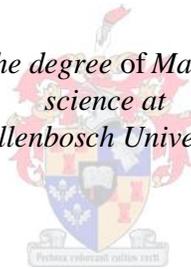


**THE EFFECT OF DIFFERENT ENERGY AND NITROGEN
SOURCES ON *IN VITRO* FIBRE DIGESTION OF HIGH AND
LOW QUALITY ROUGHAGES**

by

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*Dissertation presented for the degree of Masters in the Faculty of Animal
science at
Stellenbosch University*



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March 2015

DECLARATION

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Date: March 2015

ABSTRACT

Title: The effect of different energy and nitrogen sources on *in vitro* fibre digestion of high and low quality roughages

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Fibre digestion differs among roughages and it is also affected by the supplementation of different energy and nitrogen sources. Improving ruminal fibre digestion could increase the energy intake of ruminant animals and increase production. It is thus important to maximize forage utilization.

This thesis reports on two *in vitro* studies aimed at the improvement of DM and NDF digestibility, as well as *in vitro* true digestibility (IVTD). In the first study, four levels of starch were chosen (0, 35, 80 and 125 mg hexose equivalents) to supplement 125 mg of NDF from either lucerne hay (good quality roughage) or wheat straw (poor quality roughage). The *in vitro* procedure was a combination of the filter bag and test tube (Tilley & Terry, 1963) methods, with slight modifications to the Goering & Van Soest (1970) incubation medium. Samples were incubated at 39 °C for six or 30 hours. No significant differences were observed among levels of starch supplementation regarding fibre digestion, thus the highest inclusion level (50:50 starch HE:NDF) was used in the second study.

In the second study, different combinations of energy sources (starch, sucrose, pectin) and nitrogen sources (urea, soybean meal or no N) were studied with the two roughages. The same *in vitro* procedure was followed as in the first study, but the treatment combinations included an incubation medium that contained no nitrogen. The same digestibility parameters were measured as in the first study and pH was also recorded at the end of the fermentations. Regarding six hours DM digestibility, lucerne showed the best results with sucrose supplementation without any N. After 30 hours, sucrose with soybean meal proved to be the best combination. For wheat straw,

treatments had no effect on six hours DM disappearance, but after 30 hours, pectin and soybean meal resulted in the highest digestion values.

Similar results were observed for NDF digestibility and IVTD, where the sucrose and soybean meal combination gave the best results for lucerne hay after 30 hours of incubation. For wheat straw, the highest NDF digestibility and IVTD values were observed with the pectin and soybean meal combination at 30 hours. After six hours of fermentation, the highest pH values were observed for lucerne hay with starch and urea supplementation. After 30 hours, the same combination of starch and urea resulted in the highest pH values in both roughages.

In the second study, it was shown that pectin and sucrose as energy sources resulted in the highest *in vitro* digestibility values when supplemented to low quality (wheat straw) and high quality (lucerne hay) roughages, respectively. Soybean meal proved to be the best N supplement for both roughage sources. It was concluded that different energy sources affect *in vitro* digestibility of different roughages in different ways. This may have practical implications in ration formulation for ruminant animals.

UITTREKSEL

Titel: Die invloed van verskillende energie- en stikstofbronne op die *in vitro* veselverteerbaarheid van hoë- en laekwaliteit ruvoere

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Die graad van veselvertering verskil tussen ruvoere en dit word ook beïnvloed deur supplementering met verskillende energie- en stikstofbronne. Deur veselvertering te verhoog, kan herkouers meer energie inneem wat dus produksie kan verhoog. Dit is dus belangrik om veselbenutting te maksimeer.

Hierdie tesis handel oor twee *in vitro* studies wat daarop gemik was om droëmateriaal (DM)- en NDF-vertering, asook *in vitro* ware verteerbaarheid (IVTD) te verhoog. In die eerste studie is vier vlakke van stysel gekies (0, 35, 80 en 125 mg heksose ekwivalente) om 125 mg NDF, afkomstig van lusern (hoë kwaliteit ruvoer) en koringstrooi (lae kwaliteit ruvoer), te supplementeer. Die *in vitro*-metode wat gebruik is, is 'n kombinasie van die filtersakkiemethode en die proefbuismetode van Tilley & Terry (1963), met geringe aanpassings in die inkubasiemedium van Goering & Van Soest (1970). Monsters is vir ses of 30 ure by 39 °C geïnkubeer. Geen betekenisvolle verskille in veselvertering is waargeneem tussen die verskillende vlakke van stysel supplementering nie en daar is besluit om die hoogste insluitingsvlak (50:50 stysel:NDF) in die tweede studie te gebruik.

In die tweede studie is verskillende kombinasies van energiebronne (stysel, sukrose en pektien) en stikstofbronne (ureum, sojaboon meel, of geen N) as supplemente tot die twee ruvoere nagegaan. Dieselfde *in vitro*-prosedure is gevolg as in die eerste studie, maar die inkubasiemedium is aangepas om geen stikstof te bevat wanneer die verskillende kombinasies getoets is nie. Dieselfde verteerbaarheidsparameters is

gestoets as wat die geval in die eerste studie was, terwyl pH ook na die fermentasies gemeet is. Betreffende die ses-ure fermentasies, het lusern die beste resultate getoon met sukrosesupplementering en geen stikstof nie. Na 30 ure was sukrose en sojameel die beste kombinasie. In die geval van koringstrooi, het behandelings geen invloed op ses-ure DM-verdwynings gehad nie, maar na 30 ure het pektien en sojameel die hoogste verteerbaarheidswaardes gelewer.

Soortgelyke resultate is ten opsigte van NDF-verteerbaarheid en IVTD waargeneem, waar die sukrose- en sojameelkombinasie die beste resultate in lusern gelewer het na 30 ure fermentasie. In koringstrooi is die hoogste NDF-verteerbaarheid en IVTD-waardes na 30 ure waargeneem met die pektien- en sojameelkombinasie. Na ses ure fermentasie is die hoogste pH-waardes waargeneem vir lusernhooi met die supplementering van stysel en ureum. Na 30 ure het dieselfde kombinasie van stysel en ureum die hoogste pH-waardes tot gevolg gehad in beide ruvoere.

In die tweede studie is bevind dat pektien en sukrose as energiebronne die hoogste *in vitro*-verteerbaarheidswaardes tot gevolg gehad het wanneer dit as supplemente tot, onderskeidelik, lae-kwaliteit (koringstrooi) en hoë-kwaliteit (lusernhooi) ruvoere gebruik is. Sojameel was die beste N-aanvulling vir beide ruvoerbronne. Die gevolgtrekking is gemaak dat verskillende energiebronne die *in vitro*-verteerbaarheid van verskillende ruvoere op verskillende maniere beïnvloed. Hierdie bevindings mag praktiese implikasies in voerformulering vir herkouers inhou.

ACKNOWLEDGEMENTS

I would like to express my appreciation to all the people who contributed to my Master's degree.

- My father, Philip Strauss, for giving me finances and encouraging me to pursue a postgraduate degree
- My mother, Elsable Strauss, for emotional support and understanding
- My supervisor, Prof Cristian Cruywagen, for giving my guidance, advice, further developing me as a scientist and countless coffees
- Beverly Ellis, the laboratory supervisor, for going out of her way on more than one occasion to ensure my results are on time and accurate
- Prof Nel, for helping with the statistical analysis of the results and explaining concepts
- Katinka Neetling, for helping me with the rumen studies early in the mornings and prepping late at night
- The dairy manager, Antonie, for always ensuring that the cannulated cows are available when I needed them
- Norman Luyt and his family, for encouraging me and giving me emotional support when I was far from home
- My adored flatmate, Legh Wilkinson, for giving insight on what to expect from a post graduate degree and because she always understood the problems I was facing as a researcher
- Feedmaster Namibia for funding my research
- My Lord Father, for making all of this possible and blessing me with all these people in my life

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LIST OF ABBREVIATIONS

ADF – Acid Detergent Fibre

ADL – Acid Detergent Lignin

CO₂ – Carbon dioxide

CP – Crude Protein

DIP – Degradable Intake Protein

DM – Dry Matter

DMI – Dry Matter Intake

DMD – Dry Matter Digestibility

HE – Hexose Equivalent

ME – Metabolise Energy

N - Nitrogen

NDF – Neutral Detergent Fibre

NDFD – Neutral Detergent Fibre Digestibility

NFC – Non-neutral Detergent Fibre Polysaccharide

NH₃N – Ruminal Ammonia Nitrogen

NPN – Non-protein Nitrogen

NSC – Non Structural Carbohydrate

NSP – Non-starch Polysaccharide

OM – Organic Matter

PecNon – Pectin with no added nitrogen

PecSoy – Pectin with added soy bean meal

PecUre – Pectin with added urea

RDP – Rumen Degradable Protein

RUP – Rumen Un-degradable Protein

StaNon – Starch with no added nitrogen

StaSoy – Starch with added soy bean meal

StaUre – Starch with added urea

SucNon – Sucrose with no added nitrogen

SucSoy – Sucrose with added soy bean meal

SucUre – Sucrose with added urea

VFA – Volatile Fatty Acids

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Cattle and sheep are classified as ruminants due to the micro-organisms in their gastro intestinal tract that can digest fibre, unlike the usual mammalian enzymes in monogastric animals (Buxton & Redfearn, 1997; Holtshausen, 2004). “Fibre can be defined nutritionally as the slowly digestible or indigestible fraction of feeds that occupies space in the gastrointestinal tract of animals” (Mertens, 1997). The rumen micro-organisms are complex (Weimer *et al.*, 1999) and responsible for fibre digestion (Varga & Kolver, 1997). They synthesize and secrete the β 1-4 cellulase enzyme complex, leading to the hydrolysis of plant cell walls (Varga & Kolver, 1997). The micro-organisms work in symbiosis with each other and attach to the fibre surface (Weimer, 1998). The high fibre content of forages can thus be utilized as a main source of energy (Theander & Åman, 1980) by the rumen micro-organisms, especially by the following cellulose digesting bacteria: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *R. albus* (Weimer, 1998).

1.2. The need for roughages in a diet

The inclusion of forages in a diet has numerous advantages. Normal ruminal movements and healthy digestive flow is achieved with as little as 10 -15% forage inclusion in a diet (Russell & Wilson, 1996). This is ensured by the fibre’s ability to stimulate salivation, rumination and the formation of a normal rumen mat (Van Soest *et al.*, 1991; Chalupa *et al.*, 1996). The mat will make particles more susceptible to rumen micro-organism digestion (Nocek & Tamminga, 1991); this will aid as a filtering system that inhibits rapid passage of feed stuffs and prevents the loss of nutrients (Van Soest *et al.*, 1991). Coppock *et al.*, (1974) showed that inclusion levels of forages at less than 40% may lead to depressed milk fat percentage and higher incidences of acidosis. Forages have been shown to reduce the incidence of metabolic disorders, inhibit the decrease of milk fat percentage in dairy cows, prevent problems with rumination and cud chewing, prohibit a decrease in dry matter intake (DMI), prevent laminitis and ensure faecal consistency (Ishler & Varga, 2001). Most of which is due to a more stable rumen environment because fibre can maintain a normal pH level that will

maintain the cellulolytic micro-organisms (Van Soest *et al.*, 1991). Maintaining the cellulolytic micro-organisms will lead to a production of a high acetate: propionate fatty acid ratio hence normal lipid metabolism in the cow (Van Soest *et al.*, 1991) ensuring no depression in milk fat percentage. As a result, inclusion of fibre is necessary to ensure healthy animals as it will ensure normal rumen function and high milk fat percentage (Van Soest *et al.*, 1991). Fibre can thus be seen as a key nutrient (Miller, 1979).

1.3. Nutrients required

Only 30% of the energy and protein that dairy cattle consume is captured in the milk. The rest is lost in heat, faeces and urine (Chalupa *et al.*, 1996). Energy and protein losses during digestion and metabolism should be minimized to increase production (Chalupa *et al.*, 1996).

The two metabolic systems in the ruminant that need nutrients, are the rumen microbes and the rumen tissue (Chalupa *et al.*, 1996). These systems require different nutrients and ruminal tissue should receive favour (Chalupa *et al.*, 1996). Amino acids is one of the important nutrients and ruminal microbes that digest cellulose and hemicellulose need ammonia as a primary nutrient, whereas microbes that digest starch, sugar and pectin use not only ammonia but peptides and amino acids to enhance growth (Chalupa *et al.*, 1996). Ammonia can thus easily be a limited nutrient and 50% of the degradable protein in the diet should be soluble protein to aid in the syntheses of tissue proteins and milk proteins to increase growth and milk yield in ruminal tissue (Chalupa *et al.*, 1996). The amino acids required are provided by rumen micro-organisms and dietary protein that passed the rumen (Chalupa *et al.*, 1996). Ruminal tissues receive energy from volatile fatty acids (VFA), an end-product of carbohydrate and fat digestion or from the adipose tissue when cows are in a negative energy balance after calving (Chalupa *et al.*, 1996).

Diet components (fibre and carbohydrates) can thus affect the proportion of VFA production (primarily acetate), the amount of energy available for the ruminal tissue and the balance of rumen micro-organisms (Chalupa *et al.*, 1996). Diets high in fibre are not adequate to provide high levels of energy for milk production. This in turn decreases body weight in cows and leads to below genetic potential production (Chalupa *et al.*, 1996). A combination of fibre and concentrates are needed because

the grains in concentrates do not have enough fibre for normal rumen function (Chalupa *et al.*, 1996).

1.4. Different roughages

Forage and roughage, are the main sources of fibre, and are used as an interlinked term. Forages do not only provide ruminants with fibre, but also with energy, protein, minerals, vitamins, lipids and water (Miller, 1979). Forages can be classified as pastures, hay, silage, chopped fresh forage and haylage (Miller, 1979). These feedstuffs are evaluated to determine their value for the animal by determining the quantity of nutrients available (protein and energy) and the intake thereof by the animal (McCullough, 1969), as well as the rate and extent of digestion (Getachew *et al.*, 2004), this can be seen in Table 1.1. The quality of forages differ due to 1) plant species and varieties 2) components of the plant (leaves or stems) 3) stage of maturity 4) soil characteristics 5) climate, weather and season and 6) the changes that it undergoes during drying, storing, preserving and processing (Miller, 1979). Forage digestibility can differ between and within plant families and should not be lower than 60% (McCullough, 1969).

Ruminants fed on pasture are not labour intensive but this also has disadvantages; pastures are dependent on the season and grow unevenly throughout the year and cows can also be selective and trample feedstuffs (Miller, 1979). Hay on the other hand is a dehydrated forage, dried at a specific stage of maturity to preserve the nutrients (McCullough, 1969). The forages are dried to decrease the chances of mould formation, but over-drying can make the roughage brittle, resulting in a loss of nutrient-rich leaves (Miller, 1979). The ideal stage of maturity for harvesting hay can be an unsuitable time for drying (Miller, 1979). The most common hay is alfalfa or alfalfa-grass mixtures (Miller, 1979). Hay and silage can have energy values in the range of 7-13 MJ/kg dry matter (DM) and crude protein in the range 60-250 g/kg DM (Wilkins, Givens, Owen, Axford, & Omed, 2000). Plants can be harvested for silage at a time that is not suitable for drying to make hay, but is a more complicated process that involves machinery and is not ideally adapted to be done on small scale (Miller, 1979). Maize silage is more popular than hay crop or grass silage and grasses are easier ensiled than legumes (Miller, 1979). The dry matter of silages should be monitored thoroughly to be between 28-40% because if the moisture levels are too high, nutrients

will be lost by seepage and could also lead to high pH values (Miller, 1979) making the feedstuff unpalatable.

Feeding animals chopped fresh forages incurs no storage cost, little field loss from trampling and selective grazing, and can be harvested at the ideal stages of maturity (Miller, 1979). However, roughages will differ between seasons and be limited in certain seasons (Miller, 1979). Forages cannot be kept cut for too long since the feedstuffs would generate heat as the early stages of ensiling would begin and make the feedstuff unpalatable (Miller, 1979).

Haylage is also known as a low-moisture silage which has the qualities of hay and silage (Miller, 1979). Commonly haylage is made when crops are harvested to make hay, but due to the unfavourable weather, the harvest is tured into silage (Miller, 1979). Haylage has a dry matter content of 20-60% (Miller, 1979). This particular silage has more air trapped in the plant and it is more difficult to reach anaerobic conditions in the bunker due to the fact that the harvest cannot compact tight enough (Miller, 1979).

Typical chemical composition of some roughages is indicated in Table 1.1. Chemical composition of the roughages affects intake, more closely digestibility (Van Soest, 1965) and also has different effects on volatile fatty acid (VFA) production (Getachew *et al.*, 2004). Crude protein, neutral detergent fibre (NDF) and acid detergent fibre (ADF) has an overall negative correlation with VFA production especially with propionate and butyrate, whereas non-neutral detergent fibre polysaccharides (NFC) has a positive correlation with VFA and especially propionate and butyrate (Getachew *et al.*, 2004).

Table 1.1. Chemical composition of roughages (g/kg) (Getachew *et al.*, 2004)**1.5. Micro-organisms present in the rumen**

Starch and cellulose are both energy components in fibre and both consist of glucose molecules (Miller, 1979). These glucose molecules are linked together by α -linkage in starch and β -linkage in cellulose (Miller, 1979). Cellulose's β -linkage is much tougher than the α -linkage and the digestive system of animals do not secrete enzymes that can break this bond (Miller, 1979). Cellulose is thus digested by the micro-organisms in the rumen that can secrete the β 1-4 cellulase enzyme complex that hydrolyse the cellulose in the plant cell walls (Varga & Kolver, 1997).

The rumen consists of a diverse rumen micro-organism environment of which only a few are known to be cellulolytic organisms. Ruminants are in symbiosis with these cellulolytic organisms where the animal provides an environment for microbial growth in exchange for the fermentation end products from microbial digestion of fibre (acetate, propionate, butyrate and amino acids) (Russell & Wilson, 1996). Fibrolytic bacteria digest cellulose and hemicellulose, the structural part of the plant tissue (Holtshausen, 2004) and are in constant competition with other micro-organisms for

Roughages	Crude Protein	NDF	ADF	NFC
Alfalfa hay	262	338	286	268
Alfalfa hay	263	351	266	251
Average	262.5	344.5	276	259.5
Alfalfa silage	168	432	341	254
Alfalfa silage	240	394	333	228
Average	204	413	337	241
Wheat middling	203	385	152	310
Wheat middling	170	399	124	337
Wheat middling	179	367	134	342
Average	184	383.7	136.7	329.7
Wheat silage	70	534	374	221
Wheat silage	95	458	327	308
Average	82.5	496	350.5	264.5

nutrients (Weimer, 1996).

In the rumen, the most important fibrolytic bacteria (also known as cellulolytic bacteria) are *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens* which all secrete cellulase enzymes (Weimer, 1996), known as the β 1-4 cellulase enzyme complex (Varga & Kolver, 1997). Rumen cellulolytic bacteria attach to the surface of cellulose and thus ensure a concentrated cellulose supply for digesting (Weimer, 1996). *Ruminococcus flavefaciens* attach mostly to damaged cell surfaces (the epidermis) by means of the coat of extracellular glycoprotein, whereas *B. succinogenes* can attach to damaged cell surfaces and smoother surfaces by means of the fine fibres that extend from its exterior (Latham *et al.*, 1978). Attachment also benefits the cellulolytic bacteria ensuring that other micro-organisms cannot attach to the substrate and the rumen cellulolytic bacteria would thus have first access (Weimer, 1996). The attachment of the bacteria to the cellulose surface also protects the bacteria from rumen degradation and grazing protozoa (Weimer, 1996).

Although some cellulolytic fungi and protozoa are also present, it is mostly bacteria that are responsible for cellulose digestion (Bauchop, 1979). Ruminal fungi contribute to fibre digestion only when they are present in large numbers (Akin & Borneman, 1990). Fungi has the ability to infiltrate the cuticle layer and disrupt the lignocellulose complex (Akin & Borneman, 1990). By disrupting the complex the bacteria, which is normally too large, can now penetrate the layer (Akin & Borneman, 1990).

1.6. Motivation for current study

From the discussion above, it is apparent that roughage alone cannot provide in the nutrient requirements of high producing ruminants. Furthermore, rumen microbes cannot digest fibre to the maximum potential without energy and nitrogen supplementation. Energy sources such as sugar, starch and pectin are frequently used as supplements to roughages in ruminant diets, in order to meet the energy requirements for growth and production. However, there is a lack of information regarding the magnitude of the relationship between different carbohydrate and nitrogen sources on the one side and neutral detergent fibre (NDF) fermentation kinetics in the rumen on the other side.

The objectives of this study were to determine the impact of three energy sources (starch, sucrose and pectin) and two nitrogen sources (soybean meal and urea) on the

in vitro DM and NDF digestion of two commonly used roughage sources (lucerne hay and wheat straw).

1.7. Project objectives and hypothesis

The study is presented in two experimental chapters.

1.7.1. First experimental chapter: The effect of different starch:NDF ratios on in vitro fibre digestion of lucerne hay and wheat straw

The objective of the first experimental chapter was to determine what concentration of starch should be used to determine the effect of energy and nitrogen sources on fibre digestion (low quality and high quality roughages) in the second experimental chapter. The concentration of starch that would be chosen would also be the basis for calculation of the concentrations of the other energy sources on a hexose equivalent basis.

Hypotheses:

Ho: There will be no significant difference with the different levels of starch on fibre digestion.

Ha: There will be a significant difference with at least one of the different levels of starch on fibre digestion

1.7.2. The second experimental chapter: The effect of different energy and nitrogen sources on in vitro fibre digestibility of lucerne hay and wheat straw

The objective of the second experimental chapter was to determine the effect and interactions of three energy sources (maize starch, pectin and sucrose) and two nitrogen sources (soybean oilcake and urea) on fibre digestion using a high and low quality roughage, looking at DM degradability, NDF digestibility, *in vitro* true digestibility and pH levels.

Hypotheses:

Ho: Different energy and nitrogen sources do not significantly affect fibre digestion.

Ha: There will be a significant difference in at least one combination treatment when different energy and protein sources are combined with lucerne or wheat straw and fibre digestion is analysed

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Over the last four years an increase in animal production has been seen in South Africa (2010-2013) (“South African yearbook,” n.d.). During the last two years (2012-2013) specifically, animal production has increased with 3.6% (“South African yearbook,” n.d.). This trend has been led by the price increase in animal products (7.4% in 2012-2013). Field crop prices, however, have also increased from 2010-2014, but the actual production of field crops decreased. Feed cost is still the biggest cost involved in agriculture, it represents 20% of the total expenses; this includes fuel, farm services, maintenance and repairs, and all these expenses have increased with 11% over the last two years. The increase in animal production and decrease in available feed thus led to the increase of 10% in animal feed prices, in 2012-2013 when compared to 2010-2011 (“Trends in the Agricultural Sector,” 2013, “South African yearbook,” n.d.).

Thus, animal producers have to ensure that they gain as much nutrients as possible from their feeds to increase profit. This could be achieved by gaining the maximum amount of nutrients from roughages (the bulk of ruminant animal feed) by increasing

digestibility through the contribution of added energy and nitrogen sources without depressing digestibility.

2.2. Carbohydrates

2.2.1. Structural and non-structural carbohydrates

Carbohydrates can be grouped into two types; structural and non-structural carbohydrates as seen in Figure 2.1 (Ishler & Varga, 2001). Non-structural carbohydrates (NSC) consist of components not included in the cell wall matrix and cannot be analyzed by neutral detergent fibre procedures (Van Soest *et al.*, 1991). These include sugars, starches, fructans and organic acids (Van Soest *et al.*, 1991). Structural carbohydrates consist of pectin, β -glucans, galactans, fructans, cellulose, hemicellulose and lignin (Holtshausen, 2004). Due to the fact that pectin, β -glucans, galactans and fructans are soluble in neutral detergent solution (Van Soest *et al.*, 1991) and have rapid absorption from rumen micro-organisms (Ishler & Varga, 2001), these components do not fall in the dietary fibre category. Pectin is has the capability to be fermented in the rumen almost completely (90-100%) and therefore is not covalently linked to lignin in the cell (Nocek & Tamminga, 1991). These are known as non-starch polysaccharides (NSP) (Van Soest *et al.*, 1991). Combining the NSC and NSP components lead to a new group known as non-neutral detergent fibre polysaccharides (NFC) (Holtshausen, 2004).

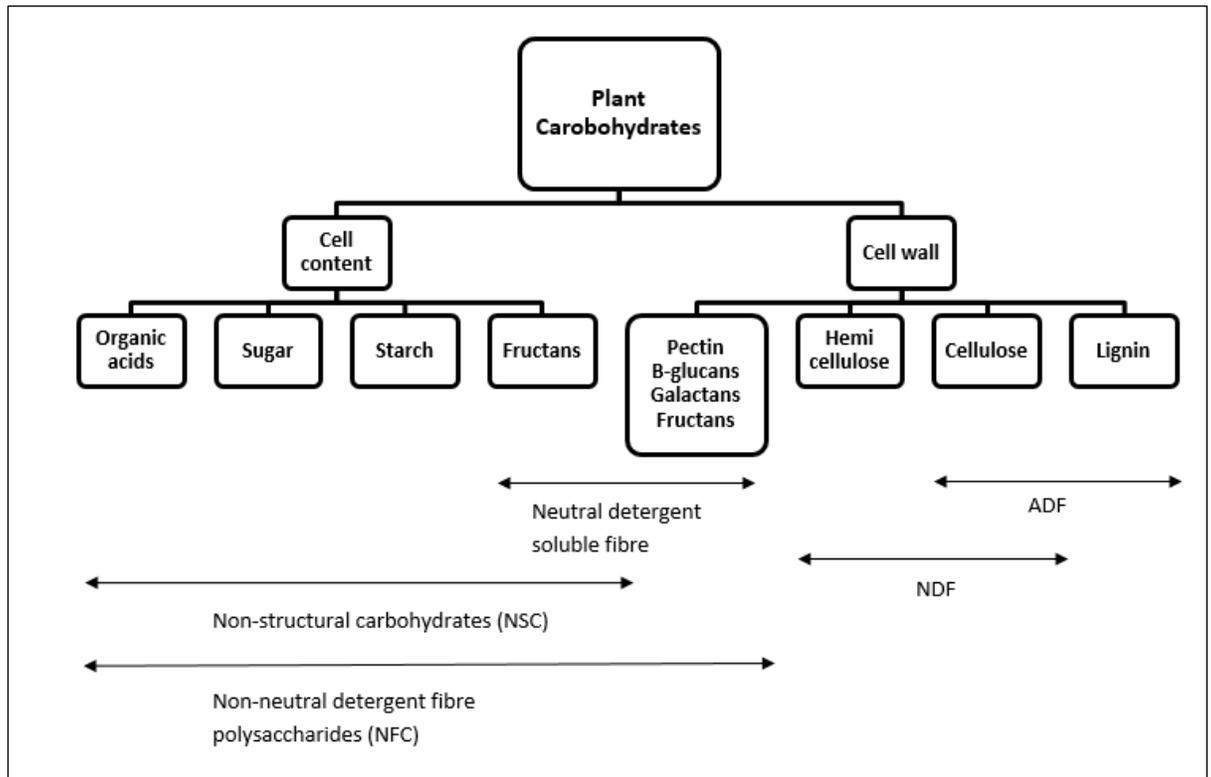


Figure 2.1. Components of plant carbohydrates.

2.2.2. Dietary Fibre

Dietary fibre is non-starch polysaccharides that can only be digested by ruminant micro-organisms and not monogastric enzymes (Holtshausen, 2004). Van Soest (1965) explained that fibre can be divided into two groups, the fibrous and soluble group. The fibrous group consist of cell wall components such as fibre bound protein and hemicelluloses that is soluble in acid detergent and cellulose, lignin and lignified nitrogenous compounds that is classified as acid detergent fibre (ADF) (as seen in Figure 2.1). The fibrous part is partly digestible depending on the amount of lignin present. The soluble group consists of cell contents and is almost completely digestible (Van Soest, 1965).

The soluble group contains components such as sugars, organic acids, water soluble matter, pectin, starch, non-protein nitrogenous compounds and soluble proteins (Van Soest, 1965). The soluble group is soluble in neutral detergent and can be called the neutral detergent fibre (NDF) portion. Increasing the NDF will also decrease the voluntary intake especially when the cell wall content makes up 50 -60% of the dry matter (Van Soest, 1965) and maturation of the plant will increase the NDF fraction of the plant (Shaver *et al.*, 1988). NDF can be seen as the chemical characteristics of fibre and particle size and density can be understood as the physical characteristics (Mertens, 1997). The physical characteristics influence the animal's health, the utilization and fermentation properties of the rumen, the animal's metabolism and milk fat production (Mertens, 1997).

Different types of dietary fibres do not always share the same characteristics and thus are not the same nutritionally, chemically and physically and, due to fermentation in the rumen by the micro-organisms, the initial composition of the diet is changed (Van Soest *et al.*, 1991). As a result of this fact it remains difficult to create a uniform model for fibres as a whole. The prediction of fibre digestion remains problematic because dietary fibre is influenced by the quality of NSC and the proportion of sugar and starch in the diet compared to NSP, this will affect the rumen environment and microbial efficiency (Van Soest *et al.*, 1991).

2.2.2.1. *Fibre digestion*

Fibre digestion is the portion of fibre ingested and not excreted (Tamminga, 1993) or the absorption of nutrients divided by the nutrient uptake (Allen & Mertens, 1988). Fibre contains an indigestible part as well as a more digestible portion. In roughages the rate of digestion varies and the total fibre digestion depends on the size of the roughage digestible portion (Allen & Mertens, 1988). In some cases the indigestible part may be more than the digestible part (Ishler & Varga, 2001). Fibre digestion has two processes. The simplest one is the transformation of monosaccharide (simple sugars) into volatile fatty acids, fermentation gasses and heat. The second process is the hydrolysis of polysaccharides with enzymes (Chesson & Forsberg, 1997). There is a difference between digestibility and digestion. Digestibility can be understood as the fibre's susceptibility to degradation and digestion can be understood as the extent of

fibre degradation (Zinn & Ware, 2007). Therefore the rate of digestion is not always compatible with the amount of digestion.

2.2.2.2. *Factors affecting fibre digestion*

Ruminal fibre digestion can vary from 13-78% in forages and non-forages (Ishler & Varga, 2001). Cellulose in fibre is not a rapidly digestible energy source and digestion thereof can vary from 20 – 70% (Varga & Kolver, 1997). This will only allow a 10 – 35% net energy intake from roughages (Varga & Kolver, 1997). There are numerous factors that affect fibre digestion.

a) Animal species

The animal size plays a role in fibre digestion. Bigger ruminants like cows do not ruminate the forage as fine as smaller animals and this has been proven to lead to a longer retention time giving more time to digest the NDF fraction (Poppi *et al.*, 1981). This shows that there is digestibility variation between species and breeds.

b) Forage types

Legumes (alfalfa) are more digestible than grasses even though legume fibre is less digestible, this is possible because legumes have almost half as much fibre as grasses (Buxton & Redfearn, 1997). Legumes have a faster fibre digestion rate but grasses have better total digestion, (Varga & Hoover, 1983) due to grasses having a longer retention time in the rumen (Ishler & Varga, 2001). The fact that grasses spend more time being digested has been shown to lead to grasses producing more gas (methane and carbon dioxide) than legumes when fermenting in the rumen (Ishler & Varga, 2001). These gasses cause the feed particles to drift to the top of the rumen environment where they get suspended in a fibre mat, decreasing the area surface for digestion from rumen micro-organisms and leading to a slower digestion rate (Ishler & Varga, 2001). Grasses have a smaller indigestible portion when compared to legumes, but due to the higher gas production of grass, the legumes have a better digestion rate when using a smaller time frame comparison (Ishler & Varga, 2001). The gas production and longer retention time with grass consumption will create gut fill and this can initiate a higher intake of legumes (Ishler & Varga, 2001). This is significant for the industry because two diets containing the same chemical composition and NDF content, with different ingredients, can differ in total digestion (Varga & Hoover, 1983). This was proven when Oba & Allen (1999) compared production parameters (dry matter intake, milk yield and body weight gain) within a forage family (within legumes or

within grasses) or across a forage family. Within a forage family an increase in NDF concentration will increase production but a decrease in production was noted across forage families (Oba & Allen, 1999). Thus it is very difficult to compare different forages with each other.

c) Forage maturity and forage components

Forage harvested later in the season is more mature and this has been shown by Welch & Smith (1969, 1970) to increase rumination time and decrease digestibility (Mowat *et al.*, 1965). This increase in rumination time could be due to the increase in structural fibre with plant age and can be seen as the most important factor affecting fibre digestion (Buxton & Redfearn, 1997). A counter argument could be that increasing the rumination time and salivation could lead to a better buffering capability (Van Soest *et al.*, 1991). Mature plants have slower fermentation rate, resulting in lower digestibility, this however could contribute to a more stable rumen environment by providing a higher ruminal pH (Van Soest *et al.*, 1991).

In immature grasses there is little difference in digestibility between the stem and the leaf (Mowat *et al.*, 1965). At a mature stage the stems show a higher concentration of structural fibre when compared to the leaves (Buxton & Redfearn, 1997). Stems have more structural tissue whereas leaves consist of thin walled mesophyll cells that will shorten the rumen retention time (Buxton & Redfearn, 1997). Even though the leaves have shorter retention time, it has still been shown to be more digestible than stems (Buxton & Redfearn, 1997). When the plant matures the stems decline in digestibility more quickly than the leaves, this combined with an increase in the stem: leaf ratio with maturity will make the whole plant less digestible (Buxton & Redfearn, 1997).

When keeping in mind that legumes are more digestible than grasses and leaves are more digestible than stems, it was shown that even though orchard grass at maturity is leafier than alfalfa, alfalfa still has better digestibility (Mowat *et al.*, 1965).

d) Concentrates

An increase in concentrates in the diet, especially NSC, has shown to decrease fibre digestibility (Hoover, 1986; Varga & Kolver, 1997). This could be due to the rapid digestion of the NSC (Varga & Kolver, 1997), a decrease in ruminal pH and/or a decrease in fibrolytic micro-organisms (Hoover, 1986). Protein supplementation in the form of mustard oil cake (Khandaker *et al.*, 2012) and sodium caseinate (Köster *et al.*, 1996) increased the digestibility of the low quality roughage and decreased the pH of

the rumen but never below the cellulytic threshold (Köster *et al.*, 1996; Khandaker *et al.*, 2012). The effect of different concentrates on fibre digestion will be clarified in detail later in the literature review.

e) Forage processing

Particle size has shown to have an influence on the intake of the forages. Pelleting and grinding of forages will reduce the particle size and increase intake (Laredo & Minson, 1975; Shaver, Nytes, Satter, & Jorgensen, 1986). This has then been shown to have an effect on the retention time of the forages in the rumen, shortening the time and hence leading to a decrease in digestibility of the forages (Laredo & Minson, 1975). Shaver *et al.* (1986) explained that the decrease in digestibility could also be because a decrease in ruminal pH was noted, that could indicate less rumination, chewing and saliva production leading to an unstable rumen environment.

f) Intake

Studies have shown that an increase in intake will decrease the digestion of the forages (Staples *et al.*, 1984; Shaver *et al.*, 1986; Bourquin *et al.*, 1990). The reason why digestibility decreases linearly with increased intake (Staples *et al.*, 1984) could be due to the increase in rumen gut fill leading to a decrease in retention time (Shaver *et al.*, 1986). The animal's intake is limited to the rumen volume (Chalupa *et al.*, 1996) and the gut fill factor depends on the NDF content of the diet and the NDF digestibility (Weimer *et al.*, 1999). This was proven when increasing the dry matter intake from 2% to 4% of body weight. The forage retention time declined from 24.7 to 15.6 hours, thus ensuring a lower digestion. Staples *et al.* (1984) had also shown a lower ruminal pH when feed intake was high. This could also be the cause of slower ruminal fibre digestion

g) Feeding strategies

In dairy cows with depressed milk fat, increasing the frequency of feedings increased the milk fat percentage, but did not increase the milk yield (Robinson, 1989). The increase in frequency increased the rumen fermentation process leading to an increase in acetate: propionate ratio and helped to obtain the body condition of high producing cows (Robinson, 1989). Studies have also shown that the sequence of feeding can influence the animal's performance (Robinson, 1989; Nocek, 1992). Dairy cows are normally fed a concentrate in the milking parlor in the mornings. Feeding forage before concentrates will decrease the dry matter intake (DMI) of the concentrates (Nocek,

1992) but feeding concentrates early in the morning will lead to animals not consuming roughages for several hours (Robinson, 1989; Chalupa *et al.*, 1996). This can have negative effects on the rumen environment. When feeding 2 types of roughages the more palatable roughage should be fed after concentrate consumption to stimulate more intake. The less palatable roughage can be fed later when the cows are hungrier (Robinson, 1989). Feeding animals more frequently and giving roughages before concentrate consumption will have positive effects on the rumen environment.

2.3. Non-neutral detergent fibre polysaccharides (NFC)

NFC (sugar, starch, organic acids, pectin, β -glucans, galactans, and fructans) are known to be the most common sources of energy for high producing ruminants like dairy cows, and are used to optimize production in an intensive system (Huntington, 1997). NFC are also more palatable and digestible when compared to NDF in dietary fibre and are practically fully fermented in the rumen (90-100%) (Van Soest *et al.*, 1991).

There are methods to improve NFC digestibility, these can be either physical (breaking, cracking, grinding, rolling and pelleting) or chemical (heat and water) (Nocek & Tamminga, 1991). Processing improves digestion of the NFC and the best processing method is applying moist heat (Nocek & Tamminga, 1991). Starch particles in the endosperm of cereal grains, is in a matrix with protein and processing destroys this matrix making starch more susceptible (Nocek & Tamminga, 1991). Processing can also decrease digestion if the extent is too extreme and gelatinization forms (Nocek & Tamminga, 1991). Processing types that causes the temperature to reach 60 - 80°C will cause gelatinization and make the protein-starch matrix more complex and indigestible (Nocek & Tamminga, 1991). This will have the opposite of the desired effect.

2.3.1. Different NFC sources

2.3.1.1. Starch

The starch content of grains vary, with wheat having the highest percentage and barley and oats the lowest (Huntington, 1997). Starch fermentation of maize in the rumen is only 40% which is abundantly less than the fermentation of barley, wheat and oats

(90%) (Orskov, 1986) and could be explained by the fact that dried grains contain plenty of insoluble starch, which digest slowly (Sniffen *et al.*, 1992). The animal however can't utilize the volatile fatty acid production of starch at 90% fermentation (Orskov, 1986).

The method of processing is important. Flaked maize is more digestible than ground or cracked maize (Orskov *et al.*, 1969). The processing of starch has a great effect on metabolic health of animals. In lambs whole grains are healthier than pelleted or rolled grains (Orskov *et al.*, 1969; Mould *et al.*, 1983) because the whole grains are fermented more slowly due to a bigger surface area being exposed (Orskov *et al.*, 1969). Whole barley only inhibits digestion when the inclusion level thereof is 75% or more, whereas pelleted barley inhibits digestion even before a 75% inclusion (Mould *et al.*, 1983). Grains are however processed because rumen micro-organisms cannot digest the grain if the pericarp is still present (Huntington, 1997). The rumen micro-organisms can only penetrate the whole grain after a few days if it was not cracked during chewing or rumination (Orskov *et al.*, 1969).

Cows on the other hand need to have the grain processed otherwise 30% of the grain will be found in the feces (Orskov, 1986). Processing grains increases the digestibility thereof, but this can also lead to metabolic disorders if the fermentation rate is too fast (Huntington, 1997). Increasing the starch intake will increase the amount of starch passing through the rumen and digested in the small intestine (Orskov, 1986). When comparing diets with just concentrates or added fibre it was noted that diets with added fibre will let more starch escape the rumen (Orskov, 1986).

The extent of starch digestion depends on the source of starch, dietary composition, amount of feed consumed, processing (mechanical and chemical) and adaptation of the rumen micro-organisms (Huntington, 1997).

2.3.1.2. *Pectin*

Pectin is commonly found in dicotyledonous species where legumes are the most important source (Van Soest *et al.*, 1991). Pectin is also found in citrus and beet pulp but at lower levels in grass forages (Van Soest *et al.*, 1991). Citrus pulp can be given to animals dried (Fegeros *et al.*, 1995), fresh (Sparkes *et al.*, 2010) or pelleted (Villarreal *et al.*, 2006). Dried citrus pulp has a high energy value, low protein value of 7.1 %

(Fegeros *et al.*, 1995; Villarreal *et al.*, 2006) and NDF value of 19.4% (Fegeros *et al.*, 1995).

Pectin does not vary as much in fermentation rates as sugar and starch because it is not as affected by the source it comes from and the quality of the carbohydrate (Van Soest *et al.*, 1991). Pectin has high fermentation rates and intestinal bacteria can degrade 99% of pectin administered abomasally (Gressley & Armentano, 2005) but pectin still provides buffering to the rumen, because it largely does not affect cellulose digestion and, due to its galacturonic acid structure, it does not yield lactic acid (Van Soest *et al.*, 1991). This provides a stable rumen environment even though pectin still has a high fermentation rate (Van Soest *et al.*, 1991).

Rumen pH that has declined to 6, will decrease pectin digestion with 53% (Strobel & Russell, 1986). The micro-organisms that digest pectin are thus particularly sensitive to pH fluctuation, even more so than sugar and starch digesting micro-organisms (Strobel & Russell, 1986). Even though pectin digestion decreases, acetate production on the other hand, does not decrease with a drop in pH, when pectin is the main carbohydrate source (Strobel & Russell, 1986). Sugar and starch will decrease the acetate production (Strobel & Russell, 1986) that will lead to a decrease in acetate: propionate ratio, leading to a decrease in milk fat percentage (Van Soest *et al.*, 1991). Increased lactate production is also common when sugar and starch are the main carbohydrate sources, but pectin does not ferment to lactate (Strobel & Russell, 1986).

2.3.1.3. Sugar

The main carbohydrate present in molasses is sugar (Heldt *et al.*, 1999) and sugars are degraded promptly by rumen micro-organisms (Sniffen *et al.*, 1992). Sugar is included in a diet to increase the utilization of rapid soluble protein or non-protein nitrogen (NPN/urea) (Hoover *et al.*, 2006) and this is measured by a decreasing ammonia concentration, hence resulting in a positive effect on nitrogen utilization (Khalili, 1993). Due to the sugar's characteristic to dissolve quickly in rumen fluid, the sugar may then skip the fermentation process, due to the higher passage rate of fluids in the rumen and have no effect on nitrogen production (Hoover *et al.*, 2006). Inulin as a source of fructans has been administered abomasally to increase the large intestine fermentation (Gressley & Armentano, 2007). Present in the large intestine, there are bacteria utilizing the energy available and this process may increase the animal's

requirement for rumen degradable protein to digest fibre (Gressley & Armentano, 2007).

2.4. Structural and non-structural carbohydrates' effect on fibre digestibility

2.4.1. Type of forage

Mould *et al.* (1983) compared different forages and the effect of energy supplements. Dried grass had the best digestibility and decreased with the smallest amount in digestibility compared to hay and straw (Mould *et al.*, 1983). The digestibility decrease was highest for the roughage that had the lowest digestibility before trials started (Mould *et al.*, 1983). When comparing hay to silage, hay maintained the levels of cellulose digestion better than silage (McCullough, 1968). This could be due to the higher ammonia nitrogen content of hay (McCullough, 1968). The same effect/result was seen when straw treated with sulphur dioxide was not as sensitive to barley inclusions, because a 70% barley inclusion did not affect organic matter digestibility (Blair-West & Brook, 1969). Treated straw has holes in the cell wall, making it possible for attached bacteria to be protected from rumen environment changes (Blair-West & Brook, 1969).

2.4.2. Starch

Researchers have found that an increase in most energy sources high in starch will decrease fibre digestion (McCullough, 1968; Mould *et al.*, 1983; Heldt *et al.*, 1999). An increase of even 10 -15% NFC decreases fibre digestion (Hoover, 1986). The decrease in fibre digestion could be due to the following five assumptions. 1) The rumen micro-organisms prefer easily fermentable carbohydrates more to the fibre components of the roughages (Hoover, 1986). 2) The micro-organisms that digest starch, produce an inhibitor that inhibits the digestion of fibre (McCullough, 1968). 3) The competition between the rumen micro-organisms for essential nutrients could lead to a decrease in fibre digestion and increase in starch digestion (Hoover, 1986). 4) Changing a diet to have a higher starch component decreases the cellulolytic organisms in the rumen. (Mould *et al.*, 1983). 5) The depletion of available nitrogen by the micro-organisms responsible for carbohydrate fermentation, will decrease the

ability of the micro-organisms to digest fibre (Heldt *et al.*, 1999). 6) The most likely reason is the drop in rumen pH (Hoover, 1986).

2.4.2.1. *pH*

Rumen micro-organisms are sensitive to a low pH, thus activity will decrease (Russell & Dombrowski, 1980) and growth of the organisms will be depressed if the rumen pH drops significantly (Burroughs *et al.*, 1949). Rumen pH varies according to the type of diet and can range from 5 to 7 (Russell & Dombrowski, 1980). The optimum pH for fibre digestion is 6.2 and a level of 6 to 6.1 is known as the cellulolytic threshold (Shriver, Hoover, Sargent, Crawford, & Thayne, 1986) meaning that lower pH levels will inhibit fibre digestion (Hoover, 1986). At a low pH the cellulolytic micro-organisms do not attach to the fibre particles and if the time period of this lower ruminal pH is extended the micro-organisms will wash out (Hoover, 1986) and be eliminated (Mould & Orskov, 1983).

2.4.2.2. *Changing micro-organism concentration*

Studies have shown that increasing the cellulose concentration in the diet will increase the cellulose micro-organism population (Weimer *et al.*, 1999). Comparing a 100% hay and a 100% barley diet showed a difference in cellulolytic count (Mould *et al.*, 1983). The hay diet (high in cellulose) had a cellulolytic count of 10^6 micro-organisms per milliliter and the barley diet (low in cellulose) had 10^4 micro-organisms per milliliter (Mould *et al.*, 1983). The micro-organisms thus adapted and changed to the extent that the amount and type of gram negative forms (cellulolytic bacteria) decreased (Mould *et al.*, 1983). Barley inclusion levels of 75 – 100% also showed an absence of protozoa (Mould & Orskov, 1983) whereas sucrose inclusions have shown high levels of protozoa (Migwi, Godwin, Nolan, & Kahn, 2011). Protozoa are sensitive to low pH and this could be the reason for their absence in a high starch diet (Migwi *et al.*, 2011).

2.4.2.3. *Inclusion of easily fermentable carbohydrates*

Providing livestock an exclusively NFC diet, decreased cellulolytic digestibility and the ruminal pH (Mould & Orskov, 1983). Mould *et al.* (1983) increased the ruminal pH with the addition of bicarbonate salt after the pH dropped below six on an all barley diet. The increase in pH did not increase the already depressed cellulolytic digestion. This could be because the pH did not affect the digestion, but the presence of easily

fermentable feedstuffs did. This has been called the “carbohydrate effect” (Mould *et al.*, 1983). Urdaneta *et al.*, (2000) provide a counter argument that addition of energy supplements to poor quality roughages will increase the digestion thereof. The roughages will have low levels of polysaccharide digestion that will increase when the rumen micro-organisms can utilize more energy from the NFC (Urdaneta *et al.*, 2000). The authors still reported a drop in ruminal pH but not below pH 6 (Urdaneta *et al.*, 2000).

2.4.3. Pectin

Increasing pelleted citrus pulp in a diet increases the total dry matter (DM) digestibility linearly, whereas the forage DM and total NDF is unaffected (Villarreal *et al.*, 2006). This could be due to a faster passage rate (Villarreal *et al.*, 2006). Supplementing maize with citrus pulp in a total mixed ration, gives higher NDF digestibility and the total carbohydrate digestibility is also higher (77% vs 72.5% respectively) (Miron *et al.*, 2002). Sparkes *et al.* (2010) also noted that the *in vitro* dry matter digestibility was 24% higher when fresh citrus pulp was used to replace a portion of the lucerne (49.8% vs 40.1%) and the gas production was also higher, indicating a higher fermentation process in the rumen.

Although citrus pulp has been shown to increase gas production, the acetate: propionate ratio is lowered (Sparkes *et al.*, 2010). This can indicate an improvement on the utilization of metabolisable energy (Sparkes *et al.*, 2010). Pectin was also shown to lead to a decrease in urinary nitrogen and an increase in fecal nitrogen, indicating that pectin increases the energy supply for microbial growth (Gressley & Armentano, 2005). Studies have also shown a decrease in ruminal pH in cows fed citrus pulp and pasture grass, but not below the cellulolytic threshold (Villarreal *et al.*, 2006).

Different results were found when pectin was administered abomasally (Gressley & Armentano, 2005; Sari *et al.*, 2009). A decrease in intake was noted in cows (Gressley & Armentano, 2005) and Saanen goats (Sari *et al.*, 2009). It is suspected that the viscosity of the pectin, causes this effect (Gressley & Armentano, 2005), resulting in a longer retention time in the rumen (Sari *et al.*, 2009). Both studies showed no effect on pH (Gressley & Armentano, 2005; Sari *et al.*, 2009). Pectin decreased the NDF digestibility and milk yield in Saanen goats (Sari *et al.*, 2009), but in cows there was no effect on digestibility or milk yield (Gressley & Armentano, 2005).

2.4.4. Sucrose

Insulin administered abomasally in cows at 0.2% of body weight did not have an effect on pH (Gressley & Armentano, 2007). However Migwi *et al.* (2011) found that giving sheep sucrose at 0.25% of bodyweight per day inter ruminally, decreased the pH, but the mean was above the cellulolytic threshold and only below 6 for three hours. Khalili (1993) also fed cows sucrose at 0.29%, 0.59% and 0.88% of body weight and found a decrease in pH but only below 6.2 for four hours at the highest inclusion levels. This extent was not long enough to negatively affect the cellulose activity of the rumen micro-organisms, because the DM and organic matter digestibility still increased (Khalili, 1993; Migwi *et al.*, 2011), but in the study by Khalili (1993) there was a decrease in NDF digestibility linearly at the two highest inclusion levels.

The increase in dry matter digestibility could be due to longer retention time and the decrease in NDF digestibility could be a result of higher rumen fill (a decrease in intake was noted) as well as a pH and “carbohydrate effect” (Khalili, 1993). In the study by Khalili (1993) there was also an addition of sodium bicarbonate with the highest sucrose inclusion level to increase the ruminal pH, and even though the sodium bicarbonate increased the pH with 5.6%, the NDF digestibility was still decreased. This could indicate that sucrose causes a decrease in digestibility due to the presence of easily fermentable carbohydrates rather than pH drop, and this conclusion can be sustained by the fact that highly volatile fatty acid production levels was found in the study to support the finding of the low pH levels (Migwi *et al.*, 2011).

2.4.5. Starch and sucrose

2.4.5.1. Intake and ruminal pH

In studies comparing the effect of starch and sucrose additions to a diet, different results were found. Stensig *et al.*, (1998) showed that when comparing the two energy sources on a 30% inclusion level, only sucrose increased intake whereas in a study by Royes *et al.*, (2001) all the substitutes (maize, soybean hulls and molasses) decreased the intake of the roughage at the highest inclusion level (30% NFC in the diet). In the same study by Royes *et al.* (2001) at the lowest inclusion level (15%) only molasses decreased the intake. At inclusion levels of only 9% sucrose had no effect on intake of grass silage and starch inclusions led to a decreased pH (Owens *et al.*, 2008).

Some studies show no effect on pH resulting from the different energy sources (Vallimont *et al.*, 2004; Hoover *et al.*, 2006). Stensig *et al.* (1998) and Hindrichsen & Kreuzer (2009) showed a decrease in pH with both starch or sucrose inclusion, especially at high inclusion levels (30%) and showed the highest depression with the inclusion of sucrose. Owens *et al.* (2008) also showed a decrease in pH when sucrose was added even at inclusion levels as low as 9%. Similar results were found when comparing maize, soybean hulls and molasses. A decrease in ruminal pH was found for all the diets but, molasses showed the least depression (Mould *et al.*, 1983; Heldt *et al.*, 1999; Royes *et al.*, 2001). pH tends to decrease with the addition of sucrose but not below the 6.2 level (Owens *et al.*, 2008) and if this does occur, the effect does not last for a significant period of time (Heldt *et al.*, 1999; Royes *et al.*, 2001).

2.4.5.2. NDF digestibility

Sucrose in diets, when compared to starch in diets have shown different results, sucrose have been shown to decrease (Mould *et al.*, 1983; Stensig *et al.*, 1998; Royes *et al.*, 2001), increase (Heldt *et al.*, 1999; Vallimont *et al.*, 2004) and have no effect (Owens 2008) on NDF digestibility. Sucrose also increased organic matter (Heldt *et al.*, 1999) or had no effect on the organic matter (Royes *et al.*, 2001) and DM digestibility (Vallimont *et al.*, 2004; Hoover *et al.*, 2006). Decreases in NDF digestibility with added sucrose was found to be at a lesser extent than added starch (Heldt *et al.*, 1999). Sucrose also had an increase in passage rate that led to a more extended decrease in NDF digestibility (Stensig *et al.*, 1998).

Another reason for the difference in NDF digestibility with the inclusion of sucrose could be due to the starch level (Hoover *et al.*, 2006). Hoover *et al.* (2006) found that if the non-structural carbohydrate component (starch like maize grain and maize silage) is also increased as the sucrose increases there was no effect, or an increase on DM or ADF digestibility. Low NSC levels in a diet with an increasing amount of sugar, decreases the fibre digestion whereas high NSC levels with an increasing amount of sugar show no effect or an increase in fibre digestion (Hoover *et al.*, 2006). NDF digestibility reacted differently to sucrose at different NSC levels (Hoover *et al.*, 2006). The same was observed by Vallimont *et al.* (2004), that increasing sugar in a diet will only have a positive effect on fibre digestion if the NSC component of the diet is higher than 240 g/kg (Huhtanen & Khalili, 1991; Vallimont *et al.*, 2004; Hoover *et al.*, 2006).

Sucrose is less digestible than starch thus supplementing starch with sucrose will not make more carbohydrates available in the diet (Hoover *et al.*, 2006).

Mould *et al.* (1983) described the decrease in digestibility when sucrose was added into the diet as the “carbohydrate effect”. Molasses showed higher ruminal pH levels when it was compared to barley and maize and this resulted in a lesser decrease in digestibility when compared (Mould *et al.*, 1983). McCullough (1968) showed in a study that when supplementing hay with maize or molasses, the molasses maintained the cellulose digestion of hay better than maize and even increased digestion.

2.4.5.3. *Microbial protein*

NDF digestibility increases with higher sucrose levels and this could be due to a change in the rumen micro-organism growth or population (Vallimont *et al.*, 2004). Starch and sugar are the major sources of carbohydrates needed by micro-organisms for growth (Hoover *et al.*, 2006). Hoover *et al.* (2006) found that there is a definite interaction between the starch and sugar components and that an increase in starch decreases the ammonia levels, but an increase in sugar increases the ammonia levels. This could be due to increased proteolysis or a decrease in the conversion of digested feed nitrogen to microbial nitrogen (Hoover *et al.*, 2006). The highest protein digestion was found at the highest sucrose inclusion level when comparing sucrose and starch at different levels (Hoover *et al.*, 2006).

An increase in sucrose, starch and pectin increases the trichloroacetic acid-precipitated crude protein, the highest increase in trichloroacetic acid-precipitated crude protein being shown by starch (Hall & Herejk, 2001). An increase in trichloroacetic acid-precipitated crude protein shows a direct increase in microbial protein indicating that NFC can influence the microbial protein yield and the animal performance accordingly (Hall & Herejk, 2001). Owens *et al.* (2008) on the other hand, showed no increase in microbial protein synthesis with addition of sucrose, but there was an increase with the addition of barley. There was no effect on both for the effectiveness of the synthesis of microbial protein and this could be due to the fact that there was not enough energy available for the micro-organisms to degrade the nitrogen in the grass silage (Owens *et al.*, 2008).

2.4.5.4. Volatile fatty acids (VFA)

The main end-product of fibre digestion is volatile fatty acids (acetate, propionate and butyrate) (Beever *et al.*, 2000). When ruminants consume fibre in ideal amounts the acetate concentration (65%) will be higher than the propionate (20%) and butyrate (15%) concentrations (Miller, 1979). Volatile fatty acids are absorbed through the rumen wall and some can be absorbed through the reticulum, omasum and large intestine where propionate gets converted to glucose and acetate is a precursor for fatty acids. (Miller, 1979). A decrease in forage intake or fine milling of forages will decrease the acetate and increase the propionate concentration thus reducing milk fat content (Miller, 1979).

VFA production seemed to be increased with soybean hulls (Royes *et al.*, 2001), barley (Owens *et al.*, 2008) and sucrose (Hindrichsen & Kreuzer, 2009), whereas the inclusion of maize and molasses had no effect (Stensig *et al.*, 1998; Royes *et al.*, 2001; Owens *et al.*, 2008).

2.4.6. Pectin and starch

Studies comparing pectin and starch have found that pectin has a positive effect on fibre digestion. In a study by Fondevila *et al.*, (2002) starch decreased the gas production of straw and pectin increased it, and in a digestion study by Poorkasegaran & Yansari (2014), pectin also increased the NDF digestibility. Pectin increased the gas production linearly with the amount of pectin added and increased the ability for rumen micro-organisms to ferment (Fondevila *et al.*, 2002). This could be explained by the longer retention time in the rumen and slower passage rate for beet pulp when compared to barely or maize addition (Poorkasegaran & Yansari, 2014). Beet pulp resulted in higher pH values than maize or barley additions in the study by Poorkasegaran & Yansari (2014) but, Fondevila *et al.* (2002) found that the pH remained constant.

Starch and pectin will increase the volatile fatty acid concentrations linearly with any of the substrate inclusions, acetate showed an increased and propionate showed a decrease, with the inclusion of pectin thus increasing the milk fat percentage, whereas starch had the opposite effect (Fondevila *et al.*, 2002; Poorkasegaran & Yansari, 2014). Beet pulp (a pectin source) also gave better ammonia nitrogen production (Poorkasegaran & Yansari, 2014). It is thus evident here that pectin inclusions affected

the rumen environment to a lesser extent than starch inclusions (Fondevila *et al.*, 2002). This could be explained by the study of Lechartier 2011, when comparing pectin and starch feedstuff against each other the same amount of VFA and lactate will be produced in the first few hours post ingestion. Pectin however will have a lower decrease in pH at first due to the fact that pectin can attract and bind hydrogen ions (Lechartier, 2011). Pectin also does not decrease the fibrolytic activity as with starch leading to more VFA production for longer. Unfortunately this will only last a short amount of time since pectin can be almost completely fermented and then the pH will drop significantly leading to the conclusion that pectin feedstuffs are more acidogenic than starch rich feed stuffs (Lechartier, 2011).

2.5. Protein

Microbial protein is formed when rumen bacteria hydrolyze urea to ammonia and this ammonia is used to synthesize cellular protein by being susceptible to rumen micro-organisms (Belasco, 1954). The rumen micro-organisms that then pass the rumen can be digested and absorbed as a protein source and are known as microbial protein (Belasco, 1954). Microbial protein will aid as an important amino acid source for ruminants (Hall & Herejk, 2001).

In a review article by Sniffen *et al.* (1992) protein is divided into 3 fractions as seen in Figure 2.2. Non-protein nitrogen (NPN) is categorized as fraction A, true protein is categorized as fraction B with 3 sub categories and unavailable protein also known as bound protein is in fraction C (Sniffen *et al.*, 1992).

Fraction B is divided into 3 sub categories according to the rate of ruminal degradation. Category B1 is rapidly degraded and is a small fraction of the total soluble protein in forages (5%) but can be double the concentration in concentrates. Most of the soluble protein found in fresh pastures is category B1. Category B2 is fermented in the rumen and in the lower gut when a portion of this protein category bypasses the rumen. The degradation rate of category B2 is strongly dependent on the relative rate of digestion and passage of the feedstuff. Category B3 is slowly degraded in the rumen and furthermore bypasses the rumen. Protein supplements contain small amounts of category B3, but it is found in large quantity in forages, fermented grains and by-products. Category B3 is insoluble in neutral detergent but soluble in acid detergent. It

is thus the proportion of neutral detergent in soluble protein minus the acid detergent soluble protein (Sniffen *et al.*, 1992).

The unavailable or bound protein known as fraction C is highly resistant to mammalian and microbial enzymes. The protein contains lignin and tannin protein complexes. This protein is widely present in hay crop silages, dehydrated alfalfa, citrus pulp, maize distiller's grains and brewer's grains. Fraction C is insoluble in acid detergent making it an acid detergent insoluble protein (Sniffen *et al.*, 1992).

Sniffen & Robinson (1987) also reviewed what affected micro-organism growth and yield in the rumen, to ensure adequate nitrogen absorption by the ruminant. Cows receive 40 -80% of their needed amino acids from the microbial protein that passes to the small intestine. Protein requirement of the ruminant can be met with only addition of urea and the microbial protein available (Sniffen & Robinson, 1987). However Hoover (1986) claimed that urea alone as a nitrogen source is not sufficient enough for fibre digestion since the micro-organisms lack amino acids.

For optimal growth, rumen micro-organisms need sufficient ammonia, peptides and amino acids and thus need sufficient degradable protein in their diet. If a diet is too high in protein the animal will waste energy by producing too much ammonia from the excess protein, thus the inclusion level is just as important as the inclusion itself. Optimal microbial growth has been achieved by increasing dry matter intake. Increasing intake creates greater flow of feed to the rumen. This in turn enhances the production of saliva that maintains the pH, improves hydration, bacterial attachment and retention time of the feedstuff. Improving the retention time gives greater microbial growth (Sniffen & Robinson, 1987).

Increase in intake also increases the liquid outflow of the rumen and feed particles in the early stages of digestion with more attached micro-organisms and thus also the bacterial nitrogen, leading to an increase in micro-organism yield. Maximum yield was found with a 70% forage inclusion and a combination of rapid and slowly digestible carbohydrates. Processing of feedstuff also increases the yield due to more sites for bacterial attachment. Silage with normally lower pH than other processed feedstuff does not however increase the micro-organism yield and this could be due to the fact that the readily digestible carbohydrates and protein is already fermented in the silo, thus leaving the rumen micro-organisms only with the more indigestible portion (Sniffen & Robinson, 1987).

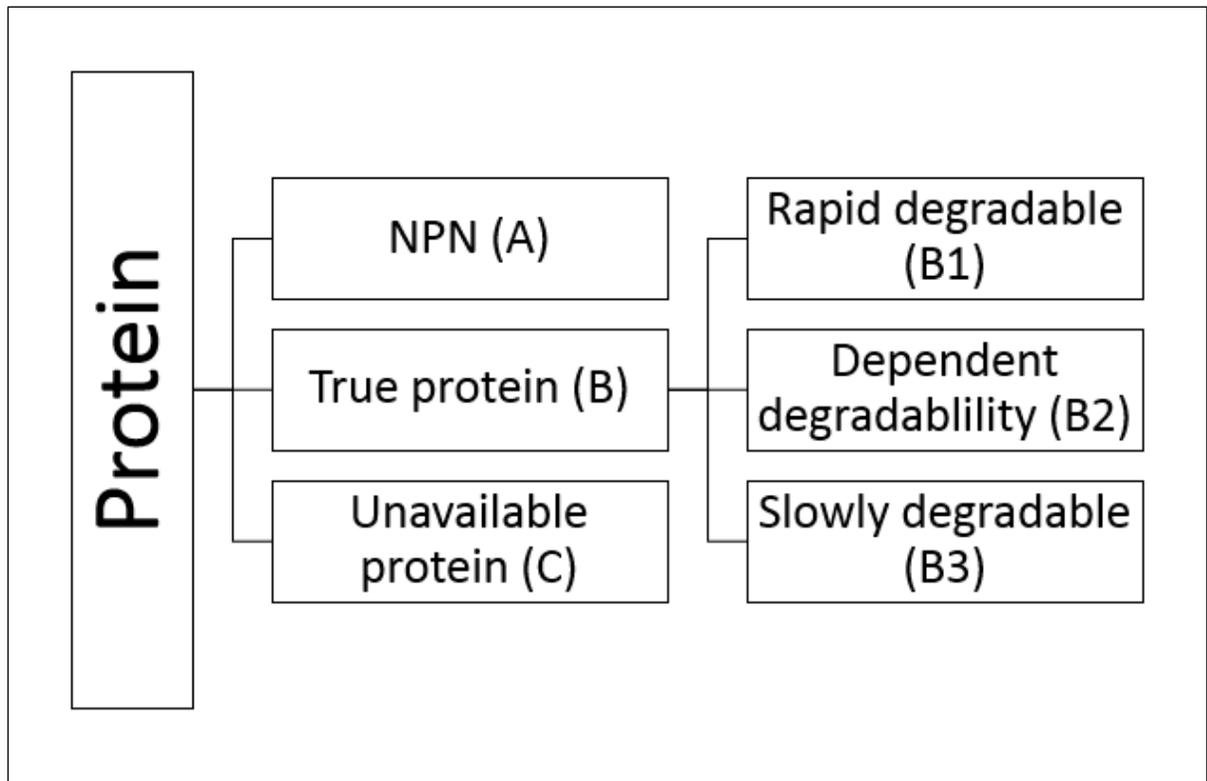


Figure 2.2. Protein divided into fractions.

2.5.1. Different protein sources

Fraction B (Sniffen *et al.*, 1992) is also known as rumen degradable protein (RDP). RDP like oil seed cakes, significantly enhances the fermentation process in the rumen, leading to an increase in nutrient utilization (Khandaker *et al.*, 2012). Degradable intake protein (DIP) is also a RDP, and the first limiting dietary component for the utilization of forages low in quality (Köster *et al.*, 1996). RDP has different levels of degradability and urea (Fraction A) can also be classified as a 100% degradable RDP.

2.6. Digestibility of Protein

2.6.1. Rumen degradable protein (RDP)

2.6.1.1. Oilseed cakes

Oil seed cakes like mustard, soybean and cotton can be supplied in a diet as a protein source (Khandaker *et al.*, 2012). Crude protein helps micro-organisms digest nutrients and increases the microbial nitrogen (Khandaker *et al.*, 2012). Inclusions of mustard oil cake in a diet (in the place of wheat bran, rice polish and molasses) combined with hay at 70, 140 and 280 g/kg levels were done, and positive results were found on digestion (Khandaker *et al.*, 2012). Even though the inclusion levels were high, the pH of the rumen never decreased to an extent that could have affected the digestibility, pH levels therefor always remained above the cellulolytic threshold (Khandaker *et al.*, 2012). Increasing oil cake levels increased the DM, organic matter, and NDF and ADF digestibility of hay to a certain extent (Khandaker *et al.*, 2012). The two highest inclusion of mustard seed oil cake showed no significant difference in NDF and ADF digestibility, thus a quadratic relationship (Khandaker *et al.*, 2012). This could be because the efficiency for nitrogen to synthesise microbial protein declined (Khandaker *et al.*, 2012).

Increasing the RDP, increased the volatile fatty acid levels and the microbial nitrogen supply (Khandaker *et al.*, 2012). A phenomenon that can be explained by the increase in ammonia, amino acids and peptides that promote the out flow of rumen micro-organisms from the rumen (Khandaker *et al.*, 2012). The RDP g/MJ metabolize energy (ME) levels of this experiment were 4.1, 6.3, 8.3 and 12.4 for the 0, 70, 140 and 280 inclusion levels respectively (Khandaker *et al.*, 2012). 12.4 RDP g/MJ ME had the greatest effect on digestibility and microbial nitrogen supply but the inclusion of 8.3 RDP g/MJ ME levels gave the best efficient microbial nitrogen supply and lower nitrogen retention and as a result can be identified as the best inclusion level (Khandaker *et al.*, 2012).

2.6.1.2. DIP (Degradable intake protein) also RDP

Casein can be given to cattle as a nitrogen source (Köster *et al.*, 1996). An increase in casein increases the forage and organic matter intake, microbial nitrogen and volatile fatty acid production (Köster *et al.*, 1996) in much the same way as mustard oil seed

cake (Khandaker *et al.*, 2012). An increase in propionate levels was found, thus a low acetate: propionate ratio that will negatively affect the milk fat percentage (Köster *et al.*, 1996). NDF digestibility also showed a decrease in variable amounts, and showed a decline with high inclusions above 11% (Köster *et al.*, 1996). This could be due to the increase in intake which led to a shortened retention time in the rumen as a result of this there was less time for cellulose digestion in the rumen (Köster *et al.*, 1996). pH showed a tendency to decrease with an increase in casein but the pH level was never below the cellulolytic threshold, the same effect was found by (Khandaker *et al.*, 2012) using mustard oilseed cakes as source (Köster *et al.*, 1996).

2.6.1.3. Urea

Lazzarini *et al.* (2009) found that, when using a mixture of urea, ammonium sulphate and albumin, the total crude protein (CP) level of the diet should be 7% to sustain a microbial population able to digest fibre from low quality roughages. Levels lower than 7% showed a decrease in microbial growth rate, a change in microbial composition and an effect on NDF digestibility but, levels near 11% CP had the best NDF digestibility (Lazzarini *et al.*, 2009). Albumin meets the requirement of micro-organisms and can supply them with essential substrates (Lazzarini *et al.*, 2009).

2.6.2. Rumen un-degradable protein (RUP)

Milis & Liamadis (2008) stated that maize gluten meal with a high RUP and low nitrogen degradability content had better organic matter (OM), NDF and ADF digestibility compared with cotton seed cake with high degradable protein. Maize gluten meal is much higher in CP compared to cotton seed cake (697 vs. 279 g/kg) but is a low protein degradability source (Milis & Liamadis, 2008). Lower protein level diets (145 g/kg CP) have better NDF and ADF digestibility compared to high protein diets (180-190 g/kg CP) (Milis & Liamadis, 2008). The same results were seen in a study by Martin & Hibberd (1990) with soybean hulls (RUP) and cotton seed cake (RDP). Soybean hulls, also a RUP, increased the organic matter digestibility of the whole diet when fed with a low quality grass, at 1kg, 2kg and 3kg level intervals instead of cotton seed meal. The NDF digestibility did not change but ADF digestibility increased (Martin & Hibberd, 1990). The increase in soybean hulls decreases the pH levels of the rumen, but this could be due to an increase in fermentation leading to an increase in volatile

fatty acid production (Martin & Hibberd, 1990). The acetate: propionate ratio decreases because microbial fermentation was enhanced (Martin & Hibberd, 1990).

2.6.3. Urea and rumen degradable protein (RDP)

Comparing urea and protein meal as a RDP, research has shown that urea results in better cellulose digestion (Belasco, 1954). Optimal levels of cellulose digestibility were reached when the protein inclusion levels were at 43% (Belasco, 1954). A further increase in protein levels resulted in a decrease in cellulose digestion from 91% to 57% (Belasco, 1954). Increasing a diet with a protein level of 13.5% to 16.1% with the addition of urea showed a decrease in dry matter intake this could be the result of a less palatable diet (Gressley & Armentano, 2007). Urea and soybean oilcake have been compared in studies to determine the effect of low nitrogen solubility against high nitrogen solubility (Jones, Stephens, & Kensett, 1975). Urea is a highly soluble nitrogen (Jones *et al.*, 1975) (100% degradable nitrogen source) and results in more protein degradation by ruminal micro-organisms, this tends to depress nitrogen retention for utilization by tissues (Jones *et al.*, 1975) leading to weaker performance parameters in animals (Majdoub, Lane, & Aitchison, 1978). Urea can increase cellulose digestion (Belasco, 1954) but decrease DMI (Gressley & Armentano, 2007) and nitrogen retention (Jones *et al.*, 1975) when compared to RDP.

Nitrogen inclusion with a combination of urea and protein meal showed better urea utilization when compared to just urea (Belasco, 1954). The utilization when the protein sources were mixed was 100% whereas urea alone was only 80-88% (Belasco, 1954).

2.7. Optimal level of nitrogen

The level of optimum ruminal ammonia nitrogen (NH_3N) for growth rate and organic matter digestion varies (Hoover, 1986). This could be due to the changes in microbial population and rumen environment or the competition between fibrolytic and non-fibrolytic organisms (Hoover, 1986). It has been shown that inclusion of high levels of NFC will encourage amylolytic organisms to grow that also need peptides and amino acids and thus might deplete these resources (Hoover, 1986). This will then limit the amount of amino acids remaining that fibrolytic organisms require (Hoover, 1986). The rumen environment will affect the level of ammonia nitrogen needed because micro-

organisms attached to fibre particles need more ammonia or are exposed to lower levels of ammonia than the free-flowing bacteria (Hoover, 1986). The optimum level for digestion is not the same optimum level as for growth rate. Digestibility of fibre needs a higher ammonia nitrogen concentration than microbial growth (Hoover, 1986; Lazzarini *et al.*, 2009). Cellulolytic organisms require amino acids in addition to ammonia to maintain fibre digestion, as a result of this protein and not only urea must be present in adequate amounts in the animal's diet (Hoover, 1986).

A diet with low protein levels causes a decrease in organic matter digestibility and this could suggest that the lower protein diet has insufficient nitrogen to support the digestion processes (Gressley & Armentano, 2007). High protein diets show an increase in ruminal ammonia with an increase in insulin (Gressley & Armentano, 2007). All of the above studies showed that the increase in fibre digestion with the increase in protein inclusions has a quadratic effect (Belasco, 1954; Köster *et al.*, 1996; Khandaker *et al.*, 2012), and that the highest inclusion of protein does not lead to the maximum increase in fibre digestion. This could be explained that by drastically increasing the protein content, the ammonia utilization by the rumen micro-organisms, cannot compete with the high rate of hydrolysis of the urea to the end products known as ammonia and carbon dioxide (Belasco, 1954).

2.8. Effect of energy and protein sources on fibre digestion

2.8.1. Intake

Nitrogen and energy have an additive effect on intake (Klevesahl *et al.*, 2003; Souza *et al.*, 2010). Nitrogen will increase intake and energy addition will decrease intake but the combined effect will be a net increase of intake since the nitrogen increase effect is greater than the energy decrease effect (Olson *et al.*, 1999; Souza *et al.*, 2010).

2.8.2. pH

The increase in digestibility shown when energy sources are replaced with protein sources could be due to the generating of a more buffered rumen and better pH control (Klevesahl *et al.*, 2003; Nousiainen, Rinne, & Huhtanen, 2009). Studies have thus shown an increase pH when protein sources are increased in the diet (Klevesahl *et al.*, 2003). Energy sources still decrease the pH of the rumen environment but never below

the cellulolytic threshold, if there is enough nitrogen available for the microbes (Klevesahl *et al.*, 2003). In a diet with increasing energy and protein sources the pH will decrease but not to an extreme extent and the cellulolytic microbes will still be able to digest cellulose (Olson *et al.*, 1999; Klevesahl *et al.*, 2003). A decrease in pH could be understood as an increase in fermentation and since the drop in pH does not fall below the cellulolytic threshold, it is possible that it is not the only reason for a decrease in fibre digestion (Olson *et al.*, 1999). Olson *et al.* (1999) showed this when the combination of starch and protein with the lowest digestion was not the combination with the lowest pH. Souza *et al.* (2010) on the other hand found a decrease in pH due to protein addition.

2.8.3. Volatile fatty acids (VFA)

Volatile fatty acids (VFA) production did not show a significant interaction for RDP and starch (Klevesahl *et al.*, 2003). RDP increases the VFA production quadratic with the maximum effect with a 50g/d inclusion (Klevesahl *et al.*, 2003). Whereas in a different study protein increases VFA production linearly and starch has no effect (Olson *et al.*, 1999).

2.8.4. Ammonia production

There is an interaction between RDP and starch for ruminal ammonia production (Klevesahl *et al.*, 2003). Ammonia production increases quadratic (Olson *et al.*, 1999) when protein is increased in the diet with or without the addition of starch (Olson *et al.*, 1999; Klevesahl *et al.*, 2003) and starch decreases ruminal ammonia production (Olson *et al.*, 1999). However the response on protein was quicker when starch was not included (Klevesahl *et al.*, 2003). It could be that there is a high demand for readily fermentable nitrogen by the amylolytic organisms (Klevesahl *et al.*, 2003). The decrease in digestibility shown when energy sources are replaced with protein sources could be due to an overcoming of a RDP deficiency, by stimulating cellulolytic bacteria from the amino acids and peptides that was added (Klevesahl *et al.*, 2003; Nousiainen *et al.*, 2009).

2.8.5. Digestibility

Research (Heldt *et al.*, 1999) showed that if there is no adequate amount of RDP there will be little positive effect of NFC on fibre digestion. An increase in digestibility from

adding protein is a response of a nitrogen deficiency (Olson *et al.*, 1999). If however the protein level was sufficient enough to allow maximum total degradability of organic matter the addition of NFC does not decrease fibre digestion but enhances organic matter and NDF digestibility (Heldt *et al.*, 1999). There is no interaction for RDP and starch on organic matter digestibility but there is an interaction on NDF digestibility (Klevesahl *et al.*, 2003; Souza *et al.*, 2010). In organic matter, RDP increases digestibility linearly and starch has no effect (Klevesahl *et al.*, 2003) but in NDF, the effect RDP has is an increase cubically (Klevesahl *et al.*, 2003) or quadratic (Belasco, 1954; Köster *et al.*, 1996; Khandaker *et al.*, 2012) but a decrease with starch inclusion (Klevesahl *et al.*, 2003; Souza *et al.*, 2010). The best NDF digestibility combination happens with no added starch and an RDP inclusion of 0.051% of body weight (50g/d) (Klevesahl *et al.*, 2003). An increase in passage rate was also noted with an increase in protein due to the increase in intake, this effect was less apparent with starch inclusion (Olson *et al.*, 1999).

2.9. Conclusion

From the literature, it is clear that interactions exist between carbohydrate supplementation, nitrogen supplementation and fibre digestion in the rumen. The aim of the current study was to investigate the effect of three carbohydrate and two nitrogen sources on the fibre digestibility in one good and one poor roughage source and to unravel some of the interactions.

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CHAPTER 3

GENERAL CHEMICAL ANALYSES AND PROCEDURES

3.1. Proximate analyses

Dry matter of the roughages and soybean oil cake was determined by drying a 2 g sample in a glass crucible, in a 105 °C oven overnight (AOAC, 2002; method 934.041). To determine the organic matter, 2 g samples of roughages and soybean oil cake were weighed into glass crucibles and were placed in a muffle furnace at 500 °C for six hours (AOAC, 2002; method 942.05). Ether extract was done by using a diethyl ether extraction on 2 g samples (roughages and soybean oil cake) weighed out in a thimble (AOAC, 2002; method 920.39).

Neutral detergent fibre (NDF) analysis of the roughage sources was done by weighing out 0.5 g samples in F57 ANKOM fibre analysis bags. These bags were sealed with an ANKOM sealer (ANKOM® 1915/1920 heat sealer; ANKOM® technology corp., Macedon, NY, USA) and NDF was determined using a ANKOM²²⁰ AUTOMATED fibre analyzer according to the ANKOM method, with the addition of sodium sulphite and heat stable α -amylase as done by Van Soest *et al.*, (1991). Acid detergent fibre (ADF) and acid detergent lignin (ADL) were also done according to the ANKOM method, using the same bags that went through the NDF procedure.

Crude protein analysis was done with the aid of a LECO Nitrogen Gas Analyzer to measure the nitrogen content. The instrument was supplied by LECO Africa (Pty) Ltd (Kempton Park, South Africa). For both the roughage and soybean oil cake samples, samples of 0.1 g were wrapped in the appropriate aluminum foil squares. The samples were ignited at 900 °C much like the AOAC Dumas method (AOAC, 2002; method 990.03) and the protein content was calculated by multiplying the nitrogen percentage with 6.25 (AOAC, 1995; method 990.03).

3.2. *In vitro* digestibility

In vitro and *in situ* (*in sacco*) NDF digestibility methods are valuable alternatives to *in vivo* methods (Weimer *et al.*, 1999). The *in vitro* technique was initially designed by Tilley & Terry (1963). It is a two stage technique done in 48 hours and using test tubes filled with rumen inoculant and a buffer medium, according to the synthetic saliva composition of McDougall (1948). Test tubes were fitted with a rubber stopper with a Bunsen valve (Tilley & Terry, 1963). The second stage was the addition of pepsin to solubilize the protein (Tilley & Terry, 1963). Goering & Van Soest (1970) modified this procedure by creating a more complicated buffer medium with a reducing agent, micro and macro minerals and a buffer component. Goering & Van Soest (1970) found that the claim of Tilley & Terry (1963) that the inoculation of rumen microbes to the fermentation process is sufficient enough to provide an anaerobic environment, to be lacking. Thus, they included a reducing agent (sodium sulphide and cysteine hydrochloride) and rezasurin as an indicator to make the change from aerobic (purple/pink) to anaerobic (clear) visible (Goering & Van Soest, 1970).

The *in vitro* technique was first designed in tubes (Tilley & Terry, 1963) but a filter bag technique in an ANKOM DAISY incubator was developed to create a less labor intensive system. The filter bag method has some disadvantages, because samples could wash out of the bag creating the illusion of high digestibility (Wilman & Adesogan, 2000). Another disadvantage is that different sample bags in the same vessel could influence the micro environment and digestibility of the samples from the soluble matter that washes out (Wilman & Adesogan, 2000). The tube method has a lower coefficient of variation, indicating more precision, but both methods can be used and the filter bag method is recommended for trials with many combinations (Wilman & Adesogan, 2000).

Roughage samples were hammer milled through a 2 mm screen and then sieved through a 106 µm sieve before the trial started to remove fine particles that could potentially be washed out of the bags. According to Wilman & Adesogan (2000), milling roughage samples through different sieve sizes (100, 150 and 150 µm) has shown not to affect the digestibility of samples in the bags. In the current study, samples were then weighed into ANKOM F57 filter bags and each sample bag was placed in a 125 ml Erlenmeyer flask for incubation.

3.2.1. *Negative aspects of the in situ method*

The *in situ* method requires animals that are surgically modified and this can raise ethical and moral issues, as well as increased costs (Mould *et al.*, 2005). The *in situ* method also has limited analytic capacity due to the fact that the rumen capacity of one cow is limited and thus there is also an inability to conduct trials with many different feeds at once (Mould, Kliem, et al., 2005).

3.2.2. *Positive aspects of the in situ method*

In situ methods are accurate for describing digestion kinetics with emphasis on animal (passage and intake) and diet (nutrient) effect (Varel & Kreikemeier, 1995). The *in situ* method has shown to have a shorter lag time (3.5 hours less), faster rate of digestion and greater digestibility when compared to *in vitro* methods (Varel & Kreikemeier, 1995).

3.2.3. *Negative aspects of the in vitro method*

The *in vitro* technique needs a rumen inoculant and can thus result in variation (Mould, Kliem, *et al.*, 2005). It could also make the method only moderately reproducible (Iantcheva *et al.*, 1999). It was shown that the *in vitro* technique had a longer lag time and this could be due to the small concentration of inoculant and thus micro-organisms present (24 ml incubation medium: 6ml rumen inoculum; Varel & Kreikemeier, 1995).

3.2.4. *Positive aspects of the in vitro method*

The *in vitro* technique requires less substrate than the *in situ* technique and many different feedstuffs can be examined at a time (Holecheck, Vavra, & Pieper, 1982; Mould, Kliem, *et al.*, 2005), making this method more favorable for comparing substrate differences (Varel & Kreikemeier, 1995). Also this method can be used to test diets that animals might be sensitive to (Iantcheva *et al.*, 1999). It is also less expensive and a more rapid technique (Getachew *et al.*, 2004). The *in vitro* technique imitates natural digestion, but in a laboratory (Holecheck *et al.*, 1982). *In vitro* techniques can also determine the rate of digestion that is closely correlated with total digestion, but only within a forage family (Holecheck *et al.*, 1982). For this study, the *in vitro* procedure was followed because there were too many treatment combinations for the *in situ* method.

3.3. Experimental animals and diet

Four multiparous lactating Holstein cows 142 ± 19 (SE) dry matter intake, and fitted with ruminal cannulae, were used as donors of rumen liquid (ethical clearance has been obtained). The cows formed part of the dairy herd and were managed with the rest of the herd. All the donor cows had free access to water and a mixture of 80% lucerne hay and 20% chopped wheat straw. They also received 24.5 kg per day (22 kg of DM) of a semi-complete lactation diet that was supplied by Afgri Animal Feeds (Malmesbury, South Africa). The semi-complete feed, which contained 357 g/kg of NDF and 165 g/kg of CP, was offered at a level of 10 kg in the morning (06:30) and 14.5 kg in the afternoon.(16:30). Before rumen fluid collection, cows were deprived of concentrate feed for 24 hours to ensure that the rumen fluid contained none of the energy sources at the time of collection.

3.4. Detailed Procedure

3.4.1. Preparing the samples

Lucerne hay and wheat straw (2 kg of each) were milled through a 2 mm screen with a 2 mm hammer mill (Cyclotec 1093 mill). Most of the roughages were then sieved through a 106 μm sieve in a stacked horizontal shaker (Retch AS 200, supplied by Wirsam Scientific, Cape Town). Roughages were sieved for 10 minutes at an amplitude setting of 80. After sieving, the top layer on the sieve was stored in airtight plastic containers and the rest was discarded. This was used in the *in vitro* experiments discussed in Chapters 4 & 5. Mack (2011) did a study on the particle loss through bags in *in vitro* studies. The hypothesis was formed from a study by Cruywagen (2003) that milling forages will create small particles that can washout of the Dacron bags giving an over estimation on the NDF disappearance. Dacron bags have a mean pore size of 53 micron but there is bigger pores visible that can allow more and bigger particle loss. Sieving with a 106 μm , 125 μm and 150 μm sieve, material loss was 11-20% and there was no difference between the NDF and crude protein content of the sieved samples compared to the control samples with lucerne (Mack, 2011).

The ANKOM F57 filter bags were used in this study. Bags were soaked in acetone for 5 minutes and left to air dry. Bags were then marked and dried overnight in a 105 °C oven. Bags were weighed using the hot weighing method (Goering & Van Soest, 1970) and weights were recorded accurately. Roughages was weighed out in prepared F57

bags in the required quantities and sealed using a hot sealer (ANKOM® 1915/1920 heat sealer; ANKOM® technology corp., Macedon, NY, USA).

3.4.2. Preparing the incubation medium

The incubation medium is created to safeguard an environment suitable for fermentation, to buffer the system and to supply the micro-organisms with necessary nutrients (Mould *et al.*, 2005). The medium contained a buffer, micro and macro minerals, nitrogen and a reducing solution (Mould, Morgan, *et al.*, 2005). The buffer solution used in this study was modified from the one described by Goering & Van Soest (1970). The water used to create the incubation medium was bubbled with nitrogen gas for one minute to ensure a faster reducing rate, especially for the incubation media without sodium sulphide and nitrogen. Different types of incubation media were used in the current study, and the compositions are presented in Table 3.1.

Table 3.1. Different types of incubation mediums used in this study

			Without sodium sulphide and with nitrogen	With sodium sulphide and nitrogen	With sodium sulphide without nitrogen
Buffer	2L				
	Ammonium bicarbonate	NH ₄ HCO ₃	8 g	8 g	0 g
	Sodium bicarbonate	NaHCO ₃	70 g	70 g	80 g
Macro	2L				
	Monosodium phosphate	NaH ₂ PO ₄	11.4 g	11.4 g	11.4 g
	Monopotassium phosphate	KH ₂ PO ₄	12.4 g	12.4 g	12.4 g
	Magnesium Sulphate	MgSO ₄ .7H ₂ O	1.17 g	1.17 g	1.17 g
Micro	100 ml				
	Calcium chloride	CaCl ₂ .2H ₂ O	13.2 g	13.2 g	13.2 g
	Manganese(ii) chloride	MnCl ₂ .4H ₂ O	10 g	10 g	10 g
	Cobalt(ii) chloride	CoCl ₂ .6H ₂ O	1 g	1 g	1 g
	Iron(iii) chloride	FeCl ₃ .6H ₂ O	8 g	8 g	8 g
Cysteine sulphide reducing	50 ml				
	Water		46 ml	48 ml	48 ml
	Cysteine HCL		0.624 mg	0.312 mg	0.312 mg
	1 N Sodium hydroxide	1 N NaOH	4 ml	2 ml	2 ml
	Sodium sulphide	Na ₂ S.9H ₂ O	0 g	0.312 mg	0.312 mg
Final Buffer	Water		500 ml	500 ml	500 ml
	Rumen buffer		250 ml	250 ml	250 ml
	Macro		250 ml	250 ml	250 ml
	Rezasurin (0.1%)		1.25 ml	1.25 ml	1.25 ml
	Micro		0.12 ml	0.12 ml	0.12 ml
	Tryptose		1.25 g	1.25 g	0 g

In the one case (last column in Table 3.1), N-sources were omitted from the buffer because the effect of N-source was investigated in the trial described in Chapter 5. The final buffer solutions were purged with CO₂ gas before addition of the reducing solution.

3.4.2.1. *Micro minerals*

The medium designed by Goering & Van Soest (1970) focused on the addition of nutrients and a reducing solution to the medium. The nutrients were added because the non-fibre fractions of the roughages limited digestion, and even more so in nitrogen-poor roughages (Grant & Mertens, 1992). The nutrients (tryptose and micro

minerals) have various functions, they promote maximum digestion, are needed for optimal cellulose digestion (Grant & Mertens, 1992) and provide all the required nutrients for the micro-organisms (Mould, Morgan, *et al.*, 2005). However Tilley & Terry (1963) claimed that the rumen inoculant will have sufficient nutrients for bacterial growth.

3.4.2.2. *Nitrogen*

The level of nitrogen required in the incubation medium depends on the digestibility of the substrate and the nitrogen content thereof; 25 mg N/g should be adequate according to Mould, Morgan, *et al.* (2005). Mould, Morgan, *et al.* (2005) created a nitrogen-free medium to witness the effect of nitrogen sources on fibre digestion. They replaced ammonium bicarbonate with sodium bicarbonate and the results claimed that the nitrogen had no effect on gas production (Mould, Morgan, *et al.*, 2005). The same was done in the current study when the effect of protein sources were studied and Tryptose was also omitted.

3.4.2.3. *Reducing solution*

Goering & Van Soest (1970) used a 40 ml:2 ml ratio (20:1) of final buffer to the cysteine reducing solution. In the current study, a ratio of 95 ml:5 ml (19:1) was used as according to Van de Vyver & Joubert (2011). Reducing solutions are designed to reduce the redox potential and thus generate an anaerobic environment (Mould, Morgan, *et al.*, 2005). Tilley & Terry (1963) did not include a reducing solution when they examined the *in vitro* technique because the authors claimed that the rumen micro-organisms would provide an anaerobic condition. Including a reducing solution (sodium sulphide nonahydrate and cysteine hydrochloride) in the medium has proved to decrease the lag time and improve the rate of digestion (Grant & Mertens, 1992). This reducing solution was used first by Goering & Van Soest (1970). Mould, Morgan, *et al.* (2005) stated that the inclusion of a reducing solution is not necessary and had no effect on gas production when cysteine hydrochloride, sodium sulphide and sodium hydroxide were replaced with urea. Morgan *et al.*, (2004) found no difference in digestion when reducing solution was added or not, except in experiments with feed or rumen inoculant low in nitrogen. This could explain why Mould, Morgan *et al.* (2005) did not see a difference when the reducing solution was left out but urea was added.

Omitting sodium sulphide from the reducing solution of Goering & Van Soest (1970) and using only cysteine hydrochloride has also shown to slowly reduce the incubation medium (Fukushima *et al.*, 2003). Sodium sulphide is toxic and precipitate essential metal ions, and some anaerobic bacteria might grow poorly in this strong reducing agent (Fukushima *et al.*, 2003). Cysteine hydrochloride has lower levels of toxicity and reduced the solution after 2- 3 hours under continuous illumination in the lab (Fukushima *et al.*, 2003). Eliminating sodium sulphide can reduce the input costs and increase the safety (Morgan *et al.*, 2004). However, low levels of sodium sulphide has shown a better colony count than solution with only cysteine hydrochloride as the reducing agent (Bryant & Robinson, 1961). A simplified medium can be designed by omitting the sodium sulphide and keeping the level of cysteine hydrochloride the same, reducing the nitrogen level to 25 mg/g by ignoring tryptose and using rezasurin as a visual indication of the redox status of the solution (Mould, Morgan, *et al.*, 2005).

In Experiment 1 (described in Chapter 4) the incubation medium used was the one without sodium sulphide but with nitrogen. This medium was purged with CO₂ for two minutes before illuminating it overnight to reduce and used the next morning. In Experiment 2 (described in Chapter 5) the reducing solution consisted of sodium sulphide and cysteine hydrochloride. The buffer medium was prepared and purged with CO₂ and kept closed overnight. The next morning before the inoculant was collected, the buffer and reducing solution were added to the 125 ml Erlenmeyer flasks and closed to reduce and kept in the warm room at 39 °C to prevent thermal shock to the rumen microbes when the inoculant was added. The medium reduced quickly and was inoculated after two hours.

3.4.3. *Collection and handling of rumen fluid*

Rumen fluid as an inoculant has shown to have better cellulolytic activity than purified preparation of rumen micro-organisms (Tilley & Terry, 1963). Rumen fluid was thus used in this study. It has been reported that the best time to sample rumen fluid is at 4 to 8 hours after feeding, when the highest concentration of different microbes are present (Dehority & Grubb, 1980; Mould, Kliem *et al.*, 2005). From 2 to 4 hours post feeding the rumen micro-organism concentration is the highest but will be diluted due to feed, water and saliva (Dehority & Grubb, 1980; Mauricio *et al.*, 1999; Mould, Kliem *et al.*, 2005). The rumen digesta will then be dominated by saccharolytic and amylolytic microbes (Mauricio *et al.*, 1999). In the current study, rumen samples were collected

after milking, before the morning feeding and 24 hours after the last commercial semi-complete feeding, but cows had *ad libitum* access to roughages. Cows were kept and fed as described above.

In Experiment 1, there were six replications of the *in vitro* fermentations, and all six started on the same day. Because there were only four cannulated cows available, rumen fluid were taken separately from each of the four cows for the first four repetitions, but for the last two replications, rumen fluid were mixed in two bathes taken from two different cows at a time. In Experiment 2, there were also six replications, but they were done in six runs and each time, two runs started on the same day. Rumen fluid was taken from all four cows, but pooled for two cows per run.

Rumen contents of the cows were extracted by hand and squeezed through two layers of cheese cloth into preheated 1L thermos flasks. The flasks were filled to the brim and then some rumen contents was pushed in on top to ensure some feed particles for the micro-organisms as well as to ensure that there are micro-organisms available that are previously attached to feed particles in the rumen inoculant.

Anaerobic conditions and warm temperatures are of importance for accuracy (Tilley & Terry, 1963). Rumen samples must be kept in anaerobic conditions but without any increase in headspace pressure, since this would force CO₂ gasses into the sample decreasing the pH and increasing fermentation where some microbes might become more dominant than others (Mould, Kliem *et al.*, 2005).

In the current study, the rumen fluid was used as soon as it was brought into the lab, which was 20 minutes after collection. Inoculation of the incubation media with rumen fluid was done 40 to 60 minutes after collection. Studies have shown that storing the inoculant in ice will inhibit the bacterial action (Dehority & Grubb, 1980) and slow down metabolism (Hervás *et al.*, 2005). After 24 hours a decrease in colony count by 8% was shown (Dehority & Grubb, 1980) and fermentation characteristics for NDF decreased when samples were stored in ice (Hervás *et al.*, 2005). Cell membranes and particles undergo damage from freezing and gram negative bacteria are sensitive to freezing and thawing (Hervás *et al.*, 2005). However, a slight increase in colony count was seen after storing for eight hours on ice (Dehority & Grubb, 1980), and no effect on fermentation characteristics was seen when samples were stored up to six hours on ice (Hervás *et al.*, 2005). After keeping the samples at room temperature for eight hours no differences were seen in colony count, and this could be due to the fact that

the growth and death processes are in equilibrium (Doetsch, Robinson, & Shaw, 1952). There are two speculated reasons for the increase in colony count when samples were put on ice for a short period. The first could be good growth and multiplication in the first 15 minutes and slow growth in the next 15- 45 minutes (Dehority & Grubb, 1980). Since rumen bacteria are mesophylls they will still be able to grow till temperature reaches 30 °C (Dehority & Grubb, 1980). The second reason is that the bacteria will be closely clumped together or to particles that will be broken down by chilling (Dehority & Grubb, 1980).

3.4.4. *Inoculant preparation*

In the current study, rumen fluid was decanted into a blender and purged with CO₂, then blended for one minute. After blending, the fluid was purged with CO₂ and then filtered through four layers of cheesecloth. The blending protocol would increase the number of bacteria that attached to the feed particles, but this method also increases small feed particles in the inoculant (Pell & Schofield, 1993). However, some studies have shown no effect on colony count when fluid was blended or not, and bacteria seem to be resistant to break down when fluid was blended (Dehority & Grubb, 1980). Rymer, Huntington & Givens (1999) also showed no significant advantages to blending. Pell & Schofield (1993) found that blending will increase the gas production in the blank vials due to the small feed particles present in the inoculant and this could give inaccurate readings. For these reasons Rymer *et al.* (1999) and Pell & Schofield (1993) recommend that blending should be left out of the protocol as it would expose the rumen content to oxygen and the advantages thereof is few.

An increase in colony count was seen after the samples were chilled when the fluid was blended and filtered through two layers of cheesecloth (Dehority & Grubb, 1980). An increase in cells can be the dislodging of cells with blending or the growth of cells during the storage period (Leedle, Bryant, & Hespell, 1982). However, if the sample was centrifuged after filtering and the larger particles was removed, no growth was seen in the sample (Dehority & Grubb, 1980). This could indicate that bacteria clump to particles and this was removed with centrifuging.

Shaking in air and keeping samples open at room temperature for four hours did not affect the colony count (Doetsch *et al.*, 1952). This could be explained that the redox potential of the rumen sample can easily be maintained by its reducing substances,

this is also seen when resazurin is reduced rapidly (Doetsch *et al.*, 1952). Thus rumen bacteria do not seem to be that sensitive to oxygen exposure, according to these authors.

3.4.4.1. *Inoculation*

Samples in the current study were placed in 125 ml Erlenmeyer flasks containing the medium two hours before inoculation with rumen fluid. If samples were to soak in the incubation medium before inoculation for longer than three hours, an increase in lag time was noted when the fermentation process started, suggesting that the soluble fraction had time to solubilize in the medium leading to a faster fermentation rate in the beginning (Rymer *et al.*, 1999).

3.4.4.2. *Proportion of inoculant and incubation medium*

Increasing the inoculant percentage from 5 to 30% increased the total amount of gas produced with a decrease in lag time but had no effect on the organic matter apparent digestibility (Pell & Schofield, 1993; Rymer *et al.*, 1999). This would assume that the concentration of inoculant would increase the rate of digestion but not the extent thereof (Rymer *et al.*, 1999). In the current study, the concentration of rumen fluid inoculated into the incubation medium was 20% (25 ml inoculant and 100 ml medium). It has been reported that 20% of the inoculant is adequate to confirm maximum fibre digestion (Tilley & Terry, 1963; Grant & Mertens, 1992; Pell & Schofield, 1993) and growth (Tilley & Terry, 1963). Calitz (2013) also used a concentration of 20% in the same lab as where this study was conducted. Mould, Morgan, *et al.* (2005) increased the level of inoculant, 100, 200 and 330 ml in fermentation tubes with 100 ml of incubation medium. No effect on the rate and extent of fermentation was found and it was concluded that if an inoculant can support microbial growth at a given volume, the concentration thereof with the incubation medium will have little effect (Mould, Morgan, *et al.*, 2005).

3.4.5. *Gassing*

In the current study, the space above the liquid in the flask was replaced with CO₂ gas. Flasks were then closed with rubber stoppers with Bunsen valves (Tilley & Terry, 1963). Grant & Mertens (1992) founded that for long term incubations, continuous

gassing would be more beneficial to maintain pH levels increasing lag times (56%) and NDF digestion (69%).

3.4.6. *Stirring*

Magnetic stirrers were placed in the Erlenmeyer flasks, which were transferred to stirrer plates. In experiment 1 the samples were stirred every hour for a minute. In experiment 2 the samples were stirred continuously, to leave out the error that the timer might not go off. All the samples were carefully swirled by hand while they were in the closed and anaerobic condition, twice in 30 hours to remove the top foam layer. Pell & Schofield (1993) published that stirred samples had half the coefficient of variation (2.1%) compared to unstirred samples.

3.4.7. *Termination of fermentation*

After the adequate time of fermentation (six or 30 hours) sample flasks were taken out of the heated room and the pH of all the sample fluids were measured. The sample bags were removed and placed directly into a manual twin-tub washing machine with cold water filled to the maximum level. Samples were washed in three cycles of 5 minutes on the delicate setting and then spin dried for five minutes. Thereafter samples were dried at 105 °C for 24 hours and weights were recorded.

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CHAPTER 4

THE EFFECT OF DIFFERENT STARCH:NDF RATIOS ON *IN VITRO* FIBRE DIGESTION OF LUCERNE HAY AND WHEAT STRAW

4.1. Introduction

Starch is almost completely degraded in the digestive system of ruminants with propionate as the major end product (Reis & Combs, 2000). An increase in dietary starch level has been shown to decrease fibre digestion (McCullough, 1968; Mould *et al.*, 1983; Heldt *et al.*, 1999). The question, however, is how different levels of starch may affect fibre digestion. Increases in dietary starch levels that varied between 12 – 60%, (Reis & Combs, 2000; Kozloski *et al.*, 2006; Sveinbjörnsson *et al.*, 2006) showed an increase in DM intake but a decrease in roughage intake, a decrease in fibre digestion, whereas no significant change in ruminal pH was noted. Van Vuuren *et al.*, (2010) even found that the pH dropped with the low starch inclusion (12%), which was under 6.2 for a longer time than with the high starch inclusion.

The objective of the current study was to determine the level of starch that would be used in the next research chapter where the effect of different energy and nitrogen sources on NDF digestion was investigated. The other energy sources (sucrose and pectin) would be supplemented in the same ratio as starch, based on a hexose equivalent basis. In the current trial, four NDF:starch ratios were used in an *in vitro* trial with NDF coming from either lucerne hay or wheat straw. The hypothesis was that starch level would not affect *in vitro* NDF digestibility.

4.2. Materials and methods

4.2.1. Location and duration

The study was done at the Stellenbosch University in the Western Cape Province of South Africa. The research was conducted from April 2014 – May 2014 in the *in vitro* lab of the Department of Animal Sciences and on the Welgevallen Experimental Farm of the Stellenbosch University.

4.2.2. Experimental animals and diets

Four multiparous lactating Holstein cows 142 ± 19 (SE) dry matter intake, and fitted with ruminal cannulae, were used as donors of rumen liquid. The cows formed part of the dairy herd and were managed with the rest of the herd. All the donor cows had free access to water and a mixture of 80% lucerne hay and 20% chopped wheat straw. They also received 24.5 kg per day (22 kg of DM) of a semi-complete lactation diet that was supplied by Afgri Animal Feeds (Malmesbury, South Africa). The semi-complete

feed, which contained 35.7 g/kg of NDF and 16.5 g/kg of CP, was offered at a level of 10 kg in the morning (06:30) and 14.5 kg in the afternoon (16:30). Before rumen fluid collection, cows were deprived of concentrate feed for 24 hours to ensure that the rumen fluid contained no starch at the time of collection.

4.2.3. Research design

In vitro digestibility trials were done with four different levels of starch with each of two roughages, viz. lucerne hay and wheat straw (Table 4.1). Substrates were incubated for either 6 or 30 hours, thus resulting in 16 treatment combinations (two forages x four levels of starch x two incubation times). The trial was replicated six times. Collection and handling of rumen liquid was as explained in Chapter 3 (general chemical analysis).

The different starch levels to be used in the trial were calculated according to the DM and NDF contents of the roughages that were used. For lucerne, the DM content was 89.9% and the NDF 45.8% of DM. For wheat straw, DM was 91.9% and NDF 80.5% of DM. The NDF:starch ratios were based on mg of NDF and mg of hexose equivalents (HE). As 1 mg of starch = 1.1111 mg HE, the desired amounts of HE were multiplied by 0.9 to yield the amount of starch required. It was decided to use the following ratios of NDF:HE, viz. 125:125, 125:80, 125:35 and 125:0. The 125 mg of NDF for the respective roughages was based on the NDF content of the forages and came to 304 mg to be weighed out for lucerne and 169 mg for wheat straw. For starch, the amounts were 112.5 mg, 72.0 mg, 31.5 mg and 0 mg.

Table 4.1. Ratios of starch hexose equivalents to NDF used in the *in vitro* trial.

	125 mg NDF	Starch	Starch	Starch inclusion
	Air dry basis	HE ¹	mg (air dry)	%
Lucerne	304	125	112.5	37

	304	80	72	24
	304	35	31.5	10
	304	0	0	0
<hr/>				
Wheat straw	169	125	112.5	67
	169	80	72	43
	169	35	31.5	19
	169	0	0	0

¹HE = Hexose equivalents

4.2.4. Sample preparation

Lucerne hay and wheat straw were milled with a hammer mill (Cyclotec 1093 mill) through a 2 mm screen, followed by sieving through a 106 µm mesh to remove dust and very fine particles. The forage substrates were incubated in Ankom F57 filter bags (Ankom Technology Corporation, Fairport, NY, USA). The bags were prepared for incubation by soaking in acetone for one hour to remove the waxy layer which could affect microbial fermentation. Bags were then air dried before placing them in an oven to dry at 105 °C for 24 hours. After drying, bags were weighed (using the hot weighing method) and marked for later identification. The appropriate amounts of the respective roughages were weighed out into the bags which were then sealed and placed into 125 ml Erlenmeyer flasks. The appropriate amounts of starch (Sigma Aldrich, catalogue number S9765) were weighed out and transferred to the Erlenmeyer flasks. The flasks were numbered and ready to use for the *in vitro* fermentation trial.

4.2.5. Chemical analyses

The composition of the buffer used in this experiment is explained in Chapter 3 (general chemical analysis). Before collecting the rumen liquid, 100 ml of the buffer solution was dispensed into each flask and flasks were gassed with CO₂ before placing them in the warm room at 39 °C. Rumen fluid (2 L per cow) was collected from two donor cows in the morning after milking. The rumen fluid was blended and pooled

under anaerobic conditions and then used to inoculate the media by adding 25 ml to each flask. More detail on the collection of rumen fluid and processing in the laboratory is provided in Chapter 3 (general chemical analyses). Flasks were incubated in a warm room at 39 °C. Half of the flasks were removed after 6 hours and the rest after 30 hours. The F57 bags were collected and washed in a normal household washing machine for 15 minutes to stop the fermentation process and to remove incubation fluids. Bags were subsequently placed in an oven at 105 °C to dry for 24 hours. After drying, the samples were weighed and these values were used to determine DM disappearance. The samples were then placed in an ANKOM²²⁰ AUTOMATED fibre analyzer (supplied by Ankom Technology Corporation, Fairport, NY, USA), sodium sulphite and heat stable α -amylase were added, and NDF determined according to the Ankom procedure. The NDF residue in the bag was used to calculate NDF disappearance.

4.2.6. *Statistical analysis*

For each incubation time (six and 30 hours) the data were subjected to a two way factorial ANOVA with substrate and starch as factors. The two way interactions were then interpreted with Bonferroni comparisons and significance was declared at $P < 0.05$.

4.3. **Results and discussion**

In vitro DM digestibility values of lucerne hay and wheat straw are presented in Table 4.2 and Figures 4.1 and 4.2. Dry matter digestibility of lucerne and wheat straw showed no substrate*starch interaction. After six hours of fermentation there was no significant difference among treatments for either of the roughages (Table 4.2). Level of starch thus had no effect on *in vitro* DM digestibility. This is also indicated in Figures 4.1 & 4.2 for six and 30 hours, respectively.

Table 4.2. The effect of starch:NDF ratio on *in vitro* DM digestibility of lucerne hay and wheat straw.

Time	Roughage	Starch:NDF ratio ¹				SEM	P
		0:125	35:125	80:125	125:125		
6 Hours	Lucerne	39.7 ^{bc}	40.5 ^b	39.1 ^{bc}	40.2 ^b	0.05	0.99
	Wheat straw	11.2 ^d	11.4 ^d	10.7 ^d	11.4 ^d	0.05	0.99
30 Hours	Lucerne	60.7 ^a	61.4 ^a	63.2 ^a	64.8 ^a	1.32	0.28
	Wheat straw	34.8 ^{bc}	34.8 ^{bc}	32.8 ^c	33.7 ^{bc}	1.32	0.28

¹Starch is in terms of hexose equivalent (mg) and NDF as derived from the respective roughage sources.

^{a-d}Per incubation time, values with different superscripts within rows and columns, differ (P<0.05).

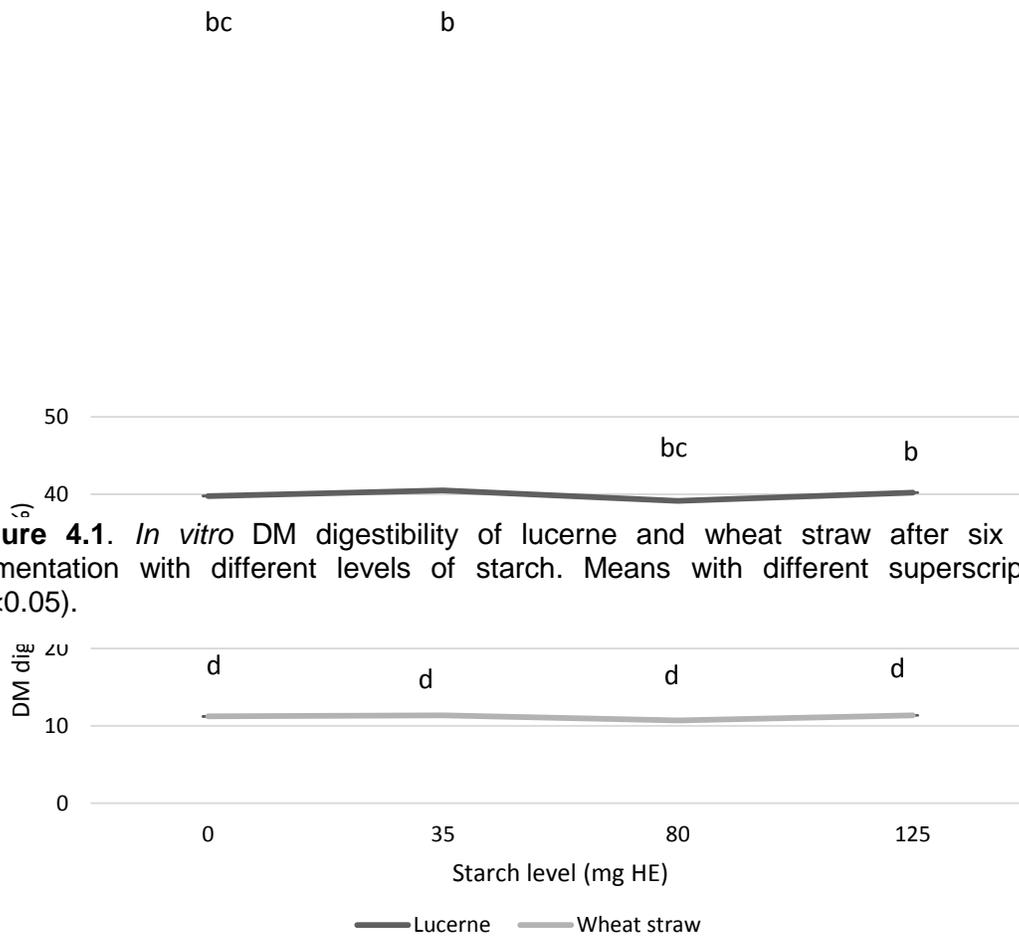


Figure 4.1. *In vitro* DM digestibility of lucerne and wheat straw after six hours of fermentation with different levels of starch. Means with different superscripts, differ (P<0.05).

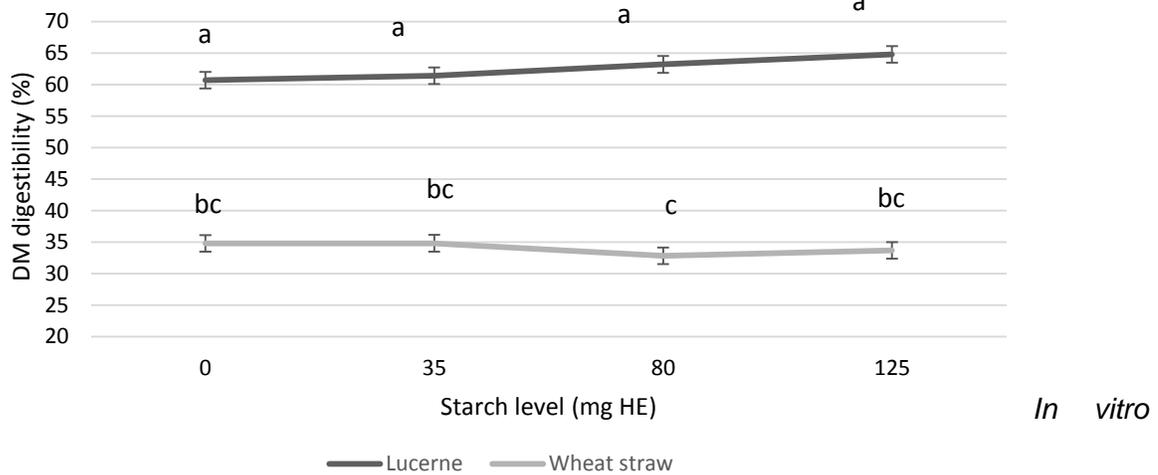


Figure 4.2. *In vitro* DM digestibility of roughages after 30 hours of fermentation with different levels of starch. Means with different superscripts, differ ($P < 0.05$).

NDF digestibility values are presented in Table 4.3 and Figures 4.3 and 4.4. Neutral detergent fibre disappearance did not show a significant forage*starch interaction (Table 4.3), as was also noted for DM disappearance. Starch level had no effect on *in vitro* NDF digestibility of neither forage at either incubation time. At the 80 and 125 mg HE starch levels, NDF digestibility differed between lucerne and wheat straw for 30 hours.

Table 4.3. The effect of starch:NDF ratio on *in vitro* NDF digestibility of lucerne hay and wheat straw.

Time	Roughages	Starch:NDF ratio ¹				SEM	P
		0:125	35:125	80:125	125:125		
6 Hours	Lucerne	4.0 ^d	3.6 ^d	4.2 ^d	4.0 ^d	0.11	0.95
	Wheat straw	0.6 ^d	0.7 ^d	0.2 ^d	0.2 ^d	0.11	0.95
30 Hours	Lucerne	34.8 ^{ab}	35.5 ^{ab}	37.2 ^a	40.3 ^a	0.95	0.42
	Wheat straw	27.0 ^{cb}	26.6 ^{cb}	25.5 ^c	25.5 ^c	0.95	0.42

¹Starch is in terms of hexose equivalent (mg) and NDF as derived from the respective roughage sources.

^{a-d}Per incubation time, values with different superscripts within rows and columns, differ ($P < 0.05$).

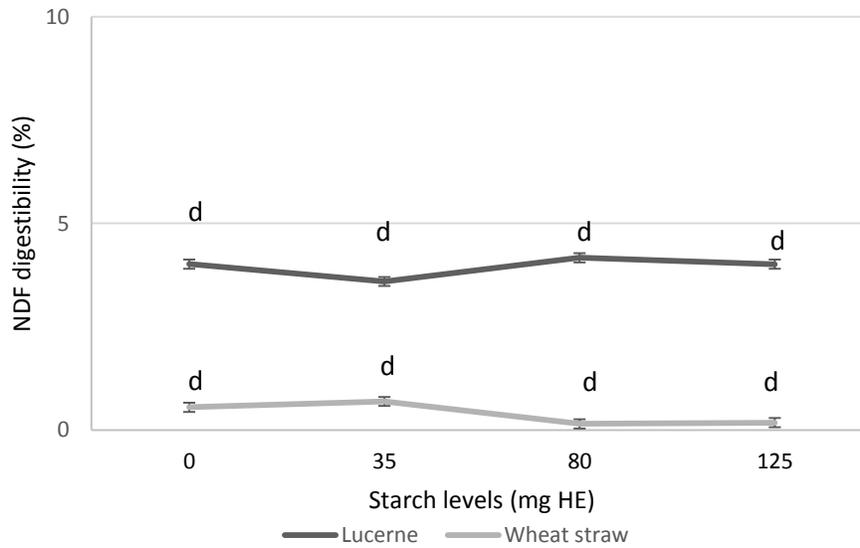


Figure 4.3. *In vitro* NDF digestibility of roughages after six hours of fermentation with different levels of starch DM. Means with different superscripts, differ ($P < 0.05$).

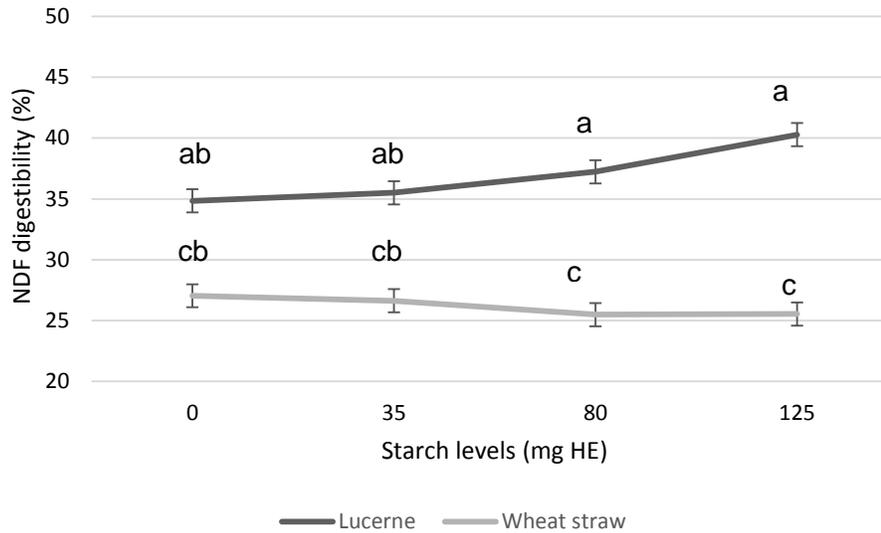


Figure 4.4. *In vitro* NDF digestibility of roughages after 30 hours of fermentation with different levels of starch. Means with different superscripts, differ ($P < 0.05$).

In vitro true digestibility values are presented in Table 4.4. Studying the *in vitro* true digestibility values in Table 4.4, it appeared that none of the starch level treatments significantly differed from each other for either of the roughages at any fermentation time, which was also noted for DM and NDF disappearances. Lucerne and wheat straw, however, differed from each other. This can be seen in more detail in Figures 4.5 & 4.6, respectively. There was also no forage*starch interaction.

Table 4.4. The effect of starch:NDF ratio on *in vitro* true digestibility of lucerne hay and wheat straw

Time	Roughages	Starch:NDF ratio ¹				SEM	P
		0:125	35:125	80:125	125:125		
6 Hours	Lucerne	55.8 ^b	55.7 ^b	55.9 ^b	55.8 ^b	0.15	0.93
	Wheat straw	19.4 ^d	19.6 ^d	19.1 ^d	19.1 ^d	0.15	0.93
30 Hours	Lucerne	70.0 ^a	70.3 ^a	71.1 ^a	72.5 ^a	0.56	0.64
	Wheat straw	40.9 ^c	40.6 ^c	39.6 ^c	39.7 ^c	0.56	0.64

¹Starch is in terms of hexose equivalent (mg) and NDF as derived from the respective roughage sources

^{a-d}Per incubation time, values with different superscripts within rows and columns, differ (P<0.05)

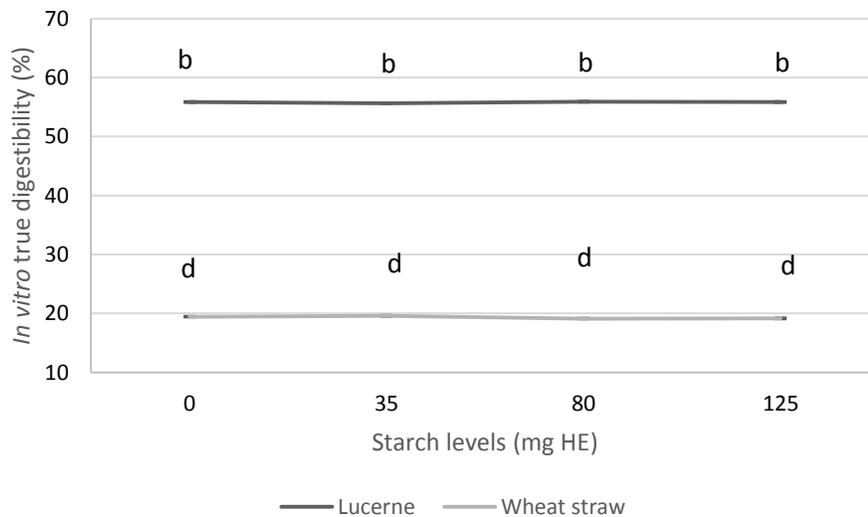


Figure 4.5. *In vitro* true digestibility of roughages after six hours of fermentation with different levels of starch. Means with different superscripts, differ (P<0.05).

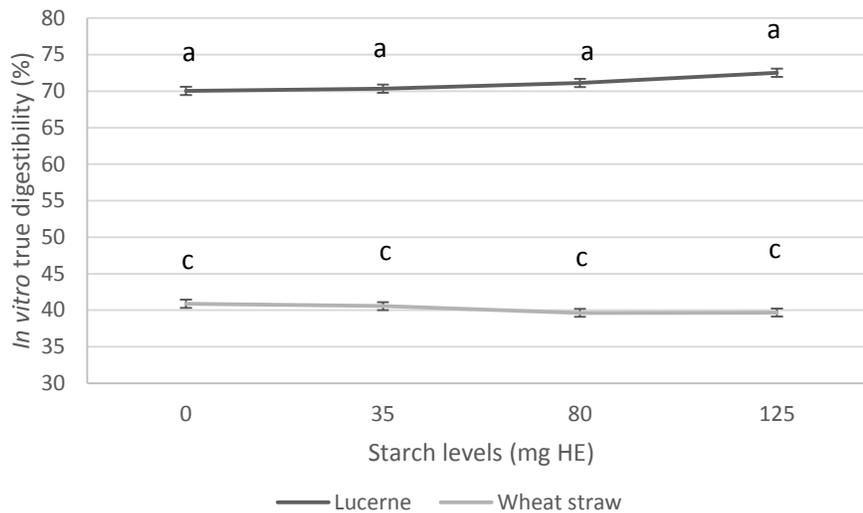


Figure 4.6. *In vitro* true digestibility of roughages after 30 hours of fermentation with different levels of starch. Means with different superscripts, differ ($P < 0.05$).

In contrast to the current study, Holtshausen (2004) reported that using 120, 80, 40 and 0 HE starch with 120 mg NDF of Bermuda grass (similar inclusion levels as the current study), showed a decrease in NDF digestibility with an increase in starch. However, similar results to the current study were reported by McCullough (1968), that tested four different levels of starch (20%, 40%, 60% and 80%) on Bermuda silage and hay. The silage showed significantly better digestibilities than the hay; in the silage, the two highest inclusion levels of maize resulted in significantly lower digestibility values. The different levels of maize on hay digestibility, however, did not differ. In the current study, the starch inclusion was a maximum of 37% in lucerne and 67% in wheat straw, and these levels had no effect on *in vitro* digestibility parameters.

4.4. Conclusion

The level at which starch was included as energy source in the *in vitro* incubation medium had no significant effect on NDF, DM or IVTD digestibility values of lucerne hay or wheat straw. The null hypothesis was thus not rejected. It was therefore decided to use a 50:50 ratio of HE:NDF in the *in vitro* fermentations in Chapter 5,

where the effect of energy and N sources on *in vitro* NDF digestibility was investigated.

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CHAPTER 5

THE EFFECT OF DIFFERENT ENERGY AND NITROGEN SOURCES ON *IN VITRO* FIBRE DIGESTIBILITY OF LUCERNE HAY AND WHEAT STRAW

5.1. Introduction

Protein added to a diet aids in digestion by increases the buffering capacity of the diet due to bicarbonate production (Lechartier & Peyraud, 2011). This would regulate pH and digestion would not decrease due to the addition of NFC. However, studies have shown different results where there is a higher pH decline when NFC is added to a diet with high RDP levels as opposed to lower RDP levels (Arroquy *et al.*, 2004). Studies have also shown that adding RDP to a diet high in NFC did not affect digestibility (Arroquy *et al.*, 2004). This could be because there is no deficiency for microbial fermentation for the protein inclusion to correct.

Inclusions of starch (McCullough, 1968; Mould *et al.*, 1983; Heldt *et al.*, 1999), pectin (Villarreal *et al.*, 2006) and sucrose (Khalili, 1993; Migwi *et al.*, 2011) have shown to decrease the pH of the rumen and starch inclusion was reported to be the reason for the decrease in roughage digestibility that was also observed. However, with the inclusion of sucrose and pectin, pH did not drop below the cellylyolytic threshold (6.1-6.0; Khalili, 1993; Villarreal *et al.*, 2006; Migwi *et al.*, 2011), indicating that the presence of the easily fermentable carbohydrates decreased digestion rather than decrease pH (Khalili, 1993). Studies have also shown that digestibility declined when NFC were added, even if the pH is kept constant with sodium bicarbonate (Khalili, 1993). According to Arroquy *et al.*, (2005), it is a “gross over-simplification to attribute the effect of NFC on ruminal fibre digestion solely to pH”.

The objective of this chapter was to determine the effect and interactions of three energy sources (maize starch, pectin and sucrose) and two nitrogen sources (soybean meal and urea) on fibre digestion of high quality (lucerne hay) and a low quality (wheat straw) roughages. The effects on DM disappearance, NDF disappearance, IVTD and pH were investigated. This was done by determining if the energy sources have an effect on digestibility and if the combination of nitrogen and energy sources have

different effects on different roughages. The hypothesis was that different energy and nitrogen sources do not affect fibre digestion and that there are no forage*energy source*nitrogen source interactions.

5.2. Materials and methods

5.2.1. Location and duration

The study was done at the Stellenbosch University in the Western Cape Province of South Africa. This research was conducted from May 2014 – July 2014 in the *in vitro* lab of the Department of Animal Sciences and on the Welgevallen Experimental Farm of the Stellenbosch University.

5.2.2. Experimental animals and diets

Four multiparous lactating Holstein cows 142 ± 19 (SE) dry matter intake, and fitted with ruminal cannulae, were used as donors of rumen liquid. The cows formed part of the dairy herd and were managed with the rest of the herd. All the donor cows had free access to water and a mixture of 80% lucerne hay and 20% chopped wheat straw. They also received 24.5 kg per day (22 kg of DM) of a semi-complete lactation diet that was supplied by Afgri Animal Feeds (Malmesbury, South Africa). The semi-complete feed, which contained 35.7 g/kg of NDF and 16.5 g/kg of CP, was offered at a level of 10 kg in the morning (06:30) and 14.5 kg in the afternoon (16:30). Before rumen fluid collection, cows were deprived of concentrate feed for 24 hours to ensure that the rumen fluid contained no starch at the time of collection.

5.2.3. Research design

Two forage substrates (lucerne hay and wheat straw) were incubated *in vitro* with different combinations of energy and nitrogen supplements. Energy supplements were either pure maize starch (Sta), sucrose (Suc) or pectin (Pec). The incubation medium was based on Goering & Van Soest (1970), with the exception that the nitrogen source in the medium was either omitted (Non) or replaced with N-equivalent amounts of soybean meal (Soy) or urea (Ure) to yield three N supplements. The different substrate combinations were incubated for six or 30 hours. The total number of treatments in this 2x3x3x2 factorial arrangement was 36 and included two substrates, three energy

sources, three nitrogen sources and two incubation times. Additional to these treatments, the two forage substrates were also incubated without any energy supplements for six and 30 hours and using the original Goering & Van Soest (1970) incubation medium. These were used as forage control treatments and brought the total number of treatments to 40. However, for the latter treatments to be included in a full factorial arrangement of treatments, the different N supplements should have been used without any energy supplements. This would have added 12 treatments to bring the total number of treatments to 48. Capacity in our laboratory (including magnetic stirrer plates), however, is limited to 40 incubations at a time. As we were primarily interested in the effect of energy source, with or without one of two N sources, it was decided to do the 2x3x3x2 factorial experiment discussed above, and to add the two forage control treatments.

The treatment combinations (Table 5.1) included 125 mg NDF or roughages with 125 mg hexose equivalents of the respective energy sources and with 21 mg of nitrogen from the respective N sources. The roughages used contained 457.8 mg/kg of NDF and 898.7 mg/kg of DM in the case of lucerne and 804.6 mg/kg of NDF and 919.2 mg/kg of DM in the case of wheat straw. The 125 mg of NDF from the roughages were provided by either 304 mg of lucerne hay or 169 mg of wheat straw.

For starch, the HE conversion factor is 0.9, thus the desired amount of starch used was $0.9 \times 125 = 112.5$ mg of starch. For sucrose, the conversion factor is 0.95, resulting in 118.8 mg of sucrose. Pectin is calculated by its galacturonic acid content that was 74% as per specification from Sigma Chemical Co. for lot SLBF4758V of P9135-500g. Thus, the conversion coefficient for pectin was 1.35 (1/74) and the pectin inclusion level was calculated to be 168.8 mg pectin.

The incubation medium according to Goering & Van Soest (1970) would provide 21 mg of N per 100 ml medium (Holtshausen, 2004). In the current study, effects of energy sources alone (no N in the medium), or energy sources in combination with N sources (from urea or soybean meal) were investigated. Because 100 ml of incubation medium was used, N sources were thus weighed out into the appropriate incubation flasks to provide 21 mg of N coming from either urea or soybean meal.

Table 5.1 The research design

	Roughage	Energy source	Nitrogen source	Incubation medium (normal or without nitrogen)
1	L	/	/	Normal
2	L	Sta	/	Without N
3	L	Sta	Soy	Without N
4	L	Sta	Ure	Without N
5	L	Suc	/	Without N
6	L	Suc	Soy	Without N
7	L	Suc	Ure	Without N
8	L	Pec	/	Without N
9	L	Pec	Soy	Without N
10	L	Pec	Ure	Without N
11	WS	/	/	Normal
12	WS	Sta	/	Without N
13	WS	Sta	Soy	Without N
14	WS	Sta	Ure	Without N
15	WS	Suc	/	Without N
16	WS	Suc	Soy	Without N
17	WS	Suc	Ure	Without N
18	WS	Pec	/	Without N
19	WS	Pec	Soy	Without N
20	WS	Pec	Ure	Without N
21	Control	/	/	Normal

L = lucerne, WS = wheat straw, Sta = maize starch, Suc = sucrose, Pec = pectin, Soy = soybean meal, Ure = urea

5.2.4. Sample preparation

Lusern hay and wheat straw was milled with a 2 mm hammer mill (cyclotec 1093 mill). The starch used was a soluble starch from Sigma-Aldrich (S9765), the pectin was a

Sigma-Aldrich (P9135) from citrus peel, and the sucrose was also a Sigma-Aldrich product (S7903). The urea was a univAR® product (637504) and the soybean oil cake was sourced from the Western Cape of South Africa (472.3 g/kg of crude protein, 0.6 g/kg of ash and 894.4 g/kg of DM). The F57 fibre bags were soaked in acetone to remove any waxy layer that may influence the fermentation of the rumen micro-organisms. The bags were dried at 105 °C for 24 hours and weighed the next day using the hot weighing technique. The adequate amount of roughages were weighed out in the bag and the bags were sealed and then put into 125 ml Erlenmeyer flasks. The starch, sucrose and soybean oilcake was weighed out and dispensed into the Erlenmeyer flasks in the correct amounts. Pectin does not easily dissolve in the buffer solution and thus was made up into a solution with water. By mixing and heating 4000 mg of pectin with 200 ml of water, the pectin was completely dissolved after 30 minutes. The solution was kept at 4 °C in a refrigerator and 8.4 ml was pipetted into each Erlenmeyer flask, where applicable. Urea was also dissolved in water to make measuring easier and urea was diluted with water to a 20 % solution, resulting in 228 microliters to be added to the appropriate sample flasks. The flasks were numbered and ready to use for the *in vitro* fermentation trials.

5.2.5. Chemical analyses

For this study, two different types of incubation media were used; with or without nitrogen. The basic composition of both incubation media was still according to Goering & Van Soest (1970) , with slight modifications as described by Van de Vyver & Joubert (2011). Since the effect of nitrogen on fibre digestion was studied, the trial combinations that did not contain any nitrogen had a buffer added without any ammonium bicarbonate and tryptose (as seen in Table 5.1.). Of the respective incubation media, 100 ml was dispensed into the flasks before rumen collection. The flasks were transferred to the warm room to reach 39 °C, purged with CO₂ and closed to maintain anaerobic conditions. Rumen fluid was collected after the morning milking, as described in Chapter 3. Following preparation (Chapter 3), the Erlenmeyer incubation flasks were kept in the warm room at 39 °C. Half of the flasks were removed after 6 hours and the rest after 30 hours of incubation. The F57 bags were collected and washed in a normal household washing machine for 15 minutes to stop the fermentation process and to remove the incubation media. Bags were then dried at 105 °C for 24 hours.

The next day, the samples were weighed and that value was used to determine the DM disappearance. The samples were then used in the ANKOM²²⁰ AUTOMATED fibre analyzer and the ANKOM method, with the addition of sodium sulphite and heat stable α -amylase, according to Van Soest *et al.* (1991) to determine the NDF residue in the bag and to calculate the NDF disappearance value

5.2.6. Statistical analyses

The statistical analyses on the substrate controls were done according to a main effects ANOVA with blocks, treatments and done per time. The statistical analyses for the treatments with energy and nitrogen sources were done on the six and 30 hour treatments, according to a three way factorial ANOVA with blocks, substrate, energy and nitrogen source as the factors. The three way interaction was significant for all four variables when comparing DM disappearance, NDF disappearance, IVTD and pH. The three way interactions were then interpreted with LSD multiple comparisons and Bonferroni tests.

5.3. Results and discussion

Nutrient disappearance values of the forage substrates alone without energy or N supplementation are presented in Table 5.2, whereas disappearances values of the forage substrates with energy and N supplementation are presented in Tables 5.3-5.5. For the latter, tables indicate means and SEM values, while statistical differences ($P < 0.05$) are indicated in the figures.

Table 5.2 The nutrient disappearance and pH values for the forage substrates alone (lucerne and wheat straw) after six and 30 hours of fermentation.

Item	Substrate and time				SEM	P
	Lucerne 6 h	Lucerne 30 h	Straw 6 h	Straw 30 h		
DMD (%)	39.4 ^a	51.2 ^b	10.9 ^c	16.9 ^d	0.884	0.004
NDFD (%)	13.8 ^a	26.4 ^b	7.9 ^c	11.5 ^{ac}	1.479	0.005
IVTD (%)	56.1 ^a	62.5 ^b	19.4 ^c	21.7 ^c	0.771	0.001
pH	6.9 ^{ab}	6.8 ^b	7.1 ^a	7.1 ^a	0.050	0.001

^{a-d}Per incubation time, values with different superscripts within rows, differ ($P < 0.05$).

5.3.1. Dry matter disappearance

Table 5.3 The DM disappearance values (\pm SEM) of lucerne and wheat straw after six and 30 hours of fermentation.

Item	Energy and nitrogen sources								
	Starch			Sucrose			Pectin		
	Soybean meal	Urea	None	Soybean meal	Urea	None	Soybean meal	Urea	None
<i>6 h</i>									
Lucerne	39.4 \pm 0.9	39.5 \pm 1.1	37.6 \pm 0.9	38.8 \pm 1.7	39 \pm 0.9	41 \pm 0.7	35.6 \pm 0.4	36.4 \pm 0.5	34.2 \pm 1.0
Wheat straw	11.5 \pm 0.6	11.8 \pm 0.7	11.3 \pm 0.3	11.1 \pm 0.4	11.2 \pm 0.2	11.6 \pm 0.3	11.8 \pm 0.2	11.4 \pm 0.1	11.3 \pm 0.2
<i>30 h</i>									
Lucerne	52.5 \pm 0.8	50.2 \pm 2.0	51.1 \pm 1.9	56.8 \pm 1.9	48.8 \pm 1.4	52.4 \pm 2.1	47.7 \pm 0.8	48.7 \pm 1.2	46.8 \pm 0.9
Wheat straw	16.5 \pm 0.8	15.6 \pm 1.1	18.5 \pm 1.5	17.1 \pm 0.8	17.1 \pm 1.1	17.3 \pm 1.0	21 \pm 0.9	16.6 \pm 0.7	18.9 \pm 0.7

For substrates alone, DM disappearance differed ($P = 0.004$) among forage type and fermentation time (Table 5.2). At each fermentation time, all the digestibility parameters were higher for lucerne than for wheat straw. This could be expected, as lucerne contains more digestible nutrients than wheat straw (NRC, 2001). Within wheat straw, NDFD and IVTD did not differ between six and 30 hours. The highest value was observed for lucerne hay after 30 hours of fermentation and the lowest value for wheat straw after six hours of fermentation.

With energy and N supplementation (Table 5.3), DM disappearance after 6 and 30 hours of fermentation was higher ($P < 0.001$) in lucerne hay than in wheat straw, regardless of energy and nitrogen sources. As in the case of forages alone, this could be expected. Forage*energy interactions ($P < 0.001$) were observed at six and 30 hours. There was also a significant interaction ($P < 0.001$) for forage*energy*nitrogen sources at 30 hours but not at six hours. Regarding lucerne hay at six hours, sucrose as energy source, without any nitrogen added to the incubation medium (SucNon), resulted in a higher ($P < 0.001$) DM disappearance than pectin as energy source, regardless of

nitrogen source. Starch and sugar as energy sources in any combination with N sources

appeared to have had similar effects on DM disappearance in lucerne. Treatment had no effect on six hours DM disappearance values of wheat straw. This can be seen in Figure 5.1.

After 30 hours, lucerne with sucrose as energy source and soybean meal as N source (SucSoy) resulted in the highest DM disappearance value. This value was higher ($P < 0.001$) than values obtained with all the other energy and N combinations, except for starch with soybean meal (StaSoy) and sugar without N supplementation (SucNon). This can be seen in Figure 5.2. Heldt *et al.* (1999), using a poor quality grass (52 g/kg of CP) also found that sucrose supplementation resulted in higher *in vitro* DM and NDF digestibilities than starch. The lowest DM disappearance value was observed with pectin as energy source without any N supplementation (PecNon), as was also

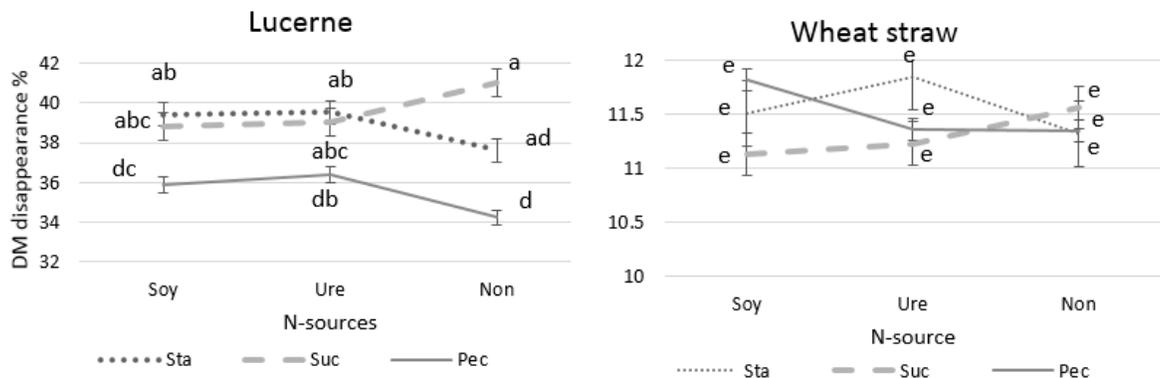


Figure 5.1. The DM disappearance of the treatment combinations of lucerne and wheat straw with different nitrogen and energy sources after six hours of fermentation. Means with different superscripts, differ ($P < 0.05$).

observed at six hours. In wheat straw, the highest DM disappearance value was observed with pectin as energy source and soybean meal as N source (PecSoy). This value was higher ($P < 0.001$) than that obtained with the StaUre combination.

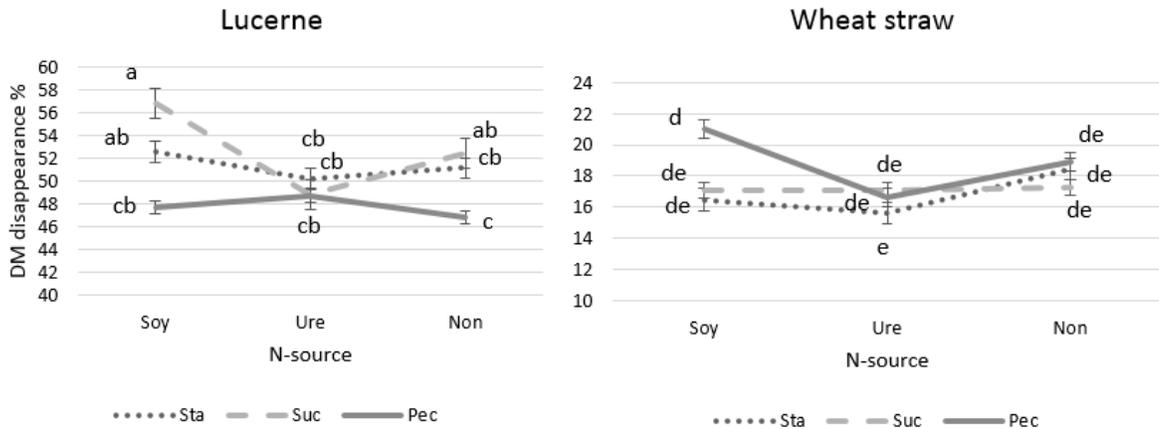
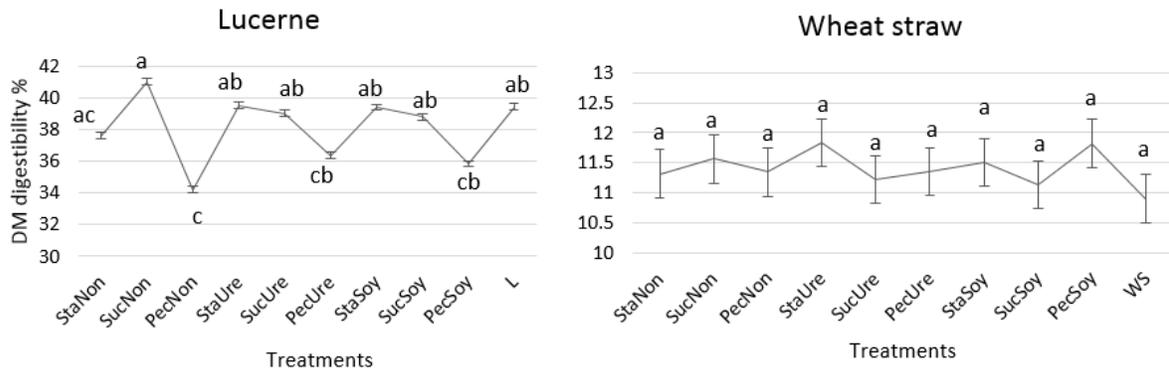


Figure 5.2. The DM disappearance of the treatment combinations of lucerne and wheat straw with nitrogen and energy sources after 30 hours of fermentation. Means with different superscripts, differ ($P < 0.05$).

As mentioned above (5.2.3), roughage sources alone, without any energy or N supplementation, were also included in the fermentations. Because of the reasons explained, the results of the roughage fermentations could not be included in the statistical analyses for a factorial arrangement to interpret substrate*energy source*N source interactions, therefore a separate main effects ANOVA was done to compare roughage alone with all the other combinations. When looking at the six hours results (Figure 5.3), it appears that pectin as energy source, without any N supplementation, suppressed *in vitro* DM digestion in lucerne ($P < 0.04$). In wheat straw, supplementation



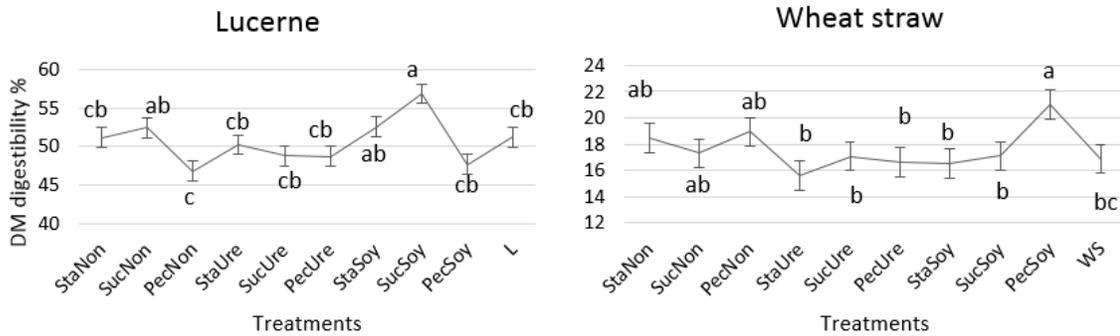
had no significant effect on DM digestibility. After 30 hours of fermentation (Figure 5.4), the SucSoy combination increased DM disappearance ($P < 0.001$) in lucerne, whereas **Figure 5.3**. The DM disappearance of roughage sources alone, compared to supplementation combinations after six hours of fermentation. Means with different superscripts, differ ($P < 0.05$).

the PecSoy combination increased DM disappearance in wheat straw ($P = 0.016$).

5.3.2. NDF disappearance

Table 5.4 The NDF disappearance values (\pm SEM) of lucerne and wheat straw at six and 30 hours fermentation

Item	Energy and nitrogen sources								
	Starch			Sucrose			Pectin		
	Soybean meal	Urea	None	Soybean meal	Urea	None	Soybean meal	Urea	None
<i>6 h</i>									
Lucerne	14.9 \pm 1.3	14.8 \pm 0.8	10.8 \pm 1.1	13.5 \pm 1.3	14.1 \pm 1.1	15.2 \pm 1.5	12.5 \pm 1.1	11.6 \pm 0.6	10.9 \pm 2.0
Wheat straw	8 \pm 0.5	8.7 \pm 0.3	8.4 \pm 0.3	8 \pm 0.6	8.4 \pm 0.6	8.1 \pm 0.3	8.1 \pm 0.4	7.5 \pm 0.4	8.6 \pm 0.2



<i>30 h</i>									
Lucerne	29 \pm 1.2	20 \pm 0.9	25.9 \pm 3.2	35.8 \pm 2.4	21.7 \pm 2.0	29.1 \pm 3.8	21.9 \pm 1.3	24.1 \pm 1.5	21.1 \pm 1.3

Figure 5.4. The DM disappearance of roughage sources alone, compared to supplementation combinations after 30 hours of fermentation. Means with different superscripts, differ ($P < 0.05$).

Wheat straw	11.6 \pm 1.2	9.6 \pm 1.0	13.5 \pm 1.7	12.4 \pm 1.1	12.6 \pm 1.7	11.9 \pm	16.5 \pm 1.0	11.7 \pm 0.8	15 \pm 0.7
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The NDF disappearance values in the substrates alone differed ($P = 0.005$) among forage type and fermentation time (Table 5.2). Lucerne NDF digestibility differed

($P < 0.005$) between six and 30 hours, whereas wheat straw showed no significant difference between six and 30 hours. After 30 hours of incubation, digestibility of wheat straw NDF did not differ from that of lucerne hay at 6 hours. The higher rate of NDF digestibility in lucerne hay compared to wheat straw is apparent (Table 5.2.).

When energy and N were supplemented, there was a forage*energy interaction at six hours ($P < 0.040$) and 30 hours ($P < 0.001$). A significant interaction was also observed for forage*energy*nitrogen at 30 hours ($P < 0.001$), but not at six hours ($P = 0.125$). After six hours of incubation, the various energy and N supplementations did not affect NDF significantly in either lucerne hay or wheat straw (Figure 5.5). There was also no difference between the substrate controls and treatments for lucerne and wheat straw at six hours (Figure 5.7.). When no N was supplemented, sucrose as energy source tended to increase lucerne NDF digestibility more than starch ($P = 0.070$) or pectin ($P = 0.096$) as energy sources. After 30 hours of incubation, lucerne hay NDF digestion was highest when sucrose and soybean meal were used as the respective energy and N sources. As was the case with DM disappearance, the SucSoy combination was also the only treatment that increased ($P < 0.002$) NDF digestibility when compared to lucerne as a substrate control (Figure 5.8). This value tended to be higher than values for the sucrose with no added nitrogen (Bonferroni $P = 0.196$; LSD $P = 0.001$) and starch with soybean meal (Bonferroni $P = 0.148$; LSD $P = 0.001$) combinations. These combinations showed the same tendencies when the DM disappearance was measured. Sucrose and soybean meal thus appears to be a good combination in terms of NDF digestibility in lucerne hay. The combinations of starch with urea (StaUre), pectin with soybean meal (PecSoy) and pectin without any N source (PecNon) resulted in the lowest NDF digestibility (Figure 5.6.)

Regarding wheat straw, pectin as energy source, combined with soybean meal as N source (PecSoy), increased the NDF digestibility ($P < 0.001$; Figure 5.8) and tended (Bonferroni $P = 0.133$; LSD $P = 0.001$) to improve NDF digestibility at 30 hours more than starch with urea. Other than that, treatment had no effect on wheat straw NDF digestibility.

Generally speaking, when looking at all the 30 hours data, it appears as if sugar was the most effective energy source and pectin the least effective one with lucerne hay. The fact that pectin has a negative effect on good quality roughages was also proven in a study by Holtshausen (2004), where the same type of energy sources was studied

at 120mg hexose equivalent to 120mg NDF of Bermuda grass, which can be classified as a good quality roughage. In the study, pectin had the lowest NDF disappearance. In the case of wheat straw, pectin appears to be the most effective and starch the least. In a study by Heldt *et al.* (1999), sucrose resulted in a higher NDF digestibility in low quality hay (5.2% protein/DM) than starch. Mould *et al.* (1983) also reported that sucrose resulted in higher DM digestibilities than starch when good or poor quality hay or straw was fed. Some other studies, however, reported that sucrose as energy source resulted in a lower NDF digestibility compared to starch when dried grass (Mould *et al.*, 1983), ammoniated hay (Royes *et al.*, 2001) or grass silage (Stensig *et al.*, 1998) was fed. Inclusion level, however, also appears to have an effect, as shown by Owens *et al.*, (2008) who found that 300 g/kg of sucrose decreased NDF

digestibility more than starch, whereas an inclusion of 200 g/kg had a similar effect as starch. Heldt *et al.* (1999) also reported that sucrose decreased NDF digestibility to a lower extent than starch. The apparent improved effects observed for pectin in the case of wheat straw at 30 hours agree with findings of Fondevila *et al.*, (2002) where pectin increased gas production in straw, and that pectin increased NDF digestibility when replacing starch (Miron *et al.*, 2002; Poorkasegaran & Yansari, 2014). In the studies of Villarreal *et al.* (2006) and Gressley & Armentano (2005), pectin had no

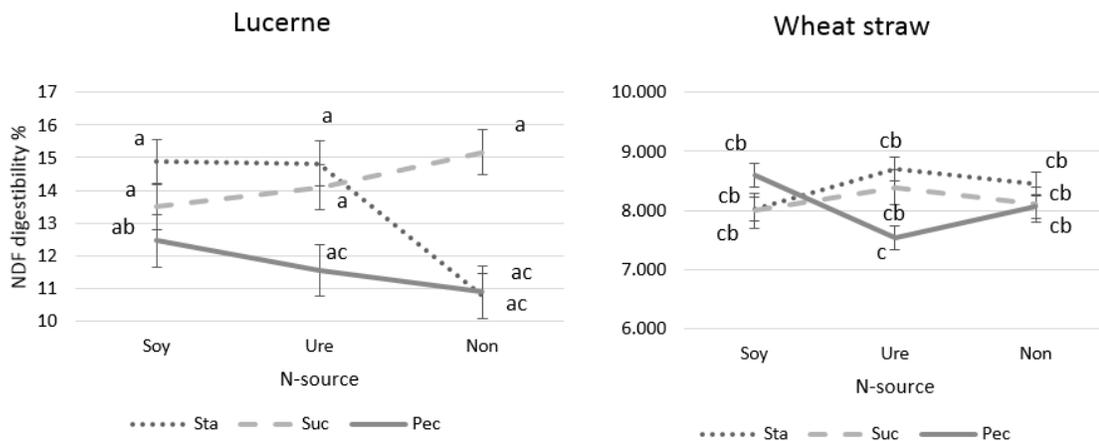
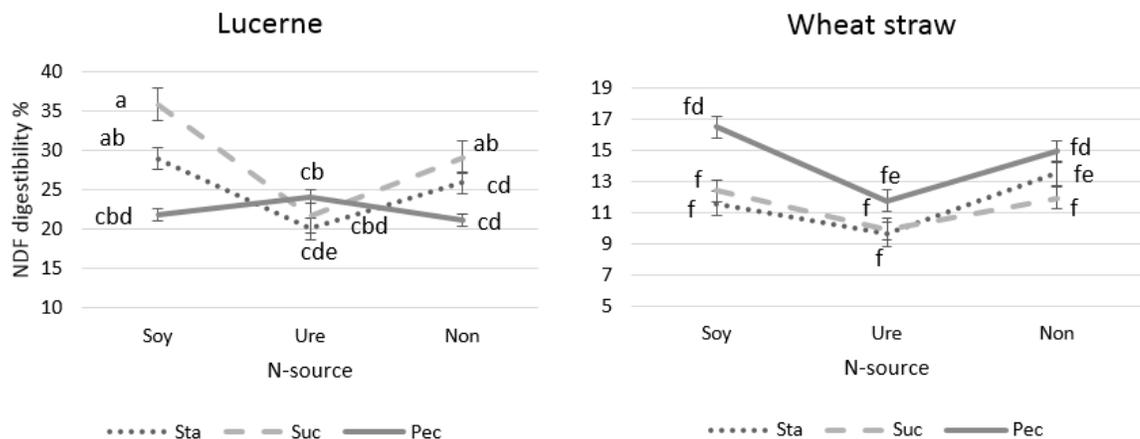


Figure 5.5. The NDF disappearance of the treatment combinations of lucerne and wheat straw with nitrogen and energy sources at six hours fermentation. Means that have different superscripts, differ ($P < 0.05$).



effect on digestion when it was added to a diet with a good quality roughage. This agrees with our results where pectin resulted in the lowest DM and NDF digestibility in good quality lucerne hay.

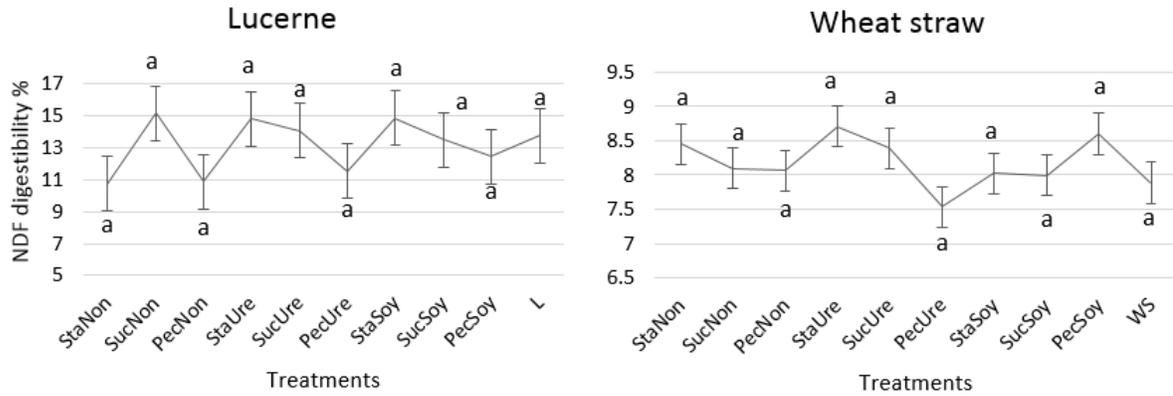


Figure 5.7 The NDF disappearance of lucerne and wheat straw at six hours as substrate controls, in combination with energy and nitrogen sources. Means with different superscripts, differ ($P < 0.05$).

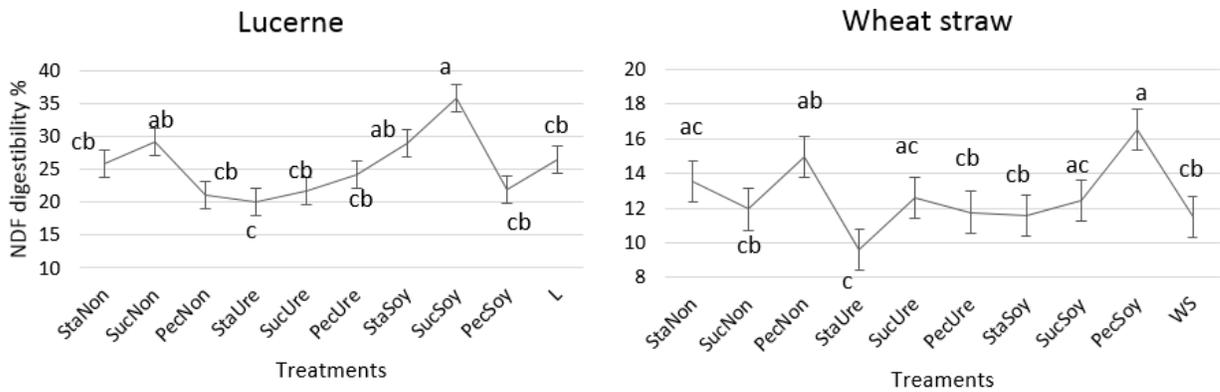


Figure 5.6. The NDF disappearance of the treatment combinations of lucerne and wheat straw with nitrogen and energy sources at 30 hours fermentation. Means that have different superscripts, differ ($P < 0.05$).

5.3.3. *In vitro* true digestibility

For the substrate control alone (Table 5.2.), *in vitro* true digestibility (IVTD) values in lucerne were higher ($P=0.001$) after 30 hours than after six hours, but in wheat straw the difference between six and 30 hours were not significantly different.

Regarding the supplementation of energy and N, forage*energy source and forage*energy*nitrogen source interactions after six hours of fermentation tended to differ ($P<0.069$ and $P<0.167$, respectively). After 30 hours, however, these interactions were significant ($P<0.001$ and $P<0.026$, respectively).

Table 5.5 *In vitro* true digestibility values (\pm SEM) of lucerne and wheat straw at six and 30 hours of fermentation

Item	Energy and nitrogen sources								
	Starch			Sucrose			Pectin		
	Soybean meal	Urea	None	Soybean meal	Urea	None	Soybean meal	Urea	None
<i>6 h</i>									
Lucerne	56.1 \pm 1.0	56.6 \pm 0.4	54.6 \pm 0.5	56 \pm 0.7	55.7 \pm 0.8	57.6 \pm 1.2	55.4 \pm 1.2	54.1 \pm 0.6	54.6 \pm 1.0
Wheat straw	19.5 \pm 0.5	20.1 \pm 0.3	19.9 \pm 0.3	19.5 \pm 0.5	19.8 \pm 0.6	19.6 \pm 0.3	20 \pm 0.3	19.1 \pm 0.3	19.5 \pm 0.2
<i>30 h</i>									

Figure 5.8 The NDF disappearance of *lucerne and wheat straw* at 30 hours as substrate controls, in combination with energy and nitrogen sources. Means with different superscripts, differ ($P<0.05$).

Lucerne	63.4 \pm 0.9	62.5 \pm 2.2	63.4 \pm 2.1	67.3 \pm 1.2	61.9 \pm 2.0	63.9 \pm 1.9	60.4 \pm 1.4	60.7 \pm 1.5	59.9 \pm 0.7
Wheat straw	23.2 \pm 1.1	22 \pm 1.2	23.5 \pm 1.8	23.9 \pm 1.4	24.7 \pm 2.4	23.6 \pm 1.1	28.1 \pm 1.9	23.6 \pm 1.4	25.9 \pm 0.9

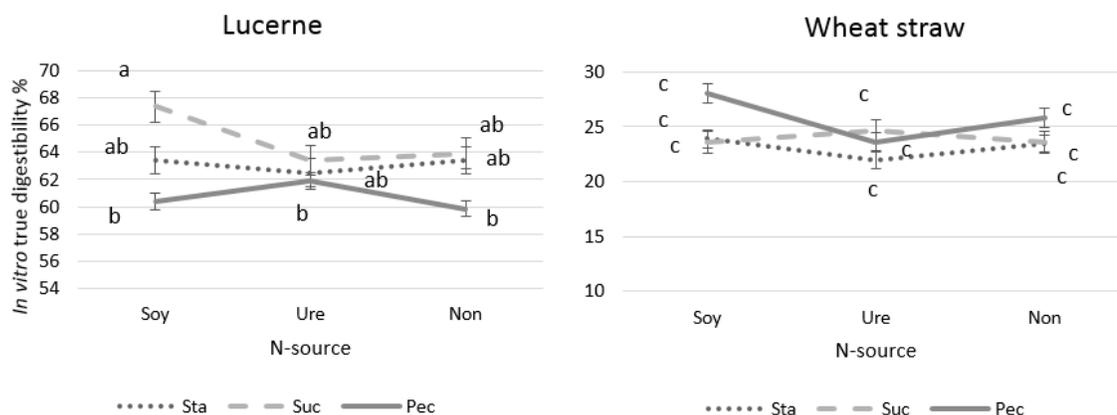
After six hours of fermentation, all the IVTD values in lucerne were between 54.1 and 57.6% (Table 5.5.). The only treatments that differed ($P=0.014$) were SucNon and PecUre In lucerne (Figure 5.9.) These differences were quite small and would likely not be of any biological significance. None of the treatments differed from lucerne as a substrate control at six hours fermentation as well (Figure 5.11). In wheat straw, no differences were observed among treatments and there was no difference between the treatments and the substrate control (Figure 5.11). IVTD values at six hours varied between 22.0 and 28.1% (Figure 5.9.). However, the six hour IVTD values of all the wheat straw treatments were lower ($P=0.001$) than those in lucerne.

After 30 hours of fermentation, the highest IVTD values in lucerne was observed for the SucSoy combination, and this value was higher ($P<0.02$) than those of all combinations with pectin as energy source (Figure 5.10). But did not differ from lucerne as a substrate control (Figure 5.12). In wheat straw, no differences were observed among treatments at 30 hours (Figure 5.10). However, pectin in combination with soybean meal (PecSoy) showed an increase in wheat straw IVTD ($P<0.001$) whereas the other treatments did not differ from the substrate control. These results can be seen in Figure 5.12.

These results showed the same pattern that was observed for DM and NDF disappearances, suggesting that sucrose as energy source with soybean meal as N source appeared to be the best combination for lucerne during the first 30 hours of *in vitro* fermentation. The combination of starch and soybean meal (StaSoy) was the second best combination and in terms of 30 h NDF disappearance, only tended to differ from the SucSoy combination. Combinations of pectin and urea resulted in the lowest lucerne digestibility values of DM, NDF and IVTD. In wheat straw, on the other hand, the best combinations appeared to have been pectin with soybean meal (PecSoy), while the starch with urea combination resulted in the lowest digestibility values after 30 hour of fermentation. Soybean meal tends to be the most favourable protein source in the treatment combinations to result in the highest substrate digestibility. Belasco (1954) reported that urea resulted in better cellulose digestion in contrast to RDP sources like soybean meal. The reasons that urea was less favourable than soybean meal in the current study could be that the inclusion of urea (a high soluble nitrogen) resulted in more protein degradation, thus depressing the nitrogen retention (Jones, Stephens, & Kensett, 1975) resulting in lower performance

parameters (Majdoub, Lane, & Aitchison, 1978). The nitrogen inclusion level of this study was 21mg of nitrogen per sample. The roughages were included at 125mg NDF resulting in 304 mg of lucerne and 169 mg of wheat straw. Thus the nitrogen percentage of the combinations was 6.91% for lucerne and 12.43% for wheat straw. These values are higher than the ideal inclusion level for urea-N of 7% for microbial growth reported by Lazzarini *et al.* (2009) and 11% for NDF digestion of low quality grass reported by Köster *et al.* (1996) and Lazzarini *et al.* (2009).

Figure 5.9. *In vitro* true digestibility of the treatment combinations of lucerne and wheat straw with energy and nitrogen sources at six hours of fermentation. Means that have different superscripts, differ ($P < 0.05$).



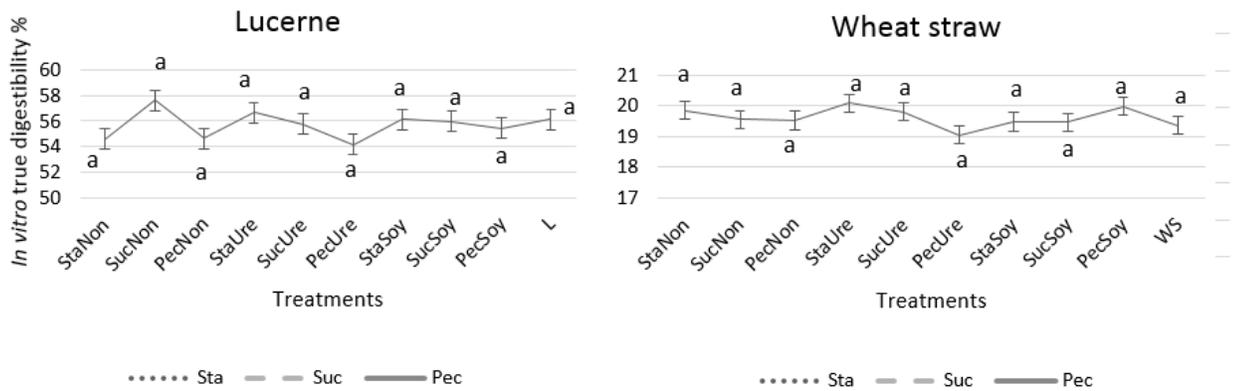


Figure 5.11. *In vitro* true digestibility of lucerne and wheat straw at six hours fermentation as substrate controls, in combination of substrate, energy and nitrogen sources. Means that have different superscripts, differ ($P < 0.05$).

Figure 5.10. *In vitro* true digestibility of the treatment combinations of lucerne and wheat straw with energy and nitrogen sources at 30 hours of fermentation. Means that have different superscripts, differ ($P < 0.05$).

5.3.4. pH

Regarding forages alone, pH after six hours of fermentation did not differ between lucerne hay and wheat straw, but after 30 h, pH was higher ($P=0.003$) for wheat straw than for lucerne (Table 5.2.). With the energy and N supplementations, the StaUre combinations resulted in the highest six hour fermentation pH values for both lucerne hay and wheat straw. At 30 hours, a significant ($P=0.045$) forage*energy interaction was observed where, in the case of wheat straw, PecSoy resulted in lower pH values than StaUre. These results can be seen in Table 5.6. and Figure 13 & Figure 14.

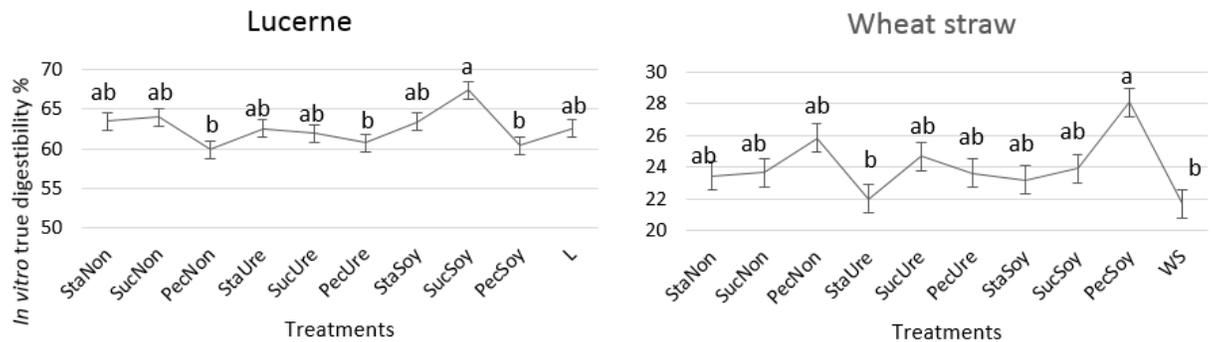


Figure 5.12. *In vitro* true digestibility of lucerne and wheat straw at 30 hours fermentation as substrate controls, in combination of substrate, energy and nitrogen sources. Means that have different superscripts, differ ($P<0.05$).

Table 5.6. The ruminal pH of lucerne and wheat straw fermentation values (\pm SEM) at six and 30 hours fermentation

Item	Energy and nitrogen sources		
	Starch	Sucrose	Pectin

	Soybean meal	Urea	None	Soybean meal	Urea	None	Soybean meal	Urea	None
<i>6 h</i>									
Lucerne	6.7±0.03	6.9±0.05	6.8±0.04	6.6±0.02	6.7±0.01	6.7±0.04	6.6±0.02	6.7±0.02	6.7±0.02
Wheat straw	6.9±0.05	7.1±0.08	6.8±0.06	6.7±0.01	6.9±0.02	6.7±0.02	6.6±0.01	6.8±0.02	6.6±0.02
<i>30 h</i>									
Lucerne	6.7±0.04	7.4±0.08	6.7±0.03	6.6±0.02	6.8±0.08	6.7±0.03	6.7±0.13	6.7±0.06	6.7±0.03
Wheat straw	6.7±0.01	6.9±0.11	6.8±0.07	6.7±0.10	6.8±0.03	6.9±0.12	6.6±0.02	6.8±0.07	6.6±0.02

In the literature, starch, compared to sucrose and pectin, has shown variable results regarding rumen pH. Sucrose resulted in higher rumen pH values than starch (Stensig *et al.*, 1998; Hindrichsen & Kreuzer, 2009), although the increase in pH also led to higher NDF digestion compared to starch (Hindrichsen & Kreuzer, 2009). However, the opposite was also found and Stensig *et al.* (1998) and Mould *et al.* (1983) reported that NDF digestion with sucrose inclusion was lower than with starch inclusion. Sucrose (molasses) compared to starch (maize) also resulted in lower ruminal pH values, which agrees with results of the current study. Mould *et al.* (1983) and Royes *et al.* (2001) reported a lower NDF digestibility of good quality hay at the lower pH values resulting from sugar, which is in contrast with results in our study.

Pectin in comparison to starch has shown a higher pH value in the study of Poorkasegaran & Yansari (2014), which is in contrast with the current study that showed pectin resulting in lower pH values. Pectin also showed an increase in straw NDF digestibility in the study by Poorkasegaran & Yansari (2014), which agrees with the results in the current study. In a study by Fondevila *et al.* (2002), pectin and starch had the same effect on pH but pectin still resulted in higher NDF digestibility of straw. This can be explained by the time period that the pH was measured. Higher amounts of lactate and volatile fatty acids will be produced in the first few hours post ingestion when pectin is compared to starch (Lechartier & Peyraud, 2011). Pectin will, however, not decrease the ruminal pH more because pectin can bind hydrogen ions and thus does not decrease the fibrolytic activity, resulting in higher NDF digestibility values (Lechartier & Peyraud, 2011). Since pectin is almost completely digested the pH will

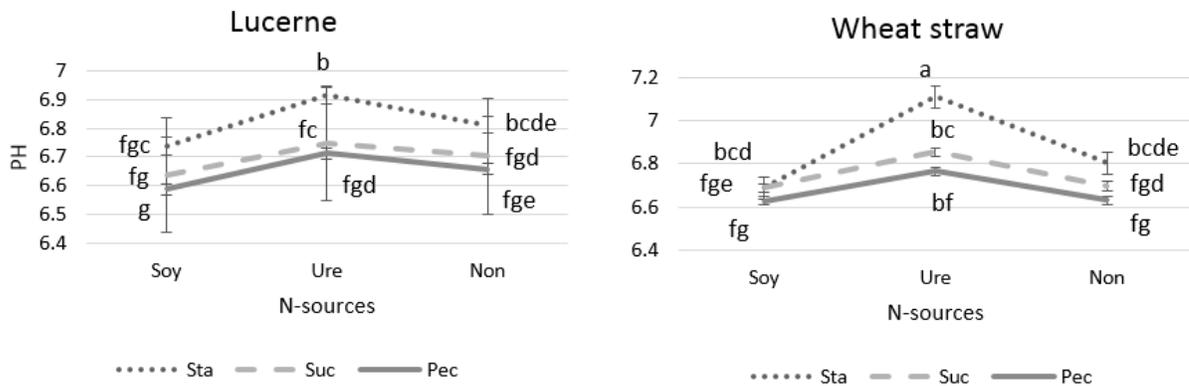
drop after a short time leading to lower pH values than starch (Lechartier & Peyraud, 2011).

The nitrogen sources used in this current study, urea and soybean meal, are both classified as rumen degradable. Although urea doesn't contain any protein, it is a source of crude protein (N x 6.25). Urea differs from soybean meal by being 100% degradable. In general, looking at all the digestibility parameters, soybean meal appeared to have been the most favourable nitrogen source for forage digestibility in the current study, especially after 30 hours of *in vitro* fermentation.

When looking at the pH values, soybean meal combinations resulted in the lowest pH values and urea in the highest. Documented studies have shown that a decrease in pH could be due to an increase in fermentation, resulting in higher NDF digestion in low quality forages (Olson *et al.*, 1999; Souza *et al.*, 2010; Khandaker, Uddin, Sultana, & Peters, 2012). This could explain why nitrogen sources that favour digestion resulted in the lowest pH values. Nitrogen sources have shown to decrease rumen pH, but not below the cellulolytic threshold (Klevesahl *et al.*, 2003; Khandaker *et al.*, 2012). This is in agreement with results of the current study where the pH value was never below 6.0-6.1. Studies have also verified that the treatment combination with a certain starch and nitrogen level resulting in the lowest digestion, is not always the treatment combination with the lowest pH (Olson *et al.*, 1999; Klevesahl *et al.*, 2003). Klevesahl *et al.* (2003) has found that nitrogen sources increased the ruminal pH, but decreased forage digestion of grass silage. Studies with urea have shown to decrease ruminal pH (Souza *et al.*, 2010), whereas studies with RDP have been reported to either increase (Nousiainen, *et al.*, 2009) or decrease ruminal pH (Olson *et al.*, 1999; Khandaker *et al.*, 2012).

5.4. Conclusion

Lucerne showed higher disappearance values than wheat straw for DM digestibility



($P < 0.004$), NDF digestibility ($P < 0.005$) and *in vitro* true digestibility ($P < 0.001$). For DM

Figure 5.13. The pH of treatment combinations of lucerne and wheat straw with energy and nitrogen sources at six hours fermentation. Means that have different superscripts, differ ($P < 0.05$).

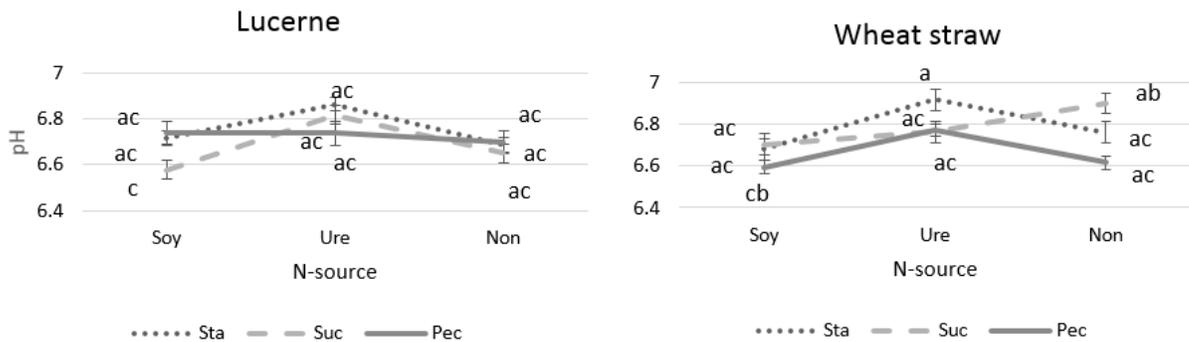


Figure 5.14. The pH of treatment combinations of lucerne and wheat straw with energy and nitrogen sources at 30 hours fermentation. Means that have different superscripts, differ ($P < 0.05$).

and NDF digestibility there was a forage*energy interaction at six and 30 hours ($P<0.001$) but only a forage*energy*nitrogen interaction at 30 hours of fermentation ($P<0.001$). IVTD showed a forage*energy ($P<0.001$) and forage*energy*nitrogen interaction ($P<0.026$) at 30 hours. pH only showed a forage*energy interaction at 30 hours ($P<0.045$).

DM digestibility of lucerne showed that the treatment combination of sucrose with no added nitrogen (SucNon) had the highest digestibility at six hours and sucrose with soybean meal (SucSoy) had the highest digestibility at 30 hours. Sucrose with soybean meal also had higher DM digestibilities than lucerne as a substrate control at 30 hours. Wheat straw at six hours fermentation showed no difference between treatments but at 30 hours pectin in combination with soybean meal (PecSoy) had the best digestibility and was higher than wheat straw as a substrate control.

NDF digestibility of lucerne at six hour fermentation showed no treatment difference as well as for wheat straw at 30 hours. Lucerne however had higher digestibility with sucrose and soybean meal (SucSoy) combination at 30 hour fermentation, this combination was also higher than lucerne as a substrate control. Even though wheat straw showed no treatment difference for six or 30 hours, there was a treatment at 30 hours that had better digestibility than wheat straw as a substrate control, this was pectin in combination with soybean meal (PecSoy). The other treatments for wheat straw at 30 hours did not differ significantly from the substrate control.

IVTD showed much the same results as NDF digestibility. Lucerne and wheat straw at six hours and wheat straw at 30 hours showed no difference between combinations. Lucerne at 30 hours showed that sucrose in combination with soybean meal had the best digestibility, this combination did however not differ from lucerne as a substrate control. Wheat straw at 30 hours showed no significant difference between treatments but pectin in combination with soybean meal had better IVTD than wheat straw as a substrate control.

The pH between lucerne and wheat straw after six hours of fermentation was not significantly different, only at 30 hours did wheat straw show significant higher pH values than lucerne ($P<0.003$). At six hour fermentation starch in combination with urea showed the highest pH values for lucerne and wheat straw. At 30 hours lucerne had no treatment combination that differed from the rest but wheat straw showed highest values for starch and urea and lowest pH for pectin and soybean meal. These

combinations did also differ from each other but not from the other treatment combinations of wheat straw at 30 hours.

The differences observed between treatment combinations thus reject the null hypothesis and show that lucerne and wheat straw favoured different treatment combinations. Sucrose with soybean meal was the best combination for lucerne hay, whereas pectin in combination with soybean meal was the most favourable combination for wheat straw. More research can be done with maize as a starch source, molasses as a sucrose source and citrus pulp as a pectin source with the same forages and nitrogen sources to evaluate the practical applications of these results.

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CHAPTER 6

GENERAL CONCLUSION

The aim of this paper was to report on two *in vitro* studies aimed at the improvement of DM and NDF digestibility, as well as *in vitro* true digestibility (IVTD). In the first study (Chapter 4), starch was supplemented at levels of 0, 35, 80 and 120 mg hexose equivalent in combination with 120 mg NDF from either lucerne hay or wheat straw. No significant differences were observed among starch supplementation regarding fibre digestion. McCullough (1968) studied four levels of maize inclusion, two inside the range used in Chapter 4, and two higher. There was found that the two lower levels of starch had no effect on silage digestibility and all four levels had no effect on hay digestibility. The levels of starch in Chapter 4 could have been higher or the fermentation procedure could be longer to give significant differences. Thus the highest inclusion level (50:50 starch HE:NDF) was used in the second study.

In the second study, different combinations of energy sources (starch, sucrose, pectin) and nitrogen sources (urea, soybean meal or no N) were studied with the two roughages at six and 30 hours of fermentation. Lucerne hay as a high quality roughage and wheat straw as a low quality roughage was used where DM digestibility, NDF digestibility, *in vitro* true digestibility and ruminal pH was measured.

Lucerne showed higher disappearance values than wheat straw for DM digestibility, NDF digestibility and *in vitro* true digestibility. Regarding six hours DM digestibility, lucerne showed the best results with sucrose supplementation without any N (SucNon). After 30 hours, sucrose with soybean meal (SucSoy) proved to be the best combination. For wheat straw, treatments had no effect on six hours DM disappearance, but after 30 hours, pectin and soybean meal (PecSoy) resulted in the highest digestion values.

NDF digestibility of lucerne was higher with sucrose and soybean meal (SucSoy) in combination at 30 hour fermentation. The treatments for wheat straw at six and 30 hours did not differ significantly from each other, as well as lucerne at six hours. IVTD showed much the same results as NDF digestibility.

pH values at six hour fermentation showed that starch in combination with urea (StaUre) had the highest values for lucerne and wheat straw. At 30 hours wheat straw showed highest values for starch and urea (StaUre) and lowest pH for pectin and

soybean meal (PecSoy). These combinations did also differ from each other but not from the other treatment combinations of wheat straw at 30 hours. Mould *et al.*, (1983) also found that starch inclusions will result in higher ruminal pH values than sucrose. Documented studies have shown that a decrease in pH could be due to an increase in fermentation, resulting in higher NDF digestion in low quality forages (Olson *et al.*, 1999; Souza *et al.*, 2010; Khandaker *et al.*, 2012). This could explain why nitrogen sources that favour digestion resulted in the lowest pH values.

It appears as if sucrose was the most effective energy source and pectin the least effective source with lucerne hay. Pectin was also the most effective energy source for wheat straw. Both of the roughages favoured soybean meal above urea as a nitrogen source. Studies by Miron *et al.*, (2002) and Poorkasegaran & Yansari, (2014) also concluded that pectin increased the NDF digestibility of straw and Holtshausen (2004) showed that pectin decreased the digestibility of Bermuda grass (a good quality roughage). Heldt *et al.*, (1999) also found that sucrose can increase digestion of poor quality roughages. It was concluded that different energy sources affect *in vitro* digestibility of different roughages in different ways. This may have practical implications in ration formulation for ruminant animals.

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