementary enzyme treatment (28.85%) was lower than the control (31.12%). However, in the *in-sacco* digestion trial, the derived seven-hour digestibility from the Ørskov degradability graphs showed no difference between the enzyme-treated groups and the control groups (Table 4.7). The milling size of the maize particles significantly influenced the soluble fraction of the maize particles. (Table 4.9) Coarser particles degrade slower and less than finer particles. At faster assumed digesta flow rates, the difference becomes more pronounced, with the fractional degradability of all three particle sizes differing significantly.

The supplemental enzyme seems to have had a negative impact on the soluble fraction A (Table 4.8). According to Cone (1990), Zinn *et al.* (1995), and Yang *et al.* (2001), it is possible that the enzyme treatment caused a pH drop below the threshold, thus limiting digestion. The insoluble fraction B seems to be improved by the supplemental enzyme treatment in MMM, but none of the starch digestibility kinetics were significantly different when the enzyme-treated cows were compared with the control cows. Contrary to the findings of Gencoglu *et al.*

(2010) and Engstrom (2013), the effective NDF degradability did not differ significantly between the enzyme-treated cows and the control cows. The current study is in agreement with the work done by Tricarico *et al.* (2005) with a supplemental exogenous amylase enzyme and Hristov *et al.* (2008) who found no significant results with amylase, xylanase, or a combination enzymatic supplement. The particle size of the maize significantly affected the soluble fraction A; this was expected to increase with the amount of processing. The particle size did not affect the insoluble fraction B.

Chapter 5

5. Conclusion

Many factors affect starch digestion, including the (i) type of grain, which dictates the endosperm content and structure (Allen *et al*., 2008), (ii) the particle size of the grain kernels (Callison *et al*., 2001; Hoffman *et al*., 2012; Gallo *et al.*, 2016; Ahmadi *et al*., 2020) and the processing methods used (Ferraretto *et al.*, 2013), (iii) the maturity of the grain kernel (Erasmus, 2003; Ngonyamo-Majee *et al.*, 2008) which influences the moisture content, (iv) the conservation of the plant structure against fungi and pests, and (v) factors pertaining to the digestion of the animal feeding on the grain kernel (Owens *et al*., 1997). An interaction exists between the two primary reasons for indigestibility in maize, (i) amylose-to-amylopectin ratio (Sajilata *et al*., 2006; Stevnebø *et al*., 2006; Owens & Soderlund, 2006) and (ii) prolamin structure protection (Hoffman & Shaver, 2010).

Although no improvement in milk production was observed with the different maize particle sizes, the increased milk protein and decreased faecal starch for the MMM group, in contrast with the CM and FM groups, indicate that more starch was digested and, therefore, more energy was available for milk protein production. The seven-hour digestibility and the fractional degradability in the *in-sacco* trial confirm this trend and indicate how a change in digesta flow rates would alter the importance of particle size. At a slow digesta flow rate, the CM and FM fractional degradability were similar, but as the flow rate increased, the starch digestion of CM was lower than that of FM. Enzyme supplementation also did not improve milk production, the reduction in milk fat compared with the control, the increase in propionic acid, the reduced acetate-to-propionate ratio, and the reduction of faecal protein are indications that ruminal starch degradation was improved. The *in-vitro* and *in-sacco* analyses did not mirror this improvement.

The above-mentioned factors affecting starch digestibility, as well as environmental and management factors, and the physiological state of the animal, influence this interaction. A better understanding of these factors and their interactions would benefit precision formulation feeds for milk production and cow longevity. More research is needed to understand all the factors affecting production responses and the mode of action of the abovementioned enzymes in order to utilise them for the benefit of the animal and the producer.

Chapter 6

6. Critical Evaluation

Although starch disappearance in the rumen is generally considered an indicator of starch digestion, in view of the literature review by Cabrita *et al.* (2006), microbial production should be viewed as an indicator of effective energy supply to the rumen.

Using only four cows for the *in-sacco* study was limiting. Although a cross-over design was used to maximise the efficiency of data collecting, the stability of the data for statistics would have been improved by repeating the full cross-over trial a second time. There would have been an improvement in the statistical reliability if measured effects of the cultivars could have been controlled or distinguished from the main features (i.e. GM or non-GM or BT maize; according to vitreousness; or samples grown in a controlled environment and tested in a broader but less variable setup).

Particle size and grinding is not a singular and exchangeable term and depends on the equipment used and the characteristics of the grain. Fragility and shear factors are also part of the influence. Comparing sizes with a difference of 1 mm between them but grinding through a screen that allows everything up to the screen size is polluting the issue. For this trial, the MMM was wet-milled to achieve the precise 1 mm particle size, but the CM and FM that were used were divided by all particles on top of a 3 mm sieve (CM) and all particles below (FM).

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APPENDICES

Appendix A: Ethical Clearance

This research trial was approved by the Department of Animal and Wildlife Sciences Research Committee and the University of Pretoria Ethics Committee, Approval Number EC048-17.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Determining the effect and interaction of any amylase enzyme and grinding size of the maize component on ruminal and total tract starch digestibility**
PROJECT NUMBER	**EC048-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**C Engelbrecht**

STUDENT NUMBER (where applicable)	**U_84384205**
DISSERTATION/THESIS SUBMITTED FOR	**MSc (Agric) Nutrition**

ANIMAL SPESIES	**Holstein cows**	
NUMBER OF ANIMALS	**4**	
Approval period to use animals for research/testing purposes		**July 2017- July 2018**
SUPERVISOR	**Prof. LJ Erasmus**	

KINDLY NOTE:

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

APPROVED	Date 31 July 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix B: Registration Certificate for Ronozyme® Rumistar 600 (CT) as a Product by DSM Nutritional Products SA (Pty) Ltd.

agriculture, forestry & fisheries

Department:
Agriculture, forestry & fisheries
REPUBLIC OF SOUTH AFRICA

FOR OFFICIAL USE ONLY

CERTIFICATE OF REGISTRATION RENEWAL: FARM FEEDS

FERTILIZERS, FARM FEEDS, AGRICULTURAL REMEDIES AND STOCK REMEDIES ACT, 1947 (ACT NO. 36 OF 1947)

1. This is to certify that the farm feed mentioned below and the label attached hereto comply with the requirements of Act No. 36 of 1947 and the regulations promulgated there-under and that it has been registered by me:

   1.1 registration Number awarded..... **V25432**

   1.2 Name of Farm Feed..... **Ronozyme RumiStar 600 (CT)**

   1.3 Name of Registration ..... **DSM Nutritional Products SA (Pty) Ltd**

2. This registration is subject to the following conditions:

   2.1 That the registration is valid until **31 March 2020**

   2.2 That only facsimiles of the attached approved label may be used.

   2.3 The type and container size must conform to the sizes as stated in the application form.

   2.4 That certificate of analyses for this farm feed is submitted to the office of the Registrar every six months.

   2.5 That the registered composition must not be changed without the approval of the Registrar.

   2.6 That the details approved for use on a label or immediate container for sale may not be altered without the approval of the Registrar.

   2.7 That the printed labels, cartons, pamphlets and package inserts be submitted within 2 (two) months from the date of registration in duplicate.

   2.8 That all adverse effects, including adverse reactions, toxicity, misuse, formulation deviation or any other undesirable effect caused by this product must be reported in writing immediately to the Registrar: Act No. 36 of 1947 by the registration holder.

3. 3.1 That the Registrar's office shall not issue the reminder notice for renewal of the registrations and instead such information shall be posted on the website of the department.

   3.2 That the registration holder may apply to renew the registration four (4) months prior to its date of expiry.

4. The granting of this registration does not exempt anybody from the requirements of any other Law.

Dept van Landbou, Bosbou en Visserye
Privaatsak/Private bag x343
2017 -03- 03
Dept of Agriculture, Forestry and Fisheries

DATE

REGISTRAR: ACT NO. 36 OF 1947

Appendix C: Appendix Tables

Table C.1: Ruminal pH values collected twice daily from cows receiving either a control buffer solution or an enzyme treatment with buffer solution for a period of 25 days.

Day	Morning			Afternoon		
	Control	Rumistar	Day Mean	Control	Rumistar	Day Mean
1	6.379^{a}±0.306 (n$^{\#}$=2)	6.596^{a}±0.306 (n=2)	6.596^{defghij}±0.216 (n=4)	5.750^{a}±0.184 (n=4)	5.775^{a}±0.184 (n=4)	5.763^{gh}±0.184 (n=4)
2	6.250^{a}±0.245 (n=4)	6.475^{a}±0.245 (n=4)	6.362^{ghijkl}±0.173 (n=8)	5.775^{a}±0.184 (n=4)	5.800^{a}±0.184 (n=4)	5.788^{fgh}±0.184 (n=4)
3	6.375^{a}±0.245 (n=4)	6.600^{a}±0.245 (n=4)	6.487^{efghijkl}±0.173 (n=8)	5.725^{a}±0.184 (n=4)	5.600±0.184 (n=4)	5.663^{gh}±0.184 (n=4)
4	6.475^{a}±0.245 (n=4)	6.100^{a}±0.245 (n=4)	6.288^{ijkl}±0.173 (n=8)	5.725^{a}±0.184 (n=4)	5.700^{a}±0.184 (n=4)	5.713^{gh}±0.184 (n=4)
5	6.350^{a}±0.245 (n=4)	6.150^{a}±0.245 (n=4)	6.250^{jkl}±0.173 (n=8)	5.825^{a}±0.184 (n=4)	5.700^{a}±0.184 (n=4)	5.763^{gh}±0.184 (n=4)
6	6.400^{a}±0.245 (n=4)	6.350^{a}±0.245 (n=4)	6.375^{ghijkl}±0.173 (n=8)	6.025^{a}±0.184 (n=4)	5.850^{a}±0.184 (n=4)	5.938^{defg}±0.184 (n=4)
7	6.725^{a}±0.245 (n=4)	6.700^{a}±0.245 (n=4)	6.713^{defg}±0.173 (n=8)	5.935^{a}±0.184 (n=4)	5.927^{a}±0.184 (n=4)	5.931^{defg}±0.184 (n=4)
8	6.575^{a}±0.245 (n=4)	6.575^{a}±0.245 (n=4)	6.575^{defghijk}±0.173 (n=8)	5.625^{a}±0.184 (n=4)	5.500^{a}±0.184 (n=4)	5.563^{h}±0.184 (n=4)
9	6.375^{a}±0.245 (n=4)	6.300^{a}±0.245 (n=4)	6.338^{hijkl}±0.173 (n=8)	5.998^{a}±0.184 (n=4)	5.750^{a}±0.184 (n=4)	5.874^{defg}±0.184 (n=4)
10	6.750^{a}±0.245 (n=4)	6.725^{a}±0.245 (n=4)	6.737^{def}±0.173 (n=8)	5.700^{a}±0.184 (n=4)	5.875^{a}±0.184 (n=4)	5.788^{fgh}±0.184 (n=4)
11	6.500^{a}±0.245 (n=4)	6.350^{a}±0.245 (n=4)	6.425^{fghijkl}±0.173 (n=8)	5.850^{a}±0.184 (n=4)	5.800^{a}±0.184 (n=4)	5.825^{efgh}±0.184 (n=4)
12	6.475^{a}±0.245 (n=4)	6.775^{a}±0.245 (n=4)	6.625^{defghi}±0.173 (n=8)	5.925^{a}±0.184 (n=4)	6.325^{a}±0.184 (n=4)	6.125^{cd}±0.184 (n=4)
13	6.950^{a}±0.245 (n=4)	6.575^{a}±0.245 (n=4)	6.763^{def}±0.173 (n=8)	6.075^{a}±0.184 (n=4)	6.025^{a}±0.184 (n=4)	6.050^{cdef}±0.184 (n=4)
14	6.750^{a}±0.245 (n=4)	7.025^{a}±0.245 (n=4)	6.888^{bcd}±0.173 (n=8)	6.053^{a}±0.184 (n=4)	6.107^{a}±0.184 (n=4)	6.080^{cde}±0.184 (n=4)
15	6.605^{a}±0.245 (n=4)	6.700^{a}±0.245 (n=4)	6.652^{defgh}±0.173 (n=8)	6.088^{a}±0.184 (n=4)	6.075^{a}±0.184 (n=4)	6.081^{cde}±0.184 (n=4)
16	6.675^{a}±0.245 (n=4)	6.675^{a}±0.245 (n=4)	6.675^{defgh}±0.173 (n=8)	6.213^{a}±0.184 (n=4)	6.057^{a}±0.184 (n=4)	6.135^{cd}±0.184 (n=4)
17	6.825^{a}±0.245 (n=4)	6.850^{a}±0.245 (n=4)	6.838^{cde}±0.173 (n=8)	6.178^{a}±0.184 (n=4)	6.280^{a}±0.184 (n=4)	6.229^{bc}±0.184 (n=4)
18	7.230^{a}±0.245 (n=4)	7.007^{a}±0.245 (n=4)	7.119^{abc}±0.173 (n=8)	6.445^{a}±0.184 (n=4)	6.452^{a}±0.184 (n=4)	6.449^{ab}±0.184 (n=4)
19	7.298^{a}±0.245 (n=4)	7.495^{a}±0.245 (n=4)	7.396^{a}±0.173 (n=8)	6.383^{a}±0.184 (n=4)	6.467^{a}±0.184 (n=4)	6.425^{ab}±0.184 (n=4)
20	7.025^{a}±0.245 (n=4)	7.425^{a}±0.245 (n=4)	7.255^{ab}±0.173 (n=8)	6.633^{a}±0.184 (n=4)	6.375^{a}±0.184 (n=4)	6.504^{ab}±0.184 (n=4)
21	7.250^{a}±0.245 (n=4)	7.100^{a}±0.245 (n=4)	7.125^{abc}±0.173 (n=8)	6.605^{a}±0.184 (n=4)	6.587^{a}±0.184 (n=4)	6.596^{a}±0.184 (n=4)
22	7.135^{a}±0.245 (n=4)	7.275^{a}±0.245 (n=4)	7.214^{ab}±0.173 (n=8)	6.525^{a}±0.184 (n=4)	6.524^{a}±0.184 (n=4)	6.475^{ab} ±0.184 (n=4)
23	7.250^{a}±0.245 (n=4)	7.250^{a}±0.245 (n=4)	7.250^{a}±0.173 (n=8)	6.700^{a}±0.184 (n=4)	6.537^{a}±0.184 (n=4)	6.619^{a}±0.184 (n=4)
24	7.505^{a}±0.245 (n=4)	7.325^{a}±0.245 (n=4)	7.188^{abc}±0.173 (n=8)	5.875^{a}±0.184 (n=4)	5.800^{a}±0.184 (n=4)	5.838^{efgh}±0.184 (n=4)
25	5.736^{a}±0.306 (n=2)	6.576^{a}±0.267 (n=3)	6.156^{jl}±0.203 (n=5)	5.959^{a}±0.184 (n=4)	6.439^{a}±0.184 (n=4)	6.199^{bcd}±0.184 (n=4)
Means	6.697^{a}±0.171	6.768^{a}±0.171		6.063^{a}±0.119	6.049^{a}±0.119	

These data are presented as pH values ± standard errors. Differences between means within rows and columns are denoted by no letters in common (P<0.05). Sample sizes (n) are indicated in parentheses for each observation.

Table C.2: Composition of the artificial saliva used to introduce the enzyme into the rumen of test cows.

Chemical	Amount
$NaHCO_3$	19.6 g
Na_2HPO_4	7.42 g
KCL	1.14 g
NaCl	0.94 g
$MgSO_4 \cdot 7H_2O$	0.24 g
$CaCl_2$	0.08 g
Made up to 2 L with laboratory-grade distilled water	

Table C.3: Composition of the preserving solution used to preserve rumen fluid for VFA analysis.

Chemical	Amount
$HgCl_2$	2 g
H_3PO_4	20 ml
$(CH_3)_2CHCH_2CH_2CO_2H$	2 g
Made up to 1 L with laboratory-grade distilled water	

Modified from Jouany (1982).

Table C.4: Composition of the buffer solutions added to the control cows and mixed with supplemental exogenous amylase enzyme for the treatment cows.

Buffer	Chemical	Amount
Buffer Solution A	KH_2PO_4	10.0 g
	$MgSO_4 \cdot 7H_2O$	0.5 g
	NaCl	0.5 g
	$CaCl_2 \cdot 2H_2O$	0.1 g
	Urea (reagent grade)	0.5 g
Buffer Solution B	Na_2CO_3	15.0 g
	$Na_2S \cdot 9H_2O$	1.0 g

The buffer solution was made according to McDougall (1948).

Description of *In-Vitro* True Digestibility using the DAISY^{II} Incubator (Gargallo *et al.*, 2006)

The apparatus used included the following:

a) DAISY^{II} Incubator
b) Filtration device: F57 Filter Bags
c) Impulse bag sealer: 1915/1920 Heat Sealer
d) Thermos
e) ANKOM $^{200/220}$ Fiber Analyzer

The *in-vitro* procedure was conducted as described below.

Preparation of Filter Bags and Sample

The F57 filter bags were pre-rinsed in acetone for three to five minutes and thoroughly air dried. The acetone rinse removes a surfactant that inhibits microbial digestion. Each F57 filter bag was weighed and recorded as (W_1). The balance was zeroed, and 0.25 g of sample (W_2) was weighed directly into the filter bag. The bag was heated, sealed closed, and placed in the DAISY^{II} Incubator digestion jar. The digestion jar could take up to 25 samples per jar, and samples were evenly distributed on both sides of the digestion jar divider. At least one sealed blank bag was included as a correction factor (C_1).

Preparation of Combined Buffer Solution (Buffer Solution A + Buffer Solution B)

For each digestion jar:

a) Both buffer solutions (A and B) were preheated to 39^{0}C. Approximately 266 ml of solution B was added to 1 330 ml of solution A (1:5 ratio) in a separate container. A and B amounts should be adjusted to obtain a final pH of 6.8 at 39^{0}C. However, no further adjustment of pH was necessary. Each digestion jar contained approximately 1 600 ml of the combined A/B mixture.
b) The digestion jars with the samples and buffer solution were placed into the DAISY^{II} Incubator, and the heat and agitation switches were turned on. The temperature of the digestion jars was allowed to equilibrate for at least 20–30 minutes.

Preparation of Inoculum and Incubation:

All glassware was maintained at 39^{0}C.

a) Two 2 L thermos bottles were preheated by filling them with 39^{0}C water. The containers were emptied just before the collection of the rumen inoculum. Using the appropriate collection procedure, at least 2 000 ml of rumen inoculum was removed

and placed in the thermos. Approximately two 'fistfuls' of the fibrous mat from the rumen was included with the rumen inoculum in the one thermos.

b) The blender was preheated by filling it with 39^0C water. The heated water was emptied from the container just prior to pouring the rumen inoculum from the thermos into the blender. The blender container was purged with CO_2 gas and blended at high speed for 30 seconds. The blending action dislodges microbes attached to the mat and assures a representative microbial population for the *in-vitro* fermentation. The blended digesta was filtered through four layers of cheesecloth into a 5 L flask (preheated to 39^0C). The remaining rumen fluid was filtered from the second thermos through four layers of cheesecloth into the same 5 L flask. The flask was continually purged with CO_2 throughout the inoculum transfer.

c) Six fibre bags were prepared per sample, and two repeats were run, giving 12 digestions per sample. This allowed us to obtain sufficient highly digestible samples to analyse after incubation.

d) One digestion jar was removed from the DAISY^{II} Incubator, and samples were incubated in a mixture of 1 L of freshly collected rumen fluid and 1 L of McDougall's artificial saliva buffer (McDougall, 1948) under anaerobic conditions at 39ºC for seven hours with constant rotation in a DAISY^{II} Incubator. The digestion jar was purged with CO_2 gas for 30 seconds, and the lid was secured.

e) The process was repeated for all the jars.

f) Samples were incubated for seven hours. A variation in the jar's temperature of greater than one degree was countered by moving the incubator to a warmer location or placing a blanket or similar insulator over the incubator.

g) After incubation, the jars were removed, and the fluid was drained. Bags were thoroughly rinsed with cold tap water until the water ran clear. Sample digestions were weighed, and the duplicates were pooled and analysed in order to get an average starch or NDF value per repeat.

h) To determine the true digestibility, the removal of microbial debris and any remaining soluble fractions was necessary. This was performed using Neutral Detergent Solution. After rinsing the bags in water, each bag was placed in the ANKOM $^{200/220}$ Fiber Analyzer, and the procedure for determining NDF was followed. Post-*in-vitro* weight was recorded as W_3.

Calculations:

%IVTD (as received basis) = $\frac{100 - (W_3 - (W_1 \times C_1))}{W_2} \times 100$

%IVTD$_{DM}$ (DM Basis) = $\frac{100 - (W_3 - W_1 \times C_1))}{(W_2 \times DM)} \times 100$

Where: W1 = Bag tare weight

W2 = Sample weight

W3 = Final Bag weight after *in-vitro* and sequential ND treatment

C1 = Blank bag correction (final oven-dried weight/original blank bag weight)

Table C.5: Effect of maize particle size and enzyme supplementation on mean milk fat percentage.

	Mean Milk Fat Percentage		
Maize Particle Size	**Control**	**Enzyme**	**Mean for Milling**
Coarse Maize (>3mm)	$4.28^{a \pm}$ 0.2	$3.40^{c} \pm 0.2$	$3.84^{a} \pm 0.2$
Fine Maize (<3 mm)	$3.69^{bc} \pm 0.2$	$3.99^{ab} \pm 0.2$	$3.85^{a} \pm 0.1$
Micro-Milled Maize (<1mm)	$4.08^{ab} \pm 0.2$	$4.07^{ab} \pm 0.2$	$4.08^{a} \pm 0.1$
Mean for Treatment	$4.02^{a} \pm 0.1$	$3.82^{a} \pm 0.1$	-

Mean percentages and standard errors are shown. Differences between means within rows and columns are denoted by no letters in common (P<0.05).

Table C.6: Effect of maize particle size and enzyme supplementation on mean milk protein percentage.

	Mean Milk Protein Percentage		
Maize Particle Size	**Control**	**Enzyme**	**Mean for Milling**
Coarse Maize (> 3mm)	$2.94^{a \pm}$ 0.01	$2.93^{a} \pm 0.05$	$2.93^{b} \pm 0.04$
Fine Maize (< 3 mm)	$2.95^{a} \pm 0.05$	$2.90^{a} \pm 0.05$	$2.93^{b} \pm 0.03$
Micro-Milled Maize (< 1mm)	$3.16^{a} \pm 0.03$	$3.07^{a} \pm 0.04$	$3.12^{a} \pm 0.03$
Mean for Treatment	$3.02^{a} \pm 0.03$	$2.97^{a} \pm 0.03$	-

Mean percentages and standard errors are shown. Differences between means within rows and columns are denoted by no letters in common (P<0.05).

Table C.7: Effect of maize particle size and enzyme supplementation on the mean manure starch content.

Maize Particle Size	Mean Manure Starch Content		
	Control	Enzyme	Mean for Milling (P<0.05)
Coarse Maize (>3mm)	4.02[a] ± 0.44	4.14[a] ± 0.44	4.08[a] ± 0.31
Fine Maize (<3 mm)	3.19[a] ± 0.27	3.66[a] ± 0.27	3.42[a] ± 0.19
Micro-Milled Maize (<1mm)	2.60[a] ± 0.26	1.93[a] ± 0.27	2.26[b] ± 0.19
Mean for Treatment	3.27[a] ± 0.19	3.24[a] ± 0.19	-

Means and standard errors are shown. Differences between means within rows and columns are denoted by no letters in common (P<0.05).