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Effect of dietary protein quality and amino acid supplementation on performance of high producing Jersey cows grazing ryegrass pasture

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Agriculturae - MSc (Agric)
Animal Science: Animal Nutrition

In the Faculty of Natural and Agricultural Sciences
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Table of Contents

Page

Page

Page

Declaration

I, Ranier van Heerden, hereby declare that this dissertation, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and effort, conducted under the supervision of Prof. L.J. Erasmus and Prof. R. Meeske and that it has not previously been submitted by me for a degree at this or any other tertiary institution.

……

R. van Heerden

Pretoria,

October, 2020

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Summary

Effect of dietary protein quality and amino acid supplementation on performance of high producing Jersey cows grazing ryegrass pasture

by

R. van Heerden

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The performance of grazing dairy cows, when supplemented with high levels of a maize-based concentrate, could potentially be improved in terms of higher milk and milk protein production by improving the essential amino acid balance of the metabolisable protein reaching the cow's small intestine. The objective of this study was to investigate the effect of supplementing rumen protected lysine or a combination of rumen protected lysine and rumen protected methionine on the production performance of high producing Jersey cows grazing ryegrass pasture in spring while receiving a maize-based concentrate. The performance parameters measured were milk production, milk composition, body weight and body condition change. The effect of supplementation on milk nitrogen fractions and plasma amino acid concentrations were also measured. A secondary objective was to evaluate a urine spot sampling technique and allantoin excretion as a non-invasive method to estimate rumen microbial protein synthesis. The study was conducted at the Outeniqua Research Farm situated near George in the Western Cape during spring. Sixty high producing multiparous Jersey cows in mid-lactation were used in a randomised complete block design experiment and blocked according pre-experimental milk production, days in milk, lactation number and randomly allocated to three groups within each block. Subsequently, each group was randomly allocated to one of three experimental treatments. These treatments were: (1) control treatment (C) supplemented with no rumen protected amino acids; (2) rumen protected lysine treatment (RPL) supplemented with 53.12 g/cow/d of RPL providing approximately 22 g of intestinally absorbable lysine/cow/d and (3) rumen protected methionine and lysine treatment (RPML) supplemented with 41.68

and 53.12 g/cow/d of RPML, providing approximately 9.3 and 22 g intestinally absorbable methionine and lysine, respectively. In addition to strip grazed ryegrass pasture the cows received 8 kg (as is) of a maize-based concentrate, fed in two equal portions in the milking parlour. The data were statistically analysed for the high (block 1 to 10) and low producing (block 11 to 20) cow groups. The supplementation of RPL did not affect milk production and composition, body weight, body condition, faecal starch content, plasma amino acid concentrations and microbial protein production ($P > 0.05$). However, compared to the C treatment RPL supplementation tended to reduce milk protein production (3.69 *vs*. 3.89 %; $P = 0.09$). Supplementation of RPL did, however, increase the plasma lysine:methionine ratio beyond the ratio represented by the C treatment (3.67 *vs*. 3.32; $P < 0.05$). The high producing group of cows (> 24 l/cow/d) did not respond to the supplementation of RPL, while the lower producing group of cows (< 20 l/cow/d) responded negatively in terms of milk protein production compared to the C treatment (3.85 *vs*. 4.16 %; $P < 0.05$). Supplementing the combination of lysine and methionine did not affect milk production and composition, faecal starch content, body weight and microbial protein production. However, in the presence of an increase in plasma methionine concentration (41.4 *vs*. 28.8 μmol/l; $P < 0.05$) cow body condition increased (+ 0.43 *vs*. + 0.31 points (Scale 1 to 5; $P < 0.05$) compared to the C treatment. In addition to an increase in plasma methionine, glycine increased (448 *vs*. 405 μmol/L; $P < 0.05$), and lysine tended to increase (109 *vs*. 95.5 μmol/l; $P = 0.09$) along with cysteine (9.45 *vs*. 8.40 μmol/l; $P = 0.07$) compared to the C treatment. Supplementation of RPML decreased the plasma lysine:methionine ratio below the ratio represented by the C treatment (2.63 *vs*. 3.32; $P < 0.05$). The higher producing group of cows (> 24 l/cow/d) and lower producing group of cows (< 20 l/cow/d) did not respond to the supplementation of RPML. Comparing the study results with predictions done with the CNCPS model gave relatively realistic and comparative predictions in terms of metabolisable energy allowable milk, metabolisable protein allowable milk, pasture dry matter intake and microbial protein synthesis, including the metabolisable protein and amino acid balance of the cows and support the findings of our study. Results indicate that the allantoin in spot urine samples are a valid method to be used to determine microbial protein synthesis for Jersey cows grazing pasture. In view of the experimental results the data indicate that the lack of positive responses in terms of milk production and milk composition when RPL or RPML were supplemented were as a result of metabolisable energy being the first limiting nutrient in addition to the cows being later in lactation and that the C diet met the cows metabolisable protein requirements. Results are interpreted to suggest that both lysine and methionine were supplied in excess of cow requirements and subsequently metabolised, repartitioning nutrients towards other metabolic pathways away from milk production and composition. The results further show that the amino acid requirements, limitations and responses in cow performance for cows grazing high-quality pasture while being supplemented with a maize-based concentrate is complex and requires further research.

List of Abbreviations

AA	amino acid/s
ADF	acid detergent fibre
ADIN	acid detergent insoluble nitrogen
ADIP	acid detergent insoluble protein
ADL	acid detergent lignin
AL	allantoin
ARC	Agricultural Research Council
BCS	body condition score
BW	body weight
CNCPS	Cornell Net Carbohydrate and Protein System
CO_2	carbon dioxide
CP	crude protein
Cr_2O_3	chromic oxide
DIM	days in milk
DM	dry matter
DMI	dry matter intake
EAA	essential amino acid/s
EE	ether extract
ECM	energy-corrected milk
ECP	endogenous CP
FME	fermentable metabolisable energy
GE	gross energy
*IV*OMD	*in vitro* organic matter digestibility
kPa	kilopascal
ME	metabolisable energy
MCP	microbial crude protein
MUN	milk urea nitrogen
MP	metabolisable protein
N	nitrogen
NAN	non-ammonia nitrogen
NCN	non-casein nitrogen
NDF	neutral detergent fibre
NDIP	neutral detergent insoluble protein
NE	net energy
NE_L	net energy for lactation

NFC	non-fibre carbohydrates
NH_3	ammonia
NH_3-N	ammonia nitrogen
NO_3	nitrate
NPN	non-protein nitrogen
NUE	nitrogen use efficiency
NRC	National Research Council
NFC	non-fibre carbohydrates
NSC	non-structural carbohydrate
OM	organic matter
PA	pasture allowance
peNDF	physically effective NDF
PDMI	pasture dry matter intake
PD	purine derivatives
RDP	rumen-degradable protein
PMR	partial mixed ration
RP_{Meter}	rising plate meter
RPAA	rumen protected amino acids
RPM	rumen protected methionine
RPL	rumen protected lysine
RUP	rumen-undegradable protein
SD	standard deviation
SEM	standard error mean
SI	small intestine
Sol CP	soluble crude protein
SR	substitution rate
TMR	total mixed ration/s
VFA	volatile fatty acids
WOL	week of lactation

List of amino acids:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine

His	Histidine
Ile	Isoleucine
Leu	Leucine
Met	Methionine
Lys	Lysine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

List of Tables

Appendix

List of Figures

Chapter 3

Chapter 4

Chapter 1: General introduction and Motivation

Pasture-based production systems in the Southern Cape region of South Africa play a major role in profitable and sustainable milk production (Meeske *et al*., 2006). These production systems collectively represent almost 60 % of all dairy producers in South Africa, supplying more than half of the total 3.4 million tonnes of milk annually produced in the country (Coetzee, 2019). While taking into account that the highest milk production densities (kg milk/km^2 land) found within the Southern Cape are mainly distributed along the coastal areas of both the Western and Eastern Cape provinces (Coetzee, 2019). As a result of this, a huge responsibility rest on the shoulders of milk producers in these provinces, which are primarily pasture-based, to supply the ever-increasing demand for dairy products.

The main reasons for pasture-based production systems to be more common within the Southern Cape region are due to the climatic conditions supporting optimal forage growth and the ability of dairy farmers to utilise irrigated pastures, which results in a distinctive seasonal pattern of pasture availability and quality. Forage species primarily used within these pasture-based production systems include kikuyu (*Pennisetum clandestinum*), Westerwolds ryegrass (*Lolium multiflorum* var. *westerwoldicum*), Italian ryegrass (*Lolium multiflorum* var. *italicum*) and perennial ryegrass (*Lolium perenne*) (Botha *et al.*, 2008; Meeske *et al.*, 2009; Van der Colf *et al.*, 2015b). Kikuyu supplies most of the dry matter (DM) consumed by the cows during summer months, but through critical winter months kikuyu may be dormant or have a very low DM production including a poor nutritive value, resulting in a winter DM shortage and a reduction in milk production (Henning *et al.*, 1995; García *et al.*, 2014). Due to this winter DM shortage, farmers normally strategically incorporate temperate forage species (i.e. ryegrass) either as a pure, mix or over-sown pasture to increase the seasonal variation in DM production and distribution, pasture quality and efficiency of utilisation (Botha *et al*., 2005). The efficiency of pasture-based systems is based on high milk production/ha land, whereas total mixed rations (TMR), on the other hand, are based on high milk production per cow (Clark & Kanneganti, 1998).

When compared to cows fed a TMR, cows grazing pasture may produce up to 30 % less milk, which is mainly attributed to nutritional challenges imposed on these cows (Kolver & Muller, 1998). Nutritional factors limiting milk production, or performance, of grazing dairy cows have been reported on by multiple authors (Fulkerson & Trevaskis, 1997; Bargo *et al.*, 2003; Kolver, 2003) and it appears that these factors usually follow along a "cascade'' of nutritional limitations, with one factor superseding the next (Roche, 2017).

The first factor most commonly identified to limit milk production from pasture is the low intake of metabolisable energy (ME) (Clark *et al.*, 1997; Bargo *et al.*, 2003; Kolver, 2003). Since ME is considered as the first limiting factor for cow performance, supplementation with an energy-rich grain to meet the high producing cow's ME requirements is necessary. Supplying more energy to meet the cow's energy requirements in the form of fermentable energy (FME) will stimulate rumen microbial activity and

proliferation, increasing pasture utilisation and efficiency (Dewhurst *et al*., 2000; Peyraud & Delaby, 2001; Verbic, 2002).

Since maize is the most widely produced crop in South Africa and readily available (AFMA, 2017; FAO, 2018), it is commonly included in concentrates fed as a supplement to grazing dairy cows (Meeske *et al.*, 2006). When milk production is high (± 25 kg milk/d) and more than 200 g of maize/kg of diet is being fed to meet the cows ME requirements the amino acid (AA) lysine (Lys) may become the next factor limiting the cows performance (Beever & Siddons, 1986; NRC, 2001; Kolver, 2003). This is because maize is low in crude protein (CP) and the AA profile is not ideal, especially for Lys (NRC, 2001; CVB, 2018). In a systematic review of the literature, Robinson (2010) reported that the Lys concentration in the duodenal digesta decreases when dairy cows are fed rations containing high levels of maize. Additionally, Kolver *et al*. (1999) concluded that methionine (Met) appears to limit cow performance across a range of pasture types and levels of intake in both early-and mid-lactation. The latter argument is supported by various authors, who indicated that the Met content in duodenal digesta flowing to the small intestine (SI) decreases when cows are fed a variety of pasture types (Van Vuuren *et al.*, 1992; Pacheco-Rios *et al.*, 2000; Robinson, 2010). This might lead to inadequate percentages of both Met and Lys in metabolisable protein (MP) flowing to the cow's SI, affecting cow performance, especially high producing multiparous cows in early- and mid-lactation which have higher AA requirements (Socha *et al.*, 2008). Thus, the nutritional factor limiting milk production just shifts from ME being first limiting to MP being first limiting, or more specifically the quality of MP reaching the cows' SI, for cows grazing high-quality pasture, supplemented with a maize-based concentrate.

Providing the grazing dairy cow, supplemented with high levels of a maize-based concentrate, with optimal concentrations of both Lys and Met in MP flowing to the cow's SI, nutritionists often make use of protein supplements high in rumen-undegradable protein (RUP). These protein supplements include soybean meal, animal protein blends, expeller soybean meal, blood meal, feather meal, maize gluten meal, heat-treated rapeseed meal and/or fish meal (Santos *et al.*, 1998; Schroeder & Gagliostro, 2000; Noftsger & St-Pierre, 2003; Danes *et al*., 2013). Several studies in which RUP were increased, or replaced rumen-degradable protein (RDP), in the diet of lactating dairy cows, have yielded inconsistent results and often resulted in an oversupply dietary CP, which could be detrimental to the animal (Susmel *et al.*, 1995; Santos *et al.*, 1998; Danes *et al*., 2013). The oversupply of dietary CP causes excessive rumen ammonia (NH_3) to be produced in the rumen, exceeding the nitrogen (N) requirements of the rumen microbes, therefore affecting microbial crude protein (MCP) production, reducing the amount and quality of MP flowing to the cow's SI (Bach *et al.*, 2005). The excessive amounts NH_3 in the rumen are absorbed into the blood and converted into urea by the liver (ureagenesis) and excreted as urinary N (Tamminga *et al.*, 1995; Bach *et al.*, 2005). This, apart from ureagenesis being an energy-expensive metabolic process, is concerning since dairy cows already have a very low nitrogen use efficiency (NUE). In addition to the low NUE of dairy cows, high-quality pasture is excessively high in CP, especially RDP, and low in ME. This resulting in

large amounts of N not being captured by rumen microbes for the synthesis of MCP and as a result excreted in urine and/or feaces (Dodd *et al*., 2019).

The ability of soils to conserve this surplus of excreted N is limited and consequently the majority of the N is lost through leaching as nitrate (NO_3) or emitted as gaseous N (NH_3, nitric oxide; nitrous oxide and dinitrogen). Nitrous oxide is currently viewed as the most potent greenhouse gas with a global warming potential 298 times higher than that of carbon dioxide (CO_2) over a 100-year period (Solomon *et al*., 2007). As a result of this, nitrous oxide currently represents the most important stratospheric ozone-depleting substance and is expected to remain the largest throughout the 21st century (Ravishankara *et al*., 2009).

Holistically, excessive N excreted from dairy cows influences water and air quality, ecosystem biodiversity, and human health. This could potentially result in aquatic ecosystem eutrophication and hypoxia, increased atmospheric matter, ozone depletion, greenhouse gas emissions and soil acidification. It could also result in the aggravation of some human respiratory diseases and methemoglobinemia in infants (Apelo *et al*., 2014). This along with the cost of dietary N in dairy rations, which represents almost 42 % of the total feed cost (St-Pierre, 2012), place dairy producers under severe social, environmental and economic pressure.

In dairy cow protein and AA nutrition the essential amino acids (EAA), Lys and Met, have consistently been identified as the two most limiting AA, as assessed from response measures of physiological AA levels, milk production, or milk component production in various production systems utilising a variety of different diets (Schwab *et al*., 1992; NRC, 2001; Rulquin *et al*., 2006; Socha *et al*., 2008, Swanepoel, 2009; Swanepoel *et al*., 2016; Whitehous *et al*., 2017; Schwab & Broderick, 2017). Measures include duodenal and ileal AA flows, MCP synthesis and milk N fractions (casein, whey, non-protein nitrogen (NPN)) (Erasmus *et al*., 1994; Lapierre *et al*., 2006; Rulquin *et al*., 2006). It has also been recognised that Lys is the first limiting AA for cows in peak- and early-lactation, but Lys along with Met become co-limiting when cows are in mid-lactation in most feeding systems (Schwab *et al*., 1992). Socha *et al*. (2005) further reported Met and Lys to be co-limiting AA for milk production and tissue anabolism for high producing dairy cows fed a maize-soybean based diet.

According to Schwab (1996), the ideal concentration of Lys and Met for milk protein synthesis in high producing cows as a percentage of MP should be 6.8 and 2.2 %, respectively, reflecting a ratio of Lys to Met of 3:1 as reported by Rulquin (1994) and the NRC (2001). Since this ratio may not be met on unsupplemented pasture alone it could explain, to some degree, why cows grazing pasture produce lower milk volume and milk protein content, or milk protein percentages than cows on a TMR system. The metabolic requirements for specific AA of high producing dairy cows are also higher than those supplied by microbial protein alone (Chalupa, 1975; Tedeschi *et al*., 2015; Lapierre *et al*. 2016; Sok *et al*., 2017; Tedeschi et al., 2017; Van Amburgh *et al*., 2017). The literature demonstrates that the balance of Met and Lys in MP reaching the grazing cows SI might be a factor limiting cow performance.

It is a major challenge for nutritionist to reach even 90 % of the estimated Lys and Met requirements, specifically the ratio of Lys:Met, of lactating dairy cows when feeding high quality protein sources alone without oversupplying dietary CP and metabolically unnecessary AA (Younge *et al.*, 2000). Therefore, it is hypothesised that an extra individual supply of post-ruminal Met and/or Lys to cows grazing high-quality ryegrass pasture while being supplemented with high levels of maize-based concentrate, will have a positive effect on cow performance.

There is a lack of literature support and experimental data available on how the quality of protein supplied from pasture and other protein sources, influences the production performance of grazing dairy cows. This is particularly true regarding the supplementation of rumen protected Met (RPM) and/or rumen protected Lys (RPL). Although, some attempts have been made to identify AA which are most likely to limit the performance of grazing dairy cows (Van Vuuren *et al.*, 1992; Kolver *et al.*, 1999; Pacheco-Rios *et al.*, 2000). Additionally, some dairy farmers also have a perception that the quality of dietary protein is not important for dairy cows.

Several studies have been conducted to determine the effects of Met and/or Lys supplementation upon cow performance with variable responses reported (Schwab *et al.*, 1976; Van Houtert, 1997; Robinson *et al.*, 1998; Pacheco-Rios *et al.*, 1999; Younge *et al.*, 2000; NRC, 2001; Swanepoel, 2009; Doepel & Lapierre, 2010, Wang *et al.*, 2010; Schwab & Broderick, 2017). In most of these studies TMR's have been fed, using different types of preserved forages and different ratios of forage to concentrate as opposed to grazed pasture (Tylutki *et al.*, 2008).

The NUE and performance of grazing dairy cows can be improved in the future by feeding diets lower in dietary CP, therefore N, this requires higher metabolic efficiency of dietary N (NRC, 2001; Kaufman e*t al*., 2018). Achieving this, cow requirements for CP must be met, taking into account the seasonal variability of the pasture-based systems. Firstly, the RDP to meet microbial demand for rumen NH_3 must be met. Secondly, optimal levels of ME to optimise MCP production and utilization of RDP must be supplied. Thirdly, the level of RUP to meet, but not exceed, the cows' MP requirements must be met. Lastly the EAA profile, specifically Met and Lys, of the MP reaching the cows SI should be optimised.

Pasture-based production systems in the Southern Cape are on the rise and also the demand for milk and milk protein (Coetzee, 2019). This emphasises the need to optimize cow performance in pasture-based production systems. Since high levels of maize are included in concentrates fed to cows on pasture (Meeske *et al.*, 2006), it may be vital to investigate whether other nutrients, beyond ME, are liming the performance of gazing dairy cows.

The industry is rapidly developing more robust and complex nutritional models for optimising dairy cow protein and AA nutrition. These models attempt to predict dietary supplies and requirements (i.e. rumen NH_3, AA for ruminal fermentation, peptides, and AA available for intestinal absorption) for dairy diets, which could improve current nutritional guidelines, improving the dietary CP feeding standards for

lactating dairy cows (Tedeschi *et al.*, 2015; Van Amburgh *et al*., 2015). However, these mechanistic types of models still need to be developed for degradable N and post ruminal AA absorption (Roche *et al.*, 2017).

In Chapter 2 a literature review of the available experimental data and literature on protein and AA nutrition of dairy cows in relation to pasture-based production systems in support of this study was undertaken. The aim of this study was to evaluate the effect of RPM and/or RPL supplementation on milk production and composition, milk N fractions, body weight (BW), body condition score (BCS) and plasma AA concentrations of high producing Jersey cows grazing ryegrass pastures during spring when fed high levels of a maize-based concentrate.

Limited data is available for high producing Jersey cows on pasture, especially with regards to RPAA supplementation, therefore the performance results obtained in this study was evaluated and compared against a current prediction model to support and aid in the interpretation of the findings.

Additionally, during the study an alternative method for estimating rumen MCP flow was attempted based on the concentration of allantoin (AL) in spot urine samples collected from the cows used in this study.

Chapter 2: Literature review:
Protein and amino acid nutrition of grazing dairy cows

2.1 Introduction

Dietary protein is most commonly referred to as CP, which is estimated by multiplying the N content of feedstuff with a factor of 6.25 (NRC, 2001). Since protein usually contains a fairly constant 16 % N. However, this estimated CP value comprises of both protein and NPN and varies to a large extent between different feedstuffs (Krishnamoorthy *et al.*, 1982). Apart from the varying dietary CP concertations and constituents, ruminal CP degradation, digestibility and AA composition could also vary considerably between different dietary sources, especially the NPN content which is rapidly, and to a large extent, degraded in the rumen (Bach *et al.*, 2005).

The chemistry of protein and AA in ruminant feeds appears to be well understood, as well as the mechanisms involved in ruminal protein degradation by rumen microbes (Broderick *et al.*, 1991; Clark *et al.*, 1992; Stern *et al.*, 1994; Schwab *et al.*, 2003; Schwab & Broderick, 2017; Savari *et al.*, 2018). Ruminant animals and their microbial processes have unique abilities. Ruminants can transform lower quality forages, protein sources and even NPN (i.e. urea and ammonium salts) into high-quality products (i.e. milk, meat and fibre) for various consumer. Dietary protein consumed by ruminants can be divided into two broad classes which include RDP and RUP (NRC, 2001). The RDP portion, representing both NPN and true protein N, is mainly degraded by rumen microbes and their proteases and is mostly utilised for the synthesis of MCP. On the other hand the RUP portion bypasses ruminal degradation to be potentially digested, or hydrolysed, by enzyme proteases in the SI into smaller polypeptide chains (Bach *et al.*, 2005). The peptides resulting from extracellular rumen proteolytic activity could further be degraded into AA by peptidases and incorporated along with the already present AA into MCP or deaminated further into volatile fatty acids (VFA), CO_2 and NH_3 (Tamminga, 1979). This evokes challenges, due to the fact that quantitatively and qualitatively the dietary AA profile consumed by the ruminant does not reflect the same AA profile leaving the rumen and reaching the cows SI (Erasmus *et al.*, 1994). Mainly as a result of extensive ruminal degradation by rumen microorganisms which are also dependent on, and vary due to, dietary characteristics (Van Soest, 1994).

All proteins consist of AA that are linked by dipeptide bonds to form polypeptide chains with a specific AA sequence, this sequence of AA determines the functionality of the proteins, as provided by the organism genetic blueprint (Bequette *et al.*, 1998). These functions include the synthesis of enzymes, hormones, immunoglobins, muscle and milk proteins, indicating the important role AA plays in terms of maintenance, growth, production and reproduction of dairy cows (Bequette & Nelson, 2006).

Protein and AA nutrition of dairy cows have evolved a lot over the last decades. In 1917, Henry & Morrison (1917), as cited by Schwab & Broderick (2017), already reported that protein quality varies due to the AA composition, although the contribution of microbial protein to the AA pool and animal requirements at that time were still poorly understood (Schwab & Broderick, 2017). Discoveries that played a significant role in protein and AA nutrition were that CP vary in characteristics (i.e. degradability and digestibility), including NPN constituents. These NPN constituents were found to vary highly in forage CP and are composed of low molecular weight compounds including amines (i.e. histamine), amides (i.e. asparagine, glutamine, urea, and uric acid), nucleic acids, NH_3, peptides and free AA (Krishnamoorthy *et al.*, 1982; Van Soest, 1994; NRC, 2001; Bach *et al.*, 2005). However, two important observations made by researchers had a large impact on protein and AA nutrition of dairy cows. The first observation was that MCP supply as a proportion of the total CP required by lactating dairy cows decrease as milk production increases (Chalupa, 1975). The second was that larger amounts of dietary CP need to escape ruminal degradation (i.e. RUP) to meet the MP demand of the cow (Santos *et al.*, 1998). As a result of these observation more focus was placed on dietary CP quality and the determination thereof, specifically the RUP and RDP fractions. This led to the migration away from the ‘‘CP system’’ towards the ‘‘MP system’’ as a denominator and the beginning to balance lactating dairy cow diets for rumen NH_3, peptides and intestinally absorbable EAA (NRC, 2001; Bach *et al.*, 2005; Tedeschi *et al.*, 2015).

The chemical composition, thus nutritional value, of forages changes as the season progresses. This change in nutrient composition (i.e. protein, carbohydrates, minerals and vitamins), thus also the ME density are influenced by the plants vegetative stage, time of day, soil fertility, N fertilisation and soil moisture content (Fulkerson *et al.*, 1998). Forages also vary to a large degree in their protein and NPN proportions, rate and extent of ruminal degradation, digestion in the SI and the AA proportions reaching the cow’s SI (Pacheco & Waghorn, 2008).

Ingested feed proteins are mostly used for protein synthesis, protein turnover in the mature animal, and for bodily proteins, with only a small fraction being deaminated for gluconeogenesis (Lapierre *et al.*, 2007). Some of the amino groups could also be used for *de novo* synthesis of other AA which are deficient or catabolised (oxidized) as an energy source to CO_2 (Schwab & Ordway, 2001; Bach *et al.*, 2005; Lapierre *et al.*, 2006). Since Lys and Met are regarded as the most limiting AA for milk production in ruminants (Schwab *et al.*, 1992), it is common practice to use protein sources high in RUP to achieve the ideal Lys to Met ratio (3:1) levels in MP reaching the SI (Rulquin, 1993; Santos *et al.*, 1998; NRC, 2001). By improving the provision of post-ruminal digestibility and/or sequentially proving AA that closely resembles that of, required for, milk protein synthesis (Noftsger & St-Pierre, 2003). Supplementing increasing amounts of RUP in dairy diets have shown to increase cow performance (Schroeder & Gagliostro, 2000; Malleson; 2008), but mostly results are inconsistent (Santos *et al.*, 1998). If the amount RUP substituted exceeds 30 to 40 % of total dietary CP, RDP may become limiting, which could result in a decrease in MCP synthesis, and non-ammonia nitrogen (NAN) flowing to the SI decreases (Santos *et al.*, 1998). Increasing RUP further

could result a reduction of diet fermentability, cow DMI and subsequently milk yield. This suggest that quantitatively the supply of RUP plays a smaller role than qualitatively, in that the AA profile of the RUP supplied is more important for milk production. As a result, it is common in practice to oversupply CP to meet the cow's AA requirements by simply balancing for RDP and RUP without taking the AA profile of the RUP into consideration. Oversupplying metabolically unnecessary AA result in deamination of the AA which is an energetically inefficient process. It has been suggested by some (Clark *et al.*, 1992; Doepel & Lapierre, 2010), that the AA profile of the RUP supplied should complement that of MCP, since MCP is considered a high-quality protein source (Sok *et al.*, 2017).

One of the major problems associated with protein nutrition of ruminants is the low NUE, for example, dairy cows secrete only approximately 250 to 350 g/kg of N ingested as milk protein (Chase, 1994, Bequette *et al.*, 1998; Dewhurst *et al.*, 2000). This is in agreement with Huhtanen & Hristov (2009) who reported in a meta-analysis that the milk NUE of lactating dairy cows might vary from 14 to 45 %. Thus, almost 70 to 80 % of ingested N is excreted from dairy cows. The low NUE observed in dairy cows may be as a result of various factors which include limiting dietary ME, reduced ruminal microbial growth, catabolism and repartitioning of specific AA, inadequate supply of AA to the SI and/or genetic constraints (Bequette *et al.*, 1998). It has also been reported in previous papers, that the NUE of lactating dairy cows decrease as days in milk (DIM) progress, thus as milk production decline naturally trough lactation the proportion of N excreted as either faecal or urinary N increases (Castillo *et al.*, 2000). This highlights the need to evaluate protein and AA supply in the range of mid-lactation not just in peak-or early-lactation. Yan *et al*. (2006) reported that the NUE of lactating dairy cows decrease with 0.67 points for every 0.1 % incremental supply of dietary CP in excess of cow requirements.

Nitrogen not retained for maintenance, reproduction, health or production and which is excreted in feaces and urine mainly consists of undigested and/or unabsorbed dietary CP and metabolites of different metabolic processes and are correlated with the level of dietary CP (Broderick, 2003). Faecal N includes both undigested microbial protein, and endogenous N. Endogenous N represent 18 to 31 % of faecal N and includes undigested protein secreted along the gastrointestinal tract (GIT), sloughed mucosal cells, and recycled urea entering the hindgut (Tamminga *et al.*, 1995). Urinary excretion of N, unlike faecal N, is highly correlated with N intake (Bequette *et al.*, 1998; Bannink *et al*., 1999).

Improving the NUE, thus CP, and performance of dairy cows, requires diets to be balanced for specific AA requirements of the lactating dairy cow. Balancing for specific AA in MP may allow for the use of diets lower in CP. Reducing the CP in dairy diets and supplying a more balanced duodenal flow of AA would reduce the metabolic cost associated with the deamination of excess AA, and excretion of excess N. The reduced proportion of CP in the diet could potentially leave space to supply more ME, including a reduction in feed cost, which may improve cow performance and farm profitability. Especially considering cows grazing pasture which are often limited in ME. Ruminant feeds which are by-products from various industries (processing), which are low in Met and Lys, could also be better utilised. Achieving an optimal

rumen efficiency and production performance while feeding minimum levels of dietary CP highlights the main goal and challenges for optimising production efficiency of dairy cows as set out by the NRC (2001) and highlighted by Schwab & Broderick, 2017.

2.2 Nutrition of grazing dairy cows: kikuyu/ryegrass pasture as feed

Pasture-based production systems are mainly found within regions where climatic characteristics support optimal forage growth. These production systems incorporate pasture in a variety of feeding systems either as a pure pasture, pasture plus a partial mixed ration (PMR) or pasture plus concentrate. Forages used to make up these pastures within different feeding systems may be further intergraded as a monoculture or a mixture of different forage species depending on the seasonal growth pattern and climatic conditions (Neal *et al.*, 2007). As mentioned, the forage species primarily utilised within these systems comprise of kikuyu, Westerwolds ryegrass, Italian ryegrass and/or perennial ryegrass.

Kikuyu is a subtropical (C_4) grass species that is highly productive, persistent, respond well to fertilisation and irrigation and is resilient to trampling (Fulkerson & Lowe, 2003). Less desirable characteristics of kikuyu include aggressive colonising behaviour resembling that of weed, low nutritive value compared to other perennial grasses, mineral imbalances, animal poisoning, and a high degree of seasonality in terms of DM production and nutritive value (Fulkerson *et al.*, 1998; Marais, 2001; Fulkerson & Lowe, 2003). The nutritive value of kikuyu is driven by its unique morphological, physiological and chemical characteristics and varies in relation to growth stage, cultivar, and environmental conditions (i.e. temperature and precipitation) (Marais, 2001; García *et al*., 2014). Kikuyu comprises the greater part of summer and autumn irrigated pastures in the Southern Cape providing most or all of the DM to the cows during that period (Botha *et al.*, 2008). Thus, playing a fundamental role in the fodder-flow planning and profitability of pasture-based dairy systems within these regions. If well managed, high DM yields could be achieved from kikuyu pasture producing almost double the annual DM compared to some temperate species, subsequently supporting higher stocking rates (Reeves, 1997; Botha *et al.*, 2008). According to Gherbin *et al*. (2007) a well-established and managed kikuyu pasture can achieve a potential annual DM production of up to 28.2 t/ha, although 12 to 14 t DM/ha of utilisable pasture is usually expected and more realistic (Van der Colf, 2010; García *et al.*, 2014). Kikuyu, which is highly influenced and dictated by temperature, grows optimally at an atmospheric temperature between 16 to 21 °C (Russel & Webb, 1976), with a maximum growth rate observed at 25 °C (Murtagh *et al.*, 1987). A decline in the growth of kikuyu is observed outside the range of 10 to 40 °C (Ivory & Whiteman, 1978). In the Southern Cape, an increase in the growth rate of kikuyu is marked by the onset of late spring (November) through to late summer (February), as atmospheric temperature tend to be higher (Botha *et al.*, 2008). During this period the soil water content is relatively low, but kikuyu is relatively tolerant to water stress. This tolerance of kikuyu makes this grass species favourable under variable South African climatic conditions, and are mostly

attributed to the relatively deep root system that kikuyu develops in well-drained soils (Marais, 2001). As the seasonal growth pattern of kikuyu pasture progresses, along with the inherent characteristics and deficiencies of this grass species, a drop in milk production (l/d) could be observed by as much as 38 % from December through to May (Henning *et al.*, 1995). This reported drop in daily milk yield is accompanied by a 34 % drop in organic matter (OM) digestibility, hence a reduced DMI. This trend observed is often referred to as an "autumn slump" (Henning *et al.*, 1995; Van der Colf, 2010). As the season progresses further and soil temperatures drop below the average minimum soil temperature of 18 °C for kikuyu the growth can be reduced in terms of DM by up to 11 kg/ha/d (Whitney, 1974), Which is similar to the drop in DM production reported by Van der Colf *et al*. (2011) for kikuyu pasture over-sown with ryegrass as the seasons progress from summer to winter. According to Bell *et al*. (2013), the rate of photosynthesis of kikuyu pasture drops from 2.0 to 1.1 mg $CO_2/m^2.s$ when the atmospheric temperature drops from 25 °C to below 18 °C. As a result, kikuyu may be dormant or have a very low DM production in winter and spring, resulting in a shortage of DM production (Marais, 2001). As the average daily maximum and minimum temperature for the Southern Cape are reported to be on average 24 and 15 °C during summer, 22 and 12 °C during autumn, 19 and 7 °C during winter and 20 and 11 °C during spring, respectively (ARC, 2018), it is evident that there might be a winter-spring shortage in DM supplied from kikuyu pasture.

To overcome this DM shortage, the strategic incorporation of temperate (C_3) grass species either as pure swards, mixtures or over-sown into perennial pastures (i.e. kikuyu) are frequently used. This increases the seasonal DM production and distribution along with the nutritive value of the pasture (Botha, 2003, García *et al.*, 2014), subsequently increasing milk production per cow. Annual Italian ryegrass is among the temperate forage species planted for winter and spring grazing in the Southern Cape, due to the atmospheric temperatures supporting optimal growth of temperate forage species during this period (Fulkerson *et al.*, 1993; Van der Colf *et al*., 2015a). Pasture-based production systems utilising ryegrass are seasonal in DM production with the highest production occurring during spring, followed by winter, and the lowest production during autumn and summer (Botha *et al.*, 2008; Van der Colf *et al.*, 2015a).

Ryegrass is high in CP, N degradability, soluble protein content and DM digestibility while it is low in non-fibre carbohydrates (NFC), physically effective neutral detergent fibre (peNDF) and ME (Kellaway & Porta, 1993; Bargo *et al.*, 2003; Botha *et al.*, 2008; Meeske *et al.*, 2009). The chemical composition of ryegrass during spring as reported by various authors is shown in Table 2.1. These values are similar to the values reported in literature review of ryegrass by Hopkins (2003). Meeske *et al*. (2006), Malleson (2008) and Roche (2017) further reported that ryegrass is low in protein quality, being low in RUP and high in RDP, which is also supported by Higgs *et al*. (2015). This may not supply the cow with the optimum ratio of 2.5:1 for RDP and RUP as recommended by the NRC (2001) for a small breed cows (i.e. Jersey) in mid-lactation producing 20 kg milk/d. Ryegrass also has a poor AA profile, which is shown by the insufficient level of Met in the total EAA content flowing to the duodenum of cows grazing ryegrass pastures (Van

Vuuren *et al.*, 1992; Van Vuuren *et al.*, 1993; Kolver *et al.*, 1999; Pacheco-Rios *et al.*, 2000). The inadequate percentage of Met in MP flowing to the duodenum could negatively effect cow performance, especially high producing cows in early-and mid-lactation that have higher AA requirements (Socha *et al.*, 2008).

Table 2.1 Summary of the nutrient composition of ryegrass pasture during spring as reported by various authors

Authors	**Nutrient composition[1] (% DM or as stated)**								
	DM	**OM**	**CP**	**NDF**	**ADF**	***IV*OMD**	**ME**	**Ca**	**P**
Fulkerson *et al.* (1998)	-	-	-	-	-	-	-	0.59	0.31
Lowe *et al.* (1999)	-	-	25.5	46.2	27.6	-	9.45	-	-
Meeske *et al.* (2006)	14.7	-	18.0	49.0	28.0	-	10.9	0.67	0.36
Fulkerson *et al.* (2006)	-	-	22.3	44.4	22.1	78.3	11.3	-	-
Fulkerson *et al.* (2007)	-	-	24.7	53.1	27.7	-	9.7	-	-
Botha *et al.* (2008)	-	-	21.8	50.1	-		11.3	0.47	0.48
Malleson (2008)	12.5	87.0	25.4	44.5	27.5	80.0	11.2	0.49	0.38
Meeske *et al.* (2009)	13.1	-	21.1	47.1	31.4	-	-	0.55	0.45
Erasmus *et al.* (2010)	12.8	-	22.6	45.2	27.3	-	10.6	0.45	0.39
Coetzee (2011)	15.5	-	23.3	51.2	30.5	76.1	10.8	0.40	0.37
Joubert (2012)	12.7	87.2	29.8	47.0	34.5	83.7	11.8	0.41	0.44
Muller (2012)	12.4	88.6	23.3	47.2	28.5	82.0	11.4	0.37	0.37
Van Wyngaard *et al.* (2013)	12.9	89.4	21.5	49.4	30.2	80.2	11.5	0.38	0.34
Steyn *et al.* (2014)	13.9	88.4	23.8	41.1	24.7	82.5	12.2	-	-
Van der Colf *et al.* (2015b)	-	-	22.7	45.9	-	-	11.0	0.41	0.39
Moller *et al.* (2016)	13.5	88.1	24.6	49.4	25.5	82.2	11.2	0.4	0.4
Clark *et al.* (2018)	-	87.7	18.4	48.2	22.5	73.9	-	-	-
Van der Vyver (2019)	16.4	89.7	19.4	43.7	27.6	-	-	0.46	0.41
Douglas (2020)	-	-	20.6	42.6	24.5	-	11.2	0.48	0.43

[1]DM – Dry matter; OM – Organic matter; CP – Crude protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; *IV*OMD – *in vitro* organic matter digestibility; ME – Metabolisable energy (MJ/kg DM); Ca – Calcium; P – Phosphorous

Even though intensively managed pastures can be of high quality as shown in Table 2.1, the large difference in production and performance between grazing dairy cows and those fed a TMR in milk production is often considered proof of the nutritional deficiency and inadequacies of pasture which limits milk production (Hills *et al.*, 2015). Milk yields from cows grazing pasture, as opposed to a TMR, without supplementation could be up to 30 % less in terms of milk production, in addition to milk components production which are also reported to be lower in fat and protein content (Fulkerson & Trevaskis, 1997; Kolver *et al.*, 2000; Bargo *et al.*, 2002). Pasture fed cows also tend to lose more, or gain less, weight and

body condition during lactation, than cow fed a TMR (Kolver & Muller, 1998). The lower milk production and milk component yield can further be attributed to greater physical energy expenditure and lower intake capacity of grazing dairy cows (NRC, 2001; Kolver, 2003). Cows grazing pasture require 10 to 30 % more energy than non-grazing cows mainly as a result of higher physical requirements (NRC, 2001; Kaufman *et al*., 2018; Tedeschi & Fox, 2015). Kolver & Muller (1998) reported a reduced milk production of 14.5 l/cow/d when high producing dairy cows were fed high quality pasture compared to a TMR. With 61 % of this reduction in milk production attributed to a lower DMI, 24 % attributed to the daily energy expenditure for grazing and walking, 12 % attributed to the excretion of surplus N, 7 % reflected the greater energy output of milk and 5 % due to the differences in the partitioning of energy between milk production and BCS. Their study also indicated that high producing dairy cows on pasture require supplemental energy to optimally exploit their genetic potential for milk production. Similarly, Bargo *et al.* (2002) investigated the effects three different feeding systems, combining pasture and a TMR or a concentrate, have on the performance of high producing Holstein cows. Milk production in their study was higher for cows fed a TMR compared to the cows fed a pasture plus concentrate diet (38 *vs*. 28 kg/cow/d). The partial TMR (i.e. PMR) and TMR resulted in higher milk component yield for fat and true protein than the pasture plus concentrate diet + 0.20 and + 0.17 %, respectively. Cows on the pasture plus concentrate diet also gained less BW (34 *vs.* 76 kg) and lost more body condition (- 0.20 points) compared to the PMR and TMR which gained 0.01 and 0.19 points, respectively. When comparing a pasture only system with a system combining pasture plus 2.65 kg DM/cow/d of a maize-based concentrate, Mckay *et al*. (2019) observed higher milk production for the pasture plus concentrate group than the pasture only group (16.8 *vs.* 15.1 kg/cow/d; $P < 0.05$), the pasture only group also had a lower milk component yield than PMR group (1.46 *vs.* 1.53 kg/cow/d; $P < 0.05$). Similarly, Reis & Combs (2000) reported lower milk production (21.8 *vs*. 30.4 l/cow/d; $P < 0.05$), milk protein percentages (3.08 *vs*. 3.89 %; $P < 0.05$) and DMI (13.9 *vs*. 19.8 kg/cow/d; $P < 0.05$) when cows were fed only ryegrass pasture compared to ryegrass pasture plus 10 kg/cow/d (as is) of a maize-based concentrate, respectively. However, compared to the pasture only diet the supplementation of a maize-based concentrate decreases milk fat percentages (3.89 *vs*. 3.05 %; $P < 0.05$).

Although pasture is usually considered a cheap source of feed (Peyraud & Delagarde, 2013), the suitability of pasture could vary as a result of the region, cost, quality and availability of alternative feed sources (i.e. crop residues and/or other forages) and the specific seasonal growth pattern of the pasture within that area (Roche *et al*., 2017; McKay *et al*., 2019).

As climatic conditions change with season, the main aim of dairy nutritionists is to supply nutrients in which pasture is limited.

2.2.1 Limiting factors of pasture-based systems

High-quality pasture has many positive nutritional attributes for lactating dairy cows, but possess some inherent nutritional imbalances and deficiencies (Clark *et al*., 1997; Kolver, 2003). Pasture is usually described as young and leafy with a DM content of 13 to 16 %, CP of 18 to 30 % DM, NDF of 41 to 50 % DM, ME of 10 to 12 MJ/kg DM (Table 2.1) and net energy for lactation (NE_L) of 1.53 to 1.67 Mcal/kg DM (Clark & Kanneganti, 1998). However, multiple studies conducted on pasture quality, post ruminal digesta flows and production-focused supplementary feeding studies have facilitated an understanding of the factors most likely to limit the production of grazing dairy cows (Leaver, 1985, Bargo *et al*., 2002; Kolver, 2003; Doyle *et al*., 2006), including two literature reviews, one by Roche *et al*. (2017) and the other by Wilkinson *et al*. (2020). These limitations from pasture include a low ME content, poor RUP content and AA profile, low levels of peNDF and an imbalance of RDP: rumen fermentable carbohydrates. Pasture CP (mainly RDP) and NDF are high, and ME and non-structural carbohydrates (NSC) are low compared to the nutritional recommendation for high producing dairy cows (Leaver, 1985; Carruthers *et al.*, 1997; NRC, 2001; Bargo *et al.*, 2003; Kolver, 2003; Hills *et al.*, 2015; Wilkinson *et al*., 2020). Another factor which is limiting in these production systems is the difficulty associated with accurately quantifying pasture DM intake (PDMI) and pasture quality, especially due to the seasonality and nutrient variability of most pastures species. Various minerals, including calcium, phosphorus, magnesium, sulphur and zinc are also frequently reported to be limiting in pasture-based production systems (Bargo *et al.*, 2003).

Consequently, these limitations may result in lower milk and milk component production and will be discussed in more depth.

2.2.1.1 Metabolisable energy

Metabolisable energy is often reported to be the first nutritional factor liming milk production from pasture (Bargo *et al.*, 2003; Kolver, 2003). Ruminants mainly utilise NSC which comprises of sugars, starches, organic acids and fructans along with structural carbohydrates comprising of cellulose, hemicellulose and lignin which represents the principal components of cell walls as main energy sources (NRC, 2001). These carbohydrate fractions undergo microbial fermentation in the rumen which result in various intermediates such as small saccharides with the end products of fermentation being VFA's. The VFA's provides approximately 60 to 80 % of the energy required by the cows, thus serving as the principle source of ME (Hutjens, 2018). However, on average structural carbohydrates are less digestible than NSC and as a result, there is a negative correlation between the structural carbohydrate content of dairy diets and the dietary energy concentration (NRC, 2001).

The composition of NSC, which is the major source of energy for dairy cows, vary greatly between feedstuffs affecting the rate and extent of ruminal fermentation and as a result the energy value of the

feedstuff (Roche *et al*., 2010). To avoid acidosis, Nocek (1997) suggested the NSC content of the diet should not exceed 30 to 40 % DM and is dependent on various factors. These factors include, 1) the effect of rapidly degradable starch on ruminal fermentation, 2) substitution of NDF for NSC, affecting VFA production, rumination and salivation, 3) site of starch digestion, 4) level of cow DMI, 5) physiological stage of the cow and 6) method of preserving the feedstuff, which alter the rate and extent of NSC digestion (see section 2.2.1.3 for fibre). However, when NFC supplied to the animal is too low (< 25 to 30 % DM), milk production also tend to be low (Roche *et al*., 2010). It is important to note, NSC and NFC cannot be used interchangeably since their concentration in various feedstuffs diffe, mainly as a result of pectins and organic acids which are considered in NFC and not NSC (NRC, 2001). The NRC (2001) reported that for 1 kg increase in NFC a 2.4 kg increase in milk yield could be expected. Considering this correlation between milk yield and NFC and the low content of NFC (15.4 % DM) in ryegrass pasture and since more than 65 % of the diet DM usually comprise of pasture, the energy deficit of cows grazing ryegrass is not surprising (Bargo *et al*., 2003; Steyn *et al.*, 2014; Van Wyngaard & Meeske, 2016). The DM and NSC content in early-and late-season pasture regrowth are low in high-quality ryegrass and as a result cow performance is influenced (Marais *et al.*, 1993). Kolver (2003) reported that a cow grazing high-quality pasture (25 % CP, 43 % NDF and *in vitro* DM digestibility of 77 %) will be limited in ME, rather than protein or AA. The average ME content reported by various authors range from 9.5 to 12.2 (MJ/kg DM), as shown in Table 2.1, when considering the cow's requirements for ME there are times where the cows ME requirements will not be met. According to Erasmus *et al.* (2000) and Peyraud & Delagarde (2013) cows in mid-lactation, as a guideline, need to consume 10.3 to 10.5 MJ ME/kg DM, this is slightly lower than the 11 to 12 MJ ME/kg DM recommended by the NRC (2001). Hutjens (2018) reported that a Jersey cow requires 11.1 MJ ME/kg DM based on values adapted from the NRC (2001). Overton & Case (2011) further recommended that a high producing Jersey cows in early to mid- lactation requires 11.2 to 11.6 MJ ME/kg DM, which is also in line with the recommendation of McDonald *et al*. (2001) for a 450 kg cow producing more than 20 kg/milk/d. The consequence of a low energy diet is that the energy required by the rumen microbes to incorporate rumen NH_3 into MCP is not met and as a result cow performance are lower and excessive amounts of NH_3 is excreted, or recycled through ureagenesis which could further increase the cow's energy deficit. This asynchronous release of energy and rumen NH_3 results in an inefficient utilisation of fermentable substrates and MCP synthesis, indicating the need for grazing dairy cows to be supplemented with ME. In several studies where increasing amounts of readily fermentable carbohydrates were fed in the diet of grazing dairy cows have led to a decrease in rumen NH_3 concentration as a result of an improved N capture by ruminal microbes in both *in vivo* and *in vitro* studies (Santos *et al.*, 1998; Hristov *et al.*, 2019). This is also true for dietary AA supply, if energy is not limiting most of the ingested AA will be transaminated or incorporated directly into MCP, in contrast, when energy is limiting the AA will be deaminated and fermented to VFA and CO_2 (Bach *et al.*, 2005).

Metabolisable energy in ruminant feedstuffs is not determined directly, as it would require the use of respiration chambers in which animals are confined to determine total energy intake and excretion. However, as an indirect method, Robinson *et al.* (2004) reported that the equation ME (MJ/kg DM) = 0.82 × (GE × *IV*OMD) is suitable to determine the ME content of ruminant feedstuffs.

2.2.1.2 Protein

Proteins in forages are primarily found in the stems and leaves and comprise of chloroplastic and cytoplasmic proteins. The cell walls also contribute to the total protein fraction provided by forages through nucleoproteins of the nucleus including extensin proteins, however, the protein contribution from the cell walls is much lower and less soluble (Van Soest, 1994). The N content and chemical composition of pastures are determined by the species, maturity and different constituents (i.e. stem, leaf or inflorescence) of the forage, including the climate, application of fertiliser and management (Pacheco & Waghorn, 2008). High pasture CP content, growth rate and production/ha (DM yield) could be achieved when ryegrass pasture is fertilised with N (Leaver, 1985). The CP response to N fertilisation is nearly linear up to high levels (i.e. 800 kg N/ha/year) of N fertilisation, which corresponds to an average 50 to 90 g CP/kg DM for every additional 100 kg of N applied/ha (Peyraud & Astigarraga, 1998). Changes in production are mainly explained by the increase in green leaf mass/ha of pasture. Pasture CP concentrations under these conditions are high (16 to 25 % DM) and most of the dietary CP is either absorbed from the reticulorumen or excreted as urea in urine with the remainder of CP, estimated 30 % or less, reaching the SI (Van Vuuren *et al.*, 1993). This is in accordance with Beever *et al.* (1976) and Beever & Siddons, (1986) who reported that NPN makes up the largest proportion of total forage CP and contribute little RUP to the ruminant animal. Thus, when dairy cows are fed forages, measures of non-ammonia N (NAN) and Non-microbial nitrogen (i.e. RUP and endogenous N) are often less than 30 % of total N intake (Beever *et al.*, 1976; Pacheco & Waghorn, 2008). The high CP intake (18.0 to 29.8 % DM), high rate of ruminal degradation of CP (70 to 80 %), high NDF (41.1 to 53.1 % DM) and low level of ME (9.5 to 12.2 MJ/kg DM) in high-quality pasture may contribute towards the lower NUE of lactating dairy cows grazing pasture (Table 2.1; Van Vuuren *et al*., 1992; Van Vuuren *et al*., 1993; Pacheco & Waghorn, 2008). This reduced NUE is mainly attributed toward inefficient capture of rumen N as MCP and the metabolic cost associated with the synthesis and excretion of urea (Van Vuuren *et al.*, 1993; Bach *et al*., 2005; Hristov *et al*., 2019). Estimates of the energy associated with the conversion of rumen NH_4 to urea have been reported to be within the range 0.015 to 0.05 MJ/g N consumed by lactating dairy cows (Van Houtert, 1997). Thus, the cost of ureagenesis for 1.2 kg excess dietary protein could potentially require 2.9 to 9.6 MJ more ME, which is equivalent to the energy required by a lactating dairy cow to produce 0.6 to 1.9 kg milk (Van Houtert, 1997). More recently Reed *et al*. (2017) evaluated the energetic cost associated with feeding excess dietary N to lactating dairy cows and reported that feeding excessive N had a large negative effect on the cow's milk gross energy production of

up to 0.22 to 0.28 MJ/g N, the cows also retained 0.018 to 0.028 MJ/g N less energy. See section 2.5 for a review of the literature on the effect that excessive AA supplementation has on the cow's energy requirements.

It has been suggested that a CP content of 16 to 18 % on a DM basis is required to meet the microbial demand for N, only one out of all the CP values reported in Table 2.1 falls close to the lower 18 % CP. Ruminal microorganisms do not utilise high pasture CP adequately, since pasture ME is usually lower than that required to optimise microbial protein synthesis (Kolver & Muller, 1998), as explained in section 2.2.1.1. As a result, maximising ruminal N capture is challenging. In addition, Van Soest *et al*. (1994) reported that under cloudy condition, which is common in temperate environments, N can accumulate in forages since energy is required for photosynthesis for nitrate reductase to be synthesis by the calls. The accumulation of N, under these climatic conditions, can further be exaggerated under high levels of N fertilization (Peyraud & Astigarraga, 1998). Meeske *et al.* (2006) reported annual ryegrass CP values that varied from 14 to 31 % DM, demonstrate the importance of regular CP analysis to make dietary adjustments accordingly to meet the cow's protein requirements. Van der Colf *et al*. (2015a) also reported CP values consistent with these, ranging from 17.9 and 32.5 % DM for kikuyu-ryegrass pasture systems during spring. Lower CP values are also expected when the pasture is poorly fertilised (Peyraud & Astigarraga, 1998). Pasture *in vivo* apparent digestibility of N can be as high as 84 %, indicating the extensive degradation of high-quality pasture (Kolver & Muller, 1998; Pacheco & Waghorn, 2008). The NRC (2001) examined the relationship between RDP as a percent of DM and milk yield, and found a quadratic relationship, with milk yield maximised when RDP equalled 12.2 % of DM. Additionally, this data set revealed a positive correlation between RDP and DMI (a two percentage unit increase in RDP increased DMI by about 1.1 kg/d). In agreement with the NRC (2001) data set, a previous review has suggested that a practical target for RDP for lactating dairy cow diets should be 11 to 12 % DM (Hoover & Miller, 1996). However, when considering RUP, high producing Jersey cows may require up to 720 to 900 g of dietary RUP according to the Table 14-2 of the NRC (2001). Thus it is clear that RUP at times would not meet the cow's requirements, since the RUP content of intensively managed ryegrass pasture may vary from 16 to 35 % CP during spring (NRC, 2001; Waghorn & Clark, 2004; Malleson 2008; Higgs *et al*., 2015; Douglas, 2020). Considering the average CP content of 22 % reported in Table 2.1 for ryegrass pasture during spring the cow will be supplied with a range of 35.2 to 77.0 g RUP/kg PDMI, indicating that the cow's requirements for RUP at times might not be met.

In the past, it was generally thought that pasture supply high levels of AA to grazing dairy cows due to the concentration of protein in pastures being high and the leaf proteins have a relatively good AA profile, however, it is well known that the total N concentration of pastures is an inadequate indicator of metabolisable AA supply (Pacheco & Waghorn, 2008). With regards to the total supply of EAA, the contribution from pasture is small, Rulquin *et al*. (1993) reported that Lys (% EAA) tends to decrease along a decrease in pasture N content, the significance is small as a large proportion of CP coming from pasture

is degraded, which are primarily associated with proteolytic rumen microorganism. As a result, the contribution of MP supply from pasture is very small contributing only 3 to 5 % of the total MP supply to the cow (Beever & Siddons, 1986). However, more recent data suggest higher values ranging from 18 to 44 % (Chaves *et al*., 2006; Higgs *et al*., 2015)

2.2.1.3 Effective fibre

Crude fibre, acid detergent fibre (ADF) and NDF are the most common measures used to quantify the fibre content of feedstuffs (NRC, 2001). Neutral detergent fibre (i.e. cellulose, hemicellulose and lignin) measures most of the structural components of forages and is the best measure to separate structural carbohydrates from NFC. The NDF content in the cow's diet is important for stimulating chewing, rumination, maintain rumen pH, and increases the acetate:propionate ratio, maintaining milk fat content and avoiding metabolic disorders (Verbic, 2002). The NDF content of ryegrass pasture is frequently reported to be within the range of 41 to 53 % DM (Table 2.1), although, only 40 to 50 % of fibre in high-quality pasture may adequately stimulate rumination (De Veth & Kolver, 2001). Thus, pasture peNDF (fibre that stimulates rumination) may be too low for cows grazing young lush pasture which lowers the rumen pH, lower than optimum, resulting in ruminal acidosis, which may cause milk fat depression and a reduction in MCP synthesis (Fulkerson *et al.*, 2007). The pastures peNDF are related to the physical characteristics of the NDF content and are inversely related to rumen pH (NRC, 2001). Ryegrass may also vary in fibre content from being excessive after seed set and/or when the soil moisture content is low during summer months (Delagarde *et al.*, 2000) to deficient between May and August, up to early November, when in a vegetative state (Fulkerson *et al.*, 1998). The peNDF of pasture may range from 17 to 78 % with a mean of 43 %. Diets with a total NDF content of less than 25 % DM with less than 16 % NDF DM coming from forages may also cause milk fat depression in dairy cows (Clark & Armentano, 1993). The already low peNDF provided from pasture may be further exacerbated when a highly fermentable energy source (i.e. maize) is included in concentrates to meet the cows ME requirements (Zebeli *et al*., 2012).

2.2.1.4 Pasture intake, substitution and allowance

Kolver & Muller (1998) reported that the DMI of dairy cows grazing high-quality pasture, as opposed to pasture ME content, per se, is the major factor liming the energy supply from pasture. Because the nutritional value (i.e. CP, AA, carbohydrates and minerals) of high-quality pasture has the potential to sustain relatively high production (Roche *et al*., 2017). However, higher producing cows have a larger milk production relative to intake capacity (Fulkerson & Trevaskis, 1997; Kolver, 2003) and therefore DMI might limit milk production (Leaver, 1985; Beever & Siddons, 1986). Pasture DMI is described as a function of grazing time, biting rate and bite mass (Bargo *et al.*, 2003), and are regulated by complex neuro-

endocrine systems integrating both physical (i.e. distention of the alimentary tract) and metabolic signals (i.e. nutrient requirements). These signals are then sent to the hypothalamus stimulating either the satiety centre or the desire to eat (Hodgson & Brookes, 1999) and are associated with the reticulo-rumen capacity, rate of forage digestion and passage, forage moisture content and grazing time (Bargo *et al.*, 2003).

High producing dairy cows, fed high quality-pasture, will typically consume approximately 3 % to 4 of their BW as pasture DM (Kolver & Muller, 1998). Leaver (1985) proposed that cows grazing pasture and are high producing may achieve a total DMI of 3.25 % BW as pasture DM. If there are no quantitative or qualitative restrictions from the grazed pasture, Mayne & Wright (1988) suggested, that the high producing dairy cow's DMI might be as high as 3.5 % BW. Total DMI for cows grazing ryegrass pasture are frequently reported to be between 3 to 4 % of BW (Fulkerson *et al*., 2006). However, estimating PDMI accurately is very important, but difficult, in pasture-based production systems as opposed to confinement systems (i.e. TMR), because DMI of pasture-based systems cannot be determined directly (Bargo *et al.*, 2003). Apart from the afore mentioned prediction equations, other methods used to quantify DMI are classified as either animal- or pasture-based. Animal-based methods are based on the ratio between the diets digestibility and faecal production, which are determined with different marker methodologies (i.e. chromium oxide (Cr_2O_3) or n-alkanes) (Reeves, 1997; Peyraud & Astigarraga; 1998, Bargo *et al.*, 2002; Fulkerson *et al.*, 2005). These methods are laborious and with unknown accuracies and as a result, pasture-based techniques are usually favoured and more routinely used (Bargo *et al.*, 2003). Pasture-based methods, for determining PDMI of grazing dairy cows, require the measurement of pre-and post-grazing pasture DM yield and then by means of the difference between the two measurements the PDMI could be determined (Stockdale & King, 1983). The main disadvantages of pasture-based methods are the variation of pasture intake measured between the different methods, and the fact that individual cow DMI is estimated from a group of cows grazing the pasture rather than individual cow intake (Reeves, 1997).

Stakelum (1993) suggested that PDMI/d will increase from 0.4 to 0.5 kg for every 1 kg increase in milk production between the range of 15 to 30 kg milk/d, after which the marginal intake response to milk production tends to plateau (McGilloway & Mayne, 1996). Van Vuuren *et al.* (1992) reported that cow DMI of highly fertilised spring pasture decreased from 16.8 to 13.3 kg/d when fertilisation increased from 250 to 500 kg N/ha/year. This was partly due to the pasture DM content dropping from 22 to 14 % DM. Voluntary PDMI and pasture moisture content are negatively correlated and are applicable at all stages of the plants maturity and a wide range of DM (12 to 25 %), according to John & Ulyatt (1987). Apart from PDMI, as a limitation, pasture-based systems usually incorporate supplementary feeding (i.e. concentrates) in their respective feeding systems, which may cause a substitution effect of pasture (Bargo, 2003).

The term substitution rate (SR) refers to the effect that supplementation has on PDMI, which usually decreases as supplementation increases, accounting for most of the observed variation in milk response to supplementation (Stockdale, 2000). Substitution rate is calculated with the following equation: SR (kg/kg) = [(PDMI unsupplemented - PDMI supplemented)/DMI of supplement]. Causes of substitution are mainly

attributed to negative associative effects in the rumen (i.e. rate of digestion) and reduced grazing time observed (McGilloway & Mayne 1996; Dixon & Stockdale, 1999). There is a negative relationship between SR and milk production response, thus the higher the SR the lower the milk response to supplementation (Stockdale, 2000; Bargo *et al.*, 2003). As a result of this relationship, factors affecting both SR and milk response were reviewed together. Because, these response variables (i.e. SR and milk response) are influenced by various relating factors. These factors include pasture-related factors (i.e. pasture allowance (PA; amount of pasture DM offered per day as kg DM/cow/d), pasture height, pasture yield and the quality of the pasture), supplement-related factors (i.e. level and chemical composition of the supplement) and animal-related factors (i.e. genetic potential, DIM and milk and milk component production), respectively (Faverdin *et al.*, 1991; Bargo *et al.*, 2003).

Bargo *et al*. (2002) reported that DMI of high producing cows grazing pasture increased 2.9 % (17.7 to 20.5 kg/d) as PA increased from 25 to 40 kg DM/cow/d, since PA and PDMI are closely correlated (Moate *et al.*, 1999). However, Hodgson & Brookes (1999) reported that PDMI increases along with PA, at a declining rate, and plateaus when PA reaches 10 to 12 % BW for a large breed of dairy cow (i.e. Holstein). Delaby *et al*. (2001) reported that when PA above 5 cm cutting height increased from 12.9 to 15 kg/cow/d, PDMI increased from 11.3 to 13 kg DM/cow/d. Additionally, increasing PA from 16.5 to 21 kg DM/cow/d increased PDMI from 13 to 15 kg/cow/d. The problem associated with to high PA is that cows tend to select higher quality leaf material and as a result the cows may consume a diet higher in nutritional value than expected (Stakelum, 1986). This could result in a pasture quality deterioration as the season progresses, due to increasing residual pasture height. Too high PA also results in reduced stocking rates and efficiency of utilisation, therefore reducing profitability per hectare (Bargo *et al.*, 2003).

Even though it is evident that PDMI is closely correlated and increases along with PA (Moate *et al.*, 1999), SR also tend to increase along with PA (Stakelum, 1986), decreasing the milk production response for cows grazing pasture and supplemented with increasing levels of concentrate (Clark & Kanneganti, 1998). Thus, when cows are supplemented on a lower PA, total DMI achieved by the cows could be increased. Bargo *et al*. (2003) suggested that an optimum PA is 3 to 5 times the total DMI that will maximise PDMI. However, due to the deterioration and reduced utilisation of pasture at too high PA, Bargo *et al*. (2003) recommended a PA twice the expected PDMI. Peyraud & Delagarde (2013) suggested a PA of 90 % of a cows voluntary DMI may be adequate to optimally utilise the pasture without losing milk production. The recommended PA and PDMI across studies may cause confusion due to various methods used to quantify the pasture DM yield. When using the rising plate meter (RP_{meter}) to determine pasture DM yield, inconsistencies could arise between studies due to different cutting heights (i.e. ground levels, above 3 cm or above 5 cm), including the regression equations used. Refer to Chapter 3, section 3.7, subsection 3.7.1 for an in depth discussion on the method used in this study to quantify pasture DM yield, PDMI and PA.

The SR per kg of concentrate fed might vary from 0.4 to 1.0 kg (Bargo *et al*., 2003). Since the amount of concentrate fed to dairy cows, independent of roughage type, incrementally increases the SR and also

tend to systematically increase with 0.093 kg/kg concentrate supplemented (Faverdin *et al.*, 1991). As a result, at high SR the milk yield response and pasture utilisation tend to be lower (Peyraud, 2001).

Conclusion

Pasture alone does not have an adequate nutritional composition, under SA conditions, to meet the nutritional requirements of high-producing dairy cows as shown in section 2.2, Table 2.1 and section 2.2.1 These sections explained the nutritional limitations of pasture-based productions systems in depth.

The seasonality of pasture quality and quantity along with the difficulty associated with accurately quantifying cow PDMI challenges the dairy nutritionist to accurately meet the nutrient requirements of grazing dairy cows. Therefore, the inclusion of feeding systems, or feeding strategies, which combine pasture with a concentrate or preserved forages are often used to improve the efficiency of utilization of the pasture, cow performance and farm profitability.

2.2.2 Improving cow efficiency on pasture

Supplementation of grazing dairy cows are commonly used to supply cyclic nutritional aid (i.e. ME, RUP and various minerals), with the main objectives being to increase total DM and energy intake relative to what is achieved when cows are grazing pasture only diets to optimise the pasture utilisation and profit per cow/ha (Stockdale, 2000; Peyraud & Delaby, 2001; Hills *et al.*, 2015). Further objectives of supplementation as summarised by Kellaway & Porta (1993) include, 1) increased cow milk production, 2) increased stocking rate and cow performance per/ha, 3) maintain adequate cow BW and BCS, 4) increased lactation length, and 5) maintain optimal levels of milk and milk components production during pasture shortages.

Supplementation of dairy cows on pasture has been reviewed and studied in depth by various authors to determine the effects on pasture intake, milk production, milk composition and ruminal and post-ruminal digestion (Leaver, 1985; Stockdale, 2000; Bargo *et al.*, 2003; Meeske *et al.*, 2006). To meet the objectives, as stated, by implementing an effective feeding strategy, a clear understanding of the nutritional and managerial factors mitigating supplementation is required. As several factors, including the diminishing return of supplementary feeding, decreased PDMI due to the substitution effect and decreased utilisation efficiency of forages, dictates the degree to which supplementation can be included in the ration. The order in which nutrients become first limiting as supplementation increases should also be considered (Kellaway & Porta, 1993, Kolver, 2003). The response to supplementation is also influenced by the nutritional value and proportion of both pasture and concentrate in the ration, including the cow's genetic profile, body condition and stage of lactation (Bargo *et al.*, 2003).

The milk production response per kg of concentrate is frequently described as curvilinear, thus as the amount of concentrate increases the milk production response per kg of additional concentrate decreases (Delagarde *et al.*, 2000). When the amount of concentrate fed to lactating dairy cows increased from 1.2 to 10 kg DM/d milk production increased linearly, with an overall response of 1kg milk/kg concentrate (Bargo *et al.*, 2003). In the trial of Meeske *et al*. (2006), for Jersey cows grazing pasture, production of fat corrected milk (FCM) for each additional one kg of concentrate at low (2.4 kg), medium (4.8 kg) and high (7.2 kg) supplementation were 1.25, 0.78 and 0.54 kg, respectively, over two full lactations. This is consistent with Hoden *et al*. (1991) who found a mean efficiency of 0.6 kg FCM/kg concentrate and Dillon *et al*. (1997) who found milk responses to vary from 0.13 to 0.98 kg milk/kg concentrate. Stakelum (1993) reported mean milk production responses over five experiments that ranged from 0.17 to 0.70 kg milk/kg of concentrate DM which ranged from 2.7 to 5 kg DM/cow/d. Roche *et al*. (2010) reported that supplements based on NSC yield 0.36 kg more milk per kg compared to supplements based on non-forage fibre-based concentrates, with the same level of ME. A greater milk production response is expected for cows of higher genetic merit (Kellaway & Porta, 1993) and in early-lactation (Dixon & Stockdale, 1999), because they partition more nutrients toward milk production as opposed to gaining BW. Supplementing energy to cows on pasture remains an important strategy to improve cow performance.

Maize is most commonly used in many dairy production systems to supply supplemental energy and to increase total cow DMI compared to pasture only systems. Supplementing grazing dairy cows with a maize-based concentrate, in mid-to late-lactation has shown higher milk and milk component production and improved NUE, as compared to pasture only systems (Meeske *et al.*, 2006, McKay *et al.*, 2019). Reis & Combs (2000) evaluated the impact that a maize-based concentrate would have on the performance and rumen fermentation of grazing dairy cows. The cows received 0, 5 and 10 kg/cow/d of a maize-based concentrate along with grazed pasture, an increase in cow performance, including milk production and composition were associated with the increased amounts of concentrate. Milk production was 21.8, 26.8 and 30.4 kg/cow/d and milk protein was 2.85, 2.95 and 3.05 %, respectively, for 0, 5 and 10 kg concentrate supplementation. However, when maize is incorporated at high levels (200 g/kg DM) in the diet, specific AA might limit cow performance or the response to increased energy supplementation (Kellaway & Porta, 1993; Kolver *et al.*, 1998). Especially when cows are high producing (± 25 kg milk/cow/d), because maize has a low protein content and a poor AA profile, especially for Lys (NRC, 2001; CVB, 2018). Note, that the "cascade" of nutritional limitation as mentioned earlier is starting to become more clearly demonstrated. After, the first factor limiting cow performance is supplied (i.e. ME), the cow's performance will only increase to a level where the second limiting factor becomes the first limiting factor (i.e. MP). However, since the percentage of Lys in MP flowing to the cow SI is also reduced (Robinson, 2010), it is considered proof that maize indeed supplies low levels of metabolisable Lys. As a result, nutritionists usually incorporate protein sources higher in RUP to meet the cow's Lys requirements. In contrast to Lys, Met is reported to be first limiting for cow performance when cows are fed forage, soybean hull-based diets and/or

when dietary RUP intake is low. Methionine has also been reported to limit cow performance when lactating dairy cows are fed a variety of diets in which the proportion of RUP is mainly supplied from soybean protein, especially animal-derived protein and/or heated soybeans (Bequette & Nelson, 2006, Robinson, 2010).

As a result of the high protein degradability of pastures (Beever & Siddons, 1986; Van Vuuren *et al*., 1993) it is expected that a higher production performance may be achieved from protein supplements higher in RUP (O'Mara *et al.*, 2000). Several studies have supplemented RUP in rations of lactating dairy cows with inconsistent responses in milk and milk component production reported (Santos *et al*., 1998; Kellaway & Porta, 1993; Schwab & Broderick, 2017). In most of the studies soybean meal was replaced with, or compared to, other common protein sources high in RUP, such as treated (heat or chemically) soybean meal, maize gluten meal, dried distiller's grains, dried brewer's grains, blood bone and/or meat meal, feather meal, fish meal or various combinations of these.

The inconsistent, or lack of, response to supplementation of protein sources higher in RUP could be attributed to the following: 1) reduced MCP synthesis, 2) poor EAA profile of the RUP source, 3) low digestibility of the RUP content, 4) the control treatment met the cow's requirement for RUP and 5) oversupplying metabolic unnecessary AA and N (Clark *et al.*, 1992; Robinson *et al.*, 1995).

Fish meal and treated soybean meal are responsible for most of the positive responses observed for milk and milk component production when fed to dairy cows. This is not surprising since soybean meal and fish meal rank the highest in EAA index compared to other protein sources (Santos *et al.*, 1998; O'Mara *et al.*, 2000), as discussed in section 2.4 and shown in Table 2.2. It is well understood that as the dietary CP increases the quantity of protein degraded in the rumen also increases (Bach *et al.*, 2005). When RDP content surpasses microbial needs (9 to 11 g CP per MJ ME consumed) large amounts NH_3 are absorbed into the blood and converted into urea by the liver and excreted as urinary N, reducing the efficiency of utilisation of dietary protein (Hristov *et al.*, 2019). This pre-duodenal loss of N usually occurs when forage CP exceeds 16 to 18 % CP, which is typically the case for temperate grasses (Muller, 1998, Fulkerson *et al.*, 2007). The NRC (2001) recommend that a small breed cow (i.e. Jersey) producing 20 kg milk with 4.5 % fat and 3.5 % true protein content in milk should consume 10.5 and 3.3 %, respectively, of RDP and RUP on a DM basis. Van Vuuren *et al.* (1992) further suggested that the maximal duodenal supply of NAN could be achieved at an N:OM ratio of 38 g/kg when lush pasture is fed to dairy cows. Malleson *et al.* (2008) supplemented fishmeal (220 *vs*. 440 g fishmeal DM/cow/d) to high producing multiparous Jersey cows, in early-to mid-lactation and which the cows were grazing ryegrass pasture. Milk production (4 % FCM) increased by 18 to 19 % compared to the control treatment (24.1 and 24.2 *vs*. 20.4 kg 4 % FCM/cow/d). They concluded that the response was most probably due to increased RUP especially due to increased supply of EAA, Met and Lys, to the SI. This is in accordance with Santos *et al.* (1998) who reported that FM supplementation consistently increased the proportion of Lys in the EAA flowing the duodenum of the cows when FM supply was greater than 4 % of diet DM. This is also in agreement with

Shroeder & Gagliostro (2000) and Schor & Gagliostro (2001) who reported milk responses to fishmeal supplementation of 6 to 18 %, respectively. O'Mara *et al*. (2000) compared the milk production of grazing Holstein cows when fed energy supplements with high levels of RUP. The cows were supplemented with 1.25 kg/d of concentrate consisting predominantly beet pulp, fishmeal or Sopralin (formaldehyde treated soyabean meal). Milk yield was 17, 18 and 19 kg/d, milk fat yield was 0.67, 67 and 0.70 kg/d, and protein yield was 0.58, 0.61 and 0.62 kg/d for the beet pulp, fishmeal and Sopralin concentrates, respectively. The authors concluded that the supplementation of RUP for cows grazing pasture may result in higher milk and milk component production when an energy source is also supplemented.

Erasmus *et al*. (1994) fed lactating Holstein cow's different protein sources on a maize-based diet. Blood meal, maize gluten meal, sunflower meal and a combination of blood meal and maize gluten meal (8, 10.5, 13 and 9.3 %, respectively, of diet DM) supplying on average 35.2 % of total dietary CP. Differences between EAA profiles of ruminal bacteria analysed from the different diets were not associated with the respective protein sources supplemented. Although, the duodenal digesta's EAA profile and duodenal flows of individual EAA correlated closely with the different treatments. In agreement with this, Robinson (2010) reported that there is a modest relationship between the proportion of Met and Lys in the duodenal digesta and the dietary protein source, indicating that the AA profile of the RUP supplemented influences the MP reaching the cow's SI. In addition to Lys decreasing in the duodenal digesta when maize CP, as a proportion of dietary CP increase, Met levels in the duodenal digesta flowing to the SI also tend to be reduced when cows are grazing pasture (Rulquin & Delaby, 1997a; Kolver *et al.*, 1999; Pacheco *et al.*, 2012). Even though the proportion of Met in pasture is relatively high compared to other forages (section 2.4, Table 2.2), most are extensively degraded in the rumen and do not reach the SI. It is evident from the literature that the grazing dairy cow's performance could be improved beyond the degree to which performance is limited by various nutritional limitations. First, enough ME must be supplied to meet the energy requirements of the rumen microbes, which would result in an improved pasture N utilisation and MCP synthesis. Secondly, a RUP source with a superior AA profile, which complement the MP flowing to the cows SI, must be supplied. Thirdly and finally, the MP reaching the cow SI must meet the cows AA requirements.

2.3 Amino acid requirements of dairy cows

Requirements for lactating dairy cows are not for protein, per se, but AA which forms the structure of proteins. The AA requirements of grazing dairy cows will be dictated by the cow's level of milk production, milk composition and stage of lactation (NRC, 2001; Lapierre *et al*., 2007). Including the cow's age, BW and body condition, maintenance and pregnancy requirements, including the extent to which body tissue is mobilized (Rulquin, 1993; Kolver, 2003) and to a certain degree the ration fed (Schwab *et al.*, 1976; Higgs *et al*., 2015).

Amino acid requirements for lactating dairy cows are mainly estimated with three different methods, which include a factorial, direct dose-response and an indirect dose-response method (NRC, 2001). The factorial method is based on mathematical equations to calculate and quantify absorbable AA requirements for individual component (i.e. protein anabolism) and nutrient integration and transfer over various digestive and metabolic pools. As a result, this method requires knowledge of net requirements of protein for production (i.e. lactation), reproduction, growth and maintenance. As well as the AA composition of the resulting products and the utilisation efficiency of the absorbed AA (O'Connor *et al.*, 1993). Determining the AA composition of various products are readily reliable, but estimating utilisation efficiencies of AA are difficult and mainly erroneous (Schwab *et al.*, 1992; Apelo *et al.*, 2014).

Alternatively, the direct dose-response method measures production responses to incremental amounts of post-ruminal infusions (abomasum and duodenum) of AA and AA flows to the SI (Rulquin *et al.*, 1990; Socha *et al.*, 1994). Lastly, the indirect dose-response method which involves various steps (Rulquin, 1993), predicting AA levels in MP for both the control and treatment groups and determining a reference production value. This production value is fitted to a linear regression model at a fixed AA concentration identified in the digesta in relation to the measured production responses. The reference value estimated for each production parameter is used as a relative response value to the calculated production responses for the control and various treatment groups, respectively (Schwab, 1995; NRC, 2001). The latter two methods attempt to estimate the optimum concentration of AA in MP flowing to the cows SI to maximise the use of MP for milk protein synthesis.

By using the dose-response techniques the NRC (2001) reported the breakpoint estimates for required Lys and Met concentration in MP for maximal milk and milk protein yield to be 7.08 and 2.35 %, respectively, and are similar to the 7.24 and 2.38 %, respectively, for Lys and Met in MP reaching the cows SI (Schwab & Ordway, 2001) and the 7.00 and 2.60 % reported by Van Amburgh *et al*. (2015). This is in agreement with the values obtained by Rulquin *et al*. (1993), which were 7.3 and 2.5 % of metabolisable AA, respectively for Lys and Met. According to Schwab (1996), the ideal concentration of Lys and Met for milk protein yield in high producing cows as a percentage of MP should be 6.8 and 2.2 % respectively. Also, optimum ratios as calculated by Sniffen *et al.* (2001) using multiple regression equations were 7.4 % and 2.2 % for Lys and Met in MP, respectively. Reflecting the ideal ratio of Lys to Met ratio of 3:1 as reported by Rulquin (1994), Schwab & Ordway (2001) and the NRC (2001). These values fall between the EAA ranges for milk protein and microbial protein reported in section 2.4, Table 2.2 which are considered to have "optimal" AA profiles. However, more recently Van Amburgh *et al*. (2015) reported that the optimal ratio of Lys:Met is 2.69:1 after estimating optimum efficiencies of these two AA from a meta-analysis utilising 40 published papers (Doepel *et al*., 2004) and the study conducted by Lapierre *et al*. (2007). As mentioned earlier these specific, or required, levels and ratios of Lys and Met flowing to the duodenum of the grazing dairy cows are very difficult to meet by simply supplying higher quality protein sources (i.e. RUP) in concentrates fed to the cows.

However, when protein and AA requirements are expressed as a fraction of the dietary concentration, there estimated requirements may be erroneous due to the high variability with feed intake. This variability in feed intake could be more problematic for grazing cows as opposed to cow fed a TMR. Dairy cows on pasture impose a challenge due to factors associated with accurately quantifying the qualitative and quantitative characteristics of the pasture. These factors include measuring pasture intake, seasonal variability of the pasture and the cow's selective behaviour. If the expected feed intake is less, higher protein or AA concentrations are required to consume the same amount of that specific nutrient, conversely the same for higher intake than estimated.

Animal requirements for AA are mostly expressed as (g/d) or as a proportion of EAA (% EAA), with the latter being preferred. Since diet formulation is more accurate and easier when the diet is formulated based on a desired profile rather than an amount (Schwab, 1995; Van Amburgh *et al*., 2013). For pasture-based production systems the dietary AA profile presented to the cow mainly comes from two sources, first the pasture and secondly the supplemented concentrate or PMR.

2.4 Dietary protein and amino acid supply

Amino acids synthesised *de novo* with metabolites from other metabolic processes (i.e. catabolism of surplus AA) by rumen bacteria or bodily cells are classified as dispensable AA and include the following: Tyrosine (Tyr), Glutamine (Gln), Glutamic acid (Glu), Alanine (Ala), Serine (Ser), Glycine (Gly), Aspartic acid (Asp), Proline (Pro), Asparagine (Asn) and Cysteine (Cys). These AA are most frequently referred to as non-essential amino acids (NEAA) (Lapierre *et al*., 2006). There are no specific requirements for these AA to be provided by the diet. Schwab *et al*. (1976) demonstrated that mixtures of NEAA supplemented to lactating dairy cows did not substitute for EAA in association with N retention for milk protein production. It is commonly known that there are twenty key AA forming protein chains, however only 10 are indispensable AA or EAA. These AA need to be supplied from dietary sources and have the potential to escape rumen degradation (i.e. RUP) and include the following: Lys, Met, Histidine (His), Phenylalanine (Phe), Leucine (Leu), Isoleucine (Ile), Threonine (Thr), Tryptophan (Trp), Arginine (Arg) and Valine (Val).

Essential amino acids are not produced by animal tissue in sufficient quantities to meet the cow's metabolic requirements, especially considering high producing dairy cows, thus EAA need to be supplemented (in the form that will escape ruminal degradation) to meet the cow's AA requirements (Schwab *et al.*, 2003; Lapierre *et al.*, 2007). Table 2.2 compares, and indicates, the AA composition of ryegrass pasture in relation to other sources, for example, MCP and milk protein, which are both considered high-quality protein sources with ideal AA profiles.

Table 2.2 Comparing essential amino acid (EAA) content of some animal products, rumen microbes and common feed sources used in dairy cow diets

	Essential amino acid										
Item	**Arg**	**His**	**Ile**	**Leu**	**Lys**	**Met**	**Phe**	**Thr**	**Trp**	**Val**	**EAA[a]**
Animal products											
Lean body tissue[1]	16.8	6.3	7.1	17.0	16.3	5.1	8.9	9.9	2.5	10.1	-
Lean body tissue[9]	6.6	2.5	2.8	6.7	6.4	2.0	3.5	3.9	0.6	4.0	-
Milk protein[1]	7.2	5.5	11.4	19.5	16.0	5.5	10.0	8.9	3.0	13.0	-
Milk protein[10]	6.8	5.3	12.1	19.1	15.7	4.8	9.8	9.1	2.6	12.9	-
Rumen microbes											
Bacteria[3]	5.7	2.0	6.3	8.7	9.5	2.9	6.3	6.1	1.9	6.2	
Bacteria[8]	10.2	4.0	11.5	16.3	15.8	5.2	10.2	11.7	2.7	12.5	-
Protozoa[1]	9.3	3.6	12.7	15.8	20.6	4.2	10.7	10.5	2.8	9.7	-
Protozoa[5]	4.5	1.8	6.5	7.8	10.8	2.2	5.6	4.97	-	5.16	-
Mixture[6]	5.1	2.0	5.7	7.9	7.9	2.6	5.1	5.8	-	6.2	-
Forages											
Lucerne hay[1]	12.5	4.7	10.3	17.9	12.4	3.8	11.6	10.6	3.6	12.7	41.2
Lucerne hay[11]	4.2	1.9	3.9	6.7	4.8	1.3	4.6	4	1.4	5.0	-
Maize silage[1]	6.2	5.7	10.6	27.2	7.9	4.8	12.1	10.1	1.4	14.1	31.6
Grass silage[1]	9.4	5.1	10.9	18.8	10.1	3.7	13.4	10.2	3.3	15.0	32.6
Grass hay[1]	11.7	4.9	10.0	18.8	10.5	3.6	11.8	10.9	3.7	13.6	33.1
Fresh grass											
Ryegrass[2]	8.4	4.2	11.3	19.3	15.2	3.6	9.3	15.1	-	13.5	-
Ryegrass[5]	1.9	0.7	0.7	1.03	1.6	0.23	0.53	0.74	-	0.9	-
Perennial Ryegrass[4]	6.0	2.2	4.7	9.4	5.6	2.2	5.6	5.2	-	6.5	-
Annual Ryegrass[7]	5.3	2.0	5.3	9.6	6.4	0.6	6.2	4.8	-	6.7	46.9
Grains											
Maize[1]	11.5	7.8	8.2	27.9	7.1	5.3	11.5	8.8	1.8	10.0	40.1
Maize[8]	10.8	7.0	8.2	29.1	7.0	5.0	11.3	8.4	1.7	11.5	42.3
Wheat[1]	13.6	7.1	9.6	19.3	8.1	4.6	13.3	8.4	3.5	12.3	34.4
Plant proteins											
Dried brewers grain[1]	14.7	5.1	9.8	20.0	10.4	4.3	11.7	9.1	2.5	12.1	39.2
Soybean meal[1]	16.2	6.1	10.1	17.2	13.9	3.2	11.6	8.7	2.8	10.2	45.3
Soybean meal[11]	7.3	2.6	4.5	7.6	6.1	1.3	5.1	3.9	1.3	4.7	-
Canola meal[11]	6.1	2.6	4.2	7.0	5.7	2.1	4.0	4.4	1.5	5.3	-
Cottonseed meal[1]	26.0	6.6	7.3	13.8	9.7	3.7	12.5	7.6	2.8	10.0	42.6
Sunflower meal[1]	20.8	6.2	9.9	15.2	8.0	5.6	11.0	8.7	2.9	11.7	42.2
Animal proteins											
Fishmeal (menhaden)[1]	13.1	6.4	9.2	16.2	17.2	6.3	9.0	9.4	2.4	10.8	44.5
Blood meal[1]	7.8	11.3	2.2	22.7	15.9	2.1	12.1	7.7	2.8	15.4	56.4
Blood meal[11]	4.3	5.9	1.1	12.3	8.7	1.2	6.8	4.6	1.4	8.2	-

[a]EAA (% of CP)
[1]AA (% of total EAA) values obtained from NRC (2001)
[2]AA (g AA/100 g total EAA) values obtained from Kolver *et al.* (1999)
[3]AA (g of AA/kg true protein) values obtained from Storm & Ørskov (1983)
[4]AA (g/100g of total AA) values obtained from Edmunds *et al.* (2013)
[5]AA (g of AA/100g of AA) values obtained from Sok *et al.* (2017)
[6]AA (g of AA/100g of AA) values obtained from Clark *et al.* (1992)
[7]AA (g of AA/100g of AA) values obtained from Malleson (2008)
[8]AA (% of total EAA) values obtained from Schwab (1995)
[9]AA (% of CP) values obtained from O'Connor *et al.* (1993)
[10]AA (% of total EAA) obtained from Schwab *et al.* (1976)
[11]AA (% of CP) values obtained from Van Amburgh *et al.* (2014)

However, from a protein intake perspective, cows grazing pasture receive dietary protein from pasture and supplementation (i.e. concentrate or PMR). The resulting fraction of post-ruminal protein and AA (i.e. MP) determine the net supply of AA reaching the cow's SI. Since Met is frequently identified as the first, Lys as the second and His as the third limiting AA for lactating dairy cows, the extent and sequence in which these EAA become liming is largely dependent on the amount of MP, and the EAA composition, reaching the cows SI (Schwab *et al*., 1992; Rulquin *et al*., 1993; Schwab & Ordway, 2001; Lapierre *et al*., 2006). However, a problem associated with protein nutrition of ruminants is due to the fact that quantitatively and qualitatively the dietary AA profile consumed by ruminants does not reflect the same AA profile leaving the rumen and reaching the SI (Erasmus *et al.*, 1994).

2.4.1 Pasture

Section 2.2, Table 2.1 and subsection 2.2.1.2 clearly demonstrate the CP supply from pasture and in section 2.4, Table 2.2 indicate, and compares, the EAA composition of high-quality pasture to other feedstuffs, including animal products and microbial protein. Please refer to those sections. Out of the information provided in those sections, it could be concluded that pasture provides excessive amounts of CP (i.e. RDP), which is highly degradable, resulting in low levels of Met in MP reaching the SI, even though the Met content of ryegrass is relatively high. In addition, multiple authors have reported that the flow of Met, as a proportion of total EAA, to the grazing dairy cow duodenum is less than 5 %. Considering the 5.3 to 5.6 % Met recommended by Schwab (1996) for optimal milk protein production, it suggests that the supply of Met might in fact be limited for cows grazing pasture. In support of this statement, various authors came to the same conclusion for cows grazing pasture (Van Vuuren *et al.*, 1993; Pacheco-Rios *et al.*, 2000; Younge *et al.*, 2000; Robinson, 2010).

2.4.2 Supplementation

The supplementation of maize and especially some high quality protein sources are discussed in more detail in subsection 2.2.2 and the AA profiles of some of the high quality protein sources are presented, and compared, in section 2.4, Table 2.2. Please refer to those sections. From these sections, it may be concluded that high levels of maize supplementation may reduce the Lys content of the MP flowing to the cow's SI, and the use of protein sources high in RUP may lead to an oversupply of CP, poor Lys:Met ratio or inadequate concentrations of a specific AA in MP reaching the cow's SI.

As a result, careful attention must be given to the supply of protein and AA post-ruminally from both pasture and supplementation.

2.4.3 Post-ruminal supply of protein and amino acids

In ruminants, the source and quantity of AA absorbed and utilised by the mammary gland are mainly supplied by MP which primarily consists of, endogenous CP (ECP), MCP, RUP and a small fraction from soluble CP (Sol CP) in feeds (i.e. peptides and free AA) which escapes rumen degradation. Rumen-undegradable protein proportionally varies in MP, including the degree of digestibility which could ranges from 50 to 100 % and is also dependent on the specific feedstuff (Santos *et al.*, 1998). Recently, Lapierre *et al*. (2020) reported that the average efficiency of utilisation of MP in lactating dairy cows is between 64.9 to 65.1 % based on a data set consisting of 807 treatment means. The RUP fraction in MP is assumed to be 100 % true protein (NRC, 2001). Endogenous N flowing to the SI is presented as 1.9 g of N/kg of DMI (kg/d) by the NRC (2001) and the true protein content passing to the duodenum is assumed to be 50 % of which 80 % is digestible. Thus 40 % of ECP is converted to MP (Ørskov *et al.*, 1986). Microbial CP is highly digestible (80 %) and constitutes of 80 % true protein, with the remainder provided by nucleic acids (Zanton, 2014).

2.4.3.1 Endogenous crude protein

Endogenous crude protein is mainly derived from sources which include, 1) enzyme secretions into the abomasum and ileum, 2) cellular debris from the omasum and abomasum, 3) cellular debris abraded from the mouth, oesophagus, and reticulo-rumen, 4) sloughed epithelial cells from the respiratory tract and 5) mucoproteins in saliva (Tamminga *et al.*, 1995). The latter three are possibly extensively degraded in the rumen and do not contribute to the passage of protein to the intestine (NRC, 2001). Numerous studies have been conducted to determine ECP flow to the SI (Hristov *et al.*, 2019). However due to the technicality and difficulties associated with distinguishing between N coming either from endogenous, microbial or dietary protein has hindered progress (Ouellet *et al.*, 2002; Hristov *et al.*, 2019).

The NRC (2001) adopted 1.9 g of N/kg of DMI as an average value for endogenous N flowing to the SI as reported by various studies. This is in line with the 1.7 g of N/kg DMI reported by Vérité & Peyraud (1989), which assumes that ECP flow is closely correlated with OM intake. Similarly, Lapierre *et al*. (2007) reported that 10.5 g MP/kg DMI of faecal endogenous losses is excreted. More recently, Lapierre *et al*. (2020) reported in a review using 807 treatment means and validated with and additional data set with 129 treatments means that lactating dairy cows my produce 11.6, 0.38 and 9.53 g/kg DMI of metabolic faecal true protein, scurf true protein and endogenous true protein loss, respectively. It is clear that the ECP fraction potentially contributes up to 150 to 250 g/kg of the total CP flowing out of the rumen, and varies substantially in AA composition compared to MCP and RUP (Bequette, 2002; Lapierre *et al*., 2020). Thus it cannot be ignored since the total contribution of AA in MP may differ in the expected delivery of EAA. Studies ignoring the contribution ECP may overestimate the EAA supply from MCP and the diet (i.e. RUP).

2.4.3.2 Microbial crude protein

The most important and sensitive indicator to optimise metabolism in the rumen of high producing dairy cows are microbial protein flow (Tas & Susenbeth, 2007). Microbial protein is derived from a mixture of bacteria, protozoa, and anaerobic fungi, representing more than 50 % (50 to 80 %) of total digestible protein reaching the cow's SI (Storm & Ørskov, 1983; NRC, 2001). The nutrient availability and utilisation efficiency of these nutrients by ruminal bacteria determine the total amount of MCP flowing to the SI (Bach *et al.*, 2005). Approximately 60 % of the total NAN reaching the duodenum in lactating dairy cows comes from microbial N (Clark *et al.*, 1992). Dewhurst *et al*. (2002) reported that MCP provides approximately 100 to 150 g of MCP/kg DMI, which has an apparent intestinal digestibility of 847 g/kg (Sok *et al.*, 2017). The AFRC (1993) reported that for every 1 MJ of FME fermented it is possible to produce 9 to 11 g of MCP. The average synthesis of MCP reported in the literature per kg PDMI by dairy cows is 81 g MCP/kg PDMI, ranging from 34 to 162 g MCP/kg PDMI, with the higher values reported for immature, lush pasture and lower values for older, matured pastures (Clark *et al*., 1992; Pacheco *et al*., 2010; Danes *et al*., 2013). Microbial crude protein has a high-quality AA profile with a fairly constant EEA composition, with only a small difference between the EAA profiles of rumen microbes and the predominant strains (Table 2.2).

Since MCP greatly influences the duodenal proportion of EAA it is expected that average microbial protein values for Lys and Met (% true protein) are similar to duodenal digesta (NRC, 2001). In general, it is accepted that MCP is highly digestible and comprises of an EAA profile representing that of milk protein and lean body tissue, supplying the majority of the AA flowing to the SI of cows (Clark *et al.*, 1992; Schwab & Broderick, 2017). Table 2.2 shows a comparison of the EAA composition of MCP with that of some animal products and common feedstuffs, including that of pasture. This clearly indicates the importance of microbial proliferation and growth to optimise cow performance. Although supplementation of RUP as a means to increase MP flowing to the duodenum is relatively effective, the resulting decrease in MCP flow contributing to total MP may result in a reduction in total N efficiency and cow performance (Santos *et al.*, 1998; Dewhurst *et al.*, 2000; Schwab *et al.*, 2003).

The growth of rumen microorganisms is affected by various factors and consequently MCP yield (Sniffen & Robinson, 1987). These factors include the availability of rapidly fermentable carbohydrates, adequate N supply, rumen environment and rumen turnover rate, as discussed in previous sections (Dewhurst *et al*., 2000; Verbic, 2002). Other factors include, DMI, Forage to concentrate ratio, feeding frequency, dilution rate and dietary fat (Stern & Hoover, 1979). Ruminal microorganism has requirements for N as dietary N ingested by ruminants are first exposed to extensive ruminal degradation, meeting the microbial N requirements of the cow (Clark *et al.*, 1992). Schwab *et al*. (2005) reported that MCP flows are maximised at a ruminal concentration of 5 to 11 mM NH_3 and are also dependent on dietary characteristics. These characteristics influence ruminal fermentation conditions which are associated with the source of N, carbohydrate type (i.e. structural and non-structural) and fermentability along with factors

affecting passage rate (De Veth & Kolver, 2001). Microbial growth and milk production are often reported to be limited by FME and N (Clark, 1992). Thus, to optimise MCP synthesis and microbial N flowing to the SI, the diet, or rather the synchronous release of carbohydrates and protein, should be optimised. As DMI is arguably the largest factor influencing the passage of microbial N to the SI, it is suggested that dietary CP should be estimated relative to DMI. Thus, cows consuming more DM may be fed a reduced CP content without affecting MCP flow and as a result the microbial AA flow to the SI (Clark *et al.*, 1992). It has also been suggested that low concentrations of AA and peptides could potentially limit MCP production when high producing cows are fed diets rich in starch which is normally associated with fine particle size and a low rumen pH (Demeyer & Fievez, 2004).

However, *in vivo* estimates of MCP synthesis in dairy cows, as in ruminants, require the use of different marker methodologies either as exogenous (i.e. ^{15}N ammonium salts or radioisotopes) or endogenous (i.e. purines or integral structural components) markers (Dewhurst *et al.*, 2000; Hristov *et al.*, 2019). These markers must be measured accurately when passing through the rumen or when entering the SI, respectively, by either omasal cannulation or intestinal cannulation. Samples of digesta have also been taken from a simple T-piece cannula (Evans *et al.*, 1981) in an automated fashion or by direct sampling through a rumen cannula and/or the reticulo-omasal orifice have also been used with these different marker systems (Huhtanen *et al.*, 1997). Determining MCP synthesis with traditional *in vivo* methods are complicated, expensive, laborious, imprecise and invasive with unknown accuracies (Clark *et al.*, 1992; Hristov *et al.*, 2019). As a result, most dairy rations are commonly formulated based on prediction for duodenal flows of microbial protein from mathematical and/or empirical models simulating ruminal fermentation to predict the amount of protein flowing to the duodenum of the cow. However, these models might predict ruminal MCP synthesis and degradability, duodenal flows and digestibility of MP (i.e. RUP, MCP and ECP) and EAA inadequately (Pacheco *et al.*, 2012; Tedeschi *et al.*, 2015). Especially considering the efficiency with which the nitrogenous compounds are utilised for milk production *(*Hanigan *et al*., 1998*;* Tylutki *et al*., 2008; Tedeschi *et al.*, 2015).

In a recent meta-analysis it was reported that metabolic models used tended to predict duodenal EAA to the SI sufficiently, especially for maize-based diets, however, this excludes diets based on low concentrate rations and forages (Pacheco *et al.*, 2012). See section 2.8 below for a discussion on the use of models to balance diets of grazing dairy cows. This section establishes that the use of nutritional models to balance for EAA in dairy diets under practical conditions are possible. However, the digestibility, composition and partial efficiency of EAA provided form MCP are sometimes erroneous or not included in some of these models. Including the effect of various feeding strategies (i.e. pasture-based systems *vs.* TMR), which could result in inaccurate estimation of EAA available for digestion (Apelo *et al.*, 2014). Thus, failing to meet the cow's AA requirements for milk protein, due to overestimating the utilisation efficiency of AA's for milk protein production and maintenance requirements. This, including the assumption that the efficiency of utilisation of AA is constant for milk protein synthesis and the single

liming AA theory, not allowing for the additive effects of EAA, could lead to an oversupply of dietary N. Since there are difficulties and inaccuracies associated with the *in vivo* or model-based estimation of ruminal MCP yield, alternative methods need to be explored to determine and monitor MCP synthesis in various production systems (Dewhurst *et al.*, 1996; Chen & Orskov, 2004; Swanepoel *et al*., 2016).

The correlation between urinary excretion of purine derivatives (PD) and microbial protein flow to the duodenum of lactating dairy cows has been well documented (González-Ronquillo *et al.*, 2004). Multiple papers have been published since Terroine & Mourot (1931), as cited by Shingfield (2000), indicated that there is a correlation between PD excretion and protein intake in sheep and the suggestion by Topps & Elliot (1965) that urinary AL could be used to estimate ruminal MCP synthesis. This correlation between urinary AL excretion and MCP synthesis was evaluated in studies using duodenal infusions of nucleic acids and duodenal and abomasal infusion of purines (Vagnoni *et al.*, 1997), different dietary CP levels (Gonda & Lindberg, 1997) and various degradabilities of feed protein sources (Gonda & Lindberg, 1997), including different dietary RDP: RUP ratios and different dietary N:OM ratios (Moorby *et al.*, 2006). These studies further supports the validity of the assumption of the technique that most purines entering the duodenum originate from MCP, which are digested and excreted as PD in urine and milk in quantifiable quantities and that microbial purine content and digestibility are limited in variation (Shingfield, 2000). However, there are various errors associated with this method, which include the following: 1) dietary contribution of nucleic acid to duodenal flow, 2) variation in microbial purine content, 3) portion of PD between renal, mammary and enteric excretion, 4) PD resulting from endogenous purine metabolism and 5) the variation in the N: purine ratio in the duodenal digesta (Chen & Gomes, 1992; Hristov *et al.*, 2019). These limitations were addressed by Tas & Susenbeth (2007), Dewhurst (2000) and Shingfield (2000) who reviewed the literature.

Dietary nucleic acids are completely ruminally degraded and as a result feed purines usually do not influence the concentration of PD excreted in urine (McAllan & Smith, 1973). Purines found in ruminant feeds are mostly in low quantities and are degraded extensively in the rumen as a result of ruminal fermentation (McAllan & Smith, 1973; Chen & Gomes, 1992). Nucleic acid derived from microbes flowing from the rumen are digested and absorbed in the SI and secreted as PD in urine and milk. The PD fractions includes uric acid, xanthine, hypoxanthine and AL, with AL being excreted in a relatively constant proportion (0.82 to 0.93) of total PD excreted (Chen & Gomes, 1992; Vagnoni *et al.*, 1997). The remainder of PD are mainly secreted as uric acid, since xanthine and hypoxanthine secretion for lactating dairy cows are negligibly small. Even though milk sampling is done more routinely and more easily than urine sampling, which is much more difficult, there is no consistent relationship between milk AL and urinary AL excretion (Gonda & Lindberg, 1997). Milk AL is extensively catabolised to uric acid in the mammary gland and excretion is not constant and positively associated with milk yield representing only a small fraction (0.63 to 1.34 %) of total AL excretion in milk and urine (Gonda & Lindberg, 1997). Thus, totally excluding milk PD from the total PD excretion calculations would result in a less than 7 % underestimation

in lactating dairy cows as suggested by (Gonzalez-Ronquillo *et al.*, 2003). Apart from the mammary secretion of PD, urinary secretion of PD accounts for up to 83 to 88 % of all PD absorbed (Susmel *et al.*, 1995).

Determining the daily output of purines requires repeated measures of total urine flow which limits the use of this method, however, it has been shown that urinary output could be accurately predicted with creatinine as a marker (Lee *et al.*, 2019) as proposed by other authors (Valadares *et al.*, 1999). Creatinine excretion is not influenced by dietary factors (i.e. NFC, NPN and CP) or DIM (Susmel *et al.*, 1995) and can be calculated from the animal's BW (i.e. muscle mass). Chen & Ørskov (2004) corrected the PD: Creatine ratio to metabolic BW by developing a PD to creatinine index as creatine production and consequently excretion is related to muscle mass and thus also a change in BW. Therefore, the PD: creatine ratio in spot urine samples could be used, in relation to total urine volume, to calculate the daily excretion of AL. Alternatively, the AL in spot urine samples could be determined by a chemical analyses described by Young & Conway (1942) as reported by Chen & Gomes (1992). Determining total urine volume, which is required to calculate the daily excretion of AL in the analysed spot urine sample, the urine specific gravity is required. Since volume has a close relationship with specific gravity (SG) and as a result could be used to calculate the total urine volume from a collected spot urine samples as reported by Burgos *et al.* (2005). Although questioned by authors due to diurnal variations associated with using the urine SG method (Shingfield & Offer, 1998), this error could be resolved by a constant sampling protocol and/or increasing the number of animals sampled. When total (24h00) PD output was compared to spot sampling there was no significant difference between the two sampling techniques (Chizzotti *et al.*, 2008). This finding is similar to that of Valadares *et al*. (1999). Vagnoni *et al*. (1997) quantified the relationship between total daily excretion of PD and total abomasal infusion plus ruminal outflow by a linear regression analysis in an abomasal purine infusion study. The relationship $Y = 0.856\ X + 103$ ($r^2 = 0.93$) was reported by the authors, the slope indicated that 85.6 % of the measured purines that reached the omasum were excreted as PD. Additionally, 98.4 % of total PD excreted was accounted for by urinary metabolite excretion. They also reported, in agreement with others, that the biological variation accounted for only 3 % of total variation. This method, which is based on urinary PD excretion is most probably the most defined non-invasive or indirect method for the estimation of MCP (Moorby *et al.*, 2006) and may be the best method to potentially measure MCP more routinely (Tas & Susenbeth, 2007).

2.4.3.3 Rumen-undegradable protein

Microbial CP presents a high-quality AA profile, but might not supply the high producing cows with sufficient amounts of AA for optimum production. Especially cows grazing pasture, since pasture is low in RUP and high in RDP, which shifts the focus towards supplementing sources high in RUP to grazing dairy cows. Common sources high in RUP such as treated (i.e. heat or chemically) soybean meal, maize gluten

meal, dried distiller's grains, dried brewer's grains, blood, bone and/or meat meal, feather meal, fish meal or various combinations of these (Santos *et al.*, 1998). However, these protein sources may not provide a balanced profile of AA that matches that of milk protein (Table 2.2). It is assumed that the protein source with the EAA profile representing that of milk protein is higher in quality. As a result, animal by-products are usually used as high RUP supplements to dairy cows, however these sources are usually expensive and not always readily available (St-Pierre, 2012; Edmunds *et al.*, 2013). Methionine is also reported to be low in forage-based diets and/or soybean meal based diets where Lys is usually low in maize-based diets (Robinson, 2010). It is therefore important that the source providing most of RUP as a percentage of the total diet CP, must be taken into account as excessive supplementation may exaggerate the limiting AA (Santos *et al.*, 1998).

Conclusion

Optimising the flow of microbial protein, RUP and the AA composition and concentration of the protein sources supplied to match the cow's EAA requirements may increase cow performance, NUE and ultimately the farm's profitability (NRC, 2001; Schwab & Broderick, 2017). Achieving this optimisation in practice is complicated and realising these benefits requires a commitment to the use of nutritional models and AA balancing tools (Tylutki *et al*., 2008; Van Amburgh *et al*., 2015). Even though, much effort has been done with regards to accurately quantifying the profile of EAA available for absorption (Lapierre *et al.*, 2005; Hristov *et al.*, 2019; Lapierre *et al*., 2020), the controls and metabolic fate of the absorbed EAA play a significant role in cow performance (Lobley, 1992; Doepel & Lapierre, 2010; Hanigan *et al*., 2018). Therefore, knowledge of post-ruminal AA metabolism is required to clearly understand how the alteration of AA supplied to the cow might influence AA metabolism in order to interpret possible responses, or lack thereof, in cow performance.

2.5 Post-ruminal amino acid metabolism

Alternating the AA concentration and spectrum within the cow's body influences AA transfer and extraction efficiencies, along with the cow's partial net energy (NE) balance (Doepel *et al.*, 2004; Lapierre *et al.*, 2005). This directly affects the cow's performance and NUE (Schwab, 1996; Lapierre *et al.*, 2005).

This section will emphasise the metabolic fate of EAA supplied by the nitrogenous compounds (i.e. ECP, RUP and MCP), as discussed in section 2.4, flowing of EAA to the SI and the net fluxes of AA across different tissues in dairy cows. It is important to characterise the utilisation of AA by the mammary gland for the synthesis of milk protein, ultimately to develop effective AA supplementation strategies (Lapierre *et al.*, 2007; Lapierre *et al*., 2020). Due to vast quantities of feedstuffs and nutritional variability in dairy rations, especially in diets of grazing dairy cows, careful attention must be given to the N balance within

the cow's, including the diet, to turn N into milk protein as efficiently as possible (Colmenero & Broderick, 2006; Apelo *et al.*, 2014).

Generally, about 80 % of protein arriving at the SI is digested resulting in peptides and AA. These peptides and AA are absorbed by specific transport proteins into the enterocytes. After being absorbed into the enterocytes the peptides are hydrolysed into AA by peptidases, and both resulting EAA and NEAA are either used for protein anabolism, oxidised, transported into the blood-stream and/or circulated back into the intestines by means of endogenous secretions (i.e. enzyme secretion) (Lapierre *et al.*, 2006). Lapierre *et al*. (2005) reported that approximately 35 % of the AA that pass through the SI are lost (i.e. oxidation, endogenous secretions, gut utilisation and non-absorption), thus only 65 % of digestible AA is recovered in the portal vein.

Intestinally absorbed AA, and the availability thereof for milk protein synthesis, are mainly modulated by splanchnic tissues (Bequette *et al.*, 1998). These tissues include the portal-drained viscera and the liver, which account for more than 50 % of the dairy cow's bodily protein synthesis (Lapierre *et al.*, 2005). These two tissue groups supply metabolites to peripheral tissues for anabolism of muscle tissue, milk proteins and fetal growth (Baumrucker, 1985; Schwab, 1996; Pacheco-Rios *et al.*, 2000). The liver performs a few key processes in AA nutrition (i.e. synthesis of NEAA, plasma proteins, gluconeogenesis, production of ketones and the conversion of NH_3 to urea). Additionally, the liver also removes roughly 45 % (range, 16 to 69 %) of the AA that flow through the portal system (Bequette *et al.*, 1998). Mepham (1982) reported that measures of overall net fluxes of EAA across tissues in dairy cows support the grouping of EAA into two separate groups, group one representing His, Met, Phe, Tyr and Trp, and group two Lys, Val, Ile and Leu. There is a lot of variation among these two groups in the degree of oxidation, liver removal and affinities, making the development of nutritional models even more challenging (Apelo *et al*., 2014). Hepatic removal of AA varies widely, with branched chain AA (BCAA) and Lys undergoing very little hepatic removal and Met and His undergoing almost 50 % of their total portal absorption (Lapierre *et al*., 2007). However, even when the availability of posthepatic (post-liver) AA are not limited, only about 30 % of these AA are effectively converted into milk protein. This may be due to factors affecting the affinity of the mammary gland for these AA, for example the balance and timing of AA reaching the mammary gland (Bequette *et al.*, 1998; Apelo *et al*., 2014).

Baumrucker (1985), Robinson *et al*. (2000) and Pacheco & Waghorn (2008) reported that supplying AA in excess of cow requirements has a negative effect on cow performance, partially due to the energetic cost associated with removing excess AA. The removal (metabolism) of excess AA has the same energy cost that urea has for the same amount of NH_3 absorbed from the rumen and SI (30 kJ ME/g N) and can be as much as the energy provided from one kg pasture DM consumed by a lactating dairy cow. When a grazing cow consumes 350 g N/day from pasture times 30 kJ/g N consumed, this equals 10.5 MJ of ME, which could represent 4 to 6 % of the total ME intake of the cow (Pacheco & Waghorn, 2008). This, in addition to the grazing cows additional energy requirements for grazing and physical activity, indicates that

care must be taken when altering the balance of AA supplied to the cow to avoid over supplying AA, which could have an impact on the grazing dairy cow's energy status. Increasing the protein supply reduces the efficiency of milk protein synthesis due to the reduced transfer efficient of the absorbed AA (Hanigan *et al*., 1998; Doepel & Lapierre, 2010). For example, when Guinard *et al*. (1994) increased the total level of duodenal casein infusion up to 762 g/d they observed that the conversion of absorbed EAA into milk protein decreased (0.44 to 0.34). Guinard & Rulquin (1994) also reported that the removal of EAA by the mammary gland increased in response to casein incrementally infused into the duodenum, but the total removal of EAA were higher (0.81) at lower infusion rates and lower (0.50) at higher infusion rates. This reduction in efficiency can be attributed to increased hepatic removal (i.e. Met and His) and increased catabolism by peripheral tissues (i.e. Lys). This is in contrast to the general idea that the recommendations for Lys and Met are presented as a function of the AA profile of milk. By doing this the impact that AA have on milk fat production and other metabolic pathways are ignored. This further suggests that Met and Lys must be evaluated separately, rather than just as a ratio between the two AA. However, when supplementing RPM and RPL in combination it is challenging to separate the effects (Robinson *et al*., 1998; Robinson *et al*., 2000) and limited studies have been published on Lys supplementation as compared to Met supplementation.

The main goal of AA nutrition of lactating dairy cows in terms of mammary metabolism is to supply the mammary gland with optimum EAA for meeting the demand for milk production and limiting AA. Apart from the total supply of CP and that of which CP comprises of, special attention must be given in which fraction each and every nitrogenous compound arrives at its site of metabolism. Simply increasing the dietary protein content or by using various, or specific, high quality protein sources would not insure a relatively constant supply of AA to the cow's SI or mammary gland, especially in the case of grazing dairy cows (Pacheco-Rios *et al.*, 2000; NRC, 2001; Schwab *et al.*, 2005).

2.6 Limiting amino acids

The two AA, Lys and Met are considered to be the first and second limiting EAA in MP, as a result of evidence from infusion studies supplying individual AA directly to the duodenum or abomasum and measuring the effects on N retention (Schwab *et al.*, 1976). To identify possible EAA limiting cow performance three methods, apart from those mentioned in section 2.3, based on AA transfer efficiencies, uptake to output ratios and excretion efficiencies have been proposed. With the latter method being regarded as the most accurate of the three methods (Nichols *et al.*, 1998). This is due to the fact that it does not only account for AA output, but also the EAA requirements in the mammary gland, including intermammary metabolism, protein degradation and synthesis, and there are no errors associated with estimates of blood flow involved. The reason being, when a method for determining AA limitations only focuses on AA output it does not distinguish between protein synthesised in the mammary gland and protein absorbed from the

blood (Piepenbrink & Schingoethe, 1998). The extraction efficiency, which is calculated as follow: Extraction efficiency = [(Arteriovenous difference (g/L) × 100) ÷ Arterial AA concentration (g/L)], inspects arteriovenous differences as a proportion of AA in the plasma (Coccygeal artery) to identify AA limitations. This method assumes that a low arterial concentration of an AA or large extraction percentage identifies an AA limiting milk production. However, Lys uptake from blood plasma for the mammary gland tend to surpass the cows net requirements for lactation, thus being "extracted" in correlation with its supply irrespective of the cow's requirements (Guinard & Rulquin, 1994; Lapierre *et al*., 2005). This is in agreement with Broderick (1974) who proposed that the variation of plasma EAA could be used as a method to identify limiting AA, by assuming that an EAA concentration would build up in the blood plasma only after the cow's requirements have been met. Additionally, Whitehouse *et al*. (2017) evaluated the plasma free AA dose-response technique and concluded that plasma free lys increases in a linear fashion to the incremental supplementation of RPL and that the dose-response technique could be used for evaluating RPAA supplements. Patton *et al*. (2015) evaluated the relationship between circulating plasma concentrations and duodenal flows of EAA in lactating dairy cows and concluded that over a wide range of protein intake, plasma EAA levels increase linearly with duodenal flow. Mulrooney *et al*. (2009) reported that the Plasma AA concentration can be used as a tool to evaluate differences in the AA supply of various diets, including RPAA supplements (Polan *et al*., 1991).

2.7 Amino acid supplementation

The supplementation of AA and the subsequent effects on animal performance have been reported in numerous studies, with some studies reporting positive responses and other none or even negative responses. Most of these studies were done with Met and/or in combination with Lys, but not Lys alone. Only a few have reported on Lys only supplementation and most of these studies were done on TMR and not pastures (Van Houtert, 1997; Robinson *et al.*, 1998; Robinson *et al.*, 2000; Younge *et al.*, 2000; Rulquin *et al.*, 2006; Třináctý *et al.*, 2009; Wang *et al.*, 2010). Responses to post ruminal infusion of Met and Lys in dairy cows have been reviewed in depth by the NRC (2001). Growing animals tend to respond in terms of feed efficiency and average daily gain. While lactating dairy cows tend to respond in terms of milk yield, milk protein content, and DMI. Robinson (2010), in a systematic review of literature, reported that the production responses of lactating dairy cows relative to dietary manipulation of metabolisable Met and/or Lys are disappointingly small, despite the fact that some responses were statistically significant. This is also in agreement with the findings of Rulquin *et al*. (1993). Responses included, 1) decreased DMI and increased milk/DMI ratio from RPL supplementation, 2) increased percentages of milk protein and milk fat along with increased milk energy output and dietary NUE from RPM supplementation and 3) increased milk, milk energy output, milk protein percentage, dietary NUE and milk/DMI ratio from the combined supplementation of RPML. However, the general nature of the response to improved supplies of Lys and

Met in MP as reviewed by multiple authors (Piepenbrink *et al.*, 1996; Schwab, 1996; Rulquin & Delaby, 1997a; NRC, 2001), further include the following: 1) milk protein is more responsive than milk production in post-peak lactation cows, 2) the increase in milk protein is as percentage (independent of milk yield), 3) casein is the protein fraction mostly influenced, 4) increase in milk protein is most predictable when the resulting predicted supply of AA are according to AA requirement, 5) response to AA are most common in early-lactation and 6) responses are greatest at realistic (14 to 18 % DM) CP diets.

Supplying the lactating dairy cow with specific AA in the ration to improve cow performance and NUE is thus not a new concept (Clark, 1975). Supplementing the lactating dairy cow with RPAA may have various benefits, that being, 1) a small amount of RPAA can substitute larger portions of RUP, 2) allowing space in the diet for the inclusion of ME, which could be beneficial for cows on pasture, 3) more constant levels of production with lower dietary CP levels and 4) improved NUE, thus reduce N pollution and alleviate milk protein and milk fat depression.

Methionine, apart from being incorporated into milk protein, is also involved in multiple metabolic pathways, thus being responsible for a large number of different metabolites and other specialised compounds, for instance, phospholipids, polyamines, carnitines and creatine (Schwab, 1995). Additionally, Met also provides methyl groups for several trans-methylation reactions involved in DNA regulation. Including the oncogene status and sulphur groups for the synthesis of Cys (Baker, 1994; Bequette *et al.*, 1998). Methionine further plays a key role in the synthesis of sulphur-containing antioxidant (taurine and glutathione), including single carbon metabolism where multiple methylation reactions acquire methyl groups (Ulrey *et al*., 2005). Methionine is also associated with lipid biosynthesis, especially the synthesis of very low-density lipoproteins, including the breakdown of fats (lipotropic). Methionine is also a methyl donor for the synthesis of choline. Choline on the other hand is essential for the synthesis of phospholipids required for synthesis of chylomicrons and very low density lipoproteins and is also possibly a limiting nutrient for milk fat synthesis (Campbell & Farrell, 2003; Arshad *et al*., 2020).

Lysine, which is a diamino acid (α- and ε-amino group) that occurs naturally as an L-isomer assists in Ca absorption, maintenance and regulation of the cow's N balance in the body and production of various other metabolic compounds. These compounds include antibodies, hormones, enzymes and collagen formation (Rulquin *et al.*, 1990). Lysine plays a significant role in the repair of bodily tissues (i.e. elastin and collagen) and protein anabolism for bodily protein (i.e. muscle proteins) (Campbell & Farrell, 2003). Another function of Lys is the formation of carnitine by providing the carbon backbone for carnitine synthesis. Carnitine plays an essential role in energy generation, proxisomal FA oxidation, BCAA oxidation (Hoppel, 2003).

Understanding the metabolic roles that both Met and Lys play could aid in balancing concentrates for grazing dairy cows to improve milk production, milk component production and the overall efficiency with which dairy cows utilise nutrients.

2.7.1 Responses to rumen-protected methionine and lysine supplementation

2.7.1.1 Milk production and milk composition

In a comprehensive meta-analysis Lean *et al*. (2018) evaluated the statistical correlation between supply of metabolisable AA, milk production, milk protein production and milk protein percentages for lactating dairy cows. The authors reported that metabolisable Met (g/d) is associated with milk protein (% and kg/d) indicating the positive effects of Met supplementation. The meta-analysis included 63 research publications with 258 treatment means in this analysis. Robinson *et al*. (1995) supplemented, in a full lactation study (40 weeks), ruminally protected Lys and Met in a diet specifically balanced to meet the requirements for microbial and digestible proteins. They reported increased responses in milk, lactose, protein, and fat with no significance difference between treatments by time relative to these parameters. The increase in gross efficiency of dietary protein and energy utilisation observed in the study by Robinson *et al*. (1995) could be explained by the dietary characteristics, making the cows more responsive to RPM and RPL supplementation. The production of lactose requires a large proportion of glycogenic AA, thus supporting milk production, but the ability of mammary tissue to convert glucose to lactose is partly mitigated by absorbed AA. Noftsger & St-Pierre (2003), supplemented RPM and increased dietary soya bean meal from 6.83 to 7.68 % DM and blood meal from 0 to 1.8 % DM and reduced meat meal by 8 % DM, while keeping $NE_{L,}$ NDF, ADF and Ash concentration constant in the diet. They observed significant improvements in milk yield (40.8 *vs.* 46.6 kg/d), milk protein (2.95 *vs.* 3.09 %), N efficiencies (29.5 *vs.* 35.0 milk N/N intake) and environmental efficiency (2.47 *vs*. 1.89 kg N excreted/kg milk N). This demonstrated that post ruminal digestibility of RUP and the AA balance reaching the SI could be more important than RUP supplementation alone. When high producing (> 36 kg/d) Holstein cows, after peak-lactation (111 ± 18 DIM), were supplemented with RPL, Bernard *et al*. (2014) reported an increase in milk yield, milk fat and a tendency towards increased milk protein percentages. However, in contrast to high producing cows, lower producers (< 36 kg/d) only had a tendency for increased milk protein percentages. This suggests that a positive response to supplementation is expected in higher producing cows rather than lower producers, which is in agreement with the statement made earlier. Chalupa *et al*. (1999) formulated dairy cow diets supplemented with AA and increased the diet's metabolisable Met from 1.89 to 2.35 % and the metabolisable Lys from 6.38 to 7.45 % resulting in a Lys:Met ratio of 3.2:1. Milk production increased by 5.1 %, milk protein 8 % and milk protein production 18 %. This study clearly demonstrates that a response to RPAA supplementation can be expected when the diet supplies less than required levels of both Lys and Met, Lys:Met ratio or reduced levels of MP. It also emphasises the importance of knowing the balance of EAA reaching the cows intestinal absorptive site and the ability to manipulate this balance.

In another comprehensive meta-analysis, Vyas & Erdman (2009) evaluated the response of milk protein (g/d) to Lys supplementation, Lys was supplemented either post-ruminally by abomasal and/or

duodenal infusions or fed in rumen protected form. They concluded that the response of milk protein to Lys supplementation decreased from 5.0 to 3.2 g per gram of metabolisable Lys intake, which could vary from 80 to 203 g/cow/d of MP. Assuming that milk protein has a Lys concentration of 2.76 g/100 g, it could imply a decreasing marginal efficiency of metabolisable Lys above maintenance from 39 to 25 %. The decrease in efficiency would be expected as the efficiency of utilisation of metabolisable AA decrease when supply approaches the cow's AA requirements (see section 2.5 for an in-depth discussion on post-ruminal AA metabolism). Thus, oversupplying Lys may overestimate the milk protein responses to Lys supplementation (Vyas & Erdman 2009). A poor conversion efficiency could also be attributed to an energy limitation, which would most probably be the case when cows are grazing high-quality ryegrass pasture (Younge *et al*., 2000). Wang *et al*. (2010) fed a diet slightly liming in MP but adequate in energy to Holstein cows in mid-lactation (120 DIM) with a concentration of Met and Lys in MP of 1.87 and 5.93 % respectively. Supplementing RPL or RPM at a rate of 0.49 and 0.15 %, respectively, improved milk yield significantly for RPL (+ 1.5 kg/d), RPM (+ 2.0 kg/d) and the combination RPML (+ 3.8 kg/d). Cows fed the RPM and RPML in combination had higher milk fat content (4.0 and 3.9 %) than the control and RPL diets (3.6 and 3.67 %), respectively. This is an important observation, since cows on pasture could be supplemented with dietary energy above their requirements and still not respond to RPAA supplementation, which could be determined by whether the concentration and/or quality of MP reaching the cows SI limited performance. Furthermore, it has been demonstrated that supplementation with RPM and RPL can play a role in alleviating the milk protein depression observed when supplementing fat to dairy diets (Cant *et al*., 1993).

In addition to increases in milk production and milk protein production, there are reports of increased fat production with Met and/or Lys supplementation. The increase in milk fat is generally associated with increases in milk protein and has proven not to be predictable. Although it is not clear why milk fat sometimes increases when Met and Lys are increased in MP. Pisulewski *et al*. (1996) suggested that the effect of Met on *de novo* synthesis of short- and medium-chain fatty acids in the mammary gland may explain the increases in milk fat. The NRC (2001) explains a different possibility that might be related to the role AA play in the hepatic and intestinal synthesis of chylomicrons, including low density lipoproteins.

This literature review confirms that greater responses in cow performance to RPAA supplementation may be achieved when: 1) the basal diet fed to the cows is imbalanced with regards to the concertation and ratio of Lys and Met in the RUP content, 2) RUP, as a proportions of MP, constitutes the largest proportion, 3) cows are high yielding and in early-lactation and 4) are supplied energy at or above cow requirements.

2.7.1.2 Body weight and body condition score

As opposed to BW, BCS is a subjective assessment of the cow's adipose and muscle stores (Roche *et al.*, 2009). Adipose tissue reserves are maintained stringently by peripheral and central produced

hormones, which are in accordance with the "lipostatic" theory (Kennedy, 1953; Roche *et al.*, 2008). Wildman *et al.* (1982) proposed a body condition scoring system with a scale from one to five at any point during the cow's lactation cycle, by palpating the cow's back and hindquarters, and by means of visual appearance. A score of one indicates a severe under condition (thin) and a score of five a severe over condition (fat).

Measuring BCS is crucial since the impact of the cows BCS on her performance, reproduction, health and welfare may be significant (Roche *et al.*, 2009). This practical-on farm management tool may be used to measure and track changes in the cow's condition which is a result of BCS mobilisation (i.e. lipolysis) and/or replenishment (i.e. lipogenesis). However, cow body condition, or change in BCS, is a result of various factors, including hormones (i.e. somatotropin, insulin, catecholamine's and leptin), metabolic pathways, physiological status (i.e. pregnancy and DIM) and multiple interrelated interaction between these factors. Supplementation studies of RPM and/or RPL in relation to effects on cow BW and/or BCS are limited and the reason for changes observed in BW could only be attributed to the metabolic pathways that Met and Lys are involved with in the cow's body (Blum *et al.*, 1999; Patton *et al.*, 2015), as discussed earlier in this section.

2.7.1.3 Plasma amino acid levels

Since the assumption is made that the cows plasma AA levels would be highly associated with the AA profile of MP reaching the intestinal absorptive site (Broderick *et al.*, 1974; Patton *et al.*, 2015; Martineau *et al.*, 2017), plasma AA levels could be used to interpret the changes in plasma AA levels relative to responses observed in cow performance.

Lee *et al.* (2015) fed Holstein cows in mid-lactation a maize-soy based TMR, deficient or adequate in MP supply with a MP balances of -281 and -24 (g/cow/d), respectively. The diets were supplemented with digestible RPM (15 g/cow/d) and/or RPL (24 g/cow/d) in combinations with these two diets. Plasma Met levels increased significantly (12.6 *vs.* 19.0 μm) when the MP adequate diet was supplemented with both RPM and RPL, although not for plasma Lys (50.9 *vs.* 58.7 μm). Plasma Lys marginally increased when the MP deficient diet was supplemented with Lys (52.2 *vs.* 60.7 μm). Treatments had no effect on total tract digestibility, milk yield and milk composition, even though the blood plasma indicated that more of Met and Lys was available for milk and milk component production. In the study of Lee *et al.* (2015) abomasal pulse doses of ladled ^{15}N-Lys and ^{13}C-Met were used to label plasma Lys and Met, analysis of decay curves showed faster extractions of Lys and Met from the blood when the MP deficient diet was fed compared to the MP adequate diet. Suggesting that the efficiency of utilisation of dietary AA increases with decreasing MP supply. Thus, the authors concluded that the main effect of duodenal EAA on plasma EAA is linear.

Patton *et al*. (2015) demonstrated that abomasal or duodenal infusions of Met and/or Lys and His, including casein revealed that Met or Lys infused alone increased the plasma concentration of the infused EAA and lowered the concentration of other EAA, particularly His. Infusion of Lys and Met or His alone was associated with increases in concentrations of EAA without affecting others. Rulquin & Delaby (1997) supplemented grazing dairy cows with 13 g/d of RPM and reported a 27 % increase in plasma Met levels. Bethiaume *et al*. (2006) also reported an increase in plasma Met concentration in response to RPM supplementation and no effect on milk production and milk protein yield, although a linear increase in true protein content was observed. This is in agreement with others (Blum *et al.*, 1999), who observed a linear increase in total splanchnic output of Ile, Leu, Phe and Thr suggesting that RPM supplementation may have triggered a homeostatic response resulting in a decrease utilisation of specific AA by the liver and GIT.

In a multilevel mixed-effects meta-analysis Martineau *et al*. (2017) evaluated the relationship between post ruminal casein infusion and milk production, and plasma AA concentrations. The data set contained 147 treatment means and one of the main objectives of their study was to review the relationship between change in estimated MP supply and the responses of various production variables (i.e. milk production and milk composition) and plasma AA concentrations. They found that changes in MP supply was positively associated with plasma AA concentrations.

As suggested by the literature reviewed, blood plasma levels could be used to identify AA limitation and determine, to a relative degree, the bioavailability of the supplemental RPAA supplied. A further suggestion is to use the plasma AA kinetics as a means of interpreting production responses, or the lack thereof, observed in AA supplementation studies.

2.7.1.4 Milk nitrogen fractions

Milk N can be divided into three broad fractions which include, 1) casein N, 2) whey protein N and 3) NPN, which constitutes approximately 77.9, 17.2 and 4.9 %, respectively, of total N found in the milk of dairy cows (Cerbulis & Farrell Jr, 1975). Casein as a proportion of total milk protein is within the range of 76 to 86 % and comprise of four main gene expressions which include α_{s1}-, α_{s2}-, β-, and κ- caseins, where γ-caseins present in milk are C-terminal fragments of β-caseins resulting from the plasmin activity in milk (Mercier & Gaye, 1982). On the other hand, whey protein N comprise of proteins which are gene expressions produced in the mammary gland that include β-lactoglobulins, α-lactalbumins and those which are transferred from the periphery, which include serum albumin and immunoglobulins. Finally, the NPN fraction found in milk protein may be up to 7 %, constituting of about 50 % urea N and the rest of the fraction is made up of ammonia, peptides, creatine, hippuric acid, uric acid and other NPN containing components (DePeters & Ferguson, 1992).

This represents a small overview of milk N fractions in milk, which has been extensively reviewed (Brunner, 1981; DePeters & Ferguson, 1992). The importance of these fractions is due to the major role

they play in the secondary dairy industry (i.e. cheese and other fermented products), including the current focus on the biological value in human nutrition and health (Pereira, 2014). Casein and whey are classified as a high-quality protein source, especially in human nutrition (i.e. AA requirements). The reason casein and whey are classified as high-quality protein is due to their high digestibility, bioavailability and metabolic activity (i.e. mineral binding capacity and bioactive peptides) and especially the role these fractions play in the prevention of some chronic human diseases (i.e. cardiovascular, cancers, obesity and diabetes) (Holt *et al.*, 2013). Schwab (1996) also reported that most of the responses to AA supplementation from lactating dairy cows have been in the casein fraction of milk protein. Similarly, Třináctý *et al*. (2009) found RPM and/or PRL supplemented to high producing dairy cows to have significantly higher percentages of casein compared to supplemented cows. Hurtaud *et al*. (1995) and Pisulewski *et al*. (1996) came to the same conclusion that RPML supplementation could improve the casein yield in milk. Various studies confirmed that milk casein is affected to a greater extent than the whey and NPN fractions in milk (DePeters & Ferguson, 1992; Schwab, 1996).

2.8 The use of models to assist in formulating diets for amino acids

The CNCPS (Fox *et al.*, 1992, O'Connor *et al.*, 1993) and CPM-dairy (Tedeschi *et al.*, 2015) models were among the first to attempt to balance diets of lactating dairy cows for AA. Using these models as tools to better understand which nutrients are limiting the grazing cow's performance and how to supply these limiting nutrients through concentrates are promising (Kolver *et al.*, 1998; Tylutki *et al*., 2008). Thus, when reformulating or balancing supplementary diets, while taking into account the nutritional deficiencies from pasture-based systems, nutritionist could potentially improve cow performance even from the same level of PDMI and supplemental concentrate level (Kolver, 2003). The CNCPS model makes use of mechanistic and empirical calculations to predict ME and MP requirements of dairy cows and the supply of these fractions from various diets, by using published data from multiple studies, which include different equations, validations and inputs (Fox *et al.*, 1992; O'Connor *et al.*, 1993; Lanzas *et al*., 2007; Van Amburgh *et al*., 2010; Higgs *et al.*, 2013). Of particular interest regarding our study is the data published, and used in this model, by Tylutki *et al*. (1994) and Diaz *et al.* (2001), regarding the AA content of milk and bodily tissues. Including work done by, Russell *et al*. (1992) and Sok *et al*. (2017) on MCP predictions and O'Connor *et al*. (1993) and Fox *et al*. (2004) on predictions for metabolisable AA available from RUP, as well as predictions of microbial protein and AA production. The main goal of the CNCPS is to serve as a functional tool for research development and feed formulation of cattle (Russell *et al*., 1992).

Kolver *et al*. (1998) evaluated the CNCPS model in production systems that utilized pasture. Data from eight pasture studies (25 treatments), conducted in NZ and the USA, were used to evaluate the CNCPS models' predictive ability and the models' ability to be used in formulating pasture-based diets. The model provided a relatively good estimates of change in BCS, blood urea nitrogen, flow of MCP, milk yield and

the cow's energy status under various grazing conditions. However, the predicted milk yield was very sensitive to changes in pasture peNDF, lignin content and fibre digestion as well as, the AA composition of the ruminal microbes. The model accurately predicted that ME was limiting milk production when high-quality pasture was fed without supplementation. Additionally, Hongerholt & Muller (1998) used the CNCPS model to compare the actual *vs*. model predicted values for their study, the model predicted ME and MP to be equally liming in milk production for cows fed a low RUP (14.7 % CP and 47 % RUP of CP) concentrate while consuming similar levels of DM (Pasture RUP was 15.8 % of CP). However, there was no difference in the observed and predicted milk yields. Similar milk yields were also observed between the observed and predicted milk yields when a concentrate higher in RUP were fed, although this time ME was limiting milk production and not MP and/or dietary AA. The model predicted ME allowable milk similar to the value that was observed, while the MP allowable milk was higher. This may help to explained why the cows did not respond to an increase in concentrate RUP content, because ME was first liming. This might also suggest that the MP reaching the SI, in the absence of ME being limiting, would have been adequate for higher milk and milk component production than observed.

Dinn *et al*. (1998) used the CNCPS model to evaluate the response of lactating dairy cows to RPML supplementation along with decreased dietary CP levels. A TMR was balanced according to degradation rates and rates of passage for both protein and carbohydrates. The dietary CP levels was (18.3, 16.7 and 15.3 % DM), respectively for diet 1, 2 and 3. Diet 1 was top dressed with no RPAA, diet 2 were top-dressed with 13 g RPM (70 % DL-Met) and 12 g RPML (15 % DL-Met and 50 % L-Lys mmonohydrochloride) and diet 3 was top dressed with 7 g RPM (70 % DL-Met) and 15 RPML (15 % DL-Met and 50 % L-Lys mmonohydrochloride). Cows receiving diet 1 had higher milk yield (34 *vs*. 33 kg/cow/d) and DMI compared to diet 2 and 3, but milk protein production was similar across treatment groups. The NUE and milk N: N intake ratio improved as the dietary CP level were reduced. The authors concluded that dietary CP could be used more efficiently with the supplementation of rumen protected AA.

The CNCPS model also takes into account differences in DMI, physical activity, cost of excreting urea, milk composition, and BW of grazing cows compared to TMR fed cows, predicts a higher milk production in the latter (Kolver *et al.*, 1998). However, it was the ability of the CNCPS model to accurately predict ME and MP adequacy which played a fundamental role in formulating diets of dairy cows which are balanced for AA (Pacheco *et al.*, 2012).

In 1993 O'Connor *et al*. (1993) made recommendation for improvements of the CNCPS model. These recommendations include, 1) Research focused on ECP, specially the AA composition of these fraction, is needed to define the AA requirements for metabolic faecal protein losses quantitatively; 2) The AA composition of tissue protein needs to be better defined in relation to various components of body proteins; 3) More research focusing on gestation is required to optimise the AA requirements of the cow during gestation; 4) The utilisation efficiency of absorbed AA for specific physiological functions needs to be better defined; 5) The AA composition of soluble and insoluble dietary protein not degraded in the rumen

needs to be defined more accurately for various natural and by-product feedstuffs; 6) The AA composition and digestibility of bacterial cell wall and non-cell wall protein fractions require more research to improve estimates of these fractions.

However, over the years new laboratory methodology and animal sampling techniques have generated the potential to improve the CNCPS model's prediction capabilities for energy, protein and AA requirements and supply. These improvements led to new and updated versions of the CNCPS model (Tedeschi *et al*., 2002; Fox *et al*., 2004; Tylutki *et al*., 2008; Higgs, 2012; Van Amburgh *et al*., 2013). More recently Higgs *et al*. (2015) and Van Amburgh *et al*. (2015) reported on updates and improvements of the CNCPS model. These updates include, 1) Chemical composition of the feeds in the feed library, estimation of digestion kinetics of the protein fraction of the feed, including the AA profiles and efficiency of EAA use for lactation and cow maintenance (Lapierre *et al*., 2007); 2) Changes to the N pool structures and assignments, including a new system to determine N recycling more mechanistically, including the capability of the model to determine the digestibility of N from indigestible N with an *in vitro* method; 3) Capabilities to characterise the unavailable fibre fraction by means of $uNDF_{240}$ rather than using the standard method of lignin × 2.4 and the expansion of the potentially digestible NDF fractions into two separate pools as opposed to one as reported by Raffrenato (2011); 4) Improved rates of passage for the NDF fraction were also included into the latest model; 5) Dynamic structure for the GIT and the expansion of the post ruminal model to include the large intestine and SI separately. The model presents the large intestine mechanistically; 6) Microbial sub-model that includes protozoa into the estimation of microbial protein yield, which improved the sensitivity of MP prediction. These improvements allowed the CNCPS model to be more robust in formulating diets.

2. 9 Conclusion and hypothesis

The reviewed literature clearly demonstrates that there might be potential to improve the grazing dairy cow's performance when supplemented with RPAA. However, to realise these potential improvements in performance, the cow's requirements for energy must be met through adequate supply of rumen fermentable carbohydrates, since pasture ME is most likely to be inadequate for the high-producing dairy cow. This will allow the cows to utilise the highly degradable CP from pasture more efficiently, thereby increasing MCP flow to the SI. When this synchronous release of energy and NH_3 in the rumen is "optimised", the AA flowing to cow's SI could be improved by the individual supply of RPAA, as opposed to supplying a high-quality protein source (i.e. fish meal and/or blood meal), which is high in RUP. The individual AA target are the EAA, specifically Met and Lys, since these two AA are frequently regarded as the two most limiting AA in dairy cow's protein and AA nutrition. This is particularly applicable to cows fed high levels of a maize-based concentrate while grazing high quality ryegrass pasture.

Interpreting this literature review has led to the hypothesis that high-producing dairy cows grazing high-quality ryegrass pasture in spring, while receiving a concentrate containing high levels of maize, might respond positively to the supplementation of RPM and/or RPL.

Chapter 3: Materials and methods:
Effect of dietary protein quality and amino acid supplementation on performance of high producing Jersey cows grazing ryegrass pasture

3.1 Ethics approval

This study and the use of animals was approved by the University of Pretoria's Animal Use and Care Ethics Committee according to the South African National Standard (SANS 10386-2008) with the approval number EC041-18.

3.2 Study objectives

The objective of this study was to investigate the effect of supplementing RPL or a combination of RPL and RPM on the production performance of high producing Jersey cows grazing ryegrass pasture and receiving a maize-based concentrate. The performance parameters measured were milk production, milk composition, body weight and body condition change. The effect of supplementation on milk N fractions and plasma AA were also measured. A secondary objective was to evaluate a urine spot sampling technique and allantoin excretion as a non-invasive method to estimate microbial protein synthesis.

3.3 Study location, climatic conditions and duration

The study was conducted at the Outeniqua Research Farm in the Western Cape Province of South Africa near George, with an altitude, latitude and longitude of 204 m above sea-level, 33°58′38′′S and 22°25′16′′E, respectively. This area is classified as having a temperate climate with a long term (52 years) mean annual rainfall of 712 mm (range 450 to 1029 mm) and a mean spring season rainfall of 70.3 mm (range 30.7 to 185 mm). The long term (18 year) mean maximum and minimum daily temperatures for the George area are 18.7 and 7.7 °C for August; 19.5 and 8.7 °C for September; 20.6 and 10.8 °C for October and 21.8 and 11.6 °C for November, respectively (ARC, 2018).

Daily average maximum and minimum temperatures for the trial period was 18.6 and 7.5 °C during September and, 23.1 and 11.9 °C during October and 21.5 and 11.5 °C for November, respectively, as recorded by an on-farm temperature port (Decagon devices., Inc., 2365 NE Hopkins Ct. Pullman, WA, USA). All climatic data are available at the Outeniqua Research Farm as measured by an on-farm weather station, recording precipitation, temperature and evaporation throughout the year. See Chapter 4, section 4.1, for a detailed description of the climatic conditions recorded during the trial in comparison with long term climatic data.

The study was conducted during spring of 2018 (27 August to 8 November), with the seasonal distribution classified as spring ranging from 1 September to 30 November, summer from 1 December to 28 February, autumn from 1 March to 30 May and winter from 1 June to 30 August (Fulkerson *et al*. 2007).

Cows entering the trial were selected on 24 August 2018 and started an adaptation period on 27 August 2018 for 14 days until 10 September 2018. Collection of samples and data took place from 10 September to 8 November 2018 for a total of 60 days.

3.4 Grazing camp design, pasture and soil

The grazing camp on which the study was conducted at the Outeniqua Research Farm consisted of 8.55 ha permanent kikuyu/Italian rye grass pasture. The camp was permanently divided by electrical fencing into 39 equal strips, 150 m in length and 15 m in width. Each strip could further be divided in 10 temporary blocks by means of temporary electrical fencing depending on the amount of pasture allocated. Each block represented 225 m^2 pasture. This area was also under permanent irrigation as shown in Figure 3.1.

Italian ryegrass (*Lolium multiflorum* var. *italicum*) cv. Fox, an annual ryegrass species, was over-sown into the permanent kikuyu (*Pennisetum clandestinum*) pasture at a seeding density of 25 kg/ha on 27 March 2018. Before seeding, the kikuyu pasture was grazed to a stubble height of 5 cm (RP_{Meter} reading of 10) and mulched (Nobili mulcher 1.6 m wide with 24 blades) to ground level (Botha, 2003). After mulching the kikuyu pasture the ryegrass seed was planted using a no-till direct drill planter (Aitchison 3116C sematic 2.4 m wide with 16 rows), after which the seed bed was lightly compacted with a 2.3 m Cambridge roller (Botha, 2002). The kikuyu pasture was assumed to be dormant during the study, thus the pasture was predominantly ryegrass.

After each grazing the pasture was top-dressed with 100 kg/ha of limestone ammonium nitrate (LAN) which contains 28 % N, resulting in 28 kg N/ha being applied on the pastures after each grazing. The pasture was irrigated according to tensiometer readings to maintain a kilopascal (kPa) level between -10 and -25 kPa (Botha, 2002). The grazing camp mainly consisted of two distinct soil types namely Estcourt and Witfontein (Soil Classification Working Group, 1991). Estcourt is found more towards the north of the grazing camp and Witfontein more to the south where the grazing camp is slightly downward sloping (Figure 3.1).

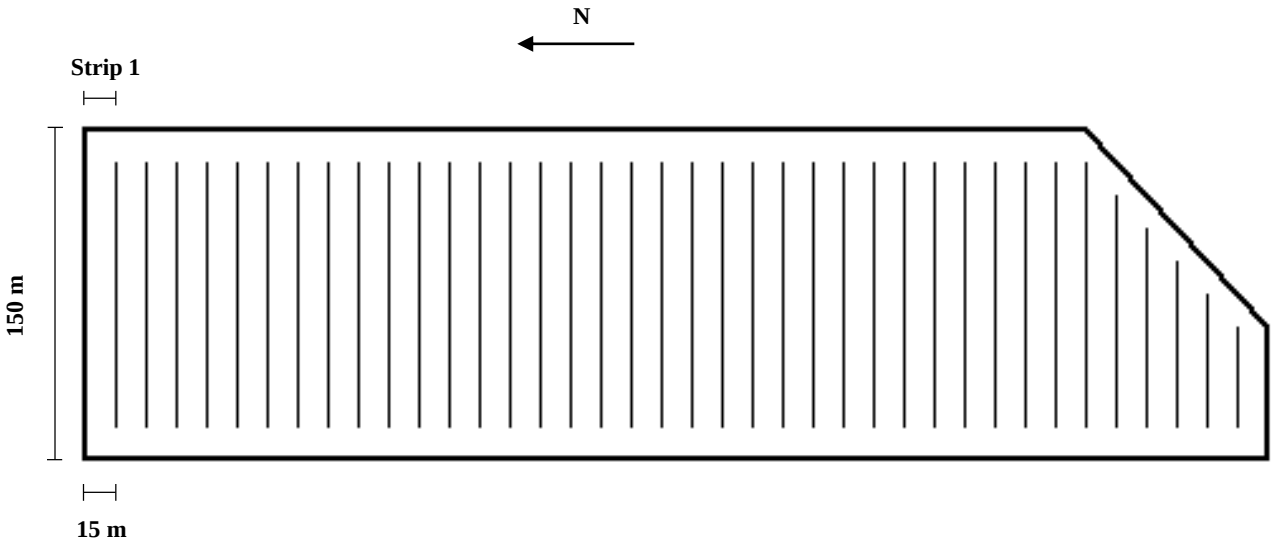

Figure 3.1 Grazing camp design of the 8.55 ha kikuyu/Italian ryegrass pasture grazed by Jersey cows during the trial at the Outeniqua Research Farm

3.5 Experimental design, cow management and cow welfare

Sixty high producing multiparous Jersey cows [BW, 408 ± 42.8 kg; milk yield, 22.1 ± 2.53 kg/d; parity 4.40 ± 1.75; DIM, 100 ± 64.8; (mean ± SD)] from the Outeniqua Research Farm herd were used in the study. The herd (367 lactating cows) average for milk production from which the study cows were selected was 17.4 ± 4.37 kg/d; (mean ± SD) in August 2018. See Appendix A Table A1.

The experimental design was a randomised complete block design, in which the cows were blocked according to pre-experimental milk production (of the previous 21 days), DIM and lactation number and randomly allocated to three groups within each block. Subsequently, each group was randomly allocated to one of three experimental treatments (Control (C), rumen protected lysine (RPL) and rumen protected Met plus rumen protected Lys (RPML)) using the RANDOM function of Microsoft® Excel (See section 3.6.1 below for treatments). After blocking, the cows within each block were balanced for body weight. The blocking of the individual cows and allocation to the respective treatments are shown in Appendix A Table A1.

Blocking of the cows resulted in 20 cows per experimental group (C, RPL and RPML) with average milk productions of 22.1 ± 2.50, 22.1 ± 2.53 and 22.1 ± 3.0 kg/d (mean ± SD), respectively, at the beginning of the study. Mean DIM on 24 August 2018 when cows were selected was 97.2 ± 68.3, 98.1 ± 63.6 and 105 ± 69.5 days (mean ± SD) and lactation number, 4.45 ± 1.93, 4.75 ± 1.77 and 4.1 ± 2.06 (mean ± SD), respectively, for the C, RPL and RPML experimental groups. Mean body weights for each experimental group was 411 ± 43.2, 414 ± 43.1 and 400 ± 42.4 (mean ± SD), respectively. See Appendix A Tables A2, A3 and A4.

The cows strip grazed pre-allocated ryegrass pasture twice a day, after each milking, so that the cows had access to fresh pasture directly after each milking (See section 3.7.1 in this chapter for allocation of pasture). Cows were milked twice a day at 0500 and 1330 h.

The average distance that the cows walked from the pasture to the milking parlour was an estimated 0.94 km (range 0.57 to 1.19 km). Cows were allowed to graze 24 hours per day, excluding milking times, and clean water was provided freely throughout the day. All the cows grazed together as a single herd to ensure equal pasture allocation and availability.

The cows in the three treatment groups each received their respective concentrate (treatment) in the milking parlour during milking. Since all the cows in the study grazed together as a single herd, they were separated before milking into their respective treatment groups. All cows in a specific treatment group entered the milking parlour together to be milked and fed their respective experimental treatments. To assist in separating the cows into the respective treatment groups each cow was marked with a coloured tag hanging from the neck, with different colours allocated to each respective treatment (Figure 3.2). The experimental concentrates were placed in colour coded bags corresponding to each treatment colour as follow: C was yellow, RPL blue and RPLM red.

Figure 3.2 Cow identification by means of a coloured tag hanging around their necks

3.6 Experimental treatments and diet

The cows were fed the experimental treatments from 27 August until 8 November 2018. The trial was 74 days in total with a 14-day adaptation and a 60-day sampling period (10 September to 8 November

2018). All the cows received 4 kg (as is) of their respective experimental concentrates at each milking (0500 and 1330 h), half of the daily allowance, for a total daily intake of 8 kg/cow/day (7.26 ± 0.41 kg DM; (mean ± SD)).

3.6.1 Experimental treatments:

Treatment 1: Control treatment (**C**), grazed ryegrass pasture plus 7.24 kg DM (8 kg as is) per day of a maize-based pelleted concentrate without any Met and/or Lys supplementation.

Treatment 2: Lys treatment (**RPL**), grazed ryegrass pasture plus 7.25 kg DM (8 kg as is) per day of concentrate pellets containing 53.12 g LysiGEM™ (Kemin industries©., Inc., USA., Reg No, V27404, Act 36 of 1947), providing approximately 22.0 g/cow/d of intestinally absorbable Lys, as per company product description.

Treatment 3: Met and Lys treatment (**RPML**), grazed ryegrass pasture plus 7.28 kg DM (8 kg as is) per day of concentrate pellets containing 41.68 g MetaSmartDRY® Dry (Adisseo., Inc., Antony, France, S.A.S., Reg No, V19417, Act 36 of 1947) and 53.12 g LysiGEM™, providing approximately 9.3 and 22 g/cow/d of intestinally absorbable Lys and Met as per company product description.

3.6.2 Experimental diet:

The concentrate was supplied to the Outeniqua Research farm by Nova feeds, George (Saagmeul St., George Industries, P.O. Box 1351, George, 6530). All the concentrates was prepared and pelleted on the same day using the same feed components by Nova feeds. Half of the concentrate were delivered at the onset of the study and the other half was stored at Nova's factory in George and delivered as required.

After arrival on the farm, the respective concentrates were weighed (4 kg as is) using a laboratory scale (Micro TZE, Bentrose, Johannesburg, RSA (maximum = 30 kg; ± 0.005 kg)) into plastic bags to ensure that each cow received the correct amount of concentrate allocation. The ingredient composition and chemical composition of the three concentrates used for the study based on analyses of the UP Nutrilab (Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria, Gauteng, RSA) are shown in Table 3.1. The EAA composition of the three concentrates as analysed at Elsenburg (Western Cape Department of Agriculture, Stellenbosch, Western Cape, RSA) is shown in Table 3.2.

The concentrates were formulated to be iso-nitrogenous with urea being used to standardize the CP concentration of the three treatments, since the effect of supplementing Met and/or Lys on dairy rations

containing equal concentration of CP was evaluated. The RPAA were mixed with feed lime and premix and then added and mixed into to the concentrate mixture.

The mean chemical composition of the ryegrass pasture that the cows grazed during the study is shown in Table 3.3. See Chapter 4, section 4.3, for a discussion on the change in nutrient composition of the ryegrass pasture over time.

Table 3.1 Ingredient and chemical composition of concentrate pellets fed to Jersey cows grazing ryegrass pasture (n = 2)

	Experimental treatment[1]		
	C	**RPL**	**RPML**
Ingredient composition DM %			
Maize meal	77.0	77.0	77.0
Soybean oilcake meal	8.00	8.00	8.00
Wheat bran	6.09	5.50	5.00
Molasses	4.88	4.88	4.88
Feed lime	2.50	2.50	2.50
LysiGEM™	0.00	0.75	0.75
MetaSmartDRY®	0.00	0.00	0.57
Mono-Calcium Phosphate	0.40	0.40	0.4
Salt	0.50	0.50	0.50
Magnesium Oxide	0.30	0.30	0.30
Urea	0.23	0.07	0.00
Premix[2]	0.10	0.10	0.10
Chemical composition[3] DM %			
DM %	90.6	90.6	91.0
Ash	5.84	6.25	6.62
OM	94.1	93.9	93.4
GE (MJ/kg DM)	16.4	16.3	16.2
ME (MJ/kg DM)	12.8	12.3	12.6
Starch	53.6	56.9	55.5
CP	12.7	13.3	12.7
NDF	9.25	9.35	9.95
ADF	3.22	3.22	3.14
ADL	1.23	1.45	1.46
NFC	70.7	69.2	67.2
EE	1.55	1.91	3.59
*IV*OMD	95.2	92.3	94.7
Ca	1.16	1.26	1.24
P	0.48	0.49	0.50
Ca:P ratio	2.42	2.58	2.48

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

[2]Premix (Advit Animal Nutrition (1 kg/t inclusion), Cape Feed and Grain – (per unit of premix); Vitamin (Vit) A: 6 million (MM) IU; Vit D3: 1 MM IU; Vit E: 8000 IU, Mn 50 g, Zn 100 g, Cu 20 g, I 1.7 g, Se 0.3 g and carrier Dolomite: 440 g)

[3]DM – Dry matter; OM – Organic matter; GE – Gross energy; ME – Metabolisable energy (ME = GE × *IV*OMD × C (C = 0.82; Robinson *et al*., 2004)); CP – Crude protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; ADL – Acid detergent lignin; NFC – Non-fibre carbohydrates (NFC = 100 – (CP + NDF + EE + ash)) (NRC, 2001); EE – Ether extract; *IV*OMD – *in vitro* organic matter digestibility; Ca – Calcium; P – Phosphorus

Table 3.2 Mean (± SD) essential amino acid (EAA) composition (% DM) of the concentrate pellets fed to Jersey cows grazing ryegrass pasture (n = 2)

	Experimental Treatments[1]		
	C	**RPL**	**RPML**
EAA			
Lysine (Lys)	0.50 ± 0.08	0.65 ± 0.03	0.60 ± 0.07
Methionine (Met)	0.26 ± 0.01	0.24 ± 0.07	0.31 ± 0.01
Arginine (Arg)	0.89 ± 0.05	0.86 ± 0.05	0.85 ± 0.03
Histidine (His)	0.35 ± 0.04	0.29 ± 0.05	0.28 ± 0.02
Isoleucine (Ile)	0.72 ± 0.04	0.67 ± 0.03	0.67 ± 0.03
Leucine (Leu)	1.30 ± 0.07	1.35 ± 0.07	1.28 ± 0.01
Phenylalanine (Phe)	0.71 ± 0.02	0.74 ± 0.09	0.77 ± 0.03
Threonine (Thr)	0.53 ± 0.06	0.46 ± 0.03	0.42 ± 0.01
Valine (Val)	0.70 ± 0.14	0.64 ± 0.05	0.62 ± 0.03
Lys:Met ratio	1.95	2.66	1.97
Total EAA	5.96	5.90	5.75

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

Table 3.3 Mean (± SD) chemical composition of the ryegrass pasture grazed by Jersey cows (n = 8)

Parameter[1]	**Mean (% DM or as stated) ± SD**
DM %	15.0 ± 1.04
Ash	10.5 ± 0.76
OM	89.5 ± 0.76
GE (MJ/kg DM)	16.3 ± 0.17
ME (MJ/kg DM)	10.3 ± 0.26
CP	18.8 ± 2.00
NDF	42.1 ± 2.83
ADF	24.9 ± 1.98
ADL	5.15 ± 2.31
NFC	25.4 ± 4.74
NDIP (% NDF)	5.61 ± 0.89
ADIP (% ADF)	6.83 ± 1.50
EE	3.23 ± 0.15
*IV*OMD	77.4 ± 4.91
Ca	0.34 ± 0.04
P	0.38 ± 0.04
Ca:P ratio	0.90 ± 0.09

[1]DM – Dry matter; OM – Organic matter; GE – Gross energy; ME – Metabolisable energy (ME = GE × *IV*OMD × C (C = 0.82; Robinson *et al*., 2004)); CP – Crude protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; ADL – Acid detergent lignin; NFC – Non fibre carbohydrates (NFC = 100 – (CP + NDF + EE + ash)) (NRC, 2001); NDIP – Neutral detergent insoluble protein; ADIP – Acid detergent insoluble protein; EE – Ether extract; *IV*OMD – *in vitro* organic matter digestibility; Ca – Calcium; P – Phosphorus

Table 3.4 Mean (± SD) essential amino acid (EAA) composition (% DM) of ryegrass pasture grazed by Jersey cows (n = 8)

Parameter	Trial period[1]
	Ryegrass pasture
EAA	
Lysine (Lys)	0.75 ± 0.06
Methionine (Met)	0.43 ± 0.03
Arginine (Arg)	1.06 ± 0.04
Histidine (His)	0.30 ± 0.02
Isoleucine (Ile)	0.89 ± 0.04
Leucine (Leu)	1.40 ± 0.04
Phenylalanine (Phe)	1.27 ± 0.05
Threonine (Thr)	0.74 ± 0.03
Valine (Val)	0.99 ± 0.03
Lys:Met ratio	1.74
Total EAA	7.82

[1](10 September to 8 November 2018)

3.7 Experimental data collection, analytical methods and calculations

3.7.1 Pasture yield, intake and allocation

Pasture yield, intake and allocation were determined using a rising plate meter (RP_{Meter}, Filip's folding plate pasture meter, Jenquip, Rd 5, Fielding, NZ). To calibrate the RP_{Meter}, regression samples were taken weekly. Three low, medium and high samples (9 in total), 3 cm from the ground, were measured with the RP_{Meter} and cut using a sampling ring and sheers (Figure 3.3). Samples were taken between 7 September and 2 November 2018 on days 1, 5, 12, 19, 26, 33, 40, 47 and 54 during the 60-day trial period.

After the pasture samples were cut and weighed using a laboratory scale (Sartorius BP8100, Göttingen, Germany (maximum = 8100 g; ± 0.1g), the samples were oven-dried in a laboratory oven (LABCON (Pty) Ltd. FSIM 16, Maraisburg, 1700, RSA) at 60 ºC for 72 hours to determine DM % (Van Vuuren *et al.*, 1992). Since the sampling ring used to sample the pasture and the plate of the RP_{Meter} used to measure pasture height had a similar circumference a fixed measurement of 0.0985 m^2 was used along with the DM value obtained to calculate pasture yield kg DM/ha. As a result, a RP_{Meter} reading could be paired with a corresponding pasture DM yield.

Since regression samples were taken on a weekly basis a composited linear regression equation could be determined to estimate DM yield, PA and PDMI throughout the study. To determine this linear regression equation (Y = mH + b) obtained during the study the LINEST Function in Microsoft® Excel were fitted to the data (pre- and -post grazing RP_{Meter} readings and DM content). Where Y = kg DM/ha, m = gradient, H = mean RP_{Meter} height (1 click on the RP_{Meter} = 0.5 cm) and b = intercept. Since the RP_{Meter} height is related to DM yield, this equation was used to determine the DM yield at a given RP_{Meter} reading. As a result, the equation Y = 91,57 (H) – 344,64 (R^2 = 0,742) was obtained, see Chapter 4, section 4.2 for

the estimation of this regression equation, PDMI and PA. However, as this could only be done after the trial, the standard equation Y = 81.05 (H) – 178.94 (R^2 = 0,831) (Van der Vyver, 2019) was used during the trial to determine daily PA. Thus, after the available DM had been calculated, the area (m^2) required for a PA of ~11 kg DM/cow/day could be estimated. A higher PA than normal (PA of 10 kg DM/cow/d) was aimed for to avoid low PDMI as pasture intake is often identified as a factor limiting milk production in pasture based systems (Bargo *et al.*, 2003). The aim was to achieve an after grazing height of between 10 to 12 on the RP_{Meter}. A reading below 10 on the RP_{Meter} indicates that availability might have been limiting. As the cows had two grazing sessions (after each milking), the area allowed for the cows to graze on was adapted accordingly.

Figure 3.3 Sampling of the ryegrass pasture grazed by Jersey cows during the study

Pre- and post-grazing heights were taken with the RP_{Meter} by taking 100 RP_{Meter} readings along each pasture strip in a randomized pattern to determine the available pasture (kg DM) pre-and post-grazing. The difference between the former two values are then assumed to be the PDMI of the cows. This PDMI value estimated could further be divided by the total amount of cows (sixty) grazing the pasture to determine average PDMI/cow/day.

Pasture intake was also estimated using different equations, including the CNCPS model. The equations included, expected PDMI kg cow/day estimated based on the assumption that a cow consumes

1.3 % of her BW (kg) as NDF (Bargo *et al*., 2003) or that a cow is only able to consume 1.5 % of BW (kg) as NDF when fed only pasture (Kolver et al., 1998). Dry matter intake was also calculate based on various assumptions made by authors that cows could only consume 3 to 4 % percentage of their live BW as DM (Mayne & Wright, 1998; Fulkerson *et al*., 2006). Substitution of pasture for concentrates was corrected for with the equation 0.093 x kg (concentrate DM/cow/d) (Faverdin *et al*., 1991). Pasture DMI was also calculated from back calculation from the cows ME requirements for a specific level of production, ME consumed from the concentrates and total ME content of pasture (Tesfa *et al*., 1995), including the equation described by the NRC (2001).

Figure 3.4 Indication of pasture allocation and cow management during the study

3.7.2 Pasture samples

Weekly ryegrass samples were taken from 10 September to 2 November 2018 before grazing on Monday, Wednesday and Friday at midday (1100 and 1300 h). Samples were taken at midday during the trial to avoid high pasture sugar content during the afternoon. Four representative samples were taken on each sampling day, amounting to twelve samples per week. The pasture was sampled by randomly throwing a 35.4 cm in diameter ring on the pasture and cutting (3 cm from the ground) the area of grass in the ring for collection in brown paper bags as was shown in Figure 3.3 earlier. These samples were weighed with a laboratory scale (Sartorius BP8100, Göttingen, Germany (maximum = 8100 g; ± 0.1g) and oven-dried (LABCON (Pty) Ltd. FSIM 16, Maraisburg, 1700, RSA) at 60 ºC for 72 hours to determine the DM % (Van Vuuren *et al*., 1992).

At each respective weighing a separate paper bag was tared along with the sample to correct for any additional weight loss. The twelve dried samples were composited weekly and milled though a 1 mm screen

(Retch GmbH 5657, Laboratory Mill, Rheinische Strobe 36, West Germany) and stored in airtight bags. This resulted in eight pooled pasture samples at the end of the 60-day trial period. The preserved samples were subsampled in duplicate and sent for proximate analysis at UP Nutrilab and AA analysis at Elsenburg.

The eight pasture samples were analysed in duplicate as follows: DM (AOAC: 2000, method 934.01), ash (AOAC: 2000, method 942.05), and OM using the equation OM = 100 - ash % (OM-basis). Samples were analysed for *in vitro* organic matter digestibility (*IV*OMD) according to Tilly & Terry (1963), using rumen fluid obtained from a cannulated Holstein dairy cow fed high quality lucerne hay and a maize-based concentrate. The fibre fractions were analysed for using an ANKOM^{2000} fibre analyser (ANKOM technology method 8: Filter bag technique), NDF was determined according to the procedure described by Van Soest & Robertson (1991). Heat-stable amylase was used to remove starch and inactivate enzymes that may degrade fibre. The ADF and acid detergent lignin (ADL) fractions were analysed for according to Goering & Van Soest (1970). Crude protein was calculated as CP = N × 6.25 (AOAC: 2000, method 968.06), where the N content was determined using a LECO Trumac™ N analyser (LECO FP-428, Leco Corporation, St Joseph, MI, USA). Neutral detergent insoluble protein (NDIP), and acid detergent insoluble protein (ADIP) were analysed according to Krishnamoorthy *et al.* (1982). Non fibre carbohydrates were calculated with the equation NFC = [100 – (NDF + ash + CP + EE)] (NRC, 2001). Samples were also analysed for ether extract (EE) (AOAC: 2000, 920.39) and for gross energy (GE) (MC – 1000 Modular Calorimeter, Operators Manual). Minerals analysed for were Calcium (Ca), samples were prepared (AOAC: 2000, 935.13) and analysed according to Giron (1973). Phosphorus (P) were also analysed for (AOAC: 2000, 965.17). Metabolisable energy (MJ/kg DM) was calculated using the equation ME = [C × GE × IVOMD] (Robinson *et al*., 2004), where C = 0.82.

Amino acids were analysed for according to the procedure described by Grace Davison (2008) as adapted by the Central Analytical Facility of Stellenbosch University (University of Stellenbosch, Matieland, 7602, RSA). Determining the AA content of the feed samples included the hydrolysis of the milled feed samples in HCL (OPA-3 pre-column derivatisation with Orto-Phthaldialdehyde, Application Service 008, Grace Davison Discovery Services, Stuttgart, Germany). Separation and detection of AA was performed using Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system fitted with a photodiode array detector and an UltraTag™ C18 column. Including Waters UV and fluorescence detection, after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Analysis of the AA was done with Waters MassLynx software (Waters Corporation, 34 Maple street, Milford, MA 01757, USA) which integrates the peaks at the defined retention times and plots calibration curves for each AA based on the peak response (peak area/internal standard peak area) against concentration.

3.7.3 Concentrate samples

Weekly concentrate samples were collected on Monday, Wednesday and Friday from 10 September to 2 November 2018 on 24 respective sampling days. Duplicate samples of each concentrate were taken on each sampling day resulting in 6 samples per week for each respective treatment. After the samples were weighed (Sartorius BP8100, Göttingen, Germany (maximum = 8100 g; ± 0.1g) in small tin containers it were oven-dried (LABCON (Pty) Ltd. FSIM 16, Maraisburg, 1700, RSA) at 60 ^{0}C for 72 hours to determine the DM % and milled through a 1 mm screen with a laboratory mill (Retch GmbH 5657, Laboratory Mill, Rheinische Strobe 36, West Germany). These samples were pooled for every four weeks resulting in 6 concentrate samples (2 per treatment). Samples were placed in airtight plastic bags and stored for analysis at UP Nutrilab and Elsenburg.

Samples were analysed for starch (AOAC: 996.11, 1984) with a Megazyme Total Starch Kit (Bray Business Park, Wicklow, Ireland), DM, ash, OM, GE, ME, NDF, ADF, ADL, CP, NDIP, ADIP, NFC, *IV*OMD, EE, Ca, P, and AA.

3.7.4 Milk production and composition

The cows, for the duration of the trial, were milked in a 20 Point Waikato swing over milking machine (Waikato SA, 20 Dobson St. PE, 6000, RSA), which is fitted with Afikim in-line, weight-all electronic milk meters including a ‘‘Milk lab’’ (Kibbutz, 1514800, Israel) (Figure 3.5). Daily milk production was recorded automatically for each cow with the Afikim milk meter-and-management system, to calculate the mean daily milk production per cow, including total and mean milk production per experimental group.

Composite milk samples (ratio 8: 16 ml, afternoon: morning milking (0500 and 1330 h)) were collected biweekly on 18/19 September, 2/3 October, 16/17 October and 30/31 October 2018 during the trial. The milk samples obtained were homogenous and preserved with potassium dichromate ($K_2Cr_2O_7$) (Ng-Kwai-Hang & Hayes, 1982) and kept in a fridge at 4°C. The milk samples, after each collection, were sent to Merieux NutriSciences (Jakaranda Centre Unit 1, 6330, Jeffreys Bay, Eastern Cape, RSA) for analysis. The preserved samples were analysed for fat, protein, lactose and milk urea nitrogen (MUN) with a Milko-Scan FT+ NIR machine (Foss Allé 1, 3400 Hillerød, Denmark).

Additional composite milk samples were collected (ratio 16: 32 ml, afternoon: morning milking (0500 and 1330 h)) twice on 2/3 October and 30/31 October 2018, thus on the second and fourth milk sampling period. Cows selected to be sampled were all three cows in every second block from block 1-20, thus ten representative blocks were selected (30 cows in total), constituting a representative portion of cow variation used within this study. This group of cows will further be referred to as “a representative group” throughout the study. See Appendix A Table A5 for a detailed blocking procedure.

After collection the milk samples were stored frozen at - 20 °C in 50 ml polyethylene containers before they were transported frozen, on ice, to Lactolab (ARC, Irene, Gauteng, RSA) to be analysed for milk nitrogen fractions (NPN, NCN, casein and whey) as reported by Rowland (1938). The NPN (AOAC: 1995; 33 2 12, 991 21) and non-casein N (NCN) (lynch *et al*., 1998) were also analysed for. A conversion factor of 6.38 was used in the calculation and N was analysed for as discussed in section 3.7.2 in this chapter.

Fat corrected milk (FCM) standardized to 4.0 % fat was calculated as FCM = [(0.4 × milk yield (kg) + (15 × milk fat (kg)] according to Gaines (1928). Energy corrected milk (ECM) was calculated by using the equation of Tyrrell & Reid (1965); ECM = [milk yield (kg/d) × (milk energy content (MJ/kg)) / 3.1]; where, milk energy content (MJ/kg) is calculated as milk energy (MJ/kg) = [(0.0384 × milk fat (g/kg)) + (0.0223 × milk protein (g/kg)) + (0.0199 × milk lactose (g/kg)) – 0.108].

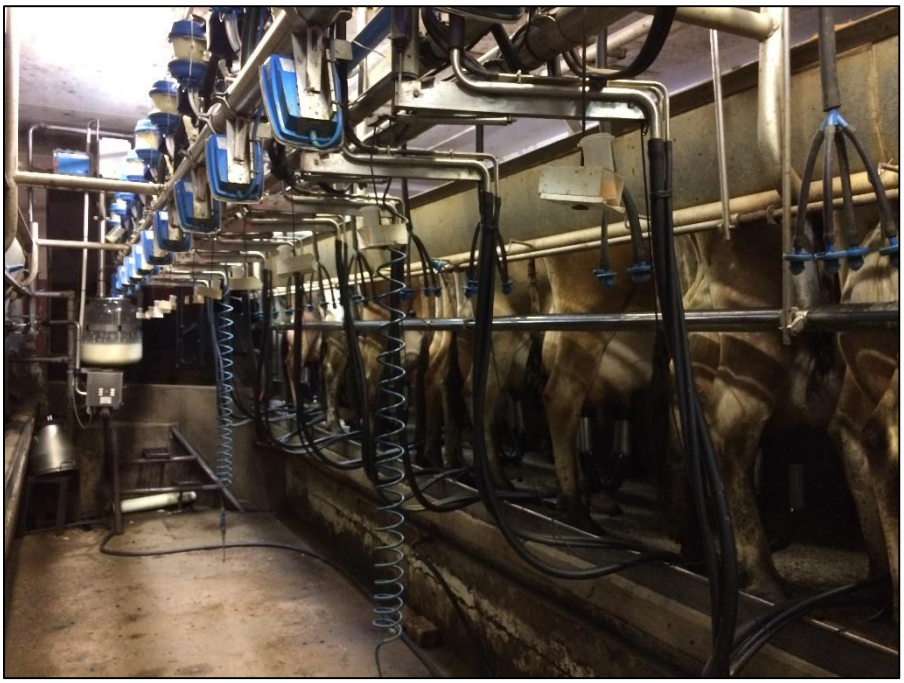

Figure 3.5 Cows being milked in a 20 Point Waikato swing over milking machine fitted with Afikim in-line, weigh-all electronic milk meters

3.7.5 Body weight and body condition score

Cows were automatically weighed daily after each milking upon exiting the milking parlour (Afikim scale). The cows, in addition to daily weighing, were also weighed on two consecutive days in the beginning of the trail on 27/28 August 2018 and at the end of the trial on 5/6 November 2018 with a fixed weighing scale (Tru-Test EziWeigh v. 1.0 scale, 0.5 accuracy, Auckland, NZ). The reason for weighing the cows twice was to eliminate any discrepancies which could have arisen from variation in weight other than that

caused by the experimental treatments, such as water consumption, urination and/or defecation. The average BW between the consecutive measurements was used for analysis.

The BCS of the cows were determined after milking at the beginning and end of the trial on the first of the two consecutive days when the cows were weighed. The cows were palpated at the back and hind quarter then scored from 1 to 5, where 1 is thin and 5 is fat (Wildman *et al*., 1982). The BCS was determined by the same trained technician at the Outeniqua Research farm for each period.

3.7.6 Faecal samples

Composite faecal samples were taken after milking from a representative group of cows (30 cows, ten per treatment) by means of rectal palpation or grab sample when cows defecated in the holding area on Monday, Wednesday and Friday on two different sampling periods. The first sampling period, was 24, 26 and 28 September 2018 and the second sampling period was 22, 24 and 26 October 2018. See Appendix A Table A5 for cow selection.

Faecal Samples were collected in tin containers and oven-dried at 60 ^{0}C for 72 h (LABCON (Pty) Ltd. FSIM 16, Maraisburg, 1700, RSA) and milled through a 1 mm screen (Retch GmbH 5657, Laboratory Mill, Rheinische Strobe 36, West Germany). After milling, the samples were pooled for both periods (Monday, Wednesday and Friday (3 × 2)) resulting in one sample for each cow (10 samples per treatment) and stored in individual airtight 500 ml polyethylene containers to be analysed for starch as an indication of rumen health and efficiency of rumen fermentation (Fredin *et al.*, 2014). Due to cost implications only specific samples were selected to be analysed for faecal starch. The samples of All three cows in blocks 1, 11 and 20 were selected, respectively, resulting in three samples per treatment, which represent cow variation.

3.7.7 Urine volume, daily allantoin excretion and estimation of rumen microbial protein production

Spot urine samples were collected from a representative group of cows (30) during the 60-day trial period (see Appendix A Table A5 for cow selection). Sampling took place during six sampling periods on 25 September; 4, 9, 18 and 23 October and 1 November 2018, respectively, thus 180 samples in total (6 × 30).

Samples were taken at the second milking at 1400 h, after the cows were milked and consumed their respective concentrates. Cows were sorted as they exited the milking parlour and were moved to a crush. Urine samples were collected either as a free flow, stimulatory plus free flow or stimulatory only. Manual stimulation was done by gently rubbing the skin (escutcheon) under the vulva stimulating of the pudendal

nerve (Figure 3.6). Most samples obtained were ‘‘mid-stream’’ samples, noted otherwise if not. Detail on samples was recorded to interpret any discrepancies in AL analyses.

The samples were collected in plastic 500 ml polyethylene screw top containers, after which the SG was immediately recorded with a hand-held pen refractometer (Pen-urine SG, Atago., Inc., Tokyo, Japan) for each sample. Before sampling, the refractometer was calibrated with deionized water as close to average cow body temperature (± 38 °C) as possible with moderately warm water. The recorded SG values were later used to calculate the total volume (l/d) of urine excreted for each cow (Burgos *et al.*, 2005). The urine SG represents the ratio of urine density to water and reflects the relative proportion of dissolved solutes to total urine volume and as a result represents a measurement of urine concentration of solutes within urine (Kasiske, 2000), which in turn could be used to determine urine volume from the urine sample collected (Burgos *et al.*, 2005). After the SG value was recorded the sample was immediately placed in a coarse salt covered ice bath to reduce the urine temperature and bacterial growth and transported to the Outeniqua Research Farm’s laboratory.

Figure 3.6 Stimulation technique used to collect spot urine samples from Jersey cows

Duplicate aliquots of 7 ml urine were transferred onto 2 ml of 100 ml/L (10 %) Sulphuric acid solution (H_2SO_4) in 50 ml sampling containers. This reduces the pH of the aliquoted sample to a pH below 2.0 thereby preventing bacterial destruction of AL and preserving the urine sample. The samples were further diluted with deionized water, preventing uric acid precipitation, to a total volume of 35 ml and placed in a freezer at -20 ºC. After the samples were stored frozen for four months, they were thawed at 4 ºC to insure sample integrity. Thawed samples were shaken thoroughly to aliquot 8 ml of each respective

sample into a 10 ml centrifuging tube and again stored frozen at -20 ºC before being transported for analysis at the University of California (UC Davis, One Shields Avenue, Davis, 95616, CA, USA).

Briefly, the urine samples were chemically analysed for the purine derivative AL according to the method described by Young & Conway (1942) as reported by Chen & Gomes (1992). This colorimetric method used is based on the principles that AL is hydrolysed under weak alkaline (0.5 M NaOH) conditions at 100 ºC to allantoic acid. After the hydrolysis of AL, the allontoate which formed is then further degraded to urea and glyoxylic acid in a weak acid (0.5 M HCL) solution. Glyoxylic acid reacts with phynylhydrazine hydrochloride (Phen) forming phenylhydrazone. This product in acid conditions forms an unstable chromophore with potassium ferricyanide (Pot-fer) resulting in the formation of colour, which could be read at a 522 nm wavelength. Results obtained were measured against four different AL standards. Working standards of 10; 20; 30 and 40 mg (AL)/L were prepared in advance. The concentration of AL in the urine sample was then calculated using the equation y = mx + c from duplicate standard curves which were included at the start and end of each respective run. To fit the standard curve, samples were diluted a total of 60 times after they were thawed and centrifuged (IEC Centra CL3, Thermo Scientific., Inc., Waltham, MA, USA) at 1200 revolutions per minute (rpm) for 15 minutes at room temperature (20 to 22 °C) to remove debris in the samples which could affect the accuracy of the colorimetric readings. Variation among runs were assessed by including two inter-run standards with each run. No inter-run corrections were used, due to all the inter-run standards being within the 5 % limit point over all run averages. Additionally, samples were analysed in duplicate with the average between the two values obtained representing the final concentration.

As the urine SG, AL concentration in the urine and cow BW is known the daily MCP flow could be calculated. See Chapter 2, section 2.4.3.2 for a detailed discussion on the prediction of MCP using this method. The daily MCP flow (g CP/d) can be calculated as follows: urine volume (l/d) is calculated from the SG value: urine volume (L) = [332.66 × (((SG – 1) × 1000) -0.884] (Burgos *et al*. (2005). The AL concentration is firstly converted from mg/l to mmol/l with the equation AL (mmol/L) = [((AL (mg/l) / (158.12 × 1000)) × 1000], since 158.12 represents the molecular mass of AL (g/mol). Using the calculated daily urinary volume and AL concentration obtained, the daily urinary AL (mmol/d) excretion can then be calculated as urinary AL excretion (mmol/d) = [AL (mmol/L) × urine volume (L/d)]. The total daily PD excretion (mmol/d) is then calculated as total PD excretion (mmol/d) = [urinary PD (mmol/d) + milk PD (mmol/d)], however the daily PD excretion in the milk and urine must first be determined. Thus, daily urinary PD excretion (mmol/d) is calculated as urinary PD (mmol/d) = AL (mmol/d) output ÷ 0.906, due to the proportion of total urinary PD excretion being expressed by the coefficient 0.906. The daily milk PD excretion (mmol/d) is then calculated as milk PD excretion (mmol/d) = urinary PD excretion (mmol/d) × 0.05. Since 5 % of total AL and uric acid are excreted in milk. Knowing the total daily PD excretion, the daily microbial purine absorption could then be calculated as follows: microbial purines absorbed (mmol/d) = [(Total daily PD excretion (mmol/d) – 0.385 × (BW (kg) $^{0.75}$)) / 0.85], where the 0.85 represents the

recovery of absorbed purines as PD in urine and 0.385 the net endogenous contribution of PD to total PD excretion and (BW) $^{0.75}$ the cows metabolic weight. The next step is calculating the intestinal flow of microbial N as the intestinal flow of microbial N (g N/d) = [(Intestinal absorption of microbial PD (mmol/d) × 70) / (0.116 × 0.83 × 1000), where 0.116 represents the ratio of purine N: total N in ruminal microbes, 70 equals the purine N content (mg N/mmol) and 0.83 is the coefficient for microbial purine digestibility. The MCP (g CP/d) production was then calculate from the intestinal flow of microbial N multiplied by the factor 6.25.

3.7.8 Blood plasma samples

Blood plasma was collected on three sampling occasions during the 60-day trial on 27 September, 11 October and 25 October 2018 from a representative group of cows (see Appendix A Table A5 for cow selection). Blood was collected from the tail vein (Coccygeal) using an 8.5 ml K2 EDTA vacutainer (Becton Dickinson, 1 Becton drive, Franklin Lakes, NJ, USA). Blood was sampled directly after the second milking (1330 h) session, after the cows have consumed their respective concentrate treatments at 1400 h. The blood samples were transported immediately to the Outeniqua Research Farm's laboratory in a sealed polystyrene cooler to be centrifuged (Heltich Lab technology, EBA 200, Labotec, Ranjespark, Midrand, Gauteng, RSA). The sequence in which the cows were sampled remained the same throughout the procedure to ensure an equal time distribution between samples. All samples were centrifuged (within 30 minutes from collection) at 4 °C for 5 minutes at 4000 rpm. After each batch (7 samples) was centrifuged, the tubes were evaluated for hemolysis and/or inadequate plasma separation and if so, the tubes were centrifuged again, no tubes were discarded due to hemolysis. Plasma was then decanted into 2 ml Cryotubes (Cryo.s™ Freezing tubes, Greiner Bio-One GmbH, Maybachsraßa, Frickenhausen, Germany) and stored frozen at -20 °C for the duration of the trial. All samples (90 in total) were transported, on ice, for analysis of physiological AA (i.e. free plasma AA) at the NWU Metabolomics department (North-West University, Potchefstroom, NW, RSA). The plasma AA concentrations obtained are assumed to represent the AA available for absorption by the mammary gland (Broderick *et al*., 1974; Munneke *et al*., 1991, Swanepoel *et al*., 2015; Zhang *et al*., 2016; Martineau *et al*., 2017; Martineau *et al*., 2019). The AA concentration of plasma withdrawal from the coccygeal vein reflect the concentrations in the arterial supply of the mammary gland (Munneke *et al*., 1991), the intestinal absorptive site, and could be used to identify AA limitation (Doepel and Lapierre, 2010).

Plasma AA were analysed for using the EZ: faast™ Free (Physiological) Amino Acid GC and MS kits (Phenomenex® KGO-7166, 411 Madrid Ave, Torrance, CA 90501-1430, USA). The procedure consists of a solid phase extraction step followed by a derivatization and a liquid/liquid extraction. The solid phase extraction was performed via a sorbent packed tip that binds AA while allowing interfering compounds such as proteins, urea, hydrolysed carbohydrates, lipids and other impurities to flow through. Amino acids

on the sorbent are then extruded into the sample vial and quickly derivatized with reagent at room temperature in an aqueous solution. The derivatized AA concomitantly migrate to the organic layer for additional separation from interfering compounds. The organic layer is then removed, evaporated, and re-suspended in re-dissolution solvent and analysed with derivatized AA by gas chromatography-mass spectrometry (GC/MS).

3.8 Statistical analysis

Data was analysed statistically as a randomised block design with the General Linear Model (Statistical Analysis System, 2019) for the average effects over time. Repeated Measures Analysis of Variance with the GLM model were used for repeated period measures. Means and standard error were calculated and significance of difference ($P < 0.05$) between means was determined by Fischer's protective test (Samuels, 1989). The data of the higher producing and lower producing groups were analysed using the same statistical model. Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$.

The base linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + B_j + e_{ij}$$

Y_{ij} = studied variable

μ = overall mean

T_i = effect of the i^{th} treatment

B_j = effect of the j^{th} block

e_{ij} = error associated with Y

3.9 Modelling the trial using the Cornel Net Carbohydrate and Protein System

Using the CNCPS model to simulate our study was mainly to evaluate, interpret and support the findings of our study. Predications of the model inputs were based on the "average cow" receiving each treatment (Table 3.5). Animal inputs were used as described in Chapter 3, section 3.3 and data from Chapter 4, section 3.4.4.1, Table 4.9 and Table 4.11. Age at first calving and calving interval, days pregnant and expected calf birth weight were based on records from the Outeniqua Research Farm. All external (i.e. environmental) parameters used in the model were the same across all treatments (Table 3.6). The relative humidity was set as measured by an on farm weather station and other relevant input was either assumed or based on the climatic conditions observed during the study (section 4.1, Figure 4.1). Concentrate intake was set at 7.2 kg DM and PDMI were adjusted for actual (9.04 kg DM/cow/d; see section 4.2, Table 4.1) and predicted total DMI. The chemical composition and AA composition of the respective concentrates

and pasture, as described in Table 3.2 and Table 3.4, were considered in the model evaluation. Model defaults were used for mud on coats, hair depth, standing time and body position changes. The physical activity of the cows was also considered in the model; it is assumed that the cows walk 4.5 km/cow/d on a mild slope.

Table 3.5 Animal inputs used in the CNCPS prediction model for the cows used in the trial

Animal Inputs	**Experimental treatment[1]**		
	C	**RPL**	**RPML**
Lactation number	4.5	4.8	4.1
Current age (months)	75	75	75
First calving (months)	24	24	24
Calving interval (months)	13	13	13
Current weight (kg)	410	415	400
Mature weight (kg)	410	415	400
Calf birth weight	25	25	25
Days pregnant	47	48	55
BCS	2.24	2.39	2.27
Production (kg)	22.26	22.27	22.33
DIM	127	128	135
Milk fat (%)	4.77	4.73	4.79
Milk protein (%)	3.93	3.86	4.03

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
BCS – Body condition score, DIM – Days in milk

Table 3.6 Inputs used in the CNCPS model for environment and management variables

Environment	
Current temperature (°C)	15.7
Current relative humidity	85
Previous temperature (°C)	14.9
Previous relative humidity	85
Wind speed (meters per second)	0
Hours in sunlight	12
Storm exposure	Yes
Min night temperature (°C)	10.3
Mud depth (cm)	0
Hair depth (tenths of cm)	0.63
Hair coat	No mud
Management	
Activity	Continuous grazing
Time standing (h/d)	18
Body position changes	6
Distance walked flat (m × 1000)	4.5
Distance walked sloped (m)	0

The chemical composition of all the raw materials used for the modelling of study for the C, RPL and RPML treatment, respectively, were provided from the NOVA feeds database, George (Saagmeul St., George Industries, P.O. Box 1351, George, 6530). The chemical composition of the raw material used (i.e.

maize, soybean oilcake, wheat bran and molasses) are representative of South African raw materials. Default values of the feed library were used if there were no values for specific nutrient parameters.

The NDS model (Nutritional Dynamic Systems, Version 3.9.8.01, RUM&N Sas via Sant'Amrogio, 4/A, 42123 Reggio Emilia, Italy) which is based on CNCPS Version 6.5.5 (Cornell Net Carbohydrate and Protein System, Cornell University, Ithaca, NY, USA) was used to model this study. The chemical composition of the ryegrass pasture grazed and the C, RPL, and RPML concentrates, as used in the NDS model, are presented in Table 3.7.

Table 3.7 Chemical composition of the ryegrass pasture and the C (DairyTest1), RPL (DairyTest 2) and RPML (DairyTest 3) treatments used in the CNCPS model

Parameter	**Diet components[1]**			
	Ryegrass	**CDairyTest1**	**RPLDairyTest2**	**RPMLDairyTest3**
DM (%)	15.0	88.9	89.1	89.1
Ash (% DM)	10.5	6.89	7.40	7.63
ME (MJ/kg)	10.6	12.5	12.5	12.5
CP (% DM)	18.8	12.5	12.4	12.4
SolCP (% DM)	11.3	3.09	2.83	2.90
RDP (% DM)	14.3	7.29	7.09	6.90
RUP (% DM)	4.53	5.16	5.35	5.54
ADF (% DM)	25.0	3.76	3.62	3.56
NDF (% DM)	42.0	-	-	-
ADIP (% CP)	0.66	0.47	0.46	0.46
NDIP (% CP)	0.85	1.11	1.08	1.07
peNDF (% DM)	35.7	2.79	2.61	2.52
Lignin (% NDF)	2.10	1.27	1.26	2.53
EE (% DM)	4.00	3.25	3.42	3.40
Starch (% DM)	2.00	57.0	56.8	56.7
Ca (% DM)	0.30	1.13	1.27	1.28
P (% DM)	0.40	0.48	0.47	0.47
Met (% CP)	2.29	1.63	1.61	3.70
Lys (% CP)	3.99	3.93	7.16	7.13
Arg (% CP)	5.64	5.56	5.48	5.40
Thr (% CP)	3.94	3.49	3.46	3.43
Leu (% CP)	7.45	9.46	9.40	9.40
Ile (% CP)	4.73	3.61	3.58	3.60
Val (% CP)	5.27	4.46	4.40	4.55
His (% CP)	1.60	2.65	2.62	2.60
Phe (% CP)	1.27	4.62	4.57	4.55
Lys:Met ratio	1.74	2.41	4.45	1.93

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

[1]DM – Dry matter; ME – Metabolisable energy; CP – Crude protein, SolCP – Soluble CP; RDP – Rumen-degradable protein; RUP – Rumen-undegradable protein; ADF – Acid detergent fibre; NDF – Neutral detergent fibre; ADIP – Acid detergent insoluble protein; NDIP – Neutral detergent insoluble protein; peNDF – physically effective NDF; EE – Ether extract; Ca – Calcium; P – Phosphorus; Met – Methionine; Lys – Lysine; Arg – Arginine; Thr – Threonine; Leu – leucine; Ile – Isoleucine; Val – Valine; His – Histidine; Phe – Phenylalanine

Chapter 4: Results and Discussion

4.1 Temperature and precipitation

The comparison between long term data and data collected during the trial for mean maximum and minimum daily temperatures, and mean monthly rainfall are presented in Figure 4.1. The mean maximum and minimum daily temperatures were 17.2 and 6.7 °C for August, 18.6 and 7.5 °C for September, 23.1 and 11.9 °C for October, and 21.5 and 11.5 °C for November, respectively. Compared to the previous 18 year (2000 to 2018) mean maximum and minimum daily temperatures, which were 18.7 and 7.7 °C for August, 19.5 and 8.7 °C for September, 20.6 and 10.8 °C for October and 21.8 and 11.6 °C for November, respectively.

Total rainfall measured during the trial period (10 September to 8 November 2018) was 145 mm. In August, September, October and November 2018 the rainfall measured was 46.5, 101, 44.6 and 96.4 mm, respectively. Compared to the previous 52 year (1967 to 2019) means of 64.0, 54.0, 78.7 and 78.4 mm for August, September, October and November.

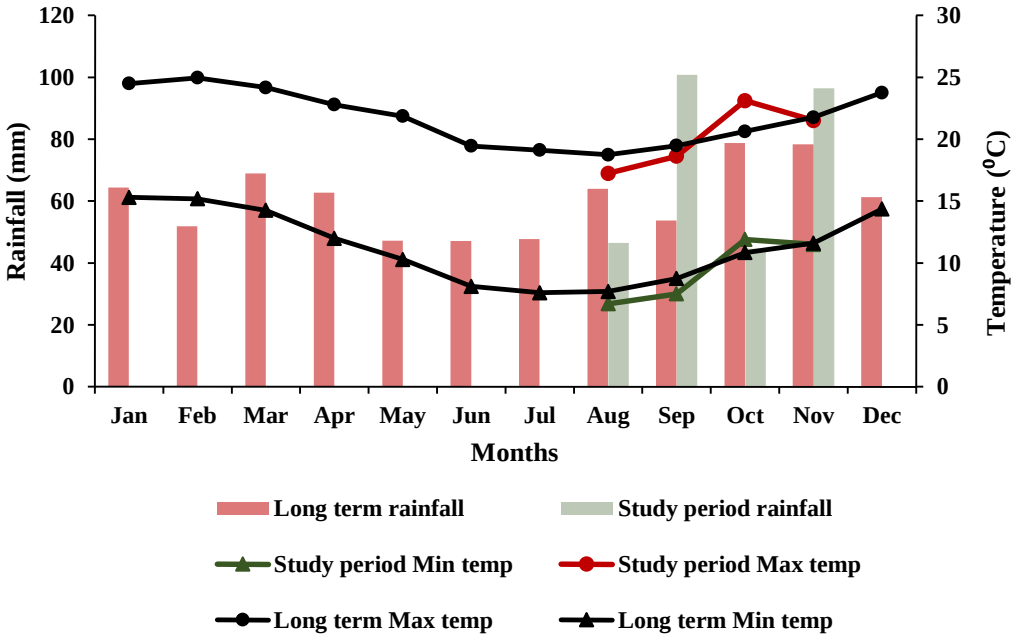

Figure 4.1 Comparing the long term mean monthly average rainfall (52 years) and long term (18 years) mean maximum and minimum monthly temperature with the rainfall and temperature observed during the study on the Outeniqua Research Farm in which cows grazing ryegrass were supplemented with rumen protected Met and/or Lys.

This data reflects on the high pasture growth rate observed from September to October, as September experienced a higher than average rainfall and October a warmer than average daily temperature. However, the lower than average rainfall observed during October could not have influenced the study, as the pasture was under permanent irrigation. The mean maximum and minimum daily temperature (21.07 and 10.3 °C) corresponds with temperatures that generally occur during spring in the George area.

4.2 Pasture intake and allocation

The mean pre-and post-grazing RP_{Meter} readings (in half cm increments) obtained during the trial (9 September to 6 November 2018) is presented in Table 4.1. Means for RP_{Meter} readings pre-and post-grazing were 27.4 and 10.9 RP_{Meter} units, respectively. Stakelum *et al*. (1997) suggested that PDMI could be maximised at a pre-grazing sward height of 9 to 13 cm (RP_{Meter} reading 18 to 26) and Fulkerson *et al*. (1998) recommended that pasture utilisation is optimised at a post-grazing stubble height of 5 to 6 cm (RP_{Meter} reading 10 to 12). Delaby *et al.* (2001) also recommended a post-grazing height equal to 45 to 50 % of the pre-grazing height to ensure optimal pasture utilisation. This indicates that the pasture was well utilised in our study, insuring optimal pasture regrowth, quality and persistence (Irvine *et al*., 2010). These values are in agreement with values reported by Meeske *et al*. (2009).

During our study a seasonal linear regression equation was used: $Y = 81.054\ H - 178.94$ ($R^2 = 0.831$), established by Van der Vyver (2019), where Y equals pasture yield (kg DM/ha) and H equals the RP_{Meter} height reading (Meeske, R., personal communication, robinm@elsenburg.com). Using this regression equation, it was calculated that, on average 2045 kg DM/ha of pasture was available pre-grazing and 703 kg DM/ha post-grazing, thus 1341 kg DM/ha of pasture on average were removed by the cows during each grazing. A PA of more than 11 kg DM/cow per day were aimed for in this study to ensure optimum pasture intake, since PDMI is one of the major factors limiting the performance of cows consuming high quality pasture (Kolver, 2003). As a result, the area allocated to the cows was adapted accordingly to avoid low pasture intake.

Visual observations, which included the leaf stage of the pasture, were done twice daily to further ensure optimum removal of the pasture to avoid over or under utilisation of the pasture. Research indicate that senescence of forages begins around 3.5 to 4 leaf stage regrowth, indicating that a decrease in growth rate and nutritive value could be expected after this stage (Fulkerson & Slack, 1994). Solomon *et al*. (2017) reported that the maximum productivity of annual ryegrass could be achieved when defoliated at a leaf stage interval of up to 4. However, it is recommended to graze ryegrass pasture at a 2.5 to 3 leaf stage or canopy closure, depending on which comes first, since the pasture ME reduces when dead leafs develop (Fulkerson & Donaghy, 2001).

The mean estimated daily PA, based on the equation described above, was 12.1 kg DM/cow/d and the mean PDMI was 7.97 kg DM/cow/d (Table 4.1).

Table 4.1 Mean (± SD) pre- and post- grazing measurements, pasture allowance and pasture dry matter intake obtained during spring for cows grazing ryegrass and fed a concentrate supplemented with rumen protected Met and/or Lys (n = 95)

Parameter	**Regression equations[2]**	
	Y = 81.054 × H – 178.94[3]	**Y = 91.574 × H – 344.64[4]**
Pasture height (1unit = 5 mm)		
Pre- grazing	27.4 ± 5.90	27.4 ± 5.90
Post- grazing	10.9 ± 1.62	10.9 ± 1.62
Pasture yield (kg DM[1]/ha)		
Pre- grazing	2045 ± 476	2354 ± 757
Post- grazing	703 ± 131	651 ± 148
Pasture removed	1341 ± 449	1702 ± 723
Pasture allowance (kg DM/cow/d)	12.12 ± 1.61	12.85 ± 1.79
Pasture Intake (kg DM/cow/d)	7.97 ± 1.48	9.04 ± 1.60
Coefficient of determination (R^2)	0.831	0.742

[1]DM = Dry matter
[2]Y = Pasture yield (kg DM/ha); H = Rising plate meter (RP_{Meter}) height
[3]Regression equation established by Van der Vyver (2019) and which was used in the current study
[4]Regression equation established during the current study

A linear seasonal regression equation Y = 91.574 H – 344.64 (R^2 = 0.742) was established at the end of the trial and is presented in Figure 4.2. This equation was fitted as a single regression equation to the pre-and post-grazing RP_{Meter} readings taken during the trial to calculate the actual pasture DM yield, PA and PDMI from the ryegrass pasture and is presented in Table 4.1. By using this regression equation, the pasture available pre-grazing was calculated to be 2354 kg DM/ha and post-grazing 651 kg DM/ha, thus 1702 kg DM/ha of pasture on average was actually removed by the cows resulting in a mean PA of 12.9 kg DM/cow/d and a mean PDMI of 9.04 kg DM/cow/d. The PA exceeded that as stated (*ca.* 11 kg DM/cow/day), this over-estimation could be as a result of a generalised seasonal regression equation that was used to determine pasture yield and thus PA. The pre-grazing pasture mass are similar to the 2467 kg DM/ha reported by Lile *et al*. (2001) and lower than the 2561 kg DM/ha reported by Clark *et al*. (2018) for annual ryegrass. Pasture DM removed (kg DM/ha) was similar to the 1674 kg DM/ha removed by Jersey cows grazing the same pasture type as reported by Van Wyngaard *et al*. (2018) and 1605 kg DM/ha reported by Fulkerson *et al*. (2006).

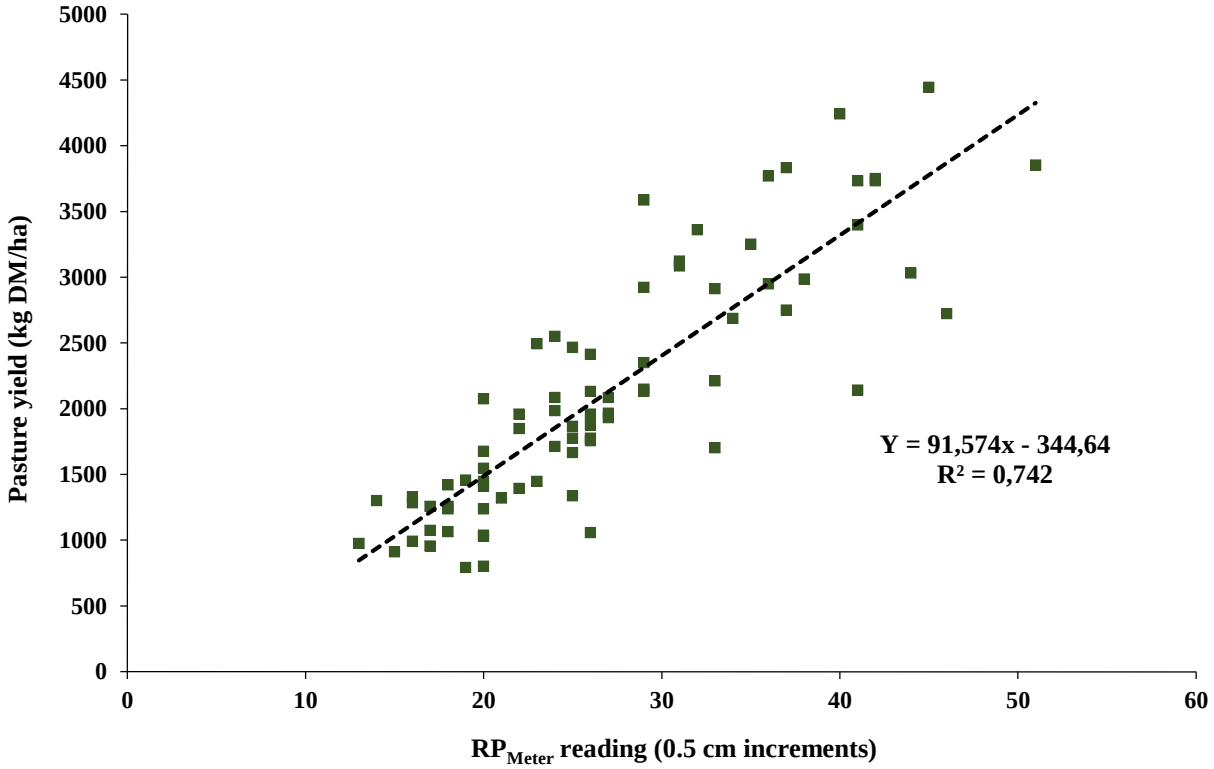

Figure 4.2 Regression equation (Y = mH + b) established during the trial using a rising plate meter (RP_{Meter}) where Y equals the pasture yield (kg DM/ha) and H equals the average RP_{Meter} reading

The post-grazing pasture mass, or grazing residual, measured in our study is in agreement with others (Fulkerson *et al.*, 1998, Malleson, 2008, Van der Colf, 2010). Some researchers, however, reported higher (Fulkerson *et al.*, 2006) and some lower (Meeske *et al.*, 2009; Van Wyngaard *et al*., 2018) post-grazing residuals for the same pasture type. The differences reported could be due to multiple factors (i.e. climatic conditions, cultivar and/or management practises). Grazing residuals might be elevated in late spring for ryegrass, since tillers move from a vegetative to reproductive stage. This leads to stem elongation and as a result increasing the amount of stem in the pasture that needs to be consumed by the cows (Fulkerson *et al.*, 2007). Figure 4.3 clearly demonstrates this, due to PDMI decreasing relative to PA. The increase in grazing residuals could be attributed to an increase in pasture NDF content and a decrease in pasture *IV*OMD (Figure 4.4), which could translate into a decrease in PDMI.

The difference between the linear regressions used during the trial and the one established after the trial, emphasises the importance to estimate calibration equations for each unique season, pasture and area combinations, rather than using standardised equations for the area. Reeves *et al*. (1996) reported that the RP_{Meter} method is an inaccurate method to determine individual cow PDMI, although this was not the purpose during the study. The RP_{Meter} was used as a management tool for PA and primarily to monitor pre- and post-grazing pasture heights. However, a few techniques to estimate PDMI will be discussed in support of the PDMI predicted in our study, since it will be used in further calculations.

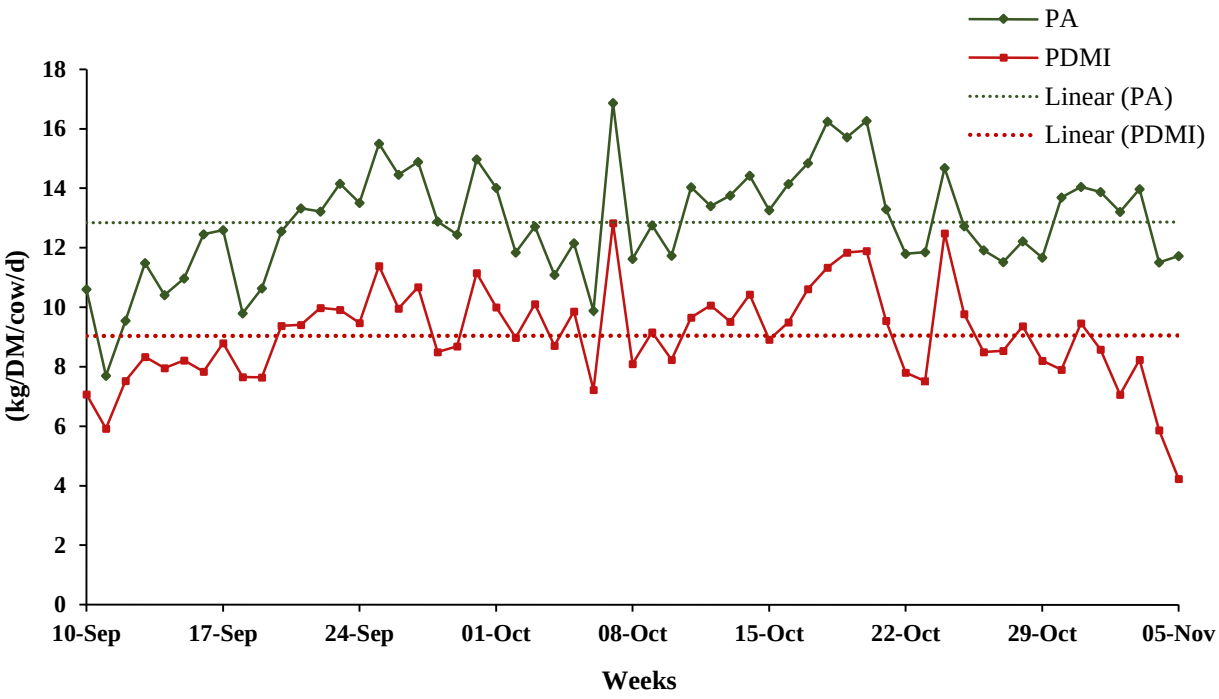

Figure 4.3 Ryegrass pasture allowance (PA) and pasture dry matter intake (PDMI) estimated using a rising plate meter (RP_{Meter}) based on the seasonal regression equation Y = 91.574 × H – 344.64 (R^2 = 0.742) where Y equals pasture yield (kg DM/ha) and H equals the average RP_{Meter} reading

Accurately determining PDMI in pasture-based production systems is important, hence different methods were used to compare against the values obtained in our study. It is expected that a lactating dairy cow may consume on average 3 % of her BW as DM (Kolver & Muller, 1998) or 3.25 % when there are no quantitative and qualitative limitations from pasture (Leaver, 1985). According to the above assumptions cows in our study may have consumed 12.3 and 13.3 kg DM/cow/d, respectively. This is an under estimation compared to the 16.2 kg DMI/cow/d observed in our study. Higher pasture intake as a percentage of the cow's live BW for pasture-based production systems have been reported. Heard *et al.* (2004) reported that maximum DMI is around 4.2 % of cow's BW, although 3.5 to 4 % is more frequently reported (Mayne & Wright, 1998; Fulkerson *et al.*, 2006). Within these values the cows in our study would have been able to consume 14 to 17 kg DM/d, which coincide with the 16.2 kg DMI reported in our study.

Bargo *et al.* (2003) further reported that a cow may consume up to 1.3 % of her BW per day as NDF, thus it is expected that the cows would have been able to consume 5.3 kg NDF/d (1.3 % of 408 kg). Considering that the cows consumed on average 7.26 kg of concentrate on a DM basis with a mean NDF concentration of 9.5 % (Table 4.4) and the pasture had a mean NDF concentration of 42.1 % (Table 4.2), the PDMI could have been calculated as 42.1 % of PDMI + 9.5 % of 7.26 kg = 5.3, therefore PDMI = 10.96 kg DM/cow/d. Cow PDMI could also be estimated with the assumption of Kolver & Muller (1998) that cows are only able to consume 1.5 % of their BW as NDF when fed only pasture and that the inclusion of concentrate in the diet of grazing dairy cows would have a SR of 0.093 per kg of concentrate fed (Faverdin

et al., 1991). As a result, it is estimated that the cows would have been able to consume 6.12 kg as NDF (1.5 % of 408 kg) or 14.5 kg PDMI (6.12 kg/42.1 % NDF; Table 4.2) on pasture only. Since the cows received 7.26 kg concentrate DM/cow/d, the SR would have been 0.67 (0.093 × 7.2), thus the PDMI would have been reduced by 4.8 kg (0.67 × 7.2) to 9.7 kg DM/cow/d. Both methods just described, are in agreement with the PDMI observed in our study.

A back calculation could also be used to predict PDMI based on the energy requirements of the cow for maintenance, production, change in BW and physiological status (Tesfa *et al.*, 1995, Reeves *et al.*, 1996). To perform this calculation the ME requirements of the cow must be known, the average ME requirements of the cows in our study were calculated to be 186.5 MJ ME/d (Appendix B Table B1). Thus, if the cows consumed on average 7.26 kg DM of concentrate per day with an average ME concentration of 12.6 MJ ME/kg DM (Table 4.4), the concentrate would have supplied approximately 91.5 MJ ME/d of the cows total ME requirements. The remaining 95 MJ ME would have been supplied from pasture. For the cows to consume this amount of ME from the ryegrass pasture containing 10.3 MJ ME/kg (Table 4.2), a PDMI of 9.2 kg DM/cow/d should have been achieved. In addition to the back calculation method, the equation described by the NRC (2001) for the prediction of PDMI could also be used. This equation requires 4 % FCM, cow BW and the cow's week of lactation (WOL), the prediction equation is as follow: DMI (kg/d) = [((0.372) (4 % FCM) + (0.0968) ($BW^{0.75}$)) (1 – $e^{(-0.192 \times (WOL + 3.67))}$)]. The average 4 % FCM across all treatments was 24.7 kg/cow/d (section 3.5), average cow BW was 408 kg (section 3.5) and average WOL was 18.6 weeks (section 3.5). According to the NRC (2001) equation, the PDMI was calculated to be 10.5 kg DM/cow/d. The comparison of the different methods suggests that the mean predicted PDMI/cow/d and that obtained during our study by using the RP_{Meter} method are in agreement with each other. As a result, I am confident that the cows did consumed 9.04 kg DM/cow/d from pasture and would be used in further calculations.

The pasture growth rate estimated during the trial was 69.5 kg DM/ha/d, which was higher than the growth rate of 63.6 kg DM/ha/d observed for the same cultivar, (*Lolium multiflorum* var. *italicum*) cv. Fox, during October 2018 in an ongoing elite evaluation trial at the Outeniqua Research Farm (S, Ammann., personal communication, sigruna@elsenburg.com). The pasture growth rate was lower than the average 75.5 kg DM/ha/d reported by van der Colf *et al*. (2015a) for the same pasture type during Spring. However, the growth rate observed in our study falls within the range of growth rates (46.1 to 86.0 kg DM/ha/d) reported by Botha *et al*. (2015) for the same pasture type during September and October, established within a two-month deviation from when the ryegrass seed was planted in our study (27 March 2018). The estimated pasture growth rate seems to be in accordance with the literature for ryegrass during Spring.

Since PDMI is related to PA, the utilisation of pasture is expressed as a proportion of pre-grazing pasture mass. The pasture utilisation during the study (70 % above 30 mm height) was slightly higher than the 64 % reported by Fulkerson *et al*. (2006) and lower than the 81 % reported by Van Wyngaard (2013) but similar to others for Jersey cows grazing the same pasture type (Malleson, 2008, Van der Vyver, 2019).

One limitation of using the RP_{Meter} method to estimate PDMI is that the growth rate between pre-and post-grazing measurements are not taken into account, including the fact that the same equation is used for both pre-and post-grazing.

The cows went through approximately 2.5 grazing cycles during the experiment, with a mean grazing cycle of 24.5 days. The stocking rate of 6.7 cows/ha used in our study falls between the range of 6.26 to 8.17 cows/ha reported by Van der Colf *et al*. (2015a). These grazing cycles and stocking rates are standard for pasture-based production systems in the Western Cape, George area (Meeske, R., personal communication, robinm@elsenburg.com).

4.3 Pasture composition

The chemical composition of the ryegrass pasture and how it changed as the season progressed, is presented in Figure 4.4. A trend in the nutritive composition of the pasture grazed by the cows in this study is visible as the season progressed. The pasture DM, NDF, ADF and ADL concentration increased and accordingly the ME and *IV*OMD decreased. As the season progressed a slight increase in pasture CP was observed, although a decrease was expected (Van Vuuren *et al.*, 1992). Variability in pasture CP concentration could be due to excessive N fertilisation, however, this is unlikely as the N fertilisation was set at 28 kg N/ha after each grazing for this study, which is considered as a low level of N fertilisation. Several agro-climatic factors, including precipitation and temperature may influence the volatility and leaching of N at the time of fertilisation, as a result, the CP concentration of the pasture (Peyraud & Astigarraga, 1998). The duration between fertilisation and sampling could also result in variable CP content, as pasture CP content rises after fertilisation, then plateaus, before slightly diminishes with older regrowth (Peyraud & Astigarraga, 1998). As with other forages species the nutritive value of ryegrass declines with increased maturity. Ryegrass in Australia showed that in winter (early growth stage) the NDF and lignin concentration were low, while the CP, *IV*OMD and ME were high. In contrast to winter, in summer (late growth stage), the NDF and lignin concentration increased, while the CP, *IV*OMD, and ME decreased (Fulkerson *et al*., 2007). The seasonal variation observed in the nutritional value of grazed pastures is primarily associated with the different stages of forage growth. In late spring (around November) the pasture is in the "flag" leaf stage prior to flowering, this indicates that the pasture is transitioning from a vegetative stage to the reproductive stage, hence an increase in stem (NDF and lignin) is observed and a lower growth rate (Stockdale, 1999). As the pasture matures a reduction in passage rate, protein degradation due to the cell wall becoming more resistant to ruminal degradation and a reduction in NDF degradation results in a decrease in nutrients in the rumen (Van Vuuren *et al*., 1992; Lowe *et al*., 1998; MacDonald *et al*., 2017). The NFC fraction also decreases in addition to the fructans as plants mature (MacDonald *et al*. 2017).

The decrease in pasture quality is also in agreement with multiple authors stating that the quality of ryegrass deteriorates as it progresses through the season (Fulkerson *et al.*, 1998; Bargo *et al.*, 2003; Van der Colf *et al.*, 2015b).

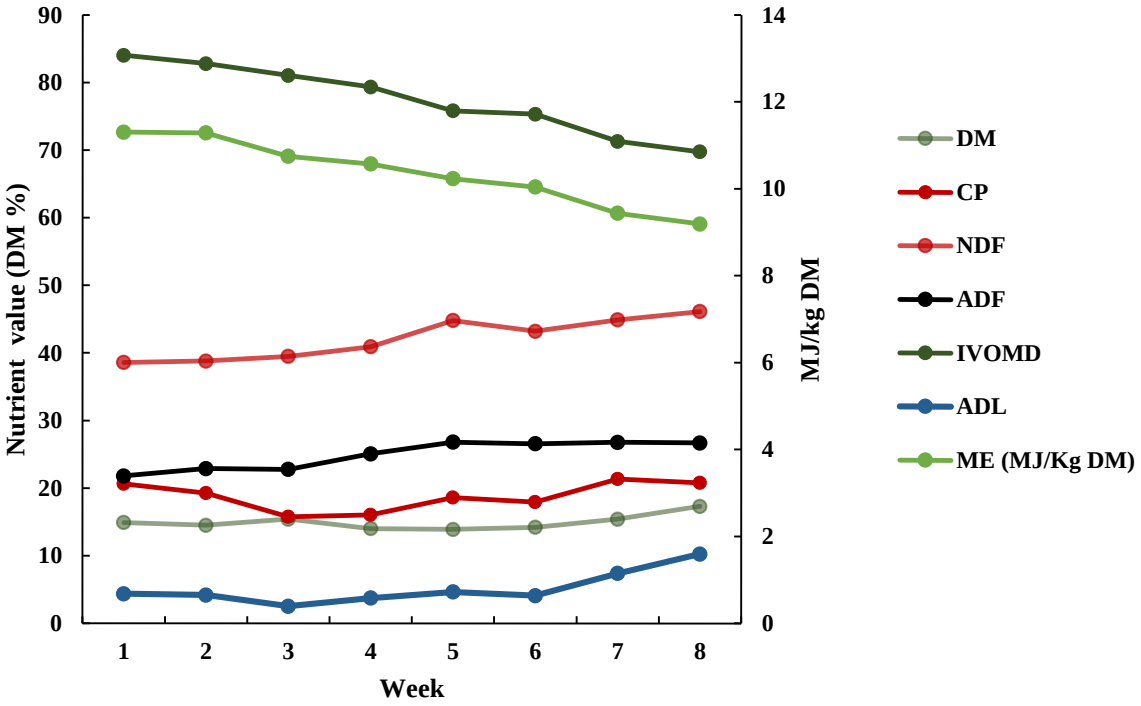

Figure 4.4 Change in nutrient parameters of the ryegrass pasture as affected by the progression from early to late spring during the study on the Outeniqua Research Farm in which cows grazing ryegrass pasture were supplemented with rumen protected Met and/or Lys.

The mean chemical composition of the ryegrass pasture is presented in Table 4.2 and the AA composition presented in Table 4.3. The mean DM concentration of 15.0 % is in agreement with the DM concentration range of 13.7 to 16.4 % reported by other researchers for annual ryegrass during spring (Meeske *et al*., 2006; Coetzee, 2011; Steyn *et al*., 2014; Van der Vyver, 2019). The mean OM concentration (100 – ash) of 89.5 % DM is similar to the 89.4 % DM reported by Van Wyngaard & Meeske (2016). The mean ash concentration of 10.5 % DM is higher than the 8.7 % DM reported by Meeske *et al*. (2006), but in agreement with the 10.3 % DM reported by Muller (2012) and Van der Vyver (2019). The pasture CP concentration are similar to the result reported by Meeske *et al*. (2006), Botha *et al*. (2008), and Clark *et al*. (2018). The mean CP concentration of 18.8 % DM was lower than expected for annual ryegrass during spring as reported by multiple authors (Chapter 2, Table 2.1), even though, the CP concentration was still above 15 % DM as recommended by the NRC (2001) for high-producing dairy cows in mid-lactation consuming a TMR.

The mean NDF concentration of 42.1 % DM, is similar to that reported by Fulkerson *et al*. (2006) and Steyn *et al*. (2014), falling between the recommended range of 40 to 50 % DM for a temperate grass pasture during spring (Muller & Fales, 1998). The NDF of the pasture is a reflection that the cows grazed

high-quality pasture. The mean ADF concentration of 24.9 % DM is within the range of 22.5 % DM (Clark *et al.*, 2018) and 28 % DM (Meeske *et al.*, 2006) and similar to the 25 % reported by Fulkerson *et al*. (1998) for annual ryegrass during spring. Moller *et al*. (2015) reported a mean ADF concentration of 25.5 % DM for the same pasture type which is in line with the values reported in this study, and above the minimum recommended 17 to 21 % DM to prevent milk fat depression (NRC, 2001). It is clear that both NDF and ADF concentrations increased, as expected, as the season progressed.

The mean *IV*OMD of 77.4 % DM is between the 78.3 % DM reported by Fulkerson *et al*. (2006) and 76.1 % DM reported by Coetzee (2011), with a general decline as the season progressed. Pasture had a mean GE of 16.3 MJ/kg DM which is lower than the 17.4 MJ/kg reported by Coetzee (2011), but in line with others (Malleson, 2008). As the ME concentration was calculated using the equation 0.82 × (GE × *IV*OMD) (Robinson *et al.*, 2004), the mean ME concentration 10.3 MJ/kg also decreased along with the decrease in OM digestibility. However, the ME concentration obtained during this study is similar to the 10.3 MJ ME/kg DM reported by Fulkerson *et al.* (2005) and 10.4 MJ ME/kg DM reported by Douglas (2020) and also within the ranges reported by others 9.45 MJ ME/kg DM (Lowe *et al*., 1999) and 10.9 MJ ME/kg DM (Meeske *et al.*, 2006), including other researchers (Fulkerson *et al*., 2007; Botha *et al.*, 2008, Erasmus, 2010) for the same pasture type during spring.

The mean EE concentration of 3.2 % DM was similar to 3.2 % DM reported by Malleson (2008) and within the range of 3 to 4 % DM reported by Muller & Fales (1998) for temperate grass species during spring. The mean Ca and P concentrations was 0.34 and 0.38 % DM, respectively, resulting in a Ca: P ratio of 0.9. Calcium was lower than the 0.39 % DM reported by Van der Colf *et al*. (2015), including the 0.55 % DM reported by Meeske *et al*. (2009). This indicates that from pasture alone the cows would have been deficient in Ca as the NRC (2001) recommend a 0.67 % Ca level in the diet of high producing dairy cows. As a result, Ca is usually supplemented to high producing dairy cows grazing ryegrass pasture as was done in this study. A P content of 0.38 % DM was observed, which is lower than the 0.45 % DM reported by Meeske (2009) and similar to the 0.37 % DM reported by Botha *et al*. (2008), including the 0.39 % DM reported by Van der Colf *et al.* (2015b). Due to the lower than normal levels of Ca and normal levels of P, the Ca:P ratio was also lower (0.9:1), lower than the 1.6:1 ratio required by dairy cows (NRC, 2001). However, the ratio was corrected by the addition of supplemental Ca in the concentrate as indicated in Table 4.6 for the total diet consumed by the cows.

The lignin (ADL) concentration of the pasture averaged 5.10 % DM and slightly increased as the season progressed, which is supported by others (Muller, 2012, Steyn *et al.*, 2014). Since the pasture progressed to a reproductive stage from a vegetative state, it led to an increase in stem content as a result of stem elongation, ultimately resulting in an increased pasture lignin and NDF content (Fulkerson *et al.*, 2007).

Fulkerson & Trevaskis (1997) reported that soluble carbohydrate levels in ryegrass may vary from less than 2 % to more than 30 % DM, with the protein content usually moving in the opposite direction, as

was observed in our study (18.8 *vs*. 22.9 % DM; Table 2.1). Furthermore, the NFC value of 25.4 % DM seems to be over-estimated based on previous research done at the Outeniqua Research Farm *(Meeske et al*., 2009, Van Wyngaard, 2016). However, Muller & Fales (1998) reported a NFC range of 12 to 20 % DM, which is lower than the NFC range of 18.8 to 30.9 % DM reported by Gehman *et al*. (2006). Other equations have also been proposed by the NRC (2001) and others (Tylutki *et al.*, 2008) to calculate NFC, since some fractions, for example NDICP are not taken into account when using the standard equation NFC = 100 – (CP % + EE % + NDF % + Ash %; NRC, 2001). This could compromise the accuracy with which NFC is calculated. In addition to various factors not taken into account when calculating the NFC content based on the equation presented by the NRC (2001) it also includes prediction errors of the other parameters used in the equation. Based on the equation above, the NFC content observed in our study followed a decreased trend as the season progressed, which is in agreement with others (Fulkerson & Trevaskis, 1997, Gehman *et al.*, 2006). The NDIP (N × 6.25), which represents the CP associated with the cell wall content that is insoluble in a neutral detergent solution, and ADIP, which represents the CP that is largely unavailable to the animal and includes lignified N and Maillard products (Krishnamoorthy *et al.*, 1982) were 5.6 % NDF and 6.8 % ADF, respectively. Krishnamoorthy *et al*. (1982) reported that these two fractions are highly variable between samples. The values observed in our study are similar to others (Steyn *et al*., 2014), but higher than that reported by Van Wyngaard & Meeske (2017) and Coetzee (2011). Bramley *et al*. (2012) reported a NDIP (% NDF) value of 8.6 % and an ADIP (% ADF) of 2.9 %. While Douglas (2020) reported a NDIP (% NDF) value of 3.7 % and an ADIP (% ADF) of 2.9 %. It seems that the ADIP content reported in our study was slightly over predicted. The NDIP value reported in our study is in line with the value reported by Higgs *et al*. (2015).

The nutritive value of the ryegrass pasture grazed by the Jersey cows in the current study corresponds with data reported by several authors as reviewed in the literature (Chapter 2, Table 2.1), representing a high-quality pasture.

The AA composition of the ryegrass grazed during the study is similar to values reported by others (Kolver *et al.*, 1998; Malleson, 2008; Edmunds *et al.*, 2013), information on the AA composition of ryegrass during different seasons, however, is limited.

Table 4.2 Mean (± SD) chemical composition of the ryegrass pasture grazed by Jersey cows supplemented with RPM and/or RPL (n = 8)

Parameter[1]	**Mean (% DM or as stated)**
DM %	15.0 ± 1.04
Ash	10.5 ± 0.76
OM	89.5 ± 0.76
GE (MJ/kg DM)	16.3 ± 0.17
ME (MJ/kg DM)	10.3 ± 0.26
CP	18.8 ± 2.00
NDF	42.1 ± 2.83
ADF	24.9 ± 1.98
NDIP (% NDF)	5.60 ± 0.89
ADIP (% ADF)	6.80 ± 1.50
ADL	5.10 ± 2.31
NFC	25.4 ± 4.74
EE	3.20 ± 0.15
*IV*OMD	77.4 ± 4.91
Ca	0.34 ± 0.04
P	0.48 ± 0.04
Ca:P ratio	0.90 ± 0.09

[1]DM – Dry matter; OM – Organic matter; GE – gross energy; ME – Metabolisable energy (ME = GE × *IV*OMD × C) (C = 0.82; Robinson *et al*., 2004); CP – Crude protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; NDIP – Neutral detergent insoluble protein; ADIP – Acid detergent insoluble protein; ADL – Acid detergent lignin; NFC – Non-fibre carbohydrates (NFC = 100 – (CP + NDF + EE + ash)) (NRC, 2001); EE – Ether extract; *IV*OMD – *in vitro* organic matter digestibility; Ca – Calcium; P – Phosphorus

Table 4.3 Mean (± SD) essential amino acid (EAA) composition (% DM) of ryegrass pasture grazed by Jersey cows supplemented with RPM and/or RPL (n = 8)

Parameter	**Period**[1]
EAA	
Lysine (Lys)	0.75 ± 0.06
Methionine (Met)	0.43 ± 0.03
Arginine (Arg)	1.06 ± 0.04
Histidine (His)	0.30 ± 0.02
Isoleucine (Ile)	0.89 ± 0.04
Leucine (Leu)	1.40 ± 0.04
Phenylalanine (Phe)	1.27 ± 0.05
Threonine (Thr)	0.74 ± 0.03
Valine (Val)	0.99 ± 0.03
Total EAA	7.82
Lys:Met ratio	1.74

[1]Period (10 September to 5 November 2018)

4.4 Concentrate composition

The chemical composition of the three concentrate treatments is presented in Table 4.4 and are representative of a typical maize-based concentrates fed to grazing Jersey cows on pasture in the Southern Cape region of South Africa (Meeske, R., personal communication, robinm@elsenburg.com). The inclusion of RPM and/or RPL in the diet was not expected to alter the chemical composition of the concentrates to a large extent only the AA profile as presented in Table 4.5. The Met and Lys values were lower than expected. This may be due to the difficulty associated with accurate sampling of concentrates containing RPAA. The RPM used is in granular form and although it might be mixed homogeneously relative to 8 kg concentrate on an as is bases, the small amount sub-sampled to be analysed might not be a representative sample.

Table 4.4 Chemical composition of the concentrate pellets fed to Jersey cows grazing ryegrass pasture supplemented with RPM and/or RPL (n = 2)

Parameter[2]	**Experimental treatment**[1]		
	C	**RPL**	**RPML**
DM %	90.6	90.6	91.0
Ash	5.84	6.25	6.62
OM	94.1	93.9	93.4
GE (MJ/kg DM)	16.4	16.3	16.2
ME (MJ/kg DM)	12.8	12.3	12.6
Starch	53.6	56.9	55.5
CP	12.7	13.3	12.7
NDF	9.25	9.35	9.95
ADF	3.22	3.22	3.14
ADL	1.23	1.45	1.46
NFC	70.7	69.2	67.2
EE	1.55	1.91	3.59
*IV*OMD	95.2	92.3	94.7
Ca	1.16	1.26	1.24
P	0.48	0.49	0.50
Ca:P ratio	2.42	2.58	2.48

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

[2]DM – Dry matter; OM – Organic matter; GE – gross energy; ME – Metabolisable energy (ME = GE × IVOMD × C) (C = 0.82; Robinson *et al*., 2004); CP – Crude protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; ADL – Acid detergent lignin; NFC – Non-fibre carbohydrates (NFC = 100 – (CP + NDF + EE + ash)) (NRC, 2001); EE – Ether extract; *IV*OMD – *in vitro* organic matter digestibility; Ca – Calcium; P – Phosphorus

Table 4.5 Mean (± SD) essential amino acid (EAA) composition (% DM) of concentrate pellets fed to Jersey cows grazing ryegrass pasture supplemented with RPM and/or RPL (n = 2)

Parameter	Experimental treatment[1]		
	C	RPL	RPML
EAA			
Lysine (Lys)	0.50 ± 0.08	0.65 ± 0.03	0.60 ± 0.07
Methionine (Met)	0.26 ± 0.01	0.24 ± 0.07	0.31 ± 0.01
Arginine (Arg)	0.89 ± 0.05	0.86 ± 0.05	0.85 ± 0.03
Histidine (His)	0.35 ± 0.04	0.29 ± 0.05	0.28 ± 0.02
Isoleucine (Ile)	0.72 ± 0.04	0.67 ± 0.03	0.67 ± 0.03
Leucine (Leu)	1.30 ± 0.07	1.35 ± 0.07	1.28 ± 0.01
Phenylalanine (Phe)	0.71 ± 0.02	0.74 ± 0.09	0.77 ± 0.03
Threonine (Thr)	0.53 ± 0.06	0.46 ± 0.03	0.42 ± 0.01
Valine (Val)	0.70 ± 0.14	0.64 ± 0.05	0.62 ± 0.03
Total EAA	5.96	5.90	5.79
Lys:Met ratio	1.95	2.66	1.97

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

4.5 Total diet composition

Cows were supplemented with 8 kg (as is) of concentrate per day, 7.24, 7.25 and 7.28 kg DM, for the C, RPL and RPML treatments, respectively. Since all cows grazed together as a single herd, pasture intake for the cows on the different concentrate treatments could not be determined by using pre-and post-grazing heights. The expected PDMI was calculated to be 9.04 kg/cow/d (Section 4.2). A diet containing 9.04 kg DM of ryegrass with the chemical composition as in Table 4.2 and Table 4.3, in addition to the experimental concentrates with the chemical composition as in Table 4.4 and Table 4.5 would have supply the cows with a total diet DM chemical composition as presented in Table 4.6.

It is assumed that the supplementation of a RPAA to the concentrate fed to grazing dairy cows should only alter the provided AA and not the chemical composition of the concentrate, this is shown in Table 4.6. Furthermore, the total diet nutrient composition is within the guidelines recommended by Erasmus *et al*. (2000) and NRC (2001) for cows in mid to late-lactation with slight variation.

The average ME concentration of the C, RPL and RPML diets were 11.4, 11.2 and 11.4 MJ ME/kg, respectively, and fall between the recommended ME concentrations of 10.5 and 11.5 MJ ME/kg for mid-lactation dairy cow (Erasmus *et al*., 2000; McDonald *et al*., 2001; NRC, 2001; Fox *et al*., 2004; Hutjens, 2018). However, due to the fact that grazing dairy cows requires 10 to 30 % additional ME as a result of higher energy requirements for physical activity (i.e. grazing and walking) the ME concentrations of the three experimental diets and the grazed pasture could have been inadequate (Bruinenberg *et al*., 2002; Kaufman *et al*., 2018). Additionally, Johnson & Johnson (1995) reported that grazing dairy cows could have an additional 2 to 12 % loss of the energy provided from the feed due to methane production.

Table 4.6 Mean chemical, Lys and Met composition (% DM) of the total diet consumed (9.04 kg ryegrass DM and approximately 7.2 kg DM concentrate/cow/d) by the Jersey cows supplemented with RPM and/or RPL

Parameter[2]	Experimental treatment[1]		
	C	RPL	RPML
DM %	48.6	48.6	48.9
OM	91.5	91.5	91.2
ME (MJ/kg DM)	11.4	11.2	11.4
CP	16.1	16.4	16.1
SolCP[3]	7.65	7.53	7.55
RDP[3]	11.2	11.1	11.0
RUP[3]	4.81	4.89	4.98
NDF	27.5	27.5	27.8
ADF	15.3	15.3	15.2
*IV*OMD	85.3	84.0	85.1
Ca	0.68	0.73	0.72
P	0.44	0.44	0.44
Ca:P ratio	1.58	1.65	1.60
EAA			
Lys	0.64	0.71	0.68
Met	0.35	0.35	0.38
Lys:Met ratio	1.84	2.15	1.84

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

[2]DM – Dry matter; OM – Organic matter; ME – Metabolisable energy (ME = GE × *IV*OMD × C) (C = 0.82; Robinson *et al*., 2004); CP – Crude protein; SolCP – Soluble CP; RDP – Rumen-degradable protein; RUP – Rumen-undegradable protein; NDF – Neutral detergent fibre; ADF – Acid detergent fibre; *IV*OMD – *in vitro* organic matter digestibility; Ca – Calcium; P – Phosphorus; EAA – Essential amino acid, Lys – Lysine; Met – Methionine

[3]Predicted using the CNCPS model

It was estimated, that the average ME supply provided from the pasture and concentrate treatments were approximately 186, 183 and 185 MJ ME/cow/d for the C, RPL and RPML diets, respectively. It was calculated, based on equations reported in the NRC (2001), as shown in Appendix B Table B1 that the cows required approximately 186, 185 and 188 MJ ME/cow/d for maintenance, lactation, physical activity and gain in BW for the C, RPL and RPML treatments, respectively. This indicates that energy could still have been first limiting, since the energy requirements of the cows could have been under predicted. Accurately assessing the ME requirements of grazing dairy cows for physical activity (i.e. walking, grazing and forage selection) has hindered the progress of research for grazing dairy cows, despite multiple methods available to determine the energy requirements of dairy cows. Kaufman *et al*. (2018) reported that the grazing cow's energy requirements for physical activity, specifically walking, is higher than cows fed a TMR ($P < 0.05$). This is in agreement with Brosh *et al*. (2010) who reported that cows grazing pasture require an additional 0.0028 MJ/kg$^{0.75}$ BW/km for grazing and 0.882 MJ/kg$^{0.75}$ BW/d for walking. However, the energy required for grazing per km can vary widely from 0.0014 to 0.0186 MJ/kg$^{0.75}$ BW/km (Tedeschi & Fox, 2018). In support of the statement that ME could have been first limiting in our study, the total dietary supply of ME provided to the cows from the pasture was calculated from an average ME value of 10.3 MJ/kg DM for the whole study period (Table 4.2). Considering the fact that the average ME concentration of the pasture

dropped from 11.3 to 9.2 MJ/kg DM over the eight-week study period (Figure 4.4), indicates that the energy deficit could have increased further as the study progressed, indicating that the ME concentrations of the diets could have been inadequate.

The CP concentration of 16.1, 16.4 and 16.1 % DM for the C, RPL and RPML treatments, respectively, is within the recommended 14 to 17 % CP required by a high producing dairy cows in mid-lactation (Erasmus *et al*., 2000; NRC, 2001; Colmenero & Broderick, 2006). According to table 14-2 of the NRC (2001) a small breed of cows (454 kg) in mid-lactation producing 20 kg milk/d with a milk fat percentage of 4.5 %, a milk true protein percentage of 3.5 to 4 %, consuming 16.5 kg DM/cow/d requires a diet containing 15 to 16 % CP, 1730 g RDP (70.4 % CP) and 720 to 900 g RUP (29 % CP). The CNCPS model predicted, based on model inputs described in section 3.9, values similar to the ranges reported by the NRC, 2001. The main goal of characterising dietary CP of lactating dairy cows is to obtain accurate estimates of RDP and RUP (Sniffen *et al*., 2001). Multiple methods have been evaluated to determine these fractions, which include *in vivo*, *in situ* and a variety of *in vitro* methods. The RDP and RUP fractions along with the rate and extent of degradation in the rumen influences the nutritive value of the protein provided by the diet to a great extent (Schwab *et al*., 2003). The amount of protein degraded in the rumen depends on the proportional content of proteins and NPN constituents (i.e. peptides, free AA, nucleic acids, amides, amines, nitrate and ammonia), the chemical and physical properties of the different protein fractions, rumen retention time and pH, including the microbial proteolytic activity (NRC, 2001; Van Amburgh *et al*., 2015).

The NRC (2001) uses an *in situ* method to estimate ruminal protein degradation, based on this method the following equation can be used to estimate the RDP and RUP fractions in the diet: RDP = A + B [K_d/(K_d + K_p)] and RUP = CP – RDP, or B[K_d /(K_d + K_P)] + C. Fraction A represents the soluble (rapidly degradable) fraction which is assumed to be completely degraded in the rumen. Fraction B represents the slowly degradable (or potentially degradable) fraction and depends on the digestion rate (K_d) of the B fraction and the passage rate (K_p) of the undigested feed. Fraction C represents the undegradable (unavailable) fraction. However, as reviewed by the NRC (2001), the soluble proteins are not equally susceptible to ruminal degradation, similarly to insoluble proteins which are not equally resistant to ruminal degradation. As a result, the CNCPS model uses a multi-chemical approach for quantifying five different N fractions (Sniffen *et al*., 1992; Fox *et al*., 2004). These fractions include an A (NPN), B_1 (rapidly degraded true protein), B_2 (moderately degraded true protein and large peptides), B_3 (slowly degraded true protein) and C (undegraded protein). As a result, the RDP and RUP is calculated as follow: RDP = A + B_1 (K_dB_1 / [K_dB_1 + K_p]) + B_2 (k_dB_2 / [k_dB_2 + K_p]) + B_3 (K_dB_3 / [K_db_3 + K_p]) and RUP = 1 – RDP. The A fraction is calculated as NPN × (0.01) × (SolCP), the B_1 fraction as SolCP - A, the B_2 fraction as 100 - A - B_1 - B_3 - C, the B_3 fraction as NDIP – ADIP (Sniffen *et al*., 1992). Unfortunately, during our study, the rumen degradability of the protein could not be measured. However, based on the CNCPS equation described (Sniffen *et al*., 1992) the RDP and RUP protein fractions could be calculated from literature values and table values for digestion and passage rate, SolCP and NPN which were not directly determined during our study.

The pasture grazed by the cows in this study had CP concentration of 18.8 % DM. Chapter 3, section 3.9 describes the inputs used for modelling the study using the CNCPS model. Concentrate treatments and PDMI were adjusted for actual and predicted total DM intake. The chemical composition and AA composition of the respective concentrate treatments and pasture, as described in Table 4.2, Table 4.3, Table 4.4, Table 4.5 were used in the model evaluation. Model defaults were used where specific nutrients were not analysed for based on average nutrient values in South Africa as used in the NDS feed library provided by NOVA feeds. As a result, the model predicted that the C, RPL and RPML treatment supplied the cows with 1818, 1804 and 1792 g/cow/d of RDP and 783, 797 and 811 g/cow/d of RUP. The increase in RUP protein was excepted due to the contribution of RUP from the RPM and RPL. This suggests that the cows were optimally supplied with RUP. The CNCPS model further predicted that the cows required on average 1756 g/cow/d of MP and that 1769 g/cow/d of MP were supplied. The MP requirement predicted by the CNCPS model is in line with the 1703 g/cow/d of MP reported by McDonald *et al*. (2001) for a mid-lactating Jersey cow. This indicated that the cows were adequately supplied in protein (i.e. RUP and MP) for the level of cow performance observed during our study across all treatments.

In the literature, digesta flow studies (i.e. duodenal sampling technique) have been used to quantify the ruminal degradation of the CP provided from pastures, including MCP flow (Young *et al*., 2000). Unlike the *in situ* bag technique frequently used, which has errors associated with the inability to accurately quantify MCP contamination, digesta flow studies use microbial markers to differentiate between the N provided from MCP and non-ammonia, non-microbial N flow (Broderick *et al*., 2010; Hristov *et al*., 2019). Van Vuuren *et al*. (1991) estimated the digestible CP fraction of plant origin escaping rumen fermentation with the equation PDCP = $(k_p/(k_d+K_p) \times D) \times CP$. Van Vuuren *et al*. (1991) further reported that ryegrass pasture has an assumed passage rate of 5 %/h with a N degradability of fresh ryegrass pastures ranging from 47 to 87 %. This is in line with various authors that quantified N flows for lactating dairy cows grazing pasture based on the duodenal sampling technique, reporting degradations rates of pasture varying from 65 to 79 % (O'Mara *et al*., 1997; Peyraud *et al*., 1997). Berzaghi *et al*. (1996) reported that cows grazing pasture while supplemented with a maze-based concentrate had a passage rate of 7.1 %/h, which is higher than that reported by Van Vuuren *et al*. (1991). Pasture *in vivo* apparent digestibility of N can be as high as 84 %, indicating the extensive degradation of high-quality pasture (Kolver & Muller, 1998; Pacheco & Waghorn, 2008).

Considering the AA composition of the concentrates fed during our study, the C diet contained 0.64 % Lys and 0.35 % Met (104 g Lys/cow/d and 57.7 g Met/cow/d), RPL diet contained 0.70 % Lys and 0.28 % Met (115 g Lys/cow/d and 56.3 g Met/cow/d) and the RPML treatment contained 0.68 % Lys and 0.38 % Met (112 g Lys/cow/d and 61.4 g Met/cow/d). These AA fractions do not represent the AA that was actually available for intestinal absorption. Duodenal sample collection would have been required to determine AA available for intestinal absorption and this was not possible in our study.

4.6 Effect of rumen protected methionine and lysine supplementation on performance of high producing Jersey cows grazing ryegrass pasture

4.6.1 Milk production and composition

The mean milk production and composition per cow and the total milk yield for each treatment group is presented in Table 4.7. Treatment had no effect on mean milk production, including total milk production between treatments (P > 0.05). The mean milk production was 22.26, 22.27 and 22.33 kg milk/cow/d and for total milk yield 1266, 1261 and 1265 kg milk for the C, RPL and RPML treatments, respectively. Figure 4.5 shows the mean daily milk yield for the C, RPL and RPML treatment groups respectively over the study period.

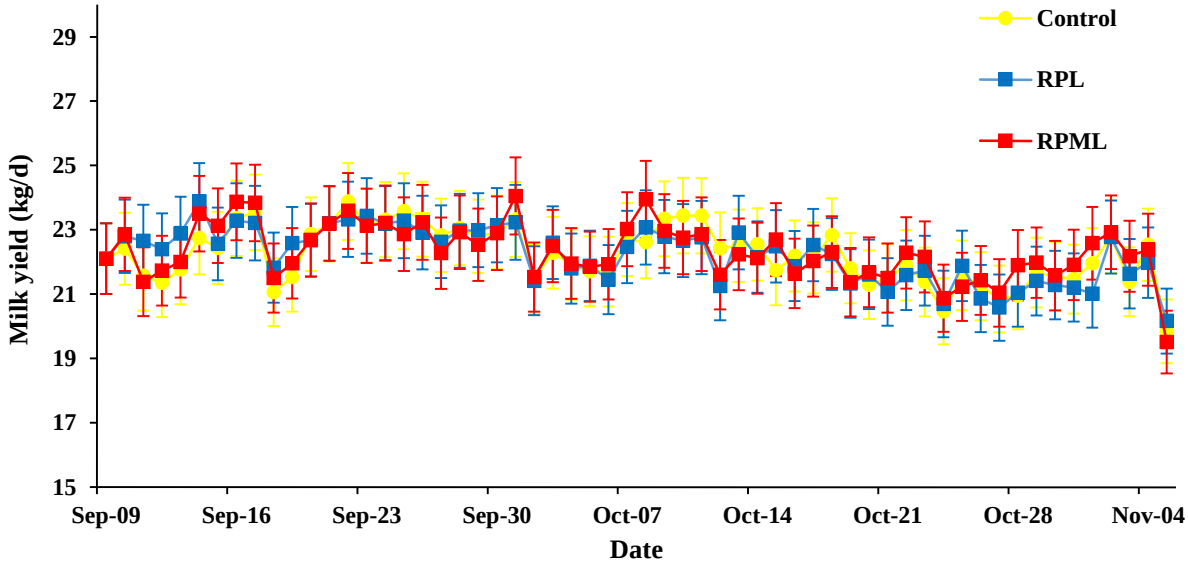

Figure 4.5 Mean daily milk yield (kg/d) with associated error bars for Jersey cows grazing ryegrass and fed a concentrate (~7.2 kg DM/cow/d) supplemented with RPL (rumen protected Lys) or RPML (ruminally protected Met and Lys) (n = 20)

This drop in milk production is expected for cows past peak-lactation grazing pasture (Garcìa & Holmes, 2005; Hutjens, 2018). This is also supported by Mostert *et al*. (2003) who derived standard lactation curves for South African Jersey cows using 1182 herds. Supplementation of RPM and/or RPL did not have an effect on 4 % FCM and ECM (P > 0.05) between the C, RPL and RPML treatment groups, with a 4 % FCM of 24.7, 24.6 and 24.9 kg/cow/d and ECM of 25.2, 25.0 and 25.6 kg/cow/d, respectively.

The mean milk protein percentage of the cows on the RPML treatment was higher than the RPL treatment (4.03 *vs.* 3.86 %, P < 0.05), although both the RPL and RPML did not differ from the control treatment (3.93; P > 0.05). Milk protein yield also tended to be higher for the RPML group compared to RPL group (0.90 *vs*. 0.85 kg/cow/d, P = 0.09), although both the RPL and RPML treatments did not differ

from the C treatment (0.87; P > 0.05). Treatment had no effect on milk fat percentage and yield, milk lactose percentage and yield, total milk solids, milk solids and MUN (P > 0.05). Milk fat percentages were 4.77, 4.73 and 4.79 %, milk fat yield was 1.05, 1.05 and 1.06 kg/cow/d, lactose percentages were 4.68, 4.64 and 4.70 %, lactose yield was 1,04, 1.03 and 1.05 kg/cow/d and MUN were 8.60, 8.74 and 9.01 mg N/dl, for the C, RPL and RPLM treatment groups, respectively.

Table 4.7 Mean milk yield and milk composition for Jersey cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 20)

Parameter	**Experimental treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[4]	C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Milk Yield (kg/d)	22.26	22.27	22.33	0.485	0.99	0.91	0.92
Total Milk Yield (kg)	1266	1262	1265	26.56	0.91	0.99	0.92
4% FCM (kg/d)	24.7	24.6	24.9	0.460	0.90	0.78	0.68
ECM (kg/d)	25.2	25.0	25.6	0.482	0.75	0.58	0.38
Protein (%)	3.93^{ab}	3.86^{a}	4.03^{b}	0.056	0.34	0.22	0.03
Protein (kg/d)	0.87^{cd}	0.85^{c}	0.90^{d}	0.017	0.62	0.23	0.09
Fat (%)	4.77	4.73	4.79	0.114	0.83	0.89	0.72
Fat (kg/d)	1.05	1.05	1.06	0.024	0.86	0.75	0.61
Lactose (%)	4.68	4.64	4.70	0.035	0.37	0.81	0.25
Lactose (kg/d)	1.04	1.03	1.05	0.030	0.78	0.87	0.66
Total MS (kg/d)[2]	2.96	2.93	3.01	0.062	0.74	0.60	0.40
MS (kg/d)[3]	1.92	1.90	1.96	0.036	0.73	0.44	0.26
MUN (mg N/dl)	8.60	8.74	9.01	0.241	0.68	0.23	0.42

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Total milk solids (Fat + Lactose + Protein)
[3]Milk solids (MS; Fat + Protein)
[4]Standard error mean
[a.b]Row means with different superscripts differ (P < 0.05)
[c.d]Row means with different superscripts tend to differ (P < 0.1)

Milk nitrogen fractions (casein, whey, NPN and NCN), true protein and casein:true protein ratio are presented in Table 4.8. The milk CP (N × 6.38) tended to be lower for the RPL compared to the C treatment (3.89 *vs.* 3.69; P = 0.09) and RPML treatment (3.91 *vs.* 3.69; P = 0.07), thus milk true protein (CP x 0.93) also tended to be lower for the RPL treatment compared to the C treatment (3.66 *vs.* 3.47; P = 0.09) and RPML treatment (3.47 *vs.* 3.67; P = 0.08). The NPN content were higher for the RPML treatment compared to the RPL treatment (0.24 *vs.* 0.21; P < 0.05), but not compared to the C treatment for both the RPL and RPML treatments (0.23; P > 0.05). The NCN content were 0.71, 0.67 and 0.67 and the whey content were 0.48, 0.43 and 0.43 for the C, RPL and RPLM treatment groups, respectively, but did not differ (P > 0.05). The casein content tended to be higher for the RPML compared to the RPL treatment (3.24 *vs.* 3.03; P = 0.06), but there was no difference between the RPML and RPL treatment compared to the C treatment (3.19; P > 0.05). When expressed as a proportion of CP the RPL and RPML treatment differed in NPN

(5.79 *vs*. 6.26 % CP; P < 0.05), but did not differ compared to the C treatment (5.96 % CP; P > 0.05). There were no differences among treatments for NCN, casein and whey protein when expressed as a proportion of CP (P > 0.05). The NCN were 18.2, 17.7 and 17.2 (% CP) and casein was 81.9, 82.6 and 82.8 (% CP) and whey protein was 12.3, 11.7 and 11.0 (% CP) for the C, RPL and RPLM treatments, respectively. The casein: true protein ratio was the same for the C, RPL and RPML treatment respectively (0.87 *vs*. 0.87 *vs*. 0.88; P < 0.05).

Table 4.8 Mean milk nitrogen fractions for Jersey cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 10)

Parameter	**Experimental treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[3]	C *vs*. RPL	C *vs*. RPML	RPL *vs*. RPML
Milk nitrogen fractions (%) or as stated							
Crude protein (CP)	3.89^{c}	3.69^{d}	3.91^{c}	0.084	0.09	0.87	0.07
NPN	0.23^{ab}	0.21^{a}	0.24^{b}	0.008	0.14	0.23	0.01
NCN	0.71	0.67	0.67	0.024	0.28	0.31	0.92
True protein[2]	3.66^{c}	3.47^{d}	3.67^{c}	0.078	0.09	0.95	0.08
Casein	3.19^{cd}	3.03^{c}	3.24^{d}	0.074	0.14	0.63	0.06
Whey	0.48	0.43	0.43	0.025	0.18	0.19	0.99
NPN (% CP)	5.96^{ab}	5.79^{a}	6.26^{b}	0.136	0.41	0.12	0.02
NCN (% CP)	18.2	17.7	17.2	0.547	0.56	0.20	0.50
Casein (% CP)	81.9	82.6	82.8	0.550	0.36	0.24	0.79
Whey protein (% CP)	12.3	11.7	11.0	0.582	0.47	0.13	0.42
Casein/true protein (% CP)	0.87	0.87	0.88	0.006	0.83	0.15	0.22

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]True protein (CP × 0.938)
[3]Standard error mean
NPN – Non-protein nitrogen; NCN – Non-casein nitrogen
[a,b]Row means with different superscripts differ (P < 0.05)
[c,d]Row means with different superscripts tend to differ (P < 0.1)

In the present study the supplementation of RPM and/or RPL did not result in any significant responses in milk production or milk composition when compared to the C treatment (P > 0.05). Even though, it was hypothesised that an individual supply of RPM and/or RPL to high-producing dairy cows grazing high-quality ryegrass pasture and supplemented with maize-based concentrate will have a positive effect on cow performance. The level of milk production observed over all treatments was similar to results observed by Malleson (2008) and Van Wyngaard *et al*. (2013), but slightly higher than that reported by Meeske *et al*. (2009) and Brossillon *et al*. (2018) for Jersey cows grazing the same pasture type during spring. This higher level of production may be due to the higher levels concentrate (8 *vs*. 6 kg concentrate/cow/d (as is)) supplemented during our trial and the fact that only high-producing cows were selected to be included in the trial (Delaby *et al*., 2001; Bargo *et al*., 2003; Clark *et al*., 2018). The milk composition observed in our study is similar to values reported by others (DePeters & Ferguson, 1992;

Carrol *et al*., 2006; Malleson, 2008; Joubert, 2012) and is representative of the Jersey breed standards in South Africa (Theron & Mostert, 2010). Cows used in our study were in fact high-producing relative to the average milk production of Jersey cows within this particular pasture-based production system and the performance was consistent with expectations based on the cow's previous lactation cycles.

In many different feeding situations, supplementation of RPM and/or RPL to lactating dairy cows has showed either unchanged (Robinson *et al.*, 1998; Třináctý *et al.*, 2009) or increased (Robinson *et al.*, 1995; Piepenbrink & Schingoethe, 1998; Chalupa *et al.*, 1999; Noftsger & St-Pierre, 2003; Wang *et al.*, 2010), milk production and/or milk composition. Although, in most of these cases the effects of RPM and RPL supplementation in combination cannot be separated, and only a limited number of studies have been published in which RPL was supplemented alone as in our study (Blauwiekel *et al.*, 1997; Robinson *et al.*, 1998; Swanepoel, 2009; Bernard *et al.*, 2014). Even less studies have been published with regards to RPM and/or Lys supplementation with regards to grazing dairy cows (Rulquin & Delaby, 1997b; Van Houtert, 1997; Pacheco-Rios *et al.*, 1999; Younge *et al.*, 2000).

The individual supplementation of RPL or combination of RPML in our study did not affect milk production and composition ($P > 0.05$). This is consistent with some studies and results reported in different meta-analysis (Robinson *et al.*, 1998; Lee *et al.*, 2015; Patton *et al.*, 2015) and inconsistent with others that reported a positive response in cow performance (Polan *et al.*, 1991; Noftsger & St-Pierre, 2003; Robinson, 2010; Wang *et al.*, 2010, Zanton *et al.*, 2014). However, several factors might influence whether cows will respond to supplementation of RPAA. These factors include the following: 1) stage of lactation and level of production, 2) chemical composition of the pasture and supplemental concentrate, 3) the balance of EAA in MP reaching the cows intestinal absorptive site, 4) the order in which nutrients become liming to cow performance and 5) the bioavailability of RPM and RPL supplement used (Polan *et al*., 1991; Chalupa *et al*., 1999; NRC, 2001; Doepel *et al*., 2004; Lapierre *et al*., 2007; Robinson, 2010; Apelo *et al*., 2014; Schwab & Broderick, 2017).

The lack of positive response in milk production observed in this study are similar to result reported by Patton (2010) and Zanton *et al*. (2014) for RPM and Robinson *et al*. (2000) for RPL. However, in contrast to our finding when Wang *et al*. (2010) fed a diet slightly limiting in MP, but adequate in energy, to Holstein cows in mid-lactation (120 DIM) supplemented with RPM and/or RPL providing an approximated concentration of Met and Lys in MP of 1.87 and 5.93 %, respectively. Milk yield improved significantly for the supplementation of RPL (+ 1.5 kg/d), RPM (+ 2.0 kg/d) and the combination RPLM (+ 3.8 kg/d). Cows fed the RPM and RPML in combination had higher milk fat content (3.95 and 3.90 %) than the control diet and RPL treatment (3.60 and 3.67 %). Similarly, Giallongo *et al*. (2016) supplemented Holstein cows fed a MP deficient diet (-54 g/d MP Balance) with RPL and reported an increase in milk protein content ($P < 0.05$), but there was no response in milk fat content ($P > 0.05$). Suggesting the MP was not limiting milk production in this study but rather the dietary ME supply.

The supplementation of RPM in this study did not affect the milk protein concentration which is in line with other studies (Apelo *et al*., 2014; Giallongo *et al*., 2015), but not with other studies that did report an increase in milk protein content when RPM was supplemented (Rulquin *et al*., 1993; Ordway *et al*., 2009). When RPL was supplemented in this study the milk protein concentration was not affected ($P > 0.05$) with the exception of tendency for reduced milk protein percentages (3.69 *vs*. 3.89; $P = 0.09$) observed in samples collected for the analysis of milk N fractions from a representative group of cows in this study (Table 4.8). This is in contrast to the positive responses observed by some (Schwab *et al*., 1992; Lapierre *et al*., 2009; Giallongo *et al*., 2016) and consistent with others (Young *et al*., 2000; Leonardi *et al*., 2003; Patton *et al*., 2015). It is suggested by Rulquin *et al*. (1993) that the response to RPM supplementation may be negative if Lys is limiting (i.e. less than 6.57 % MP). This is in accordance to (Robinson *et al*., 1996) who reported that Met supplied beyond the cow's requirements (150 to 160 %), depressed DMI and subsequently milk production when the Lys is decreased. Lapierre *at al*. (2006) suggested that an energy deficient diet may cause AA to be repartitioned away from milk protein synthesis towards oxidation for energy generation.

The individual supplementation of RPM and/or RPL in this study did not affect the milk fat content which is consistent with other studies, including a meta-analysis (Lee *et al*., 2012; Patton *et al*., 2015) and inconsistent with other studies (Watanabe *et al*., 2006; Zanton *et al*., 2014). The positive response reported in the literature in milk fat synthesis as a result of RPM supplementation has mainly been attributed to the role Met plays in providing methyl groups for several trans-methylation reactions which include the synthesis of choline. Choline is essential for the synthesis of phospholipids required for chylomicron and very low density lipoprotein synthesis (Ulrey *et al*., 2005; Giallongo *et al*., 2016). However, the positive responses observed in milk production and milk composition when diets deficient in MP and supplemented with RPM and/or RPL are in contrast to our study and could indicate that MP may not have been limiting production or that energy was still first limiting in our study. Kolver *et al*. (1999) measured the quantity and profile of AA available for absorption by grazing dairy cows while feeding the cows *ad libitum* or 75 % (restricted) ryegrass/white clover pasture in one experiment compared to *ad libitum* 100 % ryegrass pasture and a mixture of 50 % ryegrass and 50 % white clover in another experiment. All four treatments used in their experiment were found to be most limiting in Arg, Met and His, in that specific order, supplying 67, 71 and 77 % of absorbable AA requirements, respectively. The absorbable Lys met the requirements in early to mid-lactation cows across all four treatments, indicating that, although Met is frequently reported to be the first limiting AA when dairy cows are fed pasture (Kolver *et al*., 1999; Robinson, 2010), the order in which AA become limiting may differ. However, since it is speculated that ME was still first limiting establishing whether another AA was limiting production was not possible. Robinson *et al*. (1998) supplemented multiparous Holstein cows RPAA in the same manner as was done in this study, a C, RPM and/or RPL diet. The purpose of their study was to separate the effects of RPL from RPM when cows are fed a ration specifically designed to meet requirements for microbial and post-ruminal

protein. The diets contained timothy and corn silage, barley, maize and maize gluten meal and soybean meal. The RPL treatment supplied 21 g/cow/d of intestinally available Lys and the RPML treatment contained 6 and 22 g/cow/d of intestinally absorbable Met and Lys, respectively. Cow performance across all treatment groups was virtually identical, except for small numerical improvements in milk protein (+ 40 g/d) and milk fat (+ 40 g/d) for the combination of Met and Lys. However, these outcomes demonstrated that other AA (i.e. His and Ile) may have limited the performance of lactating dairy cows when fed grass-based diets. This was also confirmed by Pacheco-Rios *et al*. (1999), they supplemented Holstein cows grazing ryegrass-white clover pasture in mid and late-lactation with RPM, either orally (15 g/cow/d) or continuous intrajugular infusions of L-Met (15 g/cow/d). There were no significant differences in milk production or milk composition between treatments ($P > 0.05$). The authors concluded that apart from forage quality, Met might not have been the first limiting within this production system and that another nutrient might have limited production. Rulquin *et al*. (2001) reported that His is more likely to be first limiting in grass silage based diets and Leu in grass and barley based diets. Pacheco-Rios *et al*. (1999) concluded that His, Lys, Thr, including Phe + Tyr might be potentially limiting AA for lactating dairy cows grazing pasture. It was further demonstrated by Schwab *et al*. (1992) and Broderick *et al*. (2009) that an adequate post-ruminal supply of Lys is required for a production response to the supplementation of post-ruminal Met. Results from there experiment suggest that the lack of response in milk production and composition might be due to other AA limiting production, apart from Met and Lys (i.e. His, Ile or Arg) or that the C diet met the cow's requirements for microbial and post-ruminal protein making the cows less responsive to the supplementation of RPM and/or RPL, which could have been the case in our study. This suggestion was also made by Young *et al*. (2000) that the cows on pasture failed to respond to AA as a result of either and imbalance of AA supply, or that MP supply met the cow's requirements. However, it must be noted that some of the responses observed in our study, which are statistical significant, are because of a slightly negative effect of the RPL treatment compared to the RPML treatment. This observation is in accordance with the conclusion of Robison *et al.* (2010) in a systematic review of the literature that the response to RPL supplementation alone is mostly disappointingly small and that the general response could only be judged as negative. Furthermore, this is indicated by the lower milk CP content observed for the RPL treatment compared to both the C and RPML treatment (Table 4.8). Overall it seems possible that the contribution of EAA from microbial protein to MP might be so large that it changes the extent of Met and Lys limitation, since microbial protein represent the same AA profile of milk protein, as shown in Chapter 2, section 2.4. This is supported by other authors stating that the major contribution of MCP might supply the grazing cows with adequate levels of metabolisable AA (Fulkerson & Trevaskis, 1997; Bargo *et al.*, 2003; Kolver, 2003; Robinson, 2010).

The lack of responses in milk production could also have been as a result of cows being in a later-stage of lactation since a positive response in milk production to RPM and/or Lys supplementation is more common in early-lactation than in mid- or late-lactation cow (Schwab, 1996; Schwab *et al.*, 2003).

Swanepoel (2009) supplemented cows in early and mid-lactation with RPL which resulted in an expected intestinal absorption of lysine between 8 and 22 g/cow/d. This level of supplementation did not affect milk production, milk protein or milk lactose content. Similarly, when 204 multiparous Holstein cows grazing ryegrass pasture were supplemented with 1 kg of barley or 1 kg of barley with 17 g/d RPML or 1 kg/d of rumen protected protein meal, in either early (2 to 11 weeks), mid (12 to 22 weeks) or late (23 to 33 weeks) lactation treatment had no effect on milk production or composition across all weeks of lactation. The authors suggested that neither MP nor the concentration and profile of Met and Lys flowing to the duodenum was the first factor liming performance (Rulquin *et al*., 2001). Pacheco-Rios *et al*. (1999) came to the same conclusion for mid- and late-lactation grazing dairy cows when supplemented with RPM. However, as indicated, there are some contradictions in the literature, for example, when Socha (2005) supplemented early-lactation cows RPM or RPML on a maize-based diet. Compared to the cows receiving no RPAA, cows receiving RPML produced more ECM (45.9 *vs*. 43.8 kg/d), milk fat (1632 *vs*. 1550 g/d), milk protein (1306 *vs*. 1221 g/d) and tended to produce more 3.5 % FCM. These responses are larger than reported from the literature thus far, however, the authors concluded that the reason for the response could have been as a result of the cows being in early-lactation when the cow's metabolic requirements for absorbable AA, relative to absorbed energy, are the highest. It is further suggested by Schwab *et al*. (1992) that cows in mid to late-lactation might not respond positively to RPML supplementation, this suggestion is supported by a meta-analysis done by Patton (2010). The reason being, the cows lack the hormonal drive for increased milk and/or milk protein production (Schwab *et al*., 1992; Patton, 2010). This further suggest that the lack of response by the cows in our study may be due to the cows being more to the end of mid-lactation and start of late-lactation, thus having a lower metabolic requirement for absorbable AA relative the cow's energy consumption. A greater milk production response is expected for cows of higher genetic merit (Kellaway & Porta, 1993) and in early-lactation (Dixon & Stockdale, 1999), because they partition more nutrients toward milk production as opposed to gaining BW (Berry *et al*., 2003).

Monitoring MUN or NPN in bulk milk (tanks) has been suggested to be a reliable method for assessing dietary protein efficiency by various authors (Brunner, 1981; DePeters & Ferguson, 1992; Bashtani *et al*., 2009; Schwab & Broderick, 2017). According to literature the ideal concentration of MUN is 10 to 14 mg/dl (Jonker *et al*., 1998) and are related to dietary protein and energy (DePeters & Ferguson, 1992). Kohn (2007) and Drudik *et al*. (2007) recommended average MUN concentrations ranging from 8.5 to 11.5 mg/dl under standard conditions in which a well-balanced TMR ration is being fed to the cows. Higher MUN concentrations are normally expected for cows grazing pasture compared to cows fed a TMR (Trevaskis & Fulkerson, 1999; Bargo *et al*., 2003). Previous studies in which cows grazed pasture reported MUN values between 15 and 38 mg/dl (Bargo *et al*., 2002; Delahoy *et al*., 2003; Van Wyngaard et *al*., 2013). Compared to Holstein cows, Jersey cows normally have lower MUN concentrations (Rodriquez *et al*., 1997). This difference could be attributed to several factors including level of production, milk fat and protein concentrations, N intake, and BW (Kohn, 2007). However, the lower than expected MUN values

reported in our study (Table 4.7) could be attributed to the relatively lower pasture CP content reported in our study (Table 4.2) compared to other studies in which cows were grazing ryegrass pasture during spring (Table 2.1). It is difficult to interpret MUN result without a good and reliable baseline value for the specific production system due to various factors influencing amount of N present in the milk of individual cows. These factors include season, month, DIM, parity and MCP production (Roy *et al*., 2011). It is also known that starch provided from maize increases the efficiency with which rumen ammonia is utilized by the rumen microbes, which could result in a decreased MUN concentration (Hristov *et al*., 2005). However, the average MUN values reported in our study support the statement made earlier that it seems that protein was not limiting milk production. Milk NPN constitutes 5 to 6 % of total milk N of which MUN contributes about 50 % (Broderick *et al*., 1997). It has also been shown that MUN increases when RPAA are supplemented in excess of cow requirements as a result of AA catabolism (Doepel *et al*., 2004).

In response to the observation by Robinson *et al*, (1998), Piepenbrink *et al.* (1996) suggested that a negative response, when RPM and/or RPL are supplemented, in milk production and content could be as a result of various factors. One of these factors include the detrimental effects excessive amounts, and/or incorrect ratios of absorbable Met and Lys by feeding only RPL and not RPM might have on milk production (Robinson *et al*., 2000; Cant, 2001).

The concentration and EAA profile of MP reaching the SI of the cows, as indicated, could also have limited the response in cow performance (NRC, 2001; Lapierre *et al.*, 2007). Experimental evidence seems to be contradictory in the extent to which MP supply limits milk production in grazing dairy cows. Some studies suggest that pasture supply adequate levels of MP to sustain relatively high milk production (Beever & Siddons, 1986; Fulkerson & Trevaskis, 1997; Roche *et al.*, 2013) and since there is a poor milk production response to increase RUP (Santos *et al.*, 1998). While other studies did see an improvement in cow performance when supplemented with RUP (O'Mara *et al.*, 2000; Schroeder & Gagliostro, 2000; Malleson, 2008). As indicated in the literature review, maize has a low protein content and the AA profile is poor, especially considering Lys. However, Swanepoel (2009) reported that milk yield was not effected negatively when the contribution of maize CP to total diet CP increased, they expected a change in milk composition (i.e. protein and fat) as it is more sensitive to a change in the AA profile of the intestinally delivered protein (NRC, 2001). However, in their study there was no relationship between milk composition and the proportion of maize CP in total diet CP. Swanepoel (2009) suggested that the diet might have been balanced in AA despite the maize CP being proportionally high, possibly due to the complementary protein sources higher in RUP used and the superior AA profile, and large contribution of MCP to MP. This might also be true for our study since soybean meal, which is high in Lys and low in Met, was included at 8 % DM across all treatments and could have supplied adequate levels of Lys even though maize represented on average 34.3 % of the total diet DM. The contribution of metabolisable Met from the rumen microbes could have also been adequate, since microbial protein are very close in AA composition to milk protein and lean body tissue. The premise that providing more than 200 g/kg of the diet DM of high producing

dairy cows with a maize-based concentrate might change the factor limiting cow performance from ME as a limitation to reduced supplies of MP or a poor AA profile reaching the SI was tested by Higgs *et al.* (2013). They showed that ME was still the factor limiting production when early-lactation cows consumed 25 % of their diet as a high-starch/low- CP (i.e. maize) concentrated supplement. This is also in agreement with Roche *et al*. (2013) who reported that the response, when grazing cows are supplemented with a maize-based concentrate, was linear up to 6 kg of DM/d of concentrate (± 30 % DMI). Indeed, multiple authors reported that ME will remain the factor limiting cow's performance in studies where RUP and/or RPAA are supplemented.

The milk nitrogen fractions correspond to that reported by other studies and a literature review (Brunner, 1981; DePeters & Ferguson, 1992). Casein is the protein fraction mostly influenced by AA supplementation (Rulquin *et al.*, 1990). In a study conducted by Younge *et al*. (2000), grazing dairy cows were supplemented with RPM and RPL while grazing perennial ryegrass. The cows were allowed *ad libitum* pasture plus a 0.25 kg beet pulp or molasses for the control treatment, this same diet was then supplemented with RPM and RPL, supplying 6.2 g of absorbable Met and 8.1 g of absorbable Lys. Estimated intestinally absorbable Met and Lys were increased from sub-optimal to theoretically optimal levels expressed as proportion of total digestible AA supply (1.9 and 6.9 to 2.3 and 7.4 % MP), respectively for Met and Lys. These values are very close to the Met and Lys requirements of lactating dairy cows as indicated in Chapter 2, section 2.3. It was also calculated in that study that the cows received 117 % of ME requirement and 135 % of MP requirements. However, this did not translate to an increase in milk production and composition, specifically the casein fraction. This might have been the case in this study, the authors concluded that the lack of response could be the accumulative effect of the varying pasture characteristics, the cow's physiological status, the cows level of production, or the order that another AA other than Met and Lys might have been first limiting.

Leonardi *et al*. (2003) supplemented Holstein cows in mid-lactation, at two levels of dietary CP (16.1 *vs*. 18.8%), with RPM (0.07 g/100 g DM). They substituted 0.07 % maize (DM basis) with a RPM which increased the duodenal flow by 12 g/d on the lower (16.1 % CP) and 10 g/d on the higher (18.8 % DM) CP diets. This increase in Met in MP did not result in a significant difference for casein expressed as a fraction of skim milk N. Similar result was observed in a study by Pacheco-Rios *et al*. (1999). In contrast, Blauwiekel *et al.* (1997) reported an increase in casein N (2.64 *vs*. 2.77 %) when cows were supplemented with RPL, the authors concluded that the response might have been as a result of the 6 % higher intake of DM compared to the control treatment (23.2 *vs*. 21.8 kg DM/cow/d). This increase in DMI may have resulted in the increased MCP (319 *vs*. 296 kg microbial N/cow/d) observed in their study, which resulted in an increase of 22 g/cow/d of Lys reaching the duodenum. Socha *et al*. (1994) and Blum *et al*. (1999) reported no effect of RPM supplementation on milk protein production in multiparous cows. Casein appears to be reduced when diets provide less than 2.1 to 2.2 % Met and 6.0 to 6.5 % Lys reaching the intestinal

absorptive site (Sniffen *et al*., 2001). If these levels are reached a response in milk protein fraction, specifically casein, is not expected (Dinn *et al*., 1998).

4.6.1.1 Milk production and composition of high and low producing cow groups

Since a response in performance to RPM and/or RPL supplementation was more likely in high-producing cows, the cows were divided into a higher producing group and a lower producing group. The higher producing group are represented by the top portion (block 1-10) of the 20 blocks in the randomised complete block design used in the study as milk yield was the primary factor which was considered when the cows was blocked. Thus, the lower producers are represented by the bottom portion (block 11-20), (see appendix A for more detail). The difference in mean milk production for the high producers and the low producers for all treatments groups were on average 5.5 kg milk (24.6 *vs*. 19.9 kg milk/cow/d; $P < 0.05$) as shown in Table 4.9. This indicates that the response might have been in the higher-producing group, since the assumption was that higher producing cows are more likely to respond to AA supplementation and it is also demonstrated by the literature cited. Table 4.9 present the comparison between mean milk production for the high producers and low producers between each treatment. The difference between the higher producers and lower producers for the C, RPL and RPML treatments were -5.5, -3.8 and -4.8 kg milk less ($P < 0.05$), respectively. There was no difference in milk production between treatment for the high-producers and low-producers ($P > 0.05$). The lack of response, when the cows were separate further suggests that Met and/or Lys was not limiting production, but rather a different factor as discussed. It could also further suggest that the cows level of production was also not the cause of a lack of response.

Table 4.9 Comparison between the mean milk yield (kg/d) for higher and lower producing cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 10)

Treatment[1]	**Group**[2]			*P*-value
	A	**B**	**SEM**[3]	A vs. B
C	25.0[a]	19.5[b]	0.667	< 0.01
RPL	24.1[a]	20.3[b]	0.667	< 0.01
RPML	24.7[a]	19.9[b]	0.667	< 0.01
Average	24.6[a]	19.9[b]	0.385	< 0.01
SEM[3]	0.385	0.385	-	-
P-value				
C *vs*. RPL	0.36	0.36	-	-
C *vs*. RPML	0.73	0.62	-	-
RPL *vs*. RPML	0.57	0.68	-	-

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Group: A: Highest producing blocks; B: Lowest producing blocks
[3]Standard error mean
[a.b]Row means with different superscripts differ ($P < 0.05$)

Table 4.10 presents the mean milk composition for the higher producing group, treatment had no effect on milk protein, fat and lactose percentage and MUN (mg N/dl) (P > 0.05). Milk production can also be expressed as 4% FCM (kg/d) and ECM (kg/d) by considering the milk composition. Treatment has no effect on either 4% FCM or ECM (P > 0.05).

Table 4.10 Mean milk composition, 4 % FCM and ECM for higher producing cows[2] grazing ryegrass and fed a concentrate supplemented with RPL and/or RPL (n = 10)

Parameter[4]	**Experimental treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[3]	C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Protein (%)	3.71	3.87	3.86	0.078	0.16	0.17	0.95
Fat (%)	4.55	4.77	4.57	0.172	0.38	0.94	0.42
Lactose (%)	4.74	4.68	4.79	0.057	0.48	0.53	0.19
MUN (mg N/dl)	8.69	8.58	9.12	0.393	0.85	0.44	0.34
4% FCM (kg/d)	27.03	26.79	26.87	0.761	0.83	0.88	0.94
ECM (kg/d)	27.46	27.26	27.62	0.776	0.85	0.89	0.74

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Higer producing cows – block 1-10
[3]Standard error mean
[4]MUN - Milk urea nitrogen; 4 % FCM – 4 % Fat corrected milk; ECM – Energy corrected milk

Table 4.11 represents the mean milk composition for the lower producing group, treatment had no effect on milk fat and lactose percentages with the exception of milk protein percentage. The milk protein percentage was higher for the C and RPML group compared to the RPL which had a lower protein percentage (4.21 and 4.16 *vs*. 3.85 %, P < 0.05). This corresponds with other authors who reported that when Lys is supplied in excess it can have detrimental effects on cow performance (i.e. milk production and milk protein yield) (Piepenbrink *et al.*, 1996; Robinson *et al.*, 2000). The MUN (mg N/dl), 4 % FCM (kg/d) and ECM (kg/d) did not differ between treatment groups (P > 0.05). The milk composition observed in our study for the higher and lower producing group is similar to values reported by others (DePeters & Ferguson, 1992; Carrol *et al*., 2006; Malleson, 2008; Joubert, 2012) and is representative of the Jersey breed standards in South Africa (Moster et al., 2003; Theron & Mostert, 2010).

Table 4.11 Mean milk composition, 4 % FCM and ECM for lower producing cows[2] grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n =10)

Parameter[4]	**Experimental treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[3]	C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Protein (%)	4.16[a]	3.85[b]	4.21[a]	0.078	0.01	0.69	< 0.01
Fat (%)	4.98	4.69	5.01	0.172	0.24	0.92	0.20
Lactose (%)	4.63	4.60	4.60	0.057	0.68	0.74	0.94
MUN (mg N/dl)	8.51	8.89	8.91	0.393	0.49	0.47	0.97
4% FCM (kg/d)	22.4	22.5	22.9	0.762	0.94	0.62	0.67
ECM (kg/d)	23.0	22.7	23.5	0.776	0.83	0.59	0.45

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively.
[2]Lower producing cows – blocks 11 – 20
[3]Standard error mean
[4]MUN - Milk urea nitrogen; 4 % FCM – Fat corrected milk; ECM – Energy corrected milk
[a,b]Row means with different superscripts differ (P < 0.05)

4.6.2 Body weight and body condition

The mean cow BW and BCS for the C, RPL and RPML treatments before and after the study are presented in Table 4.12. Cows allocated to the C treatment did not differ in BW compared to the RPL and RPML (P > 0.05), but the cows allocated to the RPL and RPML did differ in BW (405 *vs.* 385; P < 0.05). The cows receiving the RPML treatment gained more BW compared to the RPL treatment (+ 30.4 *vs.* + 20.1; P < 0.05), but both the RPL and RPML treatments did not gained more BW compared to the C treatment (+ 25.2; P > 0.05). This increase in BW after peak-lactation is physiologically normal for lactating dairy cows (Stockdale, 2001; Hutjens, 2018).

At the onset of the study the cows allocated to the RPL treatment had a higher BCS than compared to both the C treatment and RPML treatment (2.20 *vs.* 2.09 and 2.05; P < 0.05), although there was no difference between the C treatment and RPML treatment (P > 0.05). Cows on the RPML treatment gained 0.12 points (1-5 scale) more body condition than those on the C treatment (+ 0.43 *vs.* + 0.31; P < 0.05), but not more than the RPL treatment (+ 0.37; P > 0.5). The increase in BCS across all treatment groups was expected as cows were past peak-lactation (Roche *et al.*, 2017).

The fact that the cows gained body condition indicate that the cows were in a positive energy balance (NRC, 2001). However, depending on the cow's stage of lactation, BW changes might not be a good indicator of the cow's energy balance due to changes in rumen fill and DMI, body fat changes *vs.* body water changes, including the cow's physiological status (i.e. BW changes related to pregnancy) (Van Amburgh *et al.*, 2014). However, Jaurena *et al.* (2005) reported that an increase in BCS of lactating dairy cows could be as a result of an accretion of both muscle and fat.

Table 4.12 Mean change in cow weight and body condition score for cows grazing ryegrass and fed a concentrate supplemented RPM and/or RPL (n = 20)

Parameter	Experimental treatment[1]			SEM[2]	*P*-value		
	C	RPL	RPML		C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Weight (kg)							
Before	397.7^{ab}	405.5^{a}	385.3^{b}	4.786	0.25	0.08	< 0.01
After	422.9^{a}	425.6^{b}	415.7^{a}	4.709	0.69	0.29	0.15
Change	+ 25.2^{ab}	+ 20.1^{a}	+ 30.4^{b}	2.422	0.14	0.14	< 0.01
BCS (Scale 1-5)							
Before	2.09^{a}	2.20^{b}	2.05^{a}	0.036	0.04	0.47	< 0.01
After	2.39^{a}	2.57^{b}	2.48^{ab}	0.042	< 0.01	0.18	0.12
Change	+ 0.31^{a}	+ 0.37^{ab}	+ 0.43^{b}	0.03	0.19	0.02	0.25

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Standard error mean
[a.b] Row means with different superscripts differ (P < 0.05)

Data on the effects that Met and/or Lys supplementation has on cow BW and BCS is lacking and inconsistent, some studies reported increases in BW and body condition upon RPM supplementation (Rulquin & Delaby, 1997a; Gomez *et al*., 2010; Swanepoel *et al*., 2015) and other no response (Robinson *et al*., 1995; Rulquin & Delaby, 1997b; Robinson *et al*., 1998; Socha *et al*., 2005; Giallongo *et al*., 2016). It is well understood that Met is a limiting AA for growing cattle (NRC, 2001), thus it could be speculated that the supplementation of Met might aid in protein deposition in the cows body (Loest *et al*., 2002; Robinson, 2010). Methionine also plays a significant role in the formation of phospholipids through the conversion of Met to S-adenosylmethionine, which might contribute in the deposition of fat (Obeid & Herrmann, 2009). Methionine is also a methyl donor for the synthesis of choline, which is essential for the synthesis of phospholipids required for the synthesis of chylomicrons and very low density lipids (Campbell & Farrell, 2003). The chylomicrons in turns carry triglycerides which aid in fat deposition and intramuscular marbling, leading to an increase in BW gain (Dodson *et al*., 2010). This was demonstrated by Gomez *et al*. (2010) in a study in which grazing Holstein heifers showed an increase in average daily gain when supplemented with a maize-based concentrate (2 kg/cow/d (as is)) containing 5 g/kg DM RPM.

The oversupply of Met to lactating dairy cows have also shown to redirect energy from other metabolic functions to synthesis of adipose tissue in lactating dairy cows (Lapierre *et al*., 2006; Swanepoel *et al*., 2010; Swanepoel *et al*., 2015). It is also commonly known that nutrients and energy are repartitioned away from milk production to BW gain for dairy cows around mid-lactation (NRC, 2001; Roche *et al*., 2009). The change might also be due to difference in PDMI between treatment groups, however, unfortunately individual DMI from pasture was not determined for individual cows in our study. Results on change in body condition should be interpreted with care, as the experimental period was limited. The body condition increase of cows on the RPML treatment was higher than that of the C treatment. However,

it does support the suggestion that Met and Lys was not limiting and that these AA were not used for milk or milk protein production but rather repartitioned along with various metabolites to other metabolic functions (i.e. protein deposition and/or lipogenesis). Otto *et al*. (1991) reported that one BCS point (Scale 1-5) equals 56 kg, and the relatively small change in BCS observed between treatments suggests a minor impact of treatment on BCS, although differences are statistically significant (P > 0.05). Similarly, Berry *et al*. (2011) performed a mixed model analyses across 11075 lactations form 7391 dairy cows to quantify the change in BW per unit change in BCS (scale 1 to 5) and reported that one point BCS point change equals 50 kg of BW with a range of 39 to 66 kg.

4.6.3 Faecal starch content

Table 4.13 presents the mean concentration of starch in the faeces (% DM) of cows on the C, RPL and RPML treatments. The mean starch concentrations in the faeces were 10.8, 10.1 and 9.58 % DM, for the C, RPL and RPML treatments, respectively. There was no difference in faecal starch concentration across all treatments (P > 0.05).

Table 4.13 Mean (± SD) starch in feaces (% DM) for cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 3)

Parameter	**Experimental treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[2]	C *vs*. RPL	C *vs*. RPML	RPL *vs*. RPML
Starch	10.8 ± 1.64	10.1 ± 0.53	9.58 ± 0.28	0.596	0.65	0.32	0.38

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Standard error mean

Malleson (2008) supplemented grazing Jersey cows with a maize-based concentrate on a similar pasture type that was used in this study, although PDMI were lower (7.6 *vs*. 9.0 kg DM/cow/d), including concentrate intake (5.5 *vs*. 7.3 kg DM/cow/d) than in our study. Faecal starch content observed in that study ranged from 0.53 to 1.05 % DM, much lower than that reported in our study. The lower level of concentrates fed in their study might have contributed to the lower faecal starch content. In accordance with our study, Granzin (2004) reported faecal starch concentrations ranging from 5.7 to 9.5 % on a DM basis for cows grazing pasture (ryegrass and prairie grass), while receiving 4.5 or 8.1 kg/cow/d of a barley-based concentrate, respectively. In the same study when the cows received a maize-based concentrate (4.5 or 8.1 kg/cow/d) grazing the same pasture, a faecal starch content ranging from 7.8 to 16.0 % DM, respectively, was reported. The latter values correspond with the values reported in our study. Thus, it might seem that the amount of concentrate fed to grazing dairy cows is positively associated with faecal starch content, thus negatively associated with starch digestibility. Reis & Combs (2000) evaluated the impact that increasing

levels (0, 5 and 10 kg DM/cow/d) of a maize-based concentrate would have on the performance, and rumen environment, of grazing dairy cows. The pasture that the cows grazed in their study composed of brome, orchardgrass, red clover and alfalfa. As the level of concentrate supplementation increased the faecal starch plus free glucose content also increased, 6.7, 9.3 and 13.8 % DM for 0, 5 or 10 kg DM of concentrate ($P < 0.01$), respectively. Indeed, faecal starch concentration is closely and linearly correlated to total-tract starch digestibility ($R^2 = 0.94$, $P < 0.01$) (Fredin *et al.*, 2014), with the digestibility of starch in dairy cows ranging from 70 to 100 % (Firkins *et al.*, 2006). Additionally, ground maize has an average starch digestion of 76.4 %, which ranges from 51.4 to 93 % (Reis & Combs, 2000). The high faecal starch observed in this study could indicate that starch was not digested efficiently in all three treatments and that the estimated ME supply could have been over predicted. This support the argument that ME might have been the first nutrient limiting cow performance.

4.6.4 Plasma amino acid concentrations

Blood or plasma AA concentrations have been used previously to assess the effectiveness of RPAA supplementation in the deliverance of AA to the systemic circulation of lactating dairy cows (Polan *et al.*, 1991, Mulrooney *et al*., 2009; Whitehouse *et al.*, 2017). Increasing the supply of AA to the SI of lactating dairy cows are expected to alter plasma AA concentrations, spectrum and possibly the availability of AA for the synthesis of milk protein in the mammary gland (Broderick *et al.*, 1974; Munneke *et al.*, 1991; Raggio *et al*., 2004; Martineau *et al*., 2017; Martineau *et al*., 2019). The AA concentration of plasma withdrawal from the coccygeal vein (tail vein) reflect the AA concentrations in the arterial supply of the mammary gland (Munneke *et al.*, 1991), the intestinal absorptive site, and could be used to identify AA limitation (Doepel and Lapierre, 2010).

Oetzel (2003) and Swanepoel *et al.* (2016) suggested that a minimum of 6 to 8 cows should be used in studies where the mean plasma AA concentrations are compared. However, in our study we selected 10 cows per treatment to collect plasma samples from.

The plasma AA concentrations (µmol/l) are presented in Table 4.14. Compared to the C treatment supplementing RPL did not have an effect on the plasma AA concentrations ($P < 0.05$). However, it should be noted that most of the plasma AA, except Phe, Thr, Trp and Tyr showed slight numerical increases when RPL was supplemented, although statistical significance was not reached. When RPML was supplemented there was no effect on the plasma AA concentrations for most of the AA with the exception of Met and Gly that increased compared to the C group (28.8 *vs*. 41.4 and 404 *vs*. 448 µmol/l; $P < 0.05$). The AA that tended to increase when RPML was supplemented include Lys compared to the C treatment (95.5 *vs*. 109 µmol/l; $P = 0.09$) and not the RPL treatment (106 *vs*. 109 µmol/l; $P = 0.79$), Thr compared to the RPL treatment (98.4 *vs*. 115 µmol/l; $P = 0.05$), but not the C treatment (104 mmol/l; $P > 0.05$) and Cys (8.40 *vs*. 9.45 µmol/l; $P = 0.07$) compared to the C treatment but not the RPL (9.05 mmol/l; $P > 0.05$) treatment.

Table 4.14 Mean physiological blood plasma amino acid concentration (µmol/l) of cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 10)

Parameter	Experimental treatment[1]			SEM[2]	*P*-value		
	C	RPL	RPML		C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Plasma amino acids							
Essential amino acid (EAA)							
Lysine (Lys)	95.5^{d}	106de	109^{e}	5.34	0.15	0.09	0.79
Methionine (Met)	28.8^{a}	29.0^{a}	41.4^{b}	1.75	0.96	< 0.01	< 0.01
Histidine (His)	62.3	64.2	70.7	4.60	0.77	0.21	0.33
Phenylalanine (Phe)	67.8	67.8	72.0	2.57	0.99	0.26	0.25
Leucine (Leu)	146	153	162	6.89	0.43	0.11	0.39
Isoleucine (Ile)	114	119	120	4.00	0.30	028	0.97
Threonine (Thr)	104de	98.4^{d}	115^{e}	5.82	0.50	0.20	0.05
Tryptophan (Trp)	35.3	33.6	36.6	1.46	0.40	0.52	0.14
Arginine (Arg)	76.3	76.9	79.2	2.31	0.85	0.36	0.40
Valine (Val)	224	230	243	5.58	0.64	0.13	0.28
Lys:Met ratio	3.32^{a}	3.67^{b}	2.63^{c}	0.11	0.04	< 0.01	< 0.01
Non- essential amino acids (NEAA)							
Tyrosine (Tyr)	68.9	68.3	69.9	3.63	0.91	0.84	0.76
Glutamine (Gln)	197	218	200	11.5	0.20	0.83	0.28
Glutamic acid (Glu)	51.6	55.7	52.6	2.26	0.21	0.77	0.34
Alanine (Ala)	281	292	306	14.4	0.59	0.23	0.50
Serine (Ser)	132	133	138	3.61	0.91	0.27	0.33
Glycine (Gly)	405^{a}	423^{ab}	448^{b}	15.2	0.39	0.04	0.26
Aspartic acid (Asp)	5.48	5.96	5.76	0.28	0.23	0.48	0.62
Proline (Pro)	112	120	122	6.30	0.36	0.26	0.84
Asparagine (Asn)	61.4	65.2	66.7	3.61	0.46	0.30	0.76
Cysteine (Cys)	8.40^{d}	9.05^{de}	9.45^{e}	0.40	0.26	0.07	0.45

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Standard error mean
[a,b,c]Row means with different superscripts differ (P < 0.05)
[d,e]Row means with different superscripts tend to differ (P < 0.1)

The basal concentration, and profile, of plasma AA observed in this study are in accordance with other studies (Dinn *et al*., 1998; Blum *et al.*, 1999; Doepel & Lapierre, 2010; Swanepoel *et al.*, 2016; Giallongo *et al*., 2016). The 43.5 % increase in plasma Met for the RPML treatment and 11.4 and 13.6 % increase in plasma Lys for the RPL and RPML treatment, respectively, suggests that both the RPM and RPL could have been effectively supplied post-ruminally and were potentially bioavailable. However, the lack of significant difference in milk protein production, which is the main response observed for dairy cows supplemented with RPM and/or RPL (Schwab, 1996), might further suggest that the AA supplemented exceeded the cows requirements. It is well known that plasma AA levels increase in response to supplementation only when supply is in excess of the cows requirements (Broderick *et al.*, 1974; Nichols *et al.*, 1998). It is therefore a possibility that Met and Lys might not have been the first

limiting factor in this study. In contrast to our study, Swanepoel (2009) reported that almost all plasma AA decreased, except Lys, when cows were fed RPL and suggested that Lys may have been first limiting and as a result its supplementation led to an increase and utilization of the other AA. Further strengthening the argument that Lys may not have been limiting in our study. The increase in plasma Lys concentration is similar than the result reported by others (Xu *et al*., 1998; Lee *et al*., 2012). Rulquin & Delaby (1997b) also demonstrated this with Met supplementation in which they reported a 27 % increase in plasma Met when RPM supplementation increased from 0 to 13 g/cow/d for cows grazing pasture. Paz *et al*. (2013) fed multiparous Holstein cows increasing amount of distillers dried grains (which is also reported to be low in lys; NRC, 2001; CVB, 2018), while supplementing 60 g/d of a RPL product. This supplementation did not result in an increase in plasma EAA, thus suggesting that Lys was not first limiting. Trinacty *et al*. (2009) supplemented high yielding Holstein cows with RPML, they reported higher milk production, protein and casein in the presence of higher plasma Met levels but not Lys ($P < 0.05$). When the supply of the most limiting AA is increased post-ruminally, other EAA in plasma should decrease as a result of an increased synthesis of milk protein and increased utilization efficiency (Broderick *et al.*, 1974; Guinard & Rulquin, 1994; Blum *et al.*, 1999), which was not the case in our study.

The cell membrane of enterocytes (absorptive cells) in the SI, including the kidney has at least four different sodium (Na^+)-dependent transport systems for AA. These systems include a polar (Thr, Tyr, Cys, Gly, Asn, and Ser), non-polar (hydrophobic), acidic (-) (Asp and Glu) and basic (+) (Lys, Arg and His) AA transport system. Some overlap may however exist between these groups (i.e. Met which is aliphatic). These membranes, including tissue cells, also contain additional transporters which are not dependent on the sodium gradient. These transporters export AA from intestinal cells into the blood and into other body cells (Saunders & Isselbacher, 1966). The transport of AA are also true for the mammary gland, as reflected in the large arteriovenous AA concentration differences across the gland (Guinard & Rulquin, 1994).

However, when Lys is supplemented, the competitive inhibition by Lys on other AA (i.e. Arg and His). which share the same transport system (i.e. cationic (γ^+) transport systems) should be considered (Shennan *et al.*, 1997). Baumrucker (1985) and Hanigan *et al*. (1998) stated that it is unlikely that the increase of Lys reaching the SI is significant enough to limit the uptake of other AA, which in turn will limit milk production, by saturating the transport system, unless the supplies of Lys to the SI vastly exceeded the cow's requirement. However, this was not the case in our study as the supplementation of RPL did not increase the plasma Arg concentration as compared to the control treatment (76.3 *vs*. 76.9 µmol/L; $P > 0.05$), including the His concertation (62.3 *vs*. 64.2 µmol/L; $P > 0.05$). Absorption of Lys by the mammary gland is usually at least 20 % greater than that observed in milk protein, suggesting that Lys play a critical role within the mammary gland (Schwab & Ordway, 2001) and could be the reason why the increase in plasma Lys when RPL were supplemented did not reach statistical significance. Since RPL was fed in both the RPL and RPML treatments an increase in the Lys concentration was expected. the fact that plasma Lys concentration did not increase ($P < 0.05$) when the RPL treatment was supplemented may

suggest that Lys was absorbed in excess, beyond cow requirements, and utilized for other metabolic functions. One possible explanation is to provide N and Carbon for the synthesis of NEAA, as uptake of NEAA by the mammary gland is often less than required for the observed milk protein output (Guinard & Rulquin, 1994; Doepel & Lapierre, 2010). Doepel & Lappierre (2010) reported that the uptake of both AA and energy-yielding nutrients by the mammary gland of the lactating dairy cow are altered in response to the balance of AA supplied. Although, even under protein deficiency they reported that the supplementation of NEAA did not translate into improve milk or milk protein production.

The increase in plasma Cys concentration, in the presence of a significant increase in plasma Met, is likely as a result of excessive Met catabolism (Baker, 1994; Bequette & Nelson, 2006). Cysteine, like Met, is one of the sulphur containing AA that is utilised for protein synthesis. Increase in blood Cys concentration have also been reported previously when RPM was supplemented to dairy cows on TMR and pasture based systems (Pacheco-Rios *et al.*, 1999). Methionine is also a methyl donor for the synthesis of choline, choline is essential for the synthesis of phospholipids required for synthesis of chylomicrons and very low density lipoproteins and is also possibly a limiting nutrient for milk fat synthesis (Campbell & Farrell, 2003; Arshad et al., 2020). Choline is also converted to Gly in one-carbon metabolic processes and could provide a methyl group for the proliferation of cells in the mammary gland (Piepenbrink & Overton, 2003). Supplementing choline (in rumen protected form) to lactating dairy cows has shown increased milk production, which is primarily associated with the role choline plays in exporting fat from the liver to the plasma of dairy cows (Piepenbrink & Overton, 2003). Glycine on the other hand plays a direct role in protein synthesis, particularly collagen and elastin and is an important gluconeogenic precursor. Interestingly, Doepel *et al*. (2002) reported that cows in a negative energy balance had an increased plasma Gly concentration and were suggested to be related to the breakdown of muscle protein, or rather the *de novo* synthesis from the AA Ser and Thr. Hence it is suggested that the plasma and milk Gly concentrations could be used as an indicator for the metabolic and energy status of lactating dairy cows (Xu *et al*., 2020). The result just discussed might support the suggestion that Met was in fact not first limiting and used for other metabolic functions (i.e. protein deposition and lipid synthesis), which might explain the increase in BCS observed in our study. In support of this statements, Iroshan *et al*. (2013) suggested that multiple AA are involved in the gluconeogenic pathway and that an oversupply of AA, not utilised for milk protein production, may result in enhanced body protein accretion, utilizing energy for increased body condition at the expense of cow performance in terms of milk component production (i.e. milk protein).

It is suggested by others that all AA should be increased proportionally, preventing competitive inhibition and the negative effects of over supplementing one AA, which is supported by studies that proposed the combined supplementation of AA which might elicit a larger response than only an individual AA (Robinson, 2010; Swanepoel *et al.*, 2010).

The lack of response to RPAA supplementation observed in this study could further be explained by the imbalance of plasma Lys: Met ratio. It is suggested that the ideal ratio of Lys:Met supplied to the SI of high producing dairy cows for milk protein synthesis should be 3:1 (NRC, 2001, Chalupa & Sniffen, 2006). It is also known that the dietary supply of AA to the SI of lactating dairy cows is expected to alter plasma AA concentrations, spectrum and possibly the availability of AA for the synthesis of milk protein in the mammary gland (Munneke *et al.*, 1991). There were differences in the Lys:Met ratio between the C, RPL and RPML treatment (3.32 *vs*. 3.67 *vs*. 2.63; $P < 0.05$). When the RPL was supplemented the plasma Lys:Met ratio increased compared to the C treatment (3.32 *vs*. 3.67; $P < 0.05$; Table 4.14), Suggesting that Lys was most probably supplied in excess of cow requirements, which is reflected by the tendency for lower milk CP levels when RPL was supplemented ($P < 0.1$; Table 4.8). The lower milk protein percentages observed were more pronounced when the lower producing cows, which it is assumed to have lower AA requirements, were supplemented with RPL ($P < 0.05$; Table 4.11). In contrast to the RPL supplementation, when the combination of RPL and RPM were supplemented the plasma Lys: Met ratio decreased (3.32 *vs*. 2.63; $P < 0.05$), indicating that Met could have been oversupplied or caused an imbalance in Lys and Met supply. This might further suggest that the C treatment could have been balanced in Met and Lys and that the supplementation of either Lys and Met caused an imbalance of AA supply.

The general recommendation is that Lys and Met reaching the intestinal absorptive site should be 3:1 (Schwab, 1996; Chalupa & Sniffen, 2006). The Lys: Met ratio and how it influences cow performance, however, is not consistent in the literature, even though the NRC (2001) provides a strong argument. Swanepoel *et al*. (2015) reduced the Lys: Met ratio from 3.80 to 2.95 and this did not translate into improved cow performance. In contrast to Swanepoel *et al*. (2015), Chalupa (1999) reduced the Lys:Met ratio from 3.4 to 3.2 and observed a positive response in cow performance. Similarly, Chen *et al*. (2011) reported an increase in cow performance when the Lys: Met ratio was decreased from 3.6 to 3.0. Rulquin & Delaby (1997) on the other hand reported an increase in milk protein content when the blood plasma ratio was decreased from 4.8 to 2.2. The plasma Lys:Met ratio of 2.2 is lower than the 2.63 reported in our study for the supplementation of RPML. Robinson *et al*. (2000) reported no effect on cow performance when the Lys:Met ratio was increased from 3.0 to 3.9, but when a theoretical imbalance was created by supplementing Met, reducing the Lys: Met ratio from 3.0 to 2.3, cow performance was negatively affected.

4.6.5 Urine volume and rumen microbial protein flow

The supplementation of RPM and/or RPL to cows grazing ryegrass pasture did not affect the urine SG, volume or excretion of AL, hence there was no response in estimated MCP synthesis across all treatments ($P > 0.05$) as shown in Table 4.15. The mean urine SG's were 1.0178, 1.0181 and 1.0164 g/cm^3 for the C, RPL and RPML treatments, respectively. Thus the mean estimated urine volumes were calculated to be 27.2, 26.9 and 28.8 l/cow/d, for the C, RPL and RPML treatments, respectively. The concentrations of AL in the urine samples were 1326, 1334 and 1227 mg/L, in combination with urine volume this

translated to urinary AL outputs of 218, 217 and 219 mmol/cow/d for the C, RPL and RPML treatments, respectively. As a result, calculated MCP values of 1166, 1158 and 1176 g/cow/day for the C, RPL and RPML treatment, respectively, were obtained.

Table 4.15 Mean microbial protein yield (CP: g/day) for cows grazing ryegrass and fed a concentrate supplemented with RPM and/or RPL (n = 10)

Parameter	**Treatment**[1]				*P*-value		
	C	**RPL**	**RPML**	**SEM**[2]	C *vs.* RPL	C *vs.* RPML	RPL *vs.* RPML
Urine analysis							
Specific gravity (g/cm^3)	1.0178	1.0181	1.0164	< 0.01	0.82	0.22	0.14
Volume[3] (L/day)	27.2	26.9	28.8	1.24	0.84	0.37	0.27
Allantoin concentrations (mg/L)[4]	1327	1335	1228	76.2	0.94	0.34	0.30
Allantoin output (mmol/day)	218	217	219	6.59	0.91	0.92	0.83
Microbial crude protein (CP: g/day)	1166	1158	1176	42.2	0.89	0.88	0.77

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively
[2]Standard error mean
[3]Urine volume predicted with the equation described by Burgos *et al.* (2004)
[4]Estimated as described by Chen & Gomes (1992)

The lack of difference in MCP production between treatments is not surprising since it was not expected that the supplementation of RPAA, either RPL and/or RPML, would have a direct effect on MCP synthesis, although, some indirect factors such as DMI may contribute to differences in MCP synthesis (Dewhurst *et al.*, 2000). This observation supports our assumption that PDMI did not differ across treatment groups. One of the main reasons for determining the MCP production in this study was to evaluate a non-invasive, easy to use, method for frequent MCP evaluation and to compare the predictions against the current CNCPS model. This was done due to the lack of experimental data available for grazing dairy cows, especially Jersey cows. Since MCP may represent > 50 % of MP flowing to the cow's SI (NRC, 2001), it is important to know the contribution of MCP to the AA pool of the animal.

Urine SG, which is the ratio of urine density to water, reflects the relative proportion of dissolved solutes to total urine volume. It therefore represents a measurement of urine concentration of solutes within urine (Kasiske, 2000), which in turn could be used to determine urine volume from a spot urine sample (Burgos *et al.*, 2005). Multiple authors have reported on urine volumes for different breeds of dairy cows (i.e. Jersey and/or Holstein) under different circumstances, from TMR to pasture based systems, however, experimental data on specifically grazing Jersey cows is very limited. Edwards *et al.* (2015) reported that mid-lactation Friesian-Jersey cross dairy cows grazing simple (i.e. perennial ryegrass-white clover pasture) or diverse (i.e. chicory, plantain, Lucerne and perennial ryegrass-white clover pasture) pastures had urination volumes ranging from 1.8 to 2.4 l/urination and a urination frequency ranging from 11.6 to 15.0

urinations/cow/day. Thus, cows excreted 20.8 to 36 l urine/cow/d, which are similar to the ranges reported by Selbie *et al.* (2015) in a review of the literature. They found an average urination volume of 2.1 l/urination and a range of 9 to 14 urinations/d, resulting in a range of 18.9 to 29.4 l urine/cow/d. The cows used in the study of Edwards *et al*. (2015) had a similar milk production (21.8 *vs*. 22.3 kg milk/cow/d) and DMI (15.8 vs 16.2 kg/DM/cow/d) to that found in our study (Section 4.2, Table 4.1). When comparing the 24-hour urine excretion (kg/d) of Holstein cows to Jersey cows, fitted urinary catheters and fed a TMR, the Jersey cows had a 28 % lower urine excretion weight (16.3 vs 22.7 kg/d) compared to the Holstein cows (Knowlton *et al*., 2010). Although both breeds (i.e. Jersey and Holstein) had relatively lower urine volumes, especially when compared to the study done by Holter & Urban (1992), who reported that cows producing an average 34.6 kg/day of milk excreted 4.5 to 35.4 l/cow/day of urine. Ravera *et al.* (2015) reported a range, higher than previously mentioned, of 24-hour urine volumes from 8.7 to 47 l/cow/d for Friesian-Jersey cross dairy cows on winter crops (kale or fodder beet). In contrast to the abovementioned values, Box *et al.* (2017) fed Friesian-Jersey cross dairy cows a perennial ryegrass-white clover pasture, or pure plantain or 50 % perennial ryegrass-white clover and 50 % plantain pasture during spring and measured total urine volumes by means of urine meter harnesses. They reported that the daily urine volume excreted by the cows was 46.5, 59.1 and 73.8 l/cow/d, respectively.

The mean urine volumes in our study for the C, RPL and RPML does seem to be biological sensible compared to the literature (27.21, 26.85 and 28.81 *vs.* 32.6 l/cow/d) and others (Pacheco, D, personal communication, pacheco@agresearch.co.nz). Although the values obtained are slightly higher than would be generally expected. In the literature there are different equations available to predict urine volume apart from the equation reported by Burgos *et al*. (2004). By using equations reported in the literature to predict urine volume, the mean urine volume across all treatments were estimated to be 21.9 (Nennich *et al.*, 2006), 20.9 (ASAE (2005) section 5.3 Lactating cow regressions of urine excretion) and 18.6 l/cow/d (Knowlton *et al*., 2010), respectively. Indicating that the values obtained were higher than what these prediction equations predicted, which takes both animal factors (i.e. BW, milk production and composition) and dietary factors (i.e. DMI and CP) into account. However, these equations are mainly estimated from Holstein cows fed a TMR ration, as a result the urine volumes predicted with these equations might have under predicted the urine volumes in our study.

Quantifying the urine volume correctly is critical since it is used to determine the daily AL output. However, when considering the urine volume, it is evident that the daily excretion of urine is highly variable (4.5 to 73.8 l/cow/d) and is highly affected by, and varies due to, multiple factors (Valadares *et al.*, 1999). These factors include, 1) amount of water consumed from water troughs, 2) moisture content of the feeds, 3) mineral intake (i.e. Na and K), 4) daily temperature and 5) chemical composition of the diet consumed (i.e. N content) (NRC, 2001; Nennich *et al.*, 2006; Ravera *et al.*, 2015).

Increasing the forage content in the diet of lactating dairy cows have shown to increase the volume of urine excreted (Dahlborn *et al.*, 1998), primarily due to the higher moisture content of fresh forage

compared to a TMR (DePeters, E, personal communication, ejdepeters@ucdavis.edu). Urine excretion has also been linked to intake of Na and K, which are usually high in ryegrass (Muller, 2001), this is not surprising due to the fact that urinary excretion is the main homeostatic regulation for Na, K and CL (Nennich *et al.*, 2006). Studies have also shown the total intake of N from pasture and concentrates to be a principle factor affecting urine excretion, as cows fed high CP diets consume more water, and excrete more urine than cows fed a lower protein diet (Bannink *et al.*, 1999; Broderick, 2003). Increasing dietary CP of dairy cows from 15.1 to 18.4 % of DM was associated with an increase of urine (6.5 l/cow/d) in the study by Broderick (2003). In addition, Sannes *et al*. (2002) reported that urine excretion increased (22.2 *vs*. 25.6 l/cow/d) along with an increase in dietary CP levels (17.2 *vs*. 19.1 % of DM). Since pasture usually supplies excessive amounts of RDP, it is expected that urine volume might be higher compared to TMR rations.

The data suggest that the method used to calculate the urine volume of cows used in this study was of acceptable accuracy and could be used to calculate the microbial protein flow for grazing Jersey cows. To calculate the microbial protein flow, as described in more detail in Chapter 3, section 3.7.7, the mean AL concertation is required, the mean AL concertation of 1326. 6, 1335.0 and 1227.9 mg/l of urine obtained in this study is lower than the 1695.9 mg/l reported Bargo *et al*. (2002) and 1992.1 mg/l reported by Carruters & Neil (1997) for cows grazing pasture. However, this can be expected since Holsteins have a larger MCP yield compared to Jersey cows (Clark *et al*., 1992; Dewhurst *et al*., 2000). Other researchers found similar AL concentrations to those found in our study (González-Ronquillo *et al.*, 2004). The daily Al output (mmol/d) reported in our study were slightly higher than the 181 mmol/d reported by Moorby *et al*. (2006) for Holstein cows fed a forage to concentrate ratio of 65:35. Moorby *et al.* (2006) further estimated the functional relationship between total PD excretion in urine and MCP flow to the cow duodenum as microbial N (g/d) = 19.9 + 0.689 x total PD (mmol/d); R^2 = 0.787. The MCP in their study was determined using marker methodology (i.e. Cys flow). By using their estimations, the average MCP observed in our study (1167 g MCP/cow/d) study resembles their MCP predictions (1200 g MCP/cow/d) relative to the same expected PD values. The values in our study for daily AL output were similar to the 206 mmol/d reported by Brossillon *et al.* (2018) for Jersey cows consuming 16.2 kg/d DM maize-soy based TMR, producing 17.1 kg/d of milk and are similar in DIM as the cows used in this study. The Al (mmol/d) values obtained in our study further agree with the range (102 to 678 mmol/l) reported by Westreicher-Kristen *et al*. (2020).

A number of authors (Chen & Gomes, 1992; Johnson *et al.*, 1998; Shingfield, 2000; González-Ronquillo *et al.*, 2003; Chen & Orskov, 2004; González-Ronquillo *et al.*, 2004; Tas & Susenbeth, 2007; Swanepoel *et al.*, 2016) have used urinary PD excretion to estimate microbial N flowing to the duodenum in ruminants. Our results support this method as a reliable method to estimate MCP synthesis.

Clark *et al*. (1992) in a review on N metabolism and AA nutrition in dairy cattle reported microbial N flows ranging from 50 to 480 g N/kg OM over a wide range of (2.5 to 22.5 kg) of OM intake levels. Considering the cows had an average OM intake of 14.9 kg DM/cow/d (section 4.5) and based on the linear

equation reported by Clark *et al*. (1992) the predicted MCP values reported in our study falls exactly between range (750 to 2062 g MCP; R^2 = 0.62) for similar OM intake levels. In a recent meta-analysis, Roman-Garcia *et al*. (2016) used data from 183 trials, 619 treatments in total, of dairy cows sampled either from the duodenum or omasum to derive different equation for the prediction of MCP flow over a large number of dietary conditions. They reported that the MCP flow was positively associated with DMI and dietary starch at a decreasing rate, including the dietary NDF content. Swanepoel *et al*. (2016) estimated MCP flow based on the diet NDF (% DM) content and the values reported in our study are also similar to the range reported in her their study for the same level of dietary NDF (% DM). The NRC (2001) uses TDN to predict MCP flow, which was reported by St-Pierre, (2003) to have significant linear bias and ignores the digestion site of starch and NDF (Firkins *et al*, 2001). Oldick *et al*. (1999) evaluated MCP flow to the duodenum of cows based on DMI and the chemical composition of the diet. In this study 213 treatment means from 55 trials, utilizing lactating and non-lactating cows with duodenal cannulas, were used. Based on the average DMI predicted from our study their prediction equation slightly over-predict MCP compared the values found in our study. Bernard *et al*. (2004) evaluated the effect of Lys supplementation on rumen fermentation and AA flow to the SI of lactating Jersey cows. The authors reported that Lys supplementation did not alter rumen fermentation, AA flow to the SI or nutrient digestibility. However, their estimated MCP synthesis (1221.8 g/cow/d) based on duodenal samples collected is higher than the average MCP synthesis observed in our study (1167 g/cow/d). Danes *et al*. (2013) evaluated the effect of protein supplementation on metabolism of Holstein × Jersey cows grazing tropical pastures (16.3 kg DMI; 18.6 % CP and 58.7 % NDF on a DM basis) in mid-lactation producing on average 20 kg Milk/cow/d while supplemented with a maze-based concentrate. They reported that the cow produced 1123 g/cow/d of MCP which is similar to the values reported in our study.

Van Vuuren (1992) and Peyraud *et al*. (1997) reported mean MCP supplied of 85.1 and 96.3 g MCP/kg DMI, respectively, and 120 and 129 g MCP/kg OM consumed, respectfully for cows consuming ryegrass fertilized at similar (100 kg/ha of LAN) levels as in our study. Although, the methods used to determine MCP flow differed from the method used in our study, Van Vuuren (1992) estimated MCP flow based on the AA profile of the grass, microbes and duodenal digesta, and Peyraud *et al*. (1997) used the diamino-pimelic acid marker method to determine the MCP flowing to the duodenum. Even though different methods were used, Chen & Ørskov (2004) demonstrated that there is a close relationship between MCP prediction using the same method used in this study compared to various methods using marker methodology (DAPA, AA profiles, RNA and ^{15}N). Slightly lower values than reported by our study, Malleson (2008) reported an average MCP of 951 g/cow/d for Jersey cows grazing ryegrass pasture fed a maize-based concentrate, as predicted by the CPM-dairy model (Version 3.0.7a; Cornell University, Ithaca, NY, University of Pennsylvania, Philadelphia, PA; Willam H. Miner Agricultural Institute, Chazy, NY). Although, the cows in her study consumed only 12.6 kg DM/d, which might explain the slight difference observed in our study. Microbial CP yield and efficiency of synthesis have often been reported to be as

high as 30 to 45 g microbial N/ kg of OM apparently digested in the rumen when lactating cows are grazing high quality pasture (Carruthers *et al.*, 1997). This is in agreement with the 27.2 g N/ kg OM apparently digested reported by Kolver and Muller (1998) and 30 to 32 g N/kg OM apparently digested reported by Dugmore (1995). Additionally, in continuous culture fermenter studies, Bach *et al*. (2005) reported that there is a quadratic relationship between the efficiency of MCP synthesis and N utilization efficiency, with a mean maximum N utilization of 69 % when 29 g of microbial N was synthesised per kg of OM fermented.

The MCP values obtained in this study seem to be biologically sensible and that this method, based on the AL concentration in spot urine samples could be used to as an alternative method to determine MCP production of grazing Jersey cows. Additionally, the MCP values observed in our study seem to support the suggestion that adequate contribution of MCP to the cows SI might supply the cow with their EAA requirements.

4.7 Modelling the trial

Table 4.16 shows the predictions of the CNCPS model for the C, RPL and RPML treatments. The use of a nutrition models when formulating concentrates fed to grazing dairy cows in terms of protein and AA nutrition could potentially allow dairy farmers to realize some production benefits in cow performance (Tylutki *et al.*, 2008). Some of these benefits include increased milk production and composition along with a decrease in dietary CP, ultimately increasing the efficiency of both pasture and dietary protein utilisation, including farm profitability. The capability of such models to be used effectively for pasture-based systems have been studied and it was concluded that such model could be used under both TMR and pasture-based conditions to accurately predict cow requirements for maintenance, growth, pregnancy and lactation, including the supply of nutrients available to meet those requirements. Parameters that are considered in the predictions include ruminal fermentation, intestinal digestion and metabolism of the different feed constituents consumed the cows (Kolver *et al.*, 1998; Pacheco *et al.*, 2012). However, models are only as accurate as the input data used in the model, this emphasises the importance of accurate measurements of the animal, dietary and climatic factors to be used as input values in the model (Chalupa & Sniffen, 2006; Lapierre *et al.*, 2007; Tedeschi *et al.*, 2015). However, it must be noted that results should be interpreted with care since average values were used for the nutrient composition of the pasture and did not take into account the reduction in ME and *IV*OMD, and the increase in NDF and Lignin, including changes in the cow's physiological status and level of production.

The model predicted that MP was fist limiting when the cows were supplemented with the C treatment. This support our hypothesis that the performance of high-producing dairy cows grazing high-quality ryegrass pasture in spring, while receiving a standard maize-based concentrate might be limited in MP rather than ME and might respond positively to the supplementation of RPM and/or RPL. For the C treatment the actual milk production compared to the predicted ME allowable milk and MP allowable milk

was 22.3, 22.5 and 22.2 kg milk/cow/d, respectively. Although the difference between actual and observed values are very small indicating that the potential benefit is very small in terms of milk production.

However, when RPL was included in the concentrate fed to the cows ME became first limiting to milk production, the actual milk production, ME allowable milk and MP allowable milk was 22.3, 22.9 and 23.0 kg milk/cow/d, respectively. It could be speculated that in our study when the RPL treatment was fed Lys was over supplied to the cows, beyond the cow's requirements. As a result, the detrimental effect excessive AA supplementation may have reduced cow performance (Piepenbrink *et al.*, 1996), which might have increased the cows energy deficit (Iroshan *et al.*, 2013). The negative effect that excessive AA supplementation has on cow performance has also been reported by others (Baumrucker, 1985; Robinson *et al.*, 2000; Pacheco & Waghorn, 2008). Baumrucker (1985) further suggested that the cationic transport system as described in section 4.6.4, used to absorb Lys uses energy, while the source of energy is unknown, it might be possible that an increase in intestinal absorbable Lys beyond the requirement of the cow could reduce the amount of energy available for production purposes (i.e. milk protein production). This supports our finding that the RPL had a slightly negative effect in milk protein.

Interestingly, when RPL and RPM were supplemented in combination to the cows in the RPML treatment MP again became the first factor liming production. The actual milk production, ME allowable milk and MP allowable milk were 22.3, 22.5 and 22.0 kg/cow/d, respectively. Table 4.14 indicated that the plasma Met increased significantly (28.8 *vs.* 41.4 mmol/d; $P < 0.05$) when the cows were supplemented the RPML treatment, indicating the Met could have been available for other metabolic function, which might include gluconeogenesis, trans-methylation reactions, single carbon metabolism and lipid biosynthesis (Baker, 1994; Schwab, 1995; Bequette *et al.*, 1998; Campbell & Farrell, 2014). Suggesting that the cow's energy states might have been improved, this could be indicated by the fact that the cows supplemented with the RPML gained more body condition compared to the C treatment (+ 0.31 *vs*. + 0.43; $P < 0.05$).

However, the model further predicted milk fat production very similar to the actual values, for the C (4.81 *vs*. 4.77 %), RPL (4.73 *vs*. 4.73 %) and RPML (4.73 *vs*. 4.79 %) treatment groups, respectively. Milk protein production are also predicted very similar to actual values observed for the C (3.92 *vs*. 3.93), RPL (3.89 *vs*. 3.86) and RPML (4.06 *vs.* 4.03) treatments groups, respectively.

The model predicted PDMI to be 9.52 kg DM/cow/d, which are very similar to the 9.04 kg DM/cow/d actually observed in our study (Chapter 4, section 4.2), further supporting the validity of the RP_{Meter} method used in our study to determine PDMI. The evidence also support the suggestion made earlier that differences in PDMI were constant across treatments. The NDF intake as a percentage of cow body weight was 1.08, 1.06 and 1.10 for the C, RPL and RPML treatment group respectively, which are very close to the 1.3 % reported by Bargo *et al*. (2003) and lower to the 1.5 % reported by Kolver & Muller (1998) when pasture only was consumed by the cows. The differences might have been as a result of the pasture and dietary characteristics used as inputs in the model.

The model predicted MCP production is similar than the values observed with the actual values reported in our study for the C (1058 *vs*. 1166 kg/cow/d), RPL (1056 *vs*. 1157 kg/cow/d) and RPML (1049 *vs*.1175 kg/cow/d) treatment groups, respectively. The fact that the model predicted slightly lower MCP value could be as a result of various factors which include DMI, rumen digestion and fermentation characteristics and the chemical composition of the diets. However, this indicating that the method used, based on the AL concentration in spot urine samples, to predict MCP production for grazing Jersey cows supplemented with a maize-based concentrate is possible, however, further research is required with regards to this method.

The diet CP and ME as predicted by the model are in accordance with the actual values reported in our study (Chapter 4; section 4.5).

Table 4.16 The CNCPS model predicted outputs for the C, RPL and RPML treatments

Parameter[2]	**Experimental Treatment**[1]		
	C	**RPL**	**RPML**
Cow performance			
Actual milk production (kg/d)	22.26	22.27	22.33
ME allowable milk (kg/d)	22.54	22.88	22.54
MP allowable milk (kg/d)	22.22	23.02	21.99
Milk fat predicted (%)	4.81	4.73	4.73
Milk fat actual (%)	4.77	4.73	4.79
Milk protein predicted (%)	3.92	3.89	4.06
Milk protein actual (%)	3.93	3.86	4.03
Intake			
DMI predicted (kg/d)	16.76	16.79	16.70
DMI actual (kg/d)	16.28	16.29	16.32
PDMI predicted (kg/d)	9.52	9.54	9.42
PDMI actual (kg/d)	9.04	9.04	9.04
NDFI (% BW)	1.08	1.06	1.10
Rumen parameters			
MP (g/d)	1753	1763	1770
MP from bacteria (g/d)	1058	1056	1049
MP from RUP (g/d)	695	707	721
Diet factors			
Diet CP (% DM)	16.0	16.0	16
Diet ME (MJ/kg DM)	11.5	11.5	11.4
Dietary AA balance			
Met (g/d)	42	42	51.2
Met (% MP)	2.41	2.38	2.89
% Required	97.6	99	116
Lys (g/d)	114	137	137
Lys (% MP)	6.53	7.76	7.73
% Required	93.2	114	109
Lys:Met ratio	2.71	3.26	2.67
Plasma Lys: Met ratio	3.32	3.67	2.63

[1]Experimental treatments: Control treatment (C) supplemented with no rumen protected amino acids (RPAA); Rumen protected Lys treatment (RPL) supplemented with 53.12 g/cow/d of RPL proving approximately 22.0 g of intestinally absorbable Lys/cow/d; Rumen protected Met and Lys treatment (RPML) supplemented with 41.68 and 53.12 g/cow/d of RPML, proving approximately 9.3 and 22.0 g intestinally absorbable Met and Lys, respectively

ME – Metabolisable energy; MP – Metabolisable protein; DMI – Dry matter intake; PDMI – Pasture DMI; NDFI – Neutral detergent fibre intake; CP – Crude protein

The AA balance predicted by the model indicate the Met and Lys were slightly limiting when cows were fed the C treatment (97.6 and 93.2 % of cow requirements), Lys exceeded cow requirements when RPL were supplemented (99 and 114 % of cow requirements) and both Met and Lys exceeded cow requirements when the RPML treatment was supplemented (116 and 109 % of cow requirements). The Met and Lys (% MP) predicted by the model for the C treatment are in accordance with the optimal values reported in the literature review (Chapter 2, section 2.3). This support the suggestion made that both Met and Lys were supplied in excess of cow requirements and support the observation reported of the plasma AA Lys:Met ratio observed in our study.

Using nutrition models such as CNCPS are promising tools to better understand pasture-based systems and how different nutrients might limit the performance of cows in these systems and to develop more efficient feeding programs around those limitations through supplemental feeding (Kolver & Muller, 1998). As a result, the use of models could aid in reformulating supplements taking into account the nutrient identified to be limiting to allow for more accurate feeding programs (Kolver, 2003, Chalupa & Boston, 2006).

Conclusion

High producing, multiparous Jersey cows in mid-lactation grazing high quality annual ryegrass pasture during spring while receiving 8 kg (as is) per day of a maize-based concentrate did not respond in terms of milk production and composition to the addition of RPL. The RPL was supplemented to the concentrate at a rate of 53.12 g/cow/d proving approximately 22.0 g of intestinally absorbable Lys/cow/d. Treatment also did not have an effect on cow body measurements, faecal starch content, plasma AA levels and MCP production. However, RPL supplementation tended to reduce milk protein production. Supplementation of RPL did increase the plasma Lys:Met ratio significantly beyond the ratio represented by the C treatment. The high producing group of cows (> 24 l/cow/d) did not respond to the supplementation of RPL, while the lower producing group of cows (< 20 l/cow/d) responded negatively in terms of milk protein production. Results from this, and others, study indicate that when RPL is supplied in excess of the cow's requirement it results in a negative effect on cow performance, especially milk protein production. Since the uptake of Lys from the blood plasma by the cow's mammary gland tend to surpass the cow's net requirements for lactation, it is speculated that Lys was extracted from the plasma in accordance with its supply irrespective of the cows requirements, being metabolised and used for other metabolic functions. Supplementing the combination of RPML to the concentrate at a rate of 41.68 and 53.12 g/cow/d of RPML, providing approximately 9.3 and 22.0 g of intestinally absorbable Met and Lys, respectively, the cows did not respond in terms of milk production and composition. Treatment also did not have an effect on faecal starch content, cow BW and MCP production. However, in the presence of a significant increase in plasma Met levels, cow BCS increased significantly when RPML were supplemented. In addition to an increase in plasma Met, Gly increased significantly, and Lys and Cys tended to increase. It is excepted that the concentration of an AA would build up in the plasma only after the cow's requirements have been met. In contrast to RPL supplementation, when RPML were supplemented the plasma Lys:Met ratio decreased significantly below the plasma Lys:Met ratio of the C treatment. The higher producing group of cows (> 24 l/cow/d) and lower producing group of cows (< 20 l/cow/d) did not respond to the supplementation RPML. The results suggest that Met was supplied beyond cow requirements, however, the increased availability of Met to the cow's mammary gland did not translate into increased milk or milk protein production but rather resulted in Met being metabolised, repartitioning nutrients towards other metabolic pathways resulting in an increase in body tissue synthesis and could explain the increase in cow BCS that was observed. Research further suggest that an energy deficient diet may cause AA to be repartitioned away from milk protein synthesis towards oxidation for energy generation and *de novo* synthesis of other AA.

In view of the experimental results the data indicate that the lack of positive responses in terms of milk production and milk composition when RPM and/or RPL were supplemented were as a result of ME being the first limiting nutrient and not MP as was hypothesised and the fact that the cows were later in lactation. The ME supply was estimated to be below cow requirements and the review of the literature

indicated that a response to AA supplementation is more frequently observed in early-lactation rather than mid- to late-lactation cows. This is due to the cow's metabolic requirements for absorbable AA, relative to absorbed energy, which is the highest in early-lactation. Additionally, the MP was estimated to be supplied beyond the cow's requirements and as a result the likelihood of a "single" AA limiting cow performance is reduced especially due to the heavy contribution of MCP as a proportion of MP and the AA profile supplied by the C diet making the cows less responsive to the supplementation of RPM and/or RPL.

Using the CNCPS model to compare the result observed in our study indicated that the study design and interpretation thereof could be aided when including model evaluations in studies where RPAA are supplemented. The model gave relatively realistic and comparative predictions in terms of ME allowable milk, MP allowable milk, PDMI, MCP production, including the MP and AA balance of the cows. Results from the model also support the finding of our study.

Evidence indicate that the AL in spot urine samples are a valid method to be used to determine MCP for Jersey cows grazing pasture.

Chapter 5: General discussion

5.1 Future research and critical evaluation

In pasture-based production systems the determination of PDMI is shown to be difficult and time consuming with variable accuracies, since individual cow PDMI is usually determined from a group estimate and not individual cow intakes or different marker-based methodologies. This could be overcome, to some degree, by using different markers (TiO, Cr_2O_3 or N-alkanes). However, it is encouraging that the model predicted PDMI levels which were relatively close to the PDMI reported in our study.

Cows could be fed individually in a basic "cut-and-carry" system or alternative system which allow for individual cow measurements. In this kind of system, ruminal/duodenal cannulated cows could be used in which more accurate prediction could be made in terms of the chemical composition of the total diet, along with complementary *in situ* studies which could further allow the evaluation of other parameters (i.e. rumen kinetics and digestion). Especially considering the potential to determine MP flowing to the grazing cows SI and the AA balance reaching the intestinal absorptive site of the cows.

Plasma AA concentrations have shown to be a useful indicator of AA absorption from the intestine, including the success with which rumen protected AA for example Lys and Met are delivered to the SI. It is also a strong indicator of whether other AA were more limiting than the ones supplemented and the metabolic fates of various other metabolites. However, more research is required on the kinetics of blood plasma AA levels in PRAA supplementation focused studies.

Since studies, in which RPAA are supplemented in the concentrates fed to grazing dairy cows are limited in the literature it is suggested that the most effective levels of RPM and/or RPL supplementation to be used could be determined using graded levels of AA supplied directly into the cow's rumen or by means of supplementary-focused studies. This could potentially indicate the level and ratio at which different AA effect cow performance and the sequence in which production parameters are influenced. Including the sequence and degree to which other AA limits cow's performance for this specific production system.

It has been shown in the literature that other AA (i.e. His and Ile), apart from Lys and Met could potentially be first limiting to cow performance, this could especially be true for cows in pasture-based production systems. This suggest that further research must also focus on AA supplementation "packages" rather than just the individual supplementation of specific AA. However, when AA supplementation to the SI is increased care must be taken to prevent competitive inhibition, as discussed earlier, this is supported by other studies, proposing the combined supplementation of AA could potentially produce larger responses to AA supplementation as compared to just individual AA supplementation.

Cows in early-lactation that are high producing are more likely to respond to the supplementation of rumen protected Met and Lys, and should be included in AA supplementation studies.

Blood non-esterified fatty acid concertations could also give an indication of the cow's energy state, and could distinguish between fat or protein turnover resulting in changes in cow's BW and/or BCS. The milk profile could also be potentially determined to observe how AA supplementation influences the fatty acid profile of the milk, since milk samples are already taken routinely this parameter could be added to the study protocol fairly easily. This is also true for blood non-esterified fatty acids concertation, since blood is already collected from the cows tail vein.

There might be added benefit to supplement cows through a whole lactation cycle, since limited data is available with regards to grazing dairy cows, especial Jersey cows. In such a study the effects of AA supplementation on cow health parameters, reproductive performance, including the cow's progeny could be measured (Ardalan *et al*., 2010, Zhou *et al*., 2016).

As indicated by this study, and others, post-experimental dietary evaluation should be considered for assessing the response of cows supplemented RPAA. For evaluations to be done more accurately along with the interpretation of study result, the chemical composition of the pasture should be determined as accurately as possible, through more rigorous sampling and analysis. It was assumed that the cows were grazing a pure spring ryegrass pasture in a ryegrass/kikuyu pasture-based system, however, in practice this is not always possible, since other species form part of the pasture. By determining the botanical composition of the pasture in pasture-focused studies a clearer indication could be gained in terms of what the cows are actually consuming.

However, based on results from our study, and others, there are potentially a few follow up studies which could complement our study. Such studies could include utilisation of a combination of preserved forages and/or silage in different feeding systems, the use of early-lactation cows that are high producing and the combination of other protein sources available in South Africa, apart from soybean meal. Since the addition of RPAA in concentrates fed to grazing dairy cows does not substitute large amounts of other raw material (i.e. maize), additional energy could also be provided in the concentrate for example rumen protected fats or fermentable carbohydrates. Additionally, due to the difficulty associated with separating production responses observed in studies which supplemented RPM and RPL in combination, a fourth treatment could potentially be used to evaluate production responses more clearly.

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Appendix A

Selection and blocking of the cows

All lactating Jersey cows in the Outeniqua Research Farm herd were subjected to the same review process to be potentially use in this study. See Chapter 3, section 3.5 for a clear discussion on how the cows were blocked and allocated to different treatments.

Table A1 Sixty Jersey cows blocked and allocating cows for the study

Cow ID	Cow name	Milk yield (kg/d)	Days in milk	Lactation number	Cow weight	Block	Treatment
12256	TARNA 36	27,65	95	4	469	1	C
12246	TPAULET 34	26,25	84	4	422	2	C
14201	TAMSA 211	25,25	49	3	368	3	C
11235	TSUSA 113	25,12	122	5	446	4	C
9160	TSANTA 21	24,24	28	8	433	5	C
8164	THES 8	23,48	27	9	374	6	C
10223	TAMSA 132	22,33	31	7	453	7	C
15230	TMAX 82	21,93	24	2	328	8	C
12226	TSUSA 126	21,83	45	4	379	9	C
13257	TETNA 31	22,68	10	3	474	10	C
12162	TSUSA 117	21,50	194	5	423	11	C
13195	TPAULET 38	21,40	130	3	455	12	C
15213	TPAULET 42	20,70	71	2	347	13	C
11232	TSANTA 29	21,07	187	5	375	14	C
13161	TMONA 20	20,00	178	4	435	15	C
12159	TMAX 62	19,72	108	5	414	16	C
13251	TSUSA 144	19,57	180	3	419	17	C
10232	TBERTA 118	19,55	218	6	413	18	C
15249	TBERTA 178	19,62	19	2	351	19	C
11181	TSUSA 109	18,62	144	5	451	20	C
10172	TSUSA 94	26,55	43	7	432	1	RPL
13192	TLIZ 53	26,30	63	4	376	2	RPL
10237	TAMSA 134	25,67	24	6	436	3	RPL
12167	TBERTA 133	24,97	152	5	437	4	RPL
12261	TAMSA 172	24,80	53	4	402	5	RPL
12199	TWANDA 43	22,80	61	4	339	6	RPL
10236	TSANTA 27	24,18	36	6	422	7	RPL
15233	TESME 13	22,10	29	2	357	8	RPL
13214	TAMSA 189	21,83	41	4	389	9	RPL
10265	TBERTA 123	21,53	78	6	449	10	RPL
10228	TSUSA 96	21,63	196	6	436	11	RPL
9253	TAMSA 117	20,97	195	7	465	12	RPL
15207	TLASS 25	21,45	70	2	351	13	RPL
13212	TSUSA 139	20,77	133	3	406	14	RPL
10263	TSUSA 101	20,58	173	6	463	15	RPL
13163	TSUSA 135	19,63	117	4	421	16	RPL
12217	TBERTA 142	19,58	202	6	428	17	RPL
10273	TLIN 45	19,13	181	5	472	18	RPL
16175	TSUSA 179	19,27	42	1	333	19	RPL
9219	TAMSA 108	18,12	73	7	458	20	RPL
12230	TPAULET 33	28,05	71	4	382	1	RPML
11163	TSUSA 106	26,25	46	6	374	2	RPML

Table A1 Sixty Jersey cows blocked and allocating cows for the study (*Continued*)

Cow ID	Cow name	Milk yield (kg/d)	Days in milk	Lactation number	Cow weight	Block	Treatment
13254	TMELBA 3	25,12	30	4	392	3	RPML
9238	TARNA 27	25,73	140	5	423	4	RPML
9180	TESME 5	24,68	29	8	409	5	RPML
15197	TLIN 57	22,72	80	2	327	6	RPML
14155	TSANTA 39	23,25	31	3	387	7	RPML
14189	TESME 9	22,30	38	3	327	8	RPML
11191	THES 15	21,97	38	6	380	9	RPML
13165	TPAULET 35	22,66	107	4	446	10	RPML
13154	TMAX 67	21,93	150	4	443	11	RPML
12213	TBERTA 141	20,83	189	4	446	12	RPML
16164	TETNA 40	20,35	70	1	350	13	RPML
12204	TWANDA 44	20,17	210	4	404	14	RPML
12187	TPAULET 31	20,05	215	4	457	15	RPML
12218	TMAX 63	19,68	71	4	379	16	RPML
12208	TAMSA 163	19,02	204	4	447	17	RPML
13170	TPAULET 36	19,42	183	4	453	18	RPML
15225	TARNA 41	19,30	61	2	327	19	RPML
11164	TMONA 14	17,97	137	6	438	20	RPML
Mean ± SD		22.09 ± 2.53	100,1 ± 64,78	4,4 ± 1.75	408,2 ±42,81		

Table A2 Mean (± SD) milk yield, days in milk, lactation number and weight for cows blocked and allocated to the control (C) treatment

Cow ID	Cow name	Milk yield (kg/d)	Days in milk	Lactation number	Cow weight	Block	Treatment
12256	TARNA 36	27,65	95	4	469	1	C
12246	TPAULET 34	26,25	84	4	422	2	C
14201	TAMSA 211	25,25	49	3	368	3	C
11235	TSUSA 113	25,12	122	5	446	4	C
9160	TSANTA 21	24,24	28	8	433	5	C
8164	THES 8	23,48	27	9	374	6	C
10223	TAMSA 132	22,33	31	7	453	7	C
15230	TMAX 82	21,93	24	2	328	8	C
12226	TSUSA 126	21,83	45	4	379	9	C
13257	TETNA 31	22,68	10	3	474	10	C
12162	TSUSA 117	21,50	194	5	423	11	C
13195	TPAULET 38	21,40	130	3	455	12	C
15213	TPAULET 42	20,70	71	2	347	13	C
11232	TSANTA 29	21,07	187	5	375	14	C
13161	TMONA 20	20,00	178	4	435	15	C
12159	TMAX 62	19,72	108	5	414	16	C
13251	TSUSA 144	19,57	180	3	419	17	C
10232	TBERTA 118	19,55	218	6	413	18	C
15249	TBERTA 178	19,62	19	2	351	19	C
11181	TSUSA 109	18,62	144	5	451	20	C
Mean ± SD		21,13 ± 2,5	97.20 ± 68.10	4.45 ± 1.93	411,45 ± 43.20		

Table A3 Mean (± SD) milk yield, days in milk, lactation number and weight for cows blocked and allocated to the ruminally protected lysine (RPL) treatment

Cow ID	Cow name	Milk yield (kg/d)	Days in milk	Lactation number	Cow weight	Block	Treatment
10172	TSUSA 94	26,55	43	7	432	1	RPL
13192	TLIZ 53	26,30	63	4	376	2	RPL
10237	TAMSA 134	25,67	24	6	436	3	RPL
12167	TBERTA 133	24,97	152	5	437	4	RPL
12261	TAMSA 172	24,80	53	4	402	5	RPL
12199	TWANDA 43	22,80	61	4	339	6	RPL
10236	TSANTA 27	24,18	36	6	422	7	RPL
15233	TESME 13	22,10	29	2	357	8	RPL
13214	TAMSA 189	21,83	41	4	389	9	RPL
10265	TBERTA 123	21,53	78	6	449	10	RPL
10228	TSUSA 96	21,63	196	6	436	11	RPL
9253	TAMSA 117	20,97	195	7	465	12	RPL
15207	TLASS 25	21,45	70	2	351	13	RPL
13212	TSUSA 139	20,77	133	3	406	14	RPL
10263	TSUSA 101	20,58	173	6	463	15	RPL
13163	TSUSA 135	19,63	117	4	421	16	RPL
12217	TBERTA 142	19,58	202	6	428	17	RPL
10273	TLIN 45	19,13	181	5	472	18	RPL
16175	TSUSA 179	19,27	42	1	333	19	RPL
9219	TAMSA 108	18,12	73	7	458	20	RPL
Mean ± SD		22,09 ± 2,53	98.10 ± 63.56	4.75 ± 1.77	413.60 ± 43.12		

Table A4 Mean (± SD) milk yield, days in milk, lactation number and body weight for cows blocked and allocated to the RPML treatment

Cow ID	Cow name	Milk yield (kg/d)	Days in milk	Lactation number	Cow weight	Block	Treatment
12230	TPAULET 33	28,05	71	4	382	1	RPML
11163	TSUSA 106	26,25	46	6	374	2	RPML
13254	TMELBA 3	25,12	30	4	392	3	RPML
9238	TARNA 27	25,73	140	5	423	4	RPML
9180	TESME 5	24,68	29	8	409	5	RPML
15197	TLIN 57	22,72	80	2	327	6	RPML
14155	TSANTA 39	23,25	31	3	387	7	RPML
14189	TESME 9	22,30	38	3	327	8	RPML
11191	THES 15	21,97	38	6	380	9	RPML
13165	TPAULET 35	22,66	107	4	446	10	RPML
13154	TMAX 67	21,93	150	4	443	11	RPML
12213	TBERTA 141	20,83	189	4	446	12	RPML
16164	TETNA 40	20,35	70	1	350	13	RPML
12204	TWANDA 44	20,17	210	4	404	14	RPML
12187	TPAULET 31	20,05	215	4	457	15	RPML
12218	TMAX 63	19,68	71	4	379	16	RPML
12208	TAMSA 163	19,02	204	4	447	17	RPML
13170	TPAULET 36	19,42	183	4	453	18	RPML
15225	TARNA 41	19,30	61	2	327	19	RPML
11164	TMONA 14	17,97	137	6	438	20	RPML
Mean ± SD		22.07 ± 3.0	105.0 ± 69,54	4.10 ± 2.06	399.6 ± 42.40		

Table A5 Mean (± SD) of cow selected for sampling of milk nitrogen fraction, blood plasma urine and faecal samples

Cow ID	Cow name	Milk yield (kg/d)	DIM	Lactation number	Cow weight	Block	Treatment
12256	TARNA 36	27,65	95	4	469	1	C
14201	TAMSA 211	25,25	49	3	368	3	C
9160	TSANTA 21	24,24	28	8	433	5	C
10223	TAMSA 132	22,33	31	7	453	7	C
12226	TSUSA 126	21,83	45	4	379	9	C
12162	TSUSA 117	21,50	194	5	423	11	C
15213	TPAULET 42	20,70	71	2	347	13	C
13161	TMONA 20	20,00	178	4	435	15	C
13251	TSUSA 144	19,57	180	3	419	17	C
15249	TBERTA 178	19,62	19	2	351	19	C
Mean ± SD		22,27 ± 2.54	89,00 ± 65.66	4.20 ± 1.89	407.7 ± 41.06		
10172	TSUSA 94	26,55	43	7	432	1	RPL
10237	TAMSA 134	25,67	24	6	436	3	RPL
12261	TAMSA 172	24,80	53	4	402	5	RPL
10236	TSANTA 27	24,18	36	6	422	7	RPL
13214	TAMSA 189	21,83	41	4	389	9	RPL
10228	TSUSA 96	21,63	196	6	436	11	RPL
15207	TLASS 25	21,45	70	2	351	13	RPL
10263	TSUSA 101	20,58	173	6	463	15	RPL
12217	TBERTA 142	19,58	202	6	428	17	RPL
16175	TSUSA 179	19,27	42	1	333	19	RPL
Mean ± SD		22.56 ± 2.44	88.00 ± 68,25	4.80 ± 1.89	409,20 ± 38,76		
12230	TPAULET 33	28,05	71	4	382	1	RPML
13254	TMELBA 3	25,12	30	4	392	3	RPML
9180	TESME 5	24,68	29	8	409	5	RPML
14155	TSANTA 39	23,25	31	3	387	7	RPML
11191	THES 15	21,97	38	6	380	9	RPML
13154	TMAX 67	21,93	150	4	443	11	RPML
16164	TETNA 40	20,35	70	1	350	13	RPML
12187	TPAULET 31	20,05	215	4	457	15	RPML
12208	TAMSA 163	19,02	204	4	447	17	RPML
15225	TARNA 41	19,30	61	2	327	19	RPML
Mean ± SD		22.37 ± 2.76	89,90	4,00 ± 1.84	397.40 ± 40.13		

Appendix B

Calculation of the cow's energy requirements

Data obtained in this study and equations from the NRC 2001 (chapter 2) were used to calculate the energy requirements of the cows used in our study. To calculate the net energy (NE) requirements for maintenance the formula 0.08 (Mcal/kg BW)$^{0.75}$ were used, mean BW used were 410.3, 415.6 and 400.5 kg for the C, RPL and RPML treatments, respectively. The value obtained needed to be converted from Mcal to MJ, this was done by multiplying with 4.184 MJ/Mcal, and the dividing it by 0.62.

The value 0.62 is used since it represents the efficiency with which NE is utilized for maintenance in dairy cows (NRC, 2001). The Gaines formula described by the NRC (2001) was used to calculate the NE requirements for lactation (Mcal/kg/kg milk) which are 0.360 + [0.0969) (fat %)]. Since 4 % FCM were used in the calculation a value of 4 were used for fat %, as a result the 4 % FCM could be multiplied by 0.749 Mcal, which represents the energy required per kg of milk. The value obtained can then be converted to MJ by multiplying with 4.184 MJ/Mcal and then to ME by dividing by 0.64 which represents the efficiency with which NE is utilized for lactation.

However, since grazing dairy cows have additional energy requirements for physical activity due to walking from the pasture to the parlour, including grazing the NE requirements for activity also needs to be calculated. The NE requirements for activity is 0.00045 Mcal of NE/kg BW per km walked + 0.0012 Mcal per kg BW. It is assumed that the cows walked on average 4.5 km/cow/d. The value obtained could then be multiplied by 4.184 MJ Mcal to convert the value from Mcal to MJ and divided by 0.62 to convert to ME. The value of 0.62 represent the same value as for the efficiency of utilization of NE for maintenance, but a figure was not provided by the NRC (2001) and thus it was assumed that the utilization efficiency of NE for activity is the same as that for maintenance.

The equation to calculate the NE requirements for gestation is [((((0.00318 (days in gestation)) - 0.0352)) (cow BW/45)]/0.0218, however this formula only represents the energy requirements for gestation during the last 100 days (190 to 279 days in gestation) of gestation. As a result, it was assumed that NE requirements for gestation equals zero since most of the cows were less than 190 days gestating.

Table 2-4 of NRC (2001) indicates that the NE requirements per kg BW gain is 4.50 Mcal/kg BW gain for a BCS of 2 and 4.9 Mcal/kg BW gain for a BCS of 2.5. The BCS scoring system of Edmundson *et al.* (1989) which are similar to Wildman *et al.* (1982) were used. The range of BCS obtained for this study (2.05 to 2.57) are similar to the values for BCS used (2.0 to 2.5) to acquire the NE requirements for BW gain. The mean change in BW across the C, RPL and RPML treatments were +25.2, +20.1 and + 30.4 kg, respectively, for the duration of the trial. To calculate the NE requirements per kg BW gain the value of 4.7 Mcal/kg BW were used, which represent the mean BCS of the cows. The cows gained 0.42, 0.34 and 0.51 kg/d for the control, RPL and RPML treatment respectively.

The efficiency with which dietary NE is converted to tissue energy for a gain in BW is presented by the value 1.12.

Table B1 Calculated energy requirements for cow grazing ryegrass pasture and fed a maize-based concentrate supplemented with RPL and/or RPML

Requirement	**Experimental Treatment**		
	C	**RPL**	**RPML**
Maintenance			
BW (kg)	410.3	415.6	400.5
NE (Mcal/d)	7.3	7.4	7.2
NE (MJ/d)	30.5	30.8	30.0
ME (MJ/d)	49.2	49.7	48.3
Lactation			
4 % FCM (kg/cow/d)	24.7	24.6	24.9
NE (Mcal/d)	18.5	18.4	18.7
NE (MJ/d)	77.4	77.1	78.0
ME (MJ/d)	120.9	120.5	121.9
Physical activity			
Distance walked (km)	4.5	4.5	4.5
NE (Mcal/d)	1.3	1.3	1.3
NE (MJ/d)	5.5	5.6	5.4
ME (MJ/d)	8.9	9.0	8.7
Gain in BW			
Daily BW gain (kg/d)	0.42	0.34	0.51
NE (Mcal/d)	2.0	1.6	2.4
NE (MJ/d)	8.3	6.7	10.0
ME (MJ/d)	7.4	6.0	9.0
Total			
ME requirements (MJ/d)	186.4	185.2	187.9