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Low protein diets based on high protein forages for dairy cows: effects on performance, metabolism and nitrogen use efficiency

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Preface

“Surely there is a lesson for you in the cattle: we give you drink, from their bellies digested food and blood, pure liquid milk for those who drink.”

Surah Al Nahl (Verse 16.66)

AL Quran

Declaration

I declare that this thesis is based on original work, which has been entirely composed by myself. Any or whole part of this thesis has not ever been submitted previously to achieve institutional qualification or award at this or any other university.

Mohammed Rashed Chowdhury

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List of Common Abbreviations

AA	amino acid(s)
ADF	acid detergent fibre
ADL	acid detergent lignin
AIA	acid insoluble ash
BCS	body condition score
BHB	β-hydroxybutyric acid
CP	crude protein
DM	dry matter
ECM	energy-corrected milk
EE	ether extract
FA	fatty acids
FCM	Fat-corrected milk
MCP	microbial protein
ME	metabolisable energy
MP	metabolisable protein
MPE	Metabolisable protein – energy limiting
MPN	Metabolisable protein – nitrogen limiting
MUFA	Monounsaturated fatty acids
MUN	milk urea nitrogen
N	nitrogen
NDF	neutral detergent fibre
NPN	non-protein nitrogen
NUE	nitrogen use efficiency
OM	organic matter
pef	physical effectiveness factor
peNDF	physically effective neutral detergent fibre
PS	particle size
PUFA	polyunsaturated fatty acids
RDP	rumen degradable protein
RP	rumen-protected
RPL	rumen-protected lysine
RPM	rumen-protected methionine

RPML	rumen-protected methionine plus lysine
RUP	rumen undegradable protein
SFA	saturated fatty acids
TMR	total mixed ration
VFA	volatile fatty acids

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Abstract

The effects of an increasing global price of soybean meal and tighter regulations on ammonia emissions, and the disposal of manure and slurry has led to renewed interest in alternative dietary protein strategies for dairy cows. The objectives of this thesis were to improve the nitrogen (N) use efficiency (NUE) of dairy cows whilst maintaining performance and reducing the environmental impact of milk production by feeding low crude protein (CP) diets based on high protein, home grown forage legumes. In Study 1, 18 early lactation Holstein-Friesian dairy cows were fed 1 of 3 diets based on 50:50 red clover to grass silage (dry matter (DM) basis) and 1 of 3 dietary CP levels: high (H) – 175 g CP/kg DM; medium (M) - 165 g CP/kg DM or low (L) – 150 g CP/kg DM. The diets were fed in a 3 x 3 Latin square design, with 3 periods of 28 days, with measurements undertaken in the final week of each period. The metabolisable protein (MP) supply was predicted to meet requirements in H and M, and be 95% of requirements in L. Cows fed L had an intake of 23.5 kg DM/d, some 1.5 kg DM/d lower than those fed H or M, but milk yield was similar across treatments, with a mean of 34.8 kg/d. The NUE was 20% higher in cows fed L than H. In Study 2, 18 multiparous Holstein-Friesian dairy cows were fed 1 of 3 dietary treatments: H50: high protein (172 g/kg DM) with 50:50 lucerne:maize silage (DM basis); L50: low protein (150 g/kg DM) with 50:50 lucerne:maize silage, and L60: low protein (150 g/kg DM) with 60:40 lucerne:maize silage. The diets were fed in a 3 x 3 Latin square design, with 3 periods of 28 days, with measurements undertaken in the final week of each period. All diets were formulated to meet MP requirements. Intake was higher in cows fed H50 vs. L50, with L60 being intermediate. Milk yield was also highest in cows fed H50 at 40.9 kg/d, and lowest in L60 at 38.9 kg/d, with L50 being intermediate. The NUE was 18% higher in cows fed L50 or L60 compared to H50. In Study 3, 56 Holstein-Friesian dairy cows were fed 1 of 4 diets based on 50:50 red clover to grass silage (DM basis). All diets were formulated to have a similar MP content and a CP concentration of 175 g/kg DM (high protein, C), 150 g/kg DM (low protein, LP), or LP supplemented with additional starch (LS) or rumen-protected methionine (LM). Reducing dietary CP from 175 to 150 g/kg DM did not affect DM intake, milk yield or composition, live weight or condition change. Reducing CP increased NUE by 20% and lowered urinary N excretion by 60 g N/d. In Study 4, a systematic review and meta-analysis was conducted to investigate the effects of dietary CP concentration on the performance, metabolism and NUE of dairy cows fed forage legume-based rations. A total of 36 studies with 102 treatment means were included. The mean CP content of the control and low CP diets was 171 and 145 g/kg DM respectively. On average, lowering dietary CP reduced DM intake by 0.6 kg/d, milk yield by 1.4 kg/d and milk protein content by 0.2 g/kg, but increased NUE by 4% units. In conclusion, low CP diets improve NUE without affecting milk performance if MP requirements are met.

CHAPTER 1: General introduction

Feeding sufficient protein to dairy cows is important to meet their requirements for milk production and to maintain health and fertility (Sinclair et al., 2014). There is considerable interest in lowering dietary crude protein (CP) concentrations and making greater use of home-grown forages in dairy cow diets due to the high and volatile costs of purchased protein feeds such as soya bean meal, and the legislative requirement to reduce nitrogen (N) and ammonia output from dairy farms (Calsamiglia et al., 2010; Johnston et al., 2020; Liu and VandeHaar, 2020a). Diets high in CP typically result in a low N use efficiency (NUE), with only around 25% of the N consumed by a cow being captured in milk, with the excess being excreted, particularly in the urine and faeces as nitrate (NO_3^-), ammonia (NH_3) and gaseous nitrous oxide (NO_2) (Lavery and Ferris, 2021). Leaching of NO_3^- to the aquatic systems has been related to the death of aquatic animals through acidification or eutrification whereas the release of gaseous nitrous oxide (NO_2) is responsible for global worming, which is almost 300 times higher potential than carbon dioxide (CO_2) (Castillo et al., 2000; Hristov et al., 2011b). The excess urinary urea is converted to NH_3 and contributes to the formation of very fine particles (2.5 μg), which is related to the respiratory and cardiovascular problems in human beings (Lavery and Ferris, 2021).

Therefore, feeding excess dietary protein is not only costly, but contributes to a negative environmental impact of milk production (Olmos Colmenero and Broderick, 2006; Hristov et al., 2015). Some studies have reported that feeding low CP diets decreases DM intake, milk yield and milk fat or protein content (Alstrup et al., 2014; Kidane et al., 2018a; Oh et al., 2019). However, others have shown that dietary protein levels can be lowered to around 140-150 g/kg dietary dry matter (DM) without affecting performance, health or fertility if the diets are formulated to meet the cows metabolisable protein (MP) requirements (Lee et al., 2012b; Bahrami-Yekdangi et al., 2014; Sinclair et al., 2014).

Soybean is widely used in dairy ration to meet the high demand for CP, and the concentration of CP in soybean meal is around 440 to 490 g/kg DM (Tadele and Getachew, 2015). Soybean is the most common transgenic crop, referred to as GM soya (genetically modified soya using genetic engineering techniques) (Flachowsky et al., 2012). However, the excessive use of GM crops, including soybean meal in animal feed can cause toxicity and adversely affect several organs and systems

(Dona and Arvanitoyannis, 2009). Therefore, there is a limitation to the use of GM soya due to public concern in the human food chain (Dona and Arvanitoyannis, 2009). The cost of soybean is, however, increasing due to growing demands, and the current year (January 2021 to August 2021) the market price is on average 431 GBP/MT, whereas it was approximately 316 GBP/MT last year and 320 GBP/MT for the previous 5 years from August 2016 to August 2021 (Ycharts.com, 2021). Furthermore, there are agronomic limitations on growing protein-rich feeds, including soybean meal in temperate climates, especially in Northern Europe, resulting in the large-scale import of soybean meal (Eurostat, 2019).

Home-grown forage legumes can contribute to the sustainability of ruminant production due to their potential ability to use atmospheric N and reduce inputs of purchased artificial fertiliser (Peyraud et al., 2009). Forage lelgumes such as lucerne and red clover are high in CP at approximately 180-200 g/kg DM and can fix N (Dewhurst et al., 2003b; Broderick, 2018). Therefore, legume silages could be an alternative vegetative protein source for high yielding diary cows. However, the protein in legume silages is rapidly released in the rumen, which lowers the digestable undegradable protein (DUP) and MP supply, particularly for high yielding dairy cows (Dewhurst et al., 2009; Westreicher-Kristen et al., 2017). Lucerne is the most popular forage legume grown globally, and is more common than grass silage in North America and many areas of Europe (Murphy-Bokern et al., 2017). Intake and milk yield are typically higher in cows fed lucerne based diets (Johansen et al., 2017a; Broderick, 2018), although high inclusion rates have been shown to reduce milk yield in recent UK based studies (Sinclair et al., 2015; Thomson et al., 2017a). In the UK, red clover is an important forage legume fed to dairy cows, and is commonly grown and ensiled with grass (Clavin et al., 2017; Johnston et al., 2020). Feeding a mixture of red clover and grass silage at inclusion rates of red clover of up to 66% of the forage DM has been shown to improve intake and milk yield (Moorby et al., 2009; Dewhurst, 2013; Johnston et al., 2020). Most research on the inclusion of forage legumes has however focussed on lower yielding dairy cows, or have fed comparatively high levels of dietary protein (Moorby et al., 2016; Schulz et al., 2018; Westreicher-Kristen et al., 2018). In contrast, there have been few studies that have fed low protein diets based on high protein legume silages, particularly red clover.

CHAPTER 2: Literature review

2.1. Protein for ruminants

2.1.1. Characterisation of dietary protein

Proteins are polymers of amino acids (AA) that are linked together by peptide bonds through a carboxylic ($^{-}$COOH) and amino (NH_3^+) group, which are popularly termed as the C and N terminal, respectively (Moran et al., 2014). Dietary protein is often referred to as crude protein (CP) and is defined as the dietary N content $\times$ 6.25 (AOAC, 2012). Crude protein is one of the nutrients required by a nutritionist to formulate rations for dairy cows. After passing through the rumen, undegraded dietary protein along with rumen microbial protein is hydrolysed by enzymes in the abomasum or the small intestine and absorbed as peptides or free AA (Schwab and Broderick, 2017). These absorbed AA's are carried via the blood circulation to body tissues, and are used as building blocks for the synthesis of body proteins, and are termed as true or available protein (Wu, 2014).

Based on growth and N balance, AA's are classified as nutritionally essential or non-essential for humans and animals (Wu, 2014). The AA's which cannot be synthesised or insufficiently synthesised *de novo* by the animal cells or organism in relation to its requirements for body growth, maintenance, development and health, are referred to as indispensable or essential AA (EAA), such as lysine, leucine, isoleucine, methionine, valine, phenylalanine, tryptophan, and threonine (Hou et al., 2015). These AA's must be provided in the diet to meet the animal requirements. Some AA's are conditionally dispensable (CEAA), which can only be synthesised from a particular EAA or synthesised to a limited extent in the body, such as cysteine (derived from methionine), tyrosine (derived from phenylalanine), histidine and arginine (Wu et al., 2014). In contrast, those AA's which can be synthesised in an adequate amounts to meet the requirements for body growth, maintenance, development and health, are referred to as dispensable or nutritionally non-essential AA (NEAA), such as alanine, asparagine, glutamine, glycine, proline and serine (Hou et al., 2015).

The role of proteins mainly depends on their three-dimensional structure as well as the AA sequence (Wu et al., 2014). Based on the covalent bond and folding patterns, there are four different structures of proteins; primary, secondary, tertiary or quaternary (McDonald, 2011). Different AA monomers are linked together by

peptide linkage and make up a polypeptide chain, which is referred to as the primary structure of the protein. The hydrogen bond in the neighbouring regions of a polypeptide chain sometimes causes a spiral or sheet-like patterns termed as α-helices or β-plated sheets. This type of folding structure is known as the secondary structure of the protein (Moran et al., 2014). The tertiary structure of protein contains multiple helices and parallel or antiparallel sheets, which shape the polypeptide chain into a three-dimensional structure (Bajaj and Blundell, 1984). In contrast, different polypeptide chains or subunits are packed together and form the quaternary structure of proteins (Klotz et al., 1970). The presence of disulfide (-S-S-) bonds in a three-dimensional protein structure may lead to a lower degradability or digestibility, such as feather meal, which is an animal source protein, high in keratin but low in digestibility (Tadele and Getachew, 2015). In contrast, some vegetable source protein contains antinutritional factors, such as trypsin inhibitor in soybean meal, limiting protein digestion, although heat-treatment or thermal processing can reduce the activity of the trypsin inhibitor and improve digestibility in the small intestine (Yin et al., 2011).

Based on the solubility or degradability, dietary protein has various fractions, including rumen-degradable protein (RDP), rumen-undegradable protein (RUP) and non-protein nitrogen (NPN) compounds (McDonald, 2011). Different isolation techniques have identified that NPN in feed is composed of numerous low molecular weight compounds including amides, amines, ammonia (NH_3), nucleic acids and different peptides group (McDonald, 2011). Different species of legumes, grass and their silages contain a higher concentration of NPN due to their source of origin (Schwab and Broderick, 2017). Feeding legume and grass silage based diets results in extensive ruminal degradation of the forage protein by rumen microbes (Bach et al., 2005). With the advancement of dairy nutrition and feeding it was determined that the degradation of dietary CP and AA produced NH_3 within the rumen (Bach et al., 2005). This NH_3 is utilised by the rumen bacteria for their protein synthesis (Cummins et al., 1983). Therefore, optimum levels of NH_3 concentration within the rumen must be maintained for maximum bacterial protein formation (Tamminga, 1979). On the other hand, a deficiency of RDP can impair microbial protein (MCP) synthesis, fibre degradation, and feed intake in dairy cows (Schwab and Broderick, 2017).

2.1.2. High and low dietary protein diets for dairy cows

Dairy cows require metabolisable protein (MP) for maintenance, growth, pregnancy and lactation, which is provided through AA absorption in the small intestine (McDonald, 2011). Dairy cows are often offered high CP diets to provide an adequate MP supply to support milk production and protein synthesis (Lee et al., 2012b). Overfeeding highly degradable CP or forage protein, including legumes (red clover, white clover and lucerne) can increase the concentration of RDP (Poppi and McLennan, 1995). This high level of RDP can lead to an excess production of NH_3 in the rumen, which may be absorbed into the bloodstream, converted to urea in the liver, and excreted in the urine (Tamminga, 1979; Bach et al., 2005). When urine comes into contact with faeces, urea nitrogen (N) is quickly hydrolysed to NH_3 and lost by volatilisation due to the labile form of N (Hristov et al., 2011b). This can cause undesirable effects on the environment (UK Clean Air Strategy, 2019). Feeding CP above 165 g/kg dry matter (DM) may contribute to low N use efficiency (NUE; Huhtanen and Hristov, 2009) and also increase feed costs that negatively affects the profit margin of the farm (Godden et al., 2001).

Feeding high CP diets (from 175 to 190 g/kg DM) to sustain improved milk production can increase urea and N excretion and decrease N utilisation (Olmos Colmenero and Broderick, 2006). Therefore, feeding a low protein diet is necessary not only to improve N balance but also reduce feed costs (Schwab and Ordway, 2004). Olmos Colmenero and Broderick (2006) reported that reducing the concentration of dietary CP from 194 to 135 g/kg DM decreased N emission in urine from 36.2 to 23.8% and increased milk N efficiency from 25.4 to 36.5% in dairy cows. Numerous authors have also reported that milk yield and milk protein content were not increased by feeding CP higher than 165 g/kg DM (Broderick, 2003; Ipharraguerre and Clark, 2005; Olmos Colmenero and Broderick, 2006). This dietary protein content is however lower than is commonly practised in the UK (Chen et al., 2020). In contrast, some authors have reported that milk yield decreased by around 4 kg/d when the dietary concentration of CP was reduced from 175 to 145 g/kg DM (Law et al., 2009; Giallongo et al., 2016). Therefore, reducing the concentration of dietary CP below 150 g/kg DM for high yielding cow is not advised, although it can improve the N efficiency and reduce purchased feed costs (Schwab and Ordway, 2004; Lee et al., 2015a).

2.1.3. Sources and composition of protein feedstuffs

There is a rising interest in using domestic protein sources for high yielding dairy cows all over the world (Peyraud et al., 2009). There are two main sources of protein feedstuffs; animal and plant origin, which are used by farmers for growing and lactating ruminants.

Different organs, viscera and muscle tissues are the primary sources of animal-based protein (Tadele and Getachew, 2015). Based on solubility, animal protein can be classified as either highly soluble for example, blood meal, serum, blood plasma, or non-soluble proteins such as wool, horn/hoofs, body hair or feathers (Meeker, 2009). These proteins, especially meat and bone meal, blood meal, feather meal and fish meal are highly digestible and concentrated sources of essential AA (Tadele and Getachew, 2015). Proteins from animal sources are rich in AA that more closely meets the requirement for milk and meat production (Jørgensen et al., 1984). However, the inclusion of animal origin protein (meat meal, feather meal, blood meal, bone meal, fish meal) in livestock feed is banned according to the EC Regulation No. 1069/2009 of the European Parliament and the Council (2009).

To meet the high demand for dietary protein, vegetable protein is commonly used in ruminants feed (Peyraud et al., 2009). Plant sources high in protein include soybean meal, sunflower meal, rapeseed or canola meal, linseed meal and forage legumes (Tadele and Getachew, 2015). Among the plant source feedstuffs, soybean meal protein is the most commonly used in ruminant production all over the world (Yin et al., 2011). The concentration of essential AA such as lysine, histidine, isoleucine, tryptophan or threonine is higher in soybean compared to maize, wheat, sorghum and other cereal grains (Tadele and Getachew, 2015; Table 2.1).

Based on the limitations of soybean meal, the demand for using home-grown high protein forage legumes has increased due to their higher concentration of CP than other forages such as grass or maize silages (Table 2.2). Also, the concentration of essential AA in legume silages is higher than in grass or maize silage (Table 2.2). These high protein forage legumes can also fix atmospheric N, reducing the requirements for artificial fertilisers (Zahran, 1999).

Table 2.1. Chemical characterisation of some plant origin protein supplements (Li et al., 2011; Heuzé et al., 2016; Stein et al., 2016).

	Feedstuffs				
	Soybean meal	Soybean meal dehulled	Cottonseed meal	Rapeseed meal	Sunflower meal dehulled
Proximate compositions (g/kg DM)					
Dry matter	879	881	922	908	915
Organic matter	929	929	930	930	931
Crude protein	518	535	450	373	387
Crude fat	20	18	23	37	21
ADF	83	59	150	184	219
NDF	137	110	237	271	307
Ash	71	71	70	70	69
Starch	94	106	46	32	21
Gross energy (MJ/kg DM)	20	20	21	18	18
Amino acids (g/100g CP)					
Lysine	2.80	2.87	1.66	2.07	1.42
Histidine	1.13	1.15	1.08	1.04	0.94
Arginine	3.18	3.12	4.54	2.21	3.27
Threonine	1.76	2.03	1.25	1.55	1.36
Glycine	2.30	2.72	2.13	1.80	2.12
Valine	2.09	2.25	1.69	1.82	1.83
Isoleucine	2.03	2.10	1.19	1.45	1.56
Leucine	3.44	3.70	2.26	2.51	2.45
Tryptophan	1.66	1.72	1.10	1.06	1.10
Phenylalanine	2.21	2.44	2.02	1.48	1.65
Cysteine	0.70	0.69	0.70	0.85	0.53
Methionine	0.60	0.64	0.66	0.71	0.79

ADF = acid detergent fibre; NDF = neutral detergent fibre.

2.2. Forages for ruminant

2.2.1. Characterisation of forage legumes

Forage legumes are the members of the Fabaceae (Leguminosae) family that are used as ruminant feeds in many countries (Phelan et al., 2015). The total number of legume species used as ruminant feed is not known, however, the Food and Agriculture Organisation (FAO) of the United Nations has listed 153 legume species as forages (www.feedipedia.org). Among the 153 species, several have received commercial importance as ruminant feeds due to their high dietary N content, symbiotic N fixation (Zahran, 1999), ability to increase forage yield, and lower impact on greenhouse gas emissions (Steinshamn, 2010; Lüscher et al., 2014; Phelan et al., 2015). The widely cultivated legume species in Europe are red clover (*Trifolium pratense L.*), lucerne/alfalfa (*Medicago sativa L.*), white clover (*Trifolium repens L.*), and subterranean clover (*Trifolium subterraneum*) (Frame et al., 1998). Production

of forage legumes rather than grasses is preferable due to their nutrient composition, amino acids profile (Table 2.3) and digestibility (Ellis and Lippke, 1976; Paulson et al., 2008). Factors influencing the high-quality forage legumes include species differences and their growth pattern (Evers, 2011). The yield of red clover and lucerne increases with maturity unlike stoloniferous species such as white clover and subterranean clover (Barnes and Gordon, 1972; Brink and Fairbrother, 1992). Therefore, the quality of red clover and lucerne are reduced with maturity because stems are less digestible by ruminants (Albrecht et al., 1987).

Grass and maize silages are also commonly used as a basal forage in dairy cow rations (Dewhurst, 2013). However, the concentration of CP and neutral detergent fibre (NDF) in grass silage is generally higher compared to maize silage, which can result in a decrease in feed intake and reduced animal performance (Paulson et al., 2008; Dewhurst, 2013). Relative to grass and maize silage, legume species may contain some anti-nutritional compounds such as tannins, saponins, cyanogenic glycosides, estrogenic flavonoid compounds and alkaloids that can reduce animal performance if present at higher concentrations (Evers, 2011).

2.2.2. Importance of forage legumes for dairy cows

Forages provide a source of carbohydrates and can form the basis of ruminant diets as either pasture or conserved feed such as hay or silage (Dynes et al., 2003). Plant carbohydrates consist of two fractions based on their solubility; cellular and cell wall fractions (Wilson, 1994). The cellular portion of a plant is often referred to as the water-soluble sugar, and is readily fermentable by the rumen microbes, while the cell wall fraction is a source of fibre, which is principally composed of cellulose, hemicellulose, pectin and lignin/phenolic acids (Jung and Allen, 1995). Ruminants have the capacity for utilisation of forage legumes through a symbiotic relationship with rumen microorganisms that degrade the plant cell wall polysaccharides and convert the NPN to AA in microbial protein (Wilson, 1994; Wilkins, 2000). The plant cell wall provides an energy source, which is required for MCP synthesis in the rumen (Nocek and Russell, 1988). The rapid digestion of the cell wall by rumen microbes in forages such as lucerne can reduce the retention time in the rumen and allow high forage intakes (Wilson, 1994).

The use of grass and legume mixtures fed as a total mixed ration (TMR) to high producing cows is a common practice in many countries, especially in Europe,

including the United Kingdom (Peyraud et al., 2009). There are some advantages of using grass and forage-legume mixtures as this provides a more balanced diet and increases nutrient use efficiency (Phelan et al., 2015). The nutritional quality and yield of grass species is generally lower than forage legumes unless high levels of artificial N are applied to the grass (Paulson et al., 2008). For example, grass silage contains more fibre and more protein than maize silage, whilst legumes contain more protein but less fibre than grass silages (Dewhurst, 2013). The AA profile along with the content of polyunsaturated fatty acid (PUFA) are also higher in legumes compared to grasses (Table 2.2 and Table 2.3). The lower concentration of cell wall (NDF) content, a higher content of dietary CP, and higher degradation rate of digestible NDF of legume silages were proposed as the main reasons for improved DM intake and milk yield compared to maize or grass silages (Paulson et al., 2008). Lignin usually presents in the plant cell wall, which is not digestible by rumen microbes due to their complex chemical constituents (Jung, 1989). In legumes, only the xylem is lignified, and other tissues of legume species are digestible (Jung and Allen, 1995; Wilson and Kennedy, 1996). In contrast, lignin in grass silage can protect the cell walls from microbial digestion and results in a lower rate of cell wall digestion compared to legumes (Steinshamn, 2010).

Table 2.2. Proximate composition, fermentation profile, and fatty acid content of different legumes, grass and maize silages (Wiking et al., 2010; Hymes-Fecht et al., 2013; Leduc et al., 2017; Tayyab et al., 2018a; b; Westreicher-Kristen et al., 2018)

	Silage				
	Grass	Maize	Red clover	White clover	Lucerne
Composition (g/kg DM)					
Dry matter g/kg	273	350	166	158	196
Organic matter	899	961	882	896	888
Crude protein	136	86.0	178	267	190
NDF	492	366	369	342	353
ADF	331	229	296	215	267
Ether extract	-	27.0	22.4	34.4	25.0
Lignin	-	-	52.3	65.3	74.0
Ash	101	39.0	90.6	104	112
Starch/sugar	21.0	291	-	-	-
ME (MJ/kg)	-	12.0	-	-	-
Fermentation (g/kg)					
pH	4.10	3.80	4.30	4.20	4.60
NH_3-N (g/kg total N)	69.5	62.0	27.0	91.0	44.0
Acetate	44.5	34.6	29.0	32.0	18.1
Propionate	0.20	1.10	-	-	-
Iso-butyrate	0.00	0.10	-	-	-
Butyrate	0.30	0.10	11.3	-	-
Lactate	127	48.0	117	133	58.3
Fatty acids (g/100 g FA)					
C16:0	3.90	4.80	17.0	18.9	20.9
C18:0	0.45	1.20	2.48	4.70	3.80
C18:1 C9	0.30	3.40	2.55	3.90	2.90
C18:2n-6	0.50	1.50	19.2	13.7	16.7
C18:3n-3	5.10	0.90	44.9	56.3	53.3
ΣFA	13.4	17.4	-	-	-

NDF = neutral detergent fibre; ADF = acid detergent fibre; ME = metabolisable energy; NH_3-N = ammonia-nitrogen.

Table 2.3. Amino acid composition of white clover, red clover, lucerne, perennial ryegrass, and maize silage (Cabrita et al., 2011; Edmunds et al., 2013; Stødkilde et al., 2019)

	Perennial ryegrass		Maize	White clover		Red clover	Lucene	
	g/16g N	g/100g TAA	g/100g TAA	g/16g N	g/100g TAA	g/16g N	g/16g N	g/100g TAA
Alanine	7.15	7.80	11.0	6.33	6.90	6.24	5.18	6.70
Arginine	5.01	6.00	6.40	5.00	5.20	5.31	4.67	5.60
Asparagine	8.18	10.4	2.30	12.4	16.6	12.5	13.5	12.7
Cysteine	0.97	1.40	2.00	0.80	1.20	0.87	1.20	1.50
Glutamic acid	9.83	12.3	15.8	10.1	11.7	10.4	9.22	11.6
Glycine	4.94	6.20	5.70	5.15	5.50	5.16	4.61	5.70
Histidine	1.88	2.20	1.90	2.08	2.30	2.16	2.19	2.50
Isoleucine	4.46	4.70	5.20	4.80	4.80	4.88	4.46	4.90
Leucine	7.63	9.40	12.2	8.03	8.70	8.21	7.22	8.70
Lysine	5.37	5.60	3.80	5.27	5.40	5.82	5.91	6.20
Methionine	1.75	2.20	0.40	1.60	1.70	1.63	1.54	1.90
Phenylalanine	4.93	5.70	4.80	5.19	5.50	5.34	4.82	5.90
Proline	4.82	6.00	9.10	4.53	4.60	4.62	4.27	6.20
Serine	4.25	5.00	4.90	4.55	5.10	4.73	4.76	5.10
Threonine	4.36	5.20	4.70	4.59	5.10	4.66	4.31	5.30
Valine	5.87	6.50	7.90	6.00	6.10	6.12	5.46	6.10
TAA1	81.40	96.6	98.1	86.4	96.4	88.7	83.3	96.6

[1]TAA = Total amino acids (all essential amino acids + all non-essential amino acids excluding tyrosine)

2.2.3. General constraints of forage digestion in dairy cows

Fibre plays a crucial role in chewing, rumination, peristalsis of the gastrointestinal tract and digestive function (McLeod and Smith, 1989). The productivity of ruminants depends on the availability of energy and protein concentration from ingested feeds (Nocek and Russell, 1988). In modern feeding system, substantial quantities of concentrates rich in cereals are commonly used in ruminant diets as a source of energy (Waldo, 1973). An inadequate supply of soluble starch or sugars can limit energy supply to the microbes and reduce fibre digestion in the rumen (Ferraretto et al., 2013). Forage digestion may also reduce due to a higher proportion of cell wall content and reduced digestion rate of fibre source diets (Wilson, 1994). The cell wall fraction is more slowly degraded by rumen microbes due to lignification (Jung and Vogel, 1986; Jung, 1989). Therefore, large fibre particles can remain in the rumen and increase retention time, which can reduce feed intake and animal performance (Allen, 2000). Alternatively, the fibre content of the diets and its physical characteristics, particle size (PS), and density of the fibre are responsible for rumen fill and animal performance (Nasrollahi et al., 2015; Tayyab et al., 2018b). Rumen pH is another a critical determinant of nutrient

availability for absorption (Dijkstra et al., 2012), and values below pH 6.0 can reduce rumen bacteria activities which can decrease fibre digestion (Tayyab et al., 2019).

2.3. Protein metabolism by ruminants

The fate of dietary protein in the rumen of dairy cows can be split into the following; degradation of protein that provides a source of N for rumen microbes and subsequent synthesis of MCP, and is carried out by different species of rumen microorganisms (Bach et al., 2005). Secondly, dietary protein that is not degraded in the rumen and is available for enzymic digestion by the cow and subsequent absorption in the small intestine (McDonald, 2011).

2.3.1. Degradation of dietary protein in the rumen

Ruminant stomach comprises of four compartments namely the rumen, reticulum, omasum and abomasum. The rumen and reticulum are linked together and called the reticulo-rumen or simply the rumen, occupying the largest part of the body cavity (Krehbiel, 2014). The rumen is the forestomach of the ruminants digestive system that contains a variety of microorganisms such as bacteria, protozoa, fungi and archaea (Castillo-González et al., 2014). These rumen microbes are responsible for extensive degradation of ingested feed proteins (Bach et al., 2005). Feed proteins are mainly composed of degradable N, non-degradable N, and NPN (Schwab and Broderick, 2017). The undegraded dietary N and some endogenous N pass directly into the small intestine for absorption (Tamminga, 1979). Feed proteins are rapidly degraded by microbial proteases secreted from many species of rumen bacteria, protozoa and fungi, producing oligopeptides, dipeptides and AA, which are then taken up by the bacterial cytoplasm to convert them into simple AA (McDonald, 2011). The AA are either integrated into bacterial protein or deaminated to NH_3, and the carbon skeleton used to produce volatile fatty acid (Figure 2.1; Bach et al., 2005). Ammonia is the primary source of N supplying 40 to 100% of N requirement for MCP synthesis (Seo et al., 2013).

In contrast to bacteria, the role of protozoa in protein degradation is not well understand, but some protozoa can consume small feed particles, and proteolysis of such proteins take place inside the protozoal cell (Belanche et al., 2012). Protozoa do not use NH_3-N, unlike rumen bacteria, however, a fraction of the insoluble-protein may return to the rumen as a soluble fraction of protein (Newbold et al., 2015). This soluble fraction, along with RDP, AA and MCP escape the rumen

and are digested and absorbed by the host animal in the small intestine (McDonald, 2011).

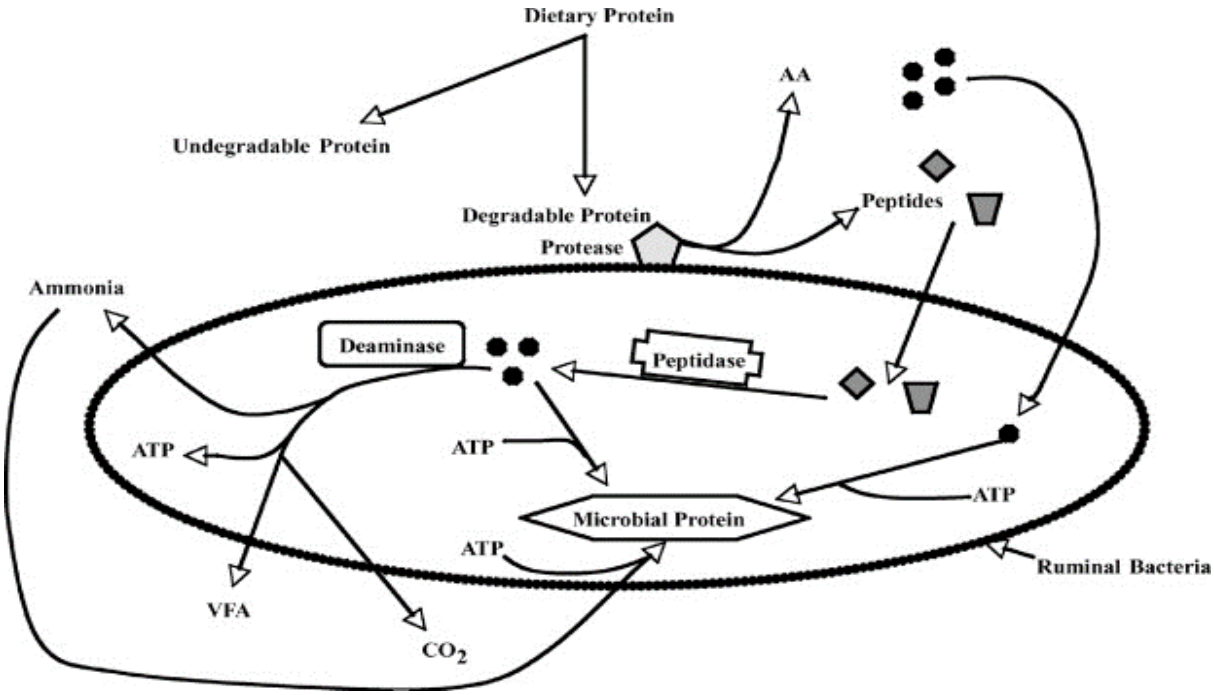


Figure 2.1. Protein metabolism in the rumen (adapted from Bach et al., 2005).

2.3.2. Microbial protein synthesis in ruminant

The MCP, along with rumen undegradable protein (RUP), provides the MP supply to the host animal (Das et al., 2014). Post-ruminal flow and absorption of microbial AA along with by-pass protein is essential for lactating cows to maintain milk and milk protein yield (Schwab and Broderick, 2017). The passage rate of MCP to the duodenum depends on the rumen-microbial ecosystem, outflow rate, N and energy availability, and the utilisation of nutrients by the rumen bacteria (Clark et al., 1992; Bach et al., 2005). The role of all these factors in MCP synthesis has been considered in several reviews (Bach et al., 2005; Rodriguez et al., 2007; Seo et al., 2013). The rumen bacteria can use simple carbohydrates as an energy source to synthesise their peptide-proteins (Seo et al., 2013). The conversion of peptides to MCP requires energy that primarily comes from the degradation of carbohydrates in the rumen by cellulolytic and amylolytic bacteria (Bach et al., 2005). For example, the inclusion of inulin at a rate of 2.40 g/d in a lucerne-based low (0 g/d) and high (1.56 g/d, sodium caseinate) RDP diet increased microbial N flow by 3.19 and 11.12%, respectively (Zhao et al., 2014). However, a high inclusion of rapidly

fermentable carbohydrate such as starch may reduce ruminal pH and lead to rumen acidosis and can disrupt fibre digestibility (Bargo et al., 2003). This was supported by Jaurena et al. (2005), who reported a 5.0% decrease in NDF digestibility when the proportion of rolled barely was increased from 0 to 450 g/kg DM in red clover silage-based rations. In contrast, the dietary supply of NPN and degradable N plays a vital role in MCP synthesis (Sinclair et al., 2012, 2014). For example, MCP synthesis was increased by 12% when the dietary CP concentration in dairy cow diets increased from 170 to 190 g/kg DM (Sannes et al., 2002). The synchronisation between fermentable carbohydrate and CP supply may also improve microbial growth (Sinclair et al., 1995; Seo et al., 2013). For example, the formation of MCP can be reduced if carbohydrates are degraded too rapidly in the rumen compared to CP (Nocek and Russell, 1988). However, balancing energy and N in the rumen may not be possible due to variations in the diet composition and the complexity of rumen ecosystems (Stern and Hoover, 1979). Additionally, intrarumen recycling of N (microbial protein synthesis and breakdown) can contribute to stabilising the efficiency of microbial production (Davies et al., 2013).

Amino acids are the primary source of building blocks for MCP synthesis (Diether and Willing, 2019). The AA in the diet along with other peptide proteins are either assimilated into microbial N or deaminated into NH_3-N (Figure 2.1; Bach et al., 2005) with 80% of microbial N usually derived from rumen NH_3-N, as reported by Atasoglu et al. (2001). Lee et al. (2012a) demonstrated an increase of 12% in microbial N synthesis in early lactation cows when fed diets deficient in MP and supplemented with rumen-protected AA (RPL+RPM). The MCP is referred to as the most important source of AA to the dairy cow as its AA composition is comparable to milk protein (Martineau et al., 2013). However, some of the AA such as histidine, methionine and lysine are limiting in certain dietary conditions, especially when high producing cows are fed maize and lucerne based diets (Giallongo et al., 2015, 2016; Lee et al., 2015a). Moreover, Lee et al. (2012) observed that the histidine concentration in the rumen bacterial protein was 27% lower than methionine.

Most dairy farmers in the EU and UK are interested in utilising home-grown forage legumes instead of using purchased protein feeds (Wilkins and Jones, 2000; Peyraud et al., 2009). However, the degradation rate of legumes protein is twice than that of grass silage (Table 2.4) which may limit the synthesis of MCP in the rumen and reduce the supply of RUP to the cow (Dewhurst et al., 2000). This

unbalance may be rectified by the inclusion of starch or sugar in forage-based legume diets that have the potentiality to improve MCP synthesis (Reynolds et al., 2001; Oba, 2011). For example, Jaurena et al. (2005) showed that increasing the amounts of rolled barley from 0 to 300 g per kg DM improved rumen bacterial protein yield by 50% in red clover silage based diets. This could also be due to an improvement in the synchronisation between ruminal N and energy supply (Sinclair et al., 1993, 1995; Seo et al., 2013)

2.3.3. *In situ* ruminal degradation of forage legumes

Legume forages have a high CP content compared to other forages (Brown et al., 2017; Damborg et al., 2018), and are frequently used in Northern America and Europe for silage production (Moorby et al., 2016). Legume silages are degraded rapidly in the rumen (Table 2.4). In general, the higher content of NPN (47.8% of total N) relative to neutral detergent insoluble CP is responsible for the rapid ruminal degradation of legume proteins (Westreicher-Kristen et al., 2017). Increasing the proportion of red clover silage from 15 to 60% in TMR diets increased effective ruminal degradability by 80 g/kg and consequently reduced the RUP and its intestinal digestibility by 220 and 240 g/kg, respectively (Westreicher-Kristen et al., 2018). However, an increased supply of RUP does not ensure the supply of all essential AA (Chowdhury et al., 2018; Stødkilde et al., 2019) because, legumes are often deficient in certain essential AA such as histidine, methionine or lysine (Schwab and Whitehouse, 2021).

In situ studies of red clover and lucerne have reported little difference between the species in the soluble fraction (a), rate of degradation (c), effective CP degradability and intestinal digestibility of RUP (Damborg et al., 2018). Damborg et al. (2018) also reported that the total tract digestibility of CP in red clover and lucerne was 888 and 900 g/kg, however, the potentially degradable CP fraction was 719 and 581 g/kg, respectively, which was 222 and 170 g/kg higher than the red clover and lucerne used in the study by Hoffman et al. (1993). The *in vivo* study by Halmemies-Beauchet-Filleau et al. (2014) noted that the substitution of grass silage by red clover silage reduced MCP synthesis by 13.0%, and as a consequence, decreased milk protein content by 1.9 g/kg (Schulz et al., 2018) indicating that the RUP and its absorption in the intestine is crucial to maintain the performance of dairy cows (Kalscheur et al., 2006).

Table 2.4. The degradation parameters, effective rumen degradability and estimated small intestinal digestibility of DM and CP of forages (Hoffman et al., 1993; Chaves et al., 2006; Damborg et al., 2018).

	RC	RC[1]	WC	LU	LU[1]	GS	PRG[2]	PRG[3]
DM degradability (g/kg)								
a	363	385	394	348	334	390	380	470
b	459	446	462	376	373	499	530	300
a + b	822	831	856	724	707	889	910	770
c (h^{-1})	0.12	0.13	0.16	0.12	0.16	0.07	0.09	0.04
ED	611	687	670	568	603	593	670	540
RUP	389	313	330	432	397	407	330	460
TTD	712		777	624		623		
CP degradability (g/kg)								
a	227	395	330	308	448	223	420	700
b	719	497	622	581	411	676	520	120
a + b	946	892	952	889	859	899	940	820
c (h^{-1})	0.17	0.30	0.19	0.18	0.23	0.11	0.10	0.09
ED	737	806	786	758	774	657	730	740
RUP	263	194	214	242	226	343	270	260
TTD	888		911	900		847		
SID	563		579	581		555		

RC = red clover, WC = white clover, LU = lucerne, GS = grass silage, PRG = perennial ryegrass
a = readily soluble fraction; b = insoluble but degradable fraction; a+b = total soluble and insoluble degradable fraction; c = the rate of fraction that are potentially degradable; ED = effective rumen degradability; RUP = rumen undegraded protein; TTD = total tract digestibility; SID = small intestinal digestibility
[1]Maturity stage 2-late bud (Hoffman et al., 1993)
[2]Perennial ryegrass leaf (Chaves et al., 2006)
[3]Perennial ryegrass stem (Chaves et al., 2006)

2.4. Effect of dietary protein on intake performance in dairy cows

2.4.1. Effect of crude protein concentration in the diet on dry matter intake

A meta-analysis reported by Huhtanen and Hetta (2012) and Owens et al. (2014) reported a positive relationship between the dietary concentration of CP (from 90 to 190 g/kg DM) and DM intake. However, data from Olmos Colmenero et al. (2006) and Law et al. (2009) demonstrated contrasting effect of dietary CP in DM intake (Figure 2.2). Therefore, different concentrations of CP can affect DM intake to varying extents (Table 2.5). For example, Giallongo et al. (2016) observed that different concentrations of CP (from 165 to 145 g/kg DM) fed in early lactation cow rations resulted in an inconsistent effect on DM intake. Broderick et al. (2015) also noted no significant change in intake in milking cows when the concentration of dietary CP was reduced from 170 to 150 g CP/kg DM. Reducing the concentration of CP usually has a beneficial effect on NUE, particularly if it does not negatively

affect DM intake or animal performance (Haque et al., 2012; Giallongo et al., 2016; Barros et al., 2017).

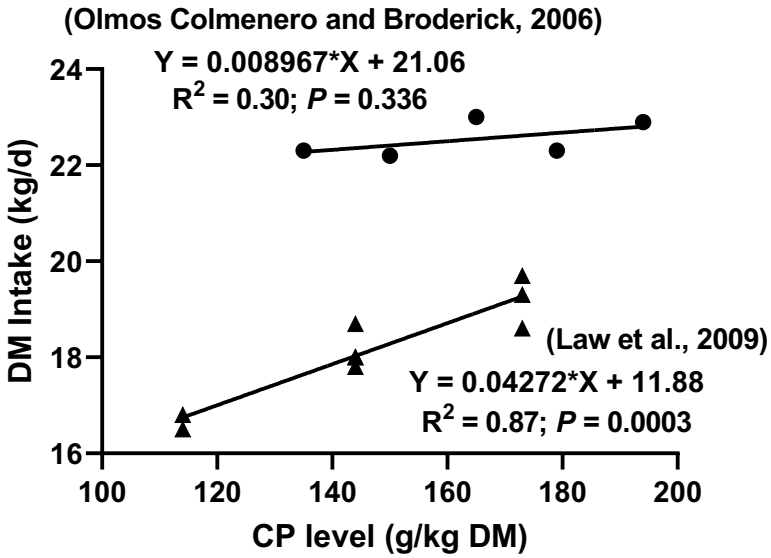


Figure 2.2. The relationship between dietary CP concentration and DM intake in lactating cows (Data from Olmos Colmenero and Broderick, 2006; Law et al., 2009).

Table 2.5. Effects of dietary crude protein concentration on dry matter intake in cows

Source	CP (g/kg DM)	Forage in TMR	Forage (%)	Days in Milk	DM intake kg/d
Oh et al. (2019)	165 155	Maize and haylage	58	123	28.4[a] 27.4[b]
Kidane et al. (2018)	175 160 145 130	Grass silage	50	126	19.1 19.6 19.9 18.9
Broderick et al. (2015)	170 150	Maize and lucerne	66	91	25.0 24.9
Alstrup et al. (2014)	157 139	Mixed silage	55	115	24.9[a] 23.8[b]
Law et al. (2009)	173 144 114	Grass and maize	45	0-150	18.6[a] 18.0[a] 16.5[b]
Olmos Colmenero et al. (2006)	194 179 165 150 135	Maize and lucerne	50	120	22.9 22.3 23.0 22.2 22.3

[a-b]Means within a column of the study with different superscripts differ ($P < 0.05$), DM = dry matter
No superscripts within column denote no significant difference between the treatments of the study.

In contrast, Oh et al. (2019) reported that reducing the dietary concentration of CP from 165 to 155 g/kg DM reduced intake by 1.0 kg DM per day but did not alter lactation cow performance. Several authors have shown that reducing the supply of dietary CP by 22 to 20 g/kg DM reduced DM intake in high yielding cows by 1.3 to 1.5 kg/d (Benefield et al., 2009; Alstrup et al., 2014; Lee et al., 2015a). The positive correlation between DM intake and dietary CP concentration could be attributed to available rumen N or RDP that can enhance MCP synthesis in the rumen (Zhao et al., 2014). Conversely, reducing the dietary CP concentration may depress rumen function by decreasing RDP and rumen NH_3 concentration (Nocek and Russell, 1988). All these factors might affect fibre degrading bacteria and increase the rumen retention time, resulting in a lower DM intake (Allen, 2000; Bach et al., 2005).

2.4.2. Amino acid supplementation and dry matter intake in dairy cows

A reduced DM intake in cows fed low CP diets results in a decrease in the post-ruminal supply of essential AA such as methionine, lysine or histidine as reported by Lee et al. (2012a) and Giallongo et al. (2016). Inclusion of RPL (100 g/cow/d) and RPM (30 g/cow/d) in lucerne and maize silage based low CP (135 g/kg DM) diets increased daily intake of cows by 0.70 kg DM, while the inclusion of RPH (50 g/cow/d) along with lysine and methionine increased DM intake up to a comparable level (24 kg/d) as the control diet (157 g/kg DM; Lee et al., 2012a).

A meta-analysis by Sinclair et al. (2014) reported that the inclusion of methionine and lysine only had a significant effect in low CP diets (≤ 150 g/kg DM) when DM intake, MP, and histidine were not limiting. However, other meta-analyses by Patton (2010) and Zanton et al. (2014) reported an inconsistent effect of RPM on the DM intake of milking cows, which may be due to the presence of other limiting AA (Patton, 2010), deficiency or excessive inclusion of protected form of methionine (Robinson et al., 2000) or the use of different synthetic sources of methionine (Zanton et al., 2014). Patton et al. (2015) demonstrated that the duodenal flow of histidine played a vital role in feed intake of dairy cows. However, post-ruminal infusion of histidine (6.5 g/d) in lactating cows did not alter DM intake but improved milk performance (Vanhatalo et al., 1999). Lee et al. (2015) concluded that histidine could be the first limiting AA for dairy cows fed grass silage and concentrate based diets. Giallongo et al. (2017) also noted that diets deficient in histidine decreased DM intake by 1.7 kg/d in dairy cows. Long and short term studies with both primiparous and multiparous cows fed histidine deficient diets below 2.5% of MP

requirements resulted in a decrease DM intake of 1.7 kg/d (Giallongo et al., 2017). Additionally, a positive relationship between DM intake and plasma histidine concentration in lactating cows has been noted by Patton et al. (2015) and Giallongo et al. (2016, 2017).

There is an assumption that rumen bacterial protein contains a constant amount of AA (Allen, 2000), and the variation in the AA composition of rumen microbes has only been explored in a few studies (Huhtanen et al., 2002; Lee et al., 2012a). In some studies, the concentration of histidine was 25 to 30% lower than methionine in MCP (Czerkawski, 1976). Therefore, the requirement of histidine is marginally higher (2.5% of MP) than methionine (Giallongo et al., 2016), but the considerable variation of histidine recommendations of between 2.4 to 3.2% of MP may be partially compensated by the contribution of carnosine, which is a dipeptide containing β-alanine and histidine for dairy cows (Lee et al., 2012a). Lee et al. (2012a) concluded that the microbial supply of histidine might not be adequate when the diet is deficient in MP.

2.5. Effects of dietary concentration of crude protein on milk performance of dairy cows

Dietary protein contributes to MP and the AA to the intestine of dairy cows (Lee et al., 2012b; Das et al., 2014; Daniel et al., 2016). Feeding low CP diets can decrease milk and milk protein yield by reducing intake, MCP synthesis and decreasing digestible RUP supply in the rumen (Huhtanen and Hristov, 2009; Hristov and Giallongo, 2014), whereas, excess dietary CP can impair health and reproduction efficiency in dairy cows (Sinclair et al., 2014). Data from several authors have demonstrated a positive (R^2 = 0.44) quadratic trend between the dietary concentration of CP and milk yield (Figure 2.3). However, the milk yield response in dairy cows depends on DM intake, and data from several experiments also showed a strong (R^2 = 0.82) relationship between feed intake and milk yield (Figure 2.4).

2.5.1. Milk yield response to dietary crude protein content

A meta-analysis by Hristov et al. (2005) demonstrated a positive relationship (R^2= 0.47) between intake of dietary CP and daily milk production in lactating cows. Olmos Colmenero and Broderick (2006) also demonstrated that milk production numerically increased by 2.0 kg/d in early lactation cows when the dietary CP

concentration was increased from 135 to 165 g/kg DM (Table 2.6). Other authors have increased the

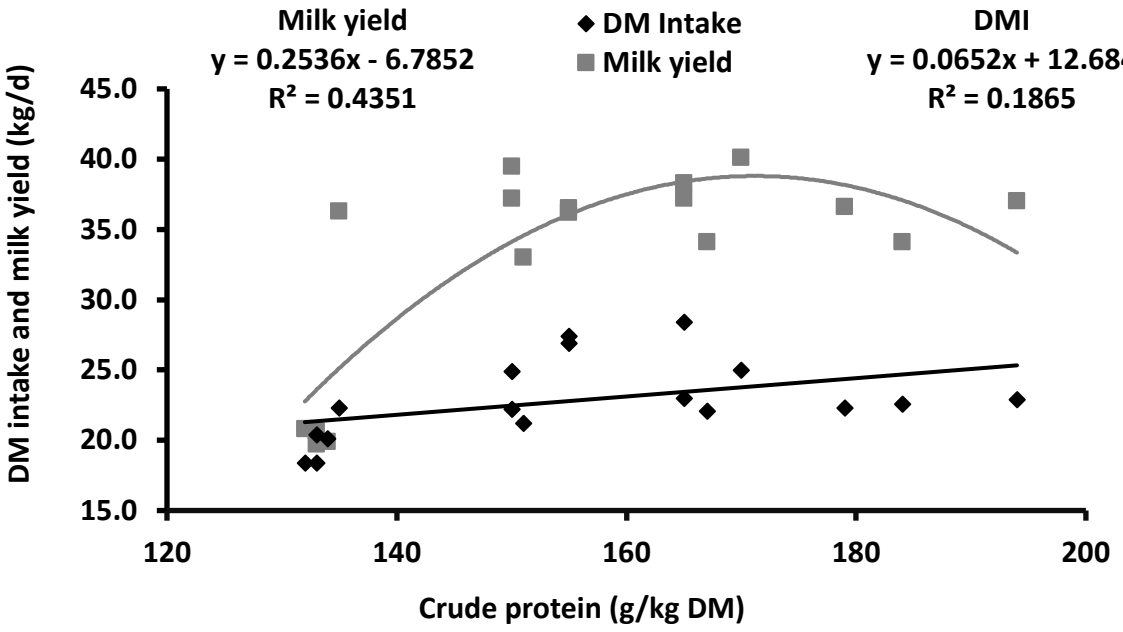


Figure 2.3. The relationship between CP concentration and milk yield (■) and DM intake (♦) in cows (Data from Broderick, 2003; Olmos Colmenero and Broderick, 2006; Whelan et al., 2011; Broderick et al., 2015; Oh et al., 2019).

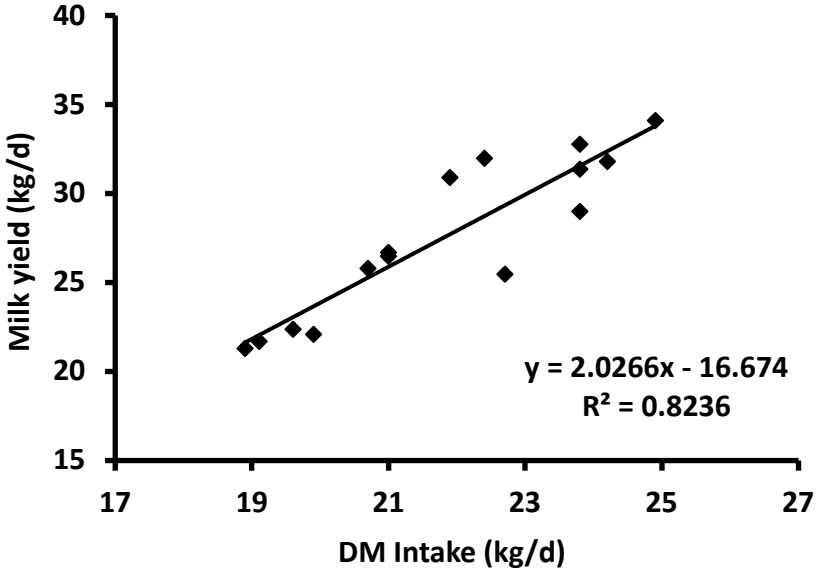


Figure 2.4. The relationship between DM intake and milk yield in dairy cows (Data from Alstrup et al., 2014; Hynes et al., 2016; Barros et al., 2017; and Kidane et al., 2018b).

concentration of CP in the diets from 165 to 189 g/kg DM but found no significant effect on daily milk yield (Bahrami-Yekdangi et al., 2014; Hynes et al., 2016).

Moreover, milk yield was numerically decreased by 1.30 kg/d when the dietary concentration of CP was increased from 165 to 194 g/kg DM in cows fed a maize and lucerne silage based diet (Olmos Colmenero and Broderick, 2006). In contrast, Ipharraguerre and Clark (2005) reported that feeding high CP diets (>170 g/kg DM) increased milk yield in dairy cows, but the rate of increase was lower compared to the optimum concentration of CP (170 to 160 g/kg DM) diets. Olmos Colmenero and Broderick (2006) recommended that the dietary concentration of CP in early lactating cows ration should not be more than 165 g/kg DM.

Hristov and Giallongo (2014) suggested that feeding a diet containing 160 or 150 g CP/kg DM does not adversely affect the yield or composition of milk in dairy cows. However, lowering the dietary CP concentration below 150 g/kg DM can negatively affect milk production (Lee et al., 2012a; Alstrup et al., 2014). It has been proposed that lowering the concentration of CP (<150 g/kg DM) can reduce the post-ruminal supply of MP and contribute to a decreased milk and milk protein yield (Hristov and Giallongo, 2014; Giallongo et al., 2016), indicating that the intestinal supply of MP and milk yield are strongly correlated (Daniel et al., 2016). For example, reducing CP (135 g/kg DM; 85% MP requirements) in dairy rations decreased milk yield by 3.6 kg per day (Lee et al., 2012a). Similarly, Lee et al. (2011) and Giallongo et al. (2016) also reported that decreasing the dietary CP concentration from 168 to 148 g/kg DM (95% MP) reduced milk yield by 3.1 and 4.3 kg/d, respectively. The adverse effect of low CP diets on milk performance could also be due to a negative energy balance, decreased feed intake, lower supply of RUP, MP, and limiting AA, especially histidine, lysine or methionine (Thomas, 2004; Hristov et al., 2005; Doepel and Lapierre, 2010). In contrast, some authors noted that feeding marginally deficient CP or MP diets did not affect milk production in dairy cows. For example, Barros et al. (2017) reported no significant change in milk yield when the dietary concentration of CP was reduced from 162 to 144 g/kg DM. Hynes et al. (2016) also found a similar response when dietary CP concentration was reduced from 181 to 141 g/kg DM. Recently, Kidane et al. (2018) reported that gradually reducing the dietary concentration of CP from 175 to 130 g/kg DM in Norwegian-red dairy cows ration did not alter feed intake or milk yield. Therefore, the effect of reducing CP concentration in dairy cow rations on milk performance is not consistent.

2.5.2. Effect of amino acids on milk performance

Hristov and Giallongo (2014) suggested that the adverse effect of low CP diets on milk performance can be alleviated by supplementation with RP-AA. For example, the inclusion of RPL (100 g/cow/d) and RPM (30 g/cow/d) together with or without RPH (50 g/cow/d) in MP deficient diets (CP 136 g/kg DM; 85% MP requirements) increased milk yield by 3.3 and 1.7 kg/d, respectively (Lee et al., 2012a). However, the response to RP essential AA such as RPM or RPL in low CP diets are variable and difficult to predict (Sinclair et al., 2014). The inclusion of RPL or RPLM in low CP (140 g/kg DM) or MP deficient (90% of MP requirements) diets did not alter mean milk yield in other studies (Lee et al., 2015a). Similarly, the addition of RPM (30 g/cow/d), RPL (130 g/cow/d), RPH (120 g/cow/d), and all three AA (130+30+120 g/cow/d, respectively) in low CP (145 to 148 g/kg DM) and MP deficient (95% of MP requirements) diets was not shown to have any effect on milk yield (Giallongo et al., 2016). However, some authors have reported that RP-AA especially RPH could increase the DM intake of cows (Lee et al., 2012a; Giallongo et al., 2015, 2016), and data from several studies has demonstrated a strong positive relationship between DM intake and milk yield (Figure 2.4). However, RPH does not show any effect on milk yield when it was supplemented to MP deficient diets (Lee et al., 2012b; Giallongo et al., 2015, 2016). Other authors also demonstrated that reducing the dietary CP or MP content did not change feed intake or lactation yield (Giallongo et al., 2015; Lee et al., 2015a).

2.5.3. Dietary crude protein intake and milk composition

Milk protein yield and composition depends on the concentration of dietary MP, and available essential AA in the mammary gland for milk protein synthesis (Hristov et al., 2005; Huhtanen and Hristov, 2009; Doepel and Lapierre, 2010). Olmos Colmenero and Broderick (2006) found a linear and quadratic effect on milk fat and protein yield, which was increased by 1.24 and 1.18 kg/d, respectively when they increased dietary CP concentration from 135 to 165 g/kg DM. Similarly, increasing the dietary concentration of CP from 130 to 175 g/kg DM in a grass silage-based ration increased milk fat and protein content by 2.6 and 0.4 g/kg, respectively (Kidane et al., 2018; Table 2.6). Broderick (2003) also noted an increase in milk protein content by 0.40 g/kg in cows fed a lucerne and maize silage-based diet containing 167 g CP/kg DM compared to 151 g CP/kg DM. The positive effect on milk protein and fat content might have been due to an increased feed intake and

higher MP content of the diets, as well as the use of a high quality protein source such as soybean meal (Law et al., 2009; Barros et al., 2017; Halmemies-Beauchet-Filleau et al., 2017). In contrast, Olmos Colmenero and Broderick (2006) observed that the dietary concentration of CP had no significant effect on milk composition except milk fat, which was increased by 1.3 g per kg of milk when dietary protein concentration was increased by 30 g/kg DM.

Table 2.6. Effects of dietary CP concentration on milk performance in dairy cows

Source	Diet CP (g/kg DM)	Forages in TMR	Milk Yield (kg/d)	Milk components (g/kg)		
				Fat	Protein	Lactose
Kidane et al. (2018)	175	Grass silage	21.7	42.0[a]	34.8[b]	44.9
	160		22.4	40.4[ab]	35.4[a]	45.4
	145		22.1	41.0[ab]	35.2[a]	45.5
	130		21.3	39.4[b]	34.4[c]	45.4
Broderick et al. (2015)	170	Maize and lucerne	40.1	40.2	30.5	48.4[b]
	150		39.5	39.9	30.5	49.1[a]
Hristov et al. (2015)	165	Maize and legume	32.2	36.6	30.8	48.0
	154		32.5	38.7	30.8	48.1
Alstrup et al. (2014)	157	Mixed silage	34.1[a]	41.0	36.3	48.5
	139		32.8[b]	41.3	36.2	48.6
Lee et al. (2012a)	157	Grass, maize and lucerne	38.8[a]	35.0	29.8	48.9
	135		35.2[b]	35.1	29.4	48.5
Olmos Colmenero and Broderick (2006)	194	Maize and lucerne	37.0	34.4[a]	31.6	49.2
	179		36.6	34.7[a]	31.8	49.1
	165		38.3	32.7[ab]	30.9	49.4
	150		37.2	32.7[ab]	31.5	48.9
	135		36.3	31.4[b]	30.9	49.1

[a-c]Means within a column of the study with different superscripts differ ($P < 0.05$).
No superscripts within a column represent no significant difference between the treatments within a study.

Increases in milk composition due to a higher concentration of dietary CP, however, is not always evident because of variable effects of dietary CP and other essential AA (Sinclair et al., 2014). For example, a recent study by Oh et al. (2019) reported that reducing the dietary CP concentration from 165 to 155 g/kg DM did not affect the milk composition. Similarly, Hristov et al. (2015) noted that milk yield and composition were not affected by diets containing 165 or 154 g CP/kg DM in Holstein cows fed maize and legume-based rations. Hynes et al. (2016) also demonstrated no effect on milk composition when dietary CP concentration was reduced from 181 to 141 g/kg DM. Milk yield and composition did not change by feeding a low (150

g/kg DM) compared to high CP (170 g/kg DM) diet except lactose content, which was increased by 0.7 g per kg of milk (Broderick et al., 2015). Additionally, feeding either high or low digestibility mixed silages with two concentrations of CP (160 or 140 g/kg DM) also had no effect on milk performance in dairy cows in the study reported by Alstrup et al. (2014). Likewise, reducing dietary concentration of CP from 180 to 156 g/kg DM did not change milk composition parameters in Holstein-Friesian multiparous cows (Bahrami-Yekdangi et al., 2014).

2.5.4. Amino acid supplementation and milk composition

The adverse effect of low CP diets on the productivity of dairy cows can be mitigated by the addition of RP essential AA (Doepel and Lapierre, 2010; Hristov and Giallongo, 2014). Inclusion of RPL (130 g/d/cow) or RPH (120 g/d/cow) alone or in combination with RPM (30 g/d/cow) in low CP (148 g/kg DM) or MP deficient (95% of MP requirements) diets increased the protein content of milk by 1.4 g/kg (Giallongo et al., 2016). These findings are consistent with other studies by Lee et al. (2012a) who noted that the inclusion of RPL (100 g/d/cow), RPM (30 g/d/cow) and RPH (50 g/d/cow) in a mixed silage-based low CP (136 g/kg DM) or MP deficient (85% of MP requirements) diets improved milk protein, lactose and 3.5% fat-corrected milk yield by 0.13, 0.17 and 1.5 kg/d, respectively. Methionine, lysine, and histidine are the key limiting AA for milk protein production in maize and lucerne based diets as reported in other studies (Giallongo et al., 2015, 2016; Lee et al., 2015a), and they independently regulate milk protein synthesis by activating the mammalian target of rapamycin (mTOR) signalling pathway in the mammary epithelial cells (Gao et al., 2015). Therefore, the correct balance of essential AA in MP is crucial for mammary protein synthesis as well as milk performance (Wu et al., 2014). The dietary requirements for high yielding dairy cows, however, can vary during lactation due to the partitioning of nutrients between body tissue and the mammary gland (Moran, 2005).

2.5.5. Low protein diets fed at different stages of lactation

Dairy cows, in general, produce more milk after 4 and 8 weeks of calving, with the yield gradually decreasing towards the end of lactation until the cow reaches the dry period (Figure 2.5; Silvestre et al., 2009). The body maintenance requirement for energy and protein does not alter significantly between different stages of lactation, however, dietary CP and energy requirement increase during the first few weeks of

lactation and gradually decline towards the end of lactation stage as requirements for production decrease (Moran, 2005).

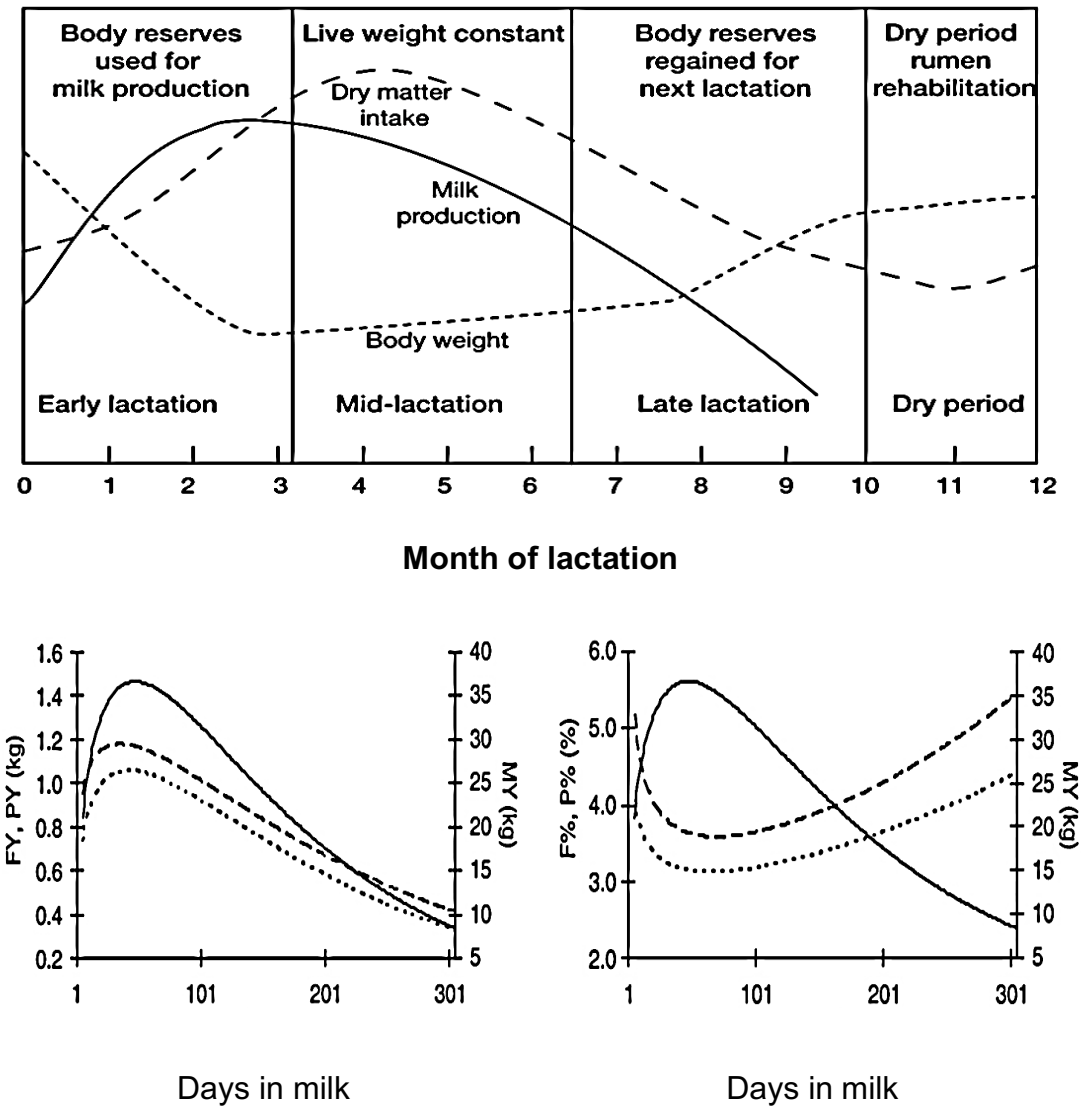


Figure 2.5. Performance curves for milk yield, milk protein, and milk fat in dairy cows (adapted from Moran, 2005; Silvestre et al., 2009).

Milk yield (MY,——); fat yield (FY,– –); protein yield (PY,·····); fat percentage (F%); protein percentage (P%).

Dairy cows usually regain their body reserves by the end of lactation (Figure 2.5; Moran, 2005) prior to mobilising body protein and fat during the pre and post-partum period (Tamminga et al., 1997; Van-Knegsel et al., 2007; van der Drift et al., 2012). However, body protein mobilisation is limited to a period ranging from between -2 and 5 weeks post-partum. Calculations have estimated the range of labile protein loss as being between 12 and 24 kg (Komaragiri et al., 1998; Chibisa et al., 2008). Therefore, there might be an opportunity to manipulate the dietary CP concentration

at different stages of lactation to maximise milk production and performance (Thomas, 2004).

Law et al. (2009) observed that reducing the concentration of dietary CP from 173 to 114 g/kg DM decreased milk, protein and fat yield by 10, 0.28, and 0.35 kg/d, respectively, during the early stages of lactation (calving to 150 day). Law et al. (2009) also conducted another experiment with late lactating cows from 151 to 305 days of lactation and found no significant difference in feed intake, milk production, milk true protein or fat yield when the CP concentration in the diet was reduced from 173 to 144 g/kg DM. These findings indicate that 114 g CP/kg DM was not sufficient for lactating cows, especially during the early stages of lactation (Thomas, 2004). Similar to Law et al. (2009), a study with late lactation cows (224 ± 54 days in milk; 85 ± 25 days pregnant) reported that reducing dietary CP concentration from 162 to 144 g/kg DM did not change milk yield (Barros et al., 2017). However, Barros et al. (2017) observed that a diet extremely low in CP (118 g/kg DM) significantly reduced milk yield 3 weeks earlier in late lactation cows compared to an another low CP diet (131 g/kg DM). Therefore, milk yield either in early or late lactation responds to CP deficient diets when the concentration of CP is lower than 144 g/kg DM but that feeding diets containing 144 g CP/kg DM is sufficient in late lactation (Law et al., 2009; Barros et al., 2017).

Feeding low CP diets (150 g/kg DM) to early lactating dairy cows decreased milk yield by 2.9 kg/d, however, the yield did not change when maize grain and RPM was added compared to the control CP (192 g/kg DM) diet (Whelan et al., 2012). However, the yield of milk fat was increased by 0.15 kg/d when the low CP diet was supplemented with RPM in early lactation cows (Whelan et al., 2012). The improved milk yield in cows fed the low CP supplemented diet might be associated with the post-ruminal supply of MP in dairy cows (Toti et al., 2018) or a more appropriate balance between lysine and methionine (Lee et al., 2015a) whereas, the higher fat yield could have been due to the formation of apolipoprotein and phosphatidyl-choline in the liver from methionine as a methylation donor (Sinclair et al., 2014). These metabolites are required for the synthesis of very-low-density lipoprotein (VLDL) and carnitine in the liver that plays a vital role in fat metabolism (McDonald et al., 2010). The VLDL transports triacylglycerol from the liver to mammary gland and carnitine transfers long-chain fatty acids (FA) across the mitochondrial membrane for subsequent β-oxidation of FA to produce acetyl CoA, which enters

into the tricarboxylic acid cycle (TCA) to produce energy (Varvikko et al., 1999; Sinclair et al., 2014).

2.6. Effect of low protein diets on nutrient metabolism in dairy cows

The performance of dairy cows primarily depends on feed intake and nutrient metabolism (McDonald et al., 2010). The dietary concentration of CP has an impact on nutrient digestion and metabolism in dairy cows (Hristov and Giallongo, 2014).

2.6.1. Effect of dietary protein concentration on nutrient digestibility in dairy cows

The concentration of CP in the diet below 165 g/kg DM can contribute to a lower nutrient digestibility, whereas feeding higher concentrations (165 to 175 g/kg DM; Thomas, 2004a) can improve apparent fibre digestibility (Olmos Colmenero and Broderick, 2006; Lee et al., 2011). This improvement could be due to more available RDP and AA that are deaminated in the rumen and increase the concentration of NH_3-N, which is utilised particularly by rumen cellulolytic bacteria and improve fibre digestibility (Kernick, 1991; Griswold et al., 1996; Carro and Miller, 1999). For example, the apparent whole-tract digestibility of DM, organic matter (OM), NDF, ADF and CP was found to be lowest at a dietary concentration of 135 g CP/kg DM and highest at 165 g CP/kg DM (Olmos Colmenero and Broderick, 2006; Table 2.7). Lee et al. (2012a) also demonstrated that decreasing the concentration of dietary CP from 157 to 135 g/kg DM reduced apparent total tract OM, CP, NDF and ADF digestibility by 21.0, 53.0, 41.0 and 37.0 g/kg, respectively in lactating cows. Similarly, the apparent whole tract NDF and ADF digestibility decreased by 48.0 and 72.0 g/kg, respectively, when the concentration of CP in the diet was reduced from 167 to 148 g/kg DM (Lee et al., 2011). Other studies (Lee et al., 2012b; Giallongo et al., 2015) have also reported a lower nutrient digestibility when a very low CP (135 to 148 g/kg DM) or MP deficient (95 to 85% of MP requirements) diets were fed to dairy cows.

In contrast, Lee et al. (2015) reported that reducing the dietary CP concentration from 155 to 137 g/kg DM did not alter apparent nutrient digestibility in dairy cows. Likewise, Niu et al. (2016) noted that the apparent whole-tract nutrient digestibility did not differ (except CP digestibility which was reduced by 63.0 g/kg) when the dietary concentration of CP was reduced from 185 to 152 g/kg DM. A recent study by Oh et al. (2019) demonstrated that reducing the concentration of dietary CP from

165 to 155 g/kg DM reduced apparent CP digestibility by 27.0 g/kg, however, DM and OM digestibility were increased by 12.0 and 14.0 g/kg, respectively in cows fed low CP (155 g/kg DM) diets, which could be due to a lower intake of DM (0.90 kg/d) and OM (0.70 kg/d) compared to the control diet. Huhtanen et al. (2009) conducted a meta-analysis and noted that the apparent whole tract OM digestibility in lactating cows was negatively correlated to DM intake.

Table 2.7. Effect of dietary protein concentration on apparent total tract nutrient digestibility in dairy cows

Source	CP (g/kg DM)	Forages in TMR	Apparent digestibility (g/kg)				
			DM	OM	CP	NDF	ADF
Oh et al. (2019)	165	Maize and haylage	689^{b}	697^{b}	665^{a}	447	378
	155		701^{a}	711^{a}	638^{b}	448	357
Niu et al. (2016)	185	Lucerne	718	726	726^{a}	463	457
	152		708	715	663^{b}	461	441
Giallongo et al. (2015)	167	Maize and lucerne	734^{a}	730^{a}	729^{a}	496	431
	148		720^{b}	717^{b}	684^{b}	470	428
Lee et al. (2015)	155	Grass, Maize and lucerne	615	628	594	307	225
	137		609	620	560	290	218
Lee et al. (2012a)	157	Grass, Maize and lucerne	672^{a}	683^{a}	625^{a}	417^{a}	386^{a}
	135		651^{b}	662^{b}	572^{b}	376^{b}	349^{b}
Lee et al. (2011)	167	Maize and legume	697^{a}	708	668^{a}	540^{a}	487^{a}
	148		684^{b}	694	636^{b}	492^{b}	415^{b}
Olmos Colmenero and Broderick (2006)	194	Maize and lucerne	724^{b}	734^{b}	704^{a}	436^{b}	422^{b}
	179		726^{b}	735^{b}	695^{a}	428^{b}	408^{b}
	165		740^{a}	749^{a}	680^{ab}	463^{a}	445^{a}
	150		746^{a}	753^{a}	671^{b}	468^{a}	454^{a}
	135		712^{c}	721^{c}	597^{c}	398^{c}	381^{c}

ADF = acid detergent fibre, CP = crude protein, DM = dry matter, NDF = neutral detergent fibre, OM = organic matter.

$^{a\text{-}c}$Means within a column of the study with different superscripts differ ($P < 0.05$).

No superscripts within a column represent no significant difference between the treatments within a study.

The negative effect of low CP diets on nutrient digestibility especially fibre could be due to a deficiency of rumen degradable N, which is required by cellulolytic bacteria to degrade ingested carbohydrates as a source of energy (Atasoglu et al., 2001). Another factor that can affect the apparent nutrient or fibre digestibility is DM intake, and reducing DM intake by 1.5 to 2.0 kg/d can decrease MCP synthesis and rumen

fermentation (Lee et al., 2012b). Several authors reported that RP essential AA have the potential to increase DM intake in lactating cows when fed low CP (140 to 148 g/kg DM) diets (Giallongo et al., 2015, 2016; Lee et al., 2015a). This can improve nutrient digestibility, however, the role of AA including methionine, lysine or histidine on feed intake and digestibility is not clear and difficult to predict (Lee et al., 2012a; Sinclair et al., 2014).

2.6.2. Effect of dietary crude protein concentration on plasma metabolites in dairy cows

Plasma metabolites in dairy cows play an essential role in milk synthesis (Wang et al., 2018). The concentration of plasma metabolites depends on nutrient intake and their absorption in the intestine, mobilisation of body reserves and their subsequent metabolism (Quigley and Bernard, 1992; McDonald, 2011), whilst others such as glucose are under homeostatic control (Rodgers et al., 2005).

Reducing the dietary CP concentration from 157 to 139 g/kg DM did not alter plasma β-hydroxybutyrate (BHB), glucose, or non-esterified FA (NEFA) concentration, but it reduced plasma urea by 1.17 mmol/l and tended to increase plasma cholesterol concentration in Holstein-Friesian dairy cows (Alstrup et al., 2014; Table 2.8). Bahrami-Yekdangi et al. (2014) also noted no significant difference in plasma glucose, BHB, NEFA or total protein, but plasma urea concentration was decreased linearly when the concentration of dietary CP was reduced from 180 to 156 g/kg DM in dairy cows. Long term studies by several authors (Giallongo et al., 2015, 2016) have also reported no difference in plasma metabolites except urea, which was decreased by 0.56, to 1.70 mmol/l, in dairy cows fed low CP (145 g CP/kg DM) in relation to the control (165 g CP/kg DM) diets.

Plasma urea concentration in dairy cows is closely related to dietary CP concentration (Recktenwald et al., 2014). In general, plasma urea concentration is increased either by oxidation of AA in the liver, or absorption of NH_3 from rumen fermentation followed by the conversion to urea in the liver and transported to the arterial vein via the hepatic circulation (Cherdthong and Wanapat, 2010). In contrast, Law et al. (2009) noted that the plasma concentration of BHB was increased by 0.08 mmol/l in cows fed 114 g CP/kg DM compared to the control CP (173 g/kg DM) diet. Likewise, Halmemies-Beauchet-Filleau et al. (2017) noted that reducing dietary CP from 171 to 156 g/kg DM in red clover and grass silage-based

fat supplemented rations increased the plasma concentration of NEFA by 0.08 mmol/l in early lactation Holstein cows.

Table 2.8. Effect of diet crude protein concentration on plasma metabolites in dairy cows

Source	Diet CP (g/kg DM)	Forages in TMR	Plasma metabolites (mmol/l)			
			Glucose	BHB[1]	Urea	NEFA[2]
Giallongo et al. (2016)	165	Maize and lucerne	3.16		6.01[b]	
	145		3.09		4.31[a]	
Halmemies-Beauchet-Filleau et al. (2017)	171	Red clover and grass silage	3.96	1.13		0.13[c]
	164		3.99	1.00		0.16[b]
	161		3.68	1.42		0.18[b]
	156		3.82	0.96		0.21[a]
Giallongo et al. (2015)	167	Maize and lucerne	3.62		4.31	
	148		3.89		3.75	
Alstrup et al. (2014)	157	Mixed silage	3.79	0.50	3.71[a]	0.35
	139		3.76	0.48	2.54[b]	0.30
Bahrami-Yekdangi et al. (2014)	180	Maize and lucerne	3.32	0.10	3.16	0.30
	172		3.36	0.20	3.02	0.30
	164		3.34	0.20	2.77	0.30
	156		3.23	0.20	2.68	0.30
Law et al. (2009)	173	Grass and maize	3.34	0.48[b]	4.32[a]	0.40
	144		3.32	0.50[ab]	2.59[b]	0.37
	114		3.33	0.56[a]	1.56[c]	0.39
Bach et al. (2000)	178	Maize and lucerne	3.67		5.87[a]	0.27
	147		3.69		3.51[b]	0.25

[1]BHB = β-hydroxybutyric acid.

[2]NEFA = non-esterified fatty acids.

[a-d]Means within a column of the study with different superscripts differ ($P < 0.05$).

No superscripts within a column represent no significant difference between the treatments within a study.

2.6.3. Effect of rumen-protected amino acids on plasma metabolites in dairy cows

Supplementation of RP essential AA to cows fed low CP or MP deficient diets can affect feed intake and milk protein synthesis (Lee et al., 2012b; Giallongo et al., 2016). However, similar to nutrient digestibility, plasma metabolites are often not affected by the inclusion of RPM, RPL or RPH in CP or MP deficient diets, except for a few discrepancies in plasma glucose and insulin (Giallongo et al., 2015, 2016). For example, Giallongo et al. (2015, 2016) demonstrated that supplementation of RPM (30 g/cow/d) did not affect the concentration of plasma insulin and glucose in

dairy cows, however, other studies have been reported an effect of RPM on increasing the concentration of plasma glucose and insulin (Krober et al., 2000; Berthiaume et al., 2001). Similarly, several studies have reported that RPL and RPH have inconsistent effects on plasma glucose concentration in milking cows (Socha et al., 2005; Weekes et al., 2006; Giallongo et al., 2016). The increased concentration of plasma glucose or insulin could be due to protein or AA which have gluconeogenic and insulinogenic effects, or the increase in dietary starch concentration that often occurs when dietary protein levels are reduced (Ranawana and Kaur, 2013; Cantalapiedra-Hijar et al., 2014). The inconsistent effects of RP-AA on plasma insulin and glucose concentrations in dairy cows may also be due to the interaction between AA (Ranawana and Kaur, 2013), dietary nutrient composition (Rius et al., 2010a), sources and dose-responses to supplemented AA (Liu et al., 2008). Therefore, the effects of RP essential AA on plasma metabolites of cows fed CP or MP deficient diets are variable (Sinclair et al., 2014).

2.7. Effect of dietary crude protein on nitrogen utilisation in dairy cows

The excretion of N to the environment from lactating dairy cows mainly depends on dietary N intake and nutrient digestion and metabolism. Excess N loss by dairy cows can contribute to environmental risk via emissions of nitrates, nitrous oxide and NH_3 from urine and faeces (Castillo et al., 2000; Hristov et al., 2011b).

2.7.1. Effect of dietary protein content on nitrogen output in dairy cows

Reducing dietary CP concentration from 165 to 135 g/kg DM in maize and lucerne-based diets reduced urinary and faecal N excretion by 77.0 and 14.0 g/d, respectively (Olmos Colmenero and Broderick, 2006; Table 2.9). Similarly, urinary and faecal N excretion was found to be lower by Oh et al. (2019), who reported 32.0 and 24.0 g/d lower excretion, respectively when lactating cows were fed a low CP (155 g/kg DM) compared to a control CP (165 g/kg DM) diet. A substantial decrease in urinary N excretion (55.0 g/d) was also reported by Giallongo et al. (2015) when dietary CP was reduced from 167 to 148 g/kg DM in dairy cow rations. These findings indicate that low CP diets extensively decrease urinary N emission rather than faecal N (Lee et al., 2012a; Niu et al., 2016; Oh et al., 2019). Nitrogen excretion mainly depends on the total N intake by diets, and there is a linear relationship between dietary N intake and output through urine or faeces (Johnston et al., 2020; Spanghero and Kowalski, 2021; Figure 2.6).

Table 2.9. Effect of dietary protein content on nitrogen excretion and efficiency in milking cows

Source	Diet CP (g/kg DM)	N output (g/d)			MUN[1] (mg/dl)	N partioning[2] (%)		
		Milk	Faecal	Urine		Milk	Faecal	Urine
Oh et al. (2019)	165	192	283[a]	150[a]	12.0[a]	27.0[b]	31.9	21.6
	155	188	259[b]	118[b]	9.47[b]	31.0[a]	41.9	18.8
Kidane et al. (2018)	175	116	132	212[a]	13.5[a]	23.0[c]	25.9[b]	41.6[a]
	160	123	131	182[b]	11.7[a]	26.9[b]	28.3[b]	39.6[ab]
	145	117	134	136[c]	9.36[b]	28.2[b]	32.2[a]	32.5[bc]
	130	114	124	95.0[d]	7.46[c]	31.7[a]	34.7[a]	26.0[c]
Hynes et al. (2016)	181	156	188	231[a]	22.5	27.0	36.3	40.2
	161	154	187	208[b]	20.9	27.1	33.2	32.7
	141	149	187	193[c]	18.9	27.0	34.5	35.6
Niu et al. (2016)	185	151	167	237[a]	17.0[a]	25.2[b]	27.8[b]	39.5[a]
	152	150	166	149[b]	11.7[b]	30.8[a]	34.2[a]	29.6[b]
Lee et al. (2012a)	157	183	233	143[a]	13.0[a]	29.4[b]	37.6[b]	23.1[a]
	135	167	213	92.0[b]	10.3[b]	34.2[a]	42.9[a]	19.0[b]
Olmos Colmenero and Broderick (2006)	194	180	210[a]	257[a]	15.6[a]	25.4[e]	29.6[d]	36.2[a]
	179	177	197[a]	213[b]	13.0[b]	27.5[d]	30.5[cd]	32.2[b]
	165	185	196[a]	180[c]	11.2[c]	30.8[c]	32.0[bc]	29.8[c]
	150	180	176[b]	140[d]	8.50[d]	34.0[b]	32.9[b]	26.6[d]
	135	173	196[a]	113[e]	7.70[d]	36.5[a]	40.3[a]	23.8[e]

[1]MUN = milk urea N; [2]Milk N use efficiency (%) = (Milk true protein/6.38)/N intake) ×100; [2]Faecal N (%) = (Faecal N/ N intake) ×100; [2]Urinary N (%) = (Urinary total N/ N intake) ×100

[a-e]Means within a column of the study with different superscripts differ ($P < 0.05$).

No superscripts within a column represent no significant difference between the treatments within a study.

Hynes et al. (2016) demonstrated that the excretion of total N through urine was higher than N output in faeces or milk. This could be due to the intake of N from the diets (Huhtanen and Hristov, 2009). For example, a dietary N intake over 400 g/cow/d can increase N output in urine compared to the faeces and milk (Castillo et al., 2000). However, the excretion of faecal and milk N were not affected by feeding a low CP (120 to 141 g/kg DM) diet to lactation cows as reported by several authors (Cantalapiedra-Hijar et al., 2014; Hynes et al., 2016; Kidane et al., 2018b). Similarly, lowering dietary CP concentration from 175 to 130 g/kg DM had no effect on faecal and milk N excretion in dairy cows, however, N as urea-N was significantly reduced (Kidane et al., 2018).

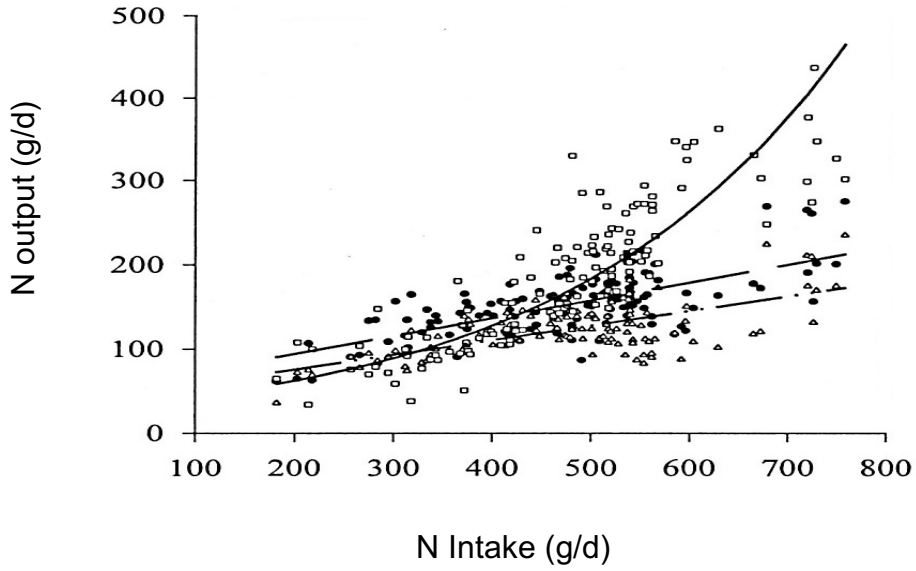


Figure 2.6. Relationship between dietary N intake and output through urine (□), faeces (●) and milk (∆). (Adapted from Castillo et al., 2000). The R^2 values for urine, faeces and milk were 0.76, 0.48, and 0.42, respectively.

Milk urea N (MUN) is highly correlated (R^2 = 0.83) with plasma urea N (Olmos Colmenero and Broderick, 2006). Increasing the dietary CP concentration can increase plasma urea concentration through urea recycling and absorption (Law et al., 2009; Alstrup et al., 2014), which is reflected in a higher concentration of MUN (Oh et al., 2019). For example, Bahrami-Yekdangi et al. (2014) reported that increasing the concentration of CP from 156 to 180 g/kg DM in maize and lucerne-based diets increased MUN by 2.20 mg/dl in dairy cows. Similarly, the concentration of MUN was increased by 4.14 mg/dl when the content of dietary CP was increased from 145 to 175 g/kg DM in grass silage-based rations (Kidane et al., 2018). Other studies have also reported similar findings when the dietary CP concentration was increased in the diet of lactating dairy cows (Lee et al., 2012a; Niu et al., 2016; Oh et al., 2019).

2.7.2. Effect of low protein diets on nitrogen use efficiency in dairy cows

Feeding low CP diets may result in a decrease in N output through the manure of dairy cows (Huhtanen and Hristov, 2009). One of the main objectives of reducing dietary CP concentrations in dairy cow rations is to increase NUE without affecting milk performance (Broderick, 2003; Hristov and Giallongo, 2014; Sinclair et al., 2014). The apparent NUE can be calculated by dividing N output in milk by N intake from the diet (Sinclair et al., 2015; Oh et al., 2019). Several studies have shown that reducing dietary N intake can increase NUE in milking cows (Olmos Colmenero and Broderick, 2006; Barros et al., 2017; Oh et al., 2019). For example, Olmos

Colmenero and Broderick (2006) noted that reducing dietary CP concentration from 165 to 135 g/kg DM increased apparent N capture in milk and faeces by 5.70% and 8.30%, respectively, and decreased N excreted in urine by 6.0% (Table 2.9). Similarly, the apparent NUE of milk was increased by 5.6% and and N partitioning in faeces and urine increased by 6.4 and 9.9%, respectively, when dietary CP concentration was reduced from 185 to 152 g/kg DM in lactating cow diets (Niu et al., 2016). Other studies have also reported similar findings when dietary CP was reduced (Lee et al., 2012a; Kidane et al., 2018b; Oh et al., 2019). Increasing the capturing of total N for milk protein synthesis can improve NUE (Huhtanen and Hristov, 2009), whereas, the higher concentration of N in urine leads to an inefficient partitioning of N. However, Hynes et al. (2016) and Oh et al. (2019) reported that faecal and urinary N partitioning did not differ significantly when lactating cows were fed low CP (141 to 155 g/kg DM) diets compared to adequate protein (165 to 181 g/kg DM) diets.

2.8. Effect of dietary concentration of crude protein on live weight, body condition score, and tissue mobilisation in dairy cows

The correlation between dietary CP concentration and live weight (LW) gain in lactating cows was reported by Hristov et al. (2005) and Sinclair et al. (2014), indicating that dietary CP can contribute to muscle protein synthesis and LW gain (Giallongo et al., 2015). The LW gain in dairy cows increased by 0.15 kg/d when dietary CP concentration was increased from 135 to 194 g/kg DM (Olmos Colmenero and Broderick, 2006). Similarly, Giallongo et al. (2015) showed that lowering the concentration of dietary CP from 167 to 148 g/kg DM decreased LW gain by 0.27 kg/d (Table 2.10). Additionally, the LW was decreased by 13.0 kg in dairy cows fed 114 g CP/kg DM compared to a control CP (173 g/kg DM) diet based on maize and grass silages (Law et al., 2009). However, most of the studies reported no significant difference in the LW of dairy cows when dietary CP concentration was altered (Giallongo et al., 2016; Hynes et al., 2016; Barros et al., 2017).

Increasing the concentration of dietary CP can improve MP and essential AA supply to the intestine of dairy cows and stimulate muscle protein synthesis when milk protein is not limiting (Proud, 2002; Wu et al., 2014). High CP diets can also improve the LW of dairy cows by increasing either plasma creatinine concentration (Ingwall et al., 1974; Whittet et al., 2004) or intramuscular-glutamine amino acid content (Wu

et al., 2011). These metabolites are required for the biosynthesis of muscle proteins (Giallongo et al., 2015).

Table 2.10. Effect of dietary concentration of crude protein on live weight and body condition score in dairy cows

Source	Diet CP (g/kg DM)	Forages in TMR	Live weight (kg)		Body condition score	
			Weight	Change[1]	Score	Change[2]
Barros et al. (2017)	162	Maize and lucerne	745	0.29	3.24	0.11
	144		731	0.45	3.28	0.07
	131		741	0.34	3.19	0.25
	118		729	0.15	3.13	0.11
Giallongo et al. (2016)	165	Maize and lucerne	585	0.20		
	145		595	0.14		
Hynes et al. (2016)	181	Ryegrass	571		2.34	
	161		582		2.30	
	141		579		2.37	
Giallongo et al. (2015)	167	Maize and lucerne	652	0.29[a]		
	148		644	0.02[b]		
Lee et al. (2012a)	157	Grass, maize and lucerne	599	0.04		
	135		591	-0.07		
Law et al. (2009)	173	Grass and maize	544[a]	0.24	2.37	0.02
	144		548[a]	0.27	2.41	0.13
	114		531[b]	0.16	2.38	−0.09

[1]Live weight change reported as kg/d,

[2]body condition score change reported within a study period of each experiment.

[a-b]Means within a column of the study with different superscripts differ ($P < 0.05$).

No superscripts within a column represent no significant difference between the treatments within a study.

Dietary energy content rather than CP concentration can have a greater effect on LW gain in early lactating dairy cows (Broderick, 2003; Pires et al., 2013). For example, feeding a high starch diet and decreasing the concentration of NDF from 360 to 280 g/kg DM significantly improved LW gain by 0.32 kg/d, while increasing CP concentration in the diet from 151 to 184 g/kg DM did not alter LW change in dairy cows (Broderick, 2003). However, LW gain in dairy cows is positively related to body condition score (BCS) as reported in other studies (Roche et al., 2007). For example, one unit change in BCS corresponds to a LW change range of 39 to 66 kg (Berry et al., 2011). Lee et al. (2012b) demonstrated that the LW and BCS of dairy cows were not affected by dietary CP concentration (140 g/kg DM) or MP

adequacy. Law et al. (2009) and Barros et al. (2017) also showed no effect on LW change and BCS in dairy cows when dietary CP concentration was reduced from 173 to 114 g/kg DM.

The response in LW gain to dietary CP or MP may vary during the pre and post-partum period of dairy cows due to the mobilisation of fat and labile protein (van der Drift et al., 2012). In general, body protein mobilisation starts between 3 to 4 weeks pre-partum and is sustained for a few weeks (3-4 weeks), while fat mobilisation continues until at least 8 weeks post-calving (Tamminga et al., 1997; Van-Knegsel et al., 2007). Maximum body tissue mobilisation occurs between -2 and 5 weeks post-partum when a dairy cow lose about 12 kg of body protein and 46 kg of fat (Komaragiri et al., 1998). The difference in tissue mobilisation between studies might be due to dietary differences, AA profile, dietary energy balance, feed intake, initial BCS, milk production, and metabolic adaptation of the cow (Komaragiri et al., 1998; van der Drift et al., 2012; Ji and Dann, 2013).

The relationship between BCS and tissue mobilisation was demonstrated by Pires et al. (2013), who reported that high BCS (>3.0) cows lost more LW (-121 kg) than thin cows (BCS<3.0; -97 kg) due to more fat mobilisation (NEFA = 500 µmol/l; BHB = 1.10 mmol/l) during the first 7 weeks post-partum, whereas low BCS (<3.0) cows showed a greater mobilisation of muscle protein as identified by a higher plasma 3-methylhistidine and creatinine ratio (3-MH:Creatinine; High BCS = 0.088 vs. low BCS = 0.111). The mobilisation of body protein is essential to support milk performance during the early stages of lactation, however, excess mobilisation of body tissues, especially lipid, may be associated with metabolic disorders, poor lactation performance, immune dysfunction, and reproduction failure (van der Drift et al., 2012; Ji and Dann, 2013). Reducing the supply of dietary CP in the first few weeks of lactation may be associated with a lower lipolytic activity that can reduce fat mobilisation (Cadórniga and López Díaz, 1995; Schor and Gagliostro, 2001). However, the evidence is unclear because, plasma NEFA concentration can vary with dietary CP concentration (Sinclair et al., 2014). Therefore, lowering the dietary CP concentration during the early stages of lactation is challenging for high yielding dairy cows (Ji and Dann, 2013).

2.9. Effect of forage legumes on dairy cows performance

Grass, legume and maize silages are commonly used in dairy cow rations in many temperate countries due to their benefits in feed intake and performance (Dewhurst, 2013). Legumes are high in CP content and can fix N which reduces N fertiliser and carbon cost (Dewhurst et al., 2009). Several studies have been conducted in Northern America and Europe to utilise forage legumes in dairy cows, however, the effect of legume silages on intake, milk performance and metabolism may depend on plant species and their inclusion rates (Lüscher et al., 2014; Phelan et al., 2015).

2.9.1. Effect of legume silages on feed intake in dairy cows

A recent meta-analysis with a dataset of 18 experiments demonstrated that DM intake increased by 1.30 kg/d in cows fed legume compared to grass silage-based diets (Johansen et al., 2017a). Similarly, Steinshamn (2010) noted that feeding red clover, white clover, lucerne, or mixed legume silages increased DM intake by 1.30, 0.60, 4.10 and 2.0 kg/d, respectively compared to grass silage based rations (Table 2.11). Feed intake of legume forages either as a herbage or silage in ruminants was reported to be 10 to 15% higher than comparable digestible-grass or grass silage (Lüscher et al., 2014). The structural differences in fibre and higher fermentation and passage rate of legume forages in the rumen can result higher in a feed intake (Phelan et al., 2015). In contrast, Dewhurst (2013) suggested that the substitution of grass silage by legumes did not affect feed intake in dairy cows.

A meta-analysis by Castro-Montoya and Dickhoefer (2017) reported that DM intake reduced gradually (approximately 115 to 88 g/kg $BW^{0.75}$) in dairy cattle when the inclusion of tropical legume silages increased from 401 to 800 g/kg DM. This could be due to the highest inclusion of forages or characteristics of the diets (RDP, NPN, AA) that can affect feed intake, MCP synthesis and nutrient digestibility (Castro-Montoya and Dickhoefer, 2017). Additionally, the negative correlation between DM intake and the inclusion of forage legumes might be attributed to a lower OM (Johansen et al., 2017a) or nutrient digestibility (Castro-Montoya and Dickhoefer, 2017) that can reduce the passage rate and decrease DM intake (Krizsan et al., 2010). Legumes such as red clover, white clover and lucerne also contain secondary compounds, including tannins, saponins and essential oils that can alter nutrient digestibility (Wallace, 2004), as well as toxic components, for example,

cyanogenic glycosides and alkaloids which are associated with a lower feed intake (D'Mello, 1992).

A meta-analysis by Johansen et al. (2017) noted that daily feed intake of red clover and lucerne silage-based diets were similar (approximately 20.5 kg DM/d) which was higher than white cover-based rations (Johansen et al., 2017a). Likewise, another meta-analysis demonstrated that DM intake was similar when lucerne and red clover silage-based diets were fed to dairy cows (Steinshamn, 2010). However, several studies have reported that the DM intake of lucerne based diets is higher than red clover based rations (Broderick et al., 2001; Dewhurst et al., 2003b; Broderick, 2018). For example, the daily DM intake was 1.70 to 2.50 kg/d higher in dairy cows fed lucerne silage-based diets compared to red clover based rations (Broderick et al., 2001). Similarly, Broderick (2018) reported that daily DM intake of a lucerne based diet was 1.20 kg/d higher compared to a red clover silage based ration.

Table 2.11. Effect of grass and legume silage on feed intake (kg DM/d) in lactating cows

	Grass silage	Legume silage			Source
		White clover	Red clover	Lucerne	
Feed intake (kg DM/d)			22.7^{b}	23.9^{a}	Broderick (2018)
	18.9^{b}	20.0^{ab}	20.0^{a}	21.0^{a}	Johansen et al. (2017a)
			25.6	24.7	Hymes-Fecht et al. (2013)
	18.3^{c}	18.9^{b}	19.6^{a}	22.4^{a}	Steinshamn (2010)
	16.7^{b}		19.0^{a}		Moorby et al. (2009)
	20.7		19.5		Vanhatalo et al. (2009)
			24.9^{a}	24.0^{b}	Broderick et al. (2007)
	18.2^{c}	19.8^{b}	20.3^{b}	20.4^{a}	Dewhurst et al. (2003b)
	17.1^{d}	20.5^{a}	19.0^{c}	19.3^{b}	Dewhurst et al. (2003a)
			23.0^{b}	25.5^{a}	Broderick et al. (2001)(1)
			21.8^{b}	23.5^{a}	Broderick et al. (2001) (2)

$^{a-d}$Means within a row of each study with different superscripts differ significantly ($P < 0.05$).
No superscripts within a row represent no significant difference between the treatments of each study.
(1) and (2) denote separate experiments reported in the same publication

The higher DM intake of lucerne silage compared to red clover silage by dairy cows might be associated with a higher concentration of CP (Broderick, 2018). For instance, red clover and lucerne silage contain 178 and 190 g CP/kg DM, respectively (Table 2.2). Additionally, Leduc et al. (2017) reported an interaction

between forage type and RDP supply and noted that DM intake was increased by 1.50 kg/d in cows fed a lucerne based high RDP (100% supply of requirements of RDP) diet compared to red clover silage-based low RDP (85% supply of requirements of RDP) diet, which was decreased by 3.40 kg/d. However, Broderick et al. (2001) demonstrated that feed intake in dairy cow was affected by forage type but not the DM or CP concentration of the diets, and noted that DM intake was increased by 2.50 kg/d in milking cows fed lucerne compared to red clover silage-based rations. The higher concentration of ADF and NDF in red clover compared to lucerne silage might result in a lower DM intake because forage fibre has the potential to reduce DM intake in cows by filling the rumen space (Allen, 2000).

Recently, several authors have attempted to utilise home-grown legume forages by substituting grass or maize silages in dairy cow rations (Sinclair et al., 2015; Moorby et al., 2016; Schulz et al., 2018). However, the DM intake of legume-based diets in dairy cows depends on the inclusion level (Moorby et al., 2009; Sinclair et al., 2015). For example, the substitution of maize with red clover silage up to 30% (red clover: maize silage = 30:45) did not alter DM intake, however, increasing the proportion of red clover up to 60% (red clover: maize silage = 60:15) reduced DM intake by 1.70 kg/d (Schulz et al., 2018; Figure 2.7). Similarly, Sinclair et al. (2015) reported that a 40% addition of lucerne silage in dairy cows grass and maize silage based diets did not affect DM intake, however, increasing the proportion of lucerne to 60% reduced DM intake by 1.10 kg/d. In contrast, Moorby et al. (2009) noted that daily feed intake increased by 2.30 kg DM when grass silage was replaced by red clover silage in dairy cow rations. The higher intake of red clover than grass silage might have been associated with the higher concentration of CP (Huhtanen and Hetta, 2012; Owens et al., 2014) and perhaps the lower rumen fill for red clover compared to grass silage (Allen, 2000).

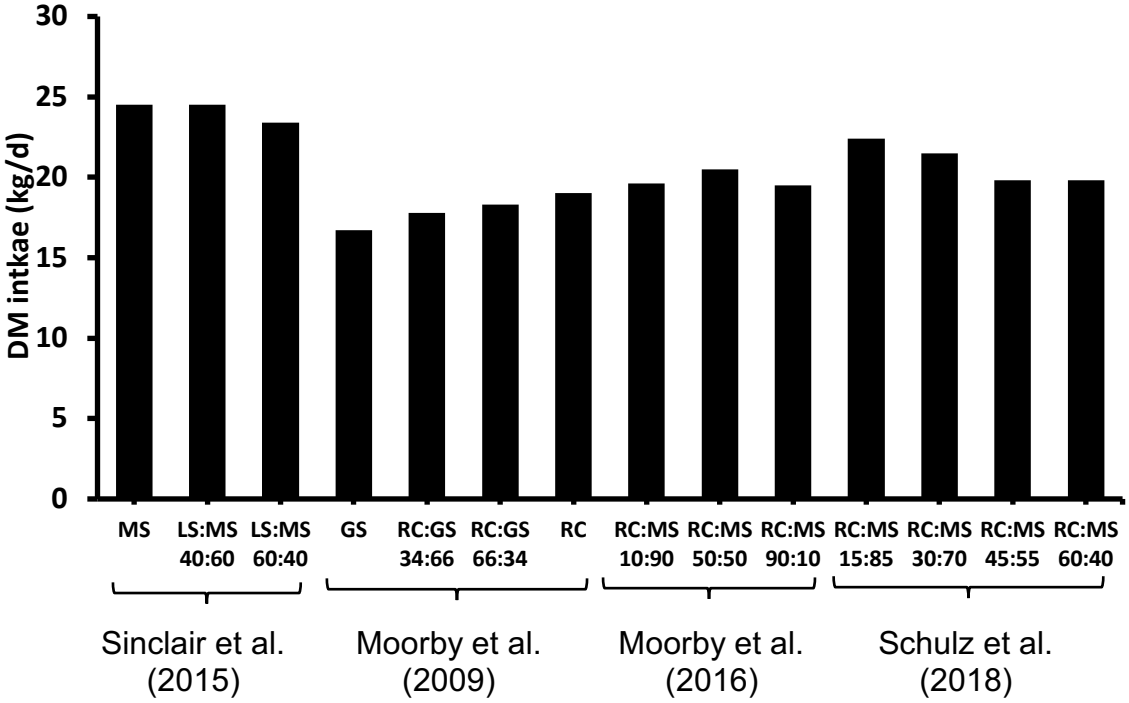


Figure 2.7. Substitution of maize (MS) or grass (GS) silage with lucerne (LS) or red clover (RC) silage in dairy cow diets: effect on DM intake.

2.9.2. Effect of legume silages on nutrient digestibility in dairy cows

The main factors that can affect the apparent digestibility of forages are fibre content, fermentation rate, retention time, PS of functional fibre, cell wall constituents and the presence of anti-nutritional compounds (Dewhurst, 2013). The apparent total tract digestibility of legume silages are comparatively lower than grass silages (Buxton and Redfearn, 1997; Dewhurst et al., 2001; Johansen et al., 2017a). For example, the total tract DM digestibility in dairy cows was found to be highest (714 g/kg) for grass silage compared to red clover (639 g/kg), white clover (673 g/kg), lucerne (636. g/kg), mixed grass-red clover (674 g/kg) or grass-white clover (707 g/kg) silages in the UK (Dewhurst et al., 2001). Johansen et al. (2017b) also noted that grass silage-based rations had the highest DM, OM, ADF and NDF digestibility compared to clover (red or white clover) based rations (Table 2.12). Similar results have also been reported by Dewhurst et al. (2003b) and Moorby et al. (2009). The higher digestibility in grass silage could be due to the structure and composition of the cell wall (Buxton and Redfearn, 1997). The NDF and ADF concentrations, including hemicellulose to cellulose ratio in grasses, are higher than legume species but legume cells, particularly the cellulose, are more lignified which can affect fibre digestion by limiting microbial access to plant polysaccharides

through a physical-barrier or by cross-linking to carbohydrates (Van-Soest et al., 1978; Dewhurst et al., 2001; Steinshamn, 2010).

Table 2.12. Effect of grass and legume silage on apparent nutrient digestibility in dairy cows.

	Grass silage	Legume silage			Source
		White clover	Red clover	Lucerne	
DM (g/kg)			652[a]	613[b]	Broderick (2018)
	774[a]	753[ab]	720[c]		Johansen et al. (2017b)
			745[a]	710[b]	Hymes-Fecht et al. (2013)
	635[a]		546[b]		Moorby et al. (2009)
	720[a]	687[b]	649[c]	640[d]	Dewhurst et al. (2003b)
OM (g/kg)			666[a]	627[b]	Broderick (2018)
	715[ab]	736[a]	694[b]	660[c]	Johansen et al. (2017a)
	789[a]	784[a]	751[b]		Johansen et al. (2017b)
			769[a]	723[b]	Hymes-Fecht et al. (2013)
	725[a]		641[b]		Moorby et al. (2009)
			635[a]	581[b]	Broderick et al. (2007)
NDF (g/kg)			495[a]	440[b]	Broderick (2018)
	745[a]	707[ab]	607[c]		Johansen et al. (2017b)
			599[a]	473[b]	Hymes-Fecht et al. (2013)
	732[a]		597[b]		Moorby et al. (2009)
			532[a]	355[a]	Broderick et al. (2007)
ADF (g/kg)			523[a]	458[b]	Broderick (2018)
	774[a]	729[ab]	622[c]		Johansen et al. (2017b)
			572[a]	484[b]	Hymes-Fecht et al. (2013)
			552[a]	406[b]	Broderick et al. (2007)
CP (g/kg)	711[b]	778[a]	716[b]		Johansen et al. (2017b)
			675	685	Hymes-Fecht et al. (2013)
	673		663		Moorby et al. (2009)
	726[a]	715[b]	650[c]	715[b]	Dewhurst et al. (2003b)

ADF = acid detergent fibre, CP = crude protein, DM = dry matter, NDF = neutral detergent fibre, OM = organic matter.

[a-d]Means within a row of the study with different superscripts differ significantly ($P < 0.05$).

No superscripts within a row represent no significant difference between the treatments of each study.

Among the legume species, the apparent total tract DM digestibility of red clover and lucerne silages was similar (approximately 638 g/kg) but 35 g/kg lower than white clover (Dewhurst et al., 2001). Similarly, a meta-analysis by Johansen et al. (2017a) reported that white clover based diets had a higher OM digestibility (736 g/kg) than red clover or lucerne based rations. Therefore, the apparent total tract nutrient digestibility can vary between legume species due to plant physiological-growth differences (Black et al., 2009). For example, white clover is a stoloniferous

plant which produces less stem during harvesting, whereas red clover and lucerne grow vertically with upward development of stems (Black et al., 2009). The NDF concentration in stems is higher than the leaf, which reduces fibre digestibility (Buxton and Redfearn, 1997). However, the flowering stage of white clover can reduce OM digestibility in milking cows due to the presence of lignin (Weisbjerg et al., 2010).

Feeding red clover silage-based diets to dairy cows increased apparent DM, N, NDF and ADF digestibility by 78.0, 100, 72.0 and 38.0 g/kg, respectively compared to lucerne based rations (Broderick et al., 2001). Similarly, a recent study by Broderick (2018) demonstrated that the apparent whole tract OM, NDF and ADF digestibility increased by 39.0, 55.0 and 65.0 g/kg, respectively in early lactation cows fed red clover compared to lucerne silage-based rations, however, total N and estimated true protein digestibility were reduced by 16.0 and 13.0 g/kg, respectively. Relative to lucerne silage based diets, the higher DM digestibility of red clover might be attributed to improved hemicellulose and NDF digestibility (Broderick et al., 2001). In contrast, the lower nutrient digestibility of lucerne silages could be related to a greater concentration of lignin, which was 41.5% higher than red clover (Table 2.2). However, the reduction in total N or estimated true protein digestibility in red clover diets might be associated with an increased concentration of acid detergent insoluble N, which was higher (1.10% of total N) in red clover than lucerne silage or due to the presence of polyphenol oxidase (PPO) enzyme, which inhibits the proteolysis of forage proteins (Broderick, 2018).

Increasing the proportion of red clover from 15 to 60% in a maize silage based ration did not alter the DM and OM digestibility, however, the apparent total tract ADF and NDF digestibility increased linearly from 484 to 577 and 504 to 596 g/kg, respectively, and N digestibility decreased from 685 to 632 g/kg (Schulz et al., 2018; Figure 2.8). These findings indicated that fibre digestibility in lactating cows fed red clover silage is comparatively higher than maize silage (Dewhurst, 2013). Moorby et al. (2009) noted that the substitution of grass silage with red clover silage (from 0 to 100%) linearly reduced apparent whole tract DM, OM and NDF digestibility, however, the apparent CP digestibility was unaffected. These results confirmed that the digestibility of grass silage was higher than red clover (Johansen et al., 2017a). Moreover, Sinclair et al. (2015) reported that the total tract apparent digestibility of DM, OM, CP, NDF and ADF was reduced by 57.0, 55.0, 57.0, 102 and 76.0 g/kg,

respectively when maize and grass silage was substituted with 60% lucerne silage. Increasing the proportion of lucerne silage may result in increased retention time in the rumen (Allen, 2000) or perhaps a greater ruminal or duodenal flow of indigestible carbohydrates, which increased sloughing of endogenous cells and reduced apparent N digestibility (Hoffman et al., 1998; Dewhurst, 2013).

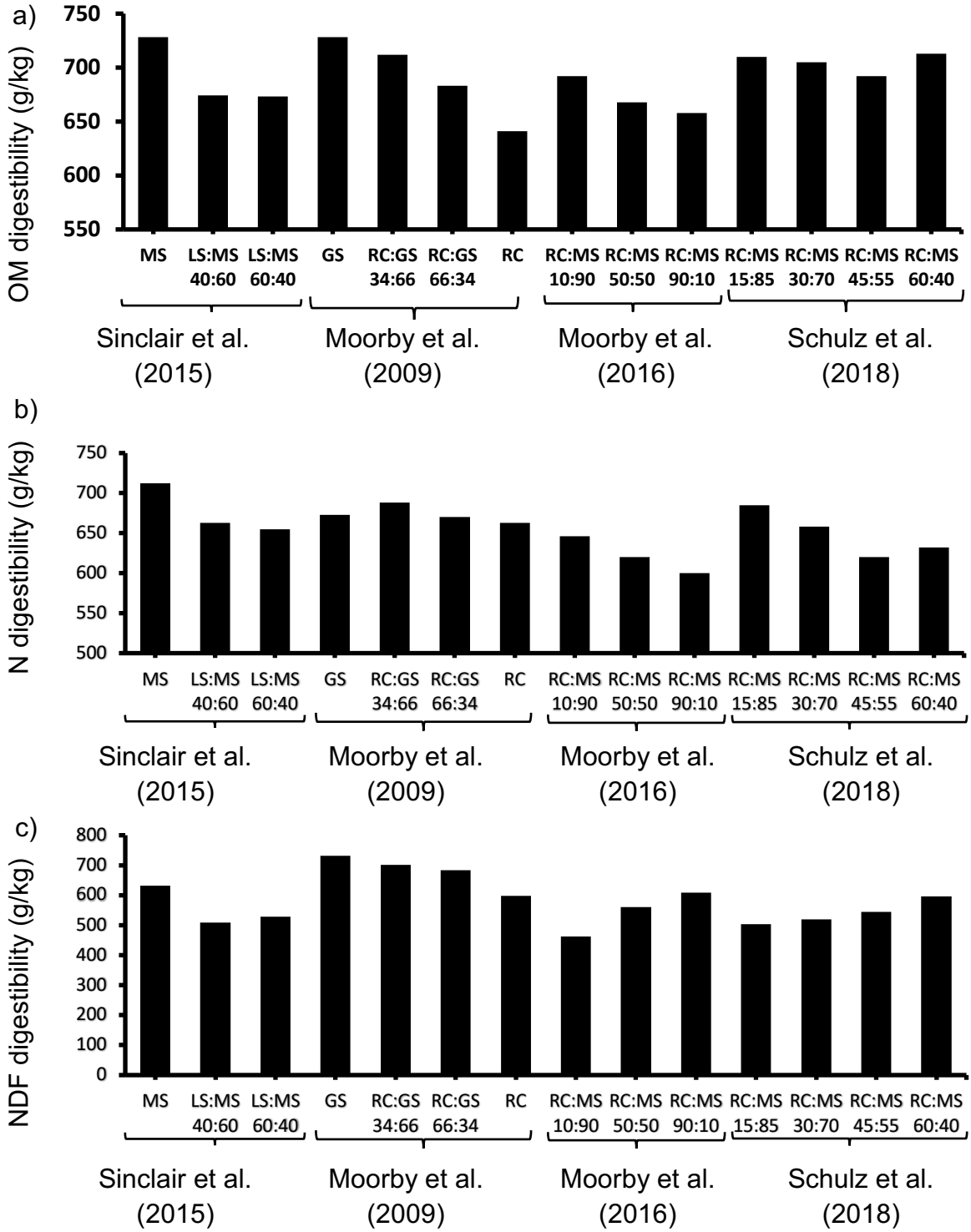


Figure 2.8. Substitution of grass (GS) or maize (MS) silage with lucerne (LS) or red clover (RC) silage in dairy cow diets: effects on OM, N and NDF digestibility.

2.9.3. Effect of legume silages on lactation performance in dairy cows

Feeding legume-based diets can improve milk yield in lactating cows compared to grass silage based rations (Dewhurst et al., 2003b; Steinshamn, 2010; Johansen et al., 2017a). For example, the lowest milk yield was reported for cows fed grass silage (26.2 kg/d) compared to clover based diets (28.2 kg/d; Steinshamn, 2010; Table 2.13). Similarly, Dewhurst et al. (2003b) noted that cows that received a grass silage based ration had a lower milk yield of 24.9 kg/d compared to those fed legume-based diets (29.1 kg/d). A meta-analysis by Johansen et al. (2017a) also reported that feeding legume silages increased milk yield by 1.60 kg/d compared to grass silage-based diets, whereas within legume species milk yield was comparable between lucerne and red clover silage (approximately, 27.5 kg/d), with the highest yield (29.6 kg/d) recorded for white clover based diets. Similarly, compared to lucerne, clover (white or red clover) based diets resulted in the highest milk yield in lactation cows (Dewhurst et al. (2003a; b). The dissimilarity in milk yield in cows fed legume silages could be due to differences in OM digestibility as reported by Johansen et al. (2017b). Moreover, the inclusion rate of forage legumes can have an inconsistent effect on the milk production of dairy cows. For example, Moorby et al. (2009) demonstrated that the inclusion of red clover silage up to 66% (red clover: grass silage = 66:34) in grass silage-based ration increased milk yield by 1.30 kg/d (Figure 2.9). In contrast, Schulz et al. (2018) suggested that the replacement of grass with red clover silage at more than 30% of the forage DM can reduce milk yield in high yielding dairy cows. However, the replacement of maize with red clover silage has not shown any major effect on milk production (Moorby et al., 2016). Similarly, Sinclair et al. (2015) demonstrated that the substitution of maize or grass silage with lucerne at up to 60% did not alter milk yield in high producing Holstein cows.

Milk composition, particularly milk fat and protein concentrations were reduced by 1.40 g/kg in cows fed legume-based rations compared to grass silage based diets (Johansen et al., 2017a). Steinshamn (2010) compared three legume forages and reported that feeding lucerne and white clover diets increased milk protein by 0.60 g/kg compared to red clover based diets. Johansen et al. (2017a) also reported similar findings where milk protein concentration was 0.5 to 1.0 g/kg lower in cows fed red clover diets compared to lucerne or white clover silage based rations. The reduction in milk true protein content in dairy cows fed red clover silage compared

to the other legumes is possibly due to the action of PPO in red clover, which can affect the plasma concentration of methionine, resulting in a reduced intestinal digestibility in dairy cows and limit the synthesis of milk protein (Lee, 2014).

Table 2.13. Effect of grass and legume silage on milk yield and milk fat, protein and lactose concentration in dairy cows

	Grass silage	Legume silage			Source
		White clover	Red clover	Lucerne	
Milk yield (kg/d)	26.2^{c}	29.6^{a}	27.3^{b}	27.7^{b}	Johansen et al. (2017a)
	26.2^{c}	28.7^{a}	27.6^{b}		Steinshamn (2010)
	29.1	30.9	32.7	32.0	Wiking et al. (2010)
	25.2^{b}		26.1^{a}		Moorby et al. (2009)
	32.1		32.7		Lee et al. (2009)
	26.4		27.6		Vanhatalo et al. (2009)
	24.9^{c}	31.5^{a}	28.1^{a}	27.7^{b}	Dewhurst et al. (2003b)
	19.7^{c}	24.0^{a}	19.9^{b}	19.4^{d}	Dewhurst et al. (2003a)
Milk fat (g/kg)	39.8^{a}	37.2^{b}	38.1^{b}	39.1^{ab}	Johansen et al. (2017a)
	40.1^{a}	39.8^{ab}	39.9^{b}	39.4^{ab}	Steinshamn (2010)
	44.0^{a}	38.6^{ab}	38.9^{ab}	40.1^{b}	Wiking et al. (2010)
	38.0^{a}		35.5^{b}		Moorby et al. (2009)
	39.7		40.0		Lee et al. (2009)
	41.1^{a}		38.4^{b}		Vanhatalo et al. (2009)
	44.5	43.9	45.2	44.2	Dewhurst et al. (2003b)
	41.3	40.2	40.0	41.3	Dewhurst et al. (2003a)
Milk protein (g/kg)			30.1^{b}	30.4^{a}	Broderick (2018)
	31.6^{a}	31.8^{a}	30.8^{b}	31.3^{a}	Johansen et al. (2017a)
	31.9^{ab}	31.8^{a}	31.2^{b}	31.8^{a}	Steinshamn (2010)
	30.0^{a}		29.3^{b}		Wiking et al. (2010)
	31.1^{b}		31.8^{a}		Moorby et al. (2009)
	32.6^{a}		30.6^{b}		Lee et al. (2009)
	32.6	32.0	31.4	32.6	Vanhatalo et al. (2009)
	33.7^{a}	33.2^{ab}	32.8^{ab}	32.8^{b}	Dewhurst et al. (2003b)
Milk lactose (g/kg)			48.6^{a}	47.9^{b}	Broderick (2018)
	45.8		45.8		Moorby et al. (2009)
	47.0^{a}		46.3^{b}		Lee et al. (2009)
	46.4^{b}		46.9^{a}		Vanhatalo et al. (2009)
	47.1	47.1	46.8	46.6	Dewhurst et al. (2003b)
	46.0^{c}	47.4^{a}	46.3^{b}	46.0^{c}	Moorby et al. (2009)

$^{a\text{-}d}$Means within a row of the study with different superscripts differ significantly ($P < 0.05$).
No superscripts within a row represent no significant difference between the treatments of each study.

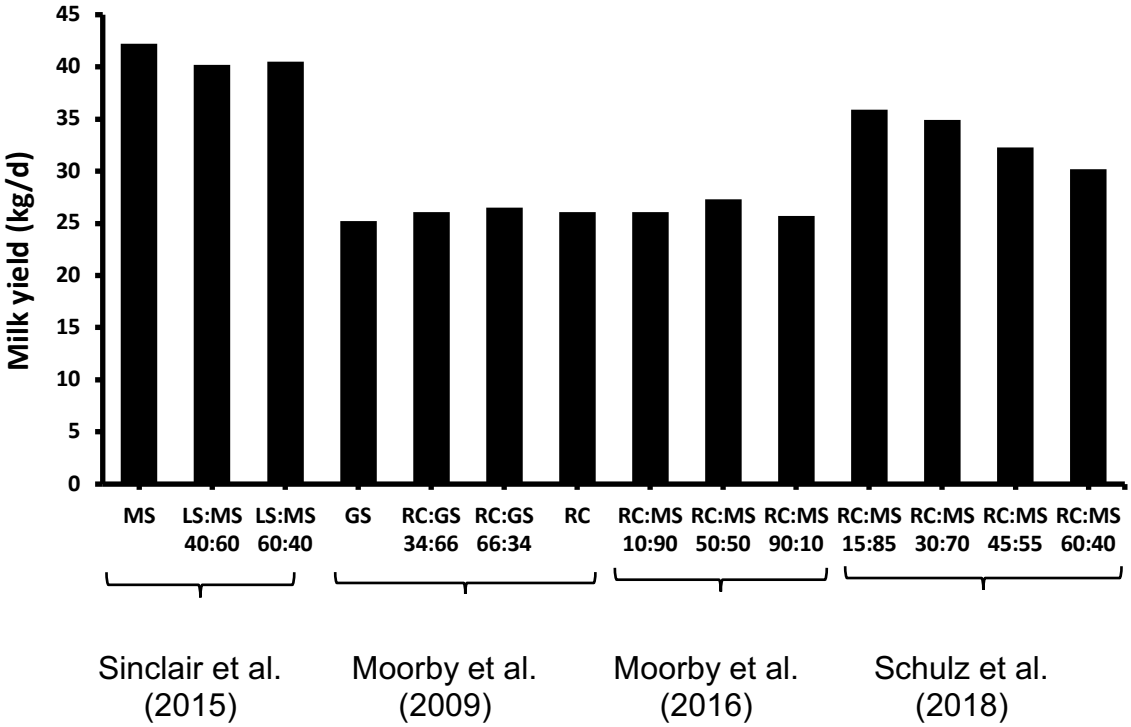


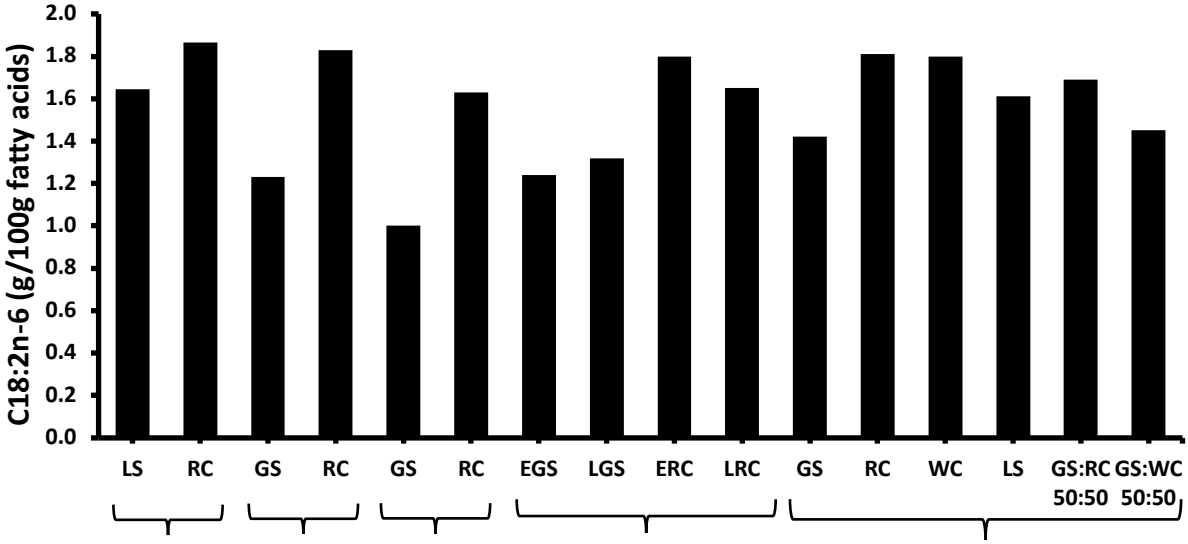
Figure 2.9. Replacement of maize (MS) or grass (GS) silage with lucerne (LS) or red clover (RC) silage in dairy cow diets: effect on milk yield.

The concentration of milk fat in dairy cows fed any of the legume silage based rations is similar, however, it is reduced in cows fed red clover silage compared to lucerne based diets (Steinshamn, 2010; Johansen et al., 2017a). Other studies have demonstrated that milk fat secretion decreased in dairy cows fed red clover silages compared to other forages (Broderick et al., 2000; Moorby et al., 2009; Broderick, 2018). The reduced milk fat concentration in red clover diets could be attributed to decreased milk FA synthesis either by a reduction in lipolysis or by intermediary compounds that were produced in the bio-hydrogenation process (Lee et al., 2007; Leduc et al., 2017).

2.9.4. Effect of legume-based diets on the milk fatty acid profile of dairy cows

The profile of milk FA usually depends on the forage species consumed (Lashkari et al., 2019). Dewhurst et al. (2006) suggested that legume silages, including white or red clover, can increase the quantity of polyunsaturated milk FA (PUFA) in dairy cows compared to grass-based rations. For instance, Dewhurst et al. (2003) demonstrated that the proportion of both C18:2n-6 and C18:3n-3 increased by 0.39 and 0.38 g per 100 g of milk fat, respectively when dairy cows were fed red clover compared to grass silage based rations (Figure 2.10). However, both PUFA were

comparable between white and red clover silage based diets (Dewhurst et al., 2003b). Likewise, a recent study by Lashkari et al. (2019) reported that the concentration of milk C18:2n-6 was similar in milking cows fed clover (white or red clover silage) based rations, however, milk C18:3n-3 was reported to be higher in cows fed white clover compared to other forage-based rations. Another study by Leduc et al. (2017) examined different milk FA in high yielding cows and noted that feeding red clover silage increased branched-chain FA (iso C13:0, iso C14:0, iso C15:0, iso C16:0, iso C17:0, anteiso C15:0, anteiso C17:0) and PUFA (C18:2n-6 and C18:3n-3) but decreased odd chain FA (C11:0, C13:0, C15:0, C17:0) compared to lucerne silage based diets. Linear odd and branched-chain FA in the milk of dairy cows can be used to predict MCP synthesis as suggested by Cabrita et al. (2003). The transfer efficiency of C18:2n-6 and C18:3n-3 in cows milk increased by 5.29 and 2.65%, respectively when red clover silage was fed compared to lucerne-based diets (Leduc et al., 2017). Similarly, the higher transfer efficiency of C18:3n-3 from feed to milk fat has been demonstrated by several authors when red clover silage was fed compared to the grass or lucerne based rations (Lee et al., 2009; Moorby et al., 2009; Halmemies-Beauchet-Filleau et al., 2014). The higher transfer efficiency of milk PUFA in cows fed red clover silage based rations could be due to the effect of PPO which reduces lipolysis and protects the bio-hydrogenation of long-chain FA (Lee et al., 2007; Lee, 2014).



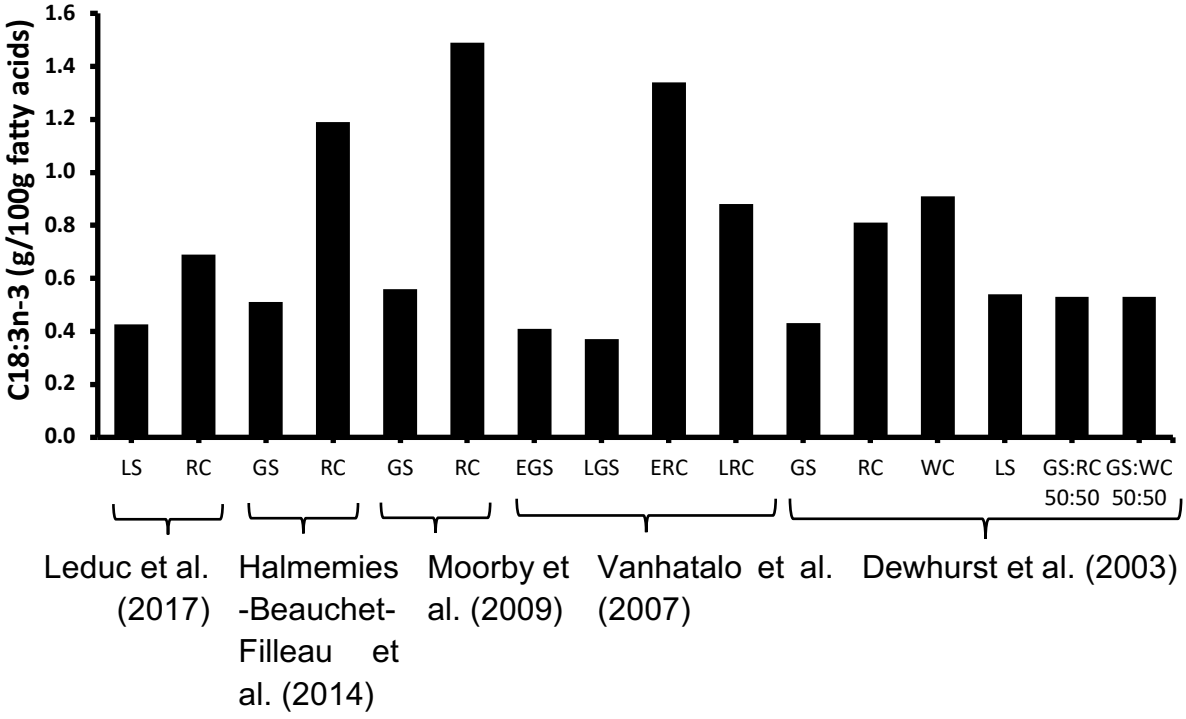


Figure 2.10. Effects of different silage (GS = grass, RC = red clover, WC = white clover, LS = lucerne, EGS = early cut grass, ERC = early cut red clover, LGS = late cut grass, LRC = late cut red clover) based diets on the proportion of C18:2n-6 and C18:3n-3 (g/100g of total FAs) in milk fat of lactating cows.

The higher concentration of linear odd chain milk FA from cows fed red clover silage based diets could be attributed to the greater concentration of valerate and propionate, which are formed by rumen microorganisms during rumen fermentation, and serve as a precursor to synthesise linear odd chain FA in milk (Vlaeminck et al., 2006; Leduc et al., 2017). Vlaeminck et al. (2006) also suggested that a higher proportion of rumen cellulolytic and amylolytic bacteria could be responsible for increasing the synthesis of iso and anteiso FA in cow milk, respectively. The higher concentration of NDF in red clover silage compared to white clover or lucerne silage may result in increased branched-chain milk FA, as the concentration of NDF is positively related to iso and anteiso FA in milk (Vlaeminck et al., 2006). According to Vlaeminck et al. (2006), milk iso or anteiso FA synthesis may, however, be related to dietary CP content, particularly NH_3 concentrations in the rumen. For example, a higher concentration of ruminal NH_3 can improve the MCP synthesis and may result in increased concentration of odd and branch chain FA in the milk of dairy cows (Leduc et al., 2017).

2.9.5. Effects of legume-based rations on live weight and condition score in dairy cows

Feeding grass silage based diets increased LW gain by 0.08 kg/d in dairy cows compared to red clover based rations (Moorby et al., 2009; Table 2.14). However, Johansen et al. (2017b) studied the effects of clover and grass silages on cow LW and reported that grass or red clover based diets did not alter the LW of dairy cows except those fed white clover, which was reduced by 13.0 kg compared to cows fed red clover. Moreover, the inclusion of clover silage (red or white clover) in grass-based diets (clover: grass = 50:50) did not alter the LW of cows (Johansen et al., 2017b). Similarly, Sinclair et al. (2015) noted that increasing the proportion of lucerne silage up to 60% of the forage DM did not alter LW gain of early lactating cows. In contrast, the inclusion of red clover silage at more than 60% (red clover: grass = 66:34) reduced LW gain by 0.28 kg/d compared to cows fed grass-based rations (Moorby et al., 2009), however, the replacement of grass silage by red clover at up to 34% (red clover: grass = 34:66) increased average daily gain by 0.11 kg (Moorby et al., 2009). Additionally, compared with lucerne silage, red clover based diets increased LW gain from 0.26 to 0.33 kg/d in high yielding dairy cows (Broderick et al., 2000, 2001, 2007). Castro-Montoya and Dickhoefer (2017) suggested that the inclusion rate of any legume silages in large ruminant diets should not be more than 400 g/kg DM to improve body condition.

Dietary energy and CP intake, body N balance, and duodenal flow of essential AA, all are positively related to BCS in milking cows (van der Drift et al., 2012; Ji and Dann, 2013; Pires et al., 2013). Halmemies-Beauchet-Filleau et al. (2014) reported that there was a tendency to reduce BCS change by 0.09 unit when dairy cows were fed red clover compared to grass silage-based rations. Similarly, the inclusion of red clover in grass silage-based diets from 0 to 100% linearly reduced both loin and tail BCS change by 0.12 and 0.13 units, respectively (Moorby et al., 2009; Figure 2.11). The lower BCS in cows fed red clover based diets could be due to the support of milk protein synthesis, as the concentration of milk protein decreased by approximately 1.0 g/kg in milk compared to cows fed other legume and grass silage based diets (Johansen et al., 2017a). The reduction in N balance, apparent total tract N digestibility, and an unbalanced duodenal supply or bioavailability of essential AA could be other reasons for the reduction in BCS in cows fed red clover silage based rations (Halmemies-Beauchet-Filleau et al., 2014).

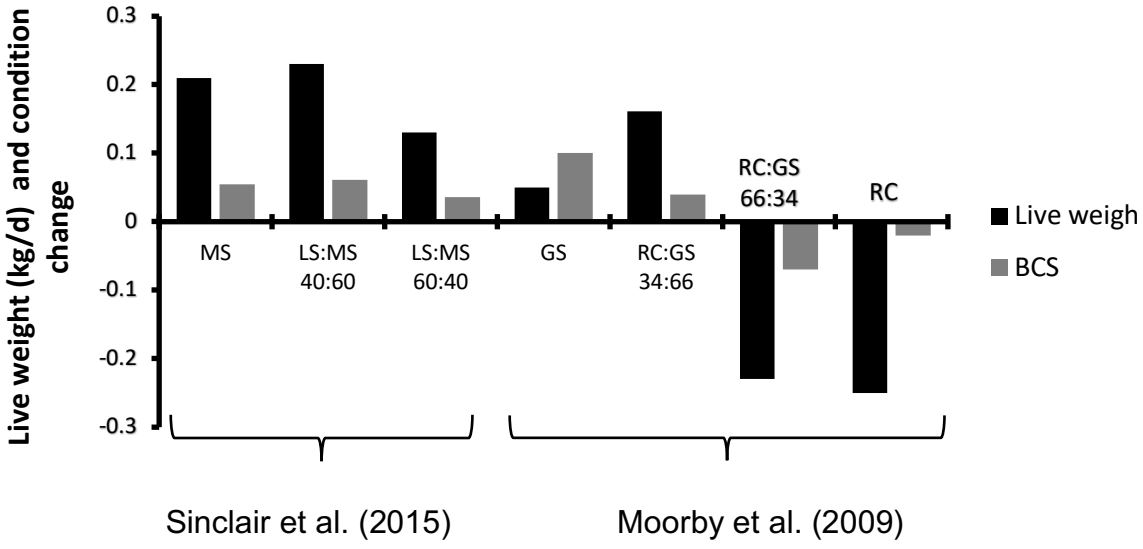


Figure 2.11. Substitution of maize (MS) or grass (GS) silage with lucerne (LS) or red clover (RC) silage in dairy cow diets: effects on live weight gain (kg/d) and condition change.

2.9.6. Effects of legume silage diets on plasma metabolites in dairy cows

The concentration of plasma metabolites in lactating cows fed different legume or grass silage based diets are not consistent (Lee et al., 2009; Vanhatalo et al., 2009; Leduc et al., 2017). For example, feeding red clover silage increased NEFA and plasma urea concentrations by 38 and 4.01 mmol/l, respectively in dairy cows compared to those fed grass silage-based rations, however, the concentration of glucose, insulin and BHB were comparable in cows fed either forage (Vanhatalo et al., 2009; Table 2.14).

Conversely, Leduc et al. (2017) demonstrated that the plasma urea concentration was 1.15 mmol/l higher in dairy cows when lucerne based diet was offered compared to red clover silage diet. Likewise, Sinclair et al., (2015) observed that increasing the proportion of lucerne up to 60% in maize silage based rations also increased plasma urea and BHB concentrations by 1.50 and 0.15 mmol/l in early lactation cows.

Table 2.14. Effect of grass and legume silages on mean plasma metabolites, live weight and condition score in dairy cows

	Grass silage	Legume silages			Source
		White clover	Red clover	Lucerne	
Glucose (mmol/l)	3.64		3.63		Vanhatalo et al. (2009)
	3.62[c]	4.24[b]	4.29[b]	4.59[a]	Dewhurst et al. (2003)
			3.19	3.06	(Broderick et al., 2001)
BHB[1] (mmol/l)	0.98		1.15		Vanhatalo et al. (2009)
	0.81[ab]	0.69[b]	0.90[a]	0.81[ab]	Dewhurst et al. (2003)
Urea (mmol/l)			2.33[b]	2.73[a]	Leduc et al. (2017)
			4.90	5.90	Hymes-Fecht et al. (2013)
	2.93[b]		7.02[a]		Vanhatalo et al. (2009)
	6.52[c]	8.73[b]	8.19[b]	10.4[a]	Dewhurst et al. (2003)
			4.47	4.55	Broderick et al. (2001)
			2.85[b]	5.14[a]	Broderick et al. (2000)
NEFA (mmol/l)	106		144		Vanhatalo et al. (2009)
Live weight change (kg/d)			0.37	0.39	Broderick (2018)
			0.03	-0.01	Hymes-Fecht et al. (2013)
	0.05[a]		-0.03[b]		Moorby et al. (2009)
			0.64	0.31	Broderick et al. (2008)
			0.03	0.38	Broderick et al. (2001)
			0.20[a]	-0.13[b]	Broderick et al. (2000)
BCS change	0.03		-0.06		Halmemies-Beauchet-Filleau et al. (2014)
	0.11[a]		-0.02[b]		Moorby et al. (2009)

[1]BHB = β-hydroxybutyric acid;

[2]BCS = body condition score;

[a-c]Means within a row of the study with different superscripts differ (P<0.05).

No superscripts within a row represent no significant difference between the treatments of each study.

The higher concentration of CP and lower capture of RDP by rumen microbes might be associated with increased plasma urea concentration in cows fed legume (lucerne, red and white clover) based rations compared to grass silage-based diets (Dewhurst et al., 2003b; Sinclair et al., 2015). The higher content of CP, particularly RDP in lucerne silage might be responsible for greater concentrations of plasma urea in cows in relation to those fed red clover silage-based diets (Broderick et al., 2000). The higher BHB concentration might have resulted from rumen fermentation, indicating a greater proportion of ruminal butyric acid (Hassanat et al., 2013). However, Lee et al. (2009) and Vanhatalo et al. (2009) demonstrated that plasma methionine concentration was reduced by 0.29 g/100 g of total AA when dairy cows

were fed red clover silages compared to grass silage based diets. Red clover silage contains PPO, which can reduce the availability of sulphur-containing AA, resulting in a lower concentration of cysteine or methionine in blood plasma (Lee, 2014).

2.9.7. Effects of legume silage-based diets on nitrogen utilisation in cows

The effects of feeding grass or clover (red clover and white clover) silages on N balance in dairy cows are variable, which may be attributed to the concentration or ruminal degradability of the forage protein (Lee et al., 2009; Moorby et al., 2009). Feeding red clover silage to lactating cows did not alter body N balance compared to a grass silage based ration (Moorby et al., 2009). However, Lee et al. (2009) reported that N retention increased by 15.1 g/d when dairy cows were fed red clover compared to a grass silage based diet. Likewise, feeding both early and late cut red clover silage increased N balance by 13 g/d compared to grass or mixed (red clover and grass silage) silage based rations (Vanhatalo et al., 2009).

The highest NUE has been reported for cows fed grass silage (25.6%) compared to clover (white or red clover, 20.7%) or lucerne (18.2%) based rations (Dewhurst et al., 2003; Table 2.15). Additionally, the substitution of grass silage with red clover at up to 90 or 100% of the forage DM decreased NUE in milking cows (Moorby et al., 2009, 2016; Figure 2.12). Similarly, Schulz et al. (2018) noted that the inclusion of red clover in maize silage based rations at up to 60% of the forage DM reduced NUE. In contrast, Sinclair et al. (2015) noted that the replacement of maize silage by lucerne did not alter NUE in early lactation cows, however, there was a tendency for an improvement when the substitution rate of lucerne was increased to 60%.

The apparent NUE may also vary between legume species. For example, several studies have reported that feeding red clover silage increased NUE compared to lucerne based rations (Broderick et al., 2001, 2007; Broderick, 2018). In contrast, feeding red clover silage decreased MUN by 2.20 mg/dl compared to milk from cows fed lucerne silage based rations (Broderick, 2018). Several authors have also reported similar findings for red clover based diets (Broderick et al., 2000, 2007; Broderick, 2018). These findings suggest that red clover silage can increase N utilisation in dairy cows compared to lucerne which could be due to a lower degradation rate of the forage CP in the rumen. The improved OM digestibility (Hymes-Fecht et al., 2013; Johansen et al., 2017a; Broderick, 2018), and decreased concentration of rumen NH_3 in dairy cows fed red clover silage-based rations could

also be responsible for a higher N efficiency compared to lucerne (Broderick, 2018) or grass silage based rations (Moorby et al., 2009; Vanhatalo et al., 2009; Halmemies-Beauchet-Filleau et al., 2014).

Table 2.15. Effect of grass and legume silages on milk urea nitrogen and nitrogen use efficiency in dairy cows.

	Grass silage	Legume silages			Source
		White clover	Red clover	Lucerne	
MUN[1] (mg/dl)			12.9[b]	15.1[a]	Broderick (2018)
			11.0	11.4	Hymes-Fecht et al. (2013)
	6.64[b]		17.0[a]		Vanhatalo et al. (2009)
			13.8[b]	17.8[a]	Broderick et al. (2007)
			10.5	9.90	Broderick et al. (2001) (1)
			9.20[b]	13.6[a]	Broderick et al. (2001) (2)
			8.70[b]	14.8[a]	Broderick et al. (2000)
NUE[2] (%)			28.5[a]	26.9[b]	Broderick (2018)
			21.0	22.3	Hymes-Fecht et al. (2013)
	28.0		25.0		Lee et al. (2009)
			22.3[a]	21.1[b]	Broderick et al. (2007)
	25.6[a]	20.5[b]	21.0[b]	18.2[c]	Dewhurst et al. (2003)
			25.0[a]	23.3[b]	Broderick et al. (2001) (1)
			24.9[a]	20.4[b]	Broderick et al. (2001) (2)

[1]MUN = milk urea N; [2]NUE = N use efficiency; (milk true protein/6.38)/N intake) × 100.
[a-c]Means within a row of the study with different superscripts differ ($P < 0.05$).
No superscripts within a row represent no significant difference between the treatments of each study.
(1) and (2) denote separate experiments reported in the same publication.

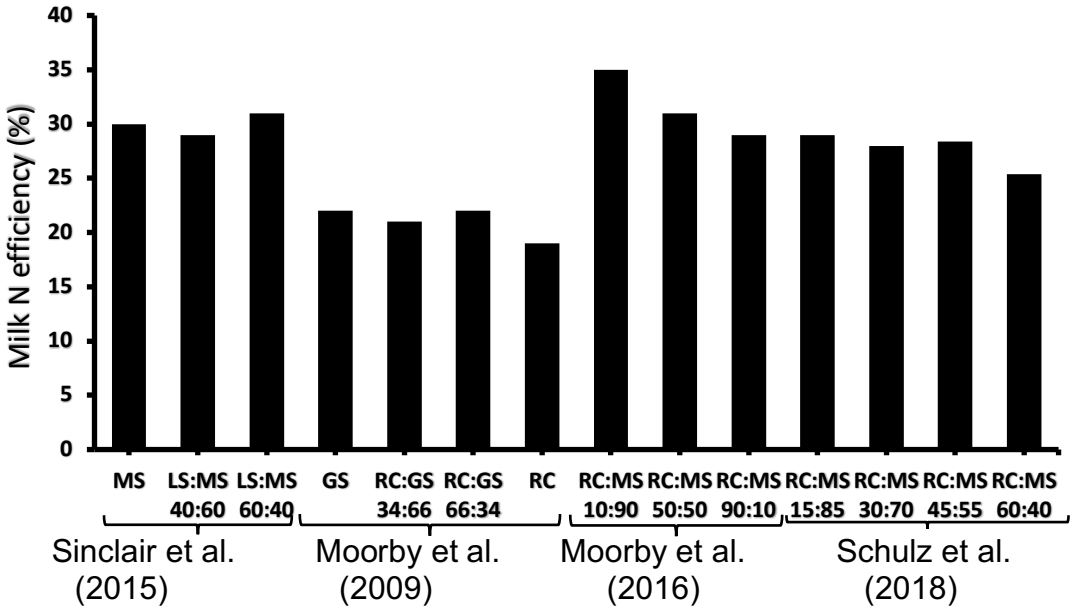


Figure 2.12. Substitution of maize (MS) or grass (GS) silage with lucerne (LS) or red clover (RC) silage in dairy cow diets: effect on apparent milk N efficiency.

2.10. Summary of literature review and knowledge gaps

Reducing the CP concentration in dairy cow diets can improve milk N efficiency and decrease N excretion in urine and faeces, however, it may reduce performance (Oh et al., 2019). Homegrown UK forage legumes may alleviate the adverse effects of low protein diets by improving feed intake and milk production when compared with grass or mixed silage based diets (Steinshamn, 2010; Johansen et al., 2017a). However, legume silages are degraded rapidely in the rumen and reduce the flow of RUP content, which may result in an inadequate supply of MP for higher yielding early lactation dairy cows (Damborg et al., 2018; Westreicher-Kristen et al., 2018). Additionally, higher inclusion rates of forage legumes (> 60% of forage DM) in dairy cow diets can reduce DM intake which may result in a decreased apparent digestibility and milk performance (Sinclair et al., 2015; Schulz et al., 2018). To rectify this, dietary strategies should aim to increase MCP synthesis in the rumen and improve the bioavailability of essential AA when low CP (≤ 150 g CP/kg DM) diets are offered to lactating cows. There is also the incentive for dairy farmers to utilise locally grown high protein forage legumes such as lucerne or red clover to reduce the reliance on artificial fertiliser and purchased protein feeds. Therefore, the challenge is to reduce the CP concentration in high yielding dairy cow diets when highly degradable high protein legume silages are fed. However, the effect of low protein diets based on legume forages in high producing cows on lactation performance, metabolism and N balance is unclear.

2.11. Hypothesis

Lowering dietary protein concentrations based on high protein legumes will not affect the performance but will improve N efficiency in dairy cows, particularly if supplemented with additional rumen available energy or RP amino acids.

2.12. Objectives and aim of the studies

The primary objective was to determine the effects of low protein diets based on legume silages including red clover and lucerne on feed intake, lactation performance, blood metabolites, apparent digestibility and N utilisation in early lactating cows. The second aim was to determine the longer-term effects of low protein diets on the performance and health of lactating cows by increasing the supply of energy available for microbial protein synthesis and balancing the essential amino acid supply.

CHAPTER 3: Materials and methods

3.1. Chemical analysis of forages, feed, and faecal samples

3.1.1. Dry matter (DM)

The dry matter content of the pooled forages, total mixed ration (TMR) and sieve fractions from the Penn State Separator was weighed using a 4-digit electric weighing balance (Fisher Scientific, UK) and dried in a forced-air oven (Binder, Cole-Palmers, UK) at 105ºC for approximately 24 h (AOAC 2012; 943.01). *In situ* forage residue and bulked faecal samples were dried in a separate hot air oven (Philip Harris Ltd, England) at 60ºC until constant weight. All dried samples were immediately placed in a desiccator for 30 min to cool down and weigh out. The DM content (g/kg) was calculated as follows;

$$DM\ (g/kg) = \frac{weight\ of\ dry\ sample\ (g)}{weight\ of\ fresh\ sample\ (g)} \times 1000 \qquad \text{[Equation 3.1]}$$

All dried samples were milled using a hammer mill (Cyclotec, Warrington, UK) with a 1 mm screen prior to subsequent laboratory analysis.

3.1.2. Crude protein (CP)

The CP content of the feed samples was calculated from their total nitrogen (N) content. Total N of the dried TMR, forage and faecal samples was determined using the Dumas method according to AOAC (2012; 988.05) using a LECO FP528 (LECO Corporation, Stockport, UK). The content of CP (g/kg) was calculated as follows;

$$CP\ (g/kg\ DM) = N\ (\%) \times 6.25 \times 10 \qquad \text{[Equation 3.2]}$$

3.1.3. Water-soluble crude protein (WSCP)

The WSCP fraction of forages was determined as described by Weisbjerg et al. (1990). Approximately 0.5 g of dried, milled forage sample was accurately weighed and transferred into a 250 ml conical flask (Fisher Scientific, UK), followed by 50 ml of cold distilled water added. The conical flask containing the sample was shaken every 15 min and soaked for 1 h. Samples were then filtered through dried pre-weighed N free filter paper and washed four times with 50 ml distilled water. After washing, the residue sample and filter paper was dried in a forced-air oven at 60ºC

for 48 h and re-weighed. Dried residues were then analysed for CP content using a LECO FP528 (Section 3.1.2). The WSCP was calculated as follows;

$$WSCP\ (g/kg\ DM) = \frac{(W1 \times CP1) - ((W2 - W3) \times CP2)}{(W1 \times forage\ CP)} \times 1000 \quad \text{[Equation 3.3]}$$

Where,

W1 = weight of the initial dried sample (g)

CP1 = crude protein of initial dried sample (g/kg DM)

W2 = weight of washed dry sample plus filter paper (g)

W3 = weight of dried filter paper before used (g)

CP2 = crude protein of washed dry sample residue (g/kg DM)

3.1.4. Ash and organic matter (OM)

The ash content of the feed and faecal samples was measured after ignition at 550ºC according to AOAC (2012; 942.05). Approximately 2.5 g of dried ground sample was weighed into pre-weighed clean and dried porcelain crucibles and then placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) with a temperature of 550ºC for 4 h. The content of ash and OM (g/kg DM) was calculated as follows;

$$Ash\ (g/kg\ DM) = \frac{Weight\ of\ ignited\ sample\ (g)}{Weight\ of\ dried\ sample\ (g)} \times 1000 \quad \text{[Equation 3.4]}$$

OM (g/kg DM) = 1000 – ash weight (g/kg DM) [Equation 3.5]

3.1.5. Ether extract (EE)

The EE content of the feed samples was determined as per the method of AOAC (2012; 920.39) using a Soxtec apparatus (FOSS, Warrington, UK). Approximately 1.0 g of dried ground sample was placed into a Soxtec extraction thimble (Whatman Plc, Maidstone, UK) and covered with a thin layer of cotton wool. The samples were boiled in 30 ml petroleum ether (Fisher Scientific, UK) for 60 min and rinsed for an additional 20 min with evaporated solvent in the Soxtec apparatus. After complete evaporation of the ether solvent, the extraction cups were then removed and reweighed when they reached room temperature. The content of EE was calculated as follows;

$$EE\ (g/kg\ DM) = \frac{W1-W2}{W3} \times 1000 \times \frac{1000}{W4}$$ [Equation 3.6]

Where,

W1 = weight of extraction cup plus ether extract (g)

W2 = weight of extraction cup (g)

W3 = weight of dry sample (g)

W4 = sample dry matter (g/kg)

3.1.6. Neutral detergent fibre (NDF)

The NDF content of the feed and faecal samples was determined using Foss hot and cold extractor Fibertec system (1020 and 1021, FOSS, Warrington, UK) according to the method described by Van Soest et al. (1991). The NDF reagent was prepared as follows; 22.8 g anhydrous-disodium-hydrogen-phosphate (Na_2HPO_4), 34 g sodium-tetra-borate ($Na_2B_4O_7.10H_2O$), 93 g of di-sodium-ethylene-diamine-tetra-acetic-acid-dehydrate (EDTA), 150 g sodium-dodecyl-sulphate (SDS), and 50 ml tri-ethylene-glycol was dissolved with hot distilled water and made up to 5 L. The pH of the prepared NDF reagent was adjusted to approximately 7.0 ± 0.1 using 0.1 M hydrochloric acid (HCl) or sodium hydroxide (NaOH) solution. To determine the NDF values exclusive of residual ash, alpha (α) amylase solution was prepared as follows; approximately 1 g of α-amylase (Sigma, Gillingham, UK; ~80EU/mg) from *Bacillus subtilis spp* was dissolved in 90 ml of distilled water and then made up to 100 ml by mixing with tri-ethylene-glycol.

Approximately 0.5 g (0.4 to 0.6 g) of dried sample was weighed into a FOSS P1 glass crucible (Soham Scientific, UK) and then fitted tightly onto the Fibertec apparatus. Accurately 25 ml of NDF reagent and 2-3 drops of octanol were added to the sample contained in each crucible and then digested for 30 min with boiling temperature. After digestion, additional 25 ml of cold NDF reagent, 0.5 g of sodium-sulphite and 2 ml of α-amylase solution were added and allowed to digest for another 30 min. Following the second digestion, the crucible containing solution was drained off through filtration, and the residue sample was washed thoroughly with 30 to 40 ml of hot (~80ºC) distilled water. After washing, an additional 25 ml of hot distilled water and 2 ml of α-amylase solution were added to the washed sample and allowed to stand for approximately 15 min. The sample was then filtered and

rewashed 3 times with hot distilled water. After complete washing, the crucibles containing the sample (residue) were removed from the Fibertec apparatus and then dried overnight in a forced air oven at 105ºC. The following day, the crucibles were removed and cooled in a desiccator for 30 min, weighed and placed in a muffle furnace to ash at 550ºC for 4 h (AOAC 2012; 942.05). The hot crucibles containing the ash were then cooled in a desiccator and, once cooled, re-weighed to give an ash weight. The NDF content (g/kg DM) was calculated as follows;

$$NDF\ (g/kg\ DM) = \frac{W1-W2}{W3} \times 100 \times \frac{1000}{W4} \quad \text{[Equation 3.7]}$$

Where,

W1 = weight of crucible plus dried residue after oven drying (g)

W2 = weight of crucible plus ash residue after ignition (g)

W3 = weight of initial dry sample added to the crucible (g)

W4 = sample dry matter (g/kg)

3.1.7. Acid detergent fibre (ADF)

The ADF content of the feed and faecal samples was determined using Foss hot and cold extractor Fibertec system (1020 and 1021, FOSS, Warrington, UK) according to the method described by Van Soest et al. (1991). The ADF reagent was prepared by dissolving 100 g cetyltrimethylammonium-bromide (CETAB; Sigma, Gillingham, UK) in 5 L of 1.0 M sulphuric-acid (H_2SO_4; Sigma, Gillingham, UK) solution.

Approximately 1.0 g (0.9 to 1.1 g) of dried ground sample was weighed into a FOSS P2 glass crucible (Soham Scientific, UK) and then fitted tightly onto the Fibertec apparatus. Then 100 ml of ADF reagent was added to the sample in each crucible and digested for 1 h by boiling. Following digestion, the solution was removed by filtration, and the residue sample washed 3 times with hot distilled water (~80ºC). After washing, the crucibles containing the sample (residue) were removed from the Fibertec apparatus and dried overnight in a forced air oven at 105ºC. The following day, the crucibles were removed from the hot air oven and cooled in a desiccator for 30 min, weighed and placed in a muffle furnace to ash at 550ºC for 4 h (AOAC 2012; 942.05). The crucibles and ash were then cooled in a desiccator and, once

cooled, re-weighed to give an ash weight. The ADF content (g/kg DM) was calculated as follows;

$$NDF\ (g/kg\ DM) = \frac{W1-W2}{W3} \times 100 \times \frac{1000}{W4} \quad \text{[Equation 3.8]}$$

Where,

W1 = weight of crucible plus dried residue after oven drying (g)

W2 = weight of crucible plus ash residue after ignition (g)

W3 = weight of initial dry sample added to the crucible (g)

W4 = sample dry matter (g/kg)

3.1.8. Acid detergent insoluble nitrogen (ADIN)

The acid detergent-insoluble nitrogen fraction of the forage samples was determined using a Foss Fibertec system (1020 and 1021, FOSS, Warrington, UK) according to the method described by Van Soest et al. (1991) with the N fraction analysed using a LECO FP528 using Dumas technique according to AOAC (2012; 988.05). Approximately 0.5 g of dried ground sample was boiled with the acid detergent solution for 1 h and then washed with hot distilled water followed by oven drying (Section 3.1.7). The dried samples were then analysed for total N using a LECO (Section 3.1.2).

3.1.9. Starch

The pooled dried and ground TMR samples were sent to Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) to determine the starch content, which was conducted by the polarimetric method according to ISO 6493 (2000).

3.2. Apparent total tract digestibility and acid insoluble ash (AIA)

Apparent total tract digestibility coefficients of DM, OM, CP, NDF, and ADF were determined using AIA as an internal marker as per the method of Van Keulen & Young (1977). Approximately 4.5 g of pooled dried and ground TMR or faecal sample was weighed into pre-weighed clean, dried porcelain crucibles and placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) for ash at 550ºC for 4 h. The ash contained in the crucibles was allowed to cool in a desiccator, weighed,

and then the residue was transferred with 10 ml of 2M HCl into 250 ml Kjeldahl digestion tubes (Foss Digestor Unit, Hilleroed, Denmark). Another 90 ml of 2M HCl was added to the Kjeldahl tube and heated at boiling point (175ºC) for 10 min. After boiling, samples were then filtered through ash-free 50 mm filter paper (Whatman number 541) and washed 3 times with hot distilled water (~80ºC) to clean the Kjeldahl tubes. Following washing, the filter paper was placed in the same pre-weighed porcelain crucible and ashed in a muffle furnace at 550ºC for 4 h. The ash contained in the crucibles was then cooled in a desiccator and re-weighed to produce the acid ash weight. The AIA content (g/kg DM) was calculated as follows;

$$AIA\ (g/kg\ DM) = \frac{W1-W2}{W3} \times 1000 \qquad \text{[Equation 3.9]}$$

Where,

W1 = weight of crucible plus acid ash (g)

W2 = weight of empty crucible (g)

W3 = weight of initial dry sample added to the crucible (g)

The apparent total tract digestibility of each nutrient was calculated as follows;

$$Digestibility\ (kg/kg) = 1 - \frac{AIA\ of\ TMR\ sample}{AIA\ of\ faecal\ sample} \qquad \text{[Equation 3.10]}$$

3.3. Forage fermentation analysis

3.3.1. Volatile fatty acids (VFAs)

The content of total volatile fatty acids (VFA), including lactate, ethanol, acetate, propionate, iso-butyrate and butyrate of pooled forages, were determined at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) by gas chromatography (SOP: S1173, Sciantec Analytical, UK) and high-performance liquid chromatography (SOP: S1155, Sciantec Analytical, UK).

3.3.2. Ammonia nitrogen (NH_3-N)

Forage ammonia nitrogen was determined using an automated titrator (Buchi Labortechnik AG CH-9230, Flawil, Switzerland and FOSS 1030, FOSS, Warrington, UK) following the method of MAFF (1986). Approximately 20 g of fresh, pooled forage samples was weighed and transferred into a 480 ml clean shaking bottle (167

× 81 mm; Fisher Scientific, UK), which contained 100 ml of cold distilled water. Samples bottles were then placed into a shaker plate (~275 shakes/min) and shaken for 60 min. Following shaking, the liquid extract was filtered through 150 mm filter paper (Whatman number 1). After the addition of 5 ml of filtrate extract and 6 ml of magnesium-oxide ($Mg(OH)_2$; 17 g of $Mg(OH)_2$ dissolved in 100 ml of deionised water) to the Kjeldahl digestion tube, the sample was then analysed by an automated titrator. The content of NH_3-N of forages was calculated as follows;

$$NH_3\text{-}N\ (g/kg\ DM) = \frac{7 \times T \times (120 - 0.02 \times W)}{W \times 10} \quad \text{[Equation 3.11]}$$

$$NH_3\text{-}N\ (g/kg\ of\ Total\ N) = \frac{NH3 - N\ (g/kg\ DM)}{Total\ N\ (g/kg\ DM)} \times 1000 \quad \text{[Equation 3.12]}$$

Where,

T = Titre reading – blank

W = sample dry matter (g/kg)

3.3.3. Forage pH

Forage pH was determined using a digital pH meter with a probe (Jenway 3505; Bibby Scientific Limited, Staffordshire, UK) following the method of MAFF (1986). Initially, the pH meter was calibrated using pH buffer 4 and 7. Approximately 50 g of fresh forage sample was transferred into a 480 ml clean glass bottle (167 × 81 mm; Fisher Scientific, UK), which contained 150 ml of cold distilled water. The sample was shaken every 15 min for 1 h, and then the pH determined. The probe of the pH meter was washed with cold distilled water before analysing the next sample.

3.4. Amino acids (AA)

The content of individual amino acids of pooled forage and TMR samples was determined at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) by ion-exchange chromatography according to ISO 13903 (2005).

3.5. *In situ* degradability

The *in situ* degradability of forage DM and CP was determined following the method reported by Huntington and Givens (1997). Three Holstein-Friesian dry cows fitted with 10.2 cm in diameter rumen cannula (#1S, Bar Diamond Inc., Parma, ID, USA)

were used in the *in situ* degradability study. *In situ* nylon bags (Sericol, Kent, UK) with a pore size of 42 µm were filled with 8 ± 0.1 g of fresh forage and placed in the rumen of dry cows (n = 3) in duplicate 30 min post-feeding, and recovered after 4, 8, 16, 24, 48 and 96 h. Following rumen incubation, all bags with residues were immediately placed in cold water to stop further microbial degradation and rinsed for 10 min using an automatic washing machine (Model WAK24210GC, Robert Bosch, Uxbridge, UK). For each forage, 0 h time points were also determined without rumen incubation. All bags were then dried for 48 h in a forced-air oven at 60°C. The dried residues were composited within time point and animal and then analysed for total N using a LECO (Section 3.1.2). *In situ* degradation of DM and CP was calculated as follows;

$$DM\ degradation\ (\%) = \frac{DM\ intake - DM\ output}{DM\ intake} \times 100 \qquad \text{[Equation 3.13]}$$

$$CP\ degradation\ (\%) = \frac{CP\ intake - CP\ output}{CP\ intake} \times 100 \qquad \text{[Equation 3.14]}$$

The *in situ* DM and CP degradability data were fitted in Sigmaplot (Jandel, Erkrath, Germany) using the following exponential equation as proposed by Ørskov and McDonald (1979).

$$p = a + b\ (1 - \exp^{-ct}). \qquad \text{[Equation 3.15]}$$

where; p is the disappearance percentage at t time, a is the immediately soluble fraction, b is the potentially degradable fraction, c is the degradation rate (/h) of b, t is the incubation period (h).

The effective rumen degradability (ED) was calculated using an 8% per hour rumen passage rate (k) as:

$$ED = a + b\ (c / (c + k)). \qquad \text{[Equation 3.16]}$$

3.6. Particle size (PS) distribution of mixed ration and forages

The PS distribution of the TMR and forage samples were determined using a Penn State Particle Separator (PSPS) with screen pore (round-shaped) sizes of 4, 8, 19, 32.9 and 44 mm (Kononoff et al., 2003; ASABE, 2007; Maulfair et al., 2010). The PSPS was operated manually by shaking the PSPS on a flat table surface with a stroke length of 17 cm and 1.1 Hz shaking frequency which was adopted from Kononoff et al. (2003). The PSPS was shaken 5 times with 2 complete turns

resulting in a total of 40 shakes for each sample. Following shaking, the DM content of each fraction was analysed (section 3.1.1).

3.7. Milk analysis

3.7.1. Milk composition

The concentrations of milk fat, protein, lactose, somatic cell count (SSC) and milk urea were analysed at National Milk Laboratories (Wolverhampton, UK) using near mid-infrared spectrophotometry (ISO 21543, 2020).

3.7.2. Milk fatty acid extraction

Milk fatty acid (FA) was extracted according to the procedure described by Feng et al. (2004). Frozen milk samples were placed in a hot water bath (40ºC) for 30 min and shaken every 10 min to dissolve the milk fat. Milk samples were weighted according to the morning and evening milk yield, and 30 ml transferred into a 50 ml plastic centrifuge tube. Samples were then centrifuged at 17,800 g for 30 min at 4°C using a Beckman Avanti 30 centrifuge. After centrifugation, approximately 1.0 g of the upper fat-cake layer was transferred to a 2 ml Eppendorf-tube and placed in an incubator (40°C) for 20 min until the cake melted. Eppendorf tubes were then centrifuged at 19,300 g for approximately 30 min at room temperature using a micro-centrifuge (MSE Micro Centaur; Sanyo Gallenkamp, Loughborough, UK). Following centrifugation, the top lipid layer was separated by pipetting from the middle fat-protein and bottom water layer into a 0.5 ml microcentrifuge tube, and stored at -20ºC prior to methylation.

3.7.3. Milk fatty acid methylation

Methylation of the separated milk lipids was conducted according to the procedure described by Christie (1982). Two reagents (methylation and termination) were prepared prior to methylation. The methylation reagent was prepared by mixing 0.4 ml 30% (1 M) sodium-methoxide (NaOMe; Fisher Scientific, UK) with 1.75 ml methanol (CH_3OH; Fisher Scientific, UK). The termination reagent was prepared as follows; 1g of oxalic acid ($C_2H_2O_4$; Fisher Scientific, UK) was weighed into a 50 ml glass reagent bottle and placed in a hot air oven at 105°C for 30 min to remove any moisture. After drying, the reagent was cooled in a desiccator and 30ml of diethyl-ether (Fisher Scientific, UK) added. The solution was then ready to use for at least 2 weeks.

The extracted lipid was melted by placing in an incubator at 60°C for 20 min. Approximately 40 mg of extracted lipid was accurately weighed and transferred into a 10 ml extraction tube that had been pre-rinsed with hexane, followed by 40 µl of methyl acetate, and 2 ml of hexane added and vortexed for 30 sec. Afterwards, 40 µl of methylation reagent was added to each tube, vortexed for 2 min and then stood for 10 min to complete the chemical reaction. Then 60 µl of termination reagent was added to each tube, vortexed for 30 sec and then a scoop (~200 mg) of calcium-chloride added. After the addition of calcium chloride, all tubes were vortexed and left to stand for a further 60 min. All tubes were then centrifuged at 2600 g for 20 min at 4ºC, and the top layer (fatty acid methyl-ester; FAME) was transferred to gas chromatography (GC) vials.

The GC vials were placed in a gas chromatography analyser (model HP6890, Germany) fitted with an automatic sampler, flame-ionization detector (FID) and a CPSil88 column (100 m × 0.25 mm i.d. × 0.2 µm film, Agilent Technologies, UK), and the FAME determined as described by Lock et al. (2006). The initial oven temperature was 70°C which was maintained for 2 min, followed by an increase of 8°C per min to reach 110°C, maintained for 4 min, then increased by 5°C per min to reach 170°C, maintained for 10 min, and finally increased by 4°C per min until the temperature reached 225°C and maintained for 15 min. Each sample took approximately 60 min for a complete run with a post run time for the next sample was 2 min at 70°C. A mixed FAME (FAME Mix C4-C24, Sigma-Aldrich, UK) was used as a reference standard to check recoveries and correction factors for individual FA, and run after every 10 samples to determine any volatile FA loss. The individual FA peaks were recognised by matching retention time with the reference standard and then corrected using the recovery factor (Sinclair et al., 2015; Tayyab et al., 2018a).

3.8. Feed fatty acids

The FA content of dried forage and TMR samples was determined as described by Jenkins (2010). The following three reagents were prepared before starting the analysis.

Sodium methoxide (CH_3NaO) 0.5 M solution: 6 ml of 30% CH_3NaO (Sigma-Aldrich, UK) was mixed with 35 ml of methanol (CH_3OH; Fisher Scientific, UK) in a 50 ml

polypropylene tube (Sigma-Aldrich, UK). The prepared solution was used within 24 h.

Potassium carbonate (K_2CO_3) 6% solution: 12 g of powdered K_2CO_3 (Sigma-Aldrich, UK) was dissolved in 200 ml of cold distilled water.

Methanolic HCl 5% solution: In a fume cabinet, 250 ml conical round bottom flask was placed in a 2.5 L beaker containing ice, ensuring no ice enters into the conical round bottom flask. A piece of magnet was then added. Afterwards, the beaker was placed on a magnetic stirrer, and 100 ml CH_3OH was added into a conical flask followed by 10 ml of acetyl chloride (CH_3COCl) was added dropwise, ensuring not a single drop comes into contact with ice. The prepared solution was used within 24 h.

Internal standard (IS) solution: 100 mg of nonadecanoic acid (C19; Sigma-Aldrich, UK) was accurately weighed and dissolved in 50 ml CH_3OH using a hot (~40ºC) water bath for 20 min.

Approximately 500 mg of dried ground sample was weighed into a 25 ml pyrex screw cap culture tube (16x125 mm) pre-rinsed with hexane, followed by 1 ml of internal standard solution and 2 ml of 0.5 M CH_3NaO added. Samples were then vortexed for 30 sec, incubated in a 50°C water bath for 10min and subsequently cooled for 5 min. After cooling, 3 ml of 5% methanolic HCl was added, vortexed lightly and incubated in a water bath at 80°C for 10 min. Samples were then removed from the hot water bath and cooled for 8 min. After cooling, 3 ml of hexane and 10 ml of 6% K_2CO_3 was added and vortexed for 5 min. One gram of anhydrous sodium sulphate and 1g of activated charcoal powder was added to each sample, followed by centrifuging at 1600 g for 10 min at 4ºC. After centrifugation, the top hexane layer containing FAME was transferred to a GC vial using a glass pipette and filtered using a syringe filter fitted with a 0.23 µm cellulose acetate membrane (Sigma-Aldrich, UK). The GC vials were placed in a gas chromatography analyser (model HP6890, Germany) fitted with an automatic sampler, flame-ionization detector (FID) and a CPSil88 column (100 m, Agilent Technologies, UK), and the FAME determined as described by Lock et al. (2006). The individual FA content of the feed samples was calculated as follows;

$$\textit{Corrected individual FA area (g/100 g)} = \frac{Individual\ FA\ area}{100 - IS\ area} \times 100 \quad \text{[Equation 3.17]}$$

Corrected total area = Total area – internal standard area [Equation 3.18]

$$\textit{Total FA content (mg)} = \frac{Corrected\ total\ area}{IS\ area} \times \textit{IS (mg) added} \times 2 \quad \text{[Equation 3.19]}$$

Individual FA content (mg/g DM)

$$= \frac{Total\ FA\ content\ (mg)}{100} \times \textit{corrected individual FA area (g/100 g)} \quad \text{[Equation 3.20]}$$

3.9. Plasma and urine metabolites

Blood samples were collected from cows by jugular venepuncture into sodium heparinised vacutainer tubes for ammonia, β-hydroxybutyrate (BHB), urea determination and into vacutainers (BD Vacutainer, Plymouth, UK) containing fluoride oxalate for glucose determination. After collection, samples were placed on ice and then centrifuged at 1600 g for 15 min at 4ºC. After centrifugation, plasma was separated into a 2 ml screw-cap microcentrifuge tube (Cole-Parmer, UK) using a 2.5 ml plastic pipette. The concentration of plasma NH_3 was analysed immediately after plasma extraction using kit-catalogue no AM 1015 and a Cobas Mira Plus Auto-analyser (ABX Diagnostics, Bedfordshire, UK), and the rest of the samples were stored at -20°C for subsequent analysis. Plasma concentrations of glucose, BHB and urea were analysed (Randox Laboratories, Antrim, UK; Kit-Catalogue no. GL 1611, RB 1008 and UR 221, respectively) using a Cobas Mira Plus Auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described by Sinclair et al. (2012). For the determination of urinary urea-N, pooled urine sample was diluted 20-fold and then analysed using kit-catalogue no. UR 221 on a Cobas Miras Plus Auto-analyser (ABX Diagnostics, Bedfordshire, UK).

3.10. Urinary nitrogen

Sub-samples of urine was bulked within cow, filtered through N free filter paper, and analysed for total N by the Kjeldahl method according to AOAC (2012; 976.06). Approximately 1 g pooled urine sample was transferred into a pre-labelled, dried Kjeldahl tube followed by a piece of N free filter paper and two Kjeltabs (Fisher Scientific, UK) added to each tube. In a fume cabinet, 15 ml of concentrated sulphuric acid was added to each tube and then heated on the digestion block (Foss Digestor Unit, Hilleroed, Denmark) at 175ºC for 1 h to evaporate water. Samples were then digested at 420ºC for 45 min in a digestion block until the content of the tubes turned green colour. Following digestion, the tubes were cooled for 15 min,

and then 75 ml of cold distilled water added. Finally, titration was completed with 0.2 M HCl by placing the tubes into an automated Kjeldahl distillation unit (Foss Kjeltec 8200 Auto Distillation Unit, Fisher Scientific, UK), and total N as a percentage was recorded for each sample.

CHAPTER 4: Low protein diets for dairy cows based on red clover and grass silage: effects on performance, nutrient digestibility, blood metabolites and nitrogen use efficiency

4.1. Introduction

The increasing global price of soybean meal in association with tighter regulations on the disposal of slurry and manure, and greater public scrutiny of the sustainability of dairy farming has led to renewed interest in alternative strategies for feeding protein to dairy cows (Liu and VandeHaar, 2020a; AHDB, 2021). Two obvious approaches are to reduce dietary protein levels in the diet of dairy cows and to increase the utilisation of high protein homegrown forage legumes (Sinclair et al., 2014).

Increasing the concentration of dietary CP can improve the supply of MP to the intestine (Imran et al., 2017), but diets high in CP (>175 g CP/kg DM) typically result in low NUE, with increased RDP leading to the production of excess ammonia in the rumen (Hristov et al., 2015). This excess ammonia is absorbed into the blood, converted to urea in the liver, and excreted in the urine (Schwab and Broderick, 2017). Excreted N can subsequently be lost to the environment contributing to climate change and the deterioration of terrestrial and aquatic ecosystems (Castillo et al., 2000; Dijkstra et al., 2013; Whelan et al., 2013). In contrast, feeding low CP diets (<165 g CP/kg DM) can reduce urinary N excretion by increasing N utilisation in dairy cows (Olmos Colmenero and Broderick, 2006). However, concentrations lower than 150 g/kg DM may decrease milk production by reducing DM intake in high yielding dairy cows (Hristov and Giallongo, 2014).

Red clover is the most popular forage legume grown globally after lucerne (Boller et al., 2010) and is common in dairy cattle diets (Moorby et al., 2016). Red clover silage contains approximately 170 to 190 g/kg DM of CP under typical UK silage management conditions compared with grass silage, which ranges from 100 to 160 g CP/kg DM (Dewhurst et al., 2010; Johnston et al., 2020). Red clover can also be grown in a range of conditions and produce high DM yields without N fertiliser input (Frame et al., 1976). Several studies have reported that the inclusion of up to 60% red clover silage in the forage component of dairy cow diets has improved DM intake, milk yield (Moorby et al., 2009; Johansen et al., 2018), and C18 PUFA content in milk (Moorby et al., 2009; Dewhurst, 2013). The use of red clover

compared to grass silage has also been reported to increase the efficiency of MCP synthesis in the rumen, and improve the omasal flow of AA (Moorby et al., 2009, 2016) except for methionine, which was lower in cows fed red clover compared to grass silage (Lee et al., 2009).

It has been shown that dietary CP concentrations can be reduced to around 140-150 g/kg DM without any significant impact on dairy cow performance, health or fertility if the diets are formulated appropriately to maximise MCP synthesis and supply sufficient MP (Sinclair et al., 2014). However, the effect of low CP diets that are based on high inclusion rates of red clover is unclear. Most studies that have evaluated the inclusion of legume silages have fed CP concentration of approximately 170 g/kg DM, and limited knowledge exists on reducing dietary protein levels in red clover silage-based diets on milk performance and N utilisation (Moorby et al., 2016; Broderick, 2018; Schulz et al., 2018). It is anticipated that such dietary conditions will result in an excess of RDP as most of the N in forage legumes is released rapidly in the rumen (Sinclair et al., 2009), will be deficient in MP and may be imbalanced in AA (Vanhatalo et al., 2009), which can lead to a reduced milk yield (Westreicher-Kristen et al., 2018). However, the RDP content of red clover silages may be less than other legumes due to the presence of PPO (Jones et al., 1995a; Cassida et al., 2000). It was hypothesised that feeding low protein diets based on red clover and grass silage will improve N utilisation in dairy cows without affecting milk performance. The objective of this study was to determine the effects of reducing dietary CP concentration in a red clover and grass silage-based diet on intake, milk performance, diet digestibility, and NUE in dairy cows.

4.2. Materials and methods

4.2.1. Animals and housing

All procedures involving animals were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (amended, 2012) and received local ethical approval. The current study was conducted from October 2018 to January 2019.

Eighteen multiparous lactating dairy cows producing (mean ± SD) 45.3 ± 5.72 kg milk/d, 71 ± 14 DIM, 690 ± 48 kg LW, and 2.6 ± 0.31 BCS (where 1 = emaciated and 5 = obese, Scored to 0.25 units; Ferguson et al., 1994) were used. All cows

were housed in the same area of an open span building fitted with free stalls and super comfort mattresses. Stalls were bedded twice weekly with sawdust and lime, and with automatic scrapers that scraped the passageways at 6 h intervals. All cows had *ad-lib* access to fresh drinking water.

4.2.2. Experimental design

The study was a 3×3 Latin square design with 3 periods and 3 dietary treatments. Experimental periods were 28 days in duration, which included a 21-day adaptation to the diets and a 7-day sampling period. Cows were blocked by milk yield, DIM and BCS, and randomly assigned to 1 of 3 dietary treatments. The experimental diets were formulated to supply 3 different levels of dietary CP: 175 (High, **H**), 165 (Medium, **M**) or 150 (Low, **L**) g CP/kg DM.

4.2.3. Diets and feeding

The animals were fed the diets as a TMR that was formulated to produce 37 kg of milk per day according to Thomas (2004a) and to be isoenergetic and to contain a similar MP content for H and M that met requirements, and 0.95 of requirements for L, with the carbohydrate source and rumen bypass protein content of the concentrates altered (Table 4.1). The forage to concentrate ratio was 52:48 (DM basis), and the red clover was fed 50:50 (DM basis) with grass silage. The red clover (*Trifolium pratense*) and first cut grass silage (*Lolium perenne*) were grown as monocultures and mown at a leafy stage on 23 May and 10 June 2018, respectively, wilted for 24 h and harvested with a self-propelled, precision-chop forage harvester (John Deere 7840i, Nottinghamshire, UK). Both forages were ensiled in separate concrete clamps using an additive (Axphast Gold; Biotal, Worcestershire, UK) applied at the rate of 2.0 litre per tonne.

The dietary ingredients were mixed for 10 minutes using a Hi-spec forage mixer calibrated to ± 0.1 kg and fed through roughage intake control (**RIC**; Insentec B.V., Marknesse, The Netherlands) feeders fitted with automatic animal identification and weighing system calibrated to ± 0.1 kg. Fresh feed was delivered once daily at approximately 0800 h at the rate of 1.05 of the previously recorded intake, with refusals collected 3 times weekly prior to feeding.

Table 4.1. Dietary ingredients and predicted chemical composition of the high (H), medium (M) or low (L) CP diet based on red clover and grass silage fed to dairy cows.

	Diet[1]		
Item	H	M	L
Dietary ingredients (g/kg DM)			
Red clover silage	262	262	262
Grass silage	262	262	262
Rolled wheat	144	156	173
Soy hulls	144	156	173
Molassed sugar beet	77.0	77.0	77.0
Soybean meal	74.9	8.33	0.00
SoyPass[2]	0.00	41.6	20.8
Rapeseed meal	12.5	4.16	0.00
RapeTech[3]	0.00	8.33	8.33
Rumen protected fat	18.7	18.7	18.7
Minerals and vitamins[4]	5.00	5.00	5.00
Predicted composition[5] (g/kg DM)			
Forage: concentrate (DM basis)	0.52	0.52	0.52
ME (MJ/kg DM)	11.9	11.9	11.9
Crude protein (CP)	175	165	150
MPE	104	104	98
MPE (% of requirements)	100	100	95
MPN	118	115	106
MPN (% of requirements)	118	111	102

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets.

[2]Xylose-treated soybean meal (KW Alternative Feeds, Staffordshire, UK)

[3]SC Feeds, Nantwich, the UK.

[4]Mineral/vitamins premix (KW Alternative Feeds, Leeds, UK) providing (g/kg) 220 calcium, 30 phosphorus, 80 magnesium, 80 sodium, (mg/kg) 760 copper, 30 selenium, 1 000 000 IU vitamin A, 300 000 IU vitamin D3, 3000 IU vitamin E, 2.5 mg/kg vitamin B12, 135 mg/kg biotin.

[5]The predicted composition was calculated using a DietCheck ration formulation software. ME = metabolisable energy; MPE = metabolisable protein-rumen energy limited; MPN = metabolisable protein-rumen nitrogen limited.

4.2.4. Experimental routine

Forage samples were collected twice weekly, dried in a forced-air oven at 105°C and the forages adjusted to achieve the desired ratio. Fresh forages and experimental diets were sampled daily during the final week of each period, stored at -20°C and pooled within period before subsequent analyses. Additional TMR samples were collected at 0, 4, 8, and 24 h post-feeding on day 1 to 3 of each sampling week. Fresh forage samples were also collected daily approximately at 1000 h from the clamps on days 22 to 24 of each period. All TMR and forage samples were separated into six fractions using a modified PSPS (Tayyab et al.,

2018a); > 44, 33 to 44, 19 to 32.9, 8 to 19, 4 to 8, and < 4 mm by manual shaking (Kononoff et al., 2003).

Cows were milked twice daily at approximately 0600 h and 1600 h in a 40-point internal rotary parlour (Westfalia, GEA Milking System, Germany). During the final week of each period, milk yield was recorded at each milking, and 4 samples were collected at 2 consecutive morning and evening milkings for subsequent analyses of milk composition. Live weight and BCS were recorded following the afternoon milking at the start and end of each study period.

Faecal grab samples (approximately 350 g/d/cow) were collected from 12 cows at 1000 and 1600 h for 5 consecutive days during the final week of each study period and stored at -20°C for subsequent analyses. Spot urine samples (approximately 250 ml/cow/sample) were collected from 12 cows on day 22, 24, 26 and 28 at 0730, 1130, 1530 and 1630 h in each study period by manual stimulation of the area around the vulva. Following pH measurement, all urine samples were immediately acidified to pH < 3 using 20% H_2SO_4 (v/v) to avoid volatilisation of N compounds before storage at -20°C for subsequent analysis of total N. This sampling routine was undertaken to account for possible diurnal and day to day variations in urinary N concentration (Schulz et al., 2018).

Blood samples were collected by jugular venepuncture from 12 representative cows into heparinised and fluoride oxalate tubes (Becton Dickinson and Company, New Jersey) over 2 consecutive days at 0800, 0900, 1100 and 1300 h in the final week of each study period. Following collection, the samples were centrifuged at 1600 × g for 15 min to separate the plasma which was immediately analysed for ammonia, with further sub-samples stored at -20°C for subsequent analysis of urea, glucose and BHB.

4.2.5. *In situ* degradability of the forages

Three rumen-cannulated Holstein-Friesian dry cows with a mean LW of 650 ± 28 kg were housed in a straw bedded metabolism unit and fed a basal ration at maintenance level with a concentrate to forage ratio of 21:79 on DM basis (Thomas, 2004). The mixed ration contained (DM basis) 264 g/kg lucerne silage, 176 g/kg maize silage, 353 g/kg chopped wheat straw, 86 g/kg spey syrup (Trident, AB Agri Ltd., Lynch Wood, UK), 90 g/kg protein blend (KW Alternative Feeds, UK), 12 g/kg magnesium chloride, 12 g/kg minerals, 4 g/kg provimi LiFT (Provimi, North

Yorkshire, UK) and 2 g/kg DM of Vistacell Ultra (AB Vista, Wiltshire, UK). Dietary ingredients were mixed with the same forage mixer and offered twice daily at 0800 and 1630 h. All cows had continuous access to fresh drinking water.

The *in situ* degradability of red clover and grass silages was determined as described in Chapter 3, Section 3.5.

4.2.6. Chemical analyses

Sub-samples of forage and TMR were bulked by study period and analysed according to AOAC (2012) for DM (934.01, intra-assay CV of 1.09%) as described in Section 3.1.1. Dried feed samples were ground in a Wiley mill (Thomas Scientific, Philadelphia) through a 1.0 mm sieve prior to analyses for ash (942.05), CP (988.05) and ether extract (920.39) with an intra-assay CV of 0.39, 0.67 and 9.80%, respectively (AOAC, 2012), and AIA (intra-assay CV of 1.93%) content as per the method of Van Keulen & Young (1977) as described in Chapter 3. Acid detergent fibre and NDF were determined as per the method of Van Soest et al. (1991) using heat-stable α-amylase for NDF analysis (Sigma, Gillingham, UK, intra-assay CV of 1.00 and 2.94% for NDF and ADF, respectively) as described in Sections 3.1.6 and 3.1.7, respectively. The water-soluble CP fraction (intra-assay CV of 0.11%) of all forages was determined (Section 3.1.3) according to Weisbjerg et al. (1990). Forage samples were also analysed for ADIN (intra-assay CV of 4.76%) as per the method described by Licitra et al. (1996; Section 3.1.8). Forage ammonia-N and pH were determined following the method of MAFF (1986; Sections 3.3.2 and 3.3.3, respectively). The content of total VFA, including lactate, ethanol, acetate, propionate, iso-butyrate and butyrate of all forages, were analysed at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) using gas and high-performance liquid chromatography (Section 3.3.1). All TMR and forage samples were analysed for PS distribution using a modified PSPS as reported by Tayyab et al. (2018a; Section 3.6).

Milk samples were analysed for fat, protein, lactose and urea by near-midinfrared at National Milk Laboratories (NML, Wolverhampton, UK; Section 3.7.1). Fatty acids in milk were analysed by extracting milk fat by centrifugation and methylation using sodium methoxide according to the method of Feng et al. (2004) as described in Sections 3.7.2 and 3.7.3, respectively. The FAME of the feed was prepared according to the protocol of Jenkins (2010) as described in Chapter 3, Section 3.8.

The individual FAME was determined by Gas-Liquid Chromatography (Hewlett Packard 6890, Wokingham, UK), fitted with a CP-Sil 88 column (100 m × 0.25 mm i.d. × 0.20 um film, Agilent Technologies, Santa Clara, California, USA) as described in Section 3.7.3.

Faecal samples were composited by cow and period, and dried in a forced-air oven at 60°C until constant weight. The dried faecal samples were then milled using an electric grinder (SG20U, Electric Grinder, UK) and analysed for AIA, total N, NDF, ADF, and ash as described in Chapter 3. Sub-samples of urine were bulked by cow for each period and pooled urine samples were then filtered through N free filter paper, and subsequently analysed for total N (976.06) by Kjeldahl (AOAC, 2012) as described in Section 3.10.

Plasma samples were analysed for ammonia (Randox Laboratories, County Antrim, UK; Kit-Catalogue no. AM 1015, intra-assay CV of 7.56%) within 1 h of collection, while BHB, glucose and urea (Randox Laboratories, County Antrim, UK; Kit-Catalogue no. RB 1008, GL 1611 and UR 221 with an intra-assay CV of 5.12, 0.87 and 4.82%, respectively) were analysed using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in Section 3.9.

4.2.7. Calculations

Dry matter intake was calculated from the daily fresh feed intake for each cow that was recorded by RIC system and the analysed DM content of the TMR (Equation 3.1). Nutrient intake, faecal output, digested nutrients, and apparent total tract digestibility coefficients of DM, OM, N, NDF, and ADF were determined (Equation 3.9) using AIA as an internal marker as per the method of Van Keulen & Young (1977). Milk yield based on 4% milk fat (4% FCM) was determined by adjusting the milk yield to 40 g of fat per kg milk, and energy corrected milk yield (ECM) was computed as (3.14 MJ/kg) = milk yield × (383 × fat (g/kg) × 100 + 242 × protein (g/kg) × 100 + 165.4 × lactose (g/kg) × 100 + 207)/3140 (Sjaunja et al., 1991). Apparent NUE was calculated as milk N output/dietary total N intake, with the N excretion in milk determined as total milk protein/6.38. The PS geometric mean ($\mathbf{X_m}$) and the standard deviation of X_m were determined using the equations by ASABE (2007);

$$\text{Geometric mean length (Xm)} = \log^{-1} \frac{\sum (Mi \ \log mXi)}{\sum Mi}$$

$$\text{Standard deviation (SDgm)} = \log^{-1}\left[\frac{\sum \text{Mi}\,(\log \text{mXi} - \log \text{Xg})2}{\sum \text{Mi}}\right]^{1/2}$$

Where M_i is a quantity on i^{th} screen, X_i is diagonal of the screen opening of the i^{th} screen, mX_i is mean geometric size of feed particles on i^{th} screen = $[X_i \times X_{i-1}]^{1/2}$, $X_{(i-1)}$. The distribution of PS was quantified by dividing each fraction weight by the total of the fractions. The physically effective fibre (**PeNDF**) was calculated by multiplying the physical effectiveness factor with the dietary NDF content (Lammers et al., 1996; Maulfair et al., 2010).

The *in situ* DM and CP degradability data were fitted in Sigmaplot (Jandel, Erkrath, Germany) using the exponential equation as described in Chapter 3, Section 3.5.

4.2.8. Statistical analysis

Data were analysed by analysis of variance (ANOVA) as a Latin rectangle design using GenStat 18th edition (VSN International Ltd, Oxford, UK) with diet and period as fixed effects and cow as random effect. All data were checked for normality using descriptive statistics before running the ANOVA model in GenStat. The model used was $Y_{ijk} = \mu + D_i + P_j + A_k + E_{ijk,}$

where Y_{ijk} and μ represent the dependent variable and total mean, and D_i, P_j, A_k, and E_{ijk} as the diet, period, animal and residual error, respectively. Plasma parameters and particle fractions were analysed by ANOVA as repeated measures that included the fixed effect of sampling time in the model. Tukey's test was conducted post hoc to determine treatment means that differed. Results are presented as the least square mean of each treatment and standard error of the mean (SEM). Values were considered significant when $P < 0.05$ and a tendency when $P < 0.10$.

4.3. Results

4.3.1. Forage and diet characteristics

The red clover silage contained a higher DM content than the grass silage, but was lower in NDF and total fat (Table 4.2). Both forages had a CP content above 160 g/kg DM, with the concentration in the red clover being 11 g/kg DM higher than the grass silage. The soluble CP content was lower in the red clover compared to the grass silage. Similarly, the acetate and lactate content of the red clover silage were

16.3 and 26.0 g/kg DM lower, respectively, than the grass silage, whilst the concentration of ammonia-N was similar in both forages, with a mean of 54.5 g/kg of total N. Both silages also had a similar long-chain PUFA content, with the content of C18:3 n-3 in the grass silage being 3.88 g FA/kg DM higher than the red clover.

Table 4.2. Nutrient composition (g/kg DM), fermentation profile, fatty acid content and particle size distribution of red clover silage, grass silage, and the high (H), medium (M) or low (L) CP diet fed to dairy cows.

Item	Forage		Diet[1]		
	Red clover silage	Grass silage	H	M	L
Dry matter (g/kg)	421	320	489	481	481
Organic matter	877	892	906	906	906
Ash	123	108	94.3	94.0	93.6
Crude protein	178	167	174	165	153
Water soluble crude protein (g/kg CP)	367	527			
Neutral detergent fibre	371	510	383	384	376
Acid detergent fibre	311	309	275	281	281
Acid detergent insoluble N	6.81	5.82			
Ether extract	14.9	35.9	25.6	26.0	26.8
Fermentation profile (g/kg DM)					
pH	4.26	3.98			
Ammonia-N (g/kg total N)	54.3	54.7			
Lactate	64.0	90.0			
Ethanol	0.99	3.74			
Acetate	12.2	28.5			
Propionate	0.25	0.75			
Iso-butyrate	0.29	-			
Butyrate	0.13	0.27			
Acetate: Propionate	0.12	0.12			
Fatty acid (g/kg DM)					
C16:0	1.86	2.43	6.11	5.46	5.89
C18:0	0.32	0.26	0.77	0.62	0.64
C18:1*C*9	0.28	0.42	4.13	3.67	3.80
C18:2n-6	2.01	2.21	3.85	3.53	3.71
C18:3n-3	4.08	7.96	3.77	3.70	3.88
ΣFA	12.0	17.5	21.9	19.4	20.6
Fractions[2] (% DM)					
> 44 (mm)	0.00	0.00	0.00	0.00	0.00
33 to 44 (mm)	4.50	4.80	2.84	2.49	2.58
19 to 32.9 (mm)	19.1	24.0	16.0	15.3	14.4
8 to 19 (mm)	50.5	56.1	45.3	44.8	45.5
4 to 8 (mm)	9.71	8.60	10.2	9.31	9.94
< 4 (mm)	16.2	6.54	25.7	28.1	27.5
X_m (mm)	15.3	18.4	12.7	12.3	12.3
SD_{gm}	2.04	1.78	2.19	2.21	2.19
pef $_{>4}$ (%)	83.8	93.5	74.3	71.9	72.5
pef $_{>8}$ (%)	74.1	84.9	64.1	62.6	62.5
peNDF $_{>4}$ (%)	31.1	47.7	28.4	27.6	27.2
peNDF $_{>8}$ (%)	27.5	43.3	24.5	24.1	23.5

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets. [2]Fractons of forages at 0 h post-feeding; DM = dry matter; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fibre.

Table 4.3. Particle size distribution of the high (H), medium (M) or low (L) CP diet based on red clover and grass silage fed to dairy cows at 0, 4, 8 and 24 h post feeding.

Item	Diet[1]			SEM	*P* value[2]		
	H	M	L		D	T	Int
Fractions[3]							
33-44 mm	2.68	2.60	2.79	0.289	0.734	0.746	0.371
0h	2.84	2.49	2.58				
4h	2.46	2.50	3.06				
8h	2.32	2.80	2.82				
24h	3.11	2.60	2.71				
19-32.9 mm	15.2	15.0	15.4	1.777	0.967	0.569	0.528
0h	16.0	15.3	14.4				
4h	13.4	14.8	15.7				
8h	15.4	15.0	15.9				
24h	15.9	15.0	15.5				
8-19 mm	44.7	45.0	45.2	1.967	0.931	0.244	0.618
0h	45.3	44.8	45.5				
4h	44.7	46.4	46.5				
8h	43.9	45.3	45.0				
24h	44.8	43.6	43.8				
4-8 mm	10.3	9.44	9.71	0.619	0.457	0.834	0.455
0h	10.2	9.31	9.94				
4h	10.5	9.27	9.42				
8h	10.7	9.11	9.25				
24h	9.66	10.1	10.2				
<4 mm	27.2	27.9	26.9	1.145	0.652	0.807	0.210
0h	25.7	28.1	27.5				
4h	29.0	27.1	25.3				
8h	27.7	27.8	27.0				
24h	26.5	28.7	27.7				
X_m mm	12.3	12.4	12.5	0.218	0.533	0.056	0.233
SD_{gm}	2.19	2.21	2.19	0.021	0.379	0.580	0.988
pef>4 (%)	72.8	72.1	73.1	1.145	0.652	0.807	0.210
pef>8 (%)	62.5	62.6	63.4	0.914	0.448	0.617	0.060
peNDF>4 (%)	27.8	27.7	27.4	0.571	0.838	0.799	0.211
peNDF>8 (%)	23.9	24.1	23.8	0.497	0.896	0.621	0.061

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets.[2]D = main effect of diet, T = main effect of time, Int = interaction between diet and time.
[3]Diets were separated into 5 fractions; 33-44, 19-32.9, 8-19, 4-8 and <4 mm; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fibre.

The mean PS of the grass silage was higher than the red clover silage, with an X_m of 18.4 and 15.3 mm for the grass and red clover silage, respectively. Also, the physically effective fibre in the grass silage was higher than the red clover silage, with a mean $peNDF_{>4mm}$ and $peNDF_{>8mm}$ of 47.7 and 43.3%, respectively. The DM, OM, NDF, ADF and EE concentration of the diets were similar, with means of 484 g/kg, 906, 381, 279 and 26.1 g/kg DM, respectively, whereas the CP concentration was 174, 165 and 153 g/kg DM in H, M and L, respectively (Table 4.2). The total FA

content in H was 1.9 g FA/kg DM higher than the other diets, which had a mean of 20 g FA/kg DM. The mean X_m, $pef_{>4mm}$, and $peNDF_{>4mm}$ were similar for all 3 diets, but there was a tendency for an interaction (P = 0.06) between diet and sampling time for $pef_{>8mm}$ (Table 4.3), the mean $pef_{>8mm}$ being lower at 4 h after the morning feed for the diet H, but at 24 h post-feeding $pef_{>8mm}$ was higher in H compared to the other 2 diets (Figure 4.1).

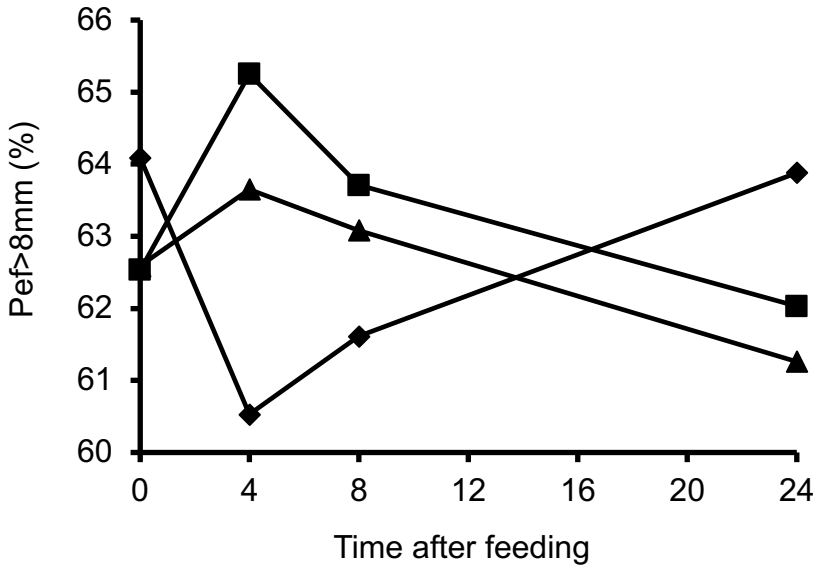


Figure 4.1. Physical effectiveness factor ($pef_{>8mm}$) of high (H, ♦); medium (M, ■); or low (L, ▲) CP diet based on red clover and grass silage. Pooled SEM = 0.914. Diet, P = 0.448, time, P = 0.617, diet × time, P = 0.060.

4.3.2. *In situ* forage degradability

There was a tendency for a higher (P = 0.094) soluble fraction (a) of DM in the red clover compared to the grass silage (Table 4.4). In contrast, the red clover silage had a lower (P = 0.014) potentially degradable fraction (b) of the DM compared to the grass silage. However, there was no effect (P > 0.05) of forage on the extent of degradation (a+b) and the rate (c) of the potentially degradable fraction of DM. The ED of DM was 7.12% higher (P = 0.030) in the red clover compared to the grass silage.

The soluble fraction of the CP was 101 g/kg higher (P = 0.011) in the grass silage compared to the red clover. In contrast, the potentially degradable fraction of CP was higher in the red clover, with a mean value of 549 g CP/kg, 109 g CP/kg higher (P = 0.004) than the grass silage. However, there was no effect (P > 0.05) of forage

on the extent of degradation and the rate of the potentially degradable fraction of CP, whilst the ED of the CP was 4.47% lower (P = 0.008) in the red clover than the grass silage.

Table 4.4. *In situ* DM and CP degradability coefficients of red clover and grass silage fed to dairy cows.

Item[1]	Forage		SEM	P value
	Red clover silage	Grass silage		
DM degradation coefficients (g/kg DM)				
a	278	246	1.1	0.094
b	565	611	0.8	0.014
a+b	844	857	0.5	0.124
c	0.08	0.07	0.007	0.243
ED	557	520	0.8	0.030
CP degradation coefficients (g/kg total N)				
a	323	424	1.6	0.011
b	549	440	1.3	0.004
a+b	872	864	0.5	0.318
c	0.10	0.09	0.009	0.416
ED	627	655	0.4	0.008

[1]DM = dry matter; CP = crude protein; a = soluble fraction; b = potentially rumen-degradable fraction; c = degradation rate of fraction b per hour; ED = effective rumen degradability at 8%/h rumen passage rate.

4.3.3. Feed intake and animal performance

The DM intake was 1.6 kg/d lower (P = 0.001) in cows receiving L at 23.5 kg DM/d than H or M, which had a mean of 25.1 kg/d (Table 4.5). However, total milk yield, ECM yield, and FCM yield were not affected by diet, with means of 34.8, 34.9, and 36.7 kg/d, respectively. Similarly, diet did not affect milk protein, fat or lactose content, with means of 31.7, 42.3 and 45.4 g/kg, respectively. In contrast, the MUN concentration was lowest (P = 0.018) in cows receiving L at 8.13 mg/dl, which was 2.07 mg/dl lower than in cows receiving H, with M having an intermediate value. Feed efficiency was 0.1 kg/kg DM higher (P < 0.05) in cows fed L compared to M, with cows receiving H having an intermediate value. Dietary treatment did not affect LW or BCS, although BCS change was numerically lower in cows fed L compared to M or H.

4.3.4. Nutrient intake and apparent total tract digestibility

The DM, OM, N and NDF intake was lower (P < 0.05) in cows receiving L compared to those receiving H or M (Table 4.6). The faecal output of DM, OM and ADF was highest (P < 0.05) in cows fed L, while the amount digested and digestibility of DM, OM, N, NDF, and ADF were lowest (P < 0.05) in cows receiving L compared with

the other 2 diets. In general, total-tract digestibility was similar between cows fed H or M, although total N digested was lower ($P < 0.001$) in cows fed M compared with those fed diet H.

4.3.5. Nitrogen output and efficiency

There was no effect of dietary treatment on milk or faecal N concentration, but urinary N content was lowest ($P < 0.001$) in cows fed L at 4.13 g/l, which was 1.38 g/l lower than those of fed H or M (Table 4.7). The N intake was lowest ($P < 0.05$) in cows fed L, whilst faecal and milk N output did not differ ($P > 0.05$) in cows fed any of the 3 diets. As a proportion of total N intake, N output in faeces was highest ($P = 0.001$) in cows fed L at 45.1%, which was 11% units higher than those fed H or M. Diet had an effect ($P < 0.001$) on the apparent NUE, which was approximately 21% higher in cows fed L than in those receiving H or M.

Table 4.5. Intake, milk performance, live weight and body condition of dairy cows fed high (H), medium (M) or low (L) CP diet based on red clover and grass silage.

Item	Diet[1]			SEM	*P* value
	H	M	L		
Intake (kg DM/d)	25.0[a]	25.2[a]	23.5[b]	0.33	0.001
Production (kg/d)					
Milk yield	35.0	34.7	34.6	0.51	0.810
ECM[2] yield	34.8	35.0	34.8	0.54	0.932
FCM[3] yield	36.1	37.0	36.8	0.75	0.692
Composition (g/kg)					
Fat	41.4	42.9	42.6	0.68	0.252
Protein	32.0	31.6	31.6	0.23	0.422
Lactose	45.2	45.6	45.4	0.15	0.164
Milk urea (mg/dl)	21.8[a]	19.7[ab]	17.4[b]	1.02	0.018
MUN[4] (mg/dl)	10.2[a]	9.20[ab]	8.13[b]	0.477	0.018
Yield (kg/d)					
Fat	1.45	1.48	1.47	0.030	0.692
Protein	1.11	1.09	1.09	0.015	0.391
Lactose	1.58	1.58	1.57	0.025	0.938
Feed efficiency					
Milk/DM intake	1.40[ab]	1.38[b]	1.48[a]	0.022	0.009
ECM/DM intake	1.39[ab]	1.38[b]	1.48[a]	0.027	0.030
Body performance					
LW[4] (kg)	685	680	684	4.5	0.713
LW change[6] (kg)	3.83	-2.77	-0.22	6.880	0.792
LW change (kg/d)	0.14	-0.10	-0.01	0.246	0.792
BCS	2.54	2.61	2.57	0.039	0.477
BCS change[6]	-0.01	0.04	-0.04	0.061	0.600

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets; [2]ECM = Energy corrected milk yield; [3]FCM = 4% fat-corrected milk yield; [4]MUN = milk urea nitrogen; [5]LW = live weight; [6]Change over the 28 days period.
Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 4.6. Intake, faecal output and apparent total-tract digestibility[1] (kg/d) of nutrients[2] in dairy cows fed high (H), medium (M) or low (L) CP diet based on red clover and grass silage.

Item		Diet[3]			SEM	*P* value
		H	M	L		
DM	Intake	25.4[a]	25.2[a]	23.6[b]	0.448	0.018
	Faecal output	6.13[b]	6.77[ab]	7.65[a]	0.408	0.050
	Digested	19.3[a]	18.5[a]	16.0[b]	0.402	<.001
	Digestibility (kg/kg)	0.759[a]	0.734[a]	0.677[b]	0.0144	0.002
OM	Intake	23.0[a]	22.9[a]	21.4[b]	0.40	0.018
	Faecal output	5.17[b]	5.72[ab]	6.49[a]	0.351	0.047
	Digested	17.9[a]	17.1[a]	14.9[b]	0.36	<.001
	Digestibility (kg/kg)	0.775[a]	0.752[a]	0.698[b]	0.0137	0.002
N	Intake	0.71[a]	0.67[a]	0.58[b]	0.012	<.001
	Faecal output	0.22	0.24	0.26	0.016	0.301
	Digested	0.48[a]	0.42[b]	0.32[c]	0.015	<.001
	Digestibility (kg/kg)	0.683[a]	0.635[a]	0.549[b]	0.0218	0.001
NDF	Intake	9.72[a]	9.68[a]	8.86[b]	0.190	0.006
	Faecal output	3.07	3.22	3.77	0.220	0.085
	Digested	6.65[a]	6.46[a]	5.09[b]	0.211	<.001
	Digestibility (kg/kg)	0.684[a]	0.669[a]	0.576[b]	0.0205	0.003
ADF	Intake	6.98	7.08	6.62	0.130	0.050
	Faecal output	2.68[b]	2.84[ab]	3.43[a]	0.193	0.031
	Digested	4.31[a]	4.25[a]	3.20[b]	0.166	<.001
	Digestibility (kg/kg)	0.616[a]	0.602[a]	0.487[b]	0.0242	0.002

[1]Measured using 12 cows (4 cows for each treatment group);
[2]DM = dry matter; OM = organic matter; N = nitrogen; NDF = Neutral detergent fibre; ADF = Acid detergent fibre. [3]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets. Means within a row with different superscript differ significantly ($P < 0.05$).

Table 4.7. Nitrogen output, efficiency, partitioning and urine pH in dairy cows fed high (H), medium (M) or low (L) CP diet based on red clover and grass silage.

Item	Diet[1]			SEM	*P* value
	H	M	L		
Concentration (g/kg)					
Diet	27.9[a]	26.4[b]	24.4[c]	0.02	<.001
Milk	5.04	4.97	4.97	0.052	0.567
Faecal	36.6	36.3	34.3	0.81	0.114
Urine (g/l)	5.76[a]	5.26[a]	4.13[b]	0.207	<.001
N output (g/d)					
Milk	175	171	174	3.1	0.597
Faecal	225	245	260	15.8	0.301
N partitioning (%)					
Faecal	31.7[b]	36.5[b]	45.1[a]	2.18	0.001
NUE[2]	24.7[b]	25.6[b]	30.4[a]	0.40	<.001
Urine pH	8.52	8.53	8.51	0.010	0.527

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets. [2]NUE = apparent nitrogen use efficiency. Means within a row with different superscripts differ significantly ($P < 0.05$)

4.3.6. Blood plasma metabolites

There was no effect of dietary treatment on the mean concentration of plasma ammonia, glucose or BHB, with means of 35.1 µmol/l, 3.69 and 0.76 mmol/l, respectively (Table 4.8). In contrast, plasma urea concentration was lowest (P = 0.011) in cows receiving L, at 3.02 mmol/l, but similar in cows receiving H or M, with a mean value of 3.79 mmol/l (Figure 4.2). There was an effect of time ($P < 0.05$) on all plasma metabolites, with plasma ammonia being lowest at 0900 and 1300 h, and highest at 1100 h (Figure 4.3a). Likewise, plasma glucose concentration decreased over time (Figure 4.3b). In contrast, plasma BHB (Figure 4.3c) and urea (Figure 4.2) concentration increased from 1 h pre-feeding to 5 h post-feeding.

Table 4.8. Plasma ammonia, β-hydroxybutyrate (BHB) and glucose concentration in dairy cows fed high (H), medium (M) or low (L) CP diet based on red clover and grass silage.

Item	Diet[1]			SEM	*P* value[2]		
	H	M	L		D	T	Int.
Ammonia (µmol/l)	35.2	35.6	34.4	2.36	0.690	<.001	0.768
BHB (mmol/l)	0.76	0.73	0.79	0.065	0.630	<.001	0.856
Glucose (mmol/l)	3.72	3.67	3.68	0.108	0.832	<.001	0.492

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets. [2]D = main effect of diet, T = main effect of time, Int. = interaction between diet and time. Means within a row with different superscripts differ ($P < 0.05$).

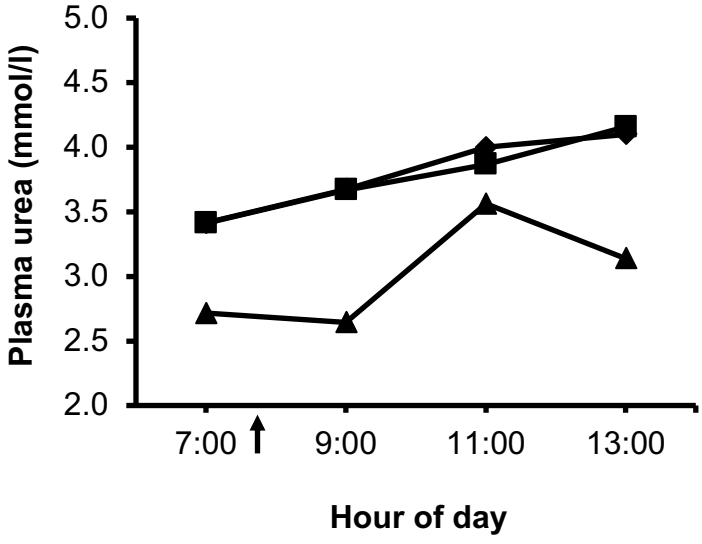


Figure 4.2. Plasma urea concentration in dairy cows fed a high (H, ♦), medium (M, ■) or low (L, ▲) CP diet based on red clover and grass silage. Pooled SEM = 0.276; diet, P = 0.011; time, P = 0.006; and diet × time, P = 0.598. Arrow indicates the feeding time.

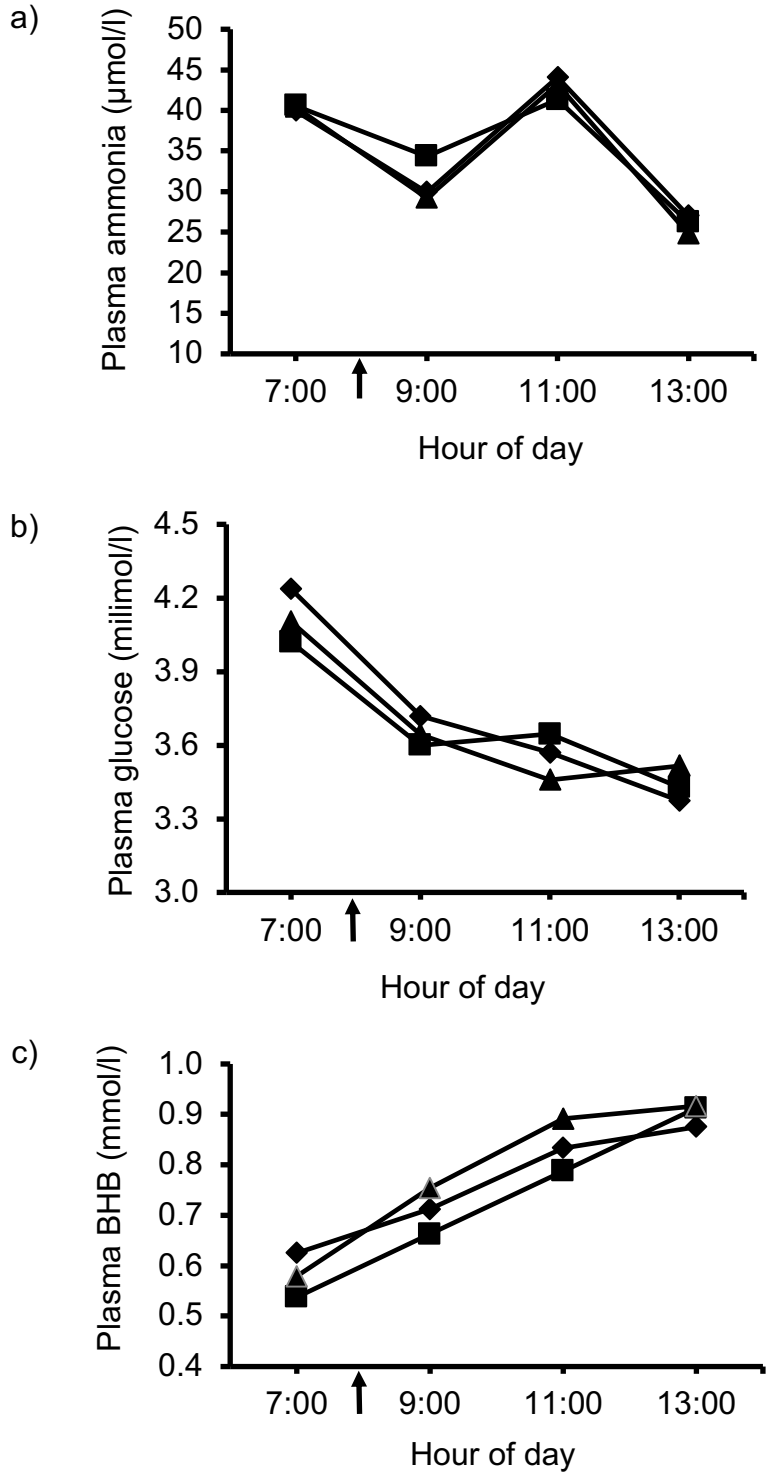


Figure 4.3. Plasma ammonia (a), glucose (b), and β-hydroxybutyrate (BHB) (c) concentration in dairy cows fed a high (H, ♦), medium (M, ■) or low (L, ▲) CP diet based on red clover and grass silage. For plasma ammonia, pooled SEM = 2.36; Diet, $P = 0.690$; time, $P < 0.001$; and diet × time, $P = 0.768$. For plasma glucose, pooled SEM = 0.065; Diet, $P = 0.630$; time, $P < 0.001$; and diet × time, $P = 0.856$. For plasma BHB, pooled SEM = 0.108; Diet, $P = 0.832$; time, $P < 0.001$; and diet × time, $P = 0.492$. Arrow indicates the feeding time.

4.3.7. Milk fatty acid profile

The highest ($P < 0.05$) concentration of milk C8:0, C11:0, C12:0, C15:0, C15:1 *c*10 and C16:1 was obtained in cows fed H, and lowest in cows fed M except for milk C15:0 and C15:1 *c*10, which were lowest in cows fed L (Table 4.9). In contrast, milk fat concentration of C18:2n-6 *c* was higher ($P = 0.002$) in cows receiving L or M than H. Similarly, milk fat content of C18:1 *t*9 and C18:1 *t*12 were highest ($P < 0.05$) in cows fed M and L, and lowest in cows fed L and H, respectively. There was no effect of diet on milk FA of chain length > C16:0 or C16:0 + 16:1, but < C16:0 was higher ($P = 0.040$) in cows when fed H compared to M. Also, dietary treatment did not affect the total milk fat content of saturated FA (SFA) or mono-unsaturated FA (MUFA), but the PUFA content was higher ($P = 0.017$) in cows receiving L than in those receiving H. The total content of milk linear odd chain FA (LOCFA) or odd-branch chain FA (OBCFA) was higher ($P < 0.05$) in cows fed H compared to L.

Table 4.9. Milk fatty acid composition (g/100 g) of dairy cows fed high (H), medium (M) or low (L) CP diet based on red clover and grass silage.

Item	Diet[1]			SEM	*P* value
	H	M	L		
C4:0	1.47	1.46	1.45	0.020	0.870
C6:0	1.39	1.37	1.39	0.011	0.348
C8:0	1.01[a]	0.98[b]	0.99[ab]	0.009	0.034
C10:0	2.54	2.47	2.54	0.048	0.448
C11:0	0.31[a]	0.29[b]	0.30[ab]	0.005	0.009
C12:0	3.39[a]	3.18[b]	3.29[ab]	0.049	0.015
C13:0	0.09	0.09	0.08	0.003	0.109
C14:0	12.08	11.78	11.94	0.088	0.073
C14:1	1.25	1.20	1.23	0.030	0.530
C15:0	1.26[a]	1.22[ab]	1.19[b]	0.016	0.014
C15:1 *c*10	0.23[a]	0.21[ab]	0.21[b]	0.001	0.023
C16:0	39.41	39.52	39.55	0.231	0.904
C16:1	1.82[a]	1.72[b]	1.73[ab]	0.030	0.031
C17:0	0.52	0.52	0.52	0.005	0.877
C17:1 *c*10	0.28	0.27	0.27	0.001	0.053
C18:0	7.68	7.79	7.90	0.130	0.479
C18:1 *t*8	0.25	0.26	0.26	0.010	0.676
C18:1 *t*9	0.11[ab]	0.14[a]	0.09[b]	0.014	0.034
C18:1 *t*10	0.99	1.01	1.04	0.039	0.702
C18:1 *t*11	0.72	0.75	0.74	0.012	0.345
C18:1 *t*12	0.15[b]	0.16[ab]	0.17[a]	0.003	0.022
C18:1 *c*9	19.15	19.51	18.97	0.220	0.228
C18:2n-6 *c*	1.96[b]	2.17[a]	2.17[a]	0.042	0.002
C18:2n-6 *t*	0.39	0.39	0.40	0.006	0.769
CLA *c*9, *t*11	0.80	0.81	0.81	0.010	0.669
CLA *t*10, *c*12	0.03	0.03	0.04	0.002	0.237
C18:3n-3	0.27	0.27	0.27	0.003	0.097
C18:3n-6	0.05	0.05	0.07	0.013	0.358
C20:0	0.01	0.01	0.01	0.001	0.965
C20:3n-3	0.13	0.13	0.13	0.003	0.907
C21:0	0.06	0.05	0.06	0.003	0.789
C22:0	0.04	0.04	0.04	0.001	0.516
EPA	0.12	0.11	0.12	0.005	0.615
DHA	0.06	0.02	0.03	0.022	0.366
<C16	25.0[a]	24.2[b]	24.6[ab]	0.20	0.040
C16 + 16:1	41.2	41.3	41.3	0.24	0.987
>C16	33.8	34.5	34.1	0.35	0.334
SFA[2]	71.3	70.8	71.3	0.25	0.301
MUFA[3]	25.0	25.2	24.7	0.24	0.309
PUFA[4]	3.81[b]	3.99[ab]	4.03[a]	0.056	0.017
LOCFA[5]	2.24[a]	2.17[ab]	2.14[b]	0.021	0.010
OBCFA[6]	2.75[a]	2.65[b]	2.62[b]	0.023	0.001

[1]Diet; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets. [2]SFA = Saturated fatty acids are defined as fatty acids with no double bonds. [3]MUFA = Monounsaturated fatty acids are defined as fatty acids with one double bond.
[4]PUFA = Polyunsaturated fatty acids are defined as fatty acids with more than one double bond.[5]LOCFA = Linear odd chain fatty acids; ∑LOCFA = (C11:0+C13:0+C15:0+C17:0+C21:0).
[6]OBCFA = Linear odd and branched chain fatty acids; ∑OBCFA = (C11:0+C13:0+C15:0+C15:1+C17:0+C17:1+C21:0).
Means within a row with different superscript differ ($P < 0.05$).

4.4. Discussion

4.4.1. Feed characteristics and particle size distribution

The chemical composition of the forages used in the current study was comparable to previous work by Broderick (2018). In line with Broderick (2018), the grass silage had the highest concentration of NDF, but the content of CP was lower than that of the red clover. Paulson et al. (2008) and Dewhurst (2013) also reported that grass silage usually contains less protein but more fibre than legume forages. The ether extract content of the red clover silage was 21 g/kg DM lower than the other forages, and was 7.5 g/kg DM lower than the second cut red clover silage used by Schulz et al. (2018). The higher pH of the red clover silage reflects a high buffering capacity due to high CP and ash content (Dewhurst et al., 2003b, 2010). The acetate and lactate content of the grass silage used in the current study was higher than that of the forages used by Sinclair et al. (2015), but relatively lower in red clover silage than reported by Dewhurst et al. (2010). A high acetate content in grass silage reflects increased stability under aerobic conditions (Danner et al., 2003). The PS distribution of the grass silages was similar to that reported by Tayyab et al. (2018b), although the mean X_m and peNDF was highest in the grass silage, mainly due to the higher content of NDF in grass silage.

The nutrient composition of the TMR was similar across the treatments except crude protein, but there was a tendency for an interaction for $pef_{>8\ mm}$, which indicated that eating behaviour differed between the diets throughout the day (Kononoff et al., 2003; Tayyab et al., 2018b). The observation that the mean $pef_{>8mm}$ was lowest at 4 h and highest at 24 h after the morning feed for the H diet could be attributed to an increase in the consumption of large PS (> 8 mm) during the first 4 h and short PS (< 8 mm) between 4 to 24 h post-feeding.

4.4.2. *In situ* degradability

The soluble CP fraction in grass silage was higher than the red clover silage, but a reverse trend was observed for DM, a finding in agreement with Purwin et al. (2014) and Hoffman et al. (1993), who examined the soluble fraction of different legumes and grass silages. The water solubility measurement of CP also reflects a higher *in situ* soluble CP fraction for grass than the red clover silage. The soluble fraction of the DM in the red clover and grass silage was 114 and 158 g/kg lower than that reported by Dewhurst et al. (2003a), at 392 and 404 g/kg, respectively. Red clover

silage has a higher concentration of the potentially degradable CP fraction compared to grass or other non-legume silages, as reported in a series of studies (Dewhurst et al., 2003a; Purwin et al., 2014; Damborg et al., 2018). Similarly, several studies (Hoffman et al., 1993; Dewhurst et al., 2003a; Damborg et al., 2018) have shown that the ED of red clover silage is greater than grass silage, with the higher content of NPN relative to neutral detergent insoluble CP being responsible for rapid ruminal degradation of legume forage proteins (Westreicher-Kristen et al., 2017). However, there was a high content of ADIN in the red clover silage that could be responsible for a lower calculated ED of CP compared to the grass silage (Nuez-Ortín and Yu, 2010). In contrast, red clover had a slightly higher ED of the DM compared to grass silage.

In relation to other legumes, red clover silage has a lower ruminal degradability of CP due to the presence of *o*-quinones that are synthesised by the PPO enzyme in red clover, which reduces protein degradation by creating a cross-linked complex with soluble and other dietary proteins (Broderick et al., 2004). In general, *o*-quinones react with red clover proteases and inhibit the function of this enzyme, resulting in reduced protein degradation in the rumen. On the other hand, the phenolic compounds were higher in red clover compared to grass silage, which are associated with a higher NDIN and ADIN content, which impair the protein breakdown (Givens et al., 2000). In the current study, the phenolic compounds were not analysed, and the content of ADIN in red clover was higher than grass silage.

4.4.3. Intake and animal performance

There was a lower DM intake in the current study when the dietary CP concentration was decreased from 175 or 165 to 150 g/kg DM, a finding that is consistent with others (Alstrup et al., 2014; Giallongo et al., 2016; Barros et al., 2017); however, most of the studies were based on lucerne and maize silage or mixtures of clover-grass. The DM intake of cows has been negatively related to the concentration of dietary CP between 140 to 220 g/kg DM (Sinclair et al., 2014). Similarly, Oh et al. (2019) reported that reducing the concentration of CP from 165 to 155 g/kg DM in a maize silage based diet decreased DM intake by 1 kg/d. In accordance with results from the current study, the DM intake was also reduced in early lactation cows when a red clover/grass silage based low CP (156 g/kg DM) diet replaced the control (171 g CP/kg DM) diet (Halmemies-Beauchet-Filleau et al., 2017). The reduced DM intake by cows fed diet L could be related to a lower supply of available N in the

rumen, which depressed the activity of fibre degrading bacteria, resulting in a lower intake (Allen, 2000). In contrast, Broderick et al. (2015) reported no significant difference in DM intake in lactating dairy cows when fed lucerne and maize silage based diets containing 170 to 150 g CP/kg DM. Likewise, Olmos Colmenero and Broderick (2006) noted that DM intake was not affected by dietary CP concentration (135 to 194 g CP/kg DM) in Holstein dairy cows.

Reducing dietary CP concentration has often been reported to reduce lactation performance (Hristov and Giallongo, 2014). For example, Olmos Colmenero and Broderick (2006) demonstrated that milk yield decreased by 2 kg/d when the dietary CP concentration was reduced from 165 to 135 g/kg DM in a lucerne and maize silage based ration. Similarly, Lee et al. (2012) and Giallongo et al. (2016) reported that decreasing the concentration of dietary CP from 165 to 135 g/kg DM (deficient in MP supply) in a lucerne and maize silage based ration reduced milk yield by approximately 2.9 to 3.9 kg/d. In contrast, milk yield and composition were not affected by dietary CP concentration in the current study, supporting Hynes et al. (2016), who also found no difference in lactation performance when the concentrate CP was reduced from 181 to 141 g CP/kg DM in dairy cows fed fresh-cut perennial grass. Barros et al. (2017) also reported similar results when the dietary CP concentration was reduced from 162 to 144 g/kg DM, and suggested that early producing cows may rely upon mobilisation of body tissues for a steady-state milk production when lucerne and maize silage based diets are marginally deficient in CP or MP. The milk yield and milk protein content can be improved by increasing the supply of MP with a balanced AA profile, including the supply of limiting AA such as lysine, methionine, or histidine (Rius et al., 2010b; Lee et al., 2012b; Giallongo et al., 2016). In the current study, the MP requirement was met for H and M, whereas L supplied 95% of MP requirement, which was not intended, but as the grass silage was high in CP, and it was not possible to supply additional DUP to keep the CP low. The concentration of bypass protein content was increased for the M and L diets to supply adequate MP, which could be the reason for the lack of difference in milk performance in high yielding cows despite of low DM intake. Despite the lower content of MP, the early lactating cows receiving L showed no negative effect on milk performance could be related to mobilisation of body protein.

The feed conversion efficiency in the current study was higher in cows fed diet L compared to M, which mainly reflects the lower DM intake. The lower concentration of MUN in cows fed L (150 g/kg DM) could reflect a lower concentration of rumen ammonia (Olmos Colmenero and Broderick, 2006). It was assumed that a marginally lower supply of MP in cows fed L would decrease milk yield (Lee et al., 2015a; Giallongo et al., 2016), but this was not supported by the milk performance in the current study. Additionally, BCS change was numerically lower in cows receiving L, indicating that body tissue mobilisation may have offset the negative impact of the lower DM intake rather than milk performance in high yielding early lactation dairy cows (Sinclair et al., 2014).

4.4.4. Apparent digestibility and nitrogen use efficiency

The apparent whole tract digestibility of DM, OM, N, NDF and ADF in the current study was similar to previous reports that have examined the effect of dietary CP or MP level in lucerne or red clover silage based diets (Broderick et al., 2008; Ouellet and Chiquette, 2016). However, feeding a low CP diet (L) to dairy cows decreased the apparent DM, OM, CP, and fibre digestibility in the current study, a finding in accordance with other lucerne and maize silage based studies (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a; Giallongo et al., 2015). The decreased nutrient digestibility with a low dietary concentration of CP could be attributed to a lower supply of RDP, which may have limited the growth of rumen microbes, resulting in a restricted intake and depressed ruminal fibre digestion (Olmos Colmenero and Broderick, 2006; Oh et al., 2019). In contrast, Niu et al. (2016) and Lee et al. (2015) reported no change in apparent nutrient digestibility, except for OM or CP digestibility, which was decreased (Niu et al., 2016) when the dietary concentration of CP was reduced from 185 to 152 and 155 to 137 g/kg DM, respectively in lucerne based ration. However, Olmos Colmenero and Broderick (2006) suggested that a CP concentration below 165 g/kg DM in a lucerne based diet could contribute to a lower nutrient digestibility, supporting the findings of the current study with red clover based ration.

Johansen et al. (2017) and Broderick (2018) reported that apparent OM digestibility was increased in dairy cows when fed red clover compared to lucerne silage-based rations. This effect was attributed to improved hemicellulose and NDF digestibility in the red clover silage (Broderick et al., 2001), or a greater lignin concentration in lucerne silage (Wedig et al., 1986). Alternatively, feeding lucerne based diets may

enhance the ruminal or duodenal flow of indigestible fibre, resulting in an increased sloughing of endogenous cells from the intestinal wall with a resultant reduction in apparent digestibility of CP (Hoffman et al., 1998; Dewhurst, 2013). In agreement, faecal DM output was also reduced by 19% when lucerne silage was replaced with red clover silage in the study of Broderick et al. (2001).

In the current study, feeding low CP diets resulted in a decreased N concentration in the urine mainly due to the differences in N intake, as there is a negative linear relationship between dietary N intake and urinary N output in dairy cows (Castillo et al., 2000; Chen et al., 2020). The negative correlation indicates that the low protein diet has the capacity to decrease N loss through manure (Lee et al., 2012a; Niu et al., 2016). However, Oh et al. (2019) noted a decreased excretion of faecal N when dairy cows were fed a low protein (155 g CP/kg DM) compared to a control (165 g CP/kg DM) diet, which may be due to a small difference in the CP content between the diets. Several studies have shown that reducing dietary N intake can increase apparent NUE in lactating dairy cows (Broderick et al., 2015; Hristov et al., 2015; Niu et al., 2016), a finding in agreement with the current results. Similarly, Kidane et al. (2018) demonstrated that reducing the dietary concentration of CP from 175 to 145 or 130 g/kg DM in grass silage based rations increased N capture in milk by 5.20 or 8.70% units without affecting milk production.

4.4.5. Plasma metabolites and milk fatty acid profile

In the current study, the blood metabolites including ammonia, BHB and glucose were not affected by dietary CP concentration, except for plasma urea, which was reduced in cows receiving the low protein diets, an effect that can be attributed to a lower content of degradable N in the rumen (Sinclair et al., 2012; Alstrup et al., 2014). Moreover, the lower concentration of MUN in cows receiving L diet in the current study also reflected a reduction in plasma urea concentration. This observation is similar to Olmos Colmenero and Broderick (2006), who noted that both milk and plasma urea N were highly correlated (R^2 = 0.83) when cows were fed lucerne and maize silage based low CP diets. Likewise, Bahrami-Yekdangi et al. (2014) demonstrated that reducing the concentration of dietary CP from 180 to 156 g/kg DM did not alter plasma glucose or other metabolites, but decreased plasma urea by 0.48 mmol/l in cows fed lucerne and maize silage based rations. A study by Alstrup et al. (2014) also reported no difference in plasma glucose or BHB, whereas urea was reduced by 1.17 mmol/l in cows fed grass-clover and maize

silage based low CP (139 g CP/kg DM) compared to a control (157 g CP/kg DM) CP diet.

Milk FA profile principally depends on the FA composition of the diet consumed by dairy cows and the degree of biohydrogenation in the rumen (Leduc et al., 2017). In the current study, milk FA content of CLA *c*9, *t*11 and CLA *t*10, *c*12 were not affected by dietary CP concentration. However, the highest concentration of total milk PUFA (mainly C18:2n-6 *c*) and C18:1 *t*12 in the milk from cows fed the low CP diet (L) could be related to a lower level of RDP that reduced rumen microbial lipolysis and subsequent biohydrogenation of long-chain FA in the rumen, increasing the duodenal supply of C18 FA (Gerson et al., 1983; Gressley and Armentano, 2007). Moreover, forage type also had an influence on milk FA content (Lashkari et al., 2019). For example, the concentration of milk PUFA, mainly C18:2n-6 *c* and C18:3n-6, in the current study was relatively higher than milk from those cows fed lucerne based diets used in the study by Leduc et al. (2017). The higher content of milk PUFA in lactating cows fed red clover based diets could be due to the action of PPO enzyme against the ruminal biohydrogenation of long-chain FA (Van-Ranst et al., 2011). The mechanism how PPO reduced biohydrogenation in the rumen is uncertain (Lee, 2014). However, one suggested mechanism is the encapsulation of plant lipids in a phenol-protein complex, which reduces the accessibility of lipids to microbial lipolysis, and lowering biohydrogenation substrates (Van-Ranst et al., 2011). Similarly, Lee et al. (2011) and Giallongo et al. (2016) reported a lower concentration of PUFA in milk fat when lucerne and maize silage based low CP (145 to 148 g/kg DM) diet was fed to dairy cows, an effect that was attributed to the inclusion of dietary heat-treated or expeller soybean meal which contained more saturated fat. The low CP diets in the current study also contained more RP protein sources compared to the control.

Milk OBCFA and < C16 FA in ruminants has been suggested as a marker to predict MCP synthesis (Vlaeminck et al., 2006; Cabrita et al., 2011). In the current study, the yield of milk < C16 (mainly C8:0, C11:0 and C12:0) and OBCFA (mainly C15:0 and C15:1) increased in cows when fed H diet compared to M or L, which may be due to a higher concentration of rumen N, a finding in agreement with previous work (Vlaeminck et al., 2006; Giallongo et al., 2016; Leduc et al., 2017) where the microbial synthesis of OBCFA was decreased with low CP diets due to the lower

supply of RDP. Indeed, there is a positive relationship between dietary N supply and MCP synthesis in ruminants (Sinclair et al., 1995; Sannes et al., 2002).

4.5. Conclusions

Reducing the dietary CP concentration from 175 or 165 to 150 g/kg DM in a red clover and grass silage-based diet decreased DM intake but had no effect on milk yield or composition in high yielding dairy cows whereas the apparent whole tract nutrient digestibility and milk FA profile were decreased. Feeding a low CP diet based on red clover and grass silage improved the apparent nitrogen use efficiency in dairy cows. Overall, the effects of the level of protein supply on animal performance and nitrogen efficiency did not differ when dietary MP supply was predicted to be similar or slightly lower to requirements.

CHAPTER 5: Low protein diets for dairy cows based on lucerne and maize silage: effects on performance, nutrient digestibility, blood metabolites and nitrogen use efficiency

5.1. Introduction

The increasing global demand for high protein soybean meal, along with its price instability, has led to renewed interest in using homegrown forage legumes in the diet of dairy cows due to their high content of CP (Broderick, 2018). Forage legumes, particularly lucerne (*Medicago sativa*), are more popular in the United States and Europe, and are widely used as a high protein silage in dairy cow diets, particularly in areas with low rainfall due to its large tap root and drought resistance (Phelan et al., 2015). Lucerne silage has a high CP and NPN content but is low in RUP, whereas maize silage has a low CP content with a range of 68 to 73 g/kg DM (Hassanat et al., 2013; Sinclair et al., 2015; Thomson et al., 2017a). Lucerne and maize silages may therefore be regarded as complementary forages in the diet of dairy cows.

Feeding optimal proportions of both silages can result in an increase in MCP synthesis in the rumen because lucerne is high in RDP and maize silage is a good source of fermentable energy due to its high starch content (Brito et al., 2006). Previous studies have reported that feeding combinations of lucerne and maize silage can increase feed intake, milk yield, and milk protein content when compared to lucerne alone (Hassanat et al., 2013), although the optimal inclusion rate of each silage is unclear. For example, a study by Sinclair et al. (2015) reported that lucerne silage could contribute up to 0.6 of the forage component (DM-basis) when fed along with maize silage without affecting performance. Increasing the inclusion rate of lucerne in a maize silage based diet often results in an increase the dietary concentration of CP and NPN, which has been associated with a lower NUE and higher urinary N excretion and manure NH_3-N emission (Brito and Broderick, 2006; Arndt et al., 2015). Reducing the dietary CP content in a high lucerne based diet may mitigate urinary N excretion and N losses to the environment (Niu et al., 2016). However, compared to maize silage, lucerne silage is degraded rapidly in the rumen and may lead to a lower supply of RUP to the cow (Hassanat et al., 2013; Thomson et al., 2017a). Therefore, purchased sources high in RUP may have to be added to the ration to meet the cows MP requirements.

To achieve a high apparent NUE in lactating cows it is essential to reduce the dietary CP concentration (Liu and VandeHaar, 2020a). An earlier study (Chapter 4; Study 1) has shown that the dietary CP concentration could be reduced to around 150 g/kg DM in red clover and grass silage-based rations without affecting milk performance if the diets are formulated to supply at or around the required MP. Several studies have been conducted in the United States to investigate the effect of low protein diets in lactating dairy cows where the diets were based on lucerne and maize silage (Olmos Colmenero and Broderick, 2006; Lee et al., 2015a; Oh et al., 2019). However, few studies have investigated the effect of feeding a low protein diet based on different inclusion rates of lucerne to maize silage on the performance and apparent NUE is unclear. The current study hypothesised that increasing the inclusion of lucerne in a low CP diet of lactating cows would improve NUE without affecting milk performance. The objective of the study was to examine the effects of feeding low CP diets with two different dietary ratios of lucerne to maize silage on feed intake, lactation performance, blood metabolites, apparent digestibility, and N utilisation in early lactation dairy cows.

5.2. Materials and methods

5.2.1. Animals and housing

All procedures involving animals were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (amended, 2012) and received local ethical approval. The study was conducted from January to April, 2019.

Eighteen multiparous lactating dairy cows yielding (mean ± SD) 46.5 ± 4.78 kg milk/d at 81 ± 13 DIM, with a mean LW of 705 ± 59 kg and BCS of 2.6 ± 0.32 (where 1 = emaciated and 5 = obese and cored on to 0.25 units; Ferguson et al., 1994) were used. All cows were housed in the same area of an open span building fitted with free stalls and super comfort mattresses. Stalls were bedded twice weekly with sawdust and lime, with automatic scrapers that scraped the passageways at 6 h intervals. All cows had *ad-lib* access to fresh drinking water.

5.2.2. Experimental design

The study was a 3×3 Latin square design with 3 periods and 3 dietary treatments. Experimental periods were 28 days in duration, which included a 21-day adaptation

to the diets and a 7-day sampling period. Cows were blocked by milk yield, DIM and BCS, and randomly assigned to 1 of 3 dietary treatments. The treatment diets were formulated to contain 175 g CP/kg DM with 50:50 lucerne to maize silage (**H50**), 150 g CP/kg DM with 50:50 lucerne to maize silage (**L50**) or 150 g CP/kg DM with 60:40 lucerne to maize silage (**L60**) and contain similar MP content.

5.2.3. Diets and feeding

The animals were fed the diets as a TMR that was formulated to produce 37 kg of milk per day according to Thomas (2004a) and to be isoenergetic and to contain a similar MP content, with the carbohydrate source and rumen bypass protein content of the concentrates altered (Table 5.1). The forage to concentrate ratio for all diets was 52:48 (DM basis). The lucerne (*Medicago sativa*) was mown at early bloom, wilted for 36 hours, harvested using a self-propelled forage harvester (John Deere 7840i, Nottinghamshire, UK) on 2nd June 2019 and ensiled in a concrete clamp with an additive added at the rate of 2.0 litre per tonne (Axcool Gold, Biotal, Cardiff, UK). The maize silage (*Zea mays L.*) was harvested on 26th October 2019 using a self-propelled forage harvester and ensiled in a concrete clamp with an additive added (Maizecool Gold, Biotal, Cardiff, UK; 2 l/tonne).

Dietary ingredients were mixed for 10 minutes using a Hi-spec forage mixer calibrated to ± 0.1 kg and fed through roughage intake control (Insentec B.V., Marknesse, The Netherlands) feeders fitted with automatic animal identification and weighing system calibrated to ± 0.1 kg. Fresh feed was delivered once daily at approximately 0800 h at the rate of 1.05 of the previously recorded intake, with refusals collected 3 times weekly prior to feeding.

Table 5.1. Dietary ingredients and predicted chemical composition of the H50, L50 or L60 diet based on lucerne and maize silage fed to dairy cows.

	Diet[1]		
Item	H50	L50	L60
Dietary ingredients (g/kg DM)			
Lucerne silage	262	262	315
Maize silage	262	262	210
Rolled wheat	129	158	163
Soy hulls	129	158	163
Molassed sugar beet	77.0	77.0	77.1
Soybean meal	95.8	29.1	7.29
SoyPass[2]	0.00	20.8	37.5
Rapeseed meal	20.8	0.00	0.00
RapeTech[3]	0.00	8.33	4.17
Rumen protected fat	18.7	18.7	18.8
Minerals and vitamins[4]	5.00	5.00	5.00
Predicted composition[5] (g/kg DM)			
Forage: concentrate (DM basis)	0.52	0.52	0.52
ME (MJ/kg DM)	12.0	11.9	11.8
Crude protein (CP)	175	150	152
MPE	109	104	104
MPE (% of requirements)	105	100	100
MPN	121	104	104
MPN (% of requirements)	116	100	100

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.
[2]Xylose-treated soybean meal (KW Alternative Feeds, Staffordshire, UK)
[3]SC Feeds, Nantwich, the UK.
[4]Mineral/vitamins premix (KW Alternative Feeds, Leeds, UK) providing (g/kg) 220 calcium, 30 phosphorus, 80 magnesium, 80 sodium, (mg/kg) 760 copper, 30 selenium, 1 000 000 IU vitamin A, 300 000 IU vitamin D3, 3000 IU vitamin E, 2.5 mg/kg vitamin B12, 135 mg/kg biotin.
[5]The predicted composition was calculated using a DietCheck ration formulation software. ME = metabolisable energy; MPE = metabolisable protein-rumen energy limited; MPN = metabolisable protein-rumen nitrogen limited.

5.2.4. Experimental routine

Lucerne and maize silages were collected twice weekly, dried in a forced-air oven at 105°C and adjusted to achieve the desired ratio. Fresh forages (lucerne and maize silages) and experimental diets were sampled daily during the final week of each period, stored at -20°C and pooled within period prior to subsequent analyses. Additional TMR samples were collected at 0, 4, 8, and 24 h post-feeding on day 1 to 3 of each sampling week. Fresh forage samples were also collected daily at approximately 1000 h from the clamps on days 22 to 24 of each period. All TMR and forage samples were separated into six fractions using a modified PSPS

(Tayyab et al., 2018a); > 44, 33 to 44, 19 to 32.9, 8 to 19, 4 to 8, and < 4 mm by manual shaking (Kononoff et al., 2003).

Cows were milked twice daily at approximately 0600 h and 1600 h in a 40-point internal rotary parlour (Westfalia, GEA Milking System, Germany). During the final week of each period, milk yield was recorded at each milking, and 4 samples were collected at 2 consecutive morning and evening milkings for subsequent analyses of milk composition. Live weight and BCS were recorded following the afternoon milking at the start and end of each study period.

Faecal grab samples (approximately 350 g/d/cow) were collected from 12 cows at 1000 and 1600 h for 5 consecutive days during the final week of each period and stored at -20°C for subsequent analyses. Spot urine samples (approximately 250 ml/cow/sample) were collected from 12 cows on day 22, 24, 26 and 28 at 0730, 1130, 1530 and 1630 h in each period by manual stimulation of the area around the vulva. Following pH measurement, all urine samples were immediately acidified to pH < 3 using 20% H_2SO_4 (v/v) to avoid volatilisation of N compounds before storage at -20°C for subsequent analysis of total N. This sampling routine was undertaken to account for possible diurnal and day to day variations in urinary N concentration (Schulz et al., 2018).

Blood samples were collected by jugular venepuncture from 12 representative cows into heparinised and fluoride oxalate tubes (Becton Dickinson and Company, New Jersey) over 2 consecutive days at 0800, 0900, 1100 and 1300 h in the final week of each period. Following collection, the samples were centrifuged at 1600 × g for 15 min to separate the plasma which was immediately analysed for ammonia, with further sub-samples stored at -20°C for subsequent analysis of urea, glucose and BHB.

5.2.5. *In situ* degradability of the forages

Three rumen-cannulated Holstein-Friesian dry cows with a mean LW of 650 ± 28 kg were housed in a straw bedded metabolism unit and fed a basal ration at maintenance level with a concentrate to forage ratio of 21:79 on DM basis (Thomas, 2004). The mixed ration contained (DM basis) 264 g/kg lucerne silage, 176 g/kg maize silage, 353 g/kg chopped wheat straw, 86 g/kg spey syrup (Trident, AB Agri Ltd., Lynch Wood, UK), 90 g/kg protein blend (KW Alternative Feeds, UK), 12 g/kg magnesium chloride, 12 g/kg minerals, 4 g/kg provimi LiFT (Provimi, North

Yorkshire, UK) and 2 g/kg DM of Vistacell Ultra (AB Vista, Wiltshire, UK). Dietary ingredients were mixed with the same forage mixer as the cows in the performance study and was offered twice daily at 0800 and 1630 h. All cows had continuous access to fresh drinking water. The *in situ* degradability of lucerne and maize silage was determined as described in Chapter 3, Section 3.5.

5.2.6. Chemical analyses

Sub-samples of forage and TMR were bulked by study period and analysed according to AOAC (2012) for DM (934.01, intra-assay CV of 1.26%) as described in Section 3.1.1. Dried feed samples were ground in a Wiley mill (Thomas Scientific, Philadelphia) through a 1.0 mm sieve prior to analyses for ash (942.05), CP (988.05) and ether extract (920.39) with an intra-assay CV of 0.73, 0.92 and 5.23%, respectively (AOAC, 2012), and AIA (intra-assay CV of 9.02%) content as per the method of Van Keulen & Young (1977) as described in Chapter 3. Acid detergent fibre and NDF were determined as per the method of Van Soest et al. (1991) using heat-stable α-amylase for NDF analysis (Sigma, Gillingham, UK, intra-assay CV of 1.11 and 0.80% for NDF and ADF, respectively) as described in Sections 3.1.6 and 3.1.7, respectively. The water-soluble CP fraction (intra-assay CV of 0.41%) of all forages was determined (Section 3.1.3) according to Weisbjerg et al. (1990). Forage samples were also analysed for ADIN (intra-assay CV of 6.51%) as per the method described by Licitra et al. (1996; Section 3.1.8). Forage ammonia-N and pH were determined following the method of MAFF (1986; Sections 3.3.2 and 3.3.3, respectively). The content of total VFA, including lactate, ethanol, acetate, propionate, iso-butyrate and butyrate of forages, were analysed at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) using gas and high-performance liquid chromatography (Section 3.3.1). All TMR and forage samples were analysed for PS distribution using a modified PSPS as reported by Tayyab et al. (2018a; Section 3.6).

Milk samples were analysed for fat, protein, lactose and urea by near-midinfrared at National Milk Laboratories (NML, Wolverhampton, UK; Section 3.7.1). Fatty acids in milk were analysed by extracting milk fat by centrifugation and methylation using sodium methoxide according to the method of Feng et al. (2004) as described in Sections 3.7.2 and 3.7.3, respectively. The FAME of the feed was prepared according to the protocol of Jenkins (2010) as described in Chapter 3, Section 3.8. The individual FAME was determined by Gas-Liquid Chromatography (Hewlett

Packard 6890, Wokingham, UK), fitted with a CP-Sil 88 column (100 m × 0.25 mm i.d. × 0.20 um film, Agilent Technologies, Santa Clara, California, USA) as described in Section 3.7.3.

Faecal samples were composited by cow and period, and dried in a forced-air oven at 60°C until constant weight. The dried faecal samples were then milled using an electric grinder (SG20U, Electric Grinder, UK) and analysed for AIA, total N, NDF, ADF, and ash as described in Chapter 3. Sub-samples of urine were bulked by cow for each period and pooled urine samples were then filtered through N free filter paper, and subsequently analysed for total N (976.06) by Kjeldahl (AOAC, 2012) as described in Section 3.10.

Plasma samples were analysed for ammonia (Randox Laboratories, County Antrim, UK; Kit-Catalogue no. AM 1015, intra-assay CV of 9.76%) within 1 h of collection, while BHB, glucose and urea (Randox Laboratories, County Antrim, UK; Kit-Catalogue no. RB 1008, GL 1611 and UR 221 with an intra-assay CV of 6.16, 1.30 and 4.83%, respectively) were analysed using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in Section 3.9.

5.2.7. Calculations

Dry matter intake was calculated from the daily fresh feed intake for each cow that was recorded by RIC system and the analysed DM content of the TMR (Equation 3.1). Nutrient intake, faecal output, digested nutrients, and apparent total tract digestibility coefficients of DM, OM, N, NDF, and ADF were determined (Equation 3.9) using AIA as an internal marker as per the method of Van Keulen & Young (1977). Milk yield based on 4% milk fat (4% FCM) was determined by adjusting the milk yield to 40 g of fat per kg milk, and energy corrected milk yield (ECM) was computed as (3.14 MJ/kg) = milk yield × (383 × fat (g/kg) × 100 + 242 × protein (g/kg) × 100 + 165.4 × lactose (g/kg) × 100 + 207)/3140 (Sjaunja et al., 1991). Apparent NUE was calculated as milk N output/dietary total N intake, with the N excretion in milk determined as total milk protein/6.38. The PS geometric mean ($\mathbf{X_m}$) and the standard deviation of X_m were determined using the equations by ASABE (2007);

$$\text{Geometric mean length (Xm)} = \log^{-1} \frac{\sum (Mi \ \log mXi)}{\sum Mi}$$

$$\text{Standard deviation (SDgm)} = \log^{-1}\left[\frac{\sum \text{Mi}\,(\log \text{mXi} - \log \text{Xg})2}{\sum \text{Mi}}\right]^{1/2}$$

Where M_i is a quantity on i^{th} screen, X_i is diagonal of the screen opening of the i^{th} screen, mX_i is mean geometric size of feed particles on i^{th} screen = $[X_i \times X_{i-1}]^{1/2}$, $X_{(i-1)}$. The distribution of PS was quantified by dividing each fraction weight by the total of the fractions. The physically effective fibre (**PeNDF**) was calculated by multiplying the physical effectiveness factor with the dietary NDF content (Lammers et al., 1996; Maulfair et al., 2010).

The *in situ* DM and CP degradability data were fitted in Sigmaplot (Jandel, Erkrath, Germany) using the exponential equation as described in Chapter 3, Section 3.5.

5.2.8. Statistical analysis

Data were analysed by ANOVA as a Latin rectangle design using GenStat 18th edition (VSN International Ltd, Oxford, UK) with diet and period as fixed effects and cow as random effect. All data were checked for normality using descriptive statistics before running the ANOVA model in GenStat. The model used was $Y_{ijk} = \mu + D_i + P_j + A_k + E_{ijk,}$

where Y_{ijk} and μ represent the dependent variable and total mean, and D_i, P_j, A_k, and E_{ijk} as the diet, period, animal and residual error, respectively. Plasma parameters and particle fractions were analysed by ANOVA as repeated measures that included the fixed effect of sampling time in the model. Tukey's test was conducted post hoc to determine treatment means that differed. Results are presented as the least square mean of each treatment and standard error of the mean (SEM). Values were considered significant when $P < 0.05$ and a tendency when $P < 0.10$.

5.3. Results

5.3.1. Forage and diet characteristics

The lucerne silage was lower in DM and OM and contained a higher NDF and ADF content than the maize silage (Table 5.2). The CP concentration of the lucerne silage was 188 g/kg DM, nearly twice that of the maize silage. The concentration of ADIN was also 2.05 g/kg DM lower in the maize compared to the lucerne silage. In contrast, the ammonia-N content of the maize silage was 10.4 g/kg of total N higher than the lucerne silage, whilst the pH of the lucerne silage was slightly lower

compared to the maize silage. The lactate content of the lucerne silage was 43.4 g/kg DM higher than the maize silage, whilst the content of acetate was 9.4 g/kg DM lower in the lucerne than the maize silage.

The content of long-chain PUFA was higher in the maize silage, with the concentration of C18:2 n-6 being 8.22 g FA/kg DM higher than the lucerne silage. In contrast, the C18:3 n-3 content of the maize silage was 4.32 g FA/kg DM lower than the lucerne silage. The PS of the lucerne silage was higher than the maize silage, with a X_m of 20.6 and 14.5 mm, respectively. The DM, OM, NDF, ADF and EE concentration of the diets were similar, with means of 445, 917, 353, 241 and 29.8 g/kg DM, respectively, whereas the CP concentration was 172, 150 and 152 g/kg DM in H50, L50, and L60, respectively. The total FA content in H50 was 1.8 g FA/kg DM higher than the other 2 diets, which had a mean of 22.1 g FA/kg DM. There was a tendency ($P < 0.10$) for the mean X_m, pef, and peNDF, to be numerically higher in L50 or L60 compared to H50 (Table 5.3). In contrast, there was a tendency ($P = 0.061$) for a higher mean of small PS fraction (< 4 mm) in H50 compared to L50 or L60. Sampling time post feeding did not affect the mean PS fractions of the diet except for the medium-length (8-19 mm) fraction, which increased ($P = 0.050$) until 8 h post-feeding.

5.3.2. *In situ* forage degradability

The maize silage had a higher ($P < 0.05$) soluble fraction of DM compared to the lucerne silage (Table 5.4). In contrast, the potentially degradable fraction of the DM was 123 g/kg higher ($P < 0.05$) in the lucerne compared to the maize silage. Likewise, the rate of degradation of the potentially degradable fraction of DM was higher ($P < 0.05$) in the lucerne compared to the maize silage. There was a difference in the ED of the DM, which was 22.1% higher ($P < 0.001$) in the maize than the lucerne silage.

Table 5.2. Nutrient composition (g/kg DM), fermentation profile, fatty acid content and particle size distribution of lucerne silage, maize silage, and the H50, L50 or L60 diet fed to dairy cows.

Item	Forage		Diet[1]		
	Lucerne silage	Maize silage	H50	L50	L60
Dry matter (g/kg)	307	340	453	442	439
Organic matter	870	964	919	920	913
Ash	130	35.8	81.1	79.9	87.2
Crude protein	188	98.2	172	150	152
Water soluble crude protein (g/kg CP)	569	599			
Neutral detergent fibre	399	380	348	355	356
Acid detergent fibre	313	198	233	243	248
Acid detergent insoluble N	6.98	4.93			
Ether extract	23.7	32.5	29.5	29.5	30.3
Fermentation profile (g/kg DM)					
pH	4.22	3.67			
Ammonia-N (g/kg total N)	66.0	76.4			
Lactate	104	60.6			
Ethanol	1.21	4.33			
Acetate	23.8	33.2			
Propionate	0.98	3.07			
Iso-butyrate	0.45	-			
Butyrate	0.16	0.15			
Acetate: Propionate	0.08	0.03			
Fatty acid (g/kg DM)					
C16:0	2.85	3.26	6.99	5.83	6.46
C18:0	0.36	0.57	0.80	0.70	0.74
C18:1*C*9	0.40	5.76	5.37	4.48	4.40
C18:2n-6	3.13	11.35	6.31	5.88	5.71
C18:3n-3	5.38	1.06	2.17	2.31	2.83
ΣFA	19.9	27.3	23.9	21.5	22.7
Fractions[2] (% DM)					
> 44 (mm)	0.00	0.00	0.00	0.00	0.00
33 to 44 (mm)	2.53	0.39	1.06	0.78	0.98
19 to 32.9 (mm)	40.2	11.7	15.8	15.6	17.0
8 to 19 (mm)	46.2	62.0	45.3	47.8	45.4
4 to 8 (mm)	4.90	14.6	12.7	13.0	12.9
< 4 (mm)	6.21	11.3	25.2	22.8	23.7
X_m (mm)	20.6	14.5	12.4	12.7	12.7
SD_{gm}	1.77	1.81	2.14	2.09	2.13
pef $_{>4}$ (%)	93.8	88.7	74.8	77.2	76.3
pef $_{>8}$ (%)	88.9	74.2	62.1	64.2	63.4
peNDF $_{>4}$ (%)	37.4	33.8	26.0	27.4	27.2
peNDF $_{>8}$ (%)	35.5	28.2	21.6	22.8	22.6

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.

[2]Fractons of forages at 0 h post-feeding; DM = dry matter; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fibre.

Table 5.3. Particle size distribution of the H50, L50 or L60 diet based on lucerne and maize silage fed to dairy cows at 0, 4, 8 and 24 h post feeding.

Item	Diet[1]				*P* value[2]		
	H50	L50	L60	SEM	D	T	Int
Fractions[3]							
33-44 mm	1.11	1.09	1.38	0.194	0.129	0.101	0.615
0h	1.06	0.78	0.98				
4h	0.91	0.93	1.39				
8h	1.20	1.30	1.77				
24h	1.27	1.37	1.41				
19-32.9 mm	14.5	15.4	17.5	1.759	0.262	0.875	0.904
0h	15.8	15.6	17.0				
4h	15.0	15.0	17.5				
8h	13.6	15.5	16.8				
24h	13.7	15.6	18.6				
8-19 mm	45.2	47.7	45.5	1.382	0.239	0.050	0.619
0h	45.3	47.8	45.4				
4h	45.4	49.7	45.1				
8h	46.4	48.3	47.6				
24h	43.6	45.0	43.8				
4-8 mm	12.8	13.0	12.4	0.564	0.521	0.499	0.739
0h	12.7	13.0	12.9				
4h	12.2	12.6	12.4				
8h	13.2	13.2	12.4				
24h	13.2	13.3	12.1				
<4 mm	26.4	22.7	23.2	1.361	0.061	0.110	0.855
0h	25.2	22.8	23.7				
4h	26.6	21.7	23.6				
8h	25.6	21.7	21.4				
24h	28.2	24.6	24.1				
X_m mm	12.1	12.8	12.9	0.370	0.085	0.448	0.832
SD_{gm}	2.14	2.09	2.13	0.022	0.196	0.063	0.653
pef>4 (%)	73.6	77.3	76.8	1.361	0.061	0.110	0.855
pef>8 (%)	60.8	64.3	64.3	1.496	0.086	0.139	0.684
peNDF>4 (%)	25.6	27.5	27.3	0.626	0.093	0.117	0.868
peNDF>8 (%)	21.2	22.8	22.9	0.627	0.094	0.145	0.709

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.
[2]D = main effect of diet, T = main effect of time, Int = interaction between diet and time.
[3]Diets were separated into 5 fractions; 33-44, 19-32.9, 8-19, 4-8 and <4 mm; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fibre.

The maize silage had a higher ($P < 0.05$) soluble fraction of CP compared to the lucerne silage. In contrast, the potentially degradable fraction of CP was 150 g CP/kg higher ($P < 0.05$) in the lucerne compared to the maize silage. Similarly, the rate of degradation of the potentially degradable fraction of CP was higher ($P < 0.05$) in the lucerne compared to the maize silage. However, the ED of the CP was comparable for both forages, with a mean value of 673 g/kg.

Table 5.4. *In situ* DM and CP degradability coefficients of lucerne and maize silage fed to dairy cows.

Item[1]	Forage		SEM	*P* value
	Lucerne silage	Maize silage		
DM degradation coefficients (g/kg DM)				
a	200	406	0.1	<.001
b	580	457	1.2	0.002
a+b	779	863	0.6	<.001
c	0.09	0.06	0.005	0.028
ED	498	608	0.6	<.001
CP degradation coefficients (g/kg total N)				
a	428	538	1.1	0.002
b	422	272	1.1	<.001
a+b	850	810	0.5	0.004
c	0.11	0.08	0.008	0.045
ED	672	673	0.3	0.843

[1]DM = dry matter; CP = crude protein; a = soluble fraction; b = potentially rumen-degradable fraction; c = degradation rate of fraction b per hour; ED = effective rumen degradability at 8%/h rumen passage rate.

5.3.3. Feed intake and animal performance

Dry matter intake was reduced in cows receiving L50, being 2 kg/d lower (P = 0.019) than in those receiving H50 (Table 5.5). Similarly, milk yield was 2 kg/d lower (P = 0.010) in cows receiving L60 compared to H50, but there was no difference in ECM or FCM yield, with mean values of 37.8 and 38.5 kg/d, respectively. Neither milk fat nor lactose content was affected by diet, with means of 38.7 and 45.2 g/kg, respectively. In contrast, milk protein concentration was 0.6 g/kg lower (P = 0.024) in cows receiving L60 than those receiving H50. The milk protein and lactose yield were also lower (P < 0.05) in cows receiving L60 compared to H50. The concentration of MUN was on average 3.35 mg/dl higher (P < 0.001) in cows when fed H50 compared to those fed L50 or L60. Feed efficiency was comparable in cows receiving all of the diets, with means of 1.51 g ECM or 1.59 g milk per kg of DM intake. There was no effect of diet on mean LW or BCS, but there was a tendency (P = 0.070) for LW gain to be highest in cows receiving H50.

5.3.4. Nutrient intake and apparent total tract digestibility

The intake of DM, OM, N and NDF was lower (P < 0.05) in cows fed L50 compared to those fed H50, with L60 being intermediate except for total N intake, which was similar to that of cows fed L50 with a mean value of 0.59 kg/d (Table 5.6). The amount of DM and OM digested were higher (P < 0.05) in cows fed H50 compared to L50, whilst those receiving L60 had an intermediate value. Similarly, digested N was 0.16 kg/d higher (P < 0.001) in cows receiving H50 than in those receiving L50

or L60. However, there was no effect of dietary treatment on apparent whole tract digestibility of DM, OM, N, NDF or ADF.

Table 5.5. Intake, milk performance, live weight and body condition of dairy cows fed H50, L50 or L60 diet based on lucerne and maize silage.

Item	Diet[1]			SEM	*P* value
	H50	L50	L60		
Intake (kg DM/d)	26.2[a]	24.2[b]	25.0[ab]	0.45	0.019
Production (kg/d)					
Milk yield	40.9[a]	39.8[ab]	38.9[b]	0.43	0.010
ECM[2] yield	38.7	37.6	37.2	0.58	0.175
FCM[3] yield	39.1	38.1	38.3	0.92	0.703
Composition (g/kg)					
Fat	38.3	38.3	39.4	0.86	0.553
Protein	30.7[a]	30.2[ab]	30.1[b]	0.14	0.024
Lactose	45.2	45.0	45.4	0.17	0.313
Milk urea (mg/dl)	24.0[a]	16.6[b]	17.0[b]	0.53	<.001
MUN[4] (mg/dl)	11.2[a]	7.76[b]	7.94[b]	0.245	<.001
Yield (kg/d)					
Fat	1.56	1.52	1.53	0.037	0.703
Protein	1.25[a]	1.20[ab]	1.17[b]	0.015	0.002
Lactose	1.85[a]	1.80[ab]	1.77[b]	0.023	0.047
Feed efficiency					
Milk/DM intake	1.57	1.64	1.56	0.031	0.158
ECM/DM intake	1.48	1.56	1.50	0.032	0.193
Body performance					
LW[5] (kg)	715	708	702	4.2	0.113
LW change[6] (kg)	18.6	-1.17	3.20	6.090	0.070
LW change (kg/d)	0.66	-0.04	0.12	0.217	0.070
BCS	2.68	2.65	2.64	0.045	0.804
BCS change[6]	0.13	0.00	0.04	0.056	0.293

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.

[2]ECM = Energy corrected milk yield;

[3]FCM = 4% fat-corrected milk yield;

[4]MUN = milk urea nitrogen;

[5]LW = live weight;

[6]Change over the 28 days period.

Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 5.6. Intake, faecal output and apparent total-tract digestibility[1] (kg/d) of nutrients[2] in dairy cows fed H50, L50 or L60 diet based on lucerne and maize silage.

Item		Diet[3]			SEM	*P* value
		H50	L50	L60		
DM	Intake	26.3[a]	23.8[b]	25.0[ab]	0.521	0.010
	Faecal output	8.04	7.32	7.78	0.388	0.433
	Digested	18.3[a]	16.4[b]	17.2[ab]	0.441	0.028
	Digestibility (kg/kg)	0.695	0.690	0.691	0.0118	0.940
OM	Intake	24.2[a]	21.9[b]	22.9[ab]	0.48	0.011
	Faecal output	6.96	6.33	6.70	0.346	0.458
	Digested	17.2[a]	15.5[b]	16.2[ab]	0.42	0.031
	Digestibility (kg/kg)	0.713	0.708	0.709	0.0116	0.956
N	Intake	0.73[a]	0.57[b]	0.61[b]	0.013	<.001
	Faecal output	0.24	0.21	0.22	0.011	0.170
	Digested	0.49[a]	0.36[b]	0.31[b]	0.028	<.001
	Digestibility (kg/kg)	0.669	0.628	0.634	0.0120	0.055
NDF	Intake	9.17[a]	8.43[b]	8.90[ab]	0.180	0.031
	Faecal output	3.92	3.60	3.76	0.205	0.556
	Digested	5.25	4.83	5.14	0.157	0.176
	Digestibility (kg/kg)	0.575	0.572	0.577	0.0176	0.982
ADF	Intake	6.15	5.76	6.18	0.120	0.050
	Faecal output	3.19	3.03	3.19	0.160	0.718
	Digested	2.96	2.73	2.99	0.118	0.278
	Digestibility (kg/kg)	0.482	0.476	0.485	0.0207	0.957

[1]Measured using 12 cows (4 cows for each treatment group);
[2]DM = dry matter; OM = organic matter; N = nitrogen; NDF = Neutral detergent fibre; ADF = Acid detergent fibre.
[3]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.
Means within a row with different superscript differ significantly ($P < 0.05$).

5.3.5. Nitrogen output and efficiency

Faecal N concentration was higher ($P = 0.002$) in cows receiving H50 than in those receiving L50 or L60 (Table 5.7). The concentration of N in urine was lowest ($P = 0.002$) in cows fed L60 compared to H50 and intermediate in L50. Similarly, N intake and milk N output were higher ($P < 0.05$) in cows receiving H50 compared to those fed L50 or L60. However, as a proportion of total N intake, faecal N output was unaffected ($P > 0.05$) by treatment. In contrast, the apparent NUE was approximately 18% higher ($P < 0.001$) in cows receiving L50 or L60 compared to H50. The pH of the urine was slightly higher ($P = 0.004$) in cows receiving L60 compared to those fed H50.

Table 5.7. Nitrogen output, efficiency, partitioning and urine pH in dairy cows fed H50, L50 or L60 diet based on lucerne and maize silage.

Item	Diet[1]			SEM	*P* value
	H50	L50	L60		
Concentrations (g/kg)					
Diet	27.6[a]	24.0[c]	24.3[b]	0.03	<.001
Milk	4.85	4.82	4.81	0.023	0.482
Faecal	30.1[a]	28.8[b]	28.7[b]	0.27	0.002
Urine (g/l)	6.12[a]	5.26[ab]	4.57[b]	0.266	0.002
N output (g/d)					
Milk	192[a]	184[ab]	180[b]	2.4	0.011
Faecal	240	210	223	10.7	0.170
N partitioning (%)					
Faecal	33.1	37.2	36.6	1.20	0.055
NUE[2]	26.5[b]	32.6[a]	29.9[a]	0.77	<.001
Urine pH	8.54[b]	8.55[ab]	8.57[a]	0.015	0.004

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.
[2]NUE = apparent nitrogen use efficiency.
Means within a row with different superscripts differ significantly ($P < 0.05$).

5.3.6. Blood plasma metabolites

Dietary treatment did not affect plasma ammonia, glucose and BHB concentration, with mean values of 41.1 µmol/l, 3.62 and 0.50 mmol/l, respectively (Table 5.8). In contrast, plasma urea concentration was lowest ($P < 0.001$) in cows fed L50 or L60, with a mean value of 3.54 mmol/l, and highest in cows fed H50, at 4.96 mmol/l (Figure 5.1). There was an effect of time ($P < 0.05$) on all plasma metabolites. Plasma urea concentration peaked at 1 h post-feeding and then gradually decreased with time (Figure 5.1). The concentration of plasma ammonia being similar at 0700 and 1300 h and highest at 1100 h (Figure 5.2a). Plasma glucose concentration decreased over time (Figure 5.2b), whereas plasma BHB increased with time (Figure 5.2c).

Table 5.8. Plasma ammonia, β-hydroxybutyrate (BHB) and glucose concentration in dairy cows fed H50, L50 or L60 diet based on lucerne and maize silage.

Item	Diet[1]				*P* value[2]		
	H50	L50	L60	SEM	D	T	Int
Ammonia (µmol/l)	40.2	41.4	41.7	4.75	0.720	0.004	0.972
BHB (mmol/l)	0.49	0.49	0.53	0.046	0.640	<.001	0.087
Glucose (mmol/l)	3.63	3.61	3.61	0.068	0.952	<.001	0.505

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.
[2]D = main effect of diet, T = main effect of time, Int = interaction between diet and time.
Means within a row with different superscripts differ (*P* < 0.05).

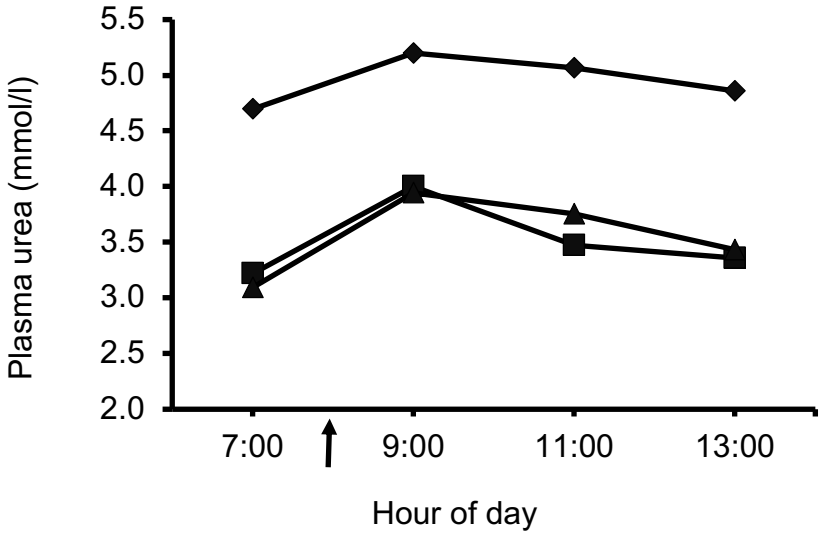


Figure 5.1. Plasma urea concentration in dairy cows fed a high CP with 50:50 lucerne to maize silage (H50, ♦) or a low CP with either a 50:50 (L50, ■) or 60:40 (L60, ▲) lucerne to maize silage ratio. Pooled SEM = 0.326; diet, *P* < 0.001; time, *P* < 0.001; and diet × time, *P* = 0.781. Arrow indicates the feeding time.

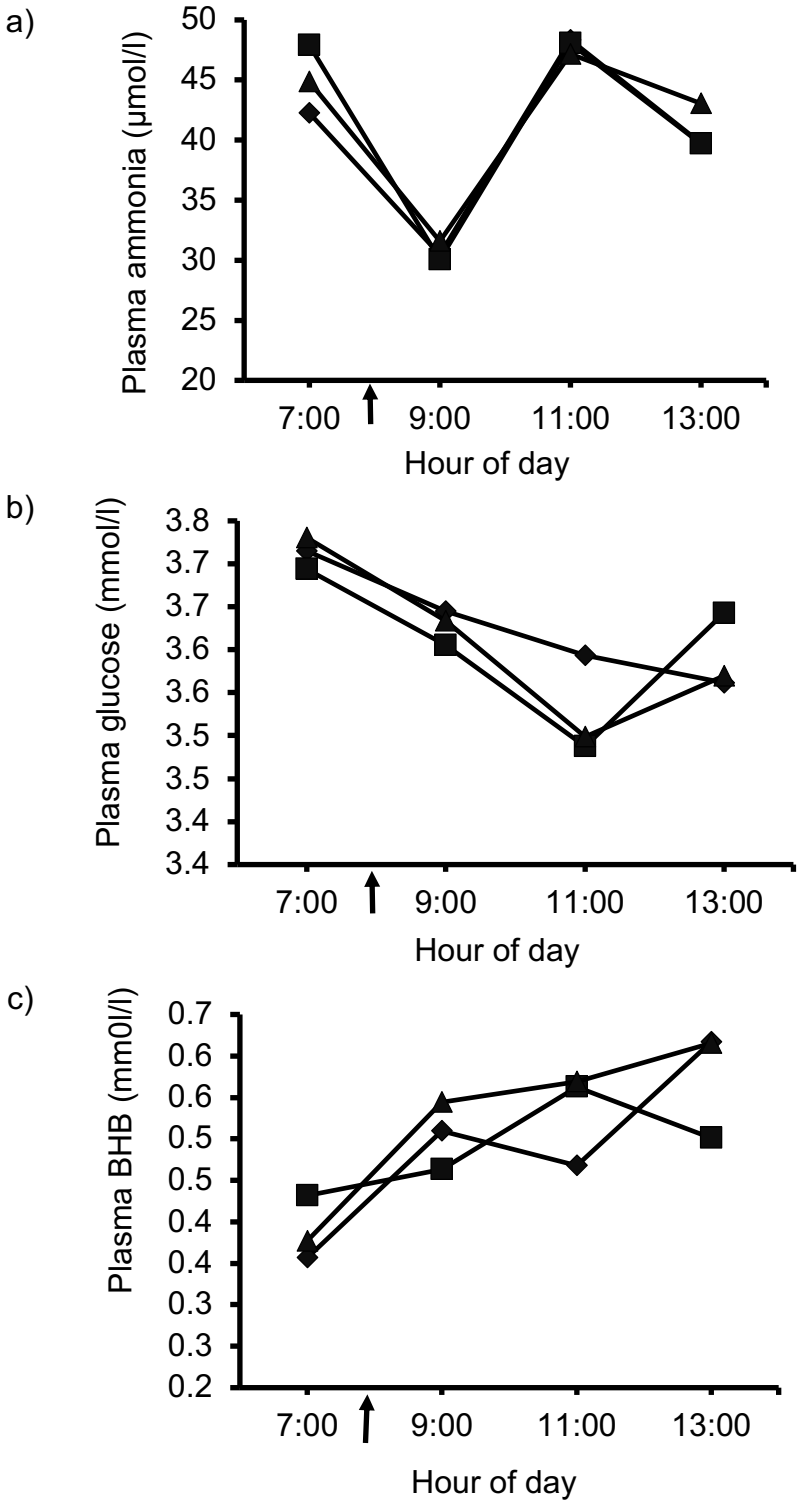


Figure 5.2. Plasma ammonia (a), glucose (b), and β-hydroxybutyrate (BHB) (c) concentration in dairy cows fed a high CP with 50:50 lucerne to maize silage (H50, ♦) or a low CP with either a 50:50 (L50, ■) or 60:40 (L60, ▲) lucerne to maize silage ratio. For plasma ammonia, pooled SEM = 4.75; Diet, $P = 0.720$; time, $P = 0.004$; and diet × time, $P = 0.972$. For plasma glucose, pooled SEM = 0.046; Diet, $P = 0.640$; time, $P < 0.001$; and diet × time, $P = 0.087$. For plasma BHB, pooled SEM = 0.068; Diet, $P = 0.952$; time, $P < 0.001$; and diet × time, $P = 0.505$. Arrow indicates the feeding time.

5.3.7. Milk fatty acid profile

There was no effect of diet on the milk FA content except for CLA *c*9, *t*11, which was highest (P = 0.019) in cows receiving L60, lowest in H50 and intermediate in those fed L50 (Table 5.9). Similarly, CLA *t*10, *c*12 tended (P = 0.071) to be higher in cows receiving L60 compared to those fed H50. Milk FA of a chain length higher or lower than C16:0, and C16:0 + 16:1 were not affected by diet. Similarly, there was no effect of dietary treatment on the total milk fat content of SFA, MUFA, PUFA, LOCFA, or OBCFA.

Table 5.9. Milk fatty acid composition (g/100 g) of dairy cows fed H50, L50 or L60 diet based on lucerne and maize silage.

Item	Diet[1]			SEM	*P* value
	H50	L50	L60		
C4:0	1.57	1.59	1.60	0.022	0.517
C6:0	1.39	1.40	1.40	0.014	0.855
C8:0	0.99	0.98	0.98	0.009	0.766
C10:0	2.50	2.37	2.41	0.040	0.091
C11:0	0.08	0.05	0.06	0.007	0.083
C12:0	3.28	3.23	3.14	0.044	0.085
C13:0	0.11	0.10	0.10	0.003	0.057
C14:0	11.7	11.7	11.5	0.09	0.202
C14:1	1.21	1.32	1.22	0.040	0.122
C15:0	1.25	1.21	1.22	0.026	0.480
C15:1 *c*10	0.18	0.21	0.21	0.020	0.256
C16:0	38.8	38.9	39.6	0.27	0.126
C16:1	1.70	1.71	1.70	0.050	0.970
C17:0	0.48	0.47	0.50	0.010	0.231
C17:1 *c*10	0.26	0.27	0.26	0.000	0.494
C18:0	8.27	8.21	8.22	0.125	0.926
C18:1 *t*8	0.15	0.14	0.15	0.009	0.816
C18:1 *t*9	0.36	0.36	0.33	0.022	0.670
C18:1 *t*10	0.59	0.57	0.61	0.022	0.482
C18:1 *t*11	0.99	0.96	0.92	0.027	0.234
C18:1 *t*12	0.32	0.35	0.34	0.011	0.115
C18:1 *c*9	19.5	19.4	19.2	0.21	0.506
C18:2n-6 *c*	2.08	2.20	2.18	0.097	0.662
C18:2n-6 *t*	0.43	0.45	0.46	0.012	0.212
CLA *c*9, *t*11	0.22[b]	0.23[ab]	0.25[a]	0.010	0.019
CLA *t*10, *c*12	0.027	0.031	0.032	0.0010	0.071
C18:3n-3	0.54	0.52	0.50	0.020	0.456
C18:3n-6	0.27	0.28	0.28	0.005	0.673
C20:0	0.04	0.04	0.04	0.001	0.534
C20:3n-3	0.16	0.16	0.15	0.004	0.240
C21:0	0.07	0.06	0.06	0.004	0.396
C22:0	0.12	0.11	0.11	0.003	0.090
EPA	0.13	0.14	0.11	0.022	0.471
DHA	0.06	0.06	0.06	0.002	0.257
<C16	24.3	24.2	23.8	0.15	0.116
C16 + 16:1	40.5	40.6	41.3	0.29	0.154
>C16	35.2	35.2	34.9	0.32	0.737
SFA[2]	70.7	66.7	71.0	2.24	0.326
MUFA[3]	25.5	24.0	25.2	0.91	0.502
PUFA[4]	3.80	3.73	3.86	0.157	0.837
LOCFA[5]	2.00	1.82	1.96	0.068	0.148
OBCFA[6]	2.44	2.27	2.43	0.081	0.259

[1]Diet; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage; [2]SFA = Saturated fatty acids are defined as fatty acids with no double bonds; [3]MUFA = Monounsaturated fatty acids are defined as fatty acids with one double bond; [4]PUFA = Polyunsaturated fatty acids are defined as fatty acids with more than one double bond; [5]LOCFA = Linear odd chain fatty acids; ∑LOCFA = (C11:0+C13:0+C15:0+C17:0+C21:0); [6]OBCFA = Linear odd and branched chain fatty acids; ∑OBCFA = (C11:0+C13:0+C15:0+C15:1+C17:0+C17:1+C21:0).
Means within a row with different superscript differ ($P < 0.05$).

5.4. Discussion

5.4.1. Forage and feed characteristics

The chemical composition of the lucerne and maize silage used in the current study was comparable to previous work by Sinclair et al. (2015) and Broderick (2018), with the maize silage having the highest concentration of NDF and lowest CP content. Dewhurst (2013) also reported that maize silage contains less protein than legume forages. The NDF and ADF contents in the lucerne silage were similar to that reported by Hassanat et al. (2013) and Sinclair et al. (2015) but lower than that used by Dewhurst et al. (2003b). The ether extract content of the lucerne and maize silage was slightly lower than that reported by Hassanat et al. (2013). The high pH of lucerne silage reflects a high buffering capacity due to the higher CP and ash content (Dewhurst et al., 2003b, 2010). The acetate content of the lucerne silage used in the current study was higher than that of the forages used by Dewhurst et al. (2003b). Similarly, the lucerne silage had the highest concentration of lactic acid, which is consistent with Sinclair et al. (2015), who reported 7 g/kg DM higher content of lactate in lucerne compared to maize or grass silage. However, a high acetate content in maize compared to lucerne silage reflects an increased stability under aerobic conditions (Danner et al., 2003). The PS distribution of the maize silage was similar to that reported by Tayyab et al. (2018b), with the mean X_m in the lucerne silage was higher than maize silage, mainly due to the greater content of the long PS (mainly 19 to 32.9 mm) fraction of lucerne silage.

The nutrient composition of the TMR was similar across the dietary treatments except for CP, as predicted. However, there was a tendency for a higher mean of X_m, pef, and peNDF in L50 or L60 than H50, with a numerically higher proportion of small (< 4 mm) and lower content of medium (8-19 mm) fractions in H50, being the major factors causing the differences. Moreover, the consumption of medium (8-19 mm) fractions by cows was lower during the first 8 h following the morning feed, mainly due to the preference of the cows for a short PS (Kononoff et al., 2003).

The content of the immediately soluble fraction was lower in the lucerne silage, but the potentially degradable fraction and the rate of degradation for DM and CP was higher than maize silage, a finding in accordance with Damborg et al. (2018), who compared degradation characteristics in legumes (clover and lucerne) and grass silage. The higher content of NPN relative to neutral detergent insoluble CP is

responsible for the rapid ruminal degradation of legume proteins (Westreicher-Kristen et al., 2017). The calculated effective DM degradability of the lucerne silage was lower than the maize silage, partially reflecting the high content of ADIN in lucerne. Due to antinutritional factors or a high polyphenolic content, some legumes have a lower ruminal degradability of CP compared to maize or grass silage (Jones et al., 1995a; Lee, 2014). The phenolic compounds are associated with a higher ADIN content, which causes the disruption of plant protein breakdown in the rumen (Givens et al., 2000). However, in the current study, the phenolic compound was not reported, and the ED of CP was comparable between two forages.

5.4.2. Intake and animal performance

In line with the results from Study 1 (Chapter 4), reducing the dietary concentration of CP from 175 to 150 g/kg DM decreased the DM intake of cows. This finding is also consistent with previous studies (Alstrup et al., 2014; Giallongo et al., 2016; Barros et al., 2017). Likewise, feeding haylage and maize silage based low CP diets (156 g/kg DM) reduced DM intake by 1 kg/d (Oh et al., 2019). A study by Sinclair et al. (2014) reported that the DM intake of cows was negatively related to the concentration of dietary CP between 140 to 220 g/kg DM. The reduced DM intake by cows fed diet L50 in the current study could be related to a lower supply of RDP in the rumen, which depressed the activity of fibre degrading bacteria, resulting in a lower intake (Allen, 2000). In contrast, Olmos Colmenero and Broderick (2006) noted that DM intake was not affected by dietary CP concentration (135 to 194 g CP/kg DM) in Holstein dairy cows fed 1:1 DM basis of lucerne to maize silage based ration. Increasing the inclusion rate of lucerne silage from 50 to 60% (L60 diet) in the current study did not alter the DM intake of lactating cows, supporting Arndt et al. (2015), who reported no change in DM intake when between 20 to 80% maize silage was replaced with lucerne silage. In contrast, Sinclair et al. (2015) observed a decrease in DM intake when maize was replaced by 60% lucerne silage in the diet of Holstein-Friesian dairy cows.

Reducing dietary CP concentration has often been reported to reduce lactation performance (Hristov and Giallongo, 2014). Olmos Colmenero and Broderick (2006) demonstrated that milk yield decreased by 2 kg/d when the dietary CP concentration was reduced from 165 to 135 g/kg DM in a lucerne and maize silage based (1:1 DM basis) ration. Similarly, Giallongo et al. (2016) reported that decreasing the concentration of dietary CP from 165 to 145 g/kg DM (5 to 10% deficient in MP

requirements) reduced milk yield by approximately 4.3 kg/d when cows were fed 1:2 (DM basis) of a lucerne haylage and maize silage based ration. In contrast, milk yield and composition were not affected by dietary CP concentration (L50 diet; 150 g CP/kg DM) in the current study, supporting Hristov et al. (2015), who also found no difference in lactation performance when the concentration of dietary CP was reduced from 165 to 154 g/kg DM in a maize and legume silage based ration. Barros et al. (2017) also reported similar results when a lucerne and maize silage based (48:52 ratio on DM) low CP diet (144 g CP/kg DM) was fed to lactating dairy cows. These findings suggest that early lactation cows may rely upon mobilisation of body tissues for milk production when the diets are marginally deficient in CP or MP. In the current study, the supply of MP was similar across the diets with the carbohydrate source and rumen-protected protein content of the concentrates altered, which could be the reason for a lack of difference in milk performance in high yielding cows when fed low CP diets. However, increasing the proportion of lucerne (from 50 to 60% DM) in the low CP diet led to a decrease in milk yield and milk protein content compared to H50. Similarly, milk yield and milk protein concentration were reduced when dairy cows were fed a high compared to a low proportion of lucerne (75:25 vs. 25:75 lucerne to maize silage ratio) based diet (Thomson et al., 2017). The decrease in milk yield and milk protein content with increasing lucerne proportion (H50 vs. L60) might have been due to the lower ME content of the lucerne silage (Steinshamn, 2010), which led to a lower supply of rumen available energy and subsequent MCP flow to the duodenum, or an imbalance between the supply of MP and rumen fermentable energy. However, several studies have reported that the replacement of maize with lucerne silage has had no consistent effect on milk yield or composition, particularly on milk fat or protein content (Hassanat et al., 2013; Arndt et al., 2015; Sinclair et al., 2015). This could be attributed to variations between diets in the supply of MP. The milk yield and milk protein content can be improved by increasing the supply of MP with a balanced AA profile, including the supply of limiting AA such as lysine, methionine, or histidine (Rius et al., 2010b; Lee et al., 2012b; Giallongo et al., 2016).

The lower concentration of MUN in cows fed the low CP diets (150 g/kg DM) in the current study (L50, L60) could reflect a lower concentration of rumen ammonia (Olmos Colmenero and Broderick, 2006) or MP (Giallongo et al., 2016). Despite there being no difference in milk yield between treatments (L50 and L60), there was a tendency for a lower LW change when the low CP diets (L50, L60) were offered,

indicating that body tissue mobilisation may have offset the negative impact of the lower DM intake rather than milk performance in high yielding early lactation dairy cows (Sinclair et al., 2014).

5.4.3. Apparent digestibility and nitrogen use efficiency

The apparent whole tract nutrient digestibility in the current study was not affected by dietary treatment. This finding is consistent with previous studies (Lee et al., 2015a; Niu et al., 2016), who reported no change in apparent nutrient digestibility, except for OM or CP digestibility, which was decreased when the dietary concentration of CP was reduced from 185 to 152 and 155 to 137 g/kg DM, respectively, in the diet of dairy cows fed lucerne and maize silage based rations (Niu et al., 2016). In contrast, Lee et al. (2012) and Giallongo et al. (2015) reported that decreasing the dietary CP concentration by 20 g/kg DM in maize and lucerne-based diets reduced nutrient digestibility in early lactation dairy cows.

The current study showed no difference in nutrient digestibility when lucerne replaced maize silage at a higher rate (L50 and L60). Likewise, Sinclair et al. (2015) and Arndt et al. (2015) reported that the replacement of maize with lucerne silage did not affect apparent nutrient digestibility, except for fibre digestibility that was increased by the inclusion rate of lucerne silage (Arndt et al., 2015). Johansen et al. (2017) and Broderick (2018) also reported that apparent OM digestibility was increased in dairy cows when fed red clover compared to lucerne silage-based rations. This effect was attributed to improved hemicellulose and NDF digestibility in the red clover silage (Broderick et al., 2001) or a greater lignin concentration in lucerne silage (Wedig et al., 1986). Alternatively, feeding lucerne-based diets may enhance the ruminal or duodenal flow of indigestible fibre, resulting in an increased sloughing of endogenous cells from the intestinal wall with a resultant reduction in apparent digestibility of CP (Hoffman et al., 1998; Dewhurst, 2013). Likewise, faecal DM output was also reduced by 19% when lucerne silage was replaced with red clover silage in the study of Broderick et al. (2001).

Feeding low CP diets resulted in a decreased N concentration in the manure mainly due to differences in N intake, which indicates that the low protein diet has the capacity to decrease N loss to the environment (Lee et al., 2012a; Niu et al., 2016). A study by Chen et al. (2020) also reported a negative linear relationship between dietary N intake and urinary N output in dairy cows. However, Oh et al. (2019) noted

a decreased excretion of faecal N when dairy cows were fed a lucerne haylage and maize silage based low protein (155 g CP/kg DM) diets compared to the control (165 g CP/kg DM), which may be due to a slight difference in the CP content between the diets. Several studies have shown that reducing dietary N intake can increase apparent NUE in lactating dairy cows fed either lucerne or lucerne and maize silage based rations (Broderick et al., 2015; Hristov et al., 2015; Niu et al., 2016), a finding in agreement with the current results. Similarly, Kidane et al. (2018) demonstrated that reducing the dietary concentration of CP from 175 to 145 or 130 g/kg DM in a grass silage based diet increased N capture in milk by 5.20 or 8.70% units without affecting milk production. There was, however, no difference in N output or NUE when lucerne replaced maize silage (L50 vs. L60) in the current study. In contrast, increasing the rate of inclusion of lucerne silage in maize silage based diets, up to 75 or 80%, has been shown to decrease NUE, which may be associated with a lower milk yield, higher RDP content, or lower dietary starch content in a high lucerne silage based diet (Arndt et al., 2015; Thomson et al., 2017a).

5.4.4. Plasma metabolites and milk fatty acid profile

In agreement with the previous study (Study 1, Chapter 4), the plasma metabolites including ammonia, BHB, and glucose were not affected by dietary CP concentration, except for plasma urea, which was reduced in cows receiving the low CP diets. The lower level of rumen degradable N might be associated with a reduced plasma urea content in cows fed low CP diets (Sinclair et al., 2012; Alstrup et al., 2014). Moreover, the lower concentration of MUN in cows receiving any of the low CP diets (L50 or L60) in the current study was also reflected by a reduction in plasma urea content. This observation is similar to Olmos Colmenero and Broderick (2006), who noted that both milk and plasma urea N were highly correlated (R^2 = 0.83) when lucerne and maize silage based diets were fed to dairy cows. Likewise, Bahrami-Yekdangi et al. (2014) demonstrated that reducing the concentration of dietary CP from 180 to 156 g/kg DM in a lucerne and maize silage based diet did not alter plasma glucose or other metabolites but decreased plasma urea by 0.48 mmol/l. A study by Alstrup et al. (2014) also reported no difference in plasma glucose or BHB, whereas urea was reduced by 1.17 mmol/l in cows when fed maize and grass-clover based low CP (139 g/kg DM) compared to a control (157 g CP/kg DM) CP diet. Increasing the proportion of lucerne silage in the current study (L50 vs. L60) did not

affect the concentration of plasma metabolites, although plasma BHB was numerically higher in cows when fed the high lucerne diet (L60). The highest concentration of plasma BHB was also associated with a high inclusion rate of lucerne (L60) in the study of Sinclair et al. (2015), an effect that may be associated with a high molar concentration of ruminal butyrate (Hassanat et al., 2013). However, the rumen fermentation metabolites were not measured in the current study.

In the current study, the milk FA content of CLA *c*9, *t*11 was not affected by dietary CP concentration (H50 vs. L50) but CLA *c*9, *t*11 and CLA *t*10, *c*12 (tendency) increased with the inclusion level of lucerne (H50 vs. L60), which could be due to a higher ruminal biohydrogenation with the high lucerne diet. Ruminal biohydrogenation was also reflected by a numerically lower concentration of C18:1 *c*9 and C18:3n-3 in milk fat when cows were fed L60 compared to the H50 or L50 diets. The lower concentration of CLA in the milk fat of cows fed H50 may partly be due to a higher DM and fibre intake, which increased the passage rate and reduced the time available for ruminal biohydrogenation (Guzatti et al., 2018).

There was no effect of diet or inclusion of lucerne silage on the total milk FA content of SFA or PUFA. However, the PUFA content in milk was increased when cows received red clover and grass silage based low CP (150 g CP/kg DM) diet in Study 1 (Chapter 4). In contrast, Lee et al. (2011) and Giallongo et al. (2016) reported a lower concentration of PUFA in milk fat when a lucerne and maize silage based low CP (145 to 148 g/kg DM) diet was fed to dairy cows, an effect that was attributed to the inclusion of dietary heat-treated or expeller soybean meal which contained more saturated fat. The low CP diets in the current study also contained more RP protein sources compared to the control.

5.5. Conclusions

Reducing the dietary CP concentration from 175 to 150 g/kg DM in a lucerne and maize silage-based diet decreased DM intake but had no effect on milk yield or composition in high yielding dairy cows. In contrast, increasing the proportion of lucerne from 50 to 60% of the forage DM in a low CP diet (L60) reduced milk yield and milk protein content compared to a control (H50) diet, but DM intake or energy corrected milk yield was unaffected. Feeding a low CP diet based on lucerne and maize silage improved the apparent nitrogen use efficiency in dairy cows. Overall,

reducing dietary protein levels in a lucerne and maize silage-based ration did not affect milk performance when dietary MP supply was predicted to be similar to requirements.

CHAPTER 6: Effects of dietary protein level and supplementation with starch or rumen-protected methionine on milk performance, metabolism and nitrogen efficiency in dairy cows fed red clover/grass silage-based diets

6.1. Introduction

There is considerable commercial interest in reducing dietary CP concentrations and making greater use of homegrown forage in dairy cow diets due to the high and volatile cost of protein feeds and the legislative requirement to reduce N and ammonia emissions from dairy cows (Broderick, 2006; Defra, 2019). Diets high in CP typically result in a low N utilisation, with excess N being excreted to the environment through manure (Dijkstra et al., 2013; Whelan et al., 2013). It has been shown that dietary protein levels can be reduced in dairy cow diets to around 140-150 g/kg dietary DM if the diet meets the cows MP requirements (Sinclair et al., 2014). However, other studies have reported that reducing the dietary CP concentration from 173 to 144 g/kg DM reduced DM intake and resulted in a decreased milk yield by 3.6 kg/d. It was also associated with lowering milk fat and protein content (Law et al., 2009; Barros et al., 2017). Hristov and Giallongo (2014) suggested that feeding diets with a CP concentration lower than 150 g/kg DM would decrease milk production in high yielding dairy cows, mainly by reducing DM intake.

In the UK, red clover is the main legume fed to dairy cows and is commonly grown and ensiled along with grass silage (Johnston et al., 2020). The protein in legume silage is rapidly degraded in the rumen (Sinclair et al., 2009), reducing the content of RUP and MP, which are required to maintain high milk yields (Damborg et al., 2018; Westreicher-Kristen et al., 2018). The benefits of red clover silage include the presence of polyphenol oxidase, which can protect the protein from microbial degradation in the rumen (Black et al., 2009; Lee, 2014), increasing its RUP content. Studies have reported that red clover silage is high in CP (170 to 200 g CP/kg DM; Dewhurst et al., 2003; Schulz et al., 2018), and inclusion at up to 66% of the forage DM improved intake, milk yield, and the proportion of PUFA in milk (Moorby et al., 2009; Dewhurst, 2013). The use of red clover silage rather than grass silage also increased the efficiency of MCP synthesis in the rumen (Merry et al., 2006; Moorby et al., 2016) and improved blood plasma concentration of AA, except for methionine (Vanhatalo et al., 2009). In our previous study (Chapter 4, Study 1), reducing dietary CP concentrations in red clover-grass silage-based rations from 175 to 150 g/kg DM

reduced DM intake without affecting milk performance, although the long-term effect on body energy reserves was unclear.

An alternative means to mitigate a potential reduction in performance due to lower dietary CP concentrations is to increase MCP synthesis in the rumen by increasing rumen fermentable energy, or to improve the duodenal flow of MP by supplementing with RP essential AA (Lee et al., 2012a; Sinclair et al., 2014; Giallongo et al., 2016). Feeding more starch has been shown to increase MCP synthesis in the rumen (Oba and Allen, 2003; Oba, 2011), increasing the capture of recycled urea and improving ruminal N utilisation (Davies et al., 2013). The effect of feeding RP-AA on dairy cow performance is variable (Robinson, 2010), although supplementation with RP-AA such as methionine, which is the first limiting in red clover silage based diets (Lee, 2014; Broderick, 2018), may improve performance and NUE in low protein (150 g CP/kg DM) diets (Sinclair et al., 2014). There have, however, been few studies that have examined the effect of dietary starch concentration or RPM in low CP diets based on red clover/grass silage. The hypothesis was therefore that supplementation with additional starch or RPM in a red clover/grass silage based low CP diet would improve N use efficiency without affecting milk performance compared to dairy cows fed a high CP diet.

6.2. Materials and methods

The study was conducted to investigate the effects of dietary CP level and supplementation of starch or RPM on performance, metabolism and NUE in early lactation dairy cows. The first study (Study 3a) was conducted from October 2019 to April 2020, and a 2nd study (study 3b) from January 2020 to March 2020 at Harper Adams University, Newport, Shropshire, UK. All procedures, including animals, care, and experimentation, were conducted according to the UK Animals (Scientific Procedures) Act 1986 (amended 2012).

6.2.1. Study 3a: Animals and housing

In study 3a, 56 Holstein-Friesian dairy cows (8 primiparous and 48 multiparous) yielding (mean ± SD) 42.3 ± 6.83 kg/d at 39 ± 13 DIM, with a mean LW of 671 ± 84 kg and BCS of 2.8 ± 0.30 (1 to 5 scale where 1 = emaciated and 5 = obese; Ferguson et al., 1994) were used. Cows were housed in the same area of an open span building fitted with free stalls and Super Comfort mattresses. Stalls were bedded

twice weekly with sawdust and lime, and automatic scrappers scraped the passageways at 6 h intervals. Cows remained on study for 14 weeks.

6.2.2. Forages

The grass silage was an equal mix of a first and second cut grass that was composed predominately of *Lolium perenne*, it was mown at a leafy stage on 25 May and 5 July 2019, respectively, wilted for 36 h and harvested with a self-propelled precision-chop forage harvester (John Deere 7840i, Nottinghamshire, UK) with an additive (Axphast Gold; Biotal, Worcestershire, UK) applied at the rate of 2.0 litre per tonne. The red clover silage (*Trifolium pretense*) was a first and second cut ley, wilted for 24 h and harvested using a self-propelled precision-chop forage harvester on 10 June and 15 July 2019, respectively, with an additive (Axphast Gold; Biotal, Worcestershire, UK) applied at the rate of 2.0 litre per tonne. The grass and red clover silages were ensiled in separate roofed concrete clamps.

6.2.3. *In situ* forage degradability

Three matured Holstein-Friesian rumen-cannulated (4″ internal diameter, Bar Diamond, Idaho, USA) dry cows with a mean LW of 650 ± 28 kg were housed in a straw bedded metabolism unit. Cows were fed a basal diet with forage to concentrate ratio of 79:21 (DM basis) at a maintenance level (Thomas, 2004). The diet contained (g/kg on DM basis) maize silage 176, lucerne silage 264, Protein blend (KW Alternative Feeds, UK) 90, Spey syrup (Trident, AB Agri Ltd., Lynch Wood, UK) 86, chopped wheat straw 353, Provimi LiFT (Provimi, North Yorkshire, UK) 4, magnesium chloride 12, Vistacell Ultra (AB Vista, Wiltshire, UK) 2, and minerals 12. All ingredients were mixed using a Hi-spec forage wagon and fed *ad-lib* twice a day at 0730 and 1600 h. All cows had continual access to drinking water.

In situ degradability of red clover and grass silages were determined as described in Chapter 3, Section 3.5.

6.2.4. Diets and feeding

Based on recordings during the week before allocation, cows were blocked by parity, DIM, and milk yield and randomly allocated to 1 of 4 experimental diets. The treatment diets were: high CP diet containing 175 g CP/kg DM (**C**); low CP diet containing 150 g CP/kg DM (**LP**); LP with added dietary starch (**LPS**) or LP with added RPM (**LPM**). The diets were fed as a total mixed ration (**TMR**) containing a

50:50 ratio (DM basis) of red clover to grass silage, and was supplemented with straight feeds to produce 37 kg of milk per day, according to Thomas (2004a; Table 6.1). The diets were formulated to contain a similar MP content but differ in their CP, starch or methionine concentration.

Table 6.1. Dietary ingredients and predicted chemical composition (g/kg DM) of the experimental diets[1] based on red clover and grass silage fed to dairy cows.

Item	Diet[1]			
	C	LP	LPS	LPM
Red clover silage	262	262	263	262
Grass silage	262	262	263	262
Barley	146	146	267	145
Soybean meal	50.0	0.00	0.00	0.00
SoyPass[2]	8.33	33.7	33.8	33.7
Soy hulls	154	154	117	154
Molassed sugar beet pulp	39.6	85.3	0.00	85.2
Rapeseed meal	41.6	0.00	0.00	0.00
NovaPro[2]	16.7	37.1	37.1	37.0
Metasmart[3]	0.00	0.00	0.00	1.66
Megalac	14.6	14.6	14.6	14.5
Minerals and vitamins[4]	5.00	5.00	5.00	5.00
Predicted composition[5]				
Forage: Concentrate (DM basis)	0.53	0.53	0.53	0.53
CP	176	152	153	152
MPE	103	100	102	100
MPN	121	110	110	110
MP (% requirement)	100	97.0	99.0	97.0
Methionine (g/100g CP)	2.00	2.00	2.00	2.30

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine; [2]SC Feeds, Nantwich, the UK; [3]Feed solution, Adisseo, France.

[4]Mineral-vitamins premix (KW Alternative Feeds, Leeds, UK) contained (DM basis) 220 g/kg calcium, 30 g/kg phosphorus, 80 g/kg magnesium, 80 g/kg sodium, 760 mg/kg copper, 30 mg/kg selenium, 1 000 000 IU vitamin A, 300 000 IU vitamin D3, 3000 IU vitamin E, 2.5 mg/kg vitamin B12, and 135 mg/kg biotin.

[5]The predicted composition was calculated using a DietCheck ration formulation software. DM = dry matter; CP = crude protein; ME = metabolisable energy; MPN = metabolisable protein-rumen nitrogen limited; MPE = metabolisable protein-rumen energy limited.5.2.4. Sampling procedure.

Dietary ingredients were mixed using a Hi-spec forage mixer wagon calibrated to ± 0.1 kg for 10 minutes and fed using RIC (Insentec B.V., Marknesse, The Netherlands) feeders fitted with automatic animal identification and weighing system calibrated to ± 0.1 kg. Fresh feed was delivered once daily at approximately 0800 h at the rate of 1.05 *ad-libitum* intake. Refusals were collected 3 times a week before the morning feed. All animals had continual access to fresh drinking water.

6.2.5. Performance and metabolism

Forage samples were collected twice per week, the DM determined, and the ratio of red clover to grass silage adjusted. A fresh sub-sample of forage was stored at -20°C for subsequent analyses. The diets were sampled weekly within 5 min of feeding, stored at -20°C and pooled for subsequent analyses. Particle length of the forages and TMR were determined using a modified Penn State Particle Separator as described by Tayyab et al. (2018).

Cows were milked twice daily at approximately 0600 h and 1600 h using a 40-point internal rotary parlour (GEA Milking System, Germany). Milk yield was recorded at each milking, and samples were collected fortnightly using a preservative (2-Bromo-2-nitropropane-1,3-diol) at morning and evening milking for later milk composition analyses. Additional milk samples were collected without a preservative during weeks 0 and 6 of the study period and stored at -20°C for milk FA profile determination. Live weight and BCS (1 to 5 scale where 1 = emaciated and 5 = obese; Ferguson et al., 1994) were recorded following the afternoon milking fortnightly.

Blood samples were collected by jugular venepuncture at 1100 h from 10 representative cows per treatment during study weeks 0, 4, 8 and 14. The samples were collected into vacutainers containing sodium heparin for BHB and urea determination or potassium oxalate for glucose determination. Immediately after collection, blood samples were centrifuged at 1600 × g for 15 min at 4°C, and the plasma then separated and stored at -20°C for subsequent analyses.

6.2.6. Study 3b: Total N balance and diet digestibility

At the end of the 14-week feeding period, 20 multiparous cows (5 blocks, resulting in 5 cows per treatment) were transferred to individual metabolism stalls for 6 days, which included 2 days adaptation and 4 days sampling. Cows had continual access to fresh drinking water at all times. The cows remained on the same dietary treatment that they received in Study 3a, which was fed at the same time and rate. Samples of the TMR were collected daily and stored at -20°C. Whilst restrained in the stalls, the cows were milked twice daily at approximately 0600 and 1600 h using a portable milking machine (Wootton Bridge Ryde Isle of Wight, UK). Milk yield was recorded at each milking, and 4 consecutive milk samples (2 in the morning and 2

in the evening milking) were collected with a preservative (2-Bromo-2-nitropropane-1,3-diol) and preserved for subsequent analysis of milk composition.

The total volume of urine was collected via a sampling device attached around the vulva of the cow into a 25 L barrel containing 1.8 L of 20% (v/v) H_2SO_4. The urine and acid were agitated throughout the day to ensure that they were well mixed. A sub-sample of approximately 1% of total urine volume was obtained, the pH immediately recorded, and, if required additional 20% H_2SO_4 was added to reduce pH below 3.0 to avoid volatilisation of N compounds, and stored at -20°C for subsequent analyses (Schulz et al., 2018). Spot faecal samples (approximately 250 g/sample per cow) were immediately collected after voiding from all cows at 0600, 1100,1600 and 2100 h for 4 consecutive days and stored at -20°C for subsequent analyses.

6.2.7. Chemical analyses

Forage and TMR samples were composited by month (Study 3a) or week (Study 3b), and sub-samples were analysed for DM (934.01, intra-assay CV of 0.12%) by drying the samples in a forced-air oven (AOAC, 2012; Section 3.1.1). Dried forage and TMR samples were milled using a hammer mill (Crompton Control Series 2000, Wakefield West Yorkshire UK) fitted with 1 mm screen before analyses of CP (990.03, intra-assay CV of 0.15%), ash (942.05, intra-assay CV of 0.50%), EE (2003.05, intra-assay CV of 1.19%) and AIA (intra-assay CV of 3.28%) content (Van-Keulen and Young, 1977) as described in Chapter 3 under Sections 3.1.2, 3.1.4, 3.1.5 and 3.2, respectively. The NDF and ADF contents were determined using heat-stable α-amylase (Sigma, Gillingham, UK, intra-assay CV of 0.73 and 1.0% for NDF and ADF respectively) and exclusive of residual ash (Sections 3.1.6 and 3.1.7, respectively) according to the method of Van Soest et al. (1991).

The soluble N fraction (intra-assay CV of 0.74%) of the two silages was determined (Section 3.1.3) as described by Weisbjerg et al. (1990). For the determination of ADIN (intra-assay CV of 1.55%; Section 3.1.8), samples were analysed according to Licitra et al. (1996). Silage ammonia-N and pH were determined using the method of MAFF (1986; Sections 3.3.2 and 3.3.3, respectively). The concentration of individual AA of the pooled TMR and silages (Section 3.4) and the total and molar proportions of VFA of red clover and grass silages were analysed (Section 3.3.1) at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) using

gas and ion-exchange chromatography. The pooled dried and ground TMR samples were sent to Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) to determine the starch content as described in Section 3.1.9.

Milk samples were analysed for fat, protein, lactose, urea, and SSC at National Milk Laboratories (NML, Wolverhampton, UK) using a near-midinfrared method calibrated by the method of AOAC (2012; Section 3.7.1). Milk and feed FAME in hexane were prepared according to the method of Jenkins (2010) and Feng et al. (2004), respectively (Sections 3.7.2, 3.7.3 and 3.8). The individual FAME was determined by gas-liquid chromatography (GLC, Hewlett Packard 6890, Wokingham, UK), fitted with a CP-Sil 88 column (100 m × 0.25 mm i.d. × 0.2 um film, Agilent Technologies, California, USA) as described by Sinclair et al. (2015).

Faecal samples were pooled by cow and dried in a forced-air oven at 60°C until constant weight and milled using an electric grinder (SG20U, Electric Grinder, UK) and analysed for AIA (Van Keulen and Young, 1977; Section 3.2), total N, ash, NDF, and ADF (Sections 3.1.2, 3.1.4, 3.1.6 and 3.1.7, respectively). Sub-samples of urine were bulked within cow, filtered with N free filter paper, and analysed for total N by the Kjeldahl method (AOAC, 2012; 976.06; Section 3.10).

Plasma samples were analysed for BHB, glucose and urea (Randox Laboratories, County Antrim, UK; Kit-Catalogue no. RB 1008, GL 1611 and UR 221 with an intra-assay CV of 4.69, 2.11 and 5.18%, respectively) using a Cobas Miras Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) as described by Sinclair et al. (2012; Section 3.9). To determine urinary urea-N concentration, pooled urine sample was diluted 20-fold and analysed using a Cobas Miras Plus autoanalyser (Kit-Catalogue no. UR 221, intra-assay CV of 5.62%; Section 3.9).

6.2.8. Calculation

Dry matter intake was calculated from the daily fresh feed intake and the measured DM content of the diets (Equation 3.1). Nutrient intake, faecal output, digested nutrient, and the apparent total tract digestibility coefficients of DM, OM, N, NDF and ADF were determined using AIA as an internal marker (Equation 3.10) as per the method of Van Keulen & Young (1977). Energy-corrected milk (ECM, 3.14 MJ/kg) yield was calculated according to Sjaunja et al. (1990) as ECM = milk yield × (38.3 × fat g/kg + 24.2 × protein g/kg + 16.5 × lactose g/kg + 207)/3,140 using milk yield and composition records. Fat-corrected milk yield was calculated by adjusting to 40

g/kg fat. The apparent NUE was estimated by dividing milk N by the total N intake, with the N excretion in milk was determined as total milk protein/6.38. The N balance was determined by subtracting N output (urinary N + faecal N + milk N) from total N intake. The X_m, PS and PeNDF content of the silage and TMR were calculated as per the method of ASABE (2007).

The *in situ* DM and CP degradability data were fitted in Sigma plot (Jandel, Erkrath, Germany) using the exponential equation as described in Chapter 3, Section 3.5.

6.2.9. Statistical analysis

Statistical analysis was conducted using GenStat (VSNI, 19th Edition, UK), with treatment (C, LP, LPS and LPM), forage (red clover and grass silage), and block as fixed effects and animal as random effect. The variables at the successive time point were analysed as repeated measures ANOVA using the model $Y_{ijkl} = \mu + T_i + P_j + B_k + A_l + E_{ijkl}$. *In situ* degradability data were also analysed by ANOVA using the model $Y_{ij} = \mu + F_i + A_l + E_{il}$; where Y is the observation, μ = overall mean, T_i = treatment, F_i = forage, P_j = experimental week, B_k = blocks, A_j = animal, and E = residual error.

All responses were evaluated for normal distribution and skewness. Milk SCC data was transformed to Log_{10} prior to statistical analysis. Week 0 was used as a covariate when appropriate. Tukey's test was conducted post hoc to determine treatments that differed significantly from each other. The results are presented as least squares means and the standard error of the mean. Means were considered different when $P < 0.05$, and a tendency when $P < 0.10$.

6.3. Results

6.3.1. Feed analysis

The red clover and grass silage had a DM content of 378 and 355 g/kg DM, respectively (Table 6.2). The red clover silage was 57 g/kg DM higher in CP but had a lower soluble N, NDF, pH and ammonia-N content compared to the grass silage. The acetate content of the red clover silage was 8.6 g/kg DM lower than the grass silage, whilst the lactate content was similar between the two forages, with a mean content of 68.8 g/kg DM. Relative to red clover, the FA content was 3.5 g/kg DM higher in grass silage, although the long-chain PUFA content was similar between the two forages, being highest in C18:3 n-3, which contributed approximately 25%

of the total FA. The Xm and peNDF was higher in the grass silage than the red clover silage. The DM, OM, NDF, ADF, and EE concentration of the experimental diets were similar with mean values of 475, 916, 386, 266 and 26.6 g/kg DM, respectively, whereas the CP concentration was 173, 151, 153 and 152 g/kg DM in diets C, LP, LPS and LPM, respectively. The total FA content of the diets was also comparable, although LPM was 1.4 g FA/kg DM lower than the other diets, which had a mean of 19.9 g FA/kg DM.

The red clover silage contained higher concentrations of all individual AA than the grass silage; however, both silages were very low in sulphur-containing AA (cysteine and methionine; Table 6.3). The higher concentrations of total acidic (aspartic and glutamic) and basic (arginine, histidine and lysine) AA were also observed in the red clover compared to the grass silage. Some essential AA concentrations were comparable between diets except the content of methionine, which was 4.73 g CP/kg DM lower in LP compared to the other diets (C, LPS or LPM), with a mean of 1.45 g CP/kg DM. Similarly, the total acidic AA concentration was 4.03 g CP/kg DM higher in LPS compared to C or LP, with a mean of 18.9 g CP/kg DM. Likewise, the total content of basic AA was 1.37 g CP/kg higher in LPS compared to C or LP, with a mean value of 9.48 g CP/kg DM.

Table 6.2. Nutrient composition (g/kg DM), fermentation profile, fatty acids and particle size of grass silage, red clover silage, and control (C), low protein (LP), low protein with added starch (LPS) or rumen-protected methionine (LPM) diets fed to dairy cows.

Item	Forages		Diet[1]			
	Grass silage	Red clover silage	C	LP	LPS	LPM
Dry matter (DM, g/kg)	378	355	484	472	470	475
Organic matter	906	881	914	915	920	913
Ash	94.3	119	85.9	84.8	79.9	86.7
Crude protein	126	183	173	151	153	152
Water-soluble crude protein (g/kg CP)	549	331				
Neutral detergent fibre	475	372	384	399	362	397
Acid detergent fibre	282	307	267	275	252	269
Acid detergent insoluble nitrogen	4.00	6.70				
Starch			134	165	187	154
Ether extract	21.7	16.5	24.6	26.9	27.7	27.0
Fermentation profile (g/kg DM)						
pH	4.11	3.96				
Ammonia-N (g/kg total N)	73.1	51.3				
Lactate	67.1	70.5				
Ethanol	2.87	0.76				
Acetate	27.5	18.9				
propionate	0.51	0.37				
Iso-butyrate		0.55				
Butyrate	0.77	0.20				
Acetate: Propionate	0.14	0.14				
Fatty acid (g/kg DM)						
C16:0	2.42	2.26	5.33	5.18	5.16	4.43
C18:0	0.23	0.29	0.65	0.62	0.60	0.56
C18:1*C*9	0.57	0.26	4.29	4.31	4.31	3.81
C18:2n-6	2.14	2.41	3.75	3.74	4.00	3.61
C18:3n-3	4.44	4.60	3.20	3.17	3.38	3.34
ΣFA	19.8	16.3	19.9	19.7	20.1	18.5
Fractions[2] (% DM)						
> 44 (mm)	0.00	0.00	0.00	0.00	0.00	0.00
33 to 44 (mm)	6.66	2.56	3.30	2.02	2.38	3.60
19 to 32.9 (mm)	31.3	32.3	20.9	19.2	18.9	22.3
8 to 19 (mm)	51.0	49.7	41.7	47.0	41.2	45.2
4 to 8 (mm)	5.14	6.66	10.8	10.7	14.4	10.3
< 4 (mm)	5.93	8.75	23.3	21.0	23.2	18.6
X_m (mm)	20.3	18.6	13.6	13.8	13.0	14.8
SD_{gm}	1.77	1.85	2.20	2.11	2.17	2.11
pef $_{>4}$ (%)	94.1	91.2	76.7	79.0	76.8	81.4
pef $_{>8}$ (%)	88.9	84.6	65.9	68.3	62.4	71.1
peNDF $_{>4}$ (%)	44.7	33.9	30.1	32.0	28.4	33.3
peNDF $_{>8}$ (%)	42.2	31.5	25.8	27.7	23.0	29.1

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine.

[2]Fractons of forages and experimental diets at 0 h post-feeding; DM = dry matter; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fibre.

Table 6.3. Amino acid composition (g/100 g of CP) of grass silage, red clover silage, and control (C), low protein (LP), low protein with added starch (LPS) or rumen-protected methionine (LPM) diets fed to dairy cows.

Item	Forage		Diet[1]			
	Grass silage	Red clover silage	C	LP	LPS	LPM
Alanine	5.46	5.08	4.66	5.05	5.28	5.40
Arginine	1.05	3.08	3.34	3.09	3.89	3.74
Aspartic	6.30	11.2	8.60	9.12	9.60	9.56
Cystine	0.84	0.62	0.60	0.70	0.70	0.69
Glutamic	5.04	8.31	10.2	10.0	13.4	12.3
Glycine	3.99	4.46	4.42	5.05	5.42	4.99
Histidine	1.05	1.69	1.79	1.82	2.09	2.08
Iso-leucine	3.15	3.85	3.46	3.79	4.03	4.02
Leucine	5.46	6.62	6.09	6.31	7.09	6.93
Lysine	3.15	4.77	4.42	4.49	4.87	4.85
Methionine	1.47	0.85	1.31	0.98	1.53	1.52
Phenylalanine	3.57	4.31	3.82	4.07	4.59	4.43
Proline	6.72	6.00	5.73	5.89	6.81	6.79
Serine	2.94	4.46	4.06	4.35	4.73	4.57
Threonine	3.15	3.85	3.58	3.65	3.89	4.02
Tyrosine	1.05	2.92	2.39	2.53	2.50	2.63
Valine	4.41	4.93	4.30	4.77	5.15	5.12

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine.

6.3.2. *In situ* degradability

In situ DM and CP degradability of red clover and grass silages are presented in Table 6.4. The grass silage had a higher ($P < 0.001$) content of soluble (a) fraction of CP and DM compared to the red clover silage. In contrast, the potential rumen degradable CP fraction (b) and the extent of CP degradation (a+b) both were higher ($P < 0.05$) in the red clover than the grass silage. Similarly, the rate of degradation (c) of the potentially degradable DM was higher ($P = 0.019$) whereas the rate of CP degradation was similar ($P > 0.05$). The calculated ED of DM and CP at a rumen outflow rate of 8% per hour was 39 and 46 g/kg higher in the grass compared to the red clover silage.

Table 6.4. In situ DM and CP degradability coefficients of red clover and grass silages fed to dairy cows.

Item[1]	Forages		SEM	*P* value
	Grass silage	Red clover silage		
DM degradation coefficient (g/kg DM)				
a	288	215	6.3	0.001
b	599	615	9.0	0.280
a+b	887	831	4.4	<.001
c	0.07	0.08	0.002	0.019
ED	559	520	2.8	<.001
CP degradation coefficient (g/kg total N)				
a	469	296	8.8	<.001
b	371	584	8.6	<.001
a+b	840	881	3.8	0.002
c	0.08	0.09	0.004	0.133
ED	648	602	5.0	0.003

[1]DM = dry matter; CP = crude protein; a = immediately soluble fraction; b = potentially rumen-degradable fraction; c = rate of per hour degradation of fraction b; ED = calculated effective rumen degradability at 0.08/h rumen passage rate.

6.3.3. Study 3a: Performance and metabolism

6.3.3.1. Intake and animal performance

Dry matter intake did not differ (*P* = 0.371) between cows fed any of the 4 diets, with a mean value of 21.5 kg/d (Table 6.5), but there was an interaction (*P* = 0.007) between diet and time (Figure 6.1), with the intake being highest in cows fed LPS in week 4 and C in weeks 9 and 14. Milk yield, 4% FCM, and ECM yield did not differ (*P* > 0.05) between cows fed any of the diets, with mean values of 37.3, 37.8 and 36.4 kg/d, respectively. Similarly, there was no effect (*P* > 0.05) of diet on milk fat, protein, lactose content or SCC, with mean values of 40.8, 30.5 and 45.8 g/kg, and 3.32 $\log_e$, respectively. In contrast, the milk urea concentration was highest (*P* < 0.001) in cows receiving C at 20.2 mg/dl, which was 6.3 mg/dl higher than those fed LP, LPS or LPM. There was no interaction between diet and time (*P* > 0.05) for milk fat, milk protein or milk urea concentration (Figure 6.2, 6.3 and 6.4, respectively). The feed conversion efficiency was not affected (*P* > 0.05) by diet, but there was a tendency (*P* = 0.082) for ECM/kg DM intake to be lower in cows fed C compared to LPM. There was no effect (*P* > 0.05) of diet on LW or BCS. However, cows receiving LPS had higher LW throughout the feeding period compared to those fed other diets (Figure 6.5). There was a tendency (*P* = 0.075) for an interaction between diet and time for BCS, being lower in cows fed C at week 12 compared to those receiving LPS (Figure 6.6). The NUE was lower (*P* < 0.001) in cows receiving C at 28.6%, compared to cows fed LP, LPS or LPM, which had a mean value of 34.2%.

Table 6.5. Feed intake, milk performance, live weight and body condition of dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3a.

Item	Diet[1]				SEM	*P* value[2]		
	C	LP	LPS	LPM		D	T	Int
Dry matter intake (kg/d)	22.0	21.1	21.7	21.2	0.59	0.371	<.001	0.007
Production (kg/d)								
Milk yield	37.9	36.3	37.8	37.1	0.73	0.170	<.001	0.957
FCM[3] yield	37.2	37.2	38.6	38.1	1.61	0.776	0.003	0.919
ECM[4] yield	36.5	35.6	37.1	36.4	1.05	0.629	0.001	0.930
Composition (g/kg)								
Fat (g/kg)	39.4	40.9	41.6	41.3	1.67	0.581	0.021	0.755
Protein (g/kg)	29.9	30.1	31.3	30.7	0.68	0.400	<.001	0.820
Lactose (g/kg)	45.7	45.8	46.1	45.5	0.44	0.672	0.273	0.872
Somatic cell count (logN)	3.24	3.17	3.29	3.59	0.290	0.641	0.236	0.678
Milk urea (mg/dl)	20.2[a]	14.8[b]	14.4[b]	12.4[b]	1.15	<.001	0.014	0.420
MUN[5] (mg/dl)	9.44[a]	6.91[b]	6.70[b]	5.77[b]	0.534	<.001	0.014	0.420
Yield (kg/d)								
Fat	1.49	1.49	1.55	1.50	0.068	0.801	0.014	0.899
Protein	1.14	1.09	1.16	1.12	0.033	0.263	0.054	0.534
Lactose	1.75	1.67	1.72	1.66	0.049	0.318	0.067	0.498
Feed efficiency								
FCM/DM intake	1.66	1.75	1.77	1.82	0.081	0.130	0.026	0.042
ECM/DM intake	1.62	1.68	1.70	1.74	0.061	0.082	0.006	0.010
Body performance								
Live weight (kg)	667	665	678	673	8.0	0.605	<.001	0.854
Condition score	2.67	2.63	2.70	2.59	0.051	0.199	0.138	0.075
NUE[6] (%)	28.6[b]	33.9[a]	34.1[a]	34.7[a]	1.31	<.001	0.011	0.003

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine;

[2]D = main effect of diet; T = main effect of time; Int = interaction between diet and time;

[3]FCM = 4% fat-corrected milk yield;

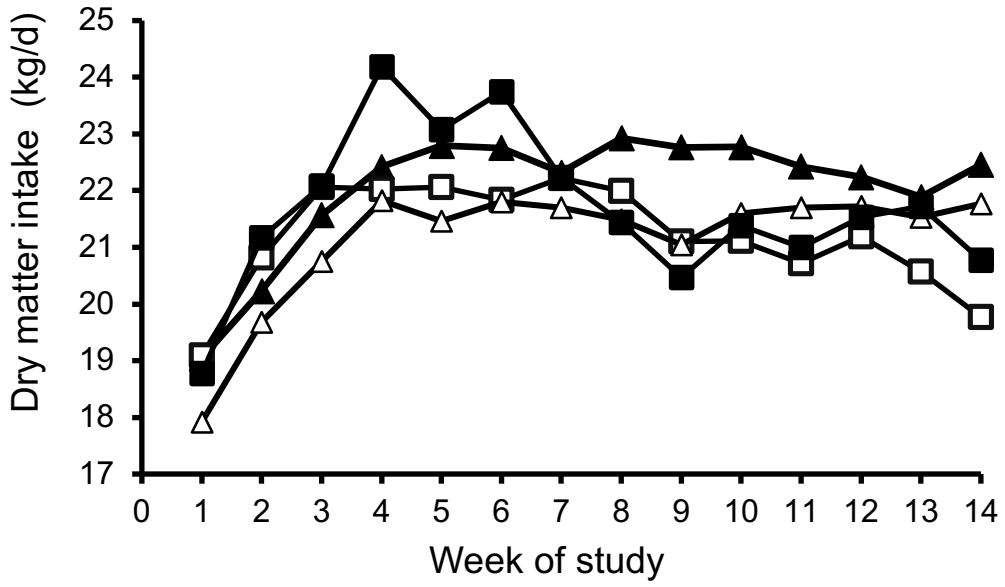
[4]ECM = Energy-corrected milk yield;

[5]MUN = milk urea nitrogen;

[6]NUE = Nitrogen use efficiency.

Week 0 was used as a covariate when appropriate.

Means within a row with a different superscript differ significantly ($P < 0.05$).



1Figure 6.1. Dry matter intake (DMI) of dairy cows offered a control (C, ▲), low CP (LP, ∆), LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 0.59; diet, *P* = 0.371; time, *P* < 0.001; and diet × time, *P* = 0.007.

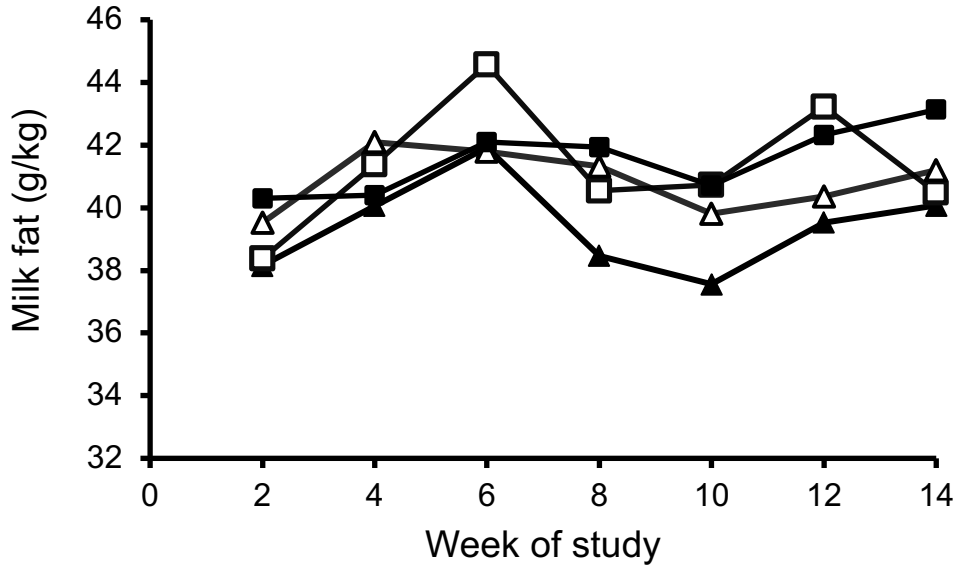


Figure 6.2. Milk fat concentration (g/kg) in dairy cows offered a control (C, ▲), low CP (LP, ∆); LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 1.67; diet, *P* = 0.581; time, *P* = 0.021; diet × time, *P* = 0.755. Week 0 was used as a covariate when appropriate.

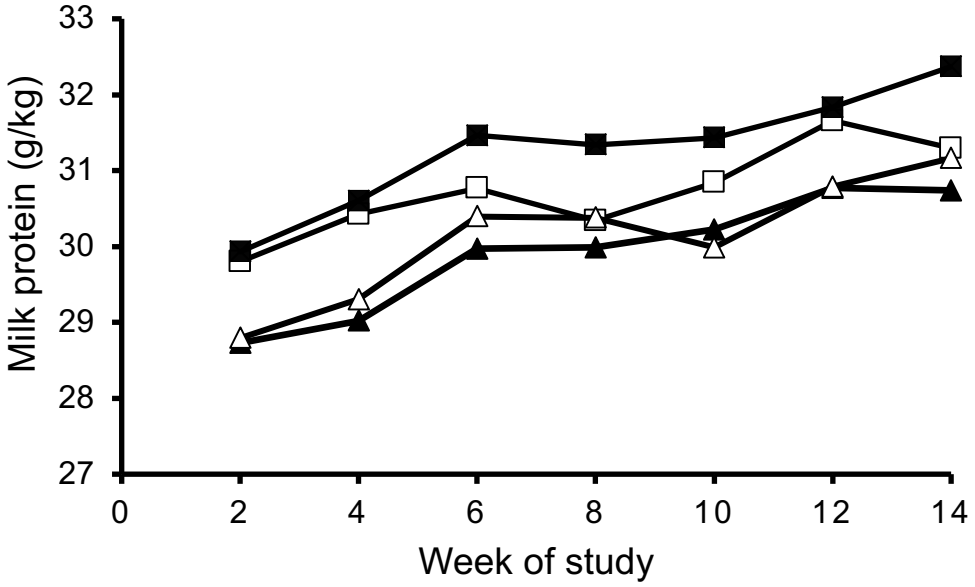


Figure 6.3. Milk protein concentration (g/kg) in dairy cows offered a control (C, ▲), low CP (LP, ∆); LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 0.68; diet, $P = 0.400$; time, $P < 0.001$; diet × time, $P = 0.820$. Week 0 was used as a covariate when appropriate.

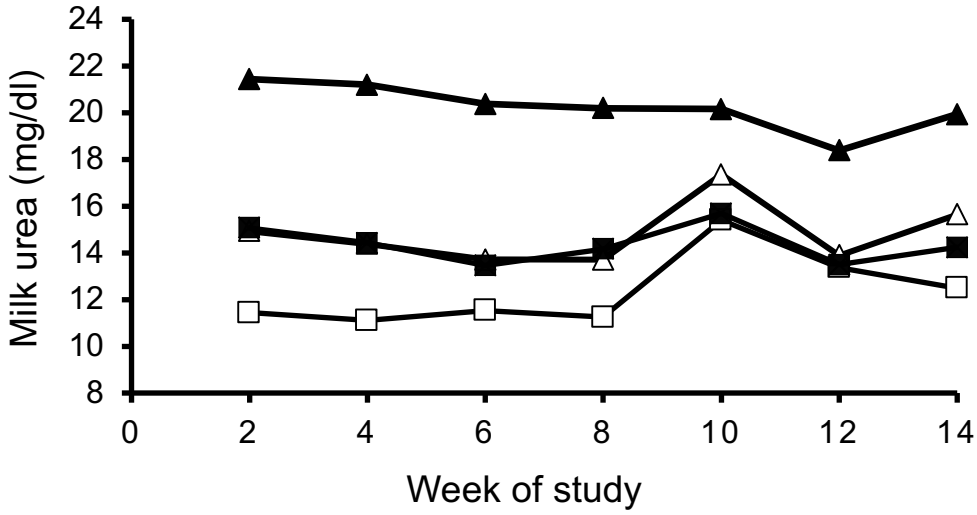


Figure 6.4. Milk urea concentration (mg/dl) in dairy cows offered a control (C, ▲), low CP (LP, ∆); LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 1.15; diet, $P < 0.001$; time, $P = 0.014$; diet × time, $P = 0.420$. Week 0 was used as a covariate when appropriate.

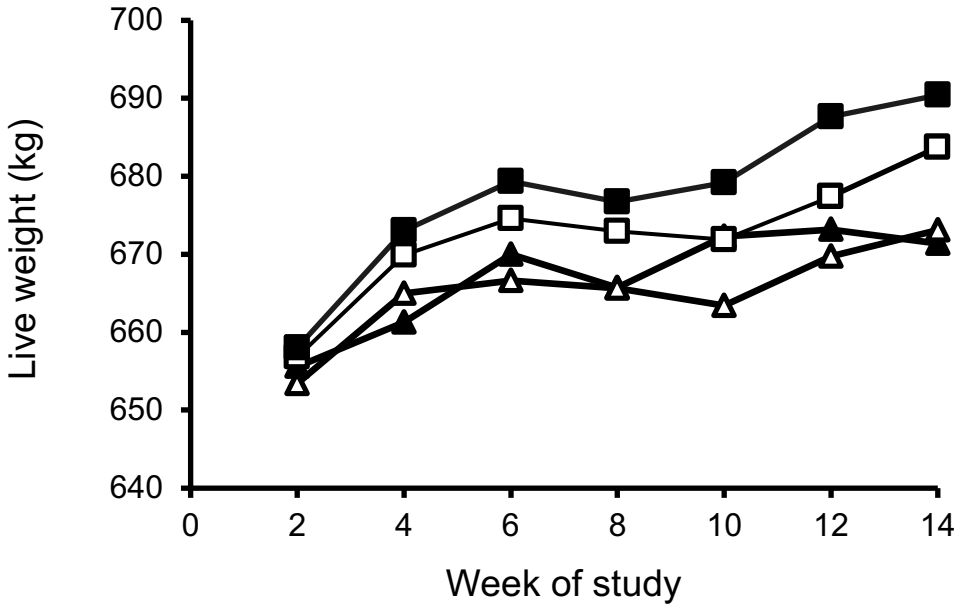


Figure 6.5. Live weight (kg) of dairy cows offered a control (C, ▲), low CP (LP, ▵); LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 8.0; diet, *P* = 0.605; time, *P* < 0.001; diet × time, *P* = 0.854. Week 0 was used as a covariate when appropriate.

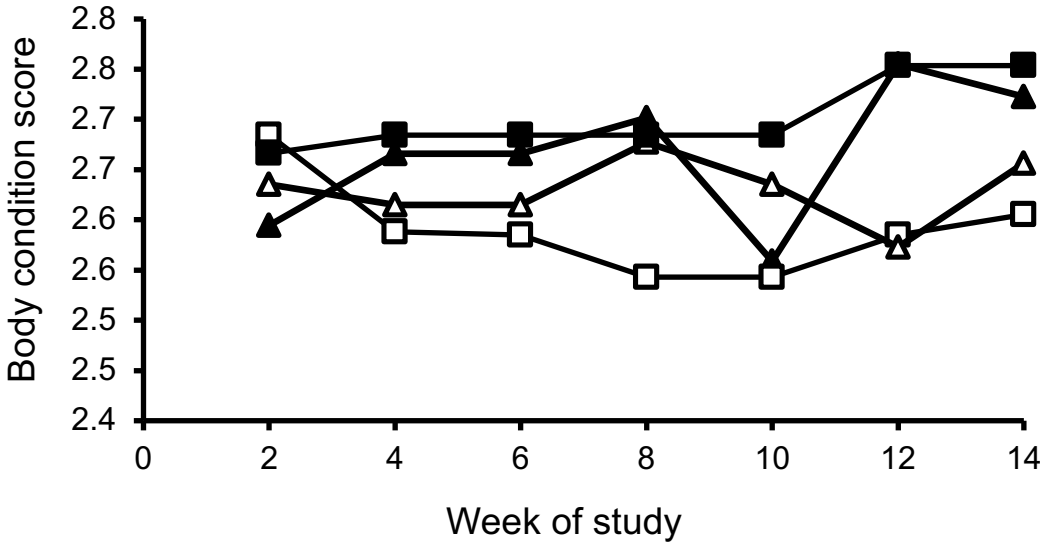


Figure 6.6. Body condition score of dairy cows offered a control (C, ▲), low CP (LP, ▵); LP with added starch (LPS, ■) or rumen-protected methionine (LPM, □) and based on red clover and grass silages in Study 3a. Pooled SEM = 0.051; diet, *P* = 0.199; time, *P* = 0.138; diet × time, *P* = 0.075. Week 0 was used as a covariate when appropriate.

6.3.3.2. Plasma metabolites

The mean concentration of plasma glucose was 3.98 mmol/l and was not affected ($P > 0.05$) by diet or week of the study (Table 6.6 and Figure 6.7a). In contrast, the mean concentration of plasma BHB was 0.22 mmol/l higher ($P = 0.003$) in cows receiving LPM compared to those receiving LPS, but was similar ($P > 0.05$) in animals receiving C or LP at 0.79 mmol/l. There was no effect of time and interaction between diet and sampling week for BHB concentration (Figure 6.7b). The mean concentration of plasma urea was 1.44 mmol/l higher ($P < 0.001$) in cows fed C compared to those fed any low protein diets (LP, LPS or LPM), with an averaged mean of 2.18 mmol/l. There was an effect of time ($P = 0.008$) and interaction between diet and sampling week ($P = 0.002$) for plasma urea, which was similar between diets at week 0 but was higher in cows fed C, at weeks 4, 8 and 14 compared to those fed any of the low protein diets, which did not differ (Figure 6.7c).

Table 6.6. Plasma concentration[1] of glucose, β-hydroxybutyrate (BHB) and urea in dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3a.

Item	Diet[2]				SEM	*P* value
	C	LP	LPS	LPM		
Glucose (mmol/l)	4.08	3.71	4.07	4.05	0.196	0.178
BHB (mmol/l)	0.80^{ab}	0.78^{ab}	0.70^{b}	0.92^{a}	0.067	0.003
Urea (mmol/l)	3.62^{a}	2.09^{b}	2.43^{b}	2.02^{b}	0.189	<.001

[1]Blood plasma was taken from 44 cows on week 0, 4, 8 and 11 (11 cows for each diet group); [2]C = Control (175g CP/kg DM); LP = low protein (150g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine. Week 0 was used as a covariate when appropriate.
Means within a row with a different superscript differ significantly ($P < 0.05$).

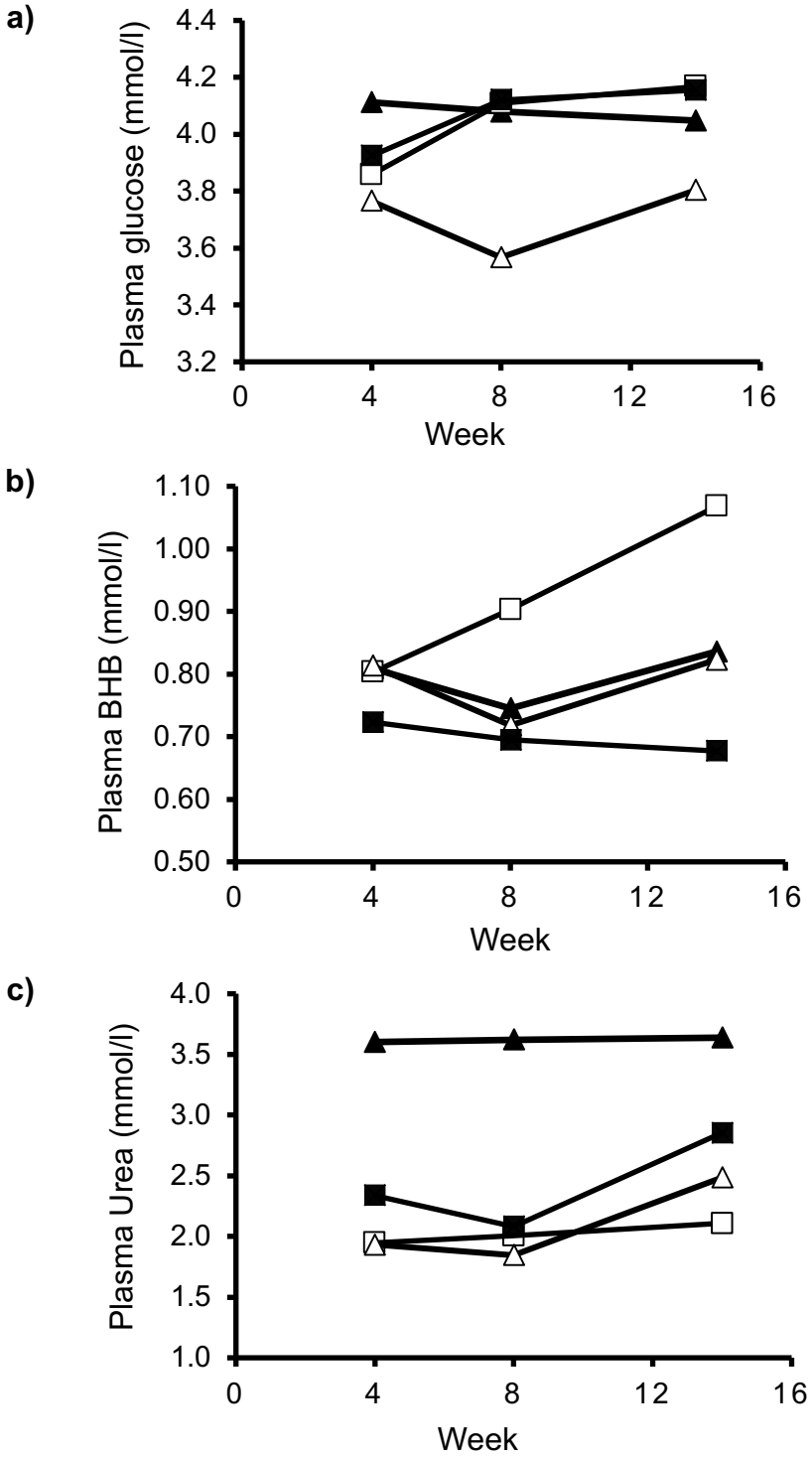


Figure 6.7. Plasma glucose (a), β-hydroxybutyrate (BHB) (b) and urea (c) concentrations in dairy cows offered a control (C, ▲), low CP (LP, △); LP with added starch (LPS, ■) or rumen-protected methionine (LM, □) and based on red clover and grass silages in Study 3a. For plasma glucose; pooled SEM = 0.196; diet, P = 0.178, time, P = 0.556 and diet × time, P = 0.802. For plasma BHB; pooled SEM = 0.067; diet, P = 0.003, time, P = 0.182 and diet × time, P = 0.321. For plasma urea; pooled SEM = 0.189; diet, P < 0.001, time, P = 0.008 and diet × time, P = 0.002. Week 0 was used as a covariate when appropriate.

6.3.3.3. Milk fatty acid profile

The milk fat concentration of C10:0 and C12:0 was higher ($P < 0.05$) in cows fed C or LPS compared to LP or LPM (Table 6.7). Likewise, milk C14:0 and C15:1*c*10 were higher ($P < 0.05$) in cows receiving C compared to those fed any of the low protein diets (LP, LPS or LPM). The lowest ($P < 0.05$) concentration of milk C17:0 and C17:1*c*10 was obtained in cows fed LPS compared to LP or LPM, with C having an intermediate value for milk C17:1*c*10. In contrast, the concentration of C18:1*t*12 was on average 0.035/100 g higher ($P < 0.001$) in the milk fat of cows when fed LPS compared to those fed C or LPM. There was a tendency towards significance ($P < 0.10$) for C18:0 and C18:1 *c*9 to be higher in milk from cows fed LP. Dietary treatment did not affect ($P > 0.05$) the concentration of CLA in the milk fat of cows, although c9, t11 CLA was numerically higher when cows received any of the low protein diets compared to C.

There was no effect of diet ($P > 0.05$) on the total milk fat content of SFA, MUFA, PUFA, linear odd chain, or odd and branch chain FA, but those with a chain length below C16:0 were higher ($P < 0.05$) in milk from cows fed C compared to those fed LP or LPM, with cows fed LPS having an intermediate value. In contrast, the concentration of FA above C16:0 was found higher in cows fed in LP compared to those receiving C or LPS, with cows fed LPM having an intermediate value.

Table 6.7. Milk fatty acid composition of dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3a.

Fatty acid (g/100 g)	Diet[1]				SEM	*P* value
	C	LP	LPS	LPM		
C4:0	1.67	1.72	1.65	1.68	0.035	0.522
C6:0	1.53	1.53	1.55	1.50	0.022	0.474
C8:0	1.10	1.07	1.13	1.05	0.021	0.062
C10:0	2.86^{a}	2.65^{b}	2.87^{a}	2.64^{b}	0.071	0.041
C11:0	0.06	0.05	0.07	0.06	0.006	0.122
C12:0	3.62^{a}	3.28^{b}	3.63^{a}	3.33^{b}	0.100	0.029
C13:0	0.10	0.08	0.11	0.10	0.006	0.076
C14:0	12.4^{a}	11.7^{b}	12.0^{ab}	11.7^{b}	0.18	0.036
C14:1	1.08	1.02	1.08	1.05	0.034	0.494
C15:0	1.21	1.09	1.15	1.19	0.039	0.158
C15:1 *c*10	0.21^{a}	0.19^{b}	0.18^{b}	0.18^{b}	0.007	<.001
C16:0	37.9	37.4	38.2	37.7	0.44	0.620
C16:1	1.35	1.40	1.47	1.46	0.073	0.661
C17:0	0.54^{a}	0.55^{a}	0.49^{b}	0.55^{a}	0.010	<.001
C17:1 *c*10	0.28^{ab}	0.30^{a}	0.25^{b}	0.29^{a}	0.007	<.001
C18:0	8.66	9.41	8.93	9.22	0.213	0.078
C18:1 *t*8	0.22	0.23	0.19	0.24	0.022	0.457
C18:1 *t*9	0.26	0.10	0.17	0.15	0.057	0.274
C18:1 *t*10	0.91	0.95	0.79	0.92	0.067	0.365
C18:1 *t*11	0.84	0.83	0.81	0.85	0.024	0.687
C18:1 *t*12	0.15^{c}	0.17^{bc}	0.20^{a}	0.18^{b}	0.005	<.001
C18:1 *c*9	19.0	20.2	18.8	19.7	0.42	0.081
C18:2n-6 *c*	2.01	2.18	2.26	2.18	0.069	0.106
C18:2n-6 *t*	0.35	0.36	0.39	0.35	0.010	0.087
CLA *c*9, *t*11	0.82	0.88	0.86	0.86	0.022	0.327
CLA t10, c12	0.03	0.03	0.03	0.03	0.002	0.386
C18:3n-3	0.33	0.34	0.32	0.33	0.009	0.302
C18:3n-6	0.06	0.07	0.07	0.07	0.002	0.241
C20:0	0.01	0.02	0.01	0.01	0.001	0.285
C20:3n-3	0.12	0.12	0.12	0.12	0.004	0.665
C21:0	0.07	0.07	0.07	0.06	0.003	0.246
C22:0	0.04	0.04	0.04	0.04	0.003	0.815
EPA	0.11	0.10	0.09	0.10	0.004	0.170
DHA	0.07	0.07	0.07	0.07	0.002	0.990
<C16:0	25.8^{a}	24.4^{b}	25.5^{ab}	24.5^{b}	0.38	0.032
C16:0 + C16:1	39.3	38.9	39.6	39.1	0.48	0.744
>C16	35.0^{b}	36.9^{a}	34.9^{b}	36.2^{ab}	0.56	0.048
SFA[2]	71.8	70.6	71.9	70.9	0.50	0.185
MUFA[3]	24.3	25.3	24.0	25.1	0.45	0.144
PUFA[4]	3.88	4.15	4.22	4.11	0.105	0.137
LOCFA[5]	1.98	1.84	1.88	1.97	0.054	0.211
OBCFA[6]	2.47	2.33	2.31	2.44	0.051	0.090

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine; [2]SFA = saturated fatty acids are defined as fatty acids with no double bonds; [3]MUFA= monosaturated fatty acids are defined as fatty acids with one double bond; [4]PUFA = polyunsaturated fatty acids are defined as fatty acids with more than one double bond; [5]LOCFA= Linear odd chain fatty acids, ∑LOCFA = C11:0+C13:0+C15:0+C17:0+C21:0. [6]OBCFA = Linear odd and branched chain fatty acid, ∑OBCFA = (C11:0+C13:0+C15:0+C15:1+C17:0+C17:1+C21:0; Week 0 was used as a covariate when appropriate; Means within a row with different superscripts differ significantly ($P < 0.05$).

6.3.4. Study 3b: Performance and digestibility

6.3.4.1. Intake and performance

There was no effect (P = 0.848) of dietary treatment on DM intake, with a mean value of 21.5 kg/d (Table 6.8). Similarly, dietary treatment did not affect (P > 0.05) milk yield, 4% FCM or ECM, with means of 36.6, 41.2 and 38.0 kg/d, respectively. The mean concentration of milk fat, protein and lactose were 45.2, 30.7 and 46.9 g/kg, respectively, whilst SCC was 3.56 $\log_e$ and was not affected (P > 0.05) by dietary treatment. In contrast, there was a difference (P = 0.004) between dietary treatment on milk urea concentration, with the milk of cows receiving C having the highest concentration at 25.5 mg/dl, which was 8.67 mg/dl higher than those fed LP, LPS or LPM, which did not differ (P > 0.05). Dietary treatment had no effect (P > 0.05) on feed conversion efficiency. Similarly, the mean LW or BCS did not differ (P > 0.05) between diets.

Table 6.8. Intake, milk and body performance of dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3b.

Item	Diet[1]				SEM	P value
	C	LP	LPS	LPM		
Intake (kg/d)	22.1	21.4	21.6	21.0	0.91	0.848
Production (kg/d)						
Milk yield	35.2	37.8	36.7	36.6	1.24	0.527
FCM[2] yield	43.3	40.5	38.4	42.5	2.76	0.607
ECM[3] yield	38.6	38.3	36.6	38.6	1.71	0.809
Composition (g/kg)						
Fat	49.5	42.7	41.9	46.6	2.43	0.155
Protein	31.8	30.3	30.0	30.9	1.09	0.688
Lactose	46.6	47.4	47.2	46.4	0.73	0.743
Somatic cell count (logN)	3.80	3.23	3.16	4.06	0.468	0.483
Milk urea (mg/dl)	25.5[a]	17.5[b]	17.9[b]	15.1[b]	1.61	0.004
Yield (kg/d)						
Fat	1.73	1.62	1.54	1.70	0.110	0.607
Protein	1.11	1.14	1.10	1.13	0.042	0.885
Lactose	1.64	1.79	1.73	1.70	0.062	0.428
Feed efficiency						
FCM/DM intake	1.97	1.89	1.79	2.04	0.112	0.472
ECM/DM intake	1.76	1.79	1.70	1.85	0.079	0.616
Body performance						
Live weight (kg)	715	667	707	665	40.5	0.746
Live weight change[4] (kg/d)	0.31	0.20	0.10	0.26	0.101	0.528
Condition score	2.80	2.55	2.80	2.60	0.162	0.591
Condition score change[4]	0.05	-0.20	-0.10	-0.25	0.078	0.079

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine; [2]FCM = 4% fat-corrected milk yield.[3]ECM = Energy-corrected milk yield; [4]Change over the 15-week feeding period.
Means within a row with a different superscript differ significantly (P < 0.05).

6.3.4.2. Apparent digestibility

There was no effect ($P > 0.05$) of dietary treatment on intake or faecal output of DM, OM, NDF and ADF but N intake was highest in cows receiving C, lowest in LP or LPM and intermediate in cows fed LPS (Table 6.9). Similarly, digestible N (g/d) was highest in cows fed C, lowest in LP, with LPS and LPM having an intermediate value. There was no difference ($P > 0.05$) between dietary treatments on apparent whole tract digestibility of DM, OM, N, NDF and ADF, with mean values of 0.766, 0.785, 0.660, 0.660 and 0.652 kg/kg, respectively.

Table 6.9. Intake, faecal output and apparent digestibility of nutrients[1] in dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3b.

Item	Diet[2]				SEM	*P* value
	C	LP	LPS	LPM		
DM (kg/d)						
Intake	22.1	21.4	21.6	21.0	0.91	0.848
Faecal output	4.95	5.82	4.66	4.67	0.482	0.324
Digested	17.2	15.6	16.9	16.3	0.90	0.626
Digestibility (kg/kg)	0.773	0.726	0.785	0.779	0.0226	0.278
OM (kg/d)						
Intake	20.3	19.7	20.0	19.2	0.84	0.818
Faecal output	4.16	4.89	4.00	3.87	0.418	0.354
Digested	16.2	14.8	16.0	15.4	0.84	0.637
Digestibility (kg/kg)	0.792	0.748	0.801	0.800	0.0216	0.308
N (kg/d)						
Intake	0.62[a]	0.52[b]	0.53[ab]	0.51[b]	0.023	0.020
Faecal output	0.18	0.21	0.17	0.17	0.019	0.343
Digested	0.44[a]	0.31[b]	0.36[ab]	0.34[ab]	0.028	0.037
Digestibility (kg/kg)	0.702	0.583	0.682	0.671	0.0384	0.191
NDF (kg/d)						
Intake	8.07	7.99	7.28	7.67	0.369	0.442
Faecal output	2.58	3.02	2.50	2.41	0.251	0.373
Digested	5.50	4.97	4.78	5.25	0.374	0.569
Digestibility (kg/kg)	0.675	0.621	0.659	0.686	0.0313	0.502
ADF (kg/d)						
Intake	5.71	5.42	5.26	5.15	0.229	0.372
Faecal output	1.88	2.19	1.72	1.69	0.190	0.269
Digested	3.83	3.22	3.54	3.46	0.274	0.495
Digestibility (kg/kg)	0.670	0.590	0.675	0.670	0.0371	0.348

[1]DM = dry matter; OM = organic matter; N = nitrogen; NDF = Neutral detergent fibre; ADF = Acid detergent fibre.
[2]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine.
Means within a row with a different superscript differ significantly ($P < 0.05$).

6.3.4.3. Nitrogen output and efficiency

The N concentration of the diets was highest ($P < 0.05$) in C and lowest in LP, LPS or LPM (Table 6.10), reflecting the CP content of the diets. Consequently, N intake

was highest ($P < 0.05$) in cows receiving C, lowest in LP or LPM and intermediate in LPS. There was a tendency ($P = 0.062$) for the urine volume to be 4.33 l/d higher in cows fed C than any low protein diets (LP, LPS or LPM), which had a mean urine output of 22.4 l/d. There was no effect ($P > 0.05$) of dietary treatment on faecal or milk N excretion, but daily urinary N output was 65.7 g higher in cows fed C compared to those fed LP or LPM, that had a mean value of 92.1 g/d, whilst cows receiving LPS had an intermediate value, at 106 g/d. There was also a difference ($P < 0.001$) between diets on apparent NUE for milk production, which was higher in cows receiving LP or LPM compared to C, with a mean value of 34.7%, approximately 6.3% units higher than in C, with LPS having an intermediate value. The mean urea-N concentration in blood plasma, milk or urine was highest in cows fed C compared to those receiving low protein diets (LP, LPS or LPM). Likewise, the daily urea-N excretion in milk and urine was increased in cows fed C. There was a positive relationship ($R^2 = 0.52$; $P < 0.001$) between milk urea concentration and urinary N output, as shown in Figure 6.8.

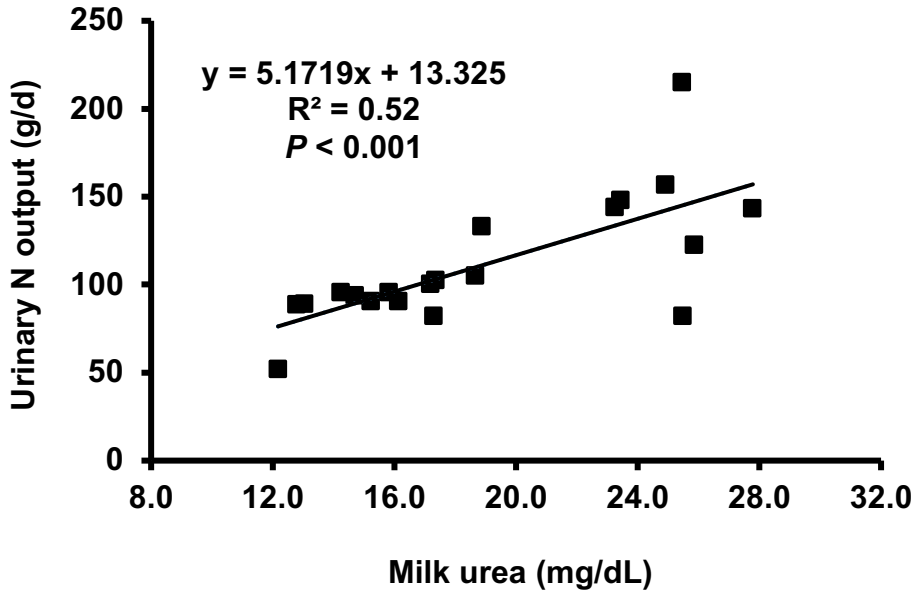


Figure 6.8. Relationship between urinary N output (g/d) and milk urea N (mg/dl) concentration in lactating dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3b.

Table 6.10. Nitrogen concentrations, excretion and partitioning in dairy cows fed control (C), low protein (LP) and low protein with added starch (LPS) or rumen-protected methionine (LPM) diets based on red clover and grass silage in Study 3b.

Item	Diet[1]				SEM	*P* value
	C	LP	LPS	LPM		
N concentration (g/kg)						
Diet	28.0[a]	24.3[b]	24.6[b]	24.4[b]	0.17	<.001
Faecal	36.7	36.7	36.3	36.3	0.77	0.962
Urine (g/l)	5.87[a]	3.91[b]	4.81[ab]	4.17[b]	0.381	0.015
Milk	4.98	4.75	4.70	4.85	0.171	0.688
N intake and excretion (g/d)						
Intake	618[a]	521[b]	530[ab]	510[b]	22.7	0.020
Faecal	182	214	169	169	19.1	0.343
Milk	174	179	173	177	6.6	0.885
Urine	156[a]	90.3[b]	106[ab]	93.9[b]	12.72	0.011
Volume of urine (l/d)	26.8	22.7	21.9	22.7	1.23	0.062
N balance[2]	106	37.3	82.0	70.6	22.00	0.226
N partitioning (%)						
Faecal	29.8	41.7	31.8	32.9	3.84	0.191
Urine	25.1[a]	17.4[b]	19.9[ab]	18.4[ab]	1.59	0.023
NUE[3]	28.4[b]	34.5[a]	32.6[ab]	34.9[a]	1.31	0.017
Urea N concentration (mg/dl)						
Milk urea N	11.9[a]	8.15[b]	8.36[b]	7.05[b]	0.753	0.004
Plasma urea N	4.76[a]	3.25[b]	3.20[b]	2.78[b]	0.319	<.001
Urine urea N	135[a]	63.1[b]	95.9[b]	78.3[b]	8.58	<.001
Urea N excretion (g/d)						
Milk urea N	4.19[a]	3.10[ab]	3.06[ab]	2.58[b]	0.330	0.029
Urine urea N	36.1[a]	14.8[b]	21.1[b]	17.7[b]	2.37	<.001

[1]C = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch; LPM = LP with added rumen-protected methionine; [2]N = Nitrogen; N balance (g/d) = Intake N – (Milk N + Faecal N + Urinary N); [3]NUE = Nitrogen use efficiency.

Means within a row with a different superscript differ significantly (*P* <0.05).

6.4. Discussion

6.4.1. Forage and diet composition

The chemical analysis of the two forages used in the current study revealed that the CP concentration was 45% higher in red clover than the grass silage, a finding similar to that report by Dewhurst et al. (2003b), who reported that homegrown UK legume silages had a CP content that was 40 to 86% higher than grass silage. The CP levels in both silages were also comparable with the forages used by Broderick (2018), but slightly lower than that reported by Dewhurst et al. (2003b). In general, legumes contain less fibre and more CP than grass silage (Dewhurst, 2013). In the current study the NDF content was 103 g/kg DM higher in the grass silage than the red clover, which was reflected in a higher PS fraction of PeNDF in the grass silage. The pH is usually higher in legume silages, which reflects a high buffering capacity compared to other forages (Dewhurst et al., 2003b); however, in the current study, the grass silage had a high pH, which could not be explained by a higher level of acetate content in the grass silage compared to the red clover silage (Dewhurst et al., 2010). The mean PS of grass silage was slightly higher than the red clover silage due to the high content of long (33-44 mm) and medium (8-19 mm) PS fraction, but lower than the long or short chop grass silage used by Tayyab et al. (2018a).

Recent studies have shown that methionine is the first limiting AA in dairy cows when fed diets based on legume silages, particularly red clover or lucerne silage (Vanhatalo et al., 2009; Broderick, 2018). In the current study there was a low concentration of methionine in both silages, which is consistent with other studies (Socha et al., 2005; Lee et al., 2009). A study by Vanhatalo et al. (2009) reported that the total concentration of basic AA in early and late cut red clover silage was 1.8 and 1.1 g/100 g of CP higher than the first and late maturity stages of grass silage, respectively, a finding in accordance with the current results. The higher concentration of methionine and acidic AA in LPM or LPS compared to LP is most probably due to the supplementation of synthetic rumen-protected methionine and barely as a source of starch, which is rich in glutamic acid (Arendt and Zannini, 2013).

6.4.2. *In situ* DM and CP degradability

The soluble fraction of DM and CP in the grass silage used in the current study was 73 and 173 g/kg, respectively higher than the red clover silage, a finding in

accordance with previous work by Dewhurst et al. (2003a). According to Purwin et al. (2014), the soluble CP fraction of red clover and grass silage was 204 and 239 g/kg; which was lower than in the current study. Several studies have shown that the potentially degradable CP fraction of red clover silage is higher than grass silage (Dewhurst et al., 2003a; Purwin et al., 2014; Damborg et al., 2018), which is consistent with the current findings and could be due to the higher rate of degradation, although the rate of CP degradation was comparable between forages. In the current study, the calculated ED of DM and CP was lower in red clover compared to the grass silage, which might be related to the presence of the PPO enzyme in red clover. This enzyme reacts with phenols in the presence of oxygen to produce quinones, which inhibit the role of proteases that degrade the forage proteins (Jones et al., 1995a; Broderick et al., 2001). Moreover, quinones reacts with red clover proteins and reduce its NPN content (Jones et al., 1995b). Alternatively, the higher concentration of ammonia and lower content of ADIN in grass silage could be the reasons for a high calculated ED compared to the red clover silage (Nuez-Ortín and Yu, 2010; Purwin et al., 2014). However, several studies (Hoffman et al., 1993; Dewhurst et al., 2003a; Damborg et al., 2018) have reported that the higher ED in red clover silage might be associated with the greater content of non-protein N relative to neutral detergent insoluble CP (Westreicher-Kristen et al., 2017).

6.4.3. Intake and animal performance

Reducing dietary CP concentration from 175 to 150 g/kg DM in the current studies (Study 3ab) did not affect the DM intake of cows fed the red clover/grass silage-based diets. A study by Broderick et al. (2015) reported that there was no decrease in DM intake when dietary CP concentration was reduced from 170 to 150 g/kg DM in a lucerne and maize silage-based diet. A similar response has also been observed by Kidane et al. (2018) and Olmos Colmenero and Broderick (2006), who fed an even lower concentration of CP (from 175 to 130 g CP/kg DM and 194 to 135 g CP/kg DM, respectively) than the current study. The addition of starch or RPM in a low CP diet did not affect DM intake, a finding in agreement with recent work that have examined the effect of MP deficient diets with supplemented starch or RP-AA alone or in combination with other limiting AA on the intake performance of Holstein dairy cows (Lee et al., 2012a; Recktenwald et al., 2014; Giallongo et al., 2016). Indeed, meta-analysis have reported no significant effect of RPM on DM intake and

noted that adequate MP supply in the diet could have resulted in a lack of a response (Patton, 2010; Robinson, 2010; Sinclair et al., 2014).

In the current study there was a higher DM intake in cows fed LPS than the other low CP diets at week 4, which could be attributed to available rumen energy from the high starch content in LPS. However, the intake difference between diet C and LPS or LPM rather than LP at week 9 and 14, respectively, could not be explained by the differences in supplementation, although mean DM intake was numerically higher in cows fed LPS than LP in Study 3a and 3b. The previous study (Study 1, Chapter 4) reported a decreased DM intake of 1.6 kg/d when dietary CP concentration was reduced from 175 to 150 g CP/kg DM in a red clover/grass silage-based diet. A similar response was observed by Huhtanen and Hetta (2012), who investigated feed intake and milk yield with in a data sets of 204 studies. Reduced feed intake due to a low CP or MP deficient diet may indicate impaired rumen function that depresses fibrolytic bacteria and decreased apparent fibre digestibility (Russell et al., 1992; Allen, 2000).

In line with earlier studies (Olmos Colmenero and Broderick, 2006; Bahrami-Yekdangi et al., 2014), there was no effect of dietary CP on milk yield or milk composition in early lactation cows. A similar result was reported by Barros et al. (2017) when the concentration of CP was reduced from 162 to 144 g/kg DM in a lucerne and maize silage based ration. There was also no effect of additional starch or RPM on total milk yield, milk fat, protein or lactose content in the current study, a finding in agreement with previous reports that have examined the effect of RP-AA on lactation performance of dairy cows (Giallongo et al., 2015, 2016; Lee et al., 2015a). The similar milk performance in cows fed the low CP diets might be associated with the supply of sufficient MP, and suggests that dietary CP concentration in red clover/grass silage-based diets can be reduced to 150 g/kg DM and still maintain performance. Hristov and Giallongo (2014) demonstrated that feeding a diet with 150 g CP/kg DM does not adversely affect the yield and composition of milk in dairy cows. However, reducing dietary CP concentration below 150 g/kg DM can negatively affect milk production (Lee et al., 2012a; Alstrup et al., 2014). It has been hypothesised that a lower concentration of CP (< 150 g/kg DM) can reduce the post-ruminal supply of MP and contribute to decreased milk and milk protein yield (Hristov and Giallongo, 2014; Giallongo et al., 2016),

indicating that the intestinal supply of MP and milk yield are strongly correlated (Daniel et al., 2016).

It was observed that feeding a low CP diet without or with starch or RPM supplementation reduced milk urea concentration. A similar response was also observed when the dietary CP concentration was reduced from 185 to 135 g/kg DM in dairy cow rations (Lee et al., 2012a; Niu et al., 2016; Oh et al., 2019). It is generally accepted that increasing dietary protein content can increase plasma urea concentration through urea absorption from the rumen which is reflected in a higher output of milk urea (Bach et al., 2000). In line with previous observations (Giallongo et al., 2016; Barros et al., 2017), feeding low CP diets did not alter LW and BCS, mostly because DM intake was not affected.

6.4.4. Digestibility and metabolism

A concentration of CP in a mixed ration below 165 g/kg DM can contribute to a lower nutrient digestibility, as suggested by Olmos Colmenero and Broderick (2006). Some studies (Lee et al., 2012b; Giallongo et al., 2015) have also reported a lower nutrient digestibility when low CP (135 to 148 g/kg DM) or MP deficient (-5 to -15% less than requirements) diets were fed to dairy cows. The effect of low protein diets on reducing nutrient digestibility could be due to a lower supply of RDP or concentration of rumen ammonia, which may decreases the growth of rumen microorganisms, resulting in a depressed feed intake and fibre digestibility (Olmos Colmenero and Broderick, 2006; Lee et al., 2011, 2012a). However, in Study 3b, no difference was observed between diets on apparent total tract nutrient digestibility, which indicates the rumen function was not impaired by feeding a low CP (150 g/kg DM) or marginally MP-deficient (-2% less than requirements) diets.

The addition of starch or RPM had no effect on nutrient digestibility, although it was numerically higher when low CP diets were supplemented (LPS, LPM) compared to the basal low CP diet (LP). It was hypothesised that dietary starch or RP-AA supplementation would improve DM intake and MP supply to the intestine and reduce the negative impact of low CP diets on apparent digestibility, particularly fibre. However, the role of RP-AA, including methionine, lysine or histidine, on feed intake and digestibility is not clear and is difficult to predict (Lee et al., 2012a; Sinclair et al., 2014; Giallongo et al., 2015). A study by Davies et al. (2013) also showed that diets supplemented with rumen degradable starch had no effect on total tract

nutrient digestibility in beef heifers. However, limited research exists with dairy cows that have examined the supplementation of dietary starch in low protein diets on nutrient digestibility and metabolism.

Feeding low CP diets either without or with supplementation decreased plasma urea concentrations in Study 3a, an effect in accordance with previous observations (Lee et al., 2012a, 2015a; Alstrup et al., 2014), who have investigated the effects of dietary protein or MP-deficient diets with the addition of RP-AA. A numerical increase in plasma urea concentration was also observed in cows when a low CP diet was offered with added starch (LPS). Similarly, Recktenwald et al. (2014) demonstrated that increasing the level of starch from 230 to 290 g/kg DM in a low CP diet (153 g CP/kg DM) increased the content of plasma urea-N in lactating dairy cows. Reduced plasma urea concentration due to feeding low CP diets can be attributed to a lower intake of N, which can lead to a decrease in urea absorption and recycling (Sinclair et al., 2012; Alstrup et al., 2014). Additionally, the lower milk urea-N concentration in cows fed any of the low protein diets (LP, LPS or LPM) in Study 3a also reflects the decrease in plasma urea-N. A study by Olmos Colmenero and Broderick (2006) confirmed that plasma and milk urea-N were highly correlated (R^2 = 0.83).

The higher concentration of plasma BHB in cows fed LPM could be attributed to a mobilisation of body reserves which might be associated with tendency towards lower BCS. Similarly, a study by Law et al. (2009) noted that the plasma concentration of BHB was increased by 0.08 mmol/l in cows fed 114 g CP/kg DM, although there was no effect of diet on BCS change. In contrast, several studies (Krober et al., 2000; Alstrup et al., 2014; Kaufman et al., 2020) have reported no effect of dietary CP on plasma BHB concentration. In line with previous reports (Bach et al., 2000; Bahrami-Yekdangi et al., 2014; Giallongo et al., 2016), there was no effect of dietary CP level on plasma glucose concentration in dairy cows. Plasma glucose level was, however, numerically higher in cows fed the low CP diets with supplementation (LPS or LPM) with either starch or AA, which have gluconeogenic effects (Ranawana and Kaur, 2013; Cantalapiedra-Hijar et al., 2014). However, plasma metabolites are often not affected by the inclusion of RPM in low CP or MP deficient diets (Krober et al., 2000; Giallongo et al., 2015, 2016).

6.4.5. Milk fatty acid, N output and efficiency

In line with Study 1 (Chapter 4), dietary CP concentration had little influence on milk FA profile. The concentration of FA in cows milk generally depends on consumed or supplemented feedstuffs, rumen fermentation, particularly biohydrogenation of long-chain PUFA, the duodenal flow of MCP, *de novo* synthesis of FA in animal tissue, and body fat mobilisation during negative energy balance (Mansbridge and Blake, 1997; Vlaeminck et al., 2006). In Study 3a, the highest concentrations of FA of chain length < C16:0 (mainly C10:0, C12:0 and C14:0) and some OBCFA (mainly C15:1, C17:0 and C17:1) was observed in the milk of cows fed C or LPM, which could be attributed to higher levels of rumen degradable N, which may lead to increased rumen microbial growth and outflow towards the intestine, or *de novo* synthesis of FA from propionate in the liver of cows (Giallongo et al., 2016). A study by Leduc et al. (2017) showed a positive relationship between RDP supply and the yield of some milk OBCFA. These FA principally derive from rumen microbial lipids and have been suggested as markers to predict MCP synthesis (Dewhurst et al., 2006; Vlaeminck et al., 2006; Cabrita et al., 2011). In line with the current findings, previous work (Giallongo et al., 2015; Lee et al., 2015b) has also reported an increased yield of OBCFA (mainly C15:0, C17:0 and C17:1) when the diet was supplemented with RPM or methionine analogue. However, recent work (Robinson et al., 2011; Giallongo et al., 2016) have shown that RP-AA, including methionine, has no effect on milk FA composition. In the current study (Study 3a), supplementation with dietary starch did not alter the content of milk C15:0 or C17:0 FA, whereas Cabrita et al. (2007) demonstrated that both FA's were increased by the addition of 250 g/kg DM starch in the diet of mid-lactation dairy cows, although the apparent recovery of C18:0 FA from feed to milk was reduced.

The sum of milk C16:0 and C16:1 FA did not differ between dietary treatment, but the concentration of milk FA of a chain length less than C16:0 and C18:2n-6*t* (tendency) were increased when cows were fed low CP diets. This might be associated with a higher supply of rumen-protected expeller rapeseed meal or a lower concentration of RDP (Mansbridge and Blake, 1997; Leduc et al., 2017). However, the higher concentration of intermediary biohydrogenation products such as C18:1*c*9 and C18:1*t*12 in the milk fat of cows fed low CP diets was not expected and may be due to a higher supply of rumen-bypass rapeseed meal, which is rich in C18:1 (Hristov et al., 2011a). In addition, the highest concentration of

biohydrogenation end products such as stearic acid (C18:0) in the milk fat of cows fed any of the low CP diets could not be explained due to low levels of RDP, which could affect ruminal biohydrogenation of long-chain FA (Leduc et al., 2017). Apart from feed FA, the other source of milk C18:0 is body adipose tissue mobilisation, but there was no differences in LW or BCS change in Study 3a, which could have indicated that a release of stearic acid had occurred (Alstrup et al., 2014).

The variation in N intake by cows in the current study (Study 3b) was due to numerical changes in DM intake. The concentration of urinary N output was observed higher in cows fed C compared to LP or LPM, which was primarily related to an increased N intake, urinary N concentration, and volume of urine. Likewise, a study by Giallongo et al. (2015) reported a substantial decrease in urinary N excretion of 55.0 g/d when dietary CP content was reduced from 167 to 148 g/kg DM in dairy cow rations. This was attributed to lower N intake and digestibility in cows fed low CP diets. There is a negative linear relationship between dietary N intake and urinary N excretion in dairy cows, as excretion of N in urine increases when the intake of N exceeds 400 g N per day (Castillo et al., 2000). One of the objectives of the current study was to improve NUE in cows by reducing the concentration of dietary CP. In agreement with previous works (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a; Kidane et al., 2018b), an increase in apparent NUE was observed when dietary CP was reduced. A meta-analysis by Huhtanen and Hristov (2009) reported that increasing the capturing of total N for milk protein synthesis can improve NUE in milk whereas, the higher concentration of N in urine leads to an inefficient partitioning of N.

The mean concentration and excretion of urea-N in milk and urine was highest in cows fed C compared to those fed any of the low CP diets, a finding in agreement with other studies (Lee et al., 2012a; Niu et al., 2016; Oh et al., 2019). Likewise, Kidane et al. (2018) demonstrated that the content of milk urea-N was increased by 4.14 mg/dl when the dietary CP concentration was increased from 145 to 175 g/kg DM in grass silage-based rations. The higher concentration of urea-N excretion in milk and urine was also reflected in a high level of plasma urea-N, a finding in accordance with previous observations (Cabrita et al., 2007; Recktenwald et al., 2014; Niu et al., 2016) who have examined urea-N recycling and excretion in cows fed different levels of dietary CP. Previous studies (Spek et al., 2013) have reported a positive relationship between milk urea-N and urinary N output in dairy cows. A

positive relationship between urinary N output (g/d) and milk urea N (mg/dl) concentration of lactating dairy cows was also observed in the current study, and the concentration of milk urea was highly correlated with the dietary level of CP (Spek et al., 2013).

6.5. Conclusions

Reducing the dietary CP concentration from 175 to 150 g/kg DM in a red clover/grass silage-based diet did not affect intake or animal performance. Feeding a low protein diet that was marginally deficient in MP with added starch or RPM had no beneficial effect on DM intake, milk yield, milk composition, LW or condition. Milk urea concentration was decreased by 7.09 mg/dl, and the apparent NUE was increased by approximately 20% in dairy cows when fed a low CP diet either without or with added starch or RPM. It is concluded that reducing the dietary CP concentration from approximately 175 to 150 g/kg DM in red clover/grass silage-based diets will reduce the environmental impact of milk production without negatively affecting dairy cow performance if the diets are formulated to maintain MP supply. However, the addition of starch or RPM to the diet may have little or no benefit. Further research on low protein diets based on red clover silage is warranted to investigate the effect of lower dietary protein/MP at different ratios of red clover to grass silage and forage to concentrate.

CHAPTER 7: Effect of reducing dietary protein level on the performance and nitrogen use efficiency of dairy cows fed legume-based diets: A systematic review and meta-analysis.

7.1. Introduction

The principle objective when reducing the CP content of the diet of dairy cows is to decrease feed costs and reduce the excretion of N from urine and manure (Sinclair et al., 2014; Broderick et al., 2015). Feeding high CP diets to dairy cows leads to inefficiency in N use, as only 20 to 35% of dietary N is currently synthesised into milk (Broderick, 2003). The lost N contributes to adverse environmental effects, including eutrophication, ammonia emissions and acidification, and subsequently has a negative public health effect, including cardiovascular and respiratory problems (Hristov et al., 2011b; Grout et al., 2020). The most effective approach to improve N utilisation and decrease N loss is to avoid overfeeding protein (Olmos Colmenero and Broderick, 2006; Huhtanen et al., 2008a; Broderick et al., 2015). As a consequence, extensive research has been conducted to determine optimal protein requirements for maximizing the production of dairy cows, including the conversion of dietary protein into milk N (Hristov et al., 2004; Huhtanen and Hristov, 2009; Lee et al., 2015a).

Feeding low CP diets to early or mid-lactation dairy cows has been widely studied, but the responses have not been consistent, possibly due to a wide variety of dietary ingredients, supplementation strategies or treatments being based on CP content rather than MP (Huhtanen et al., 2008a; Huhtanen and Hristov, 2009; Sinclair et al., 2014). Most nutritional systems employed around the world, including Feed into Milk (FiM) in the UK (Thomas, 2004), NRC (2001) in the USA or INRA (2018) in France, consider dietary CP as containing two major fractions; that which is degraded in the rumen and is available for MCP synthesis (referred to in FiM as effective degradable nitrogen (EDN) or as RDP in other rationing systems), and that which by-passes the rumen and is subsequently available for absorption in the small intestine (referred to in FiM as digestible undegradable protein (DUP) or as RUP in other systems). The combination of digestible MCP (synthesised from EDN and rumen available energy) from the rumen, along with DUP provides the MP supply to the dairy cow, which she requires for maintenance, milk performance and foetal growth (Thomas, 2004). However, most studies in the literature reported dietary CP content rather than EDN or MP supply, making responses difficult to evaluate (Sinclair et al., 2014).

A previous review of the literature and meta-analysis using a data set of 207 production trials (Huhtanen et al., 2008a) reported that the dietary CP content and rumen protein balance were essential predictors influencing the apparent milk NUE. Similarly, Sinclair et al. (2014) and Study 1, Study 2 and Study 3ab reported that dietary CP concentration could be reduced to around 140 to 150 g/kg DM without affecting performance if the diets meet the cows MP requirements. However, other dietary and animal variables such as forage source, parity and stage of lactation can also have a strong influence on nutrient use efficiency and milk performance in dairy cows (Hristov et al., 2004; Huhtanen et al., 2008b; Huhtanen and Hristov, 2009).

In addition to providing an adequate supply of MP, the amino acid content of the protein reaching the small intestine is also important. As a consequence, dietary supplementation with limiting AA such as RPL, RPM or both (RPML) to dairy cows fed low CP diets has been evaluated in a number of studies, although in most cases the performance and NUE response have not been consistent (Patton, 2010; Robinson, 2010; Lee et al., 2015a). However, more recent studies (Lee et al., 2012a; Giallongo et al., 2016) have reported that feeding MP deficient diets decreases feed intake, milk yield and milk components in lactating cows, and suggested that a combination of rumen-protected AA has the potential to improve milk performance under such dietary conditions.

Home-grown forage legume silages such as lucerne, red clover, peas or beans are attractive forages to include in the diet of dairy cows as they reduce the requirement for artificial fertilisers to grow the crop, and decrease the requirement for purchased protein sources such as soybean, rapeseed or maize gluten to meet the cows MP requirements (Halmemies-Beauchet-Filleau et al., 2018). Forage legumes are characterised by having a higher CP content than grass or maize silages (Dewhurst, 2013; Sinclair et al., 2015), although the protein is more degradable in the rumen than vegetable protein sources such as soybean or rapeseed meals (McDonald et al., 2010; Watson et al., 2017; Halmemies-Beauchet-Filleau et al., 2018), making it more difficult to meet the MP requirements of high producing dairy cows (Sinclair et al., 2014). Indeed, a recent meta-analysis showed that feed intake and milk yield response was variable between cows when different combinations of grass or legume silage were fed (Johansen et al., 2017a). The effect of reducing the dietary CP concentration on the performance of dairy cows fed legume silage-based diets, particularly red clover-based diets, has however, not been widely studied. Moreover,

there may be other factors (animal and dietary) that can influence the performance and NUE of dairy cows when a low CP diet based on forage legumes is fed. Therefore, the aim of this study was to evaluate the effect of low protein diets based on forage legumes on the performance and metabolism of early and mid-lactation cows by conducting a systematic review and meta-analysis of data from production trials.

7.2. Materials and methods

7.2.1. Literature search strategy

The systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009). A comprehensive literature search was carried out using the following electronic academic databases: Science Direct (https://www.sciencedirect.com/), Web of Science (https://apps.webofknowledge.com/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/). The studies were retrieved from January 1980 to May 2021, and the search terms included were “dairy cow”, “protein”, “milk”, “performance”, “legume silage”, “nitrogen”, and “efficiency” (Table 7.1).

Table 7.1. Database and search strategy used in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

Database	Web page link	Search term
Web of science	https://login.webofknowledge.com	TI = ((((crude protein OR dietary protein OR protein level) AND (milk OR performance OR efficiency OR nitrogen) AND (dairy OR cow) AND (legume OR red clover OR lucerne OR alfalfa))))
Science direct	https://www.sciencedirect.com/	((((crude protein OR dietary protein OR protein level) AND (milk OR performance OR efficiency OR nitrogen) AND (dairy OR cow) AND (legume OR red clover OR lucerne OR alfalfa))))
PubMed	https://www.ncbi.nlm.nih.gov/pubmed	((((crude protein [Title] OR dietary protein [Title] OR protein level [Title]) AND (milk [Title] OR performance [Title] OR efficiency [Title] OR nitrogen [Title]) AND (dairy [Title] OR cow [Title]) AND (legume [Title] OR red clover [Title] OR lucerne [Title] OR alfalfa [Title]))))

7.2.2. Study selection and inclusion criteria

A total of 580 publications were identified through the database search and were initially checked for duplicates. Around 205 duplicate studies were removed, and the title and abstracts of the remaining records were screened using pre-determined selection criteria that included dietary protein level and forage legume comparisons (Table 7.2). After carefully screening the full-text articles, this resulted in 36 studies that were included in the quantitative synthesis (meta-analysis). A PRISMA flow diagram of all of the records screened and included in the meta-analysis is shown in Figure 7.1.

The following inclusion criteria were incorporated in the meta-analysis: (1) the study or experiments within the study was conducted in early or mid-lactation dairy cows, and articles were reported in English; (2) the dairy cows in the control and treatment groups were housed in the same environment; (3) the diets were fed as a TMR or partial mixed ration (PMR) and the forage component included legume silage, or was partially replaced with grass or maize silage; (4) the CP content of the control (high protein) and treatment (low protein) diets varied from 156 to 220 and 110 to 155 g/kg DM, respectively; (5) the low protein diet was supplemented with bypass protein, AA or starch. The eligible studies consisted of 34 peer-reviewed journal articles and three unpublished studies (Study 1, 2 and 3ab). A summary of the studies included in this systematic review and the meta-analysis is presented in Table 7.3.

7.2.3. Data extraction and calculation

A systematic map was constructed to extract the data from the selected studies. The following variable data from both the control and low protein treatments were extracted for effect size estimation: feed intake, milk performance, feed efficiency, live weight, condition score, nutrient intake and apparent digestibility, urine and plasma metabolites, N output and efficiency, rumen fermentation kinetics and total milk FA. Milk yield (kg/d) data was extracted from each experiment as published in the article whereas, milk composition data reported as a percentage (%) was expressed as g/kg. In most of the studies, feed efficiency was reported as total milk yield/DM intake.

Table 7.2. PICOS terms, inclusion and exclusion criteria used in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

PICO terms	Inclusion criteria	Exclusion criteria
Population/ Participants	1. Dairy cow (all breeds including cross) 2. Lactating cow (early and mid-lactation) 3. Housed in similar environment	1. Other species including heifers and beef cattle 2. Prepartum cows 3. Cows in late lactation or producing <10 kg milk per day 4. Treatment group (Cow) exposed in different environment compared to control
Interventions/ exposure	1. Low protein/crude protein (CP)/dietary protein/metabolisable protein (MP) diet 2. Crude protein is equal or less than 155 g/kg DM in the treatment diet 3. Isoenergetic but N limiting diet 4. Dietary manipulation based on the concentration and/or source of CP 5. 100% MP requirements or low 6. Diet fed as total or partial mixed ration 7. Low CP diet added with supplementation 8. Diets based on legume forages	1. CP lower than 110 g/kg DM in the treatment diet 2. Energy restricted diet 3. Diet fed as pasture/separate
Comparator/ Comparisons	1. Control protein/high protein diet 2. CP concentration is higher than 155 g/kg DM 3. Similar dietary ingredients and composition except the level of CP 4. 100% MP requirements 5. Diets based on legume forages	1. CP concentration higher than 220 g/kg DM 2. Diet differ in roughage to concentrate ratio in a mixed ration compared to low CP/treatment diet 3. Covariate data as control
Outcomes	1. Milk (Yield, composition) 2. Performance (DMI, BW, BCS) 3. Efficiency (Feed and milk efficiency) 4. Nitrogen (Intake, output and partitioning) 5. Plasma and urine metabolites 6. Nutrient intake and apparent nutrient digestibility	1. Outcomes does not fall into inclusion criteria 2. Outcomes based on review article, systematic reviews and meta-analyses studies 3. Outcomes from non-SCI, SCOUPUS journal article 4. Outcomes with missing SD or SE 5. Outcomes from duplicate studies
Study design	1. Continuous 2. Rotation 3. In vivo	1. In vitro study

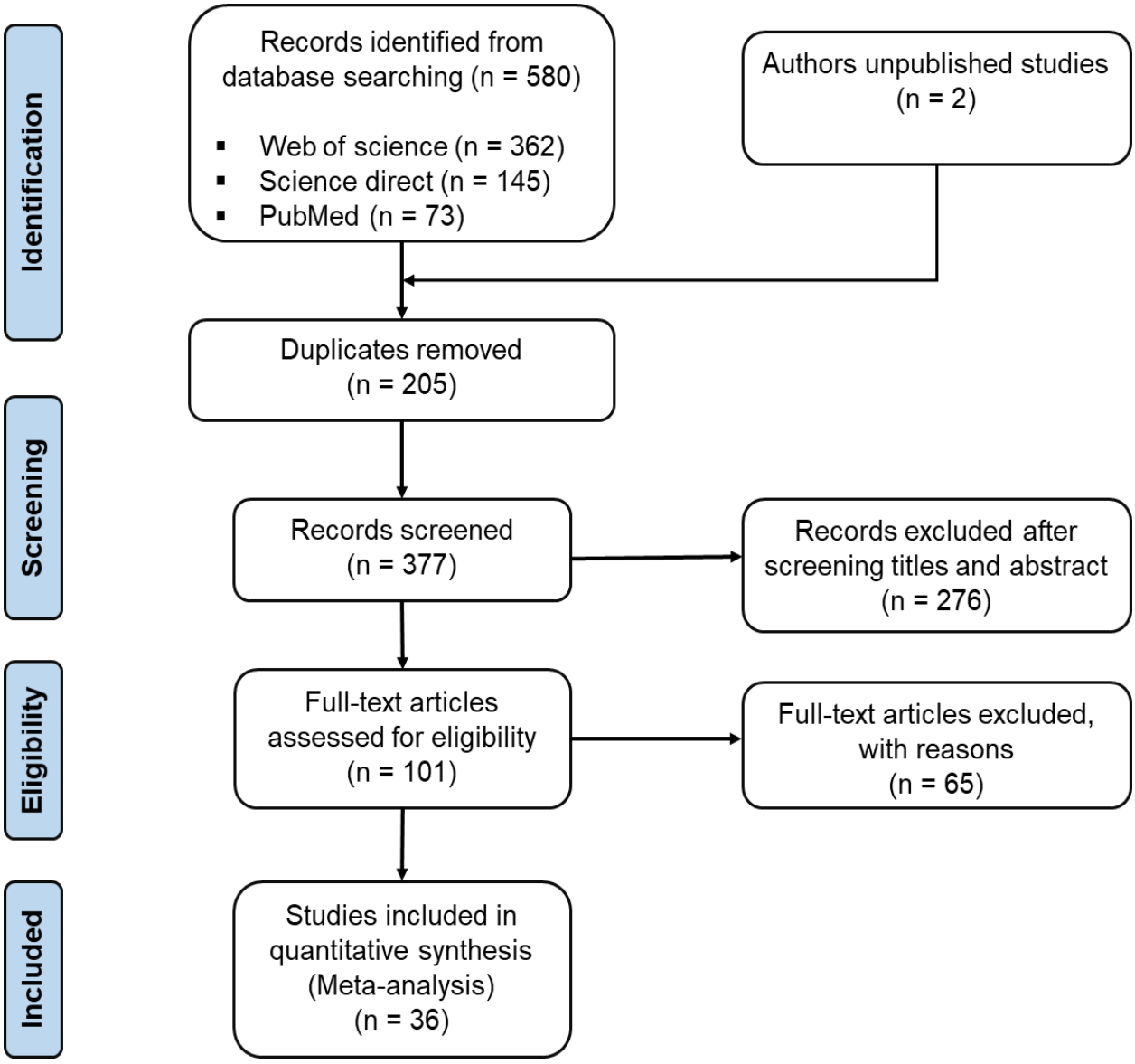


Figure 7.1. Preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram of all of the records screened and included in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

Table 7.3. Summary of the studies (n = 36) included in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

Reference	Breed[1]	n[2]	Parity	Expt. design[3]	Expt. duration	Diet type	Forage type	F:C ratio[4]	CP level (g/kg DM)
(Acharya et al., 2015)	H	16	Mixed	LSD	28	TMR	LH, MS	55:45	143, 163
(Aguerre et al., 2016)	H	24	Multiparous	LSD	21	TMR	LS, MS	51:49	153, 166
(Arieli et al., 2004)	H	220	Multiparous	RBD	85	TMR	LH, MS, WS	35:65	151, 153, 167, 173
(Arriola Apelo et al., 2014a)	H	48	Mixed	LSD	15	TMR	LH, MS	46:54	150, 169
(Bach et al., 2000)	H	4	Multiparous	LSD	10	TMR	LS, MS	51:49	147, 149, 178, 183
(Bahrami-Yekdangi et al., 2014)	H	12	Multiparous	LSD	21	TMR	LH, MS	39:61	155, 164, 172, 180
(Barros et al., 2017)	H	128	Mixed	RBD	84	TMR	LS, MS	65:35	118, 131, 144, 162
(Broderick, 2003)	H	63	Mixed	LSD	28	TMR	LS, MS	62:38	151, 167, 184
(Broderick et al., 2008)	H	24	Mixed	LSD	28	TMR	LS, MS	48:52	148, 161, 173, 186
(Broderick et al., 2015)	H	50	Mixed	LSD	21	TMR	LS, MS	66:34	150, 170
(Chen et al., 2011)	H	70	Mixed	RBD	84	TMR	LS, MS	60:40	155, 170
(Chowdhury et al., 2019; Study 1)	H	18	Multiparous	LSD	28	TMR	RCS, GS	52:48	150, 165, 175
(Chowdhury et al., 2020; Study 2)	H	18	Multiparous	LSD	28	TMR	LS, MS	52:48	150, 150, 175
(Chowdhury et al., 2021; Study 3ab)	H	56	Mixed	RBD	98	TMR	RCS, GS	53:47	150, 175
(Christensen et al., 1994)	H	5	Mixed	LSD	21	TMR	LH, MS	50:50	142, 170
(Claypool et al., 1980)	H	30	Multiparous	RBD	90	TMR	LS, MS	43:57	127, 163, 193
(Crovetto et al., 2009)	H	42	-	LSD	21	TMR	LH, RGH, MS	75:25	154, 172
(Fagundes et al., 2018)	H	8	Multiparous	LSD	21	TMR	LH, MS	60:40	152, 154, 161
(Giallongo et al., 2015)	H	60	Mixed	RBD	70	TMR	LS, MS	55:45	148, 167
(Giallongo et al., 2016)	H	72	Mixed	RBD	63	TMR	LS, MS	63:37	145, 165
(Imran et al., 2017)	H	9	Multiparous	LSD	21	TMR	LS, MS	54:46	152, 184, 209
(Ipharraguerre and Clark, 2005)	H	60	Multiparous	RBD	112	TMR	LS, MS	45:55	148, 166, 170, 185, 190
(Jaquette et al., 1987)	H	24	Multiparous	RBD	28	TMR	LH, MS	50:50	140, 220
(Lee et al., 2011)	H	36	Mixed	RBD	70	TMR	LH, MS	50:50	148, 167
(Lee et al., 2012a)	H	36	Mixed	RBD	70	TMR	LH, MS	53:47	140, 156

Table 7.3 (continued). Summary of the studies (n = 36) included in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

Reference	Breed[1]	n[2]	Parity	Expt. design[3]	Expt. duration	Diet type	Forage type	F:C ratio[4]	CP level (g/kg DM)
(Lee et al., 2012b)	H	48	Mixed	RBD	84	TMR	LH, MS	64:36	135, 157
(Lee et al., 2015a)	H	8	Multiparous	LSD	21	TMR	LH, MS	55:45	137, 156
(Liu and VandeHaar, 2020b)	H	166	Mixed	LSD	32	TMR	LS, MS	51:49	130, 140, 160, 180
(Mutsvangwa et al., 2016)	H	8	Multiparous	LSD	28	TMR	LH, BS	50:50	149, 175
(Niu et al., 2016)	H	12	-	LSD	18	TMR	LH	46:54	152, 185
(Nursoy et al., 2018)	H	36	Multiparous	LSD	28	TMR	LS, MS	60:40	110, 130, 150, 170
(Olmos Colmenero and Broderick, 2006)	H	40	Mixed	LSD	28	TMR	LH, MS	50:50	135, 150, 165, 179, 194
(Piepenbrink et al., 1996)	H	10	Multiparous	LSD	14	TMR	LS, MS	50:50	140, 180
(Rafiee-Yarandi et al., 2019)	H	8	Multiparous	LSD	21	TMR	LH, MS	40:60	148, 149, 161, 163
(Recktenwald et al., 2014)	H	6	Multiparous	LSD	14	TMR	LH, MS	60:40	151, 152, 166, 167
(Wildman et al., 2007)	H	32	Multiparous	RBD	42	TMR	LH, MS	47:53	150, 170
(Zhao et al., 2019)	H	10	Multiparous	LSD	19	TMR	LH, MS, OH	52:48	120, 160

[1]H = Holstein-Friesian dairy cows;

[2]Experimental units (cows);

[3]LSD = Latin square design; RBD = randomized block design;

[4] Forage to concentrate ratio

BS = barley silage; CP = crude protein; DM = dry matter; GS = grass silage; LH = lucerne hay; LS = lucerne silage; MS = maize silage; OH = oat hay; RCS = red clover silage; RGH = ryegrass hay; RGS = ryegrass silage; WS = wheat silage.

The DM intake data reported in either performance or digestibility studies was included. Live weight change was reported as kg/d, and BCS was based on 1 to 5 scale (Ferguson et al., 1994). All urine and plasma metabolites data were reported or converted as mmol/l except, for plasma creatinine, which was reported as mg/dl. The N excretion and its use efficiency for milk, urine and faeces were adjusted to g/d and %, respectively. The rumen fermentation parameters, including rumen pH, NH_3-N (mg/dl) and the VFA **(**mol/100 mol) data, were included in the analyses. The content of milk SFA, MUFA and PUFA reported as g/100 g of milk FA.

Most of the studies reported a pooled standard deviation (SD) or standard error (SE) for the variables in the control and low protein treatments. In the meta-analysis, only the SD was used as the measure of variance, and if SE was reported, then the SD was calculated by multiplying the SE by the square root of the sample size (Salami et al., 2020). Any variables reported without SD or SE of the mean were removed from the final analysis. The main influencing factors that may have affected the performance response and included in the analysis were parity, days in milk, experimental design and duration, forage to concentrate ratio, silage type and legume silage inclusion rate, AA supplementation of low protein diets, and level of CP in the treatment group.

7.2.4. Statistical analysis

A comprehensive meta-analysis software (CMA; version 3, Biostat Inc., Englewood, USA) was used to perform the meta-analysis and generate forest plots. The effects of low protein diets on performance variables were examined using random-effect models, assuming heterogeneity existed among the study results (Borenstein et al., 2009). The effect size of low protein diets for each or overall study was expressed as the raw mean difference (RMD) at a 95% confidence intervals (CI) level. The RMD was calculated as the mean differences between the treatment and control groups for each study. The treatment means of the random-effect model were weighted by the individual variances as per the method described by DerSimonian and Laird (1986). The significance of RMD was declared when $P < 0.05$.

Variations of the treatment effect across the studies were estimated using the chi-square (Q) and I^2 tests to define the percentage of variation due to heterogeneity (Lean et al., 2018). Types of heterogeneity were defined as follows: low, $I^2 < 25\%$;

moderate, I^2 = 25 to 50%; and high I^2 > 50%; negative I^2 value was denoted as zero (Higgins et al., 2003).

Publication bias was checked statistically with the CMA funnel plot asymmetry test using both Begg's (Begg and Mazumdar, 1994) and Egger's (Egger et al., 1997) regression test. The significance of publication bias was declared at $P < 0.05$.

A meta-regression analysis was performed using the CMA meta-regression tool with predefined categorical covariates to explore the heterogeneity among the response variables. The covariates were as follows: parity (multiparous or mixed), DIM (≥ 100 or < 100), experimental duration (≤ 50 or > 50 days), silage type (lucerne or red clover silage), legume silage inclusion rate of the forage DM (10-20, 21-40 or ≥60 %), AA supplementation in the low protein diets (RPM, RPL, RPML, or no AA), and further division of the CP content in the low CP (treatment) group (<140 or ≥140 g CP/kg DM). Meta-regression analysis was subjected to those response variables with a lack of publication bias but a high heterogeneity (I^2 > 50%) or heterogeneity test at $P < 0.05$. The adjusted R^2 value was calculated using the CMA for all covariates, representing the proportion of study variance.

Based on significant ($P < 0.05$) results of covariates in the meta-regression, the studies were divided into different groups/subgroups for each response variable, and subgroup meta-analyses were conducted using a similar random-effect model at 95% CI. A mixed model was also applied within the subgroup analysis to examine differences between groups for the effect size of each categorical covariate of respective response variables. A Bonferroni multivariate post hoc comparison test was performed for covariate "AA supplementation" to determine the effect size that differed significantly from each other. Descriptive statistics for the chemical composition of the low and high (control) protein diets among the studies were conducted using GenStat (VSNI, 19th Edition, UK). The differences in chemical composition between the diets were also evaluated using an unpaired parametric t-test in GenStat.

7.3. Results

7.3.1. Study characteristics and diet composition

A total of 36 studies with 102 treatment means were included in the meta-analysis. The studies were conducted since 1980 in eight different countries (twenty-seven from the United States, two from the United Kingdom and Iran, one each from

Canada, Italy, Israel, Pakistan and China). Holstein-Friesian dairy cows, either multiparous or mixed, were used in the studies as either crossover or continuous designs and were fed the diets as a TMR (Table 7.3). The average forage to concentrate ratio across all studies was 53:47 on a DM basis. The diets were based on either lucerne hay/silage (92%) or red clover (8.0%) silage, with an average inclusion rate of legume silage (forage DM) was 40%, except for one study (Niu et al., 2016) where lucerne hay was fed as 100% of the forage DM. Three studies incorporated in the meta-analysis included more than 30 cows per dietary treatment (Arriola Apelo et al., 2014b; Barros et al., 2017; Liu and VandeHaar, 2020b).

Descriptive statistics for the chemical composition of the diets included in the meta-analysis are presented in Table 7.4. The mean and median CP content of the control diets was 171 and 170 g/kg DM, respectively, higher ($P<0.05$) than the low CP diets, which had a mean and median of 145 and 149 g/kg DM, respectively. The highest CP level fed to cows in the control treatment was 220 g/kg DM, and the lowest in the low CP treatment was 110 g/kg DM. Similarly, the mean RDP content of the control diets was 105 g/kg DM, 12.2 g/kg DM higher than the low protein diets, whilst the mean RUP of the control diets was 62.6 g/kg DM, 11.9 g/kg DM higher than the low CP diets. This resulted in a predicted MP content of the control diets of 110 g/kg DM, 14.5 g/kg DM higher than the low CP diets. There were no other differences ($P > 0.05$) in chemical composition between the diets except for starch, which was 34 g/kg DM higher in the low CP compared to the control diets, with mean values of 226 and 260 g/kg DM, respectively.

Table 7.4. Descriptive statistics of the chemical composition of low and high (control) protein diets of studies included in the meta-analysis that investigated the response of dairy cows to dietary protein level in diets based on forage legumes.

Item[1]	Mean		Median		Maximum		Minimum		SE		N
	Control	Low CP	Control	Low CP	Control	Low CP	Control	Low CP	Control	Low CP	
DM, g/kg	542	540	532	529	679	685	379	379	9.116	9.381	61
OM, g/kg DM	930	933	928	931	951	958	906	906	1.472	1.674	67
*CP, g/kg DM	171	145	170	149	220	155	156	110	1.160	0.937	102
NDF, g/kg DM	316	316	315	316	393	415	224	219	4.178	4.440	100
ADF, g/kg DM	199	197	197	195	281	281	115	109	4.014	4.123	93
EE, g/kg DM	38.8	42.2	35.3	37.7	74.0	76.7	18.6	16.6	1.742	2.025	69
*Starch, g/kg DM	226	260	230	273	315	362	104	154	7.142	7.610	55
Ca, g/kg DM	8.92	9.01	9.00	9.55	11.7	13.3	5.87	5.13	0.224	0.244	44
P, g/kg DM	4.24	4.11	4.00	3.98	5.20	5.30	3.70	3.40	0.076	0.080	44
NE_L, g/kg DM	6.64	6.60	6.61	6.61	7.82	7.82	4.46	4.58	0.053	0.054	80
*RDP, g/kg DM	105	92.8	102	95.1	127	109	86.6	70.9	1.440	1.556	42
*RUP, g/kg DM	62.6	50.7	62.3	49.0	77.0	75.6	50.6	35.0	0.986	1.110	44
*MP, g/kg DM	110	95.5	114	93.5	120	109	94.8	86.8	2.261	1.651	16

[1]DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; EE = ether extract; NE_L = Net energy for lactation; RDP = rumen degradable protein; RUP = rumen undegradable protein; MP = metabolisable protein; SE = standard error; N = the number of comparisons between control and treatment (low CP) diets.

*means between control and low CP diet statistically differ at $P < 0.05$.

7.3.2. Feed intake and performance

The effect size estimates for DM intake, milk performance, feed efficiency, LW and BCS of the dairy cows fed the control or low CP diets are presented in Table 7.5 and Appendix 7.1. The mean DM intake of cows fed the control diets was 24.1 kg DM/d, which was 0.62 kg DM/d higher ($P < 0.001$) than in animals fed the low CP diets. Similarly, feeding low CP diets decreased ($P < 0.05$) the daily milk yield, ECM, and FCM (adjusted to 40 or 35 g fat/kg) by 1.41, 1.29, 0.73, or 1.31 kg/d, respectively. Neither milk fat nor lactose content was affected ($P > 0.05$) by dietary CP content, with means across all studies of 36.2 and 48.1 g/kg, respectively. In contrast, milk protein and MUN concentrations were 0.22 g/kg and 3.47 mg/dl, respectively lower ($P < 0.001$) in cows fed the low CP compared to the control diets. Feed efficiency tended ($P = 0.06$) to be lower when expressed as milk yield/kg DM intake and the 3.5% FCM yield efficiency was significantly lower (RMD = -0.05; $P < 0.001$) in cows fed the low compared to the control diets. The lowest ($P < 0.05$) mean BCS (RMD = -0.03; $P = 0.010$) was recorded in cows fed the low CP compared to the control diets. There was, however, no effect ($P > 0.05$) of feeding low CP diets on LW and change in LW or BCS change across the studies.

7.3.3. Nutrient intake and apparent digestibility

Differences in nutrient intake (DM intake reported in digestibility studies/table) and apparent digestibility were observed when legume-based low CP diets were fed to dairy cows (Table 7.6 and Appendix 7.2). The mean intake of DM, OM, N, NDF and ADF were 0.65, 0.59, 0.74, 0.27 and 0.19 kg/d respectively, lower ($P < 0.05$) in cows fed low CP compared to the control diets. Similarly, apparent total tract digestibility of DM, OM, N, NDF and ADF were 1.26, 1.30, 4.27, 1.87 and 2.72% units, respectively, lower ($P < 0.001$) in cows receiving low CP diets compared to those fed control, which had means of 68.4, 70.4, 66.1, 49.2 and 44.9%, respectively.

7.3.4. Urine and plasma metabolites

The effect size estimates for urine and plasma metabolites of dairy cows fed control and low CP diets based on forage legumes are presented in Table 7.7 and Appendix 7.3. No significant ($P > 0.05$) difference in urine metabolites (allantoin, uric acid and total purine derivatives) were observed between cows receiving the control or low CP diets. However, daily total urine output was 3.04 litre higher ($P < 0.001$) in cows fed the control compared to low CP diets. Plasma metabolites, including glucose,

BHB, total glycerides (TG) and creatinine level, did not differ ($P > 0.05$) between cows fed the control or low CP diets. In contrast, reducing the dietary CP content increased ($P = 0.006$) plasma concentration of NEFA, which was 0.03 mmol/l higher than those receiving the control, which had a mean value of 0.25 mmol/l. The mean concentration of PUN in cows fed the control diets was 5.6 mmol/l and was 1.85 mmol/l lower ($P < 0.001$) in cows that received the low CP diets.

7.3.5. Nitrogen intake, emissions and use efficiency

Dietary N intake was reduced ($P < 0.001$) by 107 g/d when cows received legume-based low CP diets compared to those fed the control, which had a mean value of 668 g/d (Table 7.8 and Appendix 7.4). Likewise, daily N excretion in milk, urine or faeces was 4.26, 13.6 and 69.3 g, respectively, lower ($P < 0.05$) in cows fed low CP compared to control diets. There was also a difference ($P < 0.001$) between dietary treatment in the apparent NUE, which was increased in cows receiving a low CP diet with a mean value of 32.0%, approximately 3.6% units higher than those receiving the control diet. Similarly, feeding a low CP diet increased the partitioning of faecal N (RMD = +0.47%; $P < 0.001$) but reduced urine N by 6.68% units ($P < 0.001$) compared to those receiving the control diet, which had a mean value of 35.5 % and 31.6%, respectively.

7.3.6. Rumen fermentation kinetics and milk fatty acids

The effect of low CP diets on rumen fermentation kinetics and milk FA content of dairy cows fed legume-based rations are presented in Table 7.9 and Appendix 7.5. There was no difference ($P > 0.05$) between cows on rumen pH or rumen acetate and propionate concentration, but butyrate was reduced by 0.45 mol per 100 mol of VFA when a low CP diet was fed to dairy cows. In contrast, low CP diets did not alter ($P = 0.189$) the total milk fat concentration of PUFA, but SFA was 0.68 g /100 g higher, and MUFA was 0.34 g per 100 g lower in cows compared to those receiving the control, which had a mean value of 65.8 and 27.4 g /100 g FA.

Table 7.5. Summary effect size estimates for intake, milk performance, live weight and condition of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-analysis.

Item[1]	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P* value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)	Begg's test	Egger's test	
DM intake, kg/d	24.1	0.23	-0.62	0.09	-0.80	-0.45	<0.001	176	<0.001	46.5	0.819	0.913	95
Milk yield, kg/d													
Milk	37.3	0.57	-1.41	0.15	-1.71	-1.11	<0.001	207	<0.001	51.7	0.756	0.892	101
ECM	37.4	0.52	-1.29	0.24	-1.75	-0.82	<0.001	57.9	0.155	17.1	0.843	0.483	49
4% FCM	36.3	1.24	-0.73	0.27	-1.26	-0.19	0.008	17.3	0.567	0.00	0.795	0.803	20
3.5% FCM	38.0	0.74	-1.31	0.19	-1.68	-0.93	<0.001	42.8	0.273	11.2	0.781	0.507	39
Composition, g/kg													
Fat	36.3	0.39	-0.12	0.14	-0.39	0.16	0.395	93.5	0.409	2.63	0.736	0.134	92
Protein	31.4	0.21	-0.22	0.05	-0.32	-0.12	<0.001	112	<0.001	78.1	0.750	0.906	98
Lactose	48.1	0.18	-0.02	0.03	-0.09	0.05	0.559	79.6	0.617	0.00	0.448	0.390	85
Urea, mg/dl	23.9	1.41	-6.39	0.58	-7.51	-5.26	<0.001	17.4	0.066	42.6	0.436	0.722	11
MUN, mg/dl	12.7	0.27	-3.47	0.18	-3.83	-3.11	<0.001	756	<0.001	89.6	0.381	0.111	80
Feed efficiency, %													
Milk yield/DMI	1.58	0.02	-0.01	0.01	-0.03	0.00	0.059	57.6	0.308	8.05	0.881	0.545	54
ECM/DMI	1.55	0.02	-0.02	0.01	-0.04	0.00	0.087	61.6	0.005	41.6	0.094	0.149	37
4% FCM/DMI	1.62	0.09	-0.03	0.03	-0.08	0.03	0.344	12.2	0.204	26.0	0.721	0.157	10
3.5% FCM/DMI	1.59	0.03	-0.05	0.01	-0.07	-0.02	0.001	14.7	0.796	0.00	0.506	0.793	21
Body performance													
LW, kg	652	6.70	-1.55	1.31	-4.11	1.01	0.235	44.2	0.704	0.00	0.129	0.106	51
LWC, kg/d	0.09	0.08	-0.02	0.02	-0.06	0.01	0.218	83.2	0.087	19.5	0.754	0.398	68
BCS	2.87	0.04	-0.03	0.01	-0.06	-0.01	0.010	23.5	0.911	0.00	0.300	0.203	35
BCS change	0.03	0.01	0.00	0.00	-0.001	0.00	0.284	32.8	0.137	23.7	0.947	0.166	26

[1]DM = dry matter; ECM = energy corrected milk yield; FCM = fat corrected milk yield; MUN = milk urea nitrogen; DMI = dry matter intake; LW = live weight; LWC = LW change; BCS = body condition score (1 to 5 scale).

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; Publication bias was examined using the Begg's and Egger's regression test (Funnel test).

Table 7.6. Summary effect size estimates for intake performance (intake data was included just from the digestibility studies) and apparent total tract nutrients digestibility of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-analysis.

Item[1]	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P* value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)	Begg's test	Egger's test	
Intake, kg/d													
DM	24.2	0.25	-0.65	0.09	-0.83	-0.48	<0.001	139	0.001	35.9	0.823	0.069	39
OM	22.4	0.36	-0.59	0.18	-0.95	-0.23	0.001	38.0	0.121	23.8	0.164	0.732	30
CP	3.99	0.06	-0.74	0.04	-0.82	-0.67	<0.001	240	<0.001	84.2	0.364	0.312	39
NDF	7.36	0.28	-0.27	0.07	-0.41	-0.14	<0.001	56.8	0.004	43.7	0.369	0.193	33
ADF	4.82	0.20	-0.19	0.03	-0.26	-0.13	<0.001	42.4	0.182	17.5	1.000	0.064	36
Digestibility, %													
DM	68.4	0.70	-1.26	0.31	-1.87	-0.65	<0.001	215	<0.001	78.1	0.756	0.698	48
OM	70.4	0.81	-1.30	0.28	-1.84	-0.75	<0.001	89.2	<0.001	58.5	0.960	0.773	38
CP	66.1	0.74	-4.27	0.39	-5.03	-3.52	<0.001	105	<0.001	58.1	0.953	0.897	45
NDF	49.2	1.55	-1.87	0.48	-2.82	-0.93	<0.001	138	<0.001	68.7	0.754	0.598	44
ADF	44.9	1.81	-2.72	0.58	-3.85	-1.59	<0.001	149	<0.001	75.1	0.678	0.507	38

[1]DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; Publication bias was examined using the Begg's and Egger's regression test (Funnel test).

Table 7.7. Summary effect size estimates for urine and blood metabolites of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-analysis.

Item[1]	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P* value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)	Begg's test	Egger's test	
Urine metabolites													
Allantoin, mmol/l	24.1	1.67	1.37	2.08	-2.70	5.44	0.509	0.73	1.000	0.00	1.000	0.273	18
Uric acid, mmol/l	2.37	0.23	0.21	0.26	-0.29	0.72	0.407	0.64	1.000	0.00	0.472	0.640	18
Total PD, mmol/l	26.6	1.82	1.72	2.26	-2.72	6.15	0.448	0.59	1.000	0.00	0.910	0.199	18
Urine output, l/d	25.0	1.23	-3.04	0.28	-3.59	-2.49	<0.001	41.1	0.222	14.8	0.196	0.062	36
Plasma metabolites													
Glucose, mmol/l	3.56	0.06	-0.01	0.02	-0.06	0.03	0.514	38.3	0.142	21.7	0.986	0.822	31
BHB, mmol/l	0.55	0.07	0.02	0.01	-0.01	0.05	0.131	6.39	0.846	0.00	1.000	0.652	12
NEFA, mmol/l	0.25	0.03	0.03	0.01	0.01	0.06	0.006	2.67	0.849	0.00	1.000	0.944	7
TG, mmol/l	0.15	0.02	0.01	0.01	0.00	0.02	0.140	5.02	0.890	0.00	0.312	0.156	11
Creatinine, mg/dl	1.40	0.15	-0.03	0.02	-0.08	0.01	0.150	3.89	0.565	0.00	1.000	0.884	6
PUN, mmol/l	5.64	0.23	-1.85	0.13	-2.11	-1.59	<0.001	493	<0.001	89.2	1.007	0.053	54

[1]PD = purine derivatives; BHB = β-hydroxybutyric acid; NEFA = non-esterified fatty acids; TG = total glycerides; PUN = plasma urea nitrogen.

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; Publication bias was examined using the Begg's and Egger's regression test (Funnel test).

Table 7.8. Summary effect size estimates for nitrogen intake, output and efficiency of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-analysis.

Item[1]	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P* value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)	Begg's test	Egger's test	
N intake, g/d	668	6.02	-107	7.43	-121	-92.3	<0.001	291	<0.001	82.5	0.478	0.652	52
N output, g/d													
Milk	187	2.47	-4.26	1.29	-6.78	-1.74	0.001	52.3	0.244	11.99	0.140	0.843	47
Faecal	226	5.72	-13.6	2.48	-18.5	-8.75	<0.001	67.1	0.054	25.4	0.994	0.970	51
Urine	218	6.33	-69.3	4.24	-77.6	-61.0	<0.001	435	<0.001	86.9	0.232	0.233	58
N partitioning, %													
Faecal	35.5	0.79	4.76	0.50	3.78	5.74	<0.001	169	<0.001	75.7	0.611	0.577	42
Urine	31.6	0.97	-6.68	0.49	-7.65	-5.71	<0.001	97.0	<0.001	64.9	0.173	0.144	35
NUE	28.4	0.30	3.63	0.20	3.23	4.02	<0.001	147	<0.001	50.5	0.142	0.999	74

[1]N = nitrogen; NUE = apparent N use efficiency of milk production.

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; Publication bias was examined using the Begg's and Egger's regression test (Funnel test).

Table 7.9. Summary effect size estimates for rumen fermentation kinetics and milk fatty acids of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-analysis.

Item[1]	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P* value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)	Begg's test	Egger's test	
Rumen fermentation													
Rumen pH	6.25	0.04	0.02	0.02	-0.005	0.05	0.103	28.4	0.650	0.00	0.620	0.789	33
NH_3-N, mg/dl	10.5	0.74	-3.38	0.32	-4.01	-2.75	<0.001	69.5	<0.001	61.2	0.514	0.236	28
Acetate, mol/100 mol	58.8	0.75	-0.15	0.41	-0.95	0.64	0.708	33.5	0.219	16.3	0.320	0.215	29
Propionate, mol/100 mol	23.1	0.67	-0.32	0.37	-1.04	0.39	0.376	32.9	0.165	21.0	0.819	0.959	27
Butyrate, mol/100 mol	12.0	0.28	-0.45	0.12	-0.69	-0.21	<0.001	23.3	0.717	0.00	0.561	0.956	29
Milk fatty acid (g/100 g FA)													
SFA	65.8	1.59	0.68	0.31	0.07	1.29	0.029	30.2	0.011	50.4	0.558	0.217	16
MUFA	27.4	1.03	-0.34	0.16	-0.66	-0.02	0.038	15.2	0.440	1.01	0.034	0.263	16
PUFA	4.52	0.22	-0.13	0.10	-0.33	0.07	0.189	94.8	<0.001	84.2	0.344	0.145	16

[1]NH_3-N = rumen ammonia-nitrogen; SFA = saturated fatty acid; MUFA = mono unsaturated fatty acid; PUFA = poly unsaturated fatty acid.

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; Publication bias was examined using the Begg's and Egger's regression test (Funnel test).

7.3.7. Heterogeneity, publication bias and meta-regression

A high heterogeneity was observed (I^2 > 50%; P < 0.05) for DM intake, milk yield, milk protein, MUN, PUN, CP and NDF intake, nutrients digestibility, urinary N excretion, NUE, partitioning of faecal and urine N, rumen NH_3-N level, and milk SFA and PUFA content. However, there was no substantial evidence in the Begg's and Egger's test to indicate publication bias across the studies for each of the response variables (Table 7.5 to 7.9).

The response variables which showed significant heterogeneity were subjected to meta-regression analysis using preselected covariates (described in Section 7.2.4) to identify the key sources of variation (Table 7.10). Among the covariates, the level of CP in the diet, type of legume silage and its inclusion rate, and AA supplementation were the major factors that influenced the heterogeneity of the response variables. Other covariates such as parity, DIM and experimental duration also showed a significant correlation (P < 0.05) with DM intake, milk yield, milk protein, PUN and urinary N excretion.

7.3.8. Subgroup analysis

Further analysis indicated that DM and CP intake, milk yield, MUN, urinary N excretion, and rumen NH_3-N concentration were reduced (P < 0.05) in cows receiving very low dietary CP diets containing < 140 g/kg DM compared to those fed ≥140 g CP/kg DM (Table 7.11). In contrast, feeding dietary CP < 140 g/kg DM increased (P = 0.017) the faecal partitioning of N (RMD = 6.23 vs. 3.95; P < 0.001) in cows compared to those receiving ≥140 g CP/kg DM diet.

Forage type (lucerne vs. red clover) was also an important covariate that affected the response to dietary protein level (Table 7.12). The intake of DM was reduced (P < 0.05) in cows when lucerne or red clover silage based low CP diets were fed compared to the control; however, the RMD (-0.59 and -1.12 kg/d, respectively) did not differ (P = 0.133) between forages. The NDF intake, nutrient digestibility, daily urinary N excretion, and the partitioning of urinary N were reduced (P < 0.05) in cows fed low CP diets based on red clover silage than those receiving lucerne based rations. In contrast, milk yield was reduced (RMD = -1.54 kg/d; P < 0.001) in cows fed lucerne compared to red-clover based

Table 7.10. Covariates effect on intake (kg/d), nutrient digestibility (%), milk yield (kg/d), milk protein (g/kg), milk urea N (mg/dl), plasma urea N (mmol/l), urine N (g/d), apparent milk N use efficiency (%), urine and faecal partitioning of N (%), rumen ammonia-N (mg/dl), and milk saturated and poly-unsaturated fatty acids (g/100 g) of dairy cows fed control and low CP diets based on forage legumes in a random-effect meta-regression analysis.

Variables[1]	[2]Covariates, meta-regression coefficient and associated *P* value																Adj.R^2	N
	Intercept		Forage type		Legume inclusion		AA (%)		Parity		DIM		EXPD		CP level			
	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value	Coef.	*P* value		
DM intake	-8.98	<.001	-0.59	0.047	-0.01	0.072	0.31	0.024	1.24	0.018	0.00	0.002	0.00	0.157	0.06	<.001	0.97	95
CP intake	-4.23	<.001	-0.04	0.826	0.00	0.803	0.21	0.062	0.10	0.536	0.00	0.563	0.00	0.428	0.02	<.001	0.00	39
NDF intake	0.84	0.550	-0.72	0.007	0.00	0.597	0.07	0.720	-0.27	0.253	-0.01	0.071	0.00	0.319	0.00	0.889	0.48	33
DMD	-7.82	0.123	-5.17	0.009	0.00	0.965	1.02	0.077	-0.24	0.783	-0.02	0.189	0.00	0.947	0.06	0.049	0.39	48
OMD	-3.06	0.465	-6.17	0.000	0.02	0.380	0.78	0.113	0.58	0.444	-0.04	0.022	0.01	0.581	0.03	0.253	0.63	38
CPD	-20.4	0.011	-4.60	0.080	-0.02	0.573	0.28	0.715	0.67	0.570	0.02	0.521	-0.01	0.743	0.11	0.038	0.31	45
NDFD	-7.86	0.298	-9.10	0.001	0.05	0.310	0.81	0.369	2.35	0.093	-0.03	0.196	0.03	0.285	0.04	0.409	0.38	44
ADFD	-4.51	0.694	-8.83	0.013	0.07	0.197	-0.10	0.932	2.33	0.235	-0.03	0.357	0.02	0.565	0.01	0.925	0.10	38
Milk yield	-15.4	<.001	1.57	0.002	0.02	0.129	-0.22	0.445	3.73	<.001	0.01	0.002	-0.01	0.050	0.09	<.001	0.65	101
Milk protein	-2.24	0.005	0.28	0.202	-0.01	0.001	-0.42	0.001	-1.24	0.136	0.00	0.587	-0.00	0.262	0.02	<.001	1.00	98
MUN	-15.5	<.001	1.07	0.075	-0.02	0.153	-1.41	<.001	0.73	0.060	0.01	0.037	0.00	0.869	0.09	<.001	0.45	80
PUN	-9.91	<.001	0.47	0.251	0.00	0.822	-0.90	<.001	0.94	<.001	0.01	0.031	0.01	0.008	0.05	<.001	0.49	54
Urine N	-353	<.001	-62.0	0.002	-0.63	0.028	-20.6	0.001	14.0	0.105	0.31	0.013	0.27	0.011	1.91	<.001	0.63	58
FNE	27.3	<.001	6.43	0.004	-0.03	0.386	0.97	0.374	-2.94	0.056	0.00	1.000	-0.03	0.128	-0.14	0.003	0.50	42
UNE	-10.1	0.251	-8.77	0.001	-0.04	0.324	-1.36	0.234	-1.11	0.504	-0.01	0.616	0.03	0.121	0.05	0.380	0.64	35
NUE	7.65	0.084	1.24	0.070	0.02	0.318	0.67	0.107	0.42	0.381	-0.01	0.151	0.00	0.832	-0.03	0.278	0.44	74
NH_3-N	-8.54	0.033	-	-	-0.01	0.665	-1.30	0.130	0.28	0.138	-0.02	0.060	0.01	0.734	0.06	0.025	0.33	28
SFA	59.5	0.520	1.48	0.524	-0.16	0.198	0.36	0.576	14.2	0.264	0.09	0.209	0.23	0.270	-0.53	0.460	1.00	16
PUFA	2.41	0.865	-0.06	0.717	-0.01	0.765	0.06	0.657	-0.67	0.792	-0.02	0.058	-0.02	0.687	0.00	0.978	1.00	16

[1]DM = dry matter; CP = crude protein; OMD = organic matter digestibility; CPD = crude protein digestibility; NDFD = neutral detergent fibre digestibility; ADFD = acid detergent fibre digestibility; MP = milk protein; MUN = milk urea nitrogen; PUN = plasma urea nitrogen; N = nitrogen; NUE = apparent milk N use efficiency; FNE = partitioning of faecal N; NH_3-N = Rumen ammonia N; SFA = milk saturated fatty acids; PUFA = milk poly unsaturated fatty acids.
[2]AA = amino acid; DIM = days in milk of cows; EXPD = duration of the experiment (days).
Adjusted R^2 value presented for all covariates, and N represents the number of comparisons between control and treatment (low CP) diets.

Table 7.11. Covariate (CP level: ≥140 or <140 g CP/kg DM) effect size estimates for DM and CP intake and digestibility, milk yield, milk protein, milk and plasma urea N, urinary N excretion, faecal partitioning of N, and rumen ammonia-N of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis.

Variables[1]	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P* value[2]	N
		RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)		
DM intake, kg/d DM	<140	-1.01	0.20	-1.39	-0.62	<0.001	31.8	0.016	46.5	0.033	18
	≥140	-0.53	0.10	-0.73	-0.34	<0.001	133	<0.001	42.9		77
CP intake, kg/d DM	<140	-0.92	0.06	-1.04	-0.79	<0.001	88.5	<0.001	86.4	0.001	13
	≥140	-0.67	0.04	-0.75	-0.59	<0.001	109	<0.001	77.1		26
DM digestibility, %	<140	-1.78	0.54	-2.84	-0.72	<0.001	51.6	<0.001	72.9	0.241	15
	≥140	-1.00	0.39	-1.76	-0.24	<0.001	159	<0.001	79.9		33
CP digestibility, %	<140	-5.25	0.76	-6.73	-3.77	<0.001	12.1	0.353	9.42	0.138	12
	≥140	-3.96	0.43	-4.80	-3.12	<0.001	83.2	<0.001	61.5		33
Milk yield, kg/d	<140	-2.45	0.38	-3.19	-1.71	<0.001	30.2	0.049	37.1	0.003	20
	≥140	-1.22	0.16	-1.54	-0.91	<0.001	159	<0.001	49.8		81
Milk protein, g/kg	<140	-0.39	0.13	-0.64	-0.13	0.003	37.9	0.006	49.8	0.166	20
	≥140	-0.19	0.05	-0.30	-0.09	<0.001	72.5	0.625	0.00		78
MUN, mg/dl	<140	-4.27	0.37	-5.00	-3.53	<0.001	242	<0.001	93.0	0.015	18
	≥140	-3.23	0.20	-3.63	-2.83	<0.001	470	<0.001	87.0		62
PUN, mmol/l	<140	-2.20	0.23	-2.66	-1.74	<0.001	156	<0.001	91.0	0.076	15
	≥140	-1.71	0.15	-2.00	-1.41	<0.001	281	<0.001	86.5		39
Urine N, g/d	<140	-83.1	7.97	-98.7	-67.5	<0.001	108	<0.001	87.0	0.042	15
	≥140	-64.1	4.83	-73.6	-54.7	<0.001	293	<0.001	85.6		43
FNE, %	<140	6.23	0.76	4.73	7.72	<0.001	42.9	<0.001	67.3	0.017	15
	≥140	3.95	0.58	2.81	5.08	<0.001	94.9	<0.001	72.6		27
NH_3-N, mg/dl	<140	-4.53	0.57	-5.64	-3.42	<0.001	15.8	0.007	68.3	0.017	6
	≥140	-2.95	0.34	-3.61	-2.28	<0.001	38.3	0.012	45.1		22

[1]DM = dry matter; CP = crude protein; N = nitrogen; MUN = Milk urea N; PUN = plasma urea N; FNE = faecal N/total N intake; NH_3-N = ammonia N.
[2]*P* value of a mixed model between sub-groups (< 140 vs. ≥ 140).
N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates.

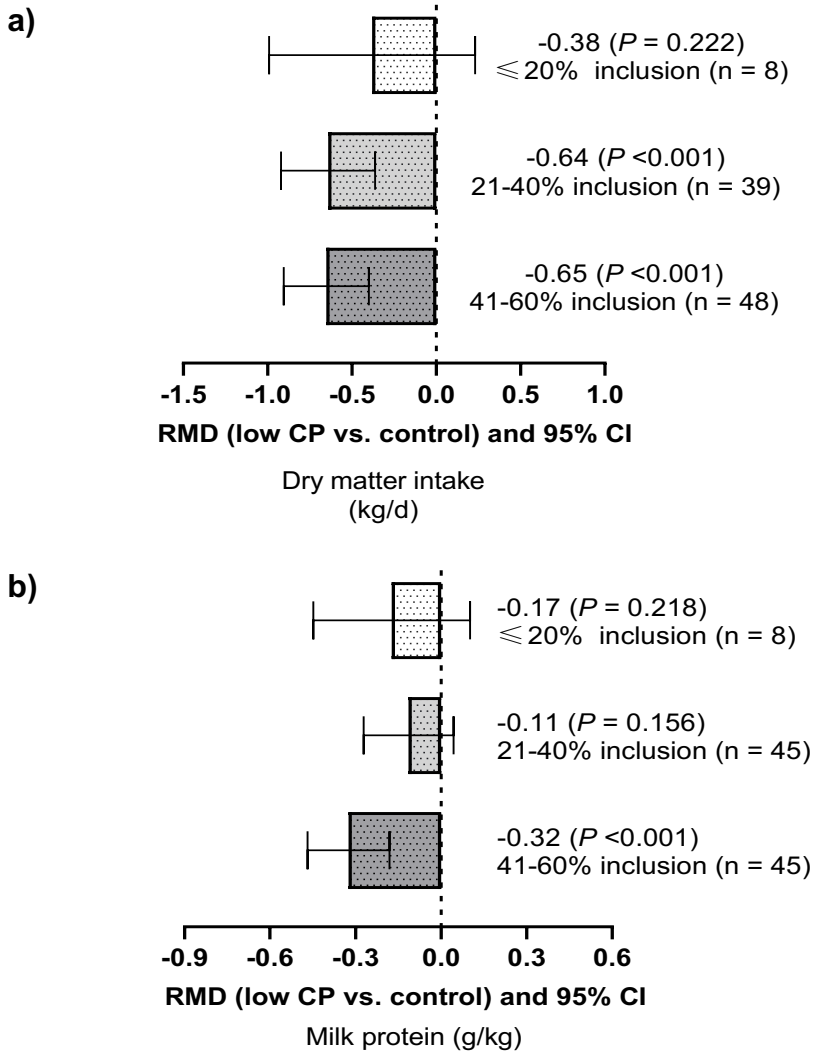
Table 7.12. Covariate (predominant silage type: lucerne (LS) or red clover (RCS)) effect size estimates for DM and NDF intake, nutrients digestibility, milk yield, urinary N excretion, urine and faecal partitioning of N of dairy cows fed control and low CP diets based on lucerne or red clover silages in a subgroup random-effect meta-analysis.

Variables[1]	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P* value[2]	N
		RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)		
DM intake, kg/d DM	LS	-0.59	0.09	-0.77	-0.41	<0.001	166	<0.001	48.3	0.133	87
	RCS	-1.12	0.34	-1.79	-0.45	0.001	3.51	0.834	0.00		8
NDF intake, kg/d DM	LS	-0.21	0.07	-0.35	-0.08	0.002	44.2	0.020	39.0	0.017	28
	RCS	-0.69	0.19	-1.07	-0.32	<0.001	2.30	0.682	0.00		5
DM digestibility, %	LS	-1.12	0.32	-1.74	-0.50	<0.001	196	<0.001	78.6	0.043	43
	RCS	-4.04	1.41	-6.80	-1.28	0.004	10.5	0.032	62.0		5
OM digestibility, %	LS	-1.15	0.27	-1.68	-0.61	<0.001	71.6	<0.001	55.3	0.015	33
	RCS	-4.16	1.21	-6.54	-1.78	0.001	9.20	0.056	56.5		5
NDF digestibility, %	LS	-1.59	0.48	-2.54	-0.64	0.001	121	<0.001	68.5	0.014	39
	RCS	-6.52	1.94	-10.3	-2.72	0.001	7.03	0.134	43.1		5
ADF digestibility, %	LS	-2.40	0.58	-3.54	-1.26	<0.001	132	<0.001	75.8	0.025	33
	RCS	-7.73	2.30	-12.2	-3.22	0.001	8.36	0.079	52.1		5
Milk yield, kg/d	LS	-1.54	0.16	-1.85	-1.23	<0.001	189	<0.001	51.4	0.005	93
	RCS	-0.08	0.50	-1.06	0.90	0.872	6.60	0.471	0.00		8
Urine N, g/d	LS	-67.3	4.20	-75.5	-59.0	<0.001	407	<0.001	86.5	0.005	56
	RCS	-137	24.6	-185	-88.7	<0.001	4.00	0.045	75.0		5
FNE, %	LS	4.55	0.51	3.56	5.54	<0.001	157	<0.001	77.1	0.057	37
	RCS	8.85	2.21	4.53	13.2	<0.001	5.16	0.271	22.5		5
UNE, %	LS	-6.33	0.45	-7.22	-5.45	<0.001	74.9	<0.001	57.3	<0.001	33
	RCS	-16.2	2.55	-21.2	-11.2	<0.001	1.63	0.201	38.8		5

[1]DM = dry matter; NDF = neutral detergent fibre; OM = organic matter; ADF = acid detergent fibre; N = nitrogen; FNE = faecal N/total N intake; UNE = urine N/total N intake; [2]*P* value of a mixed model between sub-groups.

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates.

low CP diets, with daily yield being 1.46 kg lower (P = 0.005) than those receiving red clover silage-based rations. The inclusion rate of legume silages also had an effect on the response of dairy cows to dietary protein level (Figure 7.2). Dry matter intake did not differ (P = 0.222) between cows fed the control or low CP diets when cows received ≤ 20% inclusion of legume silage, but was reduced (P < 0.001) in low CP diets when the legume silage inclusion increased from 21 to 60% of the forage DM. There was no effect (P > 0.05) of feeding low CP diets on milk protein content when dairy cows were fed up to 40% inclusion of legume silages, but beyond this level, milk protein concentration was reduced (P < 0.001). On the other hand, the urinary N excretion was decreased in cows fed up to 60% inclusion of legume silage based low CP diets. However, there was no difference (P > 0.05) between the inclusion rate (≤ 20%, 21-40%, > 60%) of legume silages on the RMD for each response variables.



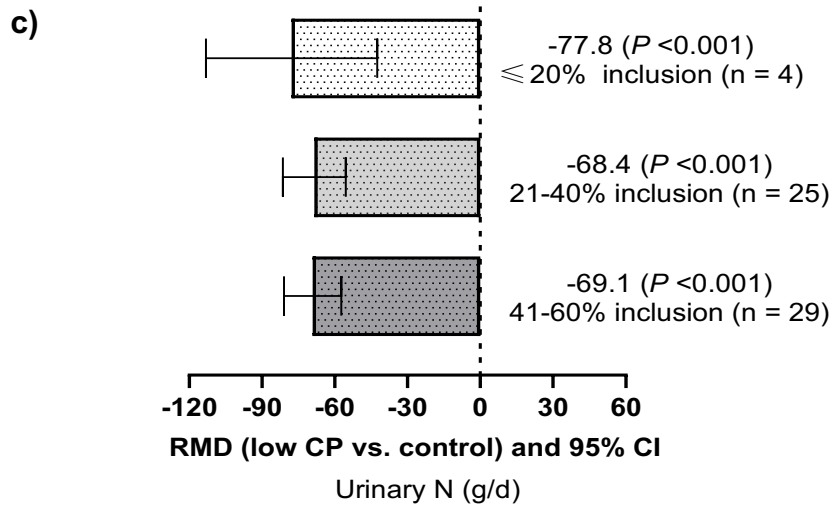
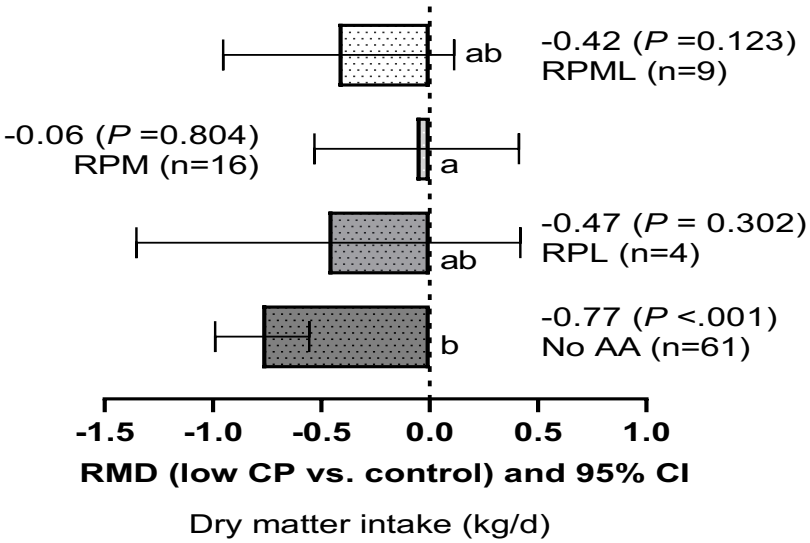


Figure 7.2. Covariate (legume silage inclusion rate on forage DM: ≤ 20%, 21 to 40% or 41 to 60%) effect size estimates for a) DM intake (kg/d), b) milk protein (g/kg) and c) urinary N excretion (g/d) of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis. RMD = raw mean differences between low CP and control diets. *P* value between groups (10-20, 21-40 or ≥60 %) for DM intake, *P* = 0.713; milk protein, *P* = 0.146; and urinary N, *P* = 0.885.

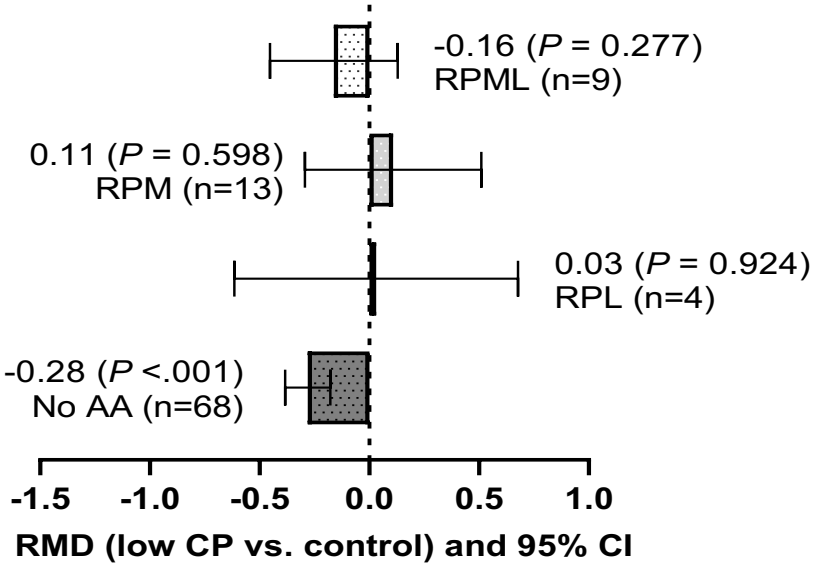
Feeding low CP diets supplemented with RPM, RPL or RPML did not affect (*P* > 0.05) DM intake or milk protein content (Figure 7.3). However, a difference was observed for the effect size on DM intake, which was 0.71 kg/d lower (*P* = 0.043) in cows receiving low CP diets without supplementation of AA compared to those with added RPM, which had a mean value of 23.8 kg/d (data not shown). Similarly, there was a tendency (*P* = 0.081) for milk protein content to be increased when a low CP diet was offered with RPM. The concentration of PUN was reduced (*P* < 0.001) in cows fed low CP diets with added RPM or without any additional AA, whereas MUN content decreased (*P* < 0.05) in cows that received low CP diets either with or without added AA. In addition, the MUN content and urinary N excretion were 2.36 mg/dl and 33.5 g/d higher (*P* < 0.05) in cows fed low CP diets with added RPL and RPM, respectively, compared to those receiving no added AA, which had a mean value of 8.83 mg/dl and 146 g/d, respectively.

a)



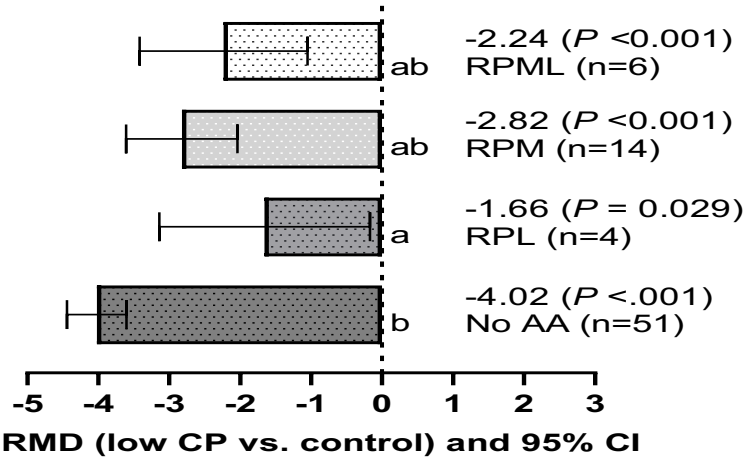
Dry matter intake (kg/d)

b)



Milk protein (g/kg)

c)



Milk urea N (mg/dL)

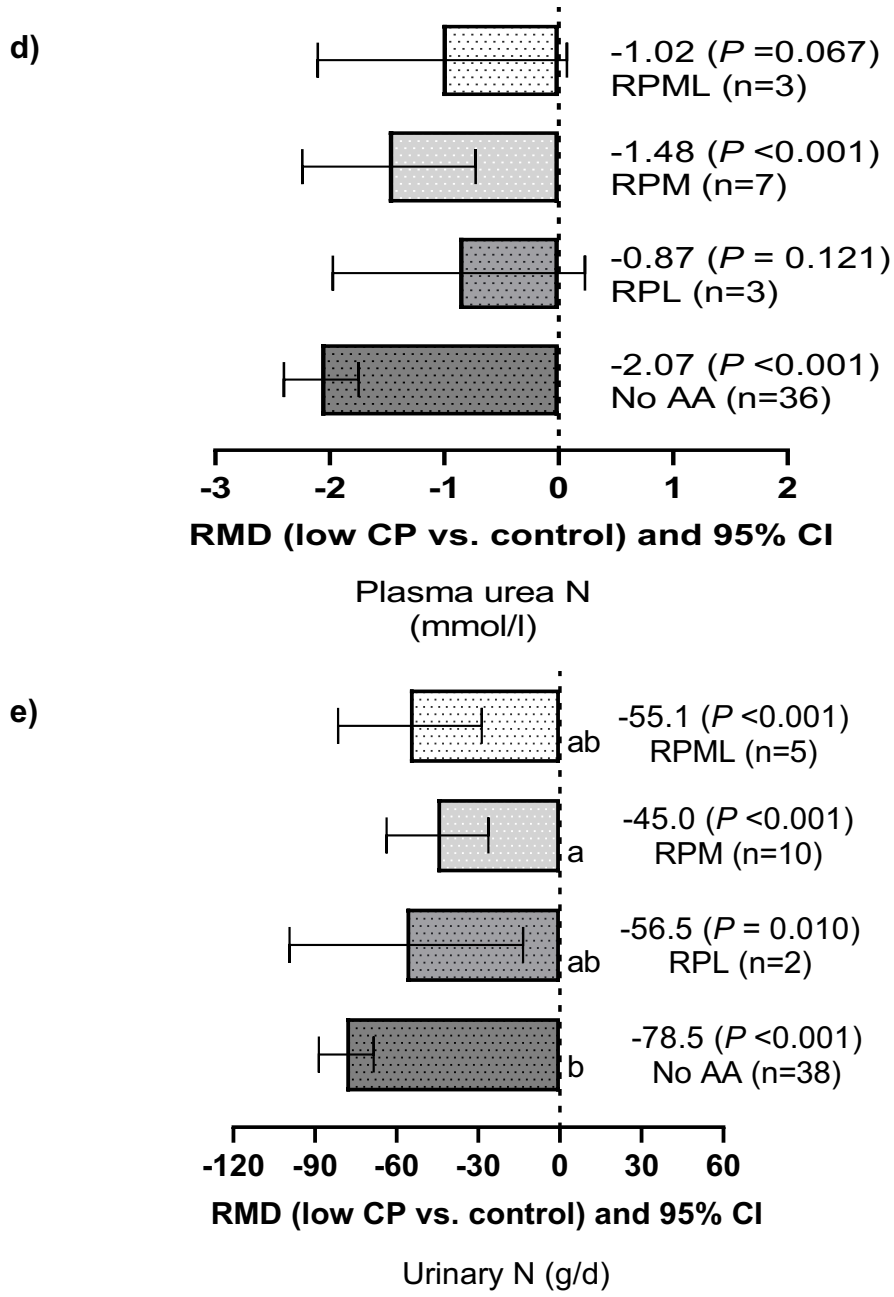


Figure 7.3. Covariate (low CP diet without (No AA) or with added amino acids: Rumen-protected lysine (RPL), Rumen-protected methionine (RPM) or Rumen-protected methionine-lysine (RPML)) effect size estimates for a) DM intake (kg/d), b) milk protein (g/kg), c) milk urea N (mg/dl), d) plasma urea N (mmol/l) and e) urinary N excretion (g/d) of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis. RMD = raw mean differences between low CP and control diets. *P* value between groups (No AA, RPL, RPM, RPML) for DM intake, $P = 0.043$; milk protein, $P = 0.081$; milk urea N, $P = 0.003$; plasma urea N, $P = 0.258$; and urinary N, $P = 0.044$. RMD with different superscripts differ significantly ($P < 0.05$).

Cows were stratified into two subgroups based on DIM (< 100 or ≥ 100 days) to evaluate the response of low protein diets (Table 7.13). The DM intake, OM digestibility, milk yield, MUN, PUN, and daily urinary N excretion were reduced (P < 0.05) in cows that were either < 100 or ≥ 100 DIM when fed low CP diets, however, there was no difference (P > 0.05) between these groups in the RMD of the response variables.

The DM intake, milk yield and PUN content were reduced (P < 0.001) in both multiparous and mixed (i.e. primiparous and multiparous) cows when low CP diets were fed compared to the control (Figure 7.4). However, there was an effect of parity on PUN level, which was 0.21 mg/dl lower (P = 0.002) in mixed parity compared to multiparous cows, which had a mean of 3.90 mg/dl. Feeding low CP diets for a short (≤ 50 days) or long period (> 50 days) both resulted in a reduction (P < 0.001) in milk yield, PUN content and urinary N excretion of dairy cows (Figure 7.5). The daily urinary N excretion was 32 g higher (P < 0.001) in cows when low CP diets were fed for a long period compared to a short period, which had a mean value of 156 g/d.

Table 7.13. Covariate (days in milk (DIM): ≥ 100 or < 100 DIM)) effect size estimates for DM intake, OM digestibility, milk yield, milk and plasma urea N, and urinary N excretion of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis.

Variables[1]	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P* value[2]	N
		RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)		
DM intake, kg/d DM	≥ 100 DIM	-0.61	0.12	-0.84	-0.38	<0.001	111	<0.001	52.2	0.850	54
	< 100 DIM	-0.65	0.15	-0.93	-0.36	<0.001	61.5	0.016	34.9		41
OM digestibility, %	≥ 100 DIM	-1.56	0.36	-2.26	-0.86	<0.001	41.7	0.001	56.9	0.253	19
	< 100 DIM	-0.92	0.43	-1.76	-0.08	0.032	41.6	0.001	56.7		19
Milk yield, kg/d	≥ 100 DIM	-1.52	0.20	-1.92	-1.12	<0.001	127	<0.001	56.8	0.415	56
	< 100 DIM	-1.26	0.24	-1.73	-0.79	<0.001	79.7	0.001	44.8		45
MUN, mg/dl	≥ 100 DIM	-3.27	0.25	-3.77	-2.78	<0.001	328	<0.001	87.8	0.264	41
	< 100 DIM	-3.68	0.26	-4.20	-3.17	<0.001	412	<0.001	90.8		39
PUN, mmol/l	≥ 100 DIM	-1.90	0.16	-2.21	-1.58	<0.001	322	<0.001	89.1	0.634	36
	< 100 DIM	-1.76	0.23	-2.22	-1.31	<0.001	153	<0.001	88.9		18
Urine N, g/d	≥ 100 DIM	-64.9	5.85	-76.4	-53.5	<0.001	320	<0.001	90.9	0.273	30
	< 100 DIM	-74.3	6.30	-86.7	-62.0	<0.001	111	<0.001	75.7		28

[1]DM = dry matter; OM = organic matter; N = nitrogen; MUN = milk urea N; PUN = plasma urea N.

[2]*P* value of a mixed model between sub-groups.

N = the number of comparisons between control and treatment (low CP) diets; RMD = the raw mean differences between control and low CP diets at 95% confidence interval; SE = standard error; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates.

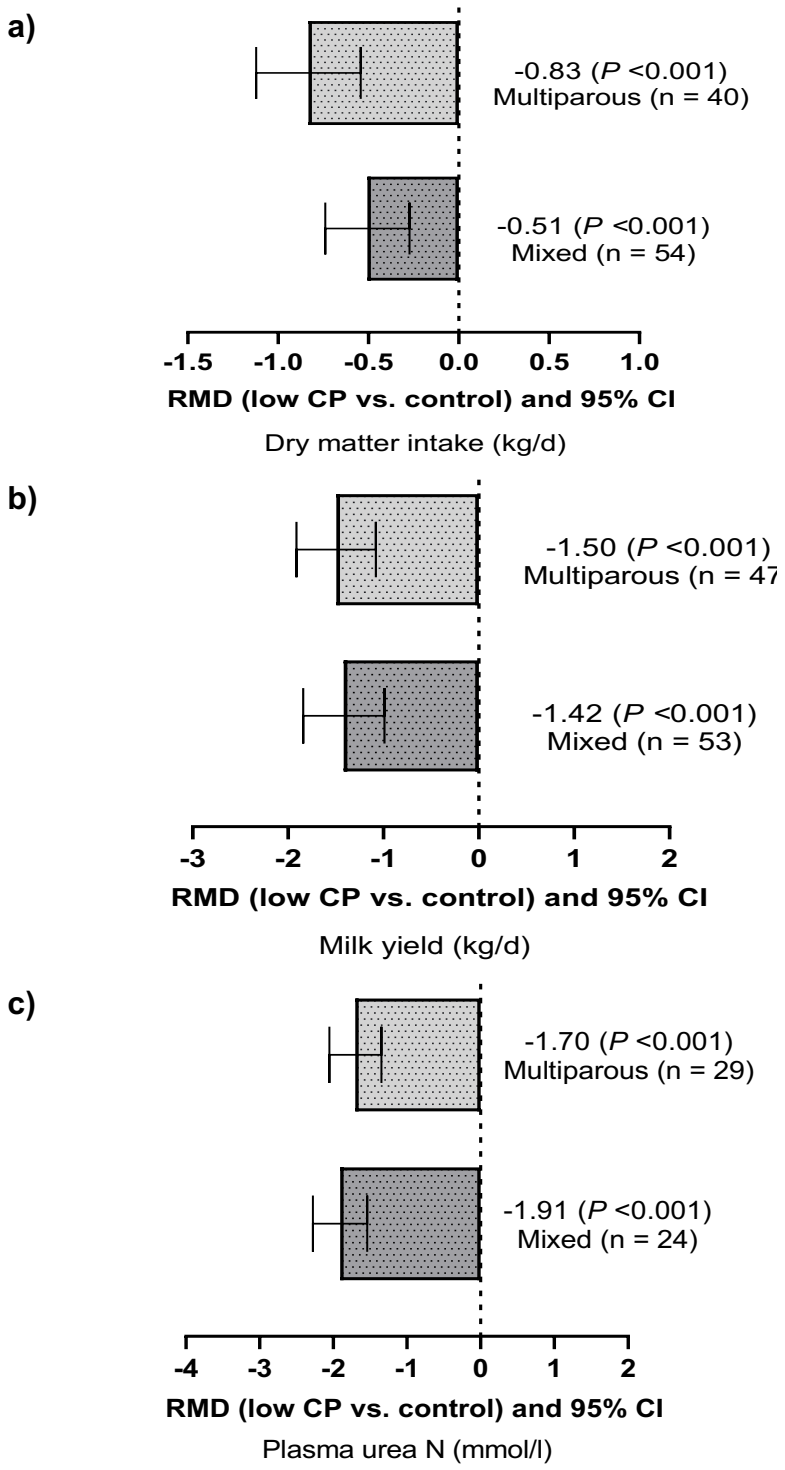


Figure 7.4. Covariate (parity: multiparous cow or mixed cow (used primiparous and multiparous)) effect size estimates for a) DM intake (kg/d), b) milk yield (kg/d), and c) plasma urea N (mmol/l) of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis. RMD = raw mean differences between low CP and control diets. *P* value between groups (multiparous vs. mixed) for DM intake, *P* = 0.168; milk yield, *P* = 0.128; and plasma urea N, *P* = 0.002.

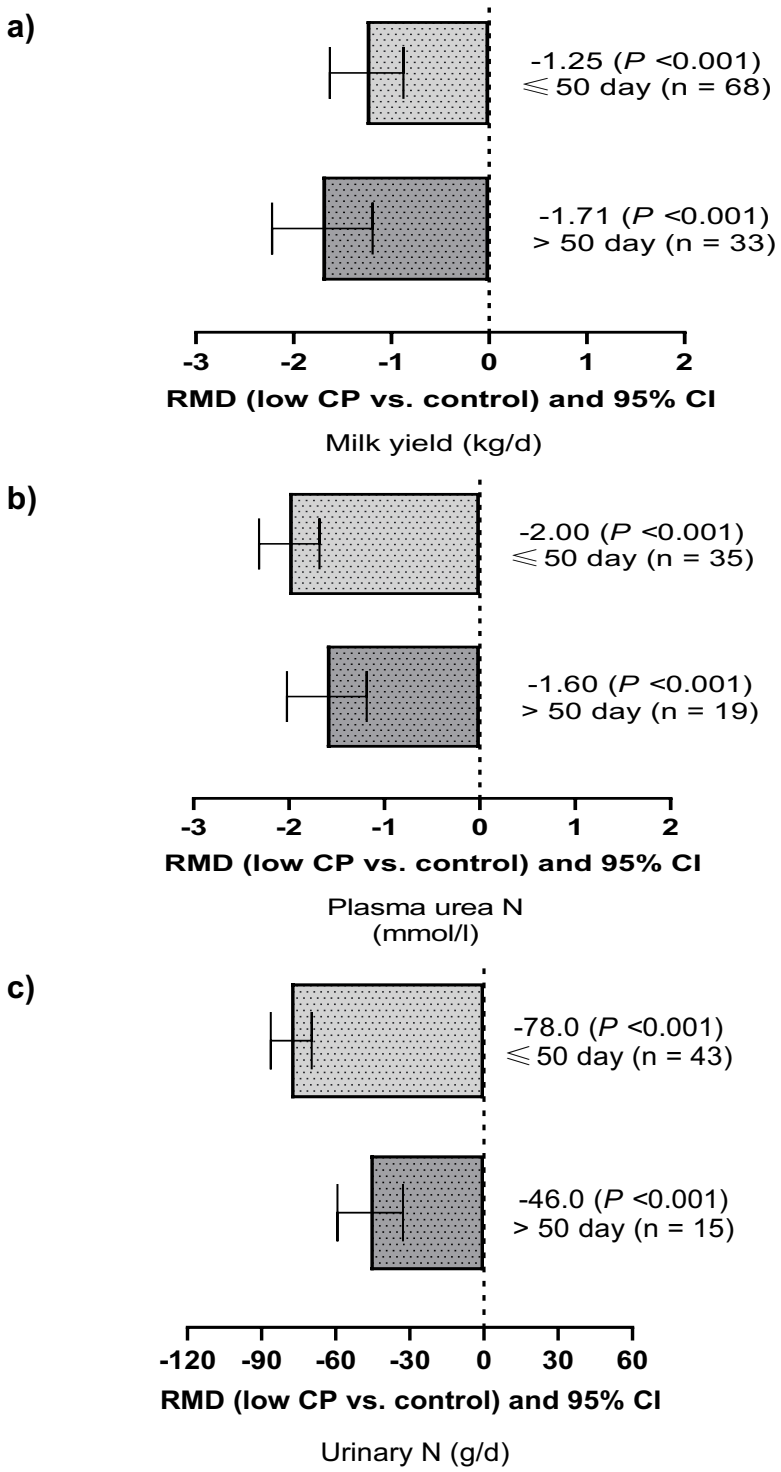


Figure 7.5. Covariate (experimental duration (days): ≤ 50 (short) or > 50 (long) days) effect size estimates for a) milk yield (kg/d), b) plasma urea N (mmol/l) and c) urinary N excretion (g/d) of dairy cows fed control and low CP diets based on forage legumes in a subgroup random-effect meta-analysis. RMD = raw mean differences between low CP and control diets. *P* value between groups (≤ 50 day vs. > 50 day) for milk yield, $P = 0.162$; plasma urea N, $P = 0.141$; and urinary N, $P < 0.001$.

7.4. Discussion

The dietary content of the CP was reduced in the treatment diet of all studies included in the systematic review and meta-analysis by decreasing the concentrations of vegetable protein, including soybean meal, heat-treated/expeller soybean or rapeseed meal, which resulted in a difference in RDP, RUP and MP content between control and low protein diets. However, some studies (42%) supplemented RP-AA in MP deficient diets to offset the negative impact on the performance of dairy cows by enhancing the post-ruminal supply of limiting essential AA (Giallongo et al., 2015, 2016; Lee et al., 2015a). The difference between diets in dietary starch content was due to the inclusion of processed/ground maize, wheat or barley in low CP diets when metabolisable energy (ME) was not limited (Ipharraguerre and Clark, 2005; Recktenwald et al., 2014; Liu and VandeHaar, 2020b).

7.4.1. Feed intake

Dry matter intake was reduced in cows fed low CP diets, which could be attributed to impaired rumen function due to an insufficient supply of RDP, which may depress fibre digestion and rumen passage rate, resulting in a lower feed intake (Allen, 2000). However, a significant heterogeneity for DM intake was observed in the current meta-analysis, and meta-regression analyses showed that the variation was due to the influence of some covariates. For example, DM intake was greatly reduced when cows received less than 140 g CP/kg DM, indicating a positive relationship between the dietary concentration of CP and DM intake, which support the findings of Barros et al. (2017), who reduced the concentration of dietary CP from 162 to 118 g/kg DM. A meta-analysis by Huhtanen and Hetta (2012) also reported a similar trend, but this response is not always evident because of the inconsistent effect of dietary CP levels on DM intake (Olmos Colmenero and Broderick, 2006; Broderick et al., 2015; Liu and VandeHaar, 2020b). According to Hristov and Giallongo (2014), the negative effect of low CP diets (< 150 g/kg DM) on DM intake is most likely to be associated with a lower supply of MP in high yielding dairy cows. Also, Sinclair et al. (2014) reported that the levels of dietary CP could be reduced to around 140 g/kg DM without affecting cow health and fertility when DM intake and MP was not limited.

Another factor that decreases the DM intake of cows fed CP deficient diets is a reduction in the post-ruminal supply of limiting essential AA, as reported by Lee et al. (2012) and Giallongo et al. (2016). In a subgroup analysis, there was no negative effect on DM intake of cows fed low CP diets supplemented with RP-AA, possibly due to the balance of available AA within MCP synthesised in the rumen. A study by Sinclair et al. (2014) concluded that dietary strategies should aim to optimise MCP synthesis to correct MP or essential AA supply, and to mitigate the anticipated reductions in DM intake by feeding low CP (≤150 g/kg DM) diets. However, a meta-analyses by Patton (2010) and Zanton et al. (2014) reported an inconsistent effect of RPM on the DM intake of milking cows, which may have occurred due to the deficiency of other rate-limiting essential AA (Patton, 2010), excessive inclusion of RPM (Robinson et al., 2000), or the use of different synthetic sources of RPM with different bioavailabilities (Zanton et al., 2014).

Broderick et al. (2001) established that feed intake in dairy cows was affected by forage type, and daily DM intake of a lucerne-based diet was 1.20 kg higher than a red clover silage-based ration (Broderick, 2018). However, the current study observed a reduced DM intake in cows fed lucerne or red clover silage-based low CP diets, but the size of the effect between forages did not alter. This finding was in agreement with the meta-analyses of Johansen et al. (2017) and Steinshamn (2010), who reported a similar DM intake when lucerne or red-clover silage-based diets were fed to lactating dairy cows. Moreover, the DM intake of cows depends on the substitution of grass or maize silages with legumes (Moorby et al., 2009; Sinclair et al., 2015; Schulz et al., 2018). The DM intake did not alter when legume silage substituted approximately 20% of non-legume forages, however, increasing the proportion up to 60% reduced intake, an effect in accordance with previous observations by Sinclair et al. (2015) and Schulz et al. (2018), who investigated the effects of different inclusion level of legume silages in the diet of dairy cows.

7.4.2. Milk performance

Milk yield was reduced when low CP diets based on forage legumes were fed to dairy cows, and the changes in milk yield were not limited to the duration of the studies, DIM or parity of cows. The decreased milk yield could be related to a reduction in DM intake. The variation in milk yield between studies was observed due to differences in dietary CP levels or forage type incorporated in the diets. The highest negative yield was found when cows received < 140 g CP/kg DM diet.

According to Lee et al. (2012) and Alstrup et al. (2014), reducing dietary CP concentration below 140 g/kg DM can negatively affect milk production. It has been hypothesised that a lower concentration of CP (< 140 g/kg DM) can reduce the post-ruminal supply of MP and contribute to decreased milk and milk protein yield (Hristov and Giallongo, 2014; Giallongo et al., 2016), indicating that the intestinal supply of MP and milk yield are strongly correlated (Daniel et al., 2016).

Feeding legume-based diets can improve milk yield in dairy cows compared to those receiving grass-silage based rations (Dewhurst et al., 2003; Steinshamn, 2010). A meta-analysis by Johansen et al. (2017) reported that feeding legume-based diets increased milk yield by 1.60 kg/d compared to grass silage-based rations, whereas cows receiving lucerne or red clover silage based rations had comparable milk production. In the current study a reduced milk yield was observed in cows fed lucerne-based ration but no significant difference was observed when cows received low CP diets based on red clover silage. One possibility for the lack of an effect on milk production when the diets are marginally deficient in CP or MP is the presence of energy reserves, which is confirmed by the lowest BCS in cows receiving low CP diets.

The concentration of milk protein was reduced in cows fed low CP diets based on legume forages, but not altered when either RPM, RPL or RPML was added to the diet, indicating that milk protein synthesis depends on the available essential AA in the mammary gland (Hristov et al., 2005; Huhtanen and Hristov, 2009; Doepel and Lapierre, 2010). Several authors (Giallongo et al., 2015, 2016; Lee et al., 2015a) have shown that methionine and lysine are the key limiting AA for milk protein production in cows fed maize and lucerne based rations. However, a decrease in milk protein content was observed with increasing proportion of legume silages in the current study, which might have been due to a limited ME content in legumes (Steinshamn, 2010) which leads to a lower supply of rumen available energy and subsequent MCP flow to the duodenum. A meta-analysis by Daniel et al. (2016) reported that the ME to MP ratio balance could improve milk performance by increasing the partition of energy to milk.

7.4.3. Nutrient intake and digestibility

Nutrient intake and apparent total-tract digestibility were reduced in cows fed low CP diets based on legume forages. The negative effect of low CP diets on nutrient

digestibility, including fibre, could be attributed to a deficiency of rumen degradable N, which is required by cellulolytic bacteria to degrade ingested carbohydrates (Atasoglu et al., 2001). The lowest concentration of rumen NH_3-N was observed in the current study when cows received less than 140 g CP/kg DM diets, confirming the limited supply of N in the rumen (Broderick, 2018). Another key factor affecting the apparent nutrient or fibre digestibility is DM intake, and reducing DM intake by 1.5 to 2.0 kg/d can decrease MCP synthesis and rumen fermentation (Lee et al., 2012b). A meta-analysis by Huhtanen et al. (2009) noted that the apparent total-tract OM digestibility in lactating cows was negatively correlated to DM intake. In addition, a significant reduction in NDF intake and apparent OM and fibre digestibility was observed in the current study when cows received red clover silage-based low CP diets, which could be attributed to a lower silage NPN, and greater concentration of ADIN or PPO. The PPO may interact with plant proteins, including proteases, and depress fibre degradation, resulting in a reduced MCP synthesis in the rumen due to a lower supply of RDP (Broderick, 2018).

7.4.4. Plasma metabolites and urea nitrogen

The mobilisation of body tissue is essential to support milk performance during the early stages of lactation but, excess mobilisation of body lipid may be associated with metabolic disorders, including the formation of ketone bodies (van der Drift et al., 2012; Ji and Dann, 2013). In the current study, the plasma concentration of BHB was numerically increased, but NEFA content was substantially increased in cows fed low CP diets, confirming mobilisation of body fat. Law et al. (2009) noted that the plasma concentration of BHB was increased by 0.08 mmol/l in cows fed 114 g CP/kg DM compared to the control CP (173 g/kg DM) diet. Similarly, Halmemies-Beauchet-Filleau et al. (2017) observed that reducing dietary CP content from 171 to 156 g/kg DM in red clover and grass silage-based rations increased plasma concentrations of NEFA by 0.08 mmol/l in early lactation Holstein cows. Sinclair et al. (2014) concluded that the plasma levels of NEFA might vary with dietary CP contents. Therefore, lowering dietary CP concentration during the early stages of lactation is challenging for high yielding dairy cows.

The decrease in total N intake and CP digestibility in dairy cows fed low CP diets reduced the concentration of PUN, which resulted in a significant reduction in MUN content and N excretion in the current study. However, a high heterogeneity was observed for both PUN and MUN contents due to the level of CP or supplementation

of RP-AA in the low CP diets. The subgroup analyses showed the lowest milk or plasma urea N content reduction occurred when cows received ≥ 140 g CP/kg DM or diets added with RP-AA. In general, the concentration of PUN in dairy cows is closely related to dietary CP level, and is increased either by deamination of AA in the liver or absorption of NH_3 from rumen fermentation followed by the conversion to urea and transportation to the arterial vein via the hepatic circulation (Recktenwald et al., 2014). On the other hand, supplementation of RP-AA to cows fed low CP or MP deficient diets had the potential to improve feed intake and milk protein synthesis, but no significant effect was observed on plasma metabolites, except for some differences in plasma glucose and insulin level as reported by Lee et al. (2012b) and Giallongo et al. (2015, 2016). The inconsistent effect of RP essential AA on plasma insulin or glucose concentration in dairy cows is possibly due to the interactions between AA's (Ranawana and Kaur, 2013), or sources and dose-responses to supplemented AA (Liu et al., 2008). Other factors that may influence the variation in PUN level in cows are DIM, parity or experimental duration. Previous studies (Peterson and Waldern, 1981; Carroll et al., 1988; Barton et al., 1996) established that multiparous cows had a higher PUN content than first lactation animals, a finding in agreement with the current result.

7.4.5. Nitrogen output and efficiency

Urinary N excretion was reduced in cows fed low CP diets based on legume forages; however, significant heterogeneity was observed due to dietary and animal factors. Nitrogen excretion mainly depends on the concentration of dietary CP or total N intake, and there is a linear relationship between dietary N intake and urinary or faecal N output (Castillo et al., 2000). Recent studies (Lee et al., 2012a; Niu et al., 2016; Oh et al., 2019) have reported that low CP diets significantly decreased urinary N emission rather than faecal N, which is consistent with the current findings. Similar to MUN, a lower excretion of urinary N was observed when animals received < 140 g CP/kg DM or a ration without added AA, indicating a positive correlation between MUN and urinary N output, which supports previous studies (Kauffman and St-Pierre, 2001; Spek et al., 2013). In contrast, feeding cows low CP diets with added RPM slightly elevated urinary N excretion compared to those fed non AA supplemented diets possibly due to a lack of change in milk protein synthesis with RPM, assuming that the excess N is excreted in the urine. A similar effect was also observed by Broderick (2018) when the diet was supplemented with RPL.

Compared with the lucerne-based diet, there was a substantial decrease in urinary N excretion and increased excretion of faecal N as a proportion of total N intake in dairy cows fed red clover silage-based rations. This effect supports the findings of Broderick (2018), who investigated N utilisation in lactating dairy cows and growing lambs by feeding lucerne or red clover based diets. The efficient utilisation of N is more important in low protein diets, and low CP diets increased the apparent milk NUE (milk N/N intake) in the current study, which relates to the reduced urinary N excretion. A meta-analysis by Huhtanen and Hristov (2009) stated that increasing the capture of N for milk protein synthesis by reducing urinary N excretion can improve NUE in milk. In contrast, the higher concentration of N in urine leads to an inefficient partitioning of N. The excretion of urinary N was slightly increased when cows were fed low CP diets for more than 7 weeks, which might be associated with urea recycling and adaptation to low CP diets.

7.4.6. Rumen fermentation and milk fatty acids

Reduced rumen NH_3-N concentration in the current study indicated a lower supply of RDP, which may impair MCP synthesis in the rumen when cows were fed with low CP diets. Feeding low CP diets reduced the rumen butyrate content in the current study, which in agreement with the findings of Cui et al. (2019), who reported a tendency (P = 0.051) towards a lower concentration of rumen butyrate in lambs fed either CP or energy-deficient diets. In contrast, several studies (Aguerre et al., 2016; Nursoy et al., 2018; Rafiee-Yarandi et al., 2019) have reported no significant changes in rumen VFA concentrations except branched-chain VFA, including valerate or iso-valerate, which might be a potential marker of rumen N deficiency or availability (Cabrita et al., 2003; Leduc et al., 2017). However, no significant interaction between CP and RDP was reported by Mutsvangwa et al. (2016) for any of the fermentation metabolites in lactation cows when fed two different dietary levels of CP (175 vs. 149 g/kg DM). Therefore, the correct balance between rumen available energy and N is crucial to maintain rumen ecosystems and the fermentation process (Sinclair et al., 1995).

Milk FA concentrations in dairy cows mainly depend on the feed FA composition and rumen biohydrogenation of long-chain FA (Lashkari et al., 2019). Some animal factors are also associated, including rumen ecosystem, *de novo* synthesis of FA within animal tissue, and FA release from body fat mobilisation during negative energy balance in early lactation (Mansbridge and Blake, 1997; Vlaeminck et al.,

2006). However, the dietary concentration of CP had little influence on milk FA profile in lactating dairy cows in the current study. Increased concentrations of milk SFA were observed and MUFA was decreased in the milk of cows fed low CP diets based on legume forages in the current study, which is consistent with the findings of others (Lee et al., 2011; Giallongo et al., 2016; Rafiee-Yarandi et al., 2019). According to Lee et al. (2011) and Giallongo et al. (2016), dietary intake of whole or heat/expeller treated soybean, which is enriched with C18:0 unsaturated FA, coconut oil/fat supplementation, and limited biohydrogenation due to a lower supply of RDP could be related to the changes in milk FA concentration. Therefore, dietary ingredient is crucial for milk FA synthesis in lactating dairy cows.

7.4.7. Limitations and strengths

The meta-analysis was limited to early and mid-lactating high yielding dairy cows, and therefore the outcomes may not be appropriate for late or low producing cows, as the lowest yield in the data analysed was 22 kg/cow/d. Most studies in the literature that have fed forage legumes were based on lucerne, and there are very few studies that have fed low protein diets containing red clover silages, which led to difficulties in conducting subgroup analyses, and could therefore lead to misinterpretation. Further studies on low CP diets based on red-clover or other legume silages such as peas or beans are therefore required. Some performance outcomes contained variations across the studies due to the level of CP in the diet, rate of legume silage inclusion, and supplementation of RP-AA in low CP diets. However, other dietary factors such as starch level, RDP and RUP content, and the concentration of MP were not included in the meta-analysis due to a very limited number of studies on legume silages that reported these values. Regardless of these limitations, the main strength of the current study was that there was no evidence of publication bias for the response variables, and there was a systematic characterisation of a pooled dataset from the literature to provide an overall summary of dairy cow performance, metabolism and N use efficiency.

7.5. Conclusions

Feeding low protein diets based on forage legumes negatively impacted the performance of dairy cows by reducing intake, milk yield, milk protein content, condition score, diet digestibility, rumen concentration of NH_3-N and butyrate, but improved apparent N use efficiency, which was associated with a reduced N

excretion in urine and decreased plasma and milk urea N content. The dietary concentration of CP, legume type and its inclusion rate, along with the supplementation of RP-AA, were strongly related to some but not all performance outcomes, and as a consequence, raised heterogeneity. Feeding very low CP content diets (<140 g/kg DM) resulted in the greatest negative impact on DM intake and milk performance. Neither DM intake nor milk protein content were altered by RP-AA supplementation although providing RPM increased DM intake compared to no AA supplement, and MUN concentration was higher in cows receiving RPL than no additional AA. Compared with red clover silage-based rations, lucerne-based low CP diets improved apparent nutrient digestibility and reduced milk yield. Future studies investigating low protein diets based on forage legumes should focus on red clover-based rations and other legumes such as peas and beans, and must report the dietary effects of RDP, RUP and particularly MP rather than just focus on CP.

CHAPTER 8: General Discussion and Conclusions

8.1. General discussion

There has been increased interest in utilising home-grown forage legumes in the UK dairy industry due to their higher CP content than other non-legume forages (Dewhurst, 2013). Over the last decade, many studies (Moorby et al., 2016; Sinclair et al., 2015; Schulz et al., 2018) have investigated the effects of replacing grass or maize silage with either red clover or lucerne silage on dairy cow performance and metabolism, although no studies have been conducted in the UK to assess the effect of different levels of CP in forage legume based diets except for comparisons between grass and legume silages (Dewhurst et al., 2003a; Dewhurst, 2013; Johnston et al., 2020) or mixtures of maize and legume silages (Dewhurst et al., 2010; Thomson et al., 2017b) on milk production and N use. In contrast, several studies (Olmos Colmenero and Broderick, 2006; Broderick et al., 2015; Lee et al., 2015a) have been undertaken in North America to investigate the effect of low CP diets on the performance of dairy cows when fed lucerne silage-based diets, but none of these studies have focused on low CP diets based on red clover silage. Therefore, the current studies were focused on the performance, metabolism and N use efficiency of high yielding Holstein-Friesian dairy cows receiving low CP diets based on home-grown (UK) red clover and lucerne forages.

This thesis has evaluated the effects of reducing dietary CP concentrations on the performance and metabolism of high yielding dairy cows by feeding either red clover/grass silage (Study 1, Chapter 4) or lucerne/maize silage (Study 2, Chapter 5) based rations for a short period, or feeding a red clover/grass silage-based diet either without or with supplementation of starch or RPM over the first 14 weeks of lactation (Study 3ab, Chapter 6). In addition, a systematic review and meta-analysis was conducted to estimate the effects of low CP diets based on forage legumes on the performance of early and mid-lactation dairy cows (Chapter 7, Study 4).

Reducing dietary CP concentration from 175 to 165 g/kg DM in Study 1 did not alter DM intake, but intake decreased by 1.5 kg/d when cows were fed 150 g CP/kg DM diet, an effect consistent with previous studies (Alstrup et al., 2014; Giallongo et al., 2016; Barros et al., 2017), who reported adverse effect of low CP diets on feed intake. A similar response was observed in Study 2 when low CP diets were based on lucerne and maize silages (both silage on an equal DM basis). These results

support the findings of a meta-analysis in Study 4 where DM intake was reduced by 0.62 kg/d in dairy cows fed legume silage-based low CP diets. However, there was no difference in DM intake when the inclusion rate of lucerne increased from 50 to 60% in a low CP (150 g/kg DM) diet in Study 2, supporting Arndt et al. (2015), who replaced 80% maize with lucerne silage in the diet of dairy cows. In contrast, Sinclair et al. (2015) reported a decreased DM intake when 60% of maize silage was replaced by lucerne silage in the diet of dairy cows. These finding support the meta-analysis (covariate = legume silage inclusion) in Study 4 where DM intake was reduced when legume silage inclusion rate increased up to 60% (DM basis). In agreement with a meta-analysis by Johansen et al. (2017), cows receiving either red clover or lucerne silage-based rations had a similar DM intake (24.6 and 24.8 kg/d in Study 1 and 2, respectively; P = 0.672), which also support the analysis in Study 4 (RMD = -0.59 and -1.12 kg/d for lucerne and red clover based diets, respectively; P = 0.133).

Feeding low CP diets based on red clover/grass silage either without or with added starch or RPM had no effect on DM intake (Study 3ab). Similarly, previous studies (Olmos Colmenero and Broderick, 2006; Broderick et al., 2015; Kidane et al., 2018b) have reported no effect on DM intake with an even lower CP concentration (130 g/kg DM) than used in the Studies 1, 2 or 3ab (150 g CP/kg DM). Also, feeding low CP diets supplemented with RP-AA did not affect DM intake in the current meta-analysis in Study 4 (Appendix 8.1), possibly due to an adequate supply of MP. Likewise, other meta-analyses (Patton, 2010; Robinson, 2010; Sinclair et al., 2014) have reported no significant effect of RPM on DM intake in dairy cows.

Reducing the concentration of CP in the diet of dairy cows did not alter milk yield or milk fat, protein or lactose content in Study 1, 2 or 3ab, which might be associated with adequate MP supply, indicating that feeding a red clover or lucerne silage-based diet with a CP content of 150 g/kg DM is sufficient to maintain milk performance. The current findings support the results of Hristov and Giallongo (2014), who reported that a diet containing 150 g CP/kg DM did not have any impact on milk yield or composition in dairy cows. Despite the reduced DM intake in Study 1 and 2, there was no difference in milk yield or milk performance, which could be attributed to the mobilisation of body energy reserves that supported milk production. However, increasing the proportion of lucerne silage in Study 2 led to a decrease in milk yield and milk protein content, an effect that is consistent with

studies in the UK by Thomson et al. (2017), who replaced 75% maize with lucerne silage in the diet of Holstein-Friesian dairy cows. The meta-analysis in Study 4 revealed that the inclusion of legume silage at up to 60% of the forage DM decreased the milk protein content in dairy cows. Feeding low CP diets based on either red clover or lucerne silage may be associated with a lack of rumen available N and energy due to the presence of PPO in red clover and a limited ME content in lucerne silage (Sinclair et al., 2015; Broderick, 2018; Johnston et al., 2020), which impaired the flow of MCP to the duodenum and decreased milk protein synthesis (Sinclair et al., 2014; Daniel et al., 2016). However, in Study 1 and 3ab, there was no effect on milk protein content when 50% of red clover silage in the forage DM was added to the diets. Previous studies have shown that some AA such as methionine, lysine, and histidine were limiting for milk protein synthesis in dairy cows when fed legume and grass/maize silage based diets (Lee et al., 2015a; Giallongo et al., 2016; Johansen et al., 2018). Reducing the dietary concentration of CP to less than 150 g/kg DM can decrease the post-ruminal supply of MP, resulting in a reduced milk or milk protein yield (Hristov and Giallongo, 2014; Giallongo et al., 2016). The meta-analysis in Study 4 also reported a similar effect on milk yield and milk protein content when cows were fed less than 140 g CP/kg DM in diets based on forage legumes (Appendix 8.2 and 8.3). However, in agreement with previous studies (Giallongo et al., 2015, 2016; Lee et al., 2015a), there was no effect of added starch or RPM on milk yield or milk composition in Study 3ab, indicating sufficient MCP and post ruminal methionine supply. The subgroup (covariate = AA supplementation) analysis in Study 4 also showed a similar result, and suggests that the role of RP-AA, including methionine and lysine, on dairy cow performance is not consistent (Lee et al., 2012a; Sinclair et al., 2014; Giallongo et al., 2015).

Nutrient intake and apparent digestibility were reduced when cows received legume-based low CP (< 156 g/kg DM) diets in Study 4. A similar response was found when red clover/grass silage-based diets were fed in Study 1. The decreased nutrient digestibility could be related to a lower supply of RDP, which may depress the growth of rumen microbes, resulting in restricted intake and digestion (Olmos Colmenero and Broderick, 2006; Oh et al., 2019). Olmos Colmenero and Broderick (2006) reported that a concentration of CP less than 165 g/kg DM in lucerne and maize silage-based rations could contribute to a lower nutrient digestibility. However, no effect on nutrient digestibility was observed in Study 2 when the ration was based on lucerne and maize silage, which may have been due to an adequate

supply of MPE. However, the OM digestibility of red clover/grass silage-based diet (Study 1) was 74.2%, approximately 3.20% units higher than the lucerne/maize silage-based ration (P = 0.065; Study 2). These findings are consistent with Johansen et al. (2017) and Broderick (2018), who reported increased OM digestibility when cows were fed red clover compared to lucerne silage-based diets. In contrast, a significant reduction in apparent OM and fibre digestibility was observed in the subgroup of forage type in Study 4, particularly in cows fed red clover silage rather than lucerne-based rations. This could be due to a lower DM intake and the action of PPO (Jones et al., 1995b), which may have impaired rumen function and depressed fibrolytic bacteria, resulting in a lower digestibility (Russell et al., 1992; Allen, 2000). However, the DM intake did not differ between treatments and there was no effect on apparent nutrient digestibility in Study 3b. Therefore, the difference between Study 1 and Study 3b on nutrient digestibility is unclear, although the faecal sampling was more robust in Study 3b.

It was hypothesised that reducing the concentration of CP in dairy cow diets would improve NUE by decreasing N excretion in manure, mainly in the urine. Relationships between N intake and total N output in milk, faeces or urine are presented in Figure 8.1 and revealed a positive relationship between milk or faecal N output and N intake (R^2 = 0.10 and R^2 = 0.29; P < 0.001, respectively). However, the relationship between N intake and urinary N output in Study 1 and 2 (R^2 = 0. 40; P < 0.0001) or in Study 3b (R^2 = 0.64; P < 0.0001) was stronger than that of milk or faecal N output (g/d). The urinary N output was calculated by difference in Study 1 and 2 and determined in Study 3b. The measured urinary N (Study 3b) showed a strong correlation (R^2 = 0.64 vs. 0.40) with N intake compared to estimated values (Study 1 and 2). This finding is consistent with the observation of a recent meta-analysis by Spanghero and Kowalski (2021), who reported urinary N excretion (g/d) was increased with the intake of total N. According to Yan et al. (2006), N intake is the single best predictor for the excretion of manure N (R^2 = 0.90) in dairy cows. The N concentration in urine was substantially lower in Study 3b when cows were fed low CP (150 g/kg DM) diets. The positive relationship between the intake of dietary N and urinary N output in dairy cows was also observed by Castillo et al. (2000), who reported that the excretion of N in urine was increased linearly when the intake of N exceeded 400 g/d.

The reduction in total N intake and CP digestibility in dairy cows fed low CP diets was associated with a lower concentration of plasma urea, and consequently milk urea in the current studies (Chapters 4, 5 and 6). The relationship between plasma or milk urea concentration and N intake across all studies was determined, which revealed that both plasma urea (R^2 = 0.37; P < 0.001) and milk urea (R^2 = 0.24; P < 0.001) were positively correlated with N intake in lactating cows (Figure 8.2). A study by Olmos Colmenero and Broderick (2006) also reported that both plasma and milk urea were highly correlated (R^2 = 0.83). Furthermore, the current meta-analysis (Study 4) demonstrated that the concentration of rumen NH_3-N was 3.38 mg/dl lower in cows fed a low CP (< 156 g/kg DM) compared to the control. The dietary level of N is the primary source of variations among the studies for both plasma and milk urea N of dairy cows (Chapter 7, Study 4).

The current studies (Study 1, 2, and 3ab) reported an average of 22.0% NUE improvement when low CP (150 g/kg DM) diets based on legume silages were fed to dairy cows, a finding in agreement with previous reports (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a; Kidane et al., 2018b). However, the efficiency of N capture was slightly lower in the current meta-analysis (Study 4) compared to previous studies (Study 1, 2 and 3ab), which could be due to the dietary and animal factors, as reported by Spanghero and Kowalski (2021). A negative relationship between N intake and NUE (R^2 = 0.54; P < 0.001) was found in the current studies (Figure 8.3). Improved NUE and decreased urinary N output could also be reflected in a lower concentration of milk urea in lactating dairy cows, indicating a positive relationship between urinary N output and milk urea content (R^2 = 0.52; P = 0.0003; Figure 8.4). Again, the measured urinary N (Study 3b) showed a strong correlation (R^2 = 0.52 vs. 0.12) with milk urea content compared to estimated urinary N (Study 1 and 2). A positive relationship between MUN and urinary N output was also reported by Spek et al. (2013) and Hynes et al. (2016). Also, MUN was associated with MP supply, which can influence the conversion of dietary N into milk N in dairy cows (Sinclair et al., 2014). Results from the current studies showed that NUE was positively related with MPN (Figure 8.5 a) or MPE (Figure 8.5 b) as % of MP requirements; however, the relationship was stronger with MPN rather than MPE (R^2 = 35 vs. 19, respectively). Moreover, the highest efficiency was observed when the balance between MPN to MPE was not more than 8 to 10 g/kg DM (Figure 8.6 a) or the proportion of MPN to MPE was 1.00 to 1.10 (Figure 8.6 b)

Reducing dietary CP concentration did not alter plasma concentrations of ammonia, BHB or glucose in the current studies. The highest plasma BHB concentration was reported in Study 3a when cows were fed a low CP diet with added RPM, which could be related to a tendency (P = 0.079) for lower BCS in cows fed the RPM compared to other diets. However, other plasma metabolites did not alter with the inclusion of RPM (Krober et al., 2000; Giallongo et al., 2015, 2016). According to Sinclair et al. (2014), plasma NEFA content in cows can vary with dietary concentration of CP. Similarly, Law et al. (2009) and Halmemies-Beauchet-Filleau et al. (2017) observed that the plasma concentration of BHB and NEFA were increased by 0.08 mmol/l in lactation cows when the CP content was reduced. These findings were confirmed by the results of the meta-analysis (Study 4) where the highest concentration of plasma NEFA was reported in dairy cows fed low CP (< 156 g/kg DM) diets.

The current studies demonstrated that the dietary concentration of CP had little influence on the milk FA content in dairy cows. The highest concentration of PUFA, including some intermediates of ruminal FA biohydrogenation, was observed in the milk of cows fed the red clover/grass silage-based low CP (150 g/kg DM) diets in Study 1, which could be related to a lower RDP supply that depressed FA biohydrogenation in the rumen, or more likely an effect of PPO in red clover silage protecting dietary PUFA from biohydrogenation in the rumen. However, an increased concentration of milk FA of a chain length less than C16:0 was reported when cows received a low CP (150 g/kg DM) diet based on red clover and grass silages in Study 3a. In contrast, feeding a high CP diet (175 g CP/kg DM) increased the yield of milk OBCFA and < C16:0 FA in the current studies, indicating synthesis of MCP in the rumen and post ruminal flow of MP to the mammary gland. However, the effect of dietary CP, RDP or PPO on milk FA synthesis in high yielding dairy cows is not consistent (Mansbridge and Blake, 1997; Vlaeminck et al., 2006; Hristov et al., 2011a).

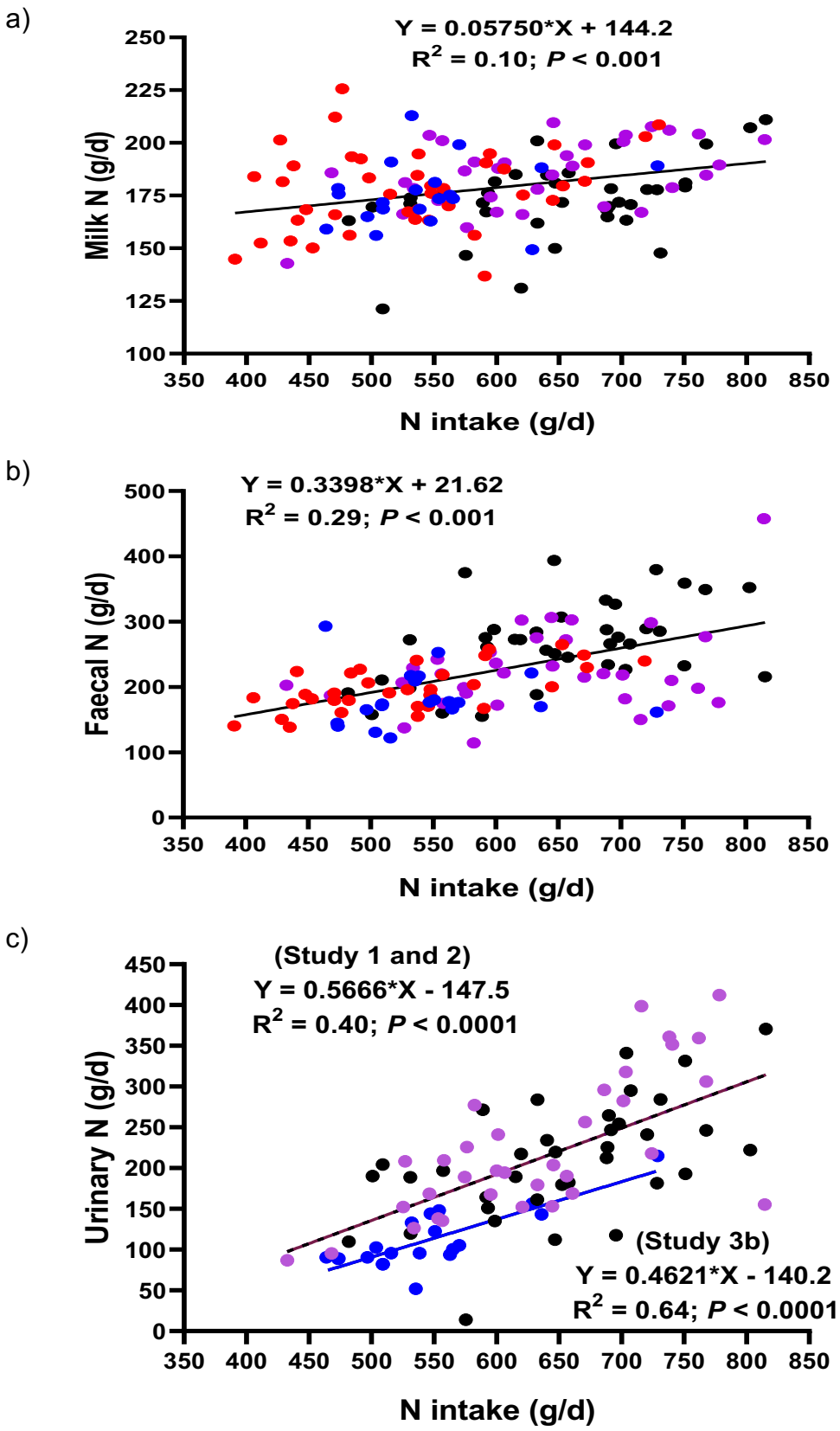
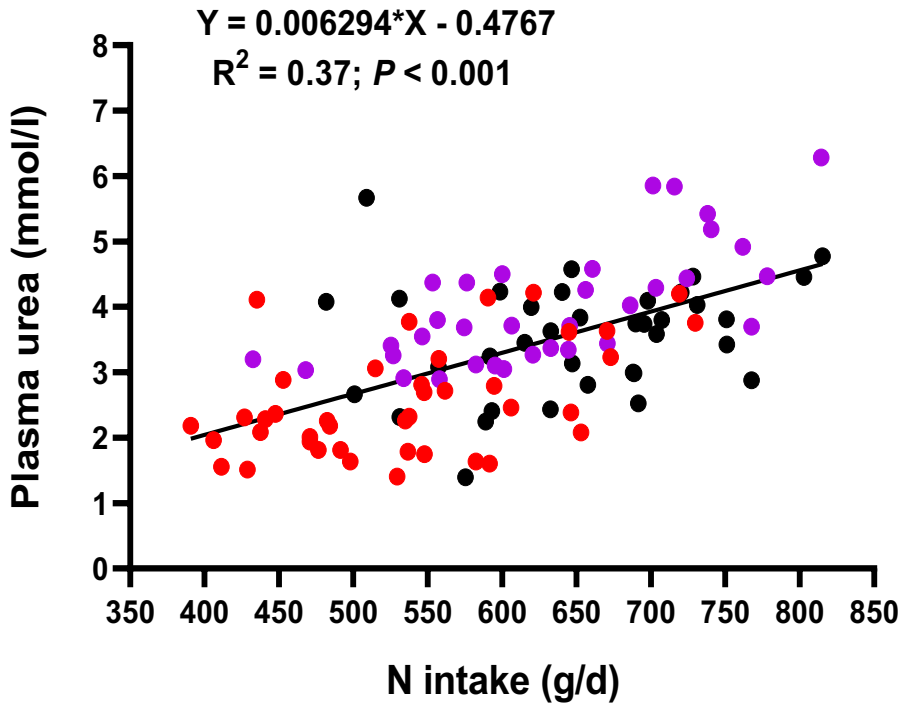


Figure 8.1. Relationship between nitrogen (N) intake (g/d) and milk N (a), faecal N (b) or urinary N (c) excretion (g/d) of dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3a (●), n = 41. For Study 3b (●), n = 20. The urinary N output was calculated by difference in Study 1 and 2, and measured in Study 3b.

a)



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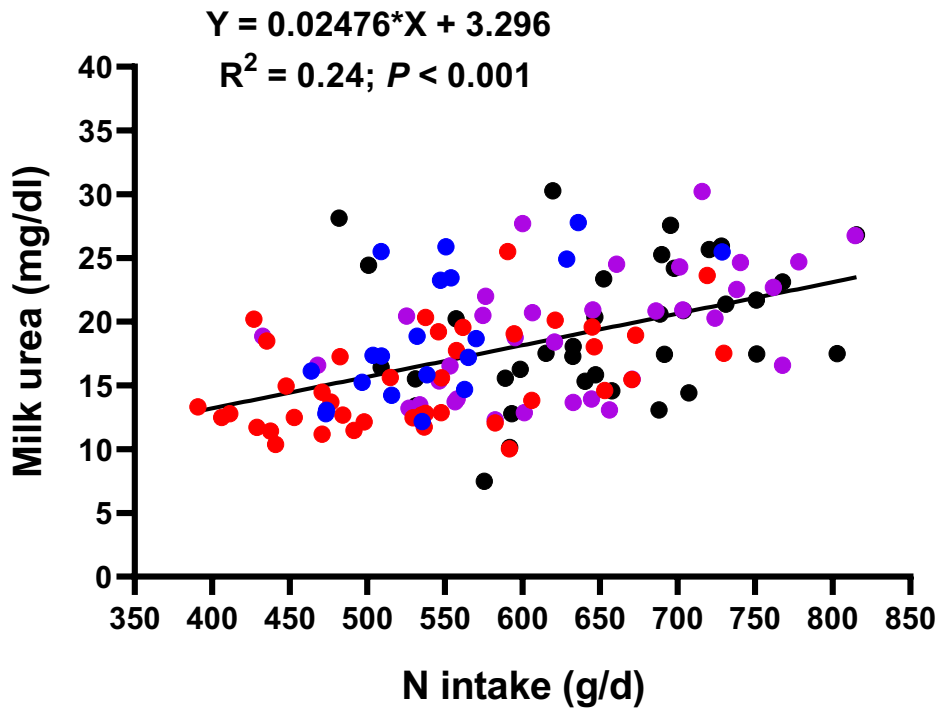


Figure 8.2. Relationship between nitrogen (N) intake (g/d) and plasma urea (a) or milk urea (b) concentration of dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3a (●), n = 41. For Study 3b (●), n = 20.

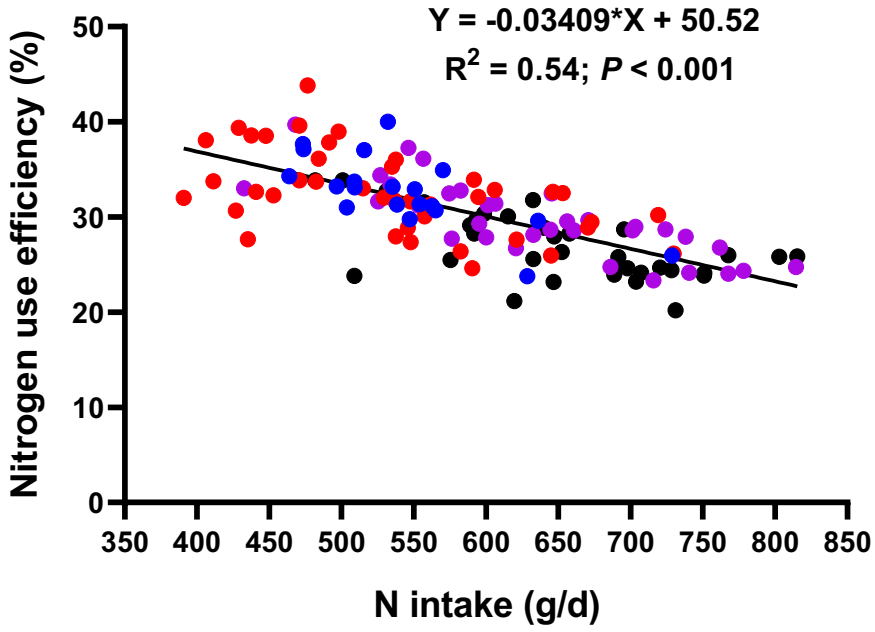


Figure 8.3. Relationship between nitrogen (N) intake (g/d) and N use efficiency (NUE, %) of dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3a (●), n = 41. For Study 3b (●), n = 20.

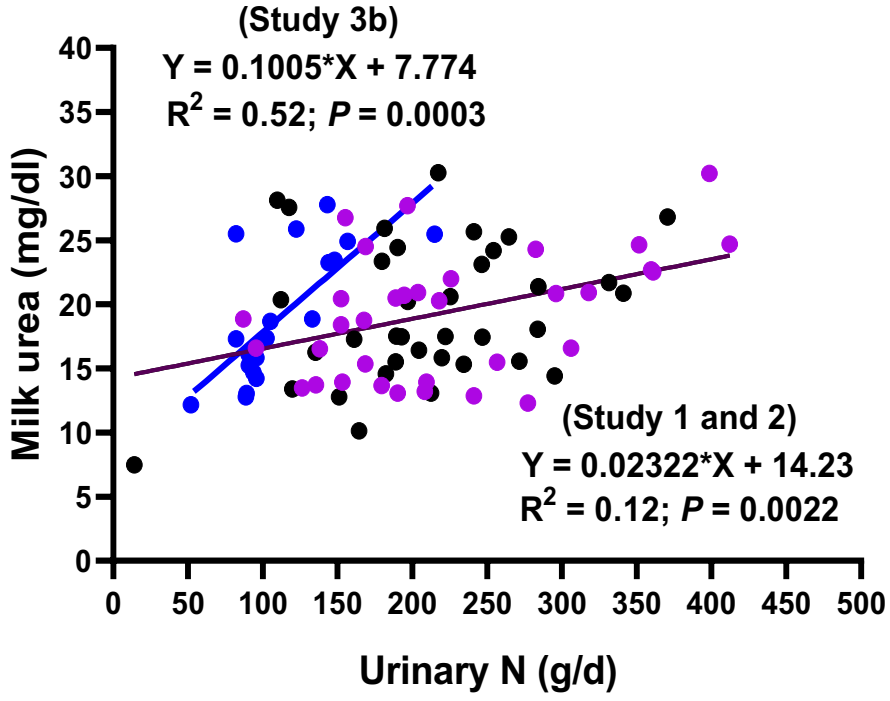
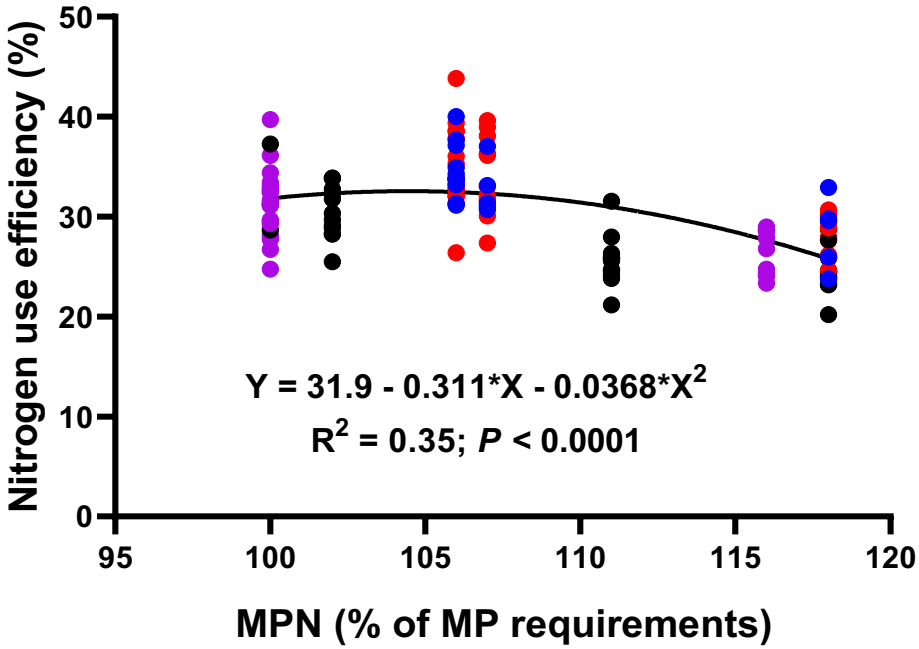


Figure 8.4. Relationship between urinary nitrogen (N) excretion (g/d) and milk urea concentration (mg/dl) of dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3b (●), n = 20. The urinary N output was calculated by difference in Study 1 and 2, and measured in Study 3b.

a)



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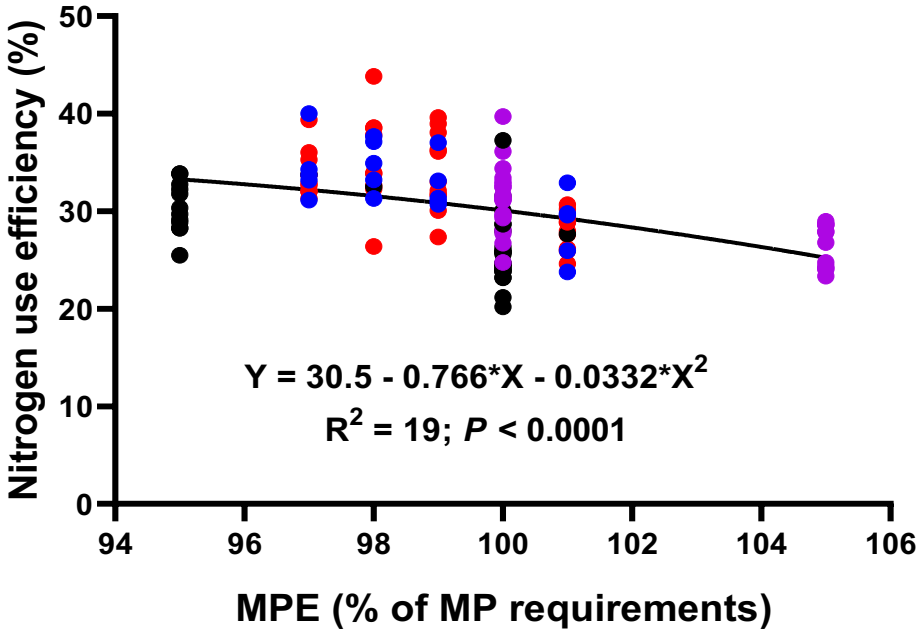

Figure 8.5. Relationship between nitrogen (N) use efficiency (%) and metabolisable protein (MP) supply as % of requirements when limiting of either rumen N (MPN, a) or rumen energy (MPE, b) in dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3a (●), n = 41. For Study 3b (●), n = 20.

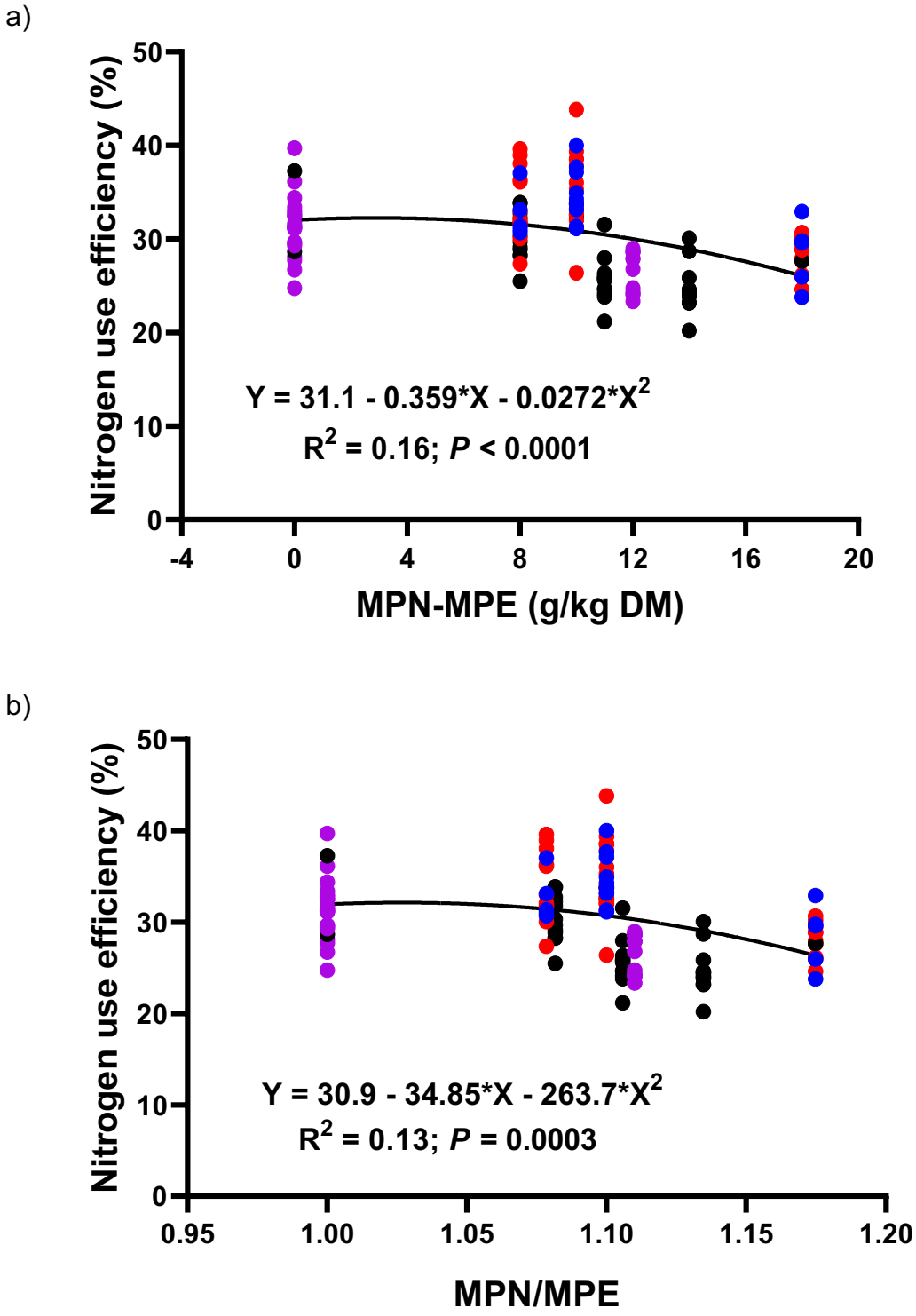

Figure 8.6. Relationship between nitrogen (N) use efficiency (%) and the balance of metabolisable protein (MP) supply when rumen N (MPN) or rumen energy (MPE) was limited (a), or the ratio of both MPN and MPE in dairy cows. For Study 1 (●), n = 36. For Study 2 (●), n = 34. For Study 3a (●), n = 41. For Study 3b (●), n = 20.

8.2. Challenges, limitation and future prospects

In the current studies, all diets were formulated based on homegrown legume silages. In general, legumes are degraded rapidly in the rumen which causes difficulty to maintain the desired MP supply in high-yielding dairy cows. In the forage dry matter, legumes (red clover or lucerne) and basal (grass or maize silage) forages were included at equal (50:50) DM ratio, but different inclusion levels of legumes had not been assessed in this project work. In Study 3ab, barley and synthetic methionine was used as dietary source of starch and rumen-protected methionine in low protein diets. Therefore, different sources and inclusion level of dietary starch, and other limiting amino acids including lysine and histidine could be supplied in low protein diets to evaluate the effect of low protein diets in lactating diary cows. The current research work was done with the high yielding early lactating dairy cows but not considered the full lactation period or late lactation cows. Additionally, in Study 3b, urine bag was attached with a barrel via hoose pipe in the metabolism unit, which was found to be more difficult to collect fresh urine for some cows. Another important challenge is to complete the final study (Study 3b) and laboratory work due to incidence of COVID-19 pandemic and campus shutdown.

It is clear from the findings of this thesis work that the dietary concentration of CP had an inconsistent effect on DM intake, which may influence lactation performance of dairy cows. Additionally, the varying effect of low CP diets that are based on red clover and grass silage on apparent nutrient digestibility in lactating cows are unclear and contradictory with published studies, although most of the studies were based on lucerne instead of red clover silage. Therefore, further studies are required to investigate the effect of different levels of dietary CP or MP on intake, milk performance and metabolism of dairy cows fed red clover and grass silage-based diets. A number of performance studies have been conducted with different proportions of legume forages but these have mainly been based on high protein (> 160 g CP/kg DM) diets. Consequently, further studies are required to investigate the effect of different inclusion levels of home-grown legume silages, including peas and beans, in low CP diets on the performance and metabolism of Holstein-Friesian dairy cows. Further investigation on the dietary supplementation of different levels of starch or limiting AA to offset the negative effect of CP or MP deficient diets on performance and metabolism of cows are also required.

8.3. Financial implications

Reducing the dietary protein content of the concentrates from 174 to 153 g/kg DM in Study 1 (L vs. H) reduced concentrate costs by £16/tonne and purchased feed costs by approximately 36p/cow/d, or 1 ppl (Table 7.1). Concentrate costs were £5/tonne and purchased feed costs 3p/cow/d lower in M compared to H.

Table 8.1. Difference in purchased feed costs[1] in dairy cows fed diets based on red clover/grass silage or lucerne/maize silage in Study 1 and 2.

Item	Study 1			Study 2		
	H	M	L	H50	L50	L60
Milk yield (kg/d)	35.0	34.7	34.6	40.9	39.8	38.9
Purchased feed costs						
£/tonne	213	208	197	248	233	232
Feed rate kg/cow/d	13.4	13.6	12.6	14.0	13.0	13.5
£/cow/d	2.85	2.82	2.49	3.09	2.68	2.76
p/kg milk	8.2	8.1	7.2	7.6	6.7	7.1
Reduction in purchased feed cost compared to control (H or H50) (p/cow/d)	0	3	36	0	41	33

[1]Diet cost was calculated during the study period: October 2018 to April 2019 for Study 1 and Study 2.
Study 1 based on red clover and grass silage; H = high (175 g CP/kg DM), M = medium (165 g CP/kg DM) and L = low (150 g CP/kg DM) CP diets.
Study 2 based on lucerne and maize silage; H50 = 175 g CP/kg DM with 50:50 lucerne to maize silage, L50 = 150 g CP/kg DM with 50:50 lucerne to maize silage, and L60 = 150 g CP/kg DM with 60:40 lucerne to maize silage.

In Study 2, reducing the dietary protein content of the concentrates from 172 to 150 g/kg DM reduced concentrate costs by £15/tonne and purchased feed costs by approximately 41p/cow/d, or 1 ppl. Concentrate costs were £16/tonne less and purchased feed costs 33 p/cow/d, or 0.5ppl lower in L60 compared to H50.

Concentrate costs in Study 3ab were highest in LM and lowest in LS, mainly due to the additional cost of the RPM, and the lower cost of barley compared to molassed sugarbeet pulp or soyhulls (Table 7.2). The increased cost of the RP soybean and rapeseed meals that were required to maintain the MP supply in the low protein diets partly compensated for the reduced rate of use protein concentrates. Feed costs per litre of milk were approximately 0.17 p/l higher in LM than C, with the lowest feed cost in cows fed LS, which was 0.63 p/l lower than in those receiving C. When expressed on a p/l fat corrected milk yield basis however, the highest concentrate costs were recorded in cows fed C, with those receiving LP or LS being 0.42 and 0.99 p/l lower respectively.

Table 8.2. Purchased feed costs[1] in dairy cows fed low protein diets based on red clover and grass silage in Study 3a.

Item	Diet			
	C	LP	LS	LM
Milk yield (kg/d)	38.2	36.5	37.8	37.1
Fat corrected milk yield (kg/d)	37.1	36.7	38.6	38.2
Fat + protein yield (kg/d)	2.66	2.56	2.73	2.66
Purchased feed costs				
£/tonne	247	239	226	253
Feed rate kg/cow/d	11.8	11.4	11.7	11.5
£/cow/d	2.92	2.73	2.65	2.90
p/kg milk	7.65	7.49	7.02	7.82
p/kg fat corrected milk yield	7.87	7.45	6.88	7.59
p/kg fat+protein yield	110	107	97	109
Reduction in purchased feed cost compared to C (p/cow/d)	--	19	27	2

[1]Diet cost was calculated during the study period: October 2019 to March 2020 for Study 3a.
C = Control (175g CP/kg DM); LP = low protein (150g CP/kg DM); LS = low protein (150g CP/kg DM) with added starch; LM = low protein (150g CP/kg DM) with added rumen-protected methionine.

8.4. Conclusions

Dietary concentration of CP was reduced from 175 to 150 g/kg DM in the current studies to assess feed intake, milk yield, milk composition, body weight and consition score, diet digestibility, N use efficiency, milk fatty acids and blood metabolites in high yielding dairy cows fed red clover or lucerne based mixed rations. The overall conclusion of this thesis has been listed below with clear direction for the UK dairy sector in relation to implanting low protein diets and the use of homegrown legumes.

1. Dietary concentration of CP could be reduced at 150 g/kg DM when the diets were based on either red clover and grass silage or lucerne and maize silage at a forage DM ratio of 1:1.
2. The inclusion rate of legume silage should not be exceeded 60% of the forage DM in low protein diets.
3. Rumen by pass protein could be useful to supply adequate MP when formulating legume-based low protein diets.
4. Feeding low protein diets (150 g CP/kg DM) based on legume silages have no negative effect on DM intake in high-yielding dairy cows unless having a long-term effect on body energy reserves.
5. Similarly, legume-based low protein (150 g CP/kg DM) diets have no negative influence on milk or body performance whilst MP supply is not limiting, however,

up to 60% inclusion of legume silages on the forage DM might reduce the milk yield and milk protein content.

6. Low protein diet (150 g CP/kg DM) has the potential to increase N use efficiency by reducing urinary N excretion, milk and plasma urea content in dairy cows.
7. Reducing the CP content of red clover or lucerne silage based diets to 150 g/kg DM whilst maintaining MP supply does not affect diet digestibility or milk fatty acids.
8. Supplementation of dietary starch or rumen-protected methionine has little effect on performance in lactating dairy cows fed low protein diets (150 g CP/kg DM) based on red clover and grass silages.
9. Feeding Holstein-Friesian dairy cows a diet containing 150 g CP/kg DM, based on homegrown red clover and grass or lucerne and maize silage that met MP requirements, might have little influence on performance but improves the apparent NUE for milk production.
10. Future studies investigating low protein diets based on forage legumes should focus on different inclision rate and source of legume-based rations, and must report the dietary effects of RDP, RUP and particularly MP rather than just focus on CP.

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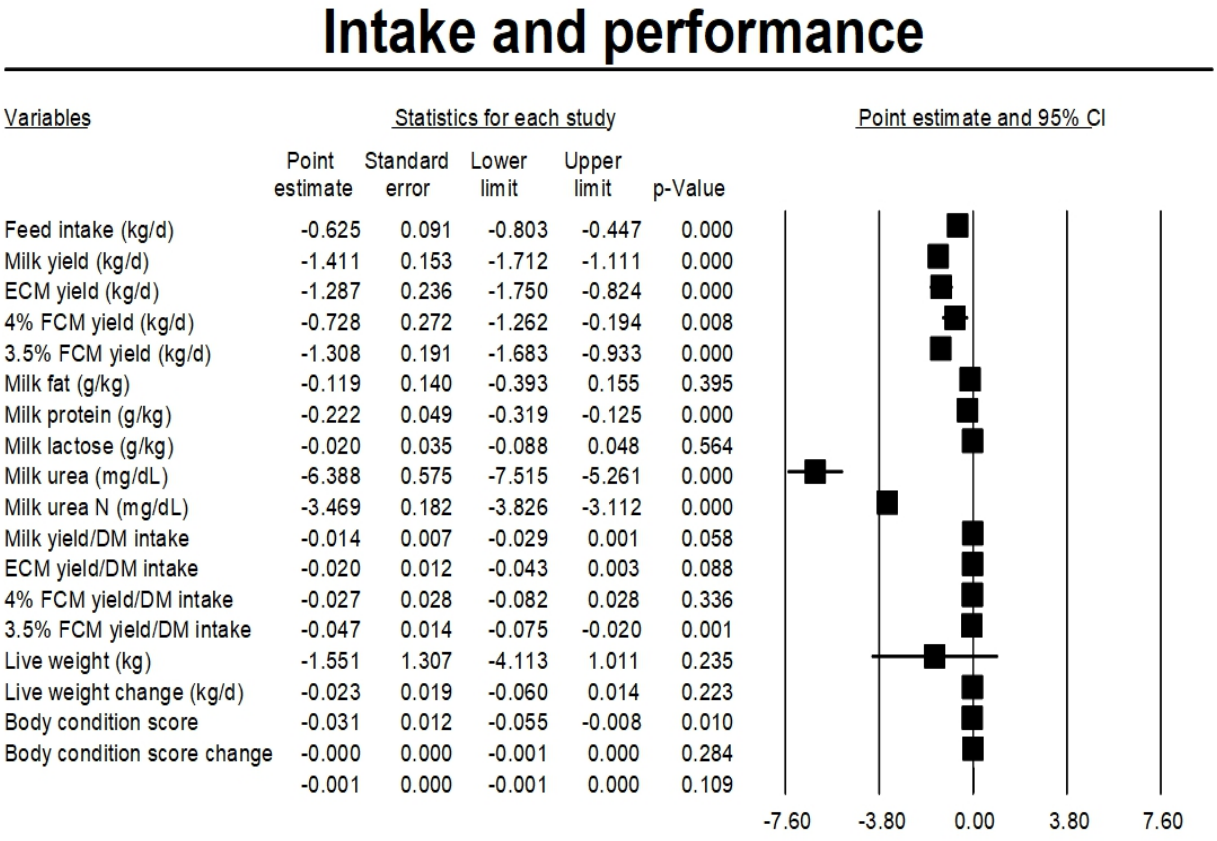
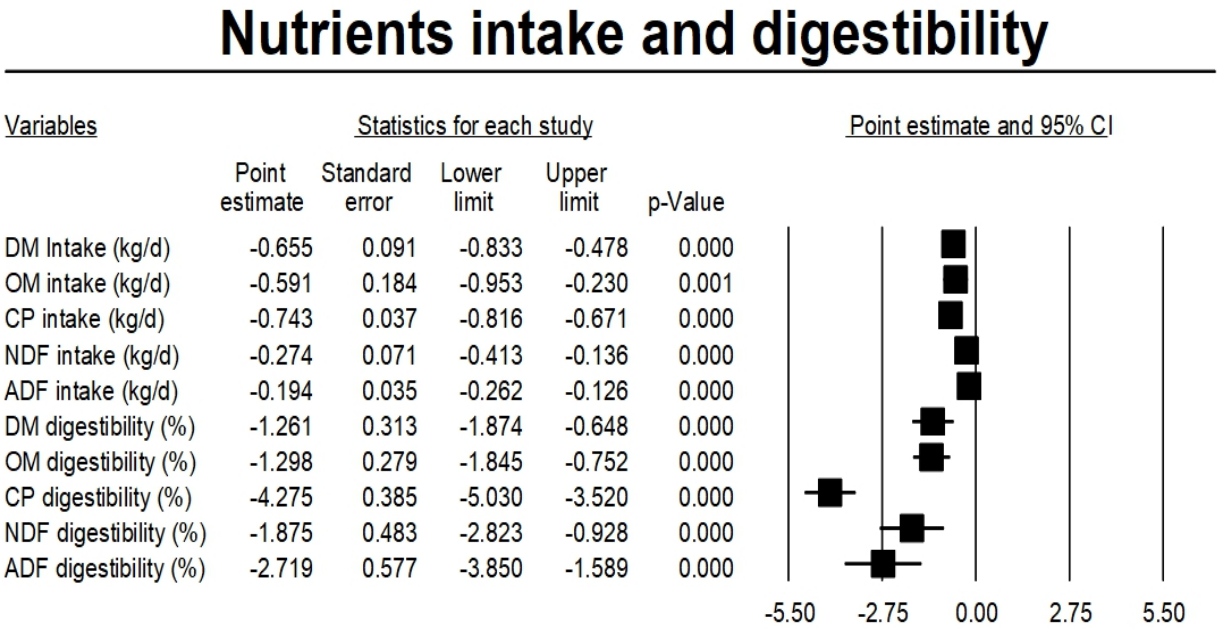
Appendices

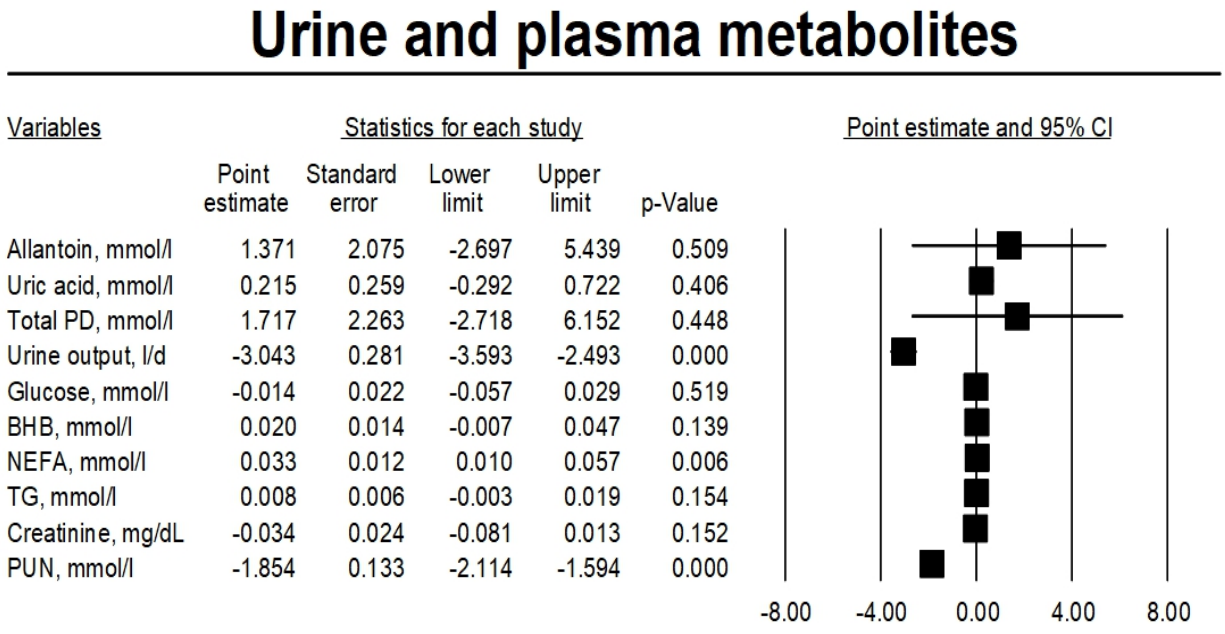
Appendix 7.1. Forest plot of the results from a random-effect meta-analysis for intake, milk performance, live weight and condition of dairy cows fed control and low CP diets based on forage legumes.



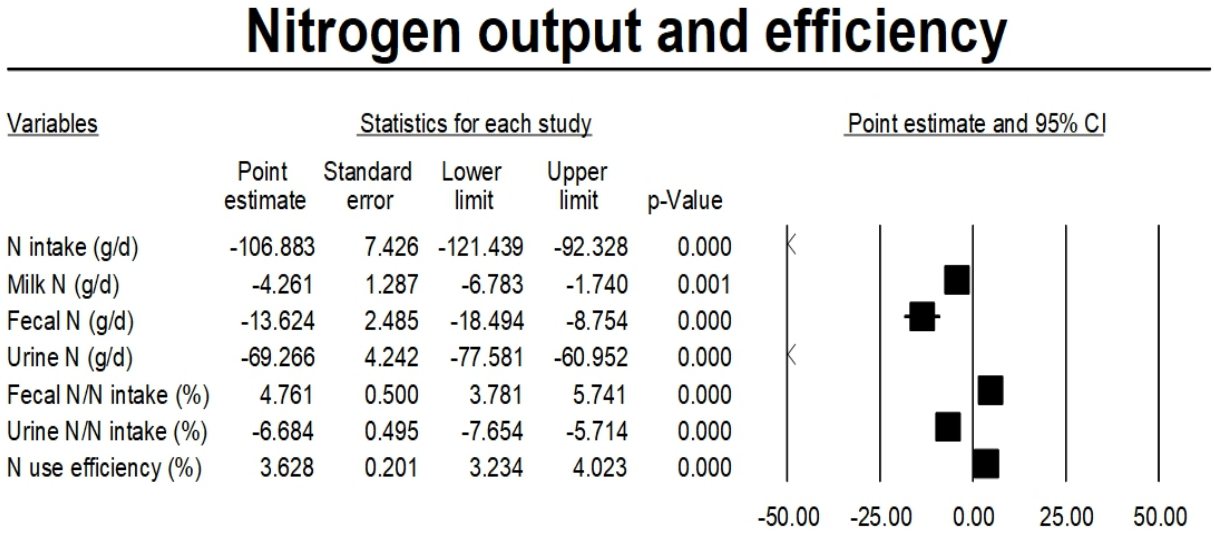
Appendix 7.2. Forest plot of the results from a random-effect meta-analysis for nutrients intake and their apparent total tract digestibility of dairy cows fed control and low CP diets based on forage legumes.



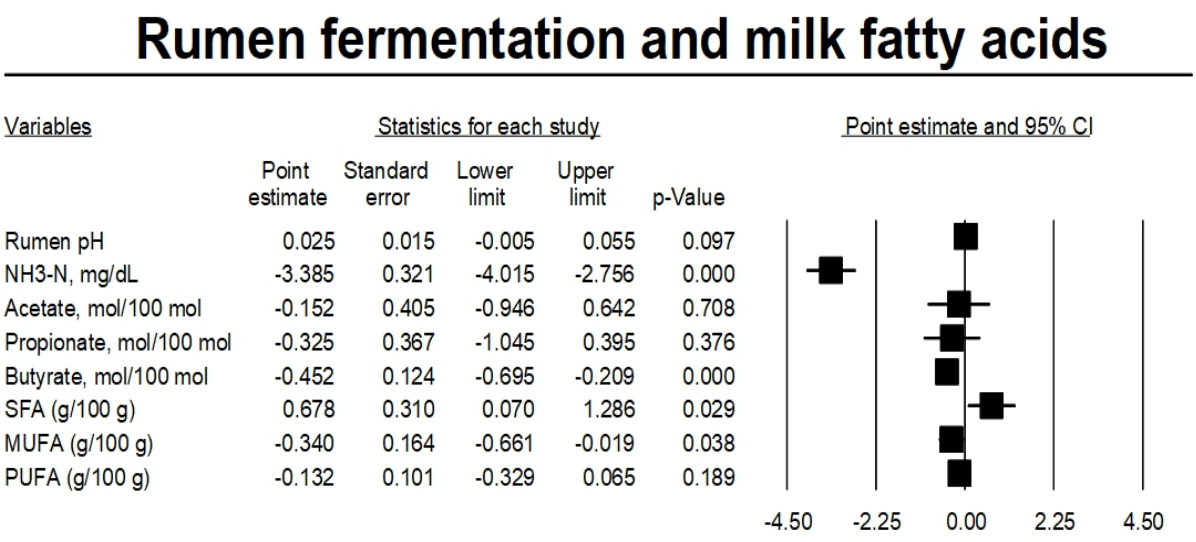
Appendix 7.3. Forest plot of the results from a random-effect meta-analysis for urine and blood plasma metabolites of dairy cows fed control and low CP diets based on forage legumes.



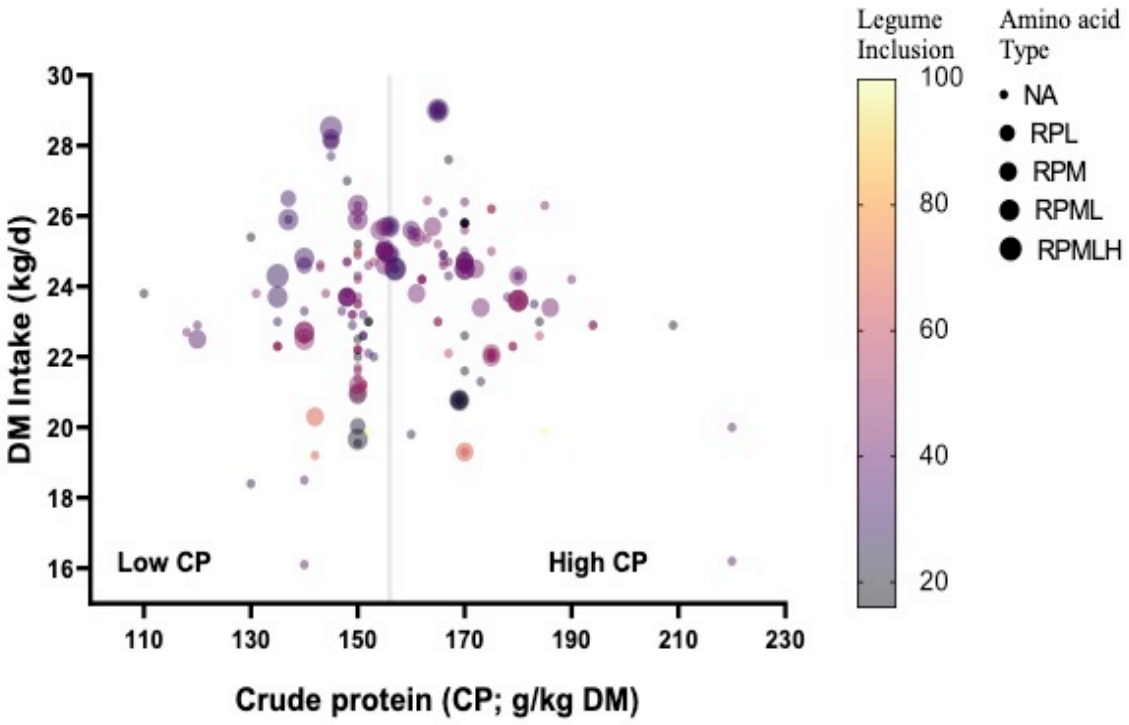
Appendix 7.4. Forest plot of the results from a random-effect meta-analysis for nitrogen intake, output and efficiency of dairy cows fed control and low CP diets based on forage legumes.



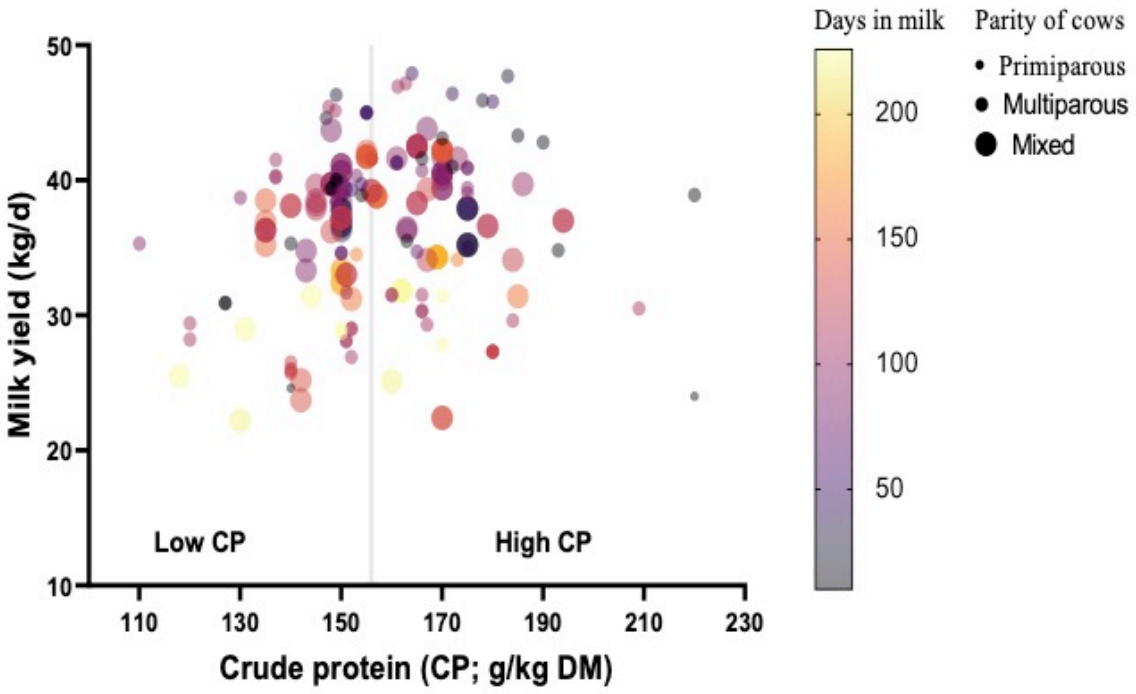
Appendix 7.5. Forest plot of the results from a random-effect meta-analysis for rumen fermentation kinetics and milk fatty acids of dairy cows fed control and low CP diets based on forage legumes.



Appendix 8.1. Effects of dietary crude protein (CP) concentration, legume silage inclusion rate on the forage dry matter (DM) and types of rumen-protected amino acid supplementation on feed intake (g/kg DM) of lactating dairy cows.



Appendix 8.2. Effects of dietary crude protein (CP) concentration, days in milk and parity of cows on milk yield (g/kg DM) of lactating dairy cows.



Appendix 8.3. Effects of dietary crude protein (CP) concentration, legume silage inclusion rate on the forage dry matter (DM) and types of rumen-protected amino acid supplementation on milk protein content (g/kg) in lactating dairy cows.