THE EFFECT OF DIFFERENT ENERGY AND NITROGEN SOURCES ON *IN VITRO* FIBRE DIGESTION AND GAS PRODUCTION KINETICS OF HIGH AND LOW QUALITY FORAGES

by
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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the faculty of Animal Science at Stellenbosch University

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Declaration

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December
2016

Abstract

Title: The effect of different energy and nitrogen sources on *in vitro* fibre digestion and gas production kinetics of high and low quality forages
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Digestibility of fibre from the roughage component of total mixed rations for dairy cows can be influenced by the source of supplemental energy and nitrogen included in the ration. By optimizing the digestion of fibre in the rumen, dairy cows can utilize the potential nutrients in roughages to its best, and thereby obtain more substrates for optimal production.

This thesis reports on two *in vitro* studies aimed to improve forage digestion. In the first study, a high quality forage (lucerne hay) and a poor quality forage (wheat straw) were incubated *in vitro* with the filter bag method with an energy source, being either maize, citrus pulp or molasses syrup and a nitrogen source, being either soybean meal, urea or no added N. The forage samples were weighed out to provide 125 mg NDF and transferred to Ankom filter bags. Bags were heat sealed and incubated in Erlenmeyer flasks for six and 30 hours at 39ºC. Energy sources were added to the incubation medium in the respective flasks and amounts were calculated to supply a metabolisable energy equivalent of 125 mg of pure starch. The amounts of the respective N sources were calculated to provide 21 mg of N per flask. Dry matter and NDF disappearance values were calculated. There was no treatment that increased DM disappearance significantly in either of the two forage sources after six or 30 hours of fermentation. The highest NDF disappearance values for *LH* treatments were observed in the *LHCU* and *LHCSB* treatments, after six and 30 hours, respectively. With *WS* as substrate, the highest NDFD values were observed in the *WSCSB* and *WSSSB,* at six and 30 hours, respectively.

In the second study, the same sources were used as in the first study. Total mixed rations were simulated in which roughage was included at 229 g DM, energy sources at 188 g DM and nitrogen sources calculated to supply 21 mg N. The sample diets were incubated

in sealed flasks with rumen fluid and incubation medium for 30 hours. During the incubation period, gas production was measured at regular intervals. After termination of the incubation the same digestion parameters were measured as in the first study. The highest DM disappearance was seen in *LHMU* and *WSSSB* for the two forage sources respectively. The combinations that showed the highest NDF disappearance values were *M*U* and *S*U* for lucerne hay, and *S*SB* for wheat straw. In both *LH* and *WS*, the highest amount of gas produced was present when *M* served as energy source and *U* as nitrogen source. It was concluded that the various combinations of forages, energy and nitrogen sources affected forage digestibility differently and knowledge thereof can be of value in formulating ruminant total mixed rations.

Uittreksel

Titel:	Die invloed van verskillende energie- en stikstofbronne op *in vitro* veselvertering en gasproduksiekinetika van hoë- en laekwaliteit ruvoere
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Verteerbaarheid van vesel uit die ruvoer komponent van totale gemengde rantsoene vir melkkoeie kan beïnvloed word deur die bron van aanvullende energie en stikstof in die rantsoen. Deur die vertering van vesel in die rumen te optimaliseer, kan melkkoeie ten volle gebruik maak van die voedingstowwe in ruvoere, om sodoende meer substrate daaruit te verkry vir optimale produksie.

Hierdie tesis handel oor twee *in vitro* studies wat gemik was op die verbetering van vesel vertering. In die eerste studie was 'n hoë kwalitiet ruvoer (lusernhooi) en 'n swak kwaliteit ruvoer (koringstrooi) *in vitro* geïnkubeer deur die filtersakkie metode met òf mielies, sitrus pulp of melassestroop as energiebron en óf sojameel, ureum of die inkubasie medium as stikstofbron. Die ruvoer monsters wat 125 mg NDF bevat het is verseël in 'n filtersakkie en geïnkubeer in flesse vir ses en 30 uur teen 39ºC, tesame met die nodige hoeveelheid van die betrokke energiebron om metaboliseerbare energie gelykstaande aan 125 mg stysel te verteenwoordig, en 21 mg stikstof voorsien deur die spesifieke stikstofbron. Die verteerbaarheidsparameters; DM verdwyning en NDF verdwyning, was bepaal. Daar was geen behandeling op enige van die twee ruvoere wat aansienlik toegeneem het in DM verdwyning na ses of 30 uur van fermentasie nie. Die hoogste NDF verdwyning waardes vir LH behandelings is gesien met behandeling *LHCU* en *LHCSB* na ses en 30 uur onderskeidelik. Onder die *WS* behandelings was die hoogste NDFD gesien met behandeling *WSCSB* en *WSSSB*, na ses uur en 30 uur onderskeidelik.

In die tweede studie is dieselfde bronne gebruik as in die eerste studie. Totale gemengde rantsoene was gesimuleer waarin ruvoer ingesluit was teen 229 g DM, energiebronne

teen 188 g DM en stikstof bronne bereken tot 21 mg N. Die monsters is geïnkubeer in verseëlde flesse met rumen vloeistof en inkubasie medium vir 30 uur. Gedurende die inkubasieperiode, is gasproduksie gemeet met gereelde tussenposes. Na beëindiging van die inkubasie is dieselfde vertering parameters gemeet as in die eerste studie. Die hoogste DM verdwyning was gesien in *LHMU* en *WSSSB* vir die twee onderskeie ruvoer bronne. Die kombinasies wat die hoogste NDF verdwyning getoon het was *M*U* en *S*U* vir lusernhooi, en *S*SB* vir koringstrooi. In beide ruvoere was die hoogste volume gas geproduseer teenwoordig toe *M* gedien het as energiebron en *U* as stikstofbron. Daar was tot die gevolgtrekking gekom dat die verskillende kombinasies van ruvoer, energie en stikstof bronne die verteerbaarheid van vesel anders kan beinvloed. Kennis daarvan kan van groot belang wees in die formulering van totale gemengde rantsoene vir herkouers.

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Abbreviations

AA	Amino acids
ADF	Acid detergent fibre
ADICP	Acid detergent insoluble crude protein
ADL	Acid detergent lignin
b	Model derived total gas production
BCS	Body condition score
BUN	Blood urea nitrogen
c	Model derived rate of gas production
C	Citrus Pulp
CP	Crude protein
DIM	Days in milk
DMI	Dry matter intake
DM	Dry matter
DMD	Apparent DM degradability percentage
ECM	Energy corrected milk
EE	Ether extract
eNDF	Effective neutral detergent fibre
FCM	Fat corrected milk
IVDMD	In-vitro dry matter digestibility
IVOMD	In-vitro organic matter digestibility
L	Model derived lag time
LH	Lucerne Hay
LW	Live weight
M	Maize
ME	Metabolisable energy
MJ	Mega Joules

MUN	Milk urea nitrogen
NDF	Neutral detergent fibre
NDFD	Neutral detergent fibre digestibility
NDICP	Neutral detergent insoluble crude protein
NDSC	Neutral Detergent Soluble Carbohydrates
NEFA	Non-esterified fatty acids
NFC	Non-fibre Carbohydrates
NH3-N	Ammonia nitrogen
NPN	Non protein nitrogen
NSC	Non-structural carbohydrates
NRC	National research council
OM	Organic matter
peNDF	Physically effective neutral detergent fibre
S	Molasses Syrup
SB	Soybean Meal
SD	Standard deviation
SEM	Standard error of the mean
TMR	Total mixed ration
R	South African Rand
RDP	Rumen Degradable Protein
U	Urea
VFA	Volatile fatty acids

CHAPTER 1: INTRODUCTION

1.1 Introduction

Carbohydrates in plants can be divided into two categories, cell walls and cell content. The cell walls provide strength and structural support to the plant, and are therefore resistant to destruction. Fibre *per se* is not digestible by mammalian digestive enzymes. However, the digestive system of the ruminant has adapted to obtain nutrients from cell walls by hosting a vast array of microorganisms in the rumen that have the ability to digest complex forms of carbohydrates, collectively known as fibre. Microorganisms in the rumen hydrolyse cell walls by the synthesis and secretion of enzymes and use the components released as nutrients (Varga and Kolver, 1997). The hemicellulose, cellulose and lignin fractions together are called neutral detergent fibre (NDF; Van Soest *et al*., 1991). NDF digestibility is generally defined as the proportion of ingested fibre that is not excreted in faeces and varies within forages (Allen and Oba, 1996) and is an important parameter in determining the quality of forage sources for use in dairy diets.

The presence of fibrous forage in the diet of lactating dairy cows is crucial for rumen function and it is generally a more affordable feed source than non-fibre carbohydrates (NFC). The longer particle length of fibrous forage causes a filling effect due to its slower passage rate, compared to fibre from non-forage origin, which is finer (Oba and Allen, 2005). Allen (1997) demonstrated that there is a strong positive correlation between ruminal pH and the amount of fibrous forage in the diet. The addition of NFC to the diet can lead to a drop in rumen pH, which could negatively affect fibre digestion. The optimal pH for fibre digestion in the rumen is 6.2, and levels below 6 tend to inhibit fibre digestion (Hoover, 1986). Differences in fibre source, forage particle size and the amount of NSC all contribute to ruminal pH and therefore, influence rumen function.

In order to achieve maximum milk yield it is necessary to supplement forage with additional energy and nitrogen. These nutrients aid in microbial efficiency by supplying additional energy and nitrogen to fibre digesting microorganisms in the rumen (Hoover and Stokes, 1991). Additional energy is provided by supplementing with feed sources high in NFC; however, the composition of NFC is highly variable. In maize the NFC portion consists mostly of starch, pectin and sugar in citrus pulp and sugar in molasses. Huhtanen and Khalili (1991) found that fibre digestibility was reduced when sugar was added to high fibre rations.

As sugar is considered to be 100% degradable in the rumen (Sniffen *et al*., 1992), Lee *et al*. (2003) reasoned that this negative effect might be due to the sugar digesting bacteria competing with the fibre digesting bacteria for available nitrogen, and that the addition of rumen degradable protein (RDP) might alleviate this phenomenon. According to the NRC (2001) total tract starch digestibility can vary from 40 to 90%, while digestibility of pectin ranges from 90 to 100%. Because rumen microorganisms require nitrogen for metabolism and there is possible competition for available nitrogen between sugar digesting bacteria and fibre digesting bacteria, it is important to supplement additional nitrogen in the diet. Most of the microbial species in the rumen are able to utilize non-protein nitrogen (NPN; e.g. urea) as the only source of nitrogen (Oltjen, 1996). Furthermore, amino acids (AA) and peptides, derived from dietary protein, can be broken down to urea or can be incorporated directly into microbial protein (Holtshausen, 2004). However, Hoover (1986) observed that proteins are superior to urea in maintaining fibre digestion.

The objective of this study was therefore to determine the effect of energy and nitrogen source on gas production and NDF digestibility of lucerne hay and wheat straw.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In the South African agricultural sector, crop production practises supply the livestock sector with ample fibre-rich forages, such as wheat straw and lucerne hay. Ruminants have the unique ability to utilise fibre due to the presence of fibre fermenting microorganisms in the rumen. In order for ruminants to maximize the nutritive value of forages, optimal utilization of the fibre fraction is required. From an economical perspective, forage tends to be a relatively low-cost feed source, compared to concentrates, and therefore make up the bulk of diets (Holter *et al.,et al.,* 1982). The neutral detergent fibre (NDF) fraction of forage is comprised of cellulose, hemicellulose and lignin, and is an important fraction to consider when balancing a ruminant diet. NDF digestibility is a function of the potentially degradable fraction, its rate of digestion and its rate of passage (Oba and Allen, 1999). As such, forage quality tends to be inversely related to NDF content.

Due to the increasing intensity in productive performance of modern ruminant systems, forages *per se* would rarely supply sufficient nutrients to meet the production standards desired by farmers (Henning, 2004) and thus forage rations need to be supplemented with energy and nitrogen sources (Kolver and Miller, 1998). Supplementary energy can be supplied in the form carbohydrates such as starch (maize), pectin (citrus) and sugar (molasses); which drive the utilization of forage NDF by microorganisms in the rumen. In addition to energy, these microorganisms also require additional nitrogen to be available in their direct environment. Therefore it is useful to supplement energy in combination with protein. Soluble protein from soybean meal is often used in dairy rations, due to its high availability and its characteristic amino acid profile. Upon entering the rumen, protein from soybean meal is gradually hydrolysed by microbial enzymes, releasing peptides and nitrogen within the rumen. Rumen microorganisms can also utilize NPN sources such as urea, which is degraded to ammonia for use in microbial protein synthesis. This form of nitrogen does not supply amino acids to microbes, but it does supply their key constituent – nitrogen. The digestibility of the NDF fraction shows a positive correlation with microbial efficiency. Enhanced NDF digestibility of forage significantly increases the intake of dry matter (DM) and milk yield. Oba and Allen (1999) showed that increasing NDF digestibility by one unit led to a 0.17 kg increase in DM intake and 0.25 kg increase in fat-corrected milk yield. Preliminary *in vitro* work at the Department of Animal Sciences, Stellenbosch

University (Cruywagen, 2009; unpublished data) indicated that there are interactions between different energy sources and NDF digestibility of different forages. Microbial efficiency is furthermore influenced by the source of nitrogen. Based on these phenomena, it is thought that there might be variation in the microbial efficiency between the different combinations of energy sources and nitrogen sources. This hypothesis has not yet been tested.

2.2 Forage classification

The Forage and Grazing Terminology Committee (1991) defines forage as "edible parts of plants, other than separate grain, that can provide feed for grazing animals or that can be harvested for feeding". The types of feeds included in the definition of forage are listed in Table 2-1. Various systems are used to feed forages. In pasture-based systems, cows graze forage crops directly from the field, whereas more intensive dairy systems incorporate dried forages in total mixed rations (TMR). Forages are often referred to as functional feeds, because the high NDF content is essential to sustain rumen health and maximize intake. Forage serves as a substrate in rumen fermentation and stimulates chewing and saliva secretion, which aids in buffering the rumen pH. Fibre originating from forages can be up to 75% more effective at maintaining rumen pH than fibre from non-forage sources such as grains (Firkins, 1997).

Table 2-1 Types of feeds included in the definition of forage (Wilkins *et al.,et al.,* 2000)

Herbage	Leaves, stems, roots of non-woody species
Hay and silage	
Browse	Buds, leaves and twigs of woody species
Straw	

There is wide variation in the nutritive quality of feedstuffs included in the definition of forages. Straw from cereal crops, such as wheat, serve as a high fibre feedstuff while being a low quality forage. It is often used to form the bulk of a diet, aiding in rumen fill (McDonald *et al.,* 2002). High quality forage, such as lucerne hay, has a lower and more digestible NDF fraction and is rich in value adding nutrients. Lucerne hay has a high protein content which declines only slightly with maturity, and its metabolisable energy (ME) content ranges from 10.2 MJ/kg DM in the pre-bud stage to 8.2 MJ/kg DM early in its flowering stage (McDonald *et al.,* 2002). The variation within the quality of a specific forage type can differ extensively

due to variation in soil type, management of the soil, season and the stage of growth at harvest. Furthermore, the quality of dried forages can be influenced by the duration of the drying period, weather conditions and mechanical handling during drying.

2.3 Carbohydrate classification

Carbohydrates can be classified as either structural or non-structural. The cell wall content is included within the structural fraction, while the cell content falls in the non-structural fraction. The cell content fraction includes organic acids, mono- and oligosaccharides, starches and fructans. The remaining cell wall fraction includes cellulose, hemicelluloses, pectin, galactans and β-glucans (Figure 2-1).

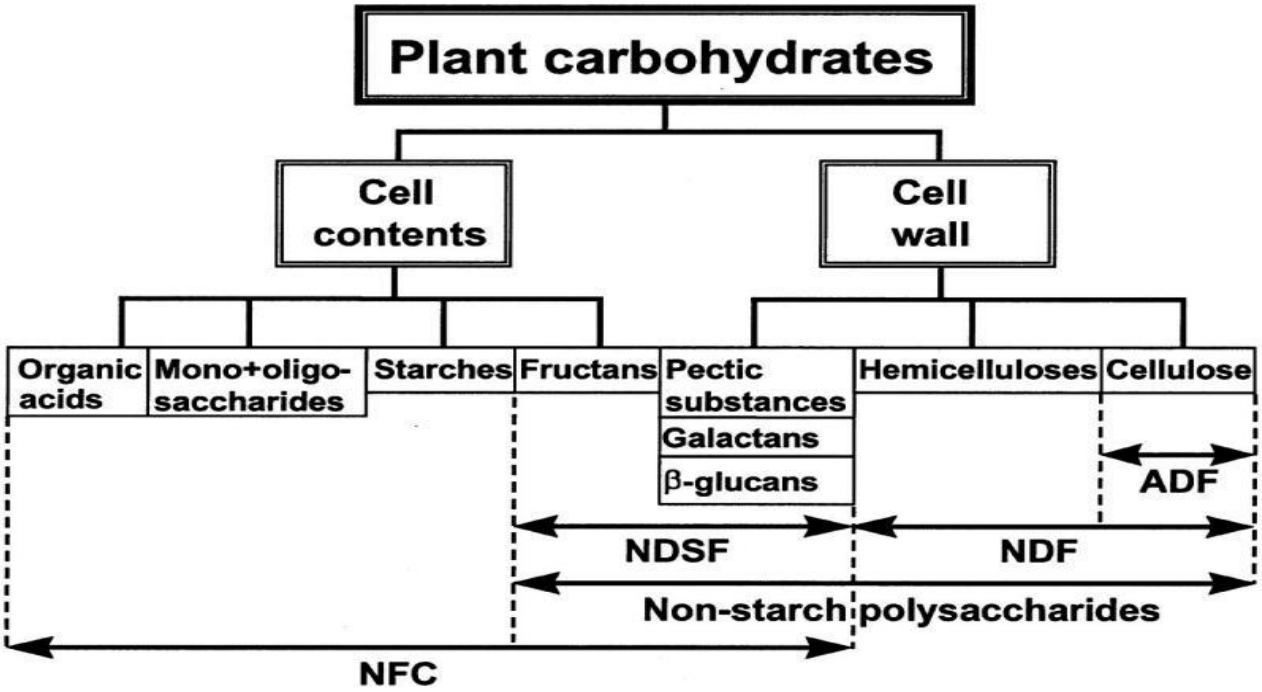

Figure 2-1 The classification of structural and non-structural carbohydrates of plants [ADF=Acid detergent fibre, NDF=Neutral detergent fibre, NDSF=Neutral detergent soluble fibre, NFC=non-NDF carbohydrates (Ishler and Varga, 2001)]

2.3.1 Non-structural carbohydrates

The inner content of the plant cell comprises predominantly of non-fibre carbohydrates (NFC). This component includes starch and simple sugars, as well as soluble fibre, such as beta-glucans, galactans and pectin from the wall of plant cells (Van Soest, 1991). The term structural carbohydrates refer to the function of this class of carbohydrates within the plant cell wall. The NFC content of feeds is calculated by subtracting the percentages of NDF, crude protein (CP), fat and ash from 100 (Mertens, 1997).

Microbial fermentation of these substrates provides volatile fatty acids (VFA) to the host animal, of which propionate is the most abundant (Allen, 1997). The structure of NFC is less complex than that of fibre, and is thus fermented rapidly upon arrival in the rumen. The rapid rate of fermentation can lead to a decline in the rumen pH, creating a favourable environment for lactate producing microorganisms, increasing the risk for acidosis (Stone, 2004). Concentrate feedstuffs such as cereal grains contain relatively large amounts of NFC compared to forages (Table 2-2), and are therefore more energy dense, yielding high VFA levels. The majority of NFC in grains such as maize and barley is starch, while pectin comprises the largest NFC content of soy hulls and citrus pulp (NRC, 2001).

Table 2-2 Composition of the NFC fraction of selected feedstuffs (NRC, 2001)

Feedstuff	Sugar	Starch	Pectin	NFC
		% of NFC		% DM
Lucerne hay	0	24.5	33	22
Barley	9.1	81.7	9.2	60.7
Maize	20.9	80	0	67.5
Soybean meal	28.2	28.2	43.6	34.4

The diets of high producing dairy cows can include up to 45% NFC on a DM basis. The optimum NFC content in diets is complicated by variation in the site of digestion and interaction with fibre sources and its digestion (NRC, 2001). Furthermore, the digestibility of NFC fractions such as starch can vary between sources and thus affect the rate of fermentation and ultimately the rumen pH (Batajoo and Shaver, 1994).

2.3.2 Structural carbohydrates

The NDF fraction of feeds includes the structural carbohydrates: hemicellulose, cellulose and lignin, while the ADF fraction excludes hemicellulose (NRC, 2001). The digestibility of the NDF fraction of various sources can differ markedly due to proportionate differences in the composition of the NDF fraction, particularly the level of lignin (Sullivan, 1966). Lignin is considered an anti-nutrient in ruminant diets since it has a negative effect on the nutritional availability of plant fibre (Moore, 2001).

The NDF content in ruminant diets is determined based on the minimum fibre required to maintain rumen health. For lactating dairy cows, it is recommended that at least 25% of the DM be compose of NDF, of which 19% is of a forage origin (NRC, 2001). According to Allen (1997), non-forage NDF is only 0.35 times as effective as forage NDF in maintaining the ruminal pH (Allen, 1997). The exact level of NDF required in dairy diets are dependent on

the forage source, the amount of starch in the diet, the length of forage particles and the inclusion of supplemental buffers.

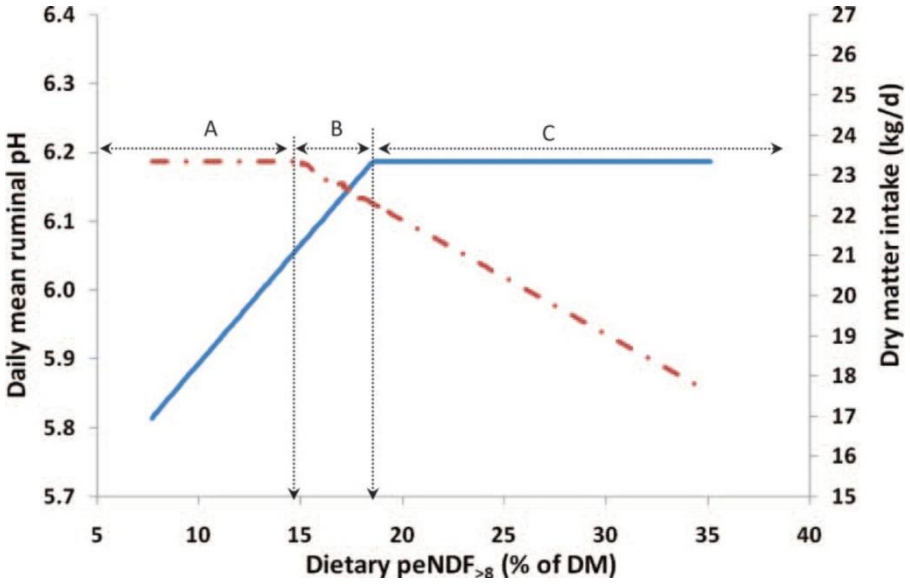

Figure 2-2 Best-fit broken line models describing the conflicting associations among dietary physically effective fibre (measured inclusive particles >8 mm; peNDF>8) with daily mean ruminal pH (solid line) and DMI (dashed dotted line) in dairy cows (Zebeli, 2012)

The NDF portion that stimulates chewing activity is referred to as physical effective fibre (peNDF). Chewing results in the secretion of salivary buffers, which prevent the rumen pH from declining severely. The dynamics of ruminal fermentation is influenced by peNDF due to its relation to fibre content, particle size and reduction in particle size. All of these factors influence the formation of the ruminal mat, where larger particles will float on a pool of liquid and smaller particles will…(Mertens, 1997). To determine the peNDF of a feed, the NDF content is multiplied by the physical effective factor. This factor may vary from zero, when a NDF is not physically effective, to one, when NDF is fully effective (Zebeli *et al.,* 2012). The physical effective factor is measured in practice by determining the proportion of DM from feed, retained on a 1.18 mm sieve following a vertical shaking procedure. Mertens (1997) proposed that particles exceeding 1.18 mm in length would be resistant to rumen passage and thus remain in the rumen to be regurgitated. He furthermore suggests that total mixed rations should contain at least 22% peNDF to stimulate sufficient chewing activity and thereby maintaining the rumen pH above six. However, Zebeli et al. (2012) recommend an average peNDF of 31.2% on a DM basis. Due to the negative correlation between NDF content and DM intake exceeding the recommended NDF content of finely chopped feeds

in order to obtain a sufficient peNDF may lower DM intake consequently resulting in lowered production.

2.4 Forage digestibility

From a nutritional point of view, the productive performance of dairy cattle is subject to forage availability, DM intake and digestibility. The digestibility of forage is a function of the proportion of the NDF fraction that is potentially digestible, the rate of fibre digestion, and rate of passage through the rumen (Allen and Mertens, 1988). The fermentation of NDF in the rumen is influenced by forage factors such as plant maturity, species, environment, and post-harvest storage procedures as well as animal factors such as dry matter intake (DMI) and the fermentation potential of the rumen environment (Mertens, 1997). Plant maturity is the primary factor influencing the rate of NDF digestion and the level of indigestible lignin polymers. Lignin is a polymer of hydroxycinnamyl alcohols, deposited in the wall of plant cells and is the chief component in limiting polysaccharide digestion in the rumen. Polysaccharides are shielded from enzymatic hydrolysis by polymers forming with phenolic acids and non-phenolic compounds by way of a free radical reaction (Jung anf Deetz, 1993). As the plant ages, the lignin content increases relative to cellulose and hemicellulose (Jung and Allen, 1995; Moore and Jung, 2001). Digestibility of grasses for example tends to exceed that of legumes, due to the higher lignin content of legumes (Jung, 1985; Buxton and Russell, 1988). Legumes have high cellulose to lignin ratios, with cellulose and hemicelluloses being more closely correlated. The higher degree of lignifications in the cell wall of legumes is compensated for by the reduced amount of overall cell walls relative to cell content. Work done by Jung (1985) indicated that natural occurring phenolic compounds in legumes could inhibit digestion of cell walls. The quality of forages from temperate environments exceeds those of forages from the tropics, due to the differences in temperature and daylight length. Van Soest (1994) listed the environmental factors which influence forage quality in order of importance as; temperature, light, water and fertilisation. Upon harvesting, forages of a high moisture climate can be ensiled and deliver a product rich in energy as well as fibre. Alternatively, chemical treatment of hays could enhance the availability of NDF by dissolving the linkages between hemicelluloses and lignin. Treating wheat straw with anhydrous ammonium increases the NDF digestibility with minor changes in the chemical composition of the forage (Sundstol *et al.,* 1986).

The microorganisms in the rumen are adapted to use the fibrous forages entering the rumen as fermentation substrates in their energy yielding processes. This in turn releases VFA, which the animal utilizes as an energy source (Hoover, 1986; Van Soest, 1991; Mertens, 1997;). Furthermore, the microbes flushed down through the digestive system supply the host with various vitamins and AA needed for protein synthesis. The composition of the microbial population can be manipulated by altering the substrates provided in the diet of the host animal so to gain maximum benefit from rumen microbes (Akin, 1979). Microbial efficiency is the ultimate determinant in the rate of NDF digestion. As mentioned, rumen microbes require energy and nitrogen to flourish and ensure maximum utilisation of NDF (Stokes *et al.,* 1991). The rate of digestion of carbohydrates is a major factor influencing microbial growth, because it controls the amount of available energy, whereas protein affects the production of microbial DM per unit of fermented carbohydrates. After feeding and extensive fermentation, the rumen content contains peptides, AA, ammonia, polymers and monomers of carbohydrates. The source of substrates fed determines the concentration of these components. Microbial protein can supply up to 60% of the protein requirement of dairy cattle (Clark *et al.,* 1992). Because forages are deficient in ruminal undegradable protein (RUP), whilst high in ruminal degradable protein (RDP), it is important to supplement forages with an additional degradable protein source, with the aim of maximizing ruminal microbial protein production. Considerable variation can be seen in microbial protein production when the digestibility of forages differs. According to Allen (1997), a 20-unit difference in forage digestibility will alter energy intake by 1.1 kg of organic DM for a high producing dairy cow consuming 23 kg DM (with 5.5 kg fibre from forage).

2.5 Dry matter intake

It is evident from various studies that an increase in NDF digestibility increases the DMI (Dado and Allen, 1996; Oba and Allen, 2000; Qiu *et al.,* 2003). In systems where forage is freely available, DMI is the most influential factor affecting the production performance of dairy cows. According to Reid (1961), up to 60% of the variation in digestible DMI is related to intake, while only up to 40% is related to digestibility differences. The NDF-energy intake system reviewed by Mertens (2009), is based on the concept that intake is regulated by both energy demand and physical capacity of the rumen (Figure 2-3).

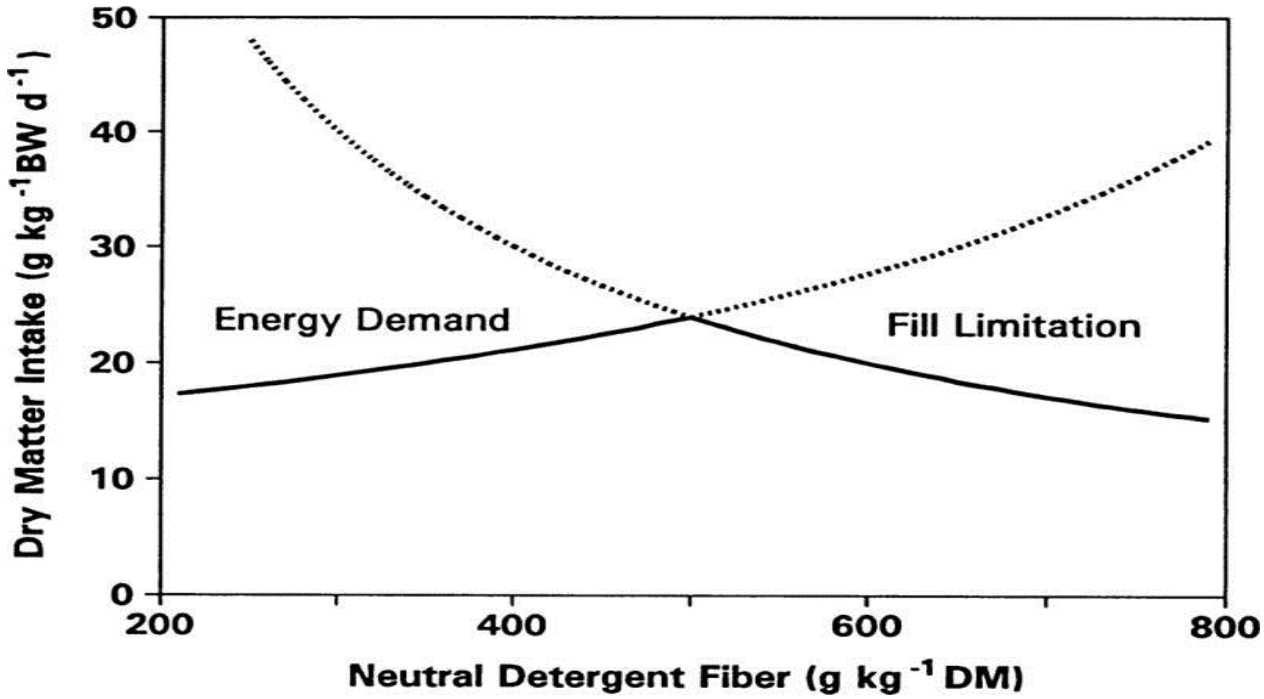

Figure 2-3 DM intake versus NDF content of forages (Mertens, 2009)

When physical fill limits DMI, an increase in the digestibility of NDF and the rate of NDF digestion may enhance DMI by speeding up the flow of digesta through the rumen (Dado and Allen, 1995). The increase in DMI may further increase the net energy available for lactation, due to the associated increase in nutrients released from the digestive system of the cow. When comparing the DMI from feeds equal in NDF content, but differing in *in vitro* NDF digestibility, it is evident that increasing the digestibility of NDF positively affects DMI. Muller et al. (1972) found a 29% increase in DMI when NDF digestibility was increased by 11% in brown mid-rib maize diets. Oba and Allen (1999) compared 13 sets of forages and concluded that DMI increased 0.17 kg/d with a one-unit increase in *in vitro* NDF digestibility. Similarly, Kendall et al. (2009) reported a 0.6 kg/d increase in DMI when NDF digestibility in a TMR increased from 41 to 77%.

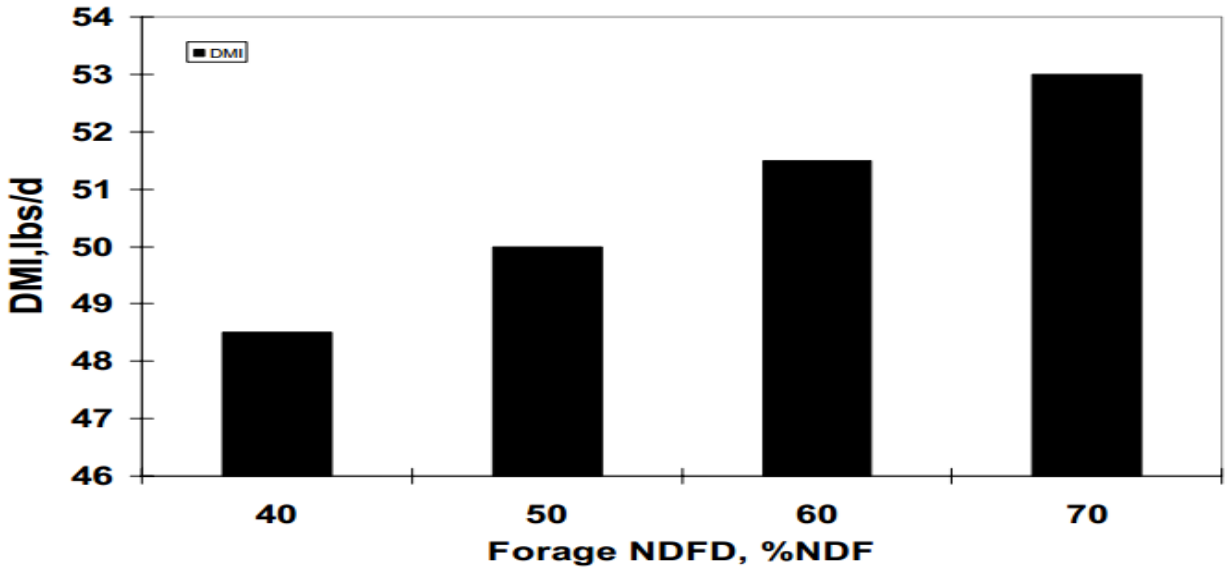

Figure 2-4 The DMI of forages varying in NDF digestibility (NDFD) expressed as a percentage of the total NDF (Oba and Allen, 1999)

2.6 The supplementation of forages

2.6.1 Energy supplementation

The NFC component forms an important part of a dairy diet. By supplying the rumen microorganisms with fermentable substrates, it ultimately supplies the cow with the energy necessary for milk production. Unfortunately, the supplementation of forage diets with readily fermentable NFC is often associated with a decrease in NDF digestion (Cameron *et al.,* 1991). It is speculated that the competition for N between NFC and fibre digesting microorganisms may be the reason for the decline in efficiency of NDF digestion, when higher levels of NFC are fed (Heldt *et al.,* 1999). It is also speculated that the decline in pH associated with the fermentation of NFC could lead to lowered NDF digestibility (Grant, 1994). In order to meet the energy requirements of high producing dairy cows, a TMR diet can contain up to 40% NFC on a DM basis (NRC, 2001). The main microbial fermentation substrates supplied by supplemental carbohydrates in diets are cellulose, hemi-cellulose, pectin, starch and soluble sugars.

2.6.1.1 Starch

Starch is a mixture of two structurally different polysaccharides, namely amylose and amylopectin (NRC, 2001). The proportion of these polysaccharides differ between starch sources; amylopectin in maize range from 70 - 75% (Wang *et al.,* 1993). The rate of

digestibility also tends to differ between the two fractions, with amylopectin degrading more rapidly. Ruminal maize starch fermentation can vary from 40 - 90% (NRC, 2001) depending on the physical form and structure of the plant (Baldwin and Allison, 1983). Total tract digestibility of ground maize averages at 93.5% (Huntington, 1997).

According to the NRC (2001), between 50 - 100% of the NSC fraction of most feedstuffs is made up of starch. In order to meet the high-energy demand of lactating dairy cows, fibrous feedstuffs are often supplemented with a starch source to supply readily fermentable energy to the rumen microorganisms. Mertens and Loften (1980) reported that supplementation of forages with starch decreased fibre utilization *in vitro*. They found that lag time of fibre digestion increased linearly upon starch addition, whilst the potential extent of digestion decreased. This observation is in line with the hypothesis proposed by El-Shazly et al. (1961) that rumen microbes would first utilize starch as an energy source, before shifting over to fibrous carbohydrates. Burroughs et al. (1949) observed that DM digestion decreased 5 - 12% in lucerne based diets when 60% starch was substituted. Various studies show that partial replacement of forage with starch in diets leads to a reduction in total tract digestibility of NDF (Putnam and Loosi, 1959; Tyrell and Moe, 1972), primarily due to the increased rate of digesta flow due to higher intake. Volume of faecal excretions increases as DM digestibility decreases, which in turn is negatively correlated to rumen retention time (Beckman and Weiss, 2005). The inconsistency in results of fibre digestion between *in vitro* and *in vivo* systems is due to the influence of pH. When starch is fed the pH in the rumen would often drop as low as 5.5 (Briggs *et al.,* 1957), whilst it is kept constant in *in vitro* by the addition of buffers. Various factors, including the peNDF fraction, could lead to a severe drop in pH *in vivo*, resulting in a reduction of cellulase activity (Stewart, 1977). Maize is comprised of approximately 717 g/kg starch and sugars on a DM basis (McDonald *et al.,* 2002).

Vast amounts of organic acids are released in the rumen upon NFC fermentation, decreasing the rumen pH. According to multiple sources (Therion *et al.,* 1982; Shi and Weimer, 1992), lowering the ruminal pH shows to have a negative effect on the proliferation of fibrolytic microorganisms. This phenomena is of significant importance considering the fact that ruminal pH in dairy cattle may fall below 6.2 for up to 80% of the day (Robinson *et al.,* 1986; Woodford and Murphy, 1988). Hoover and Stokes (1991) identified pH as one of the major factors affecting rumen fermentation. If the pH decline below 6 it lead to a decrease

in fibre digestion (Khalili and Hunhtanen, 1991a,b), yield of microbial cells (Shi and Weimer, 1992) and the efficiency of microbial protein synthesis (Russell, 1992), as well as changes in the ratio of VFAs (Sutton, 1979). With a pH as low as five, almost complete inhibition of fibre digestion has been observed (Stewart, 1977; Mould and Ørskov, 1984). Mould and Ørskov (1984) described a concept named the "carbohydrate effect" (Figure 2-5), as a possible explanation for the impaired fibre digestion observed at a pH of 6.2. The possible explanations they claimed for this phenomena is: 1) rumen microorganisms preferably utilize readily available carbohydrates ahead of fibre, 2) rumen pH decline as a result of VFA produced by fermentation of readily available carbohydrates, 3) and the population of cellulolytic microorganisms in the rumen decline when the pH drops below 5.5.

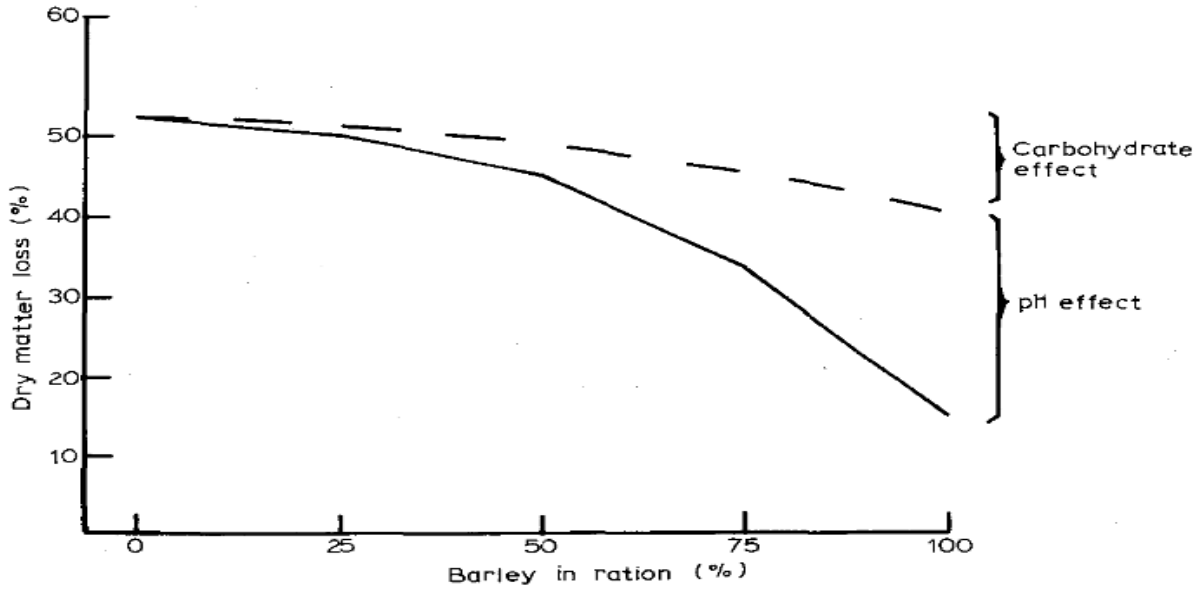

Figure 2-5 Loss in DM weight of ground hay with supplementary barley with (- - - -) and without (----) additional bicarbonate buffer addition (Mould *et al.,* 1983)

Forage and starch interaction has therefore a significant effect on the lag and rate of NDF digestion, and the extent of NDF digestion is furthermore influenced by interaction with pH. At low pH, maize starch addition has a negative effect on fibre digestion of lucerne hay (Grant 1994). *In vitro* studies carried out by Grant and Mertens (1992) found that the predicted NDF digestibility of common forages decreased when pH dropped below 6.2. The NDF digestibility of lucerne hay was decreased by 23% at pH 6.8 when maize starch was added.

2.6.1.2 Sugars

Sugars are often included in dairy rations to enhance palatability and act as a physical binding agent. The simple structure of sugar molecules makes it a readily available energy source for rumen microorganisms (Broderick and Radloff, 2004; Oba, 2011). Monosaccharides such as glucose and fructose are the most commonly found sugars in plants, followed by sucrose, which is a disaccharide. Sucrose, a six carbon sugar, is also the main energy supplying component of molasses. Sucrose is composed of one glucose and one fructose molecule linked by a glycosidic bond (Broderick *et al.,* 2008). *In vitro* and *in vivo* studies showed that fibre fermentation in the rumen could be enhanced with the addition of supplementary sugars, if adequate rumen degradable protein (RDP) is supplied (Hall, 2003). Broderick and Radloff (2004) showed that increasing the sugar content of feeds increased DMI and DM digestibility linearly and had no effect on milk yield or milk protein, in diets containing 18% CP and 0, 4, 8 or 12% dried molasses. They concluded that the optimal inclusion level for sugars is 5%, with a decrease in production when 6% inclusion is exceeded. Fermentation of sucrose lead to high butyrate yields, with a possible increase in milk fat content (Huhtanen *et al.,* 1993). Despite its rapid fermentation in the rumen, feeding sugars such as sucrose do not tend to lower the rumen pH drastically, as is evident with starch (Chamberlain *et al.,* 1993). Sucrose, specifically, provides less carbon molecules than starch per unit of mass, and thus the lack of carbon available for acid production might explain the absence of a drastic drop in ruminal pH (Hall and Herejk, 2001).

Fermentation studies done by Hall and Herejk (2001) show that incubating isolated NDF of Bermuda grass in a ratio of 60:40 with sucrose, citrus pectin or maize starch, results in different CP yield curves. When sucrose was used as an enery supplement the microbial protein yield curve peaked at an early stage and was then maintained, while the microbial protein yield when supplementeing with starch an pectin resulted in a decrease after the peak, suggesting a limitation in substrate.

The supplementation of feeds with rapidly degradable carbohydrates such as molasses can hamper cellulolysis. El-Shazly et. al. (1961) proposed that the competition for essential nutrients occurs within the microbial population and that the cellulolytic microorganisms are less competitive and as such, fail to reproduce at a rate fast enough to maintain themselves in the rumen. Huhtanen and Khalili (1991) showed supplemating forage with sucrose lowers NDF digestibility whilst increasing total diet OM digestibility. The activities of

carboxymethylcellulase and xylanase, enzymes involved in the breakdown of celluose and hemicellulose respectivly, were assayed in this study. The activities were highly correlated to NDF dissapearance in nylon bags *in vivo*. Results indicate that cellulolytic microbes prefer to utilize soluable sugars before turning towards structural carbohydrates. These findings are similar to results of Mertens (1977). This phenomena has been related to the inclusion level of both molasses and protein. Fibre digestion declines as the quantity of molasses included increases, likely due to the lowering effect of NFC fermentation on pH (Hughes-Jones and Peralta, 1981). The decline in fibre utilization can be alleviated by supplying sufficient protein for fibre fermenting bacteria to prevent their non-fibre fermenting competitors from stealing away avaliable nitrogen (discussed in more detail later). Broderick and Radloff (2004) noted an increase in total tract NDF digestibility with molasses supplementation, and peaked at an inclusion rate of 7.2 - 7.4%. Broderick et al. (2008) went further and examined at which concentration of sucrose the highest NDF digestibility was obtained. Results showed a quadratic effect of sucrose inclusion on apparent rumenal NDF digestibility, with a maximum digestibility reached at 5% sucrose inclusion. Broderick and Radloff (2004) found that the addition of dried molasses (supplying sucrose) at 2.4 - 7.2% of total sugar in diets containing 60% forage led to a 4% increase in total tract NDF digestibility in dairy cows.

2.6.1.3 Pectin

On average, citrus pulp is comprised of 223 g/kg pectin, 345 g/kg neutral detergent soluble fibre (NDSF) and 220 g/kg NDF on a DM basis (Bampidis and Robinson, 2006). Although it is part of the cell wall, it is more readily available than cellulose and hemicellulose. Baldwin and Allison (1983) identified methylesterase and polygalacturonidase as at least two of the enzymes required for the hydrolysis of pectin. Ruminants rely solely on the rumen microbial population to synthesize these enzymes and thus transform pectin into a potential energy source. The metabolites of pectin hydrolysis is utilized by some of the more eminent ruminal populations including *Fibrobacter succinogenes, Prevotella ruminicola, Butyrivibrio fibrisolvens, Streptococcus bovis* and *Lachnospira multiparus* (Czerkawski and Breckenridge, 1969; Gradel and Dehority, 1972; Baldwin and Allison, 1983). *F. succinogenes* and *P. ruminicola* are furthermore fermenters of structural carbohydrates and their presence would therefore be favourable for maximizing forage NDF digestion in rations containing a pectin source (Leng, 1970). Hall et al. (1998) established that both the sugars, NDSF, and NDF in citrus pulp ferment rapidly, but their fermentation are depressed at a low

pH (Strobel and Russell, 1986). The rapid fermentation does not lower the rumen pH as starch does (Bach *et al.,* 1999). Leiva et al. (2000) assessed the effect of NFC source on rumen pH profiles over a ten-hour period in ruminally cannulated cows. The citrus pulp and hominy diets contained 4.7 and 2.5% soluble sugars, 15.0 and 26.4% starch, and 13.8 and 8.2% soluble fibre as a percentage of diet DM, respectively. The shape of the pH by time curves differed ($P<0.05$), with citrus pulp showing a more rapid fall in pH with the lowest point reached at six hours. Hominy however showed a linear decline in pH and did not reach its lowest point within the ten-hour period. Due to its lesser effect on pH decline, pectin is often added to high concentrate feeds to lower the risk of ruminal acidosis. Thus, the impact of pectin on fibre digestion in expected to be less severe than that of other NSC components (Grigsby *et al.,* 1992).

2.6.2 Nitrogen supplementation

The work of McAllen and Smith (1976) showed that digestion of fibre could be improved with the addition of supplementary nitrogen to basal diets of concentrates and roughages. The proteolytic rumen bacteria metabolise dietary protein to ammonia, supplying cellulolytic bacteria with the necessary nitrogen for protein synthesis. Ammonia is the precursor for 60 - 80% of synthesised bacterial protein (McSweeney and Mackie, 2012). The microbial population housed inside the rumen can utilize various sources of nitrogen to fulfil their needs. Protein sources can be slowly degradable, such as soybean meal, or rapidly degradable, such as urea. In order to optimally ferment carbohydrates and thus increase the utilisation of feedstuffs, it is important to synchronise the availability of energy and nitrogen release to fibrolytic microorganisms in the rumen. Larson (2003) showed that protein degradability is affected by NFC source and that an interaction between protein degradability and NFC source could influence lactation performance through modification of the metabolisable nutrient supply. The availability of nitrogen to rumen microorganisms differs between sources. Upon arrival in the rumen, nitrogen in NPN sources is rapidly hydrolysed to ammonia. On the other hand, nitrogen from true protein sources is only partially fermented in the rumen, after which the remaining components are post ruminally digested and absorbed by the cow (NRC, 2001). In the absence of the necessary ruminal available nitrogen for microbial growth and maintenance, lactating dairy cows may enter a negative energy balance due to depressed fermentation of energy yielding carbohydrates (Koster *et al.,* 1996).

Different classes of microbial species have different preferences for nitrogen sources and are thus stimulated differently by each source (Allison, 1979). Cellulolytic bacteria require nitrogen in the form of ammonia, whereas the NSC fermenting bacteria utilize mainly AA and peptides (Steward *et al.,* 1997), with ammonia supplying only 35% of the nitrogen required (McDonald, 2011). Work done by Hungate (1966) showed that the degradation rate of AA to ammonia upon entrance in the ruman is faster than the rate at which the structural carbohydrates can be fermented. Cellulolytic bacteria do not possess the ability to utilize branch chain AA directly, and thus re-synthesize their required AA from the fermentation end products, isovalerate and isobutyrate (Nagaraja *et al.,* 1997).

2.6.2.1 Non-protein nitrogen

Research has shown that NPN sources such as urea can effectively be used to supplement nitrogen to ruminants consuming low quality forages (Currier *et al.,* 2004). It serves as an attractive alternative to natural protein due to its low cost per unit of nitrogen. Urea is composed of 281% CP, which is highly rumen degradable and is commonly used in diets to supply rumen microorganisms with rapidly available nitrogen that is hydrolysed to ammonia by urease (Satter and Slyter, 1974). When forages are substituted with NFC, the microorganisms involved in its fermentation also require a source of nitrogen. Due to its fast rate of degradation in the rumen; urea is the ideal nitrogen source to include. However, if a vast amount of urea is consumed within a short period of time the host animal would be at risk of ammonium toxicity (Bartley *et al.,* 1976). When urea makes up a major part of the protein requirements in a diet, deficiencies in sulphur-containing AA may occur; as such, it is advised that urea inclusion does not exceed 3% of the total CP in diets (NRC, 2001). Broderick and Reynal (2009) evaluated the effect of different proportions of RDP from soybean meal and urea on rumen fermentation. They found that replacing soybean meal RDP with that of urea, resulted in reduced milk yield due to depressed microbial protein formation in the rumen. Utilization of forage CP is depressed when large amounts of NPN is included (Nagel and Broderick, 1992).

Non-protein nitrogen supplementation is efficient in enhancing NDF digestibility of forages (Briggs, 2004). It changes both the type of microorganisms present in the rumen as well as their biochemical activities, resulting in higher cellulose digestion. Various studies found that supplementation of forage with urea increased cellulose digestion (Belasco, 1954; Coombe and Tribe, 1963; Raleigh and Wallace; 1963; Hemsley, 1964).The total amount of NPN that can be utilized varies according to the energy availability and the amount of ammonia

derived from salivary sources. Once the level of ammonia in the rumen exceeds 50 mg NH_3-N/l rumen fluid nothing is achieved (Satter and Slyter, 1974).

2.6.2.2 Natural protein

A vast diversity of natural protein sources are generally used in dairy diets. It includes animal proteins, legume seeds, distillery by-products and oilseed cakes. These protein sources are generally more expensive and less concentrated in CP than NPN, but contains AA and peptides that is crucial for optimal lactation performance of dairy cattle. Upon entry in the rumen, microbial proteolytic enzymes degrade the protein to release nitrogen valuable for microbial fermentation (Tamminga, 1979). The fractions that bypass the rumen by escaping proteolysis, continues past the rumen to be potentially digested post ruminally. The AA absorbed post ruminally are of advantage to the production performance of the host animal, but does not contribute to the AA available to the rumen microorganisms.

Substitution of NPN with peptides or AA has a stimulating effect on *in vitro* microbial protein yield (Russel and Strobel, 1993). Various reports show that the inclusion of true protein is superior to NPN regarding DMI and digestibility of poor quality forages (Heldt *et al.,* 1999; Jelantik, 2001). The work of Kropp et al. (1977) and Robinson et al. (1998) reported greater digestibility of low quality forages when supplemented with soybean meal than with urea. Because the energy source supplied influences nitrogen requirements of microorganisms, natural protein addresses the requirements of both starch and sugar-utilizing organisms by supplying both peptides and AA (Soto *et al.,* 1994). Given the slow rate of plant fibre degradation, it can be argued that the synchronization of energy and nitrogen release has a beneficial effect on fibre digestion. Various studies have showed a stimulatory effect on rumen microbial growth rate when AA and peptides were offered to microorganisms growing on rapidly digestible carbohydrates (Russell *et al.,* 1983; Chen *et al.,* 1987). Protein fractions vary in their susceptibility to ruminal breakdown. Degradability is affected by the surface area available for microbial attack as well as the physical and chemical nature of the proteins (Borchers, 1965). The extent of ruminal degradation is thus dependant on the innate degradability as well as the time spent in the rumen (Hoover and Stokes, 1991). Oilseed meals are commonly included in dairy rations due to its availability and relatively low cost. The nitrogen fraction of oilseed meals can consist of up to 95% true protein, with a digestibility ranging from 0.75 to 0.90 and is of good quality (NRC, 2001).

2.7 Conclusion

From the literature sited, it seems clear that various factors influence the digestibility of forage fibre and thus overall lactation performance of dairy cows due to complicated interactions between raw materials inside the rumen. By means of *in vitro* trails, this study aims to shed light on the importance of raw materials used to formulate dairy diets with the focus on optimal utilization of forages, with special reference to DM and NDF disappearance.

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CHAPTER 3: THE EFFECT OF INTERACTION BETWEEN ENERGY AND NITROGEN SOURCES ON *IN VITRO* FORAGE DM AND NDF DIGESTION

3.1 Introduction

In order to balance rations of high producing dairy herds it is inevitable for nutritionists to use an array of different feedstuffs to supply lactating cows with sufficient nutrients for milk production. Energy dense raw materials are known to have a negative effect on the rumen pH and can therefore supress fibre digestion (Khalili and Huhtanen, 1991a). Plant materials rich in either starch, sugar or pectin are commonly used as energy sources in TMR's. Diets rich in pectin sources such as citrus pulp results in less acidic rumen pH than does barley rich diets (Ben-Ghedalia *et al.,* 1989), posing a potentially improved environment for pH sensitive fibre digesting organisms. Fermentation of pectin primarily leads to the production of acetic acid, while not much lactic acid is produced. Mould and Orskov (1984) proposed that the addition of starch to a TMR reduces fibre digestion through a series of events in the rumen involving carbohydrate preferences, pH reductions and decreased cellulolytic organisms. Sinclair et al. (1991) found that the rate of starch digestion affected the utilization of nutrients by rumen microorganisms more than did protein degradability. Shabi et al. (1998) also concluded that nitrogen utilization in the rumen is limited by available energy. Belasco (1945) showed that non-protein nitrogen such as urea can markedly improve the digestion of cellulose in semi continues fermentations of rumen content. The ammonia requirement is affected by a competition between fibrolytic and non-fibrolytic organisms in the rumen. Although cellulolytic organisms primarily require ammonia as their nitrogen source, it was shown that proteins are superior to urea in the maintenance of fibre digestion thereby suggesting that amino acids or peptides are required in addition to ammonia (Belasco, 1945).

The objective of this study was thus to evaluate the effect of three different energy sources: maize (*Zea mays*); citrus pulp and molasses (*Officinarum saccharum*) in combination with either a slow- or rapidly degradable nitrogen source: soybean meal (*Glysine max*) and urea, on *in vitro* DM and NDF digestion of two forage sources, *viz.* lucerne hay and wheat straw. The hypothesis was that the digestibility of forages would not be affected by supplemental energy and nitrogen sources and that there would not be any forage*energy*nitrogen interactions.

3.2 Materials and methods

3.2.1 Study area and ethical clearance

The study was conducted at Stellenbosch University, South Africa (33.9301° S, 18.8647° E) from 14 May until 14 June 2014. Ethical clearance for the use of cannulated cows was obtained before the onset of the study (Approval code SU-ACUM13-00029).

3.2.2 Experimental animals and diets

The rumen fluid used in this study was acquired from four lactating multiparous Holstein cows, fitted with rumen cannulae. The donor cows remained part of the Stellenbosch University's dairy herd and were managed with the rest of the herd for the duration of the study. The cows received 24.5 kg of a commercial semi-complete feed/day (on an "as is" basis) which related to 22 kg DM/day. The semi-complete feed was supplied by AFGRI Animal Feeds (Klipheuwel, Western Cape, South Africa). The feed was offered twice daily, 10 kg/cow at 06:30, after the morning milking and 14.5 kg/cow at 16:30, after the afternoon milking. The semi-complete feed contained 35.7 g/kg of NDF and 16.5 g/kg of CP on a DM basis. Furthermore, cows had free access to a mixture of 20% wheat straw and 80% chopped lucerne hay, while clean drinking water was available at all times. To minimise the possibility that undigested starch could interfere with the effect of the experimental energy sources, the four donor cows received no concentrate feed for 24 hours before rumen fluid collection.

3.2.3 Simulated diets

3.2.3.1 Forage substrates

Two different forages were used as substrates in this study. Wheat straw (*WS*) represented a low quality forage and lucerne hay (*LH*) represented a high quality forage with regard to fibre digestibility as stated earlier. In order to standardize the amount of forage NDF to be weighed out into the incubation vessels, the amount of *LH* and *WS* needed were calculated to supply 125 mg NDF per incubation flask. The forages were ground through a 2 mm screen (Cyclotec 1093 mill) and sieved through a 106 µm mesh to remove extremely fine particles that would wash out of the filter bags during incubation. The NDF content was 804.6 g/kg DM for *WS* and 454.8 g/kg DM for *LH*. Consequently, 305 mg of lucerne hay (air dry basis, containing 90.1 % DM) and 169 mg of wheat straw (air dry basis, containing 92 % DM) were

used as substrates to supply 125 mg of NDF per incubation flask (Table 3-1). Forage substrates were transferred to Ankom F57 filter bags for incubation (Ankom Technology Corp., Fairport, NY, USA).

3.2.3.2 Energy sources

Three different energy sources were used: maize (*M*), citrus pulp (*C*) and molasses syrup (*S*). The *M* and *C* was ground through a 2 mm screen (Cyclotec 1093 mill). In order to standardize the amounts of energy supplied by each of the three energy sources, the quantities were calculated to be equivalent in metabolisable energy to 125 mg of pure starch. The respective ME contents of the said energy sources were as indicated in the 2001 edition of Nutrient Requirements of Dairy Cattle (NRC, 2001). The final calculated amount of each energy source was 198 mg (DM) for *M*, 111 uL of *S* and 224 mg (DM) of C. The *S* was diluted with 50% distilled water to simplify handling and thus 222 uL aliquots were used (Table 3-1).

3.2.3.3 Nitrogen sources

Two nitrogen sources, urea (*U*) and soybean meal (*SB*) were selected to represent a rapidly degradable and a slowly degradable nitrogen source, respectively. In the current study, 100 mL of the Goering and Van Soest (1970) incubation medium (O) was used per flask. This amount of incubation medium supplies 21 mg of nitrogen. Therefore, to ensure a standard level of nitrogen inclusion, the amounts of *SB* or *U* to be weighed out and added to the appropriate flasks were calculated to provide 21 mg nitrogen as well (Table 3-1).

Table 3-1 Summary of substrates, energy sources and nitrogen sources used for incubation

Parameter	Abbreviation	Inclusion level
Lucerne	*LH*	305 mg DM
Wheat straw	*WS*	169 mg DM
Maize Meal (Yellow)	*M*	198 mg DM
Molasses syrup	*S*	0.222 µL
Dried citrus pulp	*C*	224 mg DM
Urea (20% solution)	*U*	0.228 µL
Soybean meal	*SB*	44 mg DM

3.2.4 Experimental design

In order to determine the effect of a possible interaction between the various energy and nitrogen sources on *in vitro* forage fibre digestibility, two forage sources were individually incubated with the respective energy and nitrogen sources. The incubation medium was based on Goering and van Soest (1970), except for the combinations where the nitrogen source in the medium was replaced with *SB* or *U.* The substrates were incubated for either 6 h or 30 h. A total of 36 treatments were used in a 2x3x3x2 factorial design: two substrates (*WS* and *LH*), three energy sources (*M*, *S* and *C*), three nitrogen sources (*U*, *SB* and *O*) and two incubation times (6 h or 30 h). In addition, the two substrates were also incubated alone for 6 h or 30 h, without any energy source, using the standard incubation medium (*O*) of Goering and Van Soest (1970). Consequently, there were a total of 40 treatments. Each replication was repeated six times. Due to limitations in terms of incubation space and equipment, additional combinations of the forage substrates with nitrogen sources alone (no energy sources) were not included. This would have required an additional 12 treatments, bringing the total number of treatments per replication to 52, which was not possible in our laboratory. Since the primary interest in this study was the effect of energy source, with or without the two nitrogen sources, it was decided to add two forage control treatments (no energy or N sources) to the incubations. Treatments are explained in Table 3-2.

Table 3-2 Treatment description for six and 30 hour incubations

Treatment	Forage source	Amount (mg)	Energy source	Amount (mg or uL)	Nitrogen source	Amount (mg or uL)
LH	LH	305	-	0	O	
LHMO	LH	305	M	198 mg DM	O	
LHSO	LH	305	S	222 µL	O	
LHCO	LH	305	C	224 mg DM	O	
LHMU	LH	305	M	198 mg DM	U	228uL
LHMB	LH	305	M	198 mg DM	SB	44 mg DM
LHSU	LH	305	S	222 µL	U	228uL
LHSB	LH	305	S	222 µL	SB	44 mg DM
LHCU	LH	305	C	224 mg DM	U	228 µL
LHCB	LH	305	C	224 mg DM	SB	44 mg DM
WS	WS	169	-	0	O	
WSMO	WS	169	M	198 mg DM	O	
WSSO	WS	169	S	222 µL	O	
WSCO	WS	169	C	224 mg DM	O	
WSMU	WS	169	M	198 mg DM	U	228uL
WSMB	WS	169	M	198 mg DM	SB	44 mg DM
WSSU	WS	169	S	222 µL	U	228uL
WSSB	WS	169	S	222 µL	SB	44 mg DM
WSCU	WS	169	C	224 mg DM	U	228 µL
WSCB	WS	169	C	224 mg DM	SB	44 mg DM

3.2.5 Chemical analyses

The DM and ash contents of the feedstuffs were determined according to AOAC International (2002) method 934.041 and 942.05, respectively. Ether extract (EE) was determined according to method 920.39. The CP content was measured on a Leco FP-428 Nitrogen and Protein analyser (Leco Corporation, St. Joseph, MI, USA), following method 990.03 of AOAC International (2002). The NDF was determined according to the procedures described by ANKOM with the ANKOM 220 Fibre Analyser (ANKOM Technologies, Fairport, NY, USA), using F57 filter bags from ANKOM. None of the chemical

analysis was done on urea since variation in feed grade urea is accepted to be low. Table 3-3 summarizes the nutrient composition (g/kg) of the raw materials used.

Table 3-3 Nutrient composition (g/kg DM) of forages and feedstuffs

Parameter[1]	DM	ASH	CP	EE	NDF
LH	917.9	85.4	188.8	8.1	454.8
WS	905.1	34.7	37.4	3.6	804.6
M	864	5	73.1	37.7	95
S	706.8	87.3	ND	ND	ND
C	830	52	44.4	1.6	261.4
U	ND	ND	ND	ND	ND
SB	898.2	53	467.2	13.8	217

[1] DM=Dry Matter; CP=Crude protein; EE=Ether extract; NDF=Neutral detergent fibre; ND=Not determined

3.2.6 Preparation of sample substrates for *in vitro* fermentation

ANKOM F57 filter bags were pre-rinsed in acetone for five minutes after which they were left to air dry. The acetone treatment ensures that the pores of the filter bags are open, easily accessible to microorganisms and that any potential antimicrobial fabric toxins are removed. Each bag was labelled with a permanent marker and placed in a conventional oven at 105˚C overnight, where after it was weighed using the hot weighing technique. The forage samples were weighed out into the bags, which were then heat-sealed three times with an impulse heat sealer (ANKOM 1915/1920 Heat Sealer, Ankom Technology Corp., Fairport, NY, USA). The bags, along with a small magnetic stirrer, were then inserted into marked 125 ml Erlenmeyer flasks. The maize, citrus pulp and soybean meal samples were then weighed out into plastic measuring boats and transferred directly to the relevant Erlenmeyer flasks. The liquid substrates, molasses syrup and urea, were pipetted into the respective flasks. Blank bags (containing no substrates) were also prepared and incubated. The various amounts of substrates used, as well as the content of each simulated diet, are illustrated in Table 3-2.

3.2.7 Collection and preparation of rumen fluid

The rumen fluid collection took place before the morning milking, 24 hours after the last feeding of concentrate feed. This ensured that the rumen fluid was depleted of concentrate

substrates and the rumen microorganisms experienced the urgent need to consume energy sources. The donor cows were restrained in a crush and the cap of the rumen cannula was removed to gain access to the rumen by hand. The content of the rumen digesta was mixed and the fluid was collected from the ventral rumen (Weimer *et al.,* 1999). The fluid was strained through two layers of cheesecloth and collected in a pre-heated 1L thermos flask. The flask was filled to the brim with rumen fluid prior to closure, to eliminate the presence of oxygen (Maurico *et al.,* 1999). The flasks were then transported to the laboratory within 30 minutes of the first rumen fluid collection. As soon as the flasks were opened it was purged with CO_2 in order to maintain an anaerobic environment for the microorganisms (Grant and Mertens, 1992). The content of the two flasks was thoroughly mixed in a sealed industrial blender (Waring Commercial ® Heavy Duty Blender, Waring ® Corporation, New Hartford), while being continuously purged with CO_2. The fluid was then transferred from the blender to a preheated 2L Erlenmeyer flask, while being strained through further layers of cheesecloth to eliminate the presence of large particles. To maintain an anaerobic environment around the rumen fluid, the flask was left to stand for 10 minutes to allow breakdown of the frothy layer while being continuously purged with CO_2.

3.2.8 Preparation of incubation medium

An incubation medium containing a buffer, micro and macro minerals and a reducing solution, was prepared (Table 3-4). The composition of the buffer was based on the one described by Goering and van Soest (1970), with the modification that triptose and cysteine sulphide were omitted in the incubation solutions where urea and soybean oil cake were used as N sources. Work done by Poelaert et al. (2012) and Mould et al. (2005) showed that omitting the reducing agent from the incubation media did not influence fermentation kinetics. The rumen buffer solution and the micro- and macro mineral solutions were prepared in bulk and stored in a dark and dry environment. The final buffer solution was prepared before incubation by mixing the rumen buffer solution, macro mineral solution and micro mineral solution in the volumes indicated in Table 3-4. A separate final buffer solution was prepared for the treatments containing no nitrogen sources. The solution was identical to the solution described above, except that tryptose and the cysteine sulphide reducing agent were also added. The final buffer solution was then purged with CO_2 and the containers sealed and left overnight in an incubation room with an ambient temperature of 39ºC. The colour of the solutions turned from purple or pink in an oxidised state to clear

when reduced. Two hours before incubation, 100 mL of the final reduced buffer solution was pipetted into the various 125 mL Erlenmeyer flasks that contained the different substrates. The prepared flasks were then transferred to the 39ºC incubation room to adapt to the temperature before adding the rumen the fluid. When ready, 25 mL of the rumen liquid inoculant (Table3-2) was added to each of the 125 mL Erlenmeyer flasks that contained the respective forage, energy and nitrogen sources. The ratio of buffer to rumen fluid in each flask was 4:1 as recommended by Tilley and Terry (1963) and was expected to maintain a pH range in the flask similar to that found in the rumen. Flasks were purged with CO_2 before sealing. Rubber stoppers that were fitted with breather tubes were used to seal the flasks. Although the Erlenmeyer flasks were calibrated to 125 mL, the total capacity was more and allowed for enough air space between the liquid and rubber stoppers.

3.2.9 *In vitro* incubation

The prepared Erlenmeyer flasks were then placed on magnetic stirring plates inside the incubation room. Each stirrer plate could accommodate 15 flasks. The slow movement of the stirrer bars inside the flask ensured proper and continuous mixing of the incubation solution and thus exposure of the nutrients to the microorganisms. The incubation periods were six and 30 hours.

3.2.10 Residue analysis

At the end of the relevant incubation periods, the flasks were opened and the pH of each flask was measured to identify possible irregularities. The filter bags were removed with tweezers and washed in a conventional washing machine for ten minutes in order to remove attached rumen fluid and solid particles. The bags were then dried in a conventional oven at 105˚C for 24 hours after which they were accurately weighed.

3.2.10.1 DM disappearance

The % DM disappearance was used to estimate the DM degradability according to equation 3.1 as proposed by van Soest et al. (1991):

$$DMD = 100 - \frac{\left[W_3 - \left(W_1 \times C_1\right)\right]}{W_2} \times 100$$

Equation 3.1

Where: DMD = Apparent DM degradability percentage

W_1 = Empty filter bag weight (mg)

W_2 = Forage sample weight (mg DM)

W_3 = Weight of dried bag and residue after incubation (mg DM)

C_1 = Blank bag correction factor

Table 3-4 The composition of incubation medium used in the study

Solution	Amount
Buffer solution:	
Distilled water	2.0 L
NH_4HCO_3	8.0 g
$NaHCO_3$	70 g
Macro mineral solution:	
Distilled water	2.0 L
NaH_2PO_4(anhydrous)	11.4 g
KH_4PO_4(anhydrous)	12.4 g
$MgSO_4$.7 H_2O	1.17 g
Micro mineral solution:	
Distilled water	100 mL
$CaCl_2$.2 H_2O	13.2 g
$MnCl_2$.4 H_2O	10 g
$CoCl_2$.6 H_2O	1.0 g
$FeCl_3$.6 H_2O	8.0 g
Reducing solution:	
Distilled water	48.0 mL
Cysteine hydrochloride[1]	312 mg
1 N NaOH	2.00 mL
Na_2S.9 H_2O	312 mg
Final buffer solution:	
Distilled water	500 mL
Buffer solution	250 mL
Macro mineral solution	250 mL
Resazurin (0.2% w/v)	2.0 mL
Micro mineral solution	0.12 mL
Tryptose[1]	1.25 g
Reduced buffer solution:	
Final buffer solution	570 mL
Reducing solution	30 mL

[1]Omitted in the incubation solutions where urea and soybean oil cake were used as N sources.

3.2.10.2 NDF disappearance

In order to determine the NDF residue left in the filter bags after incubation, Na_2S and heat stable alpha amylase were used in the ANKOM AUTOMATED Fibre Analyser according to

the ANKOM method. The percentage NDF disappearance was used to estimate the NDF degradability according to equation 3.2, as proposed by van Soest et al. (1991):

$$NDF = 100 - \frac{[W_3 - (W_1 \times C_1)]}{W_2} \times 100$$

Equation 3.2

Where:

NDF	=	Apparent NDF degradability percentage
W_1	=	Empty filter bag weight (mg)
W_2	=	Forage sample weight (mg NDF in DM)
W_3	=	Dried weight of bag with residue after incubation (mg NDF in DM)
C_1	=	Blank bag correction factor

3.2.11 Statistical analysis

The statistical analyses on the substrate controls were done according to a main effects ANOVA. Main effects were forage, block and incubation time. For the other treatments where energy and nitrogen sources were present, the statistical analyses were done according to a three way factorial ANOVA with the following factors: block, substrate, energy source and nitrogen source. Where three way interactions were significant ($P<0.05$), effects were interpreted with LSD multiple comparisons and Bonferroni tests.

3.3 Results and discussions

3.3.1 Dry matter disappearance

Table 3-5 summarizes the DM disappearance values of the forage treatments, *LH* and *WS*, after six and 30-hours of incubation when incubated with rumen fluid and buffer solution, without additional energy and nitrogen sources.

Table 3-5 The mean DM disappearance values for lucerne hay and wheat straw, after six hours of incubation

Parameter[1]	Substrate and time				SEM	P-value
	LH 6 h	*LH* 30 h	*WS* 6 h	*WS* 30 h		
DMD (%)	39.44[a]	51.21[b]	10.90[c]	16.87[d]	0.884	0.004

[a,b] Subscript differences within rows indicate significant differences between values (P<0.05).
[1] DMD=Dry matter disappearance.

The DM disappearance (DMD) of lucerne hay differed significantly from that of wheat straw (P= 0.004). This indicates that *LH* was higher digestible than *WS* when no supplemental energy or nitrogen was added at both six and 30 hours of incubation. This observation can be explained by the higher amount of digestible nutrients present in high quality forages such as lucerne hay, compared to low quality forages such as wheat straw (NRC, 2001). Furthermore, lucerne hay used in this study had a crude protein content of 188.8 g/kg DM while that of wheat straw was only 37.4 g/kg DM (Table 3-3). The protein fraction in lucerne hay is highly digestible and are therefore readily available to the microorganisms involved in forage digestion. Results of the main effects "Energy Source" and sub-effects "Nitrogen Source" on DMD of the respective forages are shown in Table 3-6. Figures 3-1 and 3-2 show the effects on DMD per incubation time.

Table 3-6 Mean DM disappearance values (±SEM) of the various treatments after six and 30 hours of incubation

		Energy and Nitrogen Sources (% disappearance)								
		M			S			C		
		SB	U	None	SB	U	None	SB	U	None
LH	6 h	43.38±1.41	41.64±1.63	43.17±1.40	39.99±1.74	42.45±2.46	39.61±0.94	43.21±1.83	42.07±1.72	40.21±2.78
	30 h	46.75±12.16	46.43±2.49	50.12±1.14	45.09±1.79	48.00±2.38	48.23±2.23	49.52±1.51	46.60±0.44	48.47±2.07
WS	6 h	11.65±0.85	13.15±1.22	12.51±0.79	13.06±1.04	11.59±0.59	13.28±0.95	13.54±1.05	12.78±1.02	11.31±0.69
	30 h	22.92±6.84	14.59±0.86	17.15±0.88	17.52±1.74	14.75±0.50	16.64±1.07	15.36±0.96	17.83±1.42	15.95±1.86

Table 3-6 summarizes the outcome of the DMD for the various levels of energy and nitrogen treatments on lucerne hay and wheat straw as substrates. There was no DMD forage*energy*nitrogen interactions at either six hours (P=0.359) or 30 hours (P=0.127), while DMD differed between forages at both periods (P<0.0001). This outcome was expected from the results summarized in Table 3-5, showing the generally higher DMD of *LH* compared to *WS*. A possible explanation for this might be the high levels of acid detergent fibre (ADF) in wheat straw, which is mostly indigestible by rumen microorganisms (Leaver, 1995).

After six hours of incubation, the only treatment that showed a higher (P<0.05) DMD than treatment *LH* was treatment *LHCSB*, while none of the various treatments of *WS* differed from another at either six (Figure 3-1) or 30 hours (Figure 3-2). The high levels of pectin in citrus pulp and lucerne hay is highly digestible and fermentable by a vast array of microbial species in the rumen, and is digested by the same species that digests cellulose and hemicellulose (Mohney, 2002). Nevertheless, only the combination of *C* and *SB* seemed to increase DM disappearance, suggesting that soybean meal is superior to urea in this specific forage*energy source*nitrogen source combination.

After 30-hours, the *LH* alone, without supplementation, showed a higher (P<0.05) DMD than treatment *LHSSB, LHCU, LHMU* and *LHMSB* (Figure 3-2). This is similar to results found by Rezaii (2011), showing that substitution of lucerne hay with NFC may supress its digestibility. Lucerne hay has a high concentration of soluble nutrients compared to wheat straw and is also higher in nitrogen. The combination of these two properties of lucerne hay proves to serve as a good source of nutrients to rumen microorganisms who can utilize these nutrients rapidly as soon as they become available in the rumen.

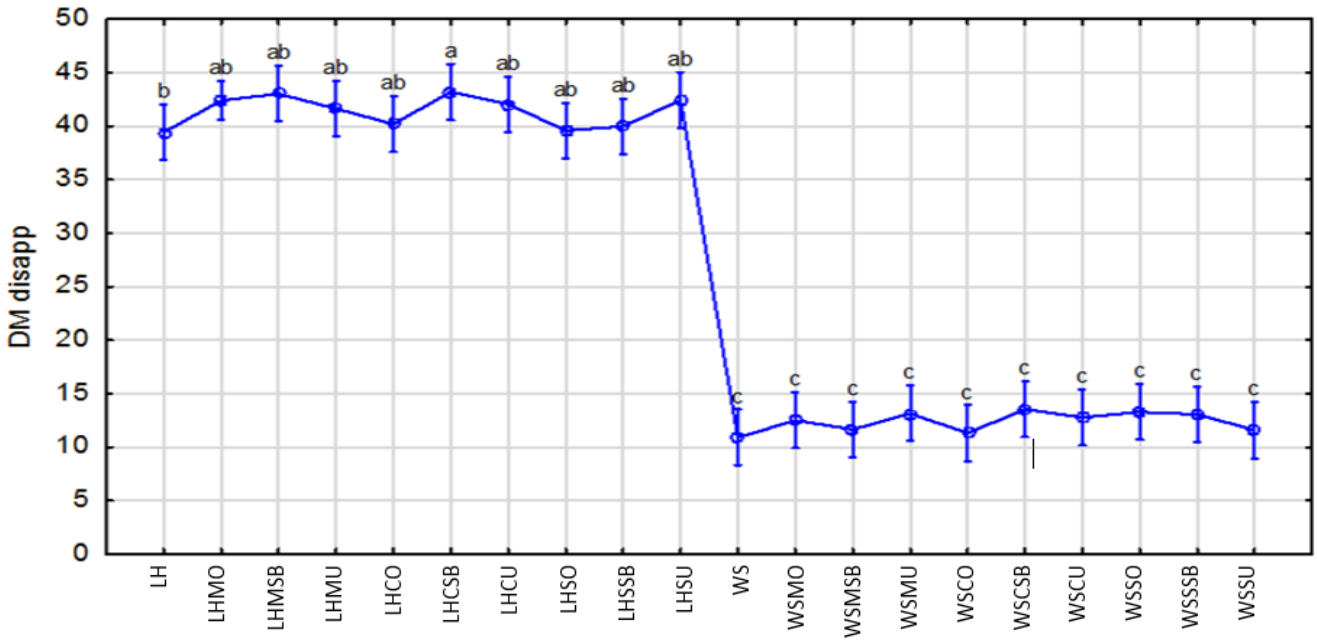

Figure 3-1 Least Square means of DMD (±SEM) for the various treatments after six hours of incubation. Means with different superscripts differ (P <0.05)

3.3.2 NDF disappearance

Table 3-7 shows a difference (P<0.05) in NDF disappearance (NDFD) at six and 30 hours for *LH* when incubated without any added energy or N sources, whilst no differences were seen between six and 30-hours for *WS*. The NDFD of *LH* at six hours did not differ from *WS* at 30 hours, confirming that NDFD is much lower in wheat straw than in lucerne hay.

Results of the main effects “Energy Source” and sub-effects “Nitrogen Source” on NDFD of the respective forages are shown in Table 3-9. Figures 3-3 and 3-4 show the effects on NDFD per incubation time.

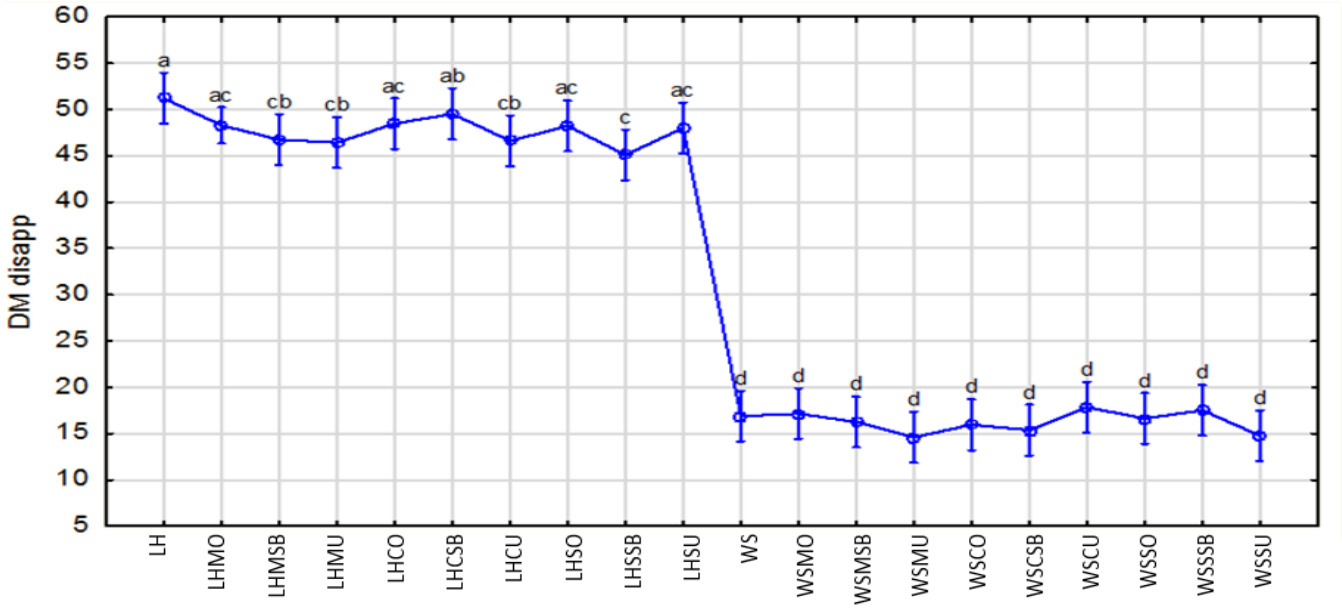

Figure 3-2 Least Square means of DMD (±SEM) for the various treatments after 30 hours of incubation. Means with different superscripts differ (P <0.05)

Table 3-7 The mean NDF disappearance values for lucerne hay and wheat straw after six and 30 hours of incubation

Parameter[1]	Substrate and time				SEM	P-value
	LH 6 h	*LH* 30 h	*WS* 6 h	*WS* 30 h		
NDFD (%)	13.77[a]	26.41[b]	7.89[c]	11.51[ac]	1.479	0.006

[a,b] Subscript differences within rows indicate significant differences between values (P<0.05).
[1] NDFD=Neutral detergent fibre disappearance.

Table 3-8 In-vitro NDF disappearance values (±SEM) for lucerne hay and wheat straw after six and 30 hours fermentation with supplemental energy and nitrogen sources

		Energy and Nitrogen Sources (% disappearance)								
		M			S			C		
		SB	U	None	SB	U	None	SB	U	None
LH	6 h	17.62±0.23	17.50±0.72	12.01±0.46	18.63±0.52	17.75±1.23	19.18±1.08	19.09±0.43	20.36±0.81	18.07±0.42
	30 h	24.61±0.48	24.16±0.08	31.29±0.411	20.73±0.98	24.25±0.48	22.29±0.66	40.07±1.51	25.98±0.79	23.14±0.42
WS	6 h	10.63±0.67	10.89±0.78	10.91±0.51	10.35±0.62	11.07±0.78	9.46±0.78	11.50±0.65	10.93±0.71	9.66±0.87
	30 h	12.36±0.63	12.76±0.42	12.31±0.63	15.86±0.17	12.41±0.56	14.16±0.68	13.00±0.37	14.64±0.65	13.47±1.76

The overall NDF digestibility was higher in treatments with lucerne hay than in treatments with wheat straw, at both six and 30 hours of fermentation, irrespective of the energy and nitrogen sources (Figure 3-3 and 3-4, respectively). A forage*energy*nitrogen interaction was present at both six hours ($P<0.01$) as well as 30 hours ($P<0.001$). There were also energy*nitrogen interactions at six hours ($P<0.05$) and at 30 hours ($P<0.0001$). Forage*energy interactions were present at six hours ($P<0.0001$) and at 30 hours ($P<0.0001$). Forage*nitrogen interactions were present at 30 hours ($P<0.001$), but not at six hours ($P>0.5$).

The treatments *LH* and *LHMO* showed a significantly lower ($P<0.05$) NDFD than the other treatments of lucerne hay at six hours of incubation (Figure 3-3). The *LHCU* treatment had the highest NDFD value at 6 h, indicating that citrus pulp and urea may stimulate the rate of NDFD early in the fermentation. Rezaii (2011) found that supplementation of lucerne hay with NFC led to a suppression of digestibility, suggesting that the addition of supplemental nitrogen sources in this case might have a more positive effect on forage digestion than does NFC supplementation alone. In the case of wheat straw, all the supplements (except citrus pulp and molasses without N) stimulated NDFD by 6 h.

After 30 hours, *LHCSB* had the highest NDFD of all the treatments (Figure 3-4). This suggests that the combination of citrus pulp and soybean meal is the most efficient in terms of NDFD among the various supplement combinations tested and that *SB* is superior to *U* in increasing NDFD of lucerne hay when supplemented with citrus pulp. Maeng and Baldwin (1976a) reported that microbial cell and protein yields were highest in *in vitro* incubations of mixed rumen bacteria when two thirds of added nitrogen came from amino acids and one third from urea. This might explain why the combination of *LH* and *SB* showed to be a good combination to stimulate NDF digestion. In the current study, lucerne hay without supplementation (*LH*) did not differ ($P<0.05$) from any of the three lucerne hay treatments where maize served as energy source, or from treatment *LHCU*. This is in line with the findings of Rezaii (2011) who showed that supplementation of lucerne hay with starch and sucrose can supress NDFD. The only treatments that had significantly higher ($P<0.05$) NDFD in wheat straw after 30 hours than non-supplemented wheat straw (WS) was *WSCU* and *WSSSB*. This is similar to the outcome of LH at six hours, where the combination of *C* and *U* also increased NDFD, showing that the fibre fraction of *WS* requires more time to be digested than that of *LH*.

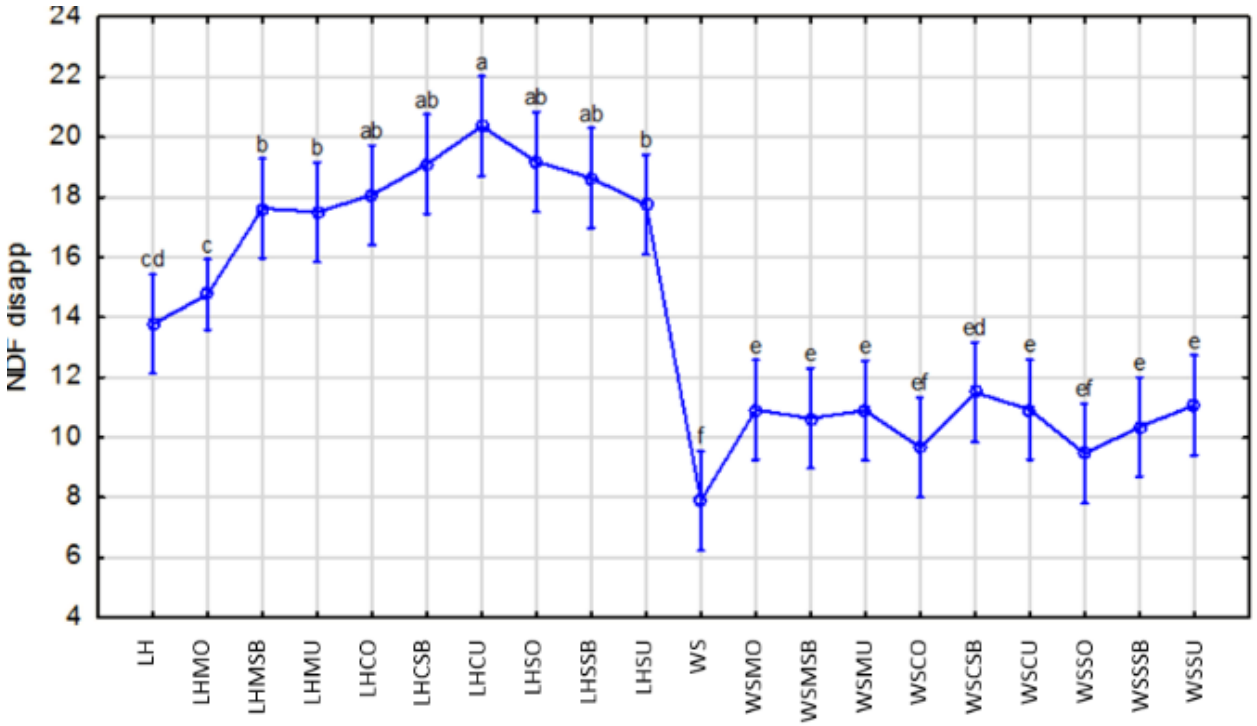

Figure 3-3 Least Square means of NDFD (±SEM) for the various treatments after six hours of incubation. Means with different superscripts differ (P<0.05)

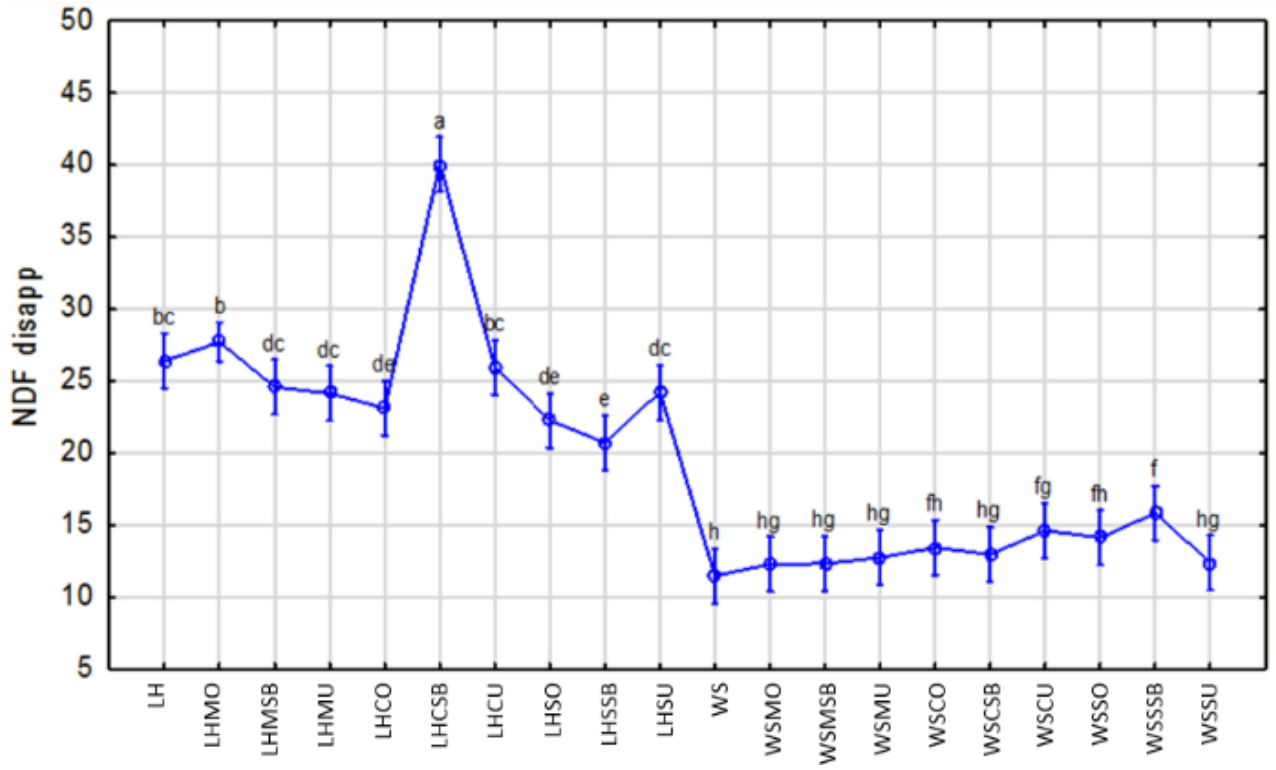

Figure 3-4 Least Square means of NDFD (±SEM) for the various treatments after 30-hours of incubation. Means with different superscripts differ (P<0.05)

3.4 Conclusion

Lucerne hay showed higher disappearance values than wheat straw for both DM digestibility ($P<0.004$) and NDF digestibility ($P<0.005$). There were no interactions for DMD after six or 30 hours of fermentation. For NDFD there were forage*energy*nitrogen and forage*energy interactions at both six and 30 hours of fermentation ($P<0.001$), while forage*nitrogen interactions were only present at 30 hours ($P<0.001$).

There was no treatment that significantly increased DMD in either of the two forage sources after six or 30 hours of fermentation. The highest NDFD value was seen with treatment *LHCU* and *LHCSB* after six and 30 hours, respectively. Among the *WS* treatments, the highest NDFD was seen with treatment *WSCSB* and *WSSSB* at six and 30 hours, respectively.

The hypothesis was that the digestibility of forages would not be affected by supplemental energy and nitrogen sources and that there would not be any forage*energy*nitrogen interactions was thus rejected.

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CHAPTER 4: THE EFFECT OF INTERACTION BETWEEN ENERGY AND NITROGEN SOURCES ON *IN VITRO* FORAGE NDF DISAPPEARANCE AND GAS PRODUCTION KINETICS OF SIMULATED DAIRY TOTAL MIXED RATIONS

4.1 Introduction

The objective in this study was to determine the effect and interactions of three energy sources: maize (*M*), citrus pulp (*C*) and molasses syrup (*S*), along with one of two nitrogen sources, urea (*U*) or soybean meal (*SB*), on fibre digestion and gas production kinetics of a high quality forage, lucerne hay (*LH*) or a low quality forage, wheat straw (*WS*) in TMR simulations.

Feedstuffs are generally assessed individually and their nutritive value are then assumed to be additive. For ruminants, this approach is not always valid, due to the complicated digestive process in the rumen where the digestion of one raw material may influence the digestion of another (Moss *et al.,* 1992). Measuring gas production aids as a powerful tool in investigating the *in vitro* degradation of feeds by rumen microorganisms (Theodouou *et al.,* 1994; Xi *et al.,* 2007). Prasad *et al.,* (1994) and Liu *et al.,* (2002) used this technique to investigate differences between gas production of substrate mixtures and gas production of substrates fermented alone, i.e. interactions between substrates.

The question under investigation was whether a specific source of energy in combination with a specific source of nitrogen resulted in differences regarding digestion of forage, with the focus on digestion of the NDF fraction. The null hypothesis was thus that the source of energy and nitrogen in in-vitro total mixed rations would have no effect on forage digestion and that there would be no forage*energy*nitrogen interactions. The second null hypothesis was that in vitro cumulative gas production and in vitro NDF disappearance of similar treatments would not be correlated.

4.2 Material and methods

4.2.1 Study area

The study was conducted at Stellenbosch University, South Africa (33° 55′ 12″ S, 18° 51′ 36″ E) during the period of March 2016. . Ethical clearance for the use of cannulated cows was obtained before the onset of the study (Approval code SU-ACUM13-00029).

4.2.2 Simulated diets

4.2.2.1 Forages

Two sources of forage were used in order to compare the differences in digestion when the sources of energy and nitrogen varied. Wheat straw (*Triticum aestivum*) represented a low quality forage source and lucerne hay *(Medicago sativa*) represented a high quality forage source. Both of the forages were representative samples that were randomly sampled and ground through a 2 mm screen of a Cyclotec 1093 mill.

4.2.2.2 Energy sources

The three energy sources which were used were yellow maize (*Zea mays*), molasses syrup, a by-product of the sugar cane (*Officinarum saccharum*) industry and dried citrus pulp, a by-product of the local citrus juice industry comprising of peels, seeds and pulp. Both the maize and dried citrus pulp were milled through a 2 mm screen of a Cyclotech 1093 mill.

4.2.2.3 Nitrogen sources

The two nitrogen sources used were soybean meal (*Glycine max*), the remaining material after oil extraction of soybeans, and feed-grade urea, a synthetic non-protein nitrogen source commonly used in animal feeds.

4.2.2.4 Final diets

A total of 12 different laboratory scale diets (Table 4.1) were prepared to represent the different combinations of the two forage sources with the three energy sources and two nitrogen sources. Equal volumes of the two forages as well as equal volumes of the energy sources was used, while inclusion of nitrogen sources was calculated to represent the same amount of nitrogen as the incubation medium. The weight of the forage samples was 229 mg DM each, the energy sources were each weighed of in 188 mg DM samples. The two forage sources were incubated individually as well, without any additional energy or nitrogen sources. To increase accuracy six replications per treatment was done.

Defining the diets:

- *LH*: Lucerne Hay as forage, no supplemental energy or nitrogen
- *LHMU*: Lucerne Hay as forage, Maize as energy source and Urea as nitrogen source

- *LHMSB* : Lucerne Hay as forage, Maize as energy source and Soybean Meal as nitrogen source
- *LHSU* : Lucerne Hay as forage, Molasses Syrup as energy source and Urea as nitrogen source
- *LHSSB* : Lucerne Hay as forage, Molasses Syrup as energy source and Soybean Meal as nitrogen source
- *LHCU* : Lucerne Hay as forage, Citrus Pulp as energy source and Urea as nitrogen source
- *LHCSB* : Lucerne Hay as forage, Citrus Pulp as energy source and Soybean Meal as nitrogen source
- *WS* : Wheat Straw as forage, no supplemental energy or nitrogen
- *WSMU* : Wheat Straw as forage, Maize as energy source and Urea as nitrogen source
- *WSMSB*: Wheat Straw as forage, Maize as energy source and Soybean Meal as nitrogen source
- *WSSU* : Wheat Straw as forage, Molasses Syrup as energy source and Urea as nitrogen source
- *WSSSB* : Wheat Straw as forage, Molasses Syrup as energy source and Soybean Meal as nitrogen source
- *WSCU* : Wheat Straw as forage, Citrus Pulp as energy source and Urea as nitrogen source
- *WSCSB* : Wheat Straw as forage, Citrus Pulp as energy source and Soybean Meal as nitrogen source

The different treatment combinations and amounts that were weighed out for in vitro incubations are indicated in Table 4-1.

Table 4-1 Treatment combinations and substrate volumes used in in-vitro fermentations

Treatment number	Treatment abbreviation	Forage source	(mg DM)	Energy Source	(mg DM)	Nitrogen Source	(mg DM)
1	LH	LH	229 mg	-		-	
2	LHMU	LH	229 mg	M	188 mg	U	10 mg
3	LHMSB	LH	229 mg	M	188 mg	SB	62 mg
4	LHSU	LH	229 mg	S	188 mg	U	10 mg
5	LHSSB	LH	229 mg	S	188 mg	SB	62 mg
6	LHCU	LH	229 mg	C	188 mg	U	10 mg
7	LHCSB	LH	229 mg	C	188 mg	SB	62 mg
8	WS	WS	229 mg	-		-	
9	WSMU	WS	229 mg	M	188 mg	U	10 mg
10	WSMSB	WS	229 mg	M	188 mg	SB	62 mg
11	WSSU	WS	229 mg	S	188 mg	U	10 mg
12	WSSSB	WS	229 mg	S	188 mg	SB	62 mg
13	WSCU	WS	229 mg	C	188 mg	U	10 mg
14	WSCSB	WS	229 mg	C	188 mg	SB	62 mg

4.2.3 Chemical analysis

The DM content of the feedstuffs was determined according to AOAC International's official 934.041 method. Ether extract was determined according to Method 920.39 and ash according to method 942.05. The crude protein content was determined with the aid of a Leco FP-428 Nitrogen and Protein analyser (Leco Corporation, St. Joseph, MI, USA), following method 990.03 of AOAC International (2002). The NDF was determined according to the procedures described by ANKOM with the ANKOM^{220} Fibre Analyser (ANKOM Technologies, Fairport, NY, USA), using F 57 filter bags from ANKOM.

The nutrient composition of the raw materials used in this trail is presented in Table 4.2.

Table 4-2 Nutrient composition of forages and feedstuffs

PARAMETER[1]	DM	ASH	CP	EE	NDF
LH	91.79	8.54	18.88	0.81	45.48
WS	90.51	3.47	3.74	0.36	80.46
M	86.40	0.50	7.31	3.77	9.5
S	70.68	8.73	ND	ND	ND
C	83	5.20	4.44	0.16	26.14
U	ND	ND	ND	ND	ND
SB	89.82	5.30	46.72	1.38	21.7

[1]DM=Dry Matter; CP=Crude Protein; EE=Ether Extract; NDF=Neutral Detergent Fibre; ND=Not Determent; Values are expressed on a DM basis as g/kg.

4.2.4 Preparation of incubation medium

The incubation medium was prepared according to the Goering and van Soest (1970) protocol. The medium was made up by macro minerals, micro minerals, cysteine sulphide reducing solution and a buffer solution (See Chapter 3, Table 3-4). The content of the buffer solution differed between the treatments according to the source of forage used (Table 4-3). This was done to ensure that the total amount of nitrogen present in each treatment was equal.

Table 4-3 Quantity of ammonium bicarbonate and sodium bicarbonate used in the buffer solutions for fermentation of lucerne hay and wheat straw, respectively

Forage source	Ammonium Bicarbonate (g.litre)	Sodium Bicarbonate (g.litre)
Lucerne Hay	0	39.2
Wheat Straw	2.8	36.275

4.2.5 Preparation of samples

ANKOM F57 filter bags were used in this study to isolate the forage samples for NDF analysis after fermentation. The filter bags were soaked in acetone for five minutes where after they had been air-dried. The bags were then individually marked with a permanent marker pen and left to dry overnight in a dry oven at 105 ^{o}C. The weight of each bag was then recorded, using the hot weighing method (Goering and van Soest, 1970). The forage samples were then weighed out in weighing boats and carefully transferred to the marked filter bags. The filter bags containing the forage samples were heat-sealed three times to ensure that no particles could escape from the weighed out samples. The bags containing the forage samples were then placed in 100 ml glass vials of which the exact volumes were known. The energy and nitrogen sources were weighed out and added to the appropriate vials containing the forage samples. Equal amounts of the energy sources were used in the various diets. The amounts of the nitrogen sources, however, were calculated to supply the required amount of 21 mg total nitrogen per 100 ml of final incubation medium. Blank vials that only contained rumen fluid and incubation medium, without any dietary substrates, were also prepared in order to correct for gas production from rumen fluid alone. A small magnetic stirrer with a known volume was added to each vial.

4.2.6 Collection of rumen fluid

Rumen fluid was collected from four ruminally cannulated lactating multiparous Holstein cows from the University of Stellenbosch dairy herd. The cows were fed a total mixed ration once daily, which comprised of oat silage and a commercial concentrate mix from AFGRI Animal Feeds. The ration contained 17.5 kilograms of commercial concentrate feed plus 2 kg of molasses meal, 7.5 kg of lucerne hay, 7 kg of oat silage, and 0.5 kg of wheat straw. Collection of rumen fluid for this trail was performed in the morning before feeding to ensure that the rumen content was free of freshly fed concentrate particles that could influence the rumen fluid content and incubation. The rumen fluid was removed by hand and squeezed through three layers of cheese cloth to separate the solids from the collected liquid. Pre-heated thermos flasks were used to collect the filtrate and closed tightly after being filled to the brim to ensure an anaerobic environment. The flasks containing the rumen fluid were immediately transferred to the laboratory where it was handled inside a temperature controlled room at 39° C. Rumen fluid was blended in a preheated blender for 15 seconds

and filtered for a second time through three layers of cheesecloth. The environment around the fluid was kept anaerobic through continues purging with carbon dioxide.

4.2.7 Inoculation

The incubation medium was prepared and 40 ml thereof added to each vial. The vials containing the medium were transferred to an insulated heated room with a temperature of 39° C and remained there overnight to prevent a thermal shock for the microbes upon incubation. Two hours before the rumen fluid was added to the vials, 2 ml of the cysteine reducing solution were added to each vial, to allow sufficient time for the incubation medium to reduce its redox potential and ensure the environment is kept anaerobic for optimal fermentation. After addition of the reducing solution and 10 ml of rumen fluid inoculant, the vials were purged with carbon dioxide and sealed with a rubber stopper.

4.2.8 *In vitro* incubation

Upon addition of the various components to each vial, the vials were sealed with a rubber stopper. The stoppers were crimp sealed, and the vials were placed on a magnetic stirrer plate, where it was continuously stirred at a slow speed to facilitate dispersal of the particles in the vials. Throughout the process, the vials were kept at 39°C. Due to laboratory limitations the incubation was only done at one time interval, being 30 hours.

4.2.9 Measuring gas production

The pressure inside the vials was measured individually by insertion of a 21-gauge needle, which was attached to a digital pressure gauge. The pressure readings were taken at 1, 2, 3, 5, 6, 21, 23, 25, 27, and 30 hours after initiation of the fermentations. To prevent excessive gas accumulation, gas was released after the 6 h reading was taken.

4.2.10 Analysis of residue

After 30 hours of fermentation, the vials were removed from the temperature controlled room and the crimp seals removed with tongs. The rubber stoppers were removed and the filter bags pulled out of the vials with tweezers. The filter bags were rinsed in water until clean, to stop the fermentation process. The bags were then dried for 24 hours at 105°C. The following day, the bags were weighed using the hot weighing method (Goering and Van Soest, 1970). These values were used to determine the disappearance of DM after the 30

hours fermentation period. To determine the NDF residue after the fermentation period, the ANKOM method was used where the filter bags were immersed in an NDF solution in the ANKOM^{220} AUTOMATED fibre analyzer with the addition of heat stable α-amylase and sodium sulfite. The filter bags containing the residual NDF were dried again at 105°C for 24 hours after which they were weighed. DM and NDF disappearance values were calculated with the same formulae as discussed in the previous chapter.

4.2.11 Estimating kinetic coefficients

Gas pressure was converted to volume (ml) with the aid of a regression developed in our lab and finally expressed as ml of gas per g OM. Kinetic coefficients for gas production were derived from the gas volume data, using of the solver tool in Excel to estimate the first derivatives of the following non-linear model:

Model 1: $Y = b(1 - e^{-c(t-L)})$

Where: Y = gas volume at time t
b = total gas production
c = rate of gas production
t = Incubation time
L = lag time

4.2.12 Statistical analysis

The experiment was a three way cross-classification with the factors forage, energy and nitrogen used in a factorial ANOVA in Statistica 13.1 (2016). In cases where no interaction was observed, the main effects were interpreted with LSD mulitiple comparisons and Benferroni tests. Signaficance was declared at $P < 0.05$ %.

4.3 Results and discussions

4.3.1 *In vitro* DM disappearance

Dry matter disappearance values of *LH* and *WS* alone (without any supplements) after 30 h of incubation are presented in Table 4-4.

Table 4-4 Dry Matter disappearance (±SEM) of wheat straw and lucerne hay after 30 hours of fermentation without supplementation

FORAGE SOURCE	*LH*	*WS*
% DMD	46.77±8.60	22.90±5.86

Dry matter disappearance of *LH* was higher than that of *WS* (P<0.001) (Table 4.5). This could be explained by the higher proportion of digestible nutrients in *LH* (NRC, 2001), the lower NDF content, as well as the NDF structure of lucerne hay. Samples sizes of 228 mg (DM) were used for both forage sources, implying that, on a DM basis, the *LH* samples contained 103.7 mg of NDF and *WS* samples 183.5 mg of NDF. The *WS* samples thus contained 77% more NDF than the *LH* samples. This difference in nutrient composition probably affected DM disappearance due to the higher amount of NDF present in *WS* treatments versus *LH* treatments.

Dry matter disappearance values of *LH* and *WS*, supplemented with different energy and N sources, are presented in Tables 4-5 and 4-6, respectively.

Table 4-5 Dry matter disappearance (±SEM) of lucerne hay as forage source after 30 hours of fermentation, supplemented with different energy and nitrogen sources

ENERGY SOURCE	*M*		*C*		*S*	
NITROGEN SOURCE	*U*	*SB*	*U*	*SB*	*U*	*SB*
% DMD	54.61±2.57	51.79±4.27	44.88±3.85	47.52±2.39	50.73±6.00	31.10±3.70

Table 4-6 Dry matter disappearance (±SEM) of wheat straw as forage source after 30 hours of fermentation, supplemented with different energy and nitrogen sources

ENERGY SOURCE	*M*		*C*		*S*	
NITROGEN SOURCE	*U*	*SB*	*U*	*SB*	*U*	*SB*
% DMD	25.38±3.78	22.04±0.36	14.08±3.19	19.78±7.78	25.22±2.36	30.93±2.94

Dry matter disappearance in *LH* was the highest when supplemented with *M* as energy source (Table 4-5) and in *WS* with *S* (Table 4-6). DM disappearance was higher in *LH* and *WS* when supplemented with *U* than with *SB*, when combined with *M*. The highest DM disappearance for *WS* as forage was present when supplemented with *S* and *SB*, while *M* and *U* supplementation led to the highest DM disappearance in *LH* (Figure 4-1).

The DM disappearance values of *LH* and *WS,* together with the various forage treatments, are illustrated in Figure 4-1.

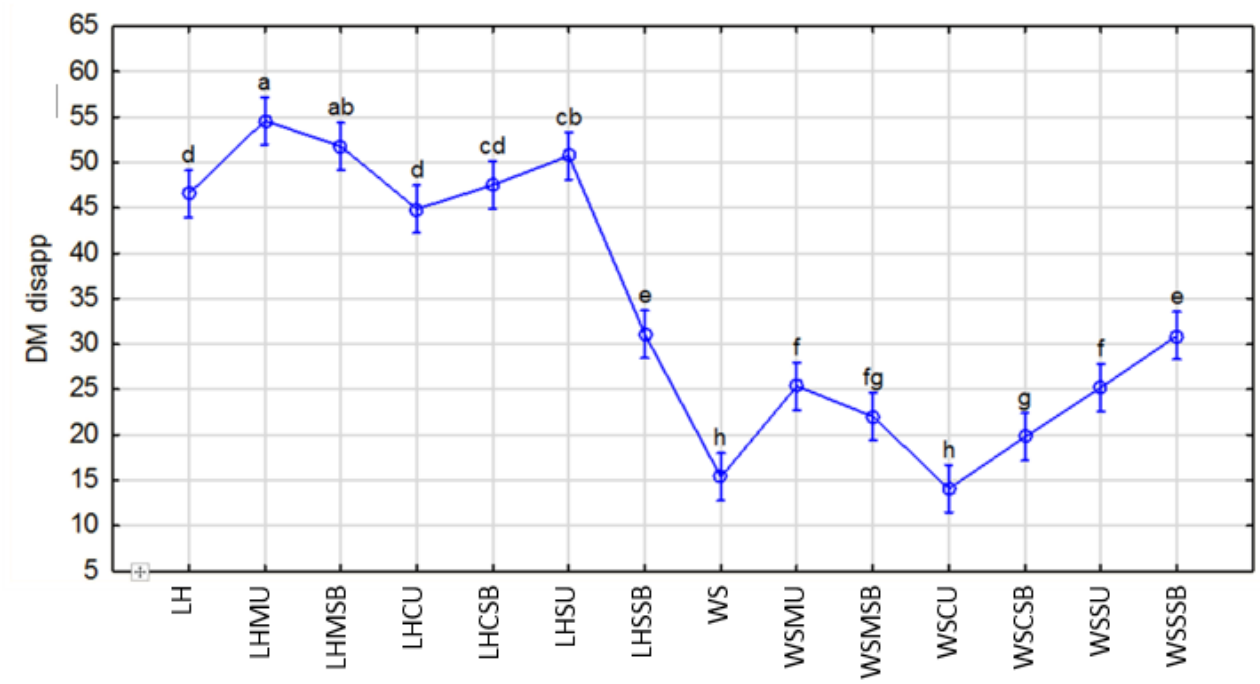

Figure 4-1 Dry matter disappearance (%) of forages alone or supplemented with different energy and nitrogen sources after 30 hours of fermentation. Means with different subscripts differ (P<0.05)

It is evident in Figure 4-1 that supplementation of *LH* with *C* had no effect on DM disappearance when compared to *LH* alone. With *M* as energy source, the DM

disappearance was higher than that of *LH* without supplementation, irrespective of the nitrogen source. Supplementation of *LH* with *S* and *SB* suppressed DM disappearance to an average of 31.10±3.70 %. Similar to the results of *LH*, the combination of *C* and *U* had no effect on DM disappearance of *WS* either. Treatments with M as energy source led to an increase in DM disappearance with both *SB* and *U* as nitrogen source. With *C* as energy source, *SB* led to a higher DM disappearance in *WS* than urea did. The combination of *S* and *U* led to similar results as *M* and *U* in *WS*. The combination of *S* and *SB* led to the highest DM disappearance of all the *WS* treatments, and did not differ from the DM disappearance of *LH* with the same energy and nitrogen source supplementations.

4.3.2 In-Vitro NDF Disappearance

The NDF disappearance values determined after 30 hours of fermentation for treatment *LH* and treatment *WS* are presented in Table 4-7.

Table 4-7 NDF Disappearance (±SEM) of wheat straw and lucerne hay after 30 hours of fermentation without supplementation

FORAGE SOURCE	*LH*	*WS*
% NDFD	26.84±7.06	17.06±6.37

NDF disappearance was higher in *LH* than in *WS* ($P<0.001$) when no supplemental energy and nitrogen was included (Table 4.7). This might be contributed to the higher ADF content of *WS* when compared to *LH* (NRC, 2001).

The NDF disappearance values of *LH* and *WS*, supplemented with different energy and N sources, are presented in Tables 4-8 and 4-9, respectively.

Table 4-8 NDF Disappearance (±SEM) of lucerne hay as forage source after 30 hours of fermentation, supplemented with different energy and nitrogen sources

ENERGY SOURCE	*M*		*C*		*S*	
NITROGEN SOURCE	*U*	*SB*	*U*	*SB*	*U*	*SB*
% NDFD	33.07±1.81	23.43±1.26	28.97±1.49	26.66±0.10	34.83±1.00	14.05±1.19

The NDF disappearance of treatments *LHMU* and *LHSU* was higher than that of treatment *LH,* suggesting that the combination of urea and maize as well as the combination of urea with molasses syrup had a positive effect on NDF disappearance of lucerne hay. *In vitro* studies conducted by Belasco (1954) showed that urea is superior to soybean meal in promoting cellulose digestion. The NDF disappearance in *LH* supplemented with the combination of *M* and *SB* was significantly lower (P<0.05) than the combination of *M* and U*.* Heldt et al. (1999) suggested that microorganisms responsible for carbohydrate fermentation may deplete nitrogen sources leading to a decrease in fibre digestion. The lower rumen digestibility of soybean meal might have led to a nitrogen depletion, while urea in contrast is 100% rumen degradable (Larson, 2003).

Nitrogen source had no effect on the NDF disappearance when C served as energy source. This result is in line with studies done by Gressley and Armentano (2005) who showed that pectin supplementation did not affect digestion in good quality hay.

Table 4-9 NDF disappearance (±SEM) of wheat straw as forage source after 30 hours of fermentation, supplemented with different energy and nitrogen sources

ENERGY SOURCE	*M*		*C*		*S*	
NITROGEN SOURCE	*U*	*SB*	*U*	*SB*	*U*	*SB*
% NDFD	18.13±0.81	15.89±0.54	5.60±2.10	16.27±0.54	20.13±0.54	26.34±1.71

Of the three energy sources, *S* had the biggest effect on NDF disappearance in *WS* (Table 4-9). McCullough (1968) found that cellulose digestion of hay is better maintained by molasses than maize. The only treatment that lowered NDF disappearance to below the control value was the combination of *C* and *U*. Hoover (1986) noted that urea as sole protein source is not sufficient to optimize fibre digestion due to the microorganisms' requirements for amino acids.

The combination of *S* and *SB* led to a DM disappearance percentage in line with those found in *LH* treatments (Figure 4-2). Protein supplementation in the form of oilcakes increased the digestibility of low quality forages (Khandaker *et al.,* 2012)

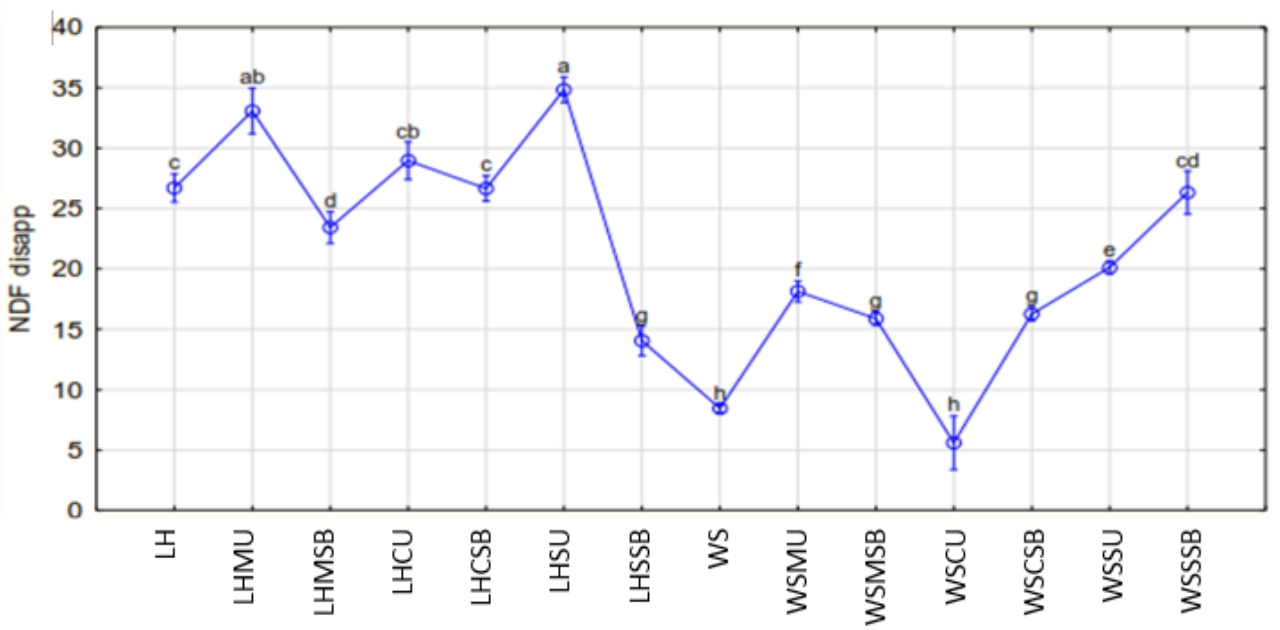

Figure 4-2 NDF disappearance of forages alone or supplemented with different energy and nitrogen sources after 30 hours of fermentation. Means with different subscripts differ (P<0.05)

NDF Disappearance in *LH* was higher when supplemented with *U* than with *SB*, and *U* also had a larger effect on NDF disappearance in *LH* than in *WS.* This may in part be due to the naturally higher nitrogen levels found in legumes such as lucerne, which contributed to an overall higher nitrogen availability. *WS* had a higher NDF disappearance when supplemented with *SB* than with *U* (P<0.05). The highest NDF disappearance values were seen when *LH* was supplemented with *S* as energy source and U as nitrogen source and for *WS* when it was supplemented with *S* as energy source and *SB* as nitrogen source. Heldt et al. (1999) found that sugar supplementation increased NDF digestion provided that sufficient nitrogen is available. Holsthausen and Hall (2002) also found that the rate of NDF digestion was increased with sucrose supplementation when nitrogen was not limited. Work done by Broderick *et al.,* (2004) found that 5% sucrose addition is the optimum level for fibre digestion.

4.3.3 Kinetic coefficients of in-vitro gas production

4.3.3.1 Forages without supplementation

The kinetic coefficient values for forages, *LH* and *WS* respectively, are summarized in Table 4-10. The total gas production (b), rate of gas production (c) and lag time (L) was derived from the gas volume data as explained in section 4.2.11.

Table 4-10 Kinetic coefficient values (b, c and L) for lucerne hay and wheat straw without supplementation

Parameter[1]	Forages			
	LH	*WS*	SEM	P
b	384.357	336.000	18.215	0.065
c	0.106	0.112	0.008	0.560
L	0.480	0.435	0.093	0.737

[1]b=Total gas production; c=Rate of gas production; L=Lag time

Without supplementation, the total gas production (*b*) for *LH* was not significantly higher than that of *WS* (P=0.065), although there was a strong tendency (P=0.065). The rate of gas production (*c*) also did not differ between the forages, neither did the lag time (L).

4.3.3.2 Forages supplemented with different energy and nitrogen sources

Results of the main effects "Energy Source" and sub-effects "Nitrogen Source" on kinetic coefficients, b, c and L of the respective forages are shown in Table 4-11.

Table 4-11 Kinetic coefficients (±SEM) for in-vitro gas production of lucerne hay and wheat straw supplemented with different energy and nitrogen sources

ENERGY SOURCE		*M*		*C*		*S*	
NITROGEN SOURCE		*SB*	*U*	*SB*	*U*	*SB*	*U*
LH	b	365.52±84.13	562.58±219.51	367.61±53.74	353.95±146.72	412.56±113.31	243.92±77.70
	c	0.085±0.029	0.057±0.024	0.063±0.040	0.144±0.045	0.164±0.063	0.121±0.034
	L	0.053±0.129	1.287±0.816	1.444±0.771	0.034±0.052	0.010±0.025	0.051±0.082
WS	b	384.54±53.90	528.44±74.75	282.43±142.90	351.23±47.64	292.03±76.40	177.30±88.14
	c	0.071±0.012	0.072±0.011	0.131±0.057	0.128±0.054	0.151±0.042	0.120±0.094
	L	0.767±0.767	0.448±0.360	0.250±0.612	0.346±0.848	0.047±0.115	0.755±0.968

4.3.3.2.1 Total gas production (b)

As stated earlier, the total gas production of the forage treatments without supplementation (treatment *LH* and treatment *WS*) did not differ from each other (Table 4.10). The results show fewer differences between the two forage sources than what had been observed with DM and NDF disappearance. It is clear from Figure 4-3 that supplementation of the two

forages with energy and nitrogen significantly increased the total gas production. An energy*nitrogen interaction was present (P<0.0005). The total gas production of the treatments containing *U* differed from each other. There were no differences in total gas production between the different energy sources supplemented with *SB* when *LH* served as forage source. The combination of *M* as energy source and *U* as nitrogen source showed the highest volume of gas produced with both of the forage sources. This suggests that, if gas production would be an indicator of potential NDF digestion, the *M*U* combination would be the combination of choice when the aim is to enhance NDF digestion of *WS* or *LH*.

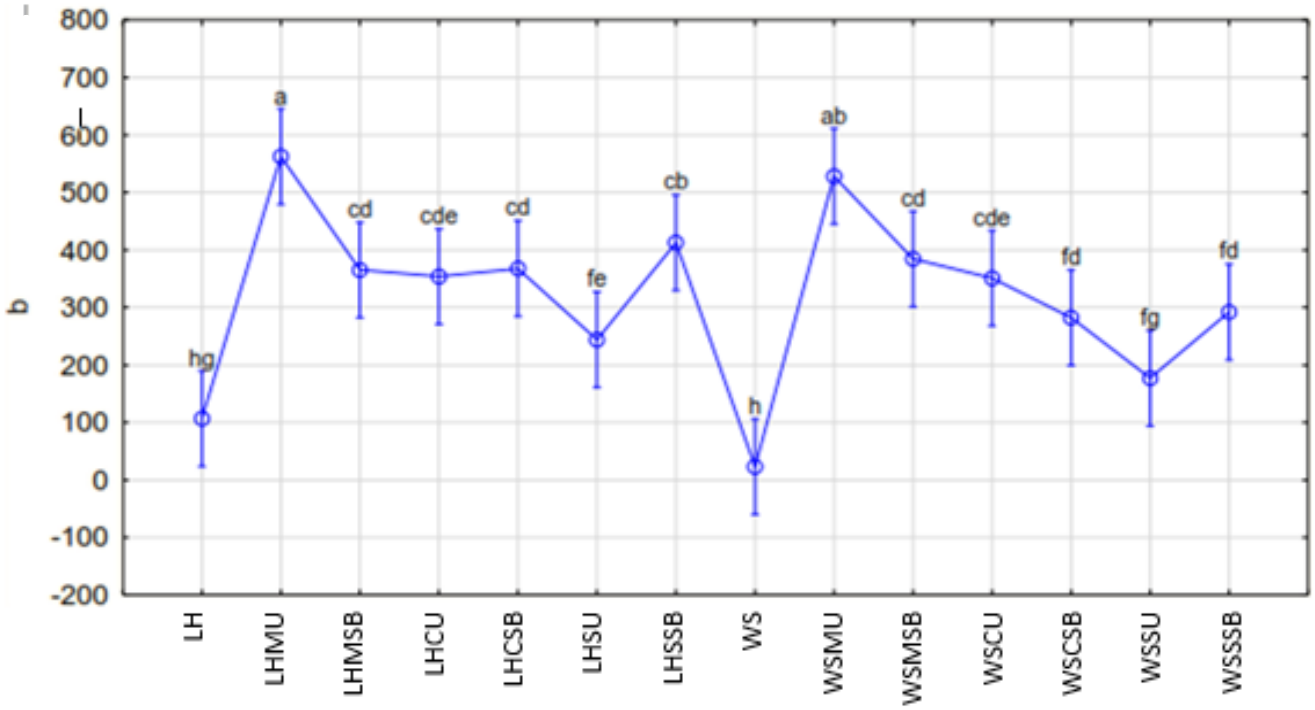

Figure 4-3 Total gas production (b) values for treatments. Means with different subscripts differ (P<0.05)

4.3.3.2.2 Rate of gas production (c)

The rate of gas production for the various energy*nitrogen combinations are compared in Table 4-12.

Table 4-12 Rate of gas production for energy*nitrogen combinations

ENERGY SOURCE	M		C		S		SEM	P
NITROGEN SOURCE	U	SB	U	SB	U	SB		
c	0.065^{a}	0.078^{a}	0.136^{bc}	0.971^{ab}	0.121^{bc}	0.157^{c}	0.014	0.025

Values with different superscripts differ (P<0.05).

As stated earlier, there was no significant difference between the rates of gas production of the two forage sources (Table 4.12). There was an energy*nitrogen interaction present which affected the rate of gas production (P<0.05). In both *WS* and *LH* as forage sources, the highest rate of gas production was present with the combination of *S***SB* (Table 4-11).

Table 4-13 Rate of gas production for energy sources

ENERGY SOURCE	M	C	S	SEM	P
c	0.07[a]	0.166[b]	0.139[b]	0.010	<0.01

Values with different superscripts differ (P<0.05).

A significant difference (P<0.001) was present in the rate of gas production of the three energy sources, with *C* and *S* having higher rates of gas production compared to *M* (Table 4-12).

4.3.3.2.3 Lag time (L)

The lag time was affected by a forage*energy*nitrogen interaction (P<0.001), as well as an energy*nitrogen interaction (P<0.005). Both *M* and *C* as energy sources increased lag time of *LH*, while *S* had no effect. In *WS*, maize had the highest effect on lag time. There was no difference in the effect of nitrogen source *per se* on lag time. Considering the interaction of energy and nitrogen sources, *U* had the largest increase in lag time when combined with *M*, while *SB* had the largest increase in lag time when combined with *C.* The lag time in *LH* was elevated only by the combination of *M* and *U* and of *C* and *SB*. The remaining combinations all had a suppressing effect on lag time. In *WS*, all of the combinations of energy and nitrogen sources increased lag time, except for *S* and *SB*, which had no effect.

4.3.4 Cumulative *in vitro* gas production

Upon incubation, substrates are partially solubilised, with the soluble components being rapidly fermented. The insoluble nutrients are hydrated and colonized by the rumen microorganisms prior to fermentation. The rate at which this processes occurs is dependent on the composition of the microbial population together with their ability to colonize, ferment and utilize the substrates. The extent to which substrates are resistant to these processes influences their gas production profiles (Groot *et al.,* 1996).

The total cumulative gas production measured in ml gas/g OM over a 30-hour period for LH and WS is illustrated in Figures 4-4 and 4-5, respectively.

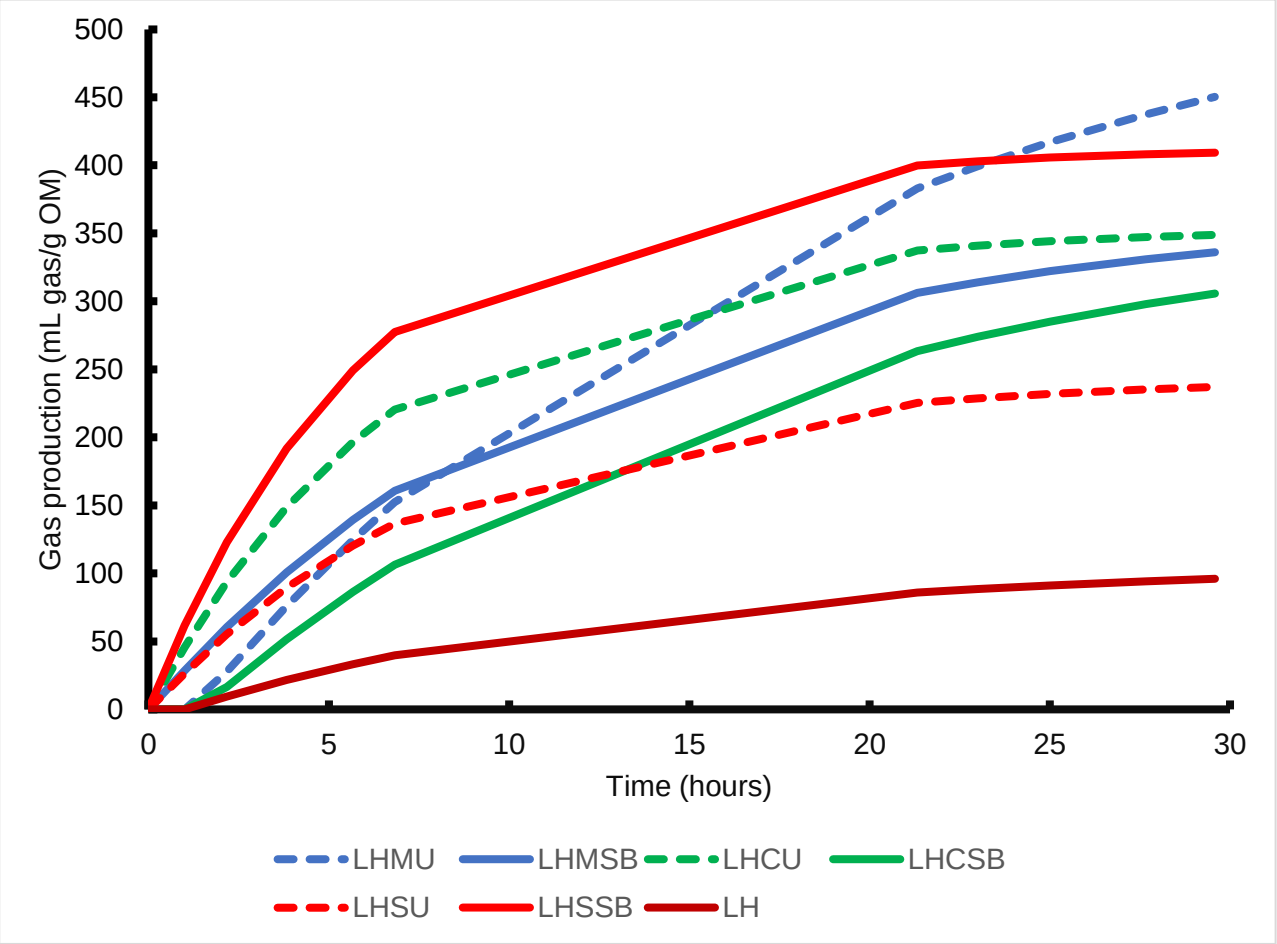

Figure 4-4 In-vitro gas production (mL gas/g OM) curves for lucerne hay without supplementation and when supplemented with different energy and nitrogen sources

Without supplemental energy and nitrogen, *LH* peaked at 96 ml gas/g OM at 30 hours. An increase in gas production was present when *LH* was supplemented with an energy-nitrogen combination. *LHSSB* initially had the highest rate of gas production. One possible explanation might be the high amount of readily available energy supplied by molasses in this treatment. After 20 hours of incubation the rate of gas production decreased, which indicated depletion of available energy to microorganisms. The *LHMU* treatment had a lower initial rate of gas production, but began to exceed that of *LHMSB* between 5 and 10 hours, leading to a higher gas production value at 30 hours. This might be an indication that the microorganisms required more time to gain access to the nutrients within *M*, whereas *S* consisted of easier accessible nutrients for fermentation. A noticeable difference was seen in the gas production profiles of *LHSU* and *LHSSB*, considering that the only difference between these two treatments was the nitrogen source. When *U* served as nitrogen source

the rate of gas production was slower and the total amount of gas produced after 30 hours of fermentation was considerably lower. This might be due to the early depletion of supplemental nutrients because both *S* and *U* are readily fermented upon contact with microorganisms.

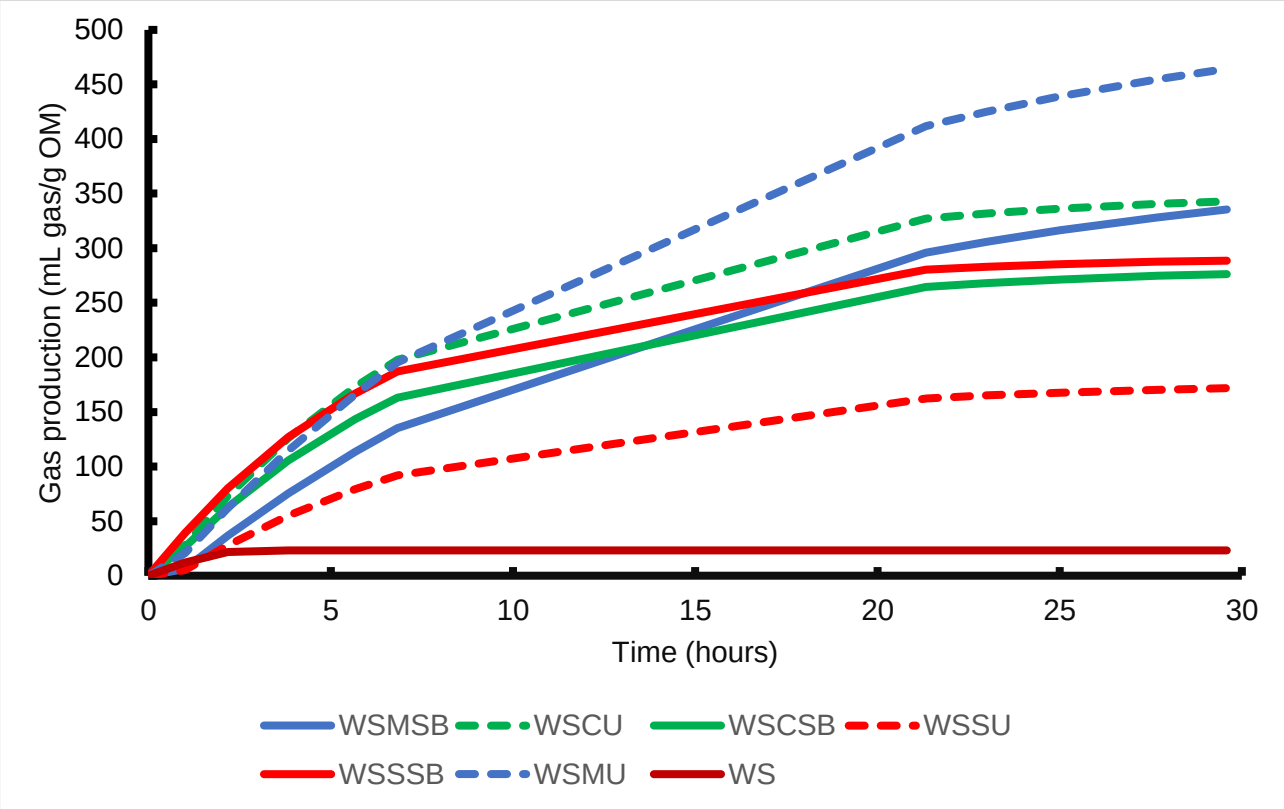

Figure 4-5 In-vitro gas production (mL gas/g OM) curves for wheat straw when supplemented with different energy and nitrogen sources

The cumulative 30-hour gas production curves for *WS* treatments are presented in Figure 4-5. It was evident that the gas production of *WS* alone without supplementation reached a plateau shortly after incubation was initiated, suggesting that *WS* alone without supplementation did not serve well as a fermentation source to the microorganisms present in the incubation medium. The combination of *M* and *U* yielded the highest amount of gas at 30 hours, similar to the results seen in *LH* as forage source (Figure 4.4). Also similar to the *LH* gas production curves, the combination of *S* and *U* had a slower rate of gas production as well as a lower net gas production value at 30 hours of fermentation, supporting the suggestion that combining two readily available nutrient sources with forage is not ideal for optimal fermentation.

There was no clear relationship between the cumulative in vitro gas production and NDF disappearance, implying that gas production alone does not appear to be a reliable indicator of NDF digestibility.

4.4 Conclusion

In the control treatments, both DM and NDF disappearance was higher in *LH* than in *WS* after 30 hours of fermentation. When the forages were supplemented with energy and nitrogen a forage*energy*nitrogen interaction was observed for both DM and NDF disappearance. The combination of *M* and *U* led to the highest DM disappearance value in *LH*, while the combination of *S* and *SB* led to the highest DM disappearance in *WS*. The combination of *M* and *U* and the combination of *S* and *U* led to the highest NDF disappearance in *LH*, while the combination of *S* and *SB* showed a lower NDF disappearance than *LH* without supplementation. However, the same combination of *S* and *SB* showed the highest NDF disappearance in *WS*. The total gas production was influenced by an energy*nitrogen interaction. In both *LH* and *WS*, the highest amount of gas produced was present when *M* served as energy source and *U* as nitrogen source. All the energy and nitrogen treatments enhanced gas production compared to untreated forages. An energy*nitrogen interaction was also present in the rate of gas production, with both forages having showed the highest rate of gas production when supplemented with *S* and *SB*. The lag time for gas production was the highest for both forages when *M* served as energy source.

The null hypothesis, stating that the source of energy and nitrogen in in-vitro total mixed rations would have no effect on forage digestion or would not yield any forage*energy*nitrogen interactions, was rejected. The second null hypothesis, stating that there would not be a relationship between cumulative gas production and NDF disappearance, was not rejected.

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CHAPTER 5: GENERAL CONCLUSION

This thesis reported on two *in vitro* studies aimed at improving forage digestion. In the first study, a high quality forage (lucerne hay) and a poor quality forage (wheat straw) were incubated *in vitro* with the filter bag method with an energy source, being either maize, citrus pulp or molasses syrup and a nitrogen source, being either soybean meal, urea or no added N. Forage samples were weighed out to supply 125 mg of NDF, while energy sources were calculated to represent an energy equivalent of 125 mg of pure starch. Nitrogen sources were added in an amount which equals the nitrogen content of the incubation medium used in control treatments. There was no treatment that increased DM disappearance significantly in either of the two forage sources after six or 30 hours. The highest NDF disappearance values for *LH* treatments were observed with the combination of *C*U* as well as *C*SB*, after six and 30 hours, respectively. With *WS* as substrate, the highest NDFD values were observed with the combination of *C*SB* and *S*SB,* at six and 30 hours, respectively. It is thus evident that *SB* had the largest effect on NDFD at 30 hours and is therefore a better choice of nitrogen source than *U* when the aim is to enhance NDFD over a longer period. The use of *C* showed to be the best of the three energy sources to combine with *LH*.

In the second study, the same sources were used as in the first study. Total mixed rations were simulated in which roughage was included in the fermentation vessels at 229 mg DM, energy sources at 188 mg DM and nitrogen sources calculated to supply 21 mg N. After termination of the incubation, the same digestion parameters were measured as in the first study. The highest DM disappearance was seen with the combination of *M*U* and *S*SB* for the two forages, *LH* and *WS*, respectively. The combinations that showed the highest NDF disappearance was *M*U* and *S*U* for *LH,* and *S*SB* for *WS*. In both *LH* and *WS*, the highest amount of gas produced was present when *M* served as energy source and *U* as nitrogen source. There is no clear relationship between gas production and fibre digestibility. It was concluded that the various combinations of forages, energy and nitrogen sources affected forage digestibility differently and knowledge thereof might be of importance in formulating ruminant total mixed rations.