DIET EFFECT ON NITROGEN PARTITIONING AND ISOTOPIC FRACTIONATION ($\delta^{15}N$) IN RUMINANTS

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ABSTRACT of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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by L. Cheng

The main objective of this PhD study was to investigate the relationship between nitrogen (N) partitioning and isotopic fractionation ($\delta^{15}N$) in ruminants consuming diets with varying levels of N and/or water soluble carbohydrate (WSC). Nitrogen balance studies were conducted for all studies with measuring $\delta^{15}N$ from various N sinks (e.g., milk, plasma, urine and faeces). Concentrations of blood (BUN) or milk urea N (MUN) and urinary excretion of purine derivative (PD) were also measured to provide indications to N metabolism in the whole body and rumen levels. Treatment effects were tested using ANOVA; the relationships between measured variables were analysed by linear regression analysis.

Studies of lactating cows (Chapter 4) and lactating goats (Chapter 5) explored the manipulation of WSC/N effect on N-use efficiency (NUE; milk N output/N intake), whilst studies of non-lactating sheep (Chapter 3) and lactating goats (Chapter 5) explored the manipulation of WSC/N effect on urinary N output proportion to N intake (UN/NI) and urinary N output proportion to faecal N output (UN/FN).

The increase in WSC/N increased NUE of lactating cows by 56 % through increasing energy intake and milk N output (MN), and decreasing N intake. On the other hand, the increase in NUE and MN were not shown in lactating goats study. It is believed the lack of response was mainly due to the high dietary N concentration and very high dietary WSC concentration in conjunction with unchanged DMI, compared with lactating cows study. No change in MCP was detected in lactating goats study; but a possibility of rumen acidosis resulted from excessive WSC intake should not be excluded. No change in

UN/NI was observed from both the non-lactating sheep and lactating goats studies. Little dietary effect on UN/NI in lactating goats may be explained by similar reasons (i.e., high dietary N and WSC concentration and fixed DMI) led to lack of N partitioning response to dietary treatment. Calculated microbial crude protein production per unit of fermentable ME (MCP/FME) and PD analysis suggested no improvement in MCP was achieved in non-lactating sheep study. No change in UN/FN was observed in both lactating goats and non-lactating sheep studies. The opposite effect from dry matter digestibility (increased) and N digestibility (decreased) resulted in little changes in non-lactating sheep FN when WSC/N increased. On the other hand, lack of changes in MCP synthesis resulted in no changes in UN in lactating goats study.

Plasma, milk, faeces, muscle, wool and liver were enriched in $\delta^{15}N$ compared with feed, while urine was depleted in $\delta^{15}N$ relative to feed. It is suggested that the main reason for the enrichment of $\delta^{15}N$ in faeces is the presence of enriched endogenous material. The majority of N in wool, liver, muscle, milk and plasma exists as true protein, which has been reported to be enriched in $\delta^{15}N$. In contrast, urea is the main N source of urine and is reported to be depleted in $\delta^{15}N$. Regression analysis demonstrated that urine and faecal $\delta^{15}N$ were related to feed $\delta^{15}N$ (Chapter 5), milk $\delta^{15}N$ and plasma $\delta^{15}N$ were related to each other (Chapter 4), and muscle $\delta^{15}N$ - feed $\delta^{15}N$ was related to urine $\delta^{15}N$ - feed $\delta^{15}N$ (Chapter 3). It is believed that these observed relationships related to N isotopic fractionation during digestion and absorption of feed components, such as WSC and N or a possible common effect of the endogenous N contribution to various N sinks from feed N.

A major observation from this PhD project was the linear relationship between N isotopic fractionation and N partitioning from both non-lactating sheep (Chapter 3) and lactating cows studies (Chapter 4). Two plausible sites (i.e., liver and rumen) of N isotopic fractionation that may be related to N partitioning were discussed in Chapter 6. Overall, under the condition of these studies (high dietary N); the relationships between N isotopic fractionation and N partitioning was believed to be mainly driven by deamination or transamination in the liver, rather than MCP synthesis in the rumen.

Results confirmed there is a potential of using N isotopic fractionation as an easy to conduct indicator of N partitioning. However, further research on the accuracy of using N

isotopic fractionation predicting N partitioning at different feeding system (e.g., low vs high N intake); animal variations contribute to changes in N isotopic fractionation; and the contribution from different body parts (e.g., rumen, liver and intestines) are needed.

Keywords: sustainability, nitrogen use efficiency, stable isotope, water soluble carbohydrate, nitrogen metabolism, urea nitrogen, purine derivatives, microbial protein synthesis, rumen

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LIST OF COMMONLY USED TERMS

Blood urea nitrogen	BUN
Crude protein	CP
Dry matter	MD
Dry matter intake	DMI
Dry matter digestibility	DMD
Fermentable metabolisable energy	FME
Faecal nitrogen output	FN
Milk nitrogen output	MN
Microbial protein synthesis	MCP
Metabolisable protein	MP
Milk urea nitrogen	MUN
Metabolisable energy	ME
Nitrogen	N
Nitrogen balance	NB
Nitrogen isotopic fractionation	$\delta^{15}N$
Nitrogen-use efficiency	NUE
Nitrogen intake	NI
Nitrogen digestibility	ND
Rumen degradable protein/nitrogen	RDP/RDN
Retained nitrogen	RN
Urinary excretion of purine derivative	PD
Urinary nitrogen output	UN
Water soluble carbohydrate	WSC

CHAPTER 1

INTRODUCTION

It has been reported that temperate pasture fed to ruminant generally contains a high concentration of nitrogen (N) relative to energy supply (Litherland and Lambert, 2007). This can lead to a low efficiency of incorporating feed N into usable N (e.g., milk, meat); and large outputs of surplus N to the environment (Pacheco and Waghorn, 2008). Any investigation into factors affecting N partitioning, such as dry matter intake, dietary composition, energy/N ratio in feed, physiological status of the animal, individual animal genetic differences, requires a simple, cheap and non-invasive method of determining N partitioning between the various N sinks (e.g., milk, meat, urine and faeces).

The classic approach to establish N partitioning has been an N balance study. However, it is subject to large experimental errors and difficult to conduct with grazing animals (Spanghero and Kowalski, 1997; Cheng et al., 2011). Milk or blood urea N has also been widely used to indicate N partitioning. However, it is susceptible to diurnal variations and limited in its use when dietary N is high (Geerts et al., 2004). Therefore, a novel and simple method is needed to give a good indication of N partitioning from large groups of grazing animals. Wattiaux and Reed (1995) suggested that N isotopic fractionation could potentially be used to quantify N partitioning of ruminants. However, there is limited information on the utility of this technique and it is not known how dietary changes affect N isotopic fractionation.

This thesis presents a series of studies which measured the N partitioning of a range of ruminants (lactating goats, lactating cows and non-lactating sheep) offered various levels of energy and N intake; and these data sources were also used to test the value of using N isotopic fractionation as a simple indicator of N partitioning.

The objectives of thesis were:

1. Examine the N partitioning among feed, urine and faeces of non-lactating sheep offered three levels of N to water soluble carbohydrate ratio in the diet, and evaluate the use of N isotopic fractionation as a simple indicator of N partitioning.

2. Evaluate the effects of supplying additional urea as a source of rumen degradable nitrogen on N isotopic fractionation in pasture-fed dairy cows. At the same time, evaluate the usefulness of using N isotopic fractionation to indicate N-use efficiency (milk N output/N intake) of lactating cows.

3. Examine the N partitioning among milk, urine and faeces of lactating goats offered a consistently high N diet with increasing levels of water soluble carbohydrate; and further evaluate the use of N isotopic fractionation as a simple indicator of N partitioning.

CHAPTER 2

LITERATURE REVIEW

2.1. The importance of studying nitrogen metabolism and partitioning in ruminants

Nitrogen (N) is an essential feed component of ruminant's diets and thus farm profitability (Pfeffer and Hristov, 2005). Compared with other animal species, ruminants are unique for having a forestomach (i.e., three compartments – the rumen, reticulum and omasum) before the true stomach (i.e., abomasum). Therefore, they can utilise relatively poor quality feed (e.g., straw) to synthesis usable energy and protein in order to support metabolism and production. On the other hand, N related-substrates that would be better absorbed directly in the small intestine, such as essential amino acids may be converted to ammonia which can cause major environmental concern (Litherland and Lambert, 2007). Thus an understanding of N metabolism and its net effect on N partitioning within the ruminant body is crucial in order to maximise production and minimise environmental pollution. Ruminant nutritionists agree that N metabolism and partitioning within the ruminant can be divided into two components; ruminal N metabolism and whole body N metabolism. These two components of N metabolism and major factors influencing N partitioning are discussed separately.

2.1.1. Nitrogen metabolism in the rumen

Ruminants differ from other animals in that they can use complex carbohydrates in the rumen. Simply speaking, the rumen is a fermenter that turns feed energy into volatile fatty acids (VFAs); which is needed for maintenance and production. Furthermore, microbial protein synthesis in the rumen provides the majority of protein supplied to the small intestine, accounting for 50-80 % of total absorbable protein (Storm and Ørskov, 1983). Energy and protein metabolism in the rumen is the result of metabolic activity of ruminal microorganisms, such as bacteria, fungi and protozoa (Pfeffer and Hristov, 2005).

In the rumen, microbes degrade and ferment carbohydrates and protein to VFAs, methane (CH_4), carbon dioxide (CO_2) and ammonia (NH_3) to support their maintenance and

growth. Figure 2.1.2 shows that crude protein (CP) is degraded by rumen microbes and 70-90 % of CP is rumen degradable protein (RDP). The RDP consists of two fractions: the quickly degradable protein (QDP) and the slowly degradable protein (SDP), according to their rates of degradation in the rumen. Both QDP and SDP can be synthesised given sufficient energy to produce microbial crude protein (MCP). Around 70-80 % of MCP is microbial true protein (MTP), and the rest is microbial non-protein nitrogen (MNPN; mostly in the form of nucleic acids). Nitrogen (i.e., in the form of NH_3) that is not synthesised into MCP builds up in the rumen then overflows into blood stream and finally being converted into urea in the liver. However, some of the urea can be recycled back to the gut in saliva or across the intestinal mucosa for metabolisable protein (MP) production (Pacheco and Waghorn, 2008). Urea recycling acts as a survival mechanism to support ruminant production, particularly when N intake is insufficient compared with the maintenance requirement (Reynolds and Kristensen, 2008). Un-degradable dietary protein (UDP) is also known as "bypass" protein, and it does not contribute to the growth of microbes in the rumen but if digestible it can be absorbed from the small intestine as MP. As a result of this, MP supply is a combination of digestible MTP and digestible UDP.

Ruminant nutritionist believes the availability of RDP and fermentable metabolisable energy (FME; ME less the ME contained in fat and fermentation acids) largely determines the MCP yield or the utilisation of N in the rumen (AFRC, 1993). When the RDP supply is not limiting, the yield of MCP is considered to be dependent on FME supply and *vice versa*. The estimated values for MCP yield ranges from 8-12 g MCP/MJ FME depending on intake level, it increases as the feeding level increases (AFRC, 1993). There is some evidence that MCP yield can be manipulated by changing the synchrony of RDP and FME supply in the rumen. Supplementation with fermentable energy significantly increased MCP flow to the duodenum, and also increased the efficiency of MCP synthesis in sheep (Chamberlain et al., 1993). In comparison, Henning et al. (1993) indicated that synchronisation between RDP and FME release in the rumen did not affect the MCP flow and microbial growth in sheep given wheat-straw based diets. There is a big debate about whether animal itself has ability to cope with dietary imbalance and make the best use of diet to synthesis MCP (Cabrita et al., 2006). It has been reported that temperate pasture contains relatively high ratio of RDP to FME, which generally results in N in excess to ruminal microorganism and animal requirements (Litherland and Lambert, 2007). Therefore, the excessive N in the rumen is absorbed across the rumen wall and converted

into urea in the liver, then excreted in the urine. This excretion of urine with high urea level may cause environmental pollution (Edwards et al., 2007).

2.1.2. Nitrogen metabolism and partitioning in ruminant body

Nitrogen metabolism in the ruminant body is based on the amino acids absorbed from the small intestine as MP to support maintenance and production need of the host animal. Digestible UDP passes through the rumen to abomasums and is absorbed into bloodstream through the small intestine wall; contributing to MP production together with digestible MTP. The utilisation of MP by the animal is depending on two major factors, namely MP-use efficiency (kn) and MP supply. AFRC (1993) suggested the kn values for growth, lactation and pregnancy are 0.59, 0.68 and 0.85 respectively which is mainly driven by the amino acid profiles of the MP. When MP supply from the feed is excess of requirement of the animal (maintenance + production; as determined by genetic potential and ME for production), excess MP has to be detoxified in the liver and excreted in the urine mainly as urea. This detoxification process of excess N come from rumen ammonia or MP is associated with an energy cost (30 KJ ME/g of N) in converting excess N to urea in the liver (Tyrrell et al., 1970).

The result of N metabolism is N being partitioned to different body sinks (i.e., fetus, muscle, wool, milk, faece and urine). It has been recognised that product N (i.e., fetus, muscle, wool and milk) is derived from MP; the more MP supplied, and the more usable product can be produced given sufficient energy available (Fox et al., 2004). Faecal N consists of undigested N from UDP, MTP and endogenous N. Urinary N can cause environmental pollution through nitrate leaching and green house gas (e.g., ammonia and nitrous oxide) emission (Edwards et al., 2007). Urinary N is mainly generated by liver detoxification of un-captured ammonia from microbial protein catabolism in the rumen and also the excessive MP that cannot be used for metabolism in the body. Eisemann et al. (1989) suggested that the protein synthesis in the liver accounts for 9-13 % of whole body protein synthesis in sheep and cattle. In temperate pasture feeding system, the facts of relatively high level of N to available ME in the feed is the major cause of high urinary N excretion. A pasture feeding based trial indicated when the effective CP degradability in the rumen increased (from 0.55 to 0.85), the proportion of effective RDP in excess as the total protein in excess to animal needs also increased dramatically (from 4 to 90 %; Rusdi

and van Houtert, 1997). Furthermore, Pacheco and Waghorn (2008) reported that the dietary N requirements (% of DM) for grazing animals are about 1.8, 2.2 and 3.0 % for maintenance, growing and lactation respectively. When dietary N concentration matches animal requirements, it is no longer limiting for production and further N intake will not benefit performance and simply excreted in urine.

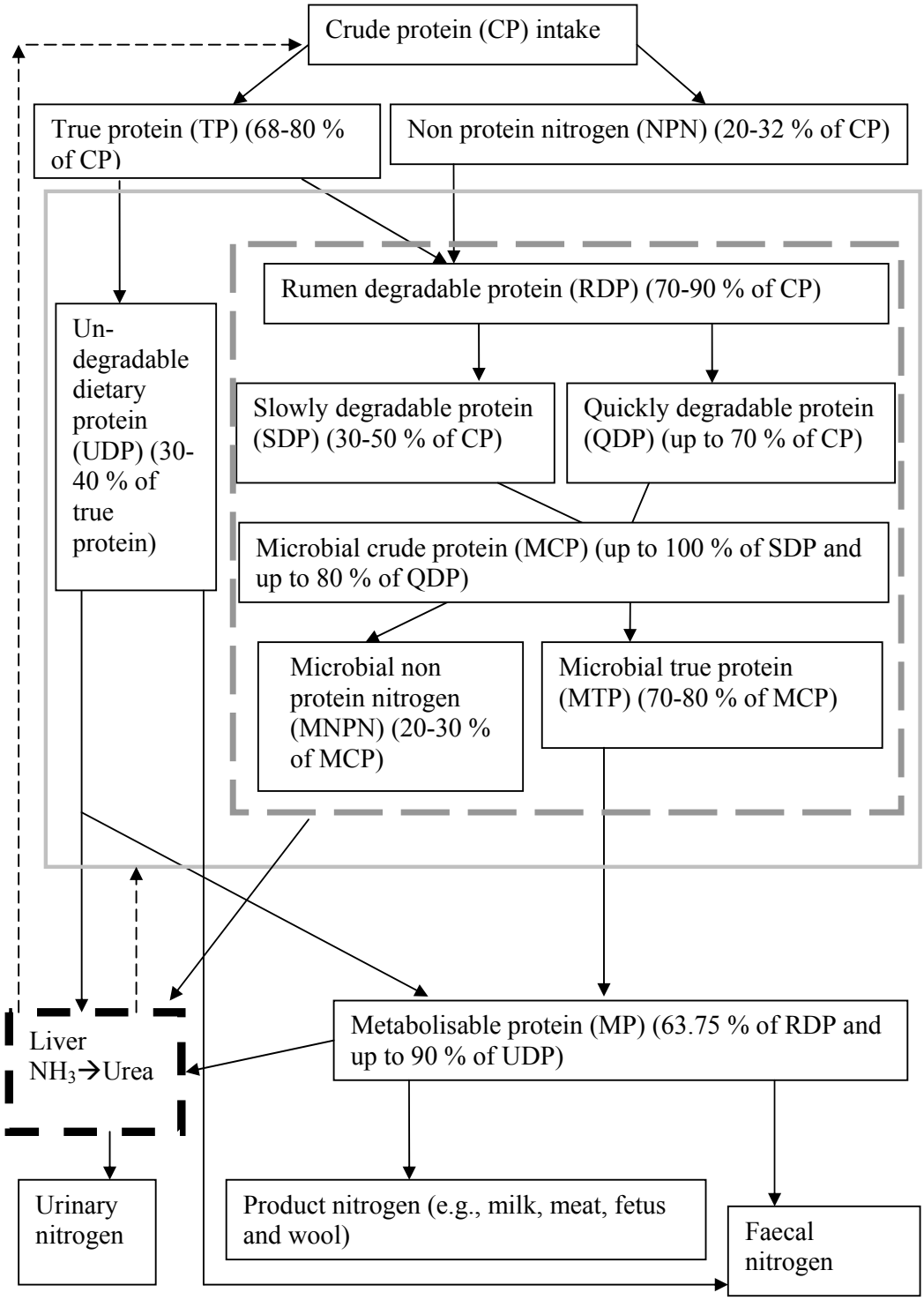

Figure 2.1.2. Flow chart of the metabolisable protein system (base on AFRC, 1993 and Pacheco and Waghorn, 2008). (Intestine N absorption: grey solid box; Ruminal N utilisation: grey broken box; Liver N metabolism: broken black box; Urea recycling: black broken arrow)

2.1.3. Major factors influencing nitrogen partitioning

Nitrogen partitioning is the distribution of N among feed, urine, faeces, milk, body gain, fetus and wool. There are number of factors which influence N partitioning, they are both dietary and animal based and will be discussed here.

Dietary effects

AFRC (1993) suggested differences in protein degradability affect the amount of N which is available for digestion and absorption in the small intestine, thus influencing MP supply to the body and N partitioning (Rusdi and van Houtert, 1997). Figure 2.1.2 shows the level of RDP from fresh pasture is between 70 to 90 % of CP, which is high compared with the animal requirement (60-70 % of CP; Brookes and Nicol, 2007). Other forage based product such as Lucerne pellet/cube is produced through dehydration process, which has a slightly reduced RDP level (between 65 to 70 % of CP; Mustafa et al., 2001) compared with fresh pasture, it better matches animal's requirement. However, it is important to note that high RDP intake is generally associated with high total N intake in pasture based feeding system and they are the major factors lead to change in N partitioning. Castillo et al. (2000) illustrated that as N intake increased regardless of ME intake, the proportion of dietary N converted into milk or faecal N declined, but that into urine increased (Figure 2.1.3). Quantitatively, it has been reported that increasing total N intake by a factor of four increased N excretion in faeces and milk by similar proportions, but urinary N excretion increased over 12 times (Hof et al., 1994). Cheng et al. (2011) illustrated that when N intake increased from 359 g/day to 626 g/day across nine dietary treatments, urinary N output and faecal N output increased linearly as N intake increased. A N intake of 410 g/day was recommended as the maximum for dairy cow before excessive urinary N output could be a potential cause of environmental pollution. Low N-use efficiency (NUE; milk N output/N intake) in grazing dairy cows is mainly due to the supply of N exceeding the supply of ME available to the animal. Furthermore, Dewhurst et al. (2003a) emphasised that dietary difference, particularly in N concentration of the feed, is the main source of variation in N partitioning by dairy cows.

It has been documented that the synchronisation of the available N and ME is crucial to both rumen function and N partitioning. Altering the feeding frequency or the feeding patterns was proposed to manipulate rumen function and N partitioning of dairy cows (Oldham, 1984). Diurnal changes in grass WSC concentrations mean that concentrations are higher in the afternoon than in the morning, when the plant has had time for photosynthesis. Orr et al. (2001) showed positive milk N output benefits from allocating fresh grass after afternoon milking, rather than after morning milking, using a strip grazing system. Although DMI was not different between animals offered fresh grass morning or afternoon, milk yield was 5 % higher from animals offered fresh grass after afternoon milking, probably as a result of increased DM % and WSC concentration of the grass. Similarly, Bryant et al. (2010) showed feed cows on longer regrowth pasture with afternoon allocation reduced urinary N concentration and increased milk N output, which potentially has its positive effect on reducing environmental pollution. However, Henning et al. (1991) in an *in vitro* study looked at synchrony effect on rumen function. They found synchrony decreased rumen ammonia concentration but no effect on microbial protein synthesis and microbial growth. Cabrita et al. (2006) reviewed the effects of synchronisation by changing dietary ingredients, feeding pattern or feeding frequency on dairy cows. They concluded that the evidence of synchronising N and ME supply to the rumen benefits on animal production is contradictory, both for efficient microbial protein synthesis and for maximising milk production.

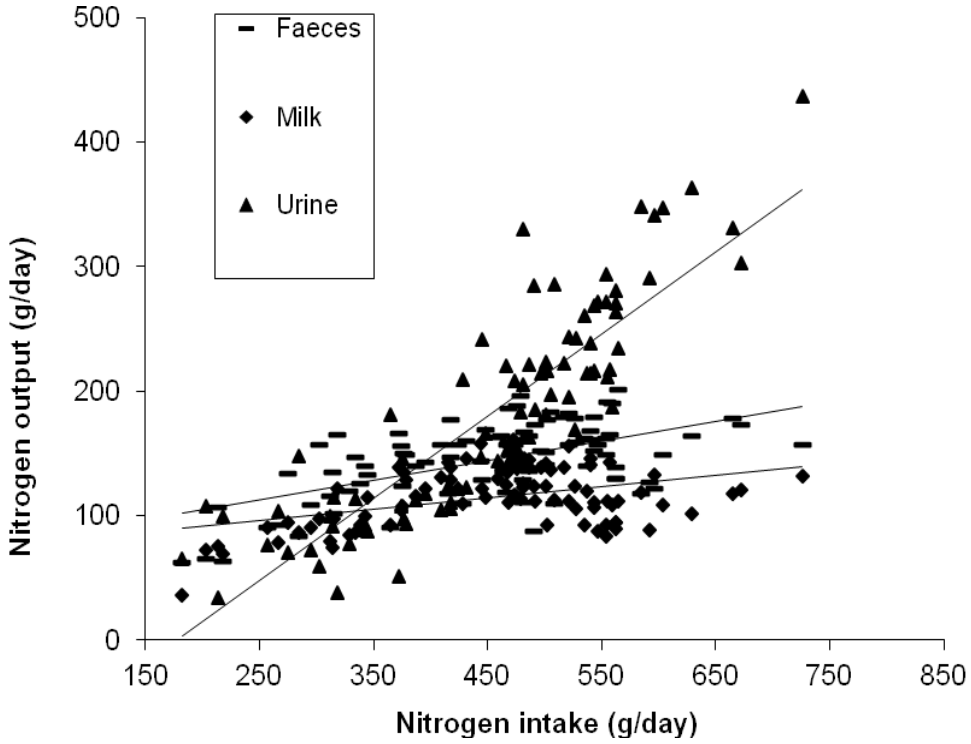

Figure 2.1.3. The relationship between total nitrogen intake and nitrogen excretion in milk, urine and faeces of lactating cows (Castillo et al., 2000)

Animal effects

Animal variation in N partitioning has long been recognised. Animal age, breed, lactation status, as well as health condition all contribute to utilisation of feed N. Dillon et al. (2003; 2006) observed the variation in milk production from four different European dairy cow breeds that were fed on seasonal grass based system and suggested that milk yield, feed intake and energy balance are heritable traits. Berry et al. (2003) used 8591 multiparous Holstein-Friesian cows to establish the genetic relationships among body condition score (BSC), body weight, milk yield, and fertility in dairy cows. The results from the study indicated that it is possible to select animals with increased milk production and NUE without any adverse effects on fertility or average BSC. Results with high genetic merit dairy cows (over 10000 kg milk/lactation) showed the efficiency of N utilisation and specifically the excretion of N in urine; were higher and lower respectively compared with average yielding herds respectively (Castillo et al., 2000). A recent pasture

fed experiment showed cows with higher genetic merit converted feed N into milk N more efficiently and also increased its N partition from urine to faeces (Woodward et al., 2011). Genotype of animal affects NUE by determining the partitioning of N among maintenance, milk production and other metabolic functions in the body. The animal variations reported may relate to the difference in eating pattern, digestibility, tissue metabolism and nutrient absorption.

2.2. Current methods for measuring and predicting nitrogen partitioning

Nitrogen balance study is a direct approach to measure N partitioning, but it is subject to large experimental errors and difficult to be conducted in grazing animals. Urea N is widely used to indicate N partitioning. However, it is susceptible to diurnal variations and limited in its use when dietary N is high.

2.2.1. Nitrogen balance

The best known technique for measuring N partitioning is a N balance (NB) study. It has traditionally been used all over the world in order to investigate N metabolism in human and animals. It is also used in ruminant to evaluate feedstuffs and study how to reduce N excretion. A complete NB involves measuring the partitioning of total N intake into N outputs in faeces, milk, urine, weight change, fetus (quantitatively important only in the last two months of lactation or after the sixth month of gestation) and scurf and dermal (DSN) losses. Kristensen et al. (1998) explained that most of the time, DSN losses are ignored, since they are difficult to collect and relatively small when compare with other losses. The NB is a quantitative analysis that is used to define ruminant N requirements, at the same time considers environmental problems in relation to ruminant production (MacRae et al., 1993).

There are four limitations encountered with the NB method. Firstly, to complete a NB study in ruminant requires a large amount of labour. A total recording of the N intake, and collection of the N outputs in faeces, milk, urine, weight change, fetus and DSN losses must be carried out over a week. This is almost impossible in a grazing situation. Secondly, throughout the procedures from faeces collection to measurement of N content, incomplete collection of material, volatile losses of ammonia and final drying of the samples can contribute significantly to the underestimation of faecal N output. Spanghero and Kowalski (1997) pointed out that dried faecal samples have a significantly lower N concentration than the wet samples, regardless they were dried in an oven or a freeze-drier. Thirdly, volatile N losses from urine collections can also add to the underestimation of N outputs. Lastly, the actual feed wastage is often higher than measured in both indoor and grazing systems, which results in an overestimation of N intake. Spanghero and Kowalski (1997) reviewed NB calculations from 125 different diets, to address the

experimental errors (i.e., underestimation of the N outputs and the overestimation of the N intake) of the NB technique. Their review showed that on average an early lactating dairy cow retained almost 40 g N/day, which converted to a total lean tissue gain of around 1000 g/d (by adopting a coefficient of 6.25, assuming a body protein content of 16 % and a ratio of 1 part protein: 3 parts water in the body). This estimation appears to be too high, especially for high yielding dairy cows in their early lactation, which are likely to be losing weight, rather than gaining. The results from conventional NB measurements were compared with slaughter measurements (SM) in growing lambs; it showed a net protein overestimation of about 24 % using NB in comparison with SM (MacRae et al., 1993). In New Zealand context, the grazing based ruminant industry is ideally to develop methods that can indicate N partitioning on large number of animals or herds. The methods should be easy to use, requiring as few samples as possible and minimal labour for analysis, so this rules out NB, although it is a useful method to calibrate against.

2.2.2. Urea nitrogen

Ammonia in the body originates from ruminal ammonia not incorporated into MCP (Section 2.1.1) and from the metabolism of amino acids in various organs and tissues (Pacheco and Waghorn, 2008). In the ruminant, urea is synthesised from ammonia in the liver and then enters circulatory system through hepatic sinuses, which drains into the hepatic vein and become part of the pool of blood urea before it is filtered by the kidneys and excreted in urine. Because of the constant urea filtration rate in the kidneys, the total volume of blood that is cleared by the kidneys for detoxification remains similar, thus urinary urea (major form of urinary N) excretion is expected to be proportional to blood urea N concentration (BUN) (Hof et al., 1997). Urea equilibrates in aqueous solutions through diffusion; therefore, milk urea N concentration (MUN) is highly correlated with BUN. As a result of this correlation, MUN and BUN are proposed to be good indicators of changes in N metabolism and N partitioning within the ruminant body. However, because it is more practical and less invasive to obtain milk for wide scale testing than blood; MUN is becoming a convenient management tool for monitoring protein status and urinary N output (UN) (Broderick and Clayton 1997; Jonker et al., 1998).

Milk urea N was found to be correlated with rumen ammonia concentration, which in turn is affected by dietary changes in N and ME (Section 2.1.1). Increased N intake was

reported to be related to increased MUN (Tas, 2006), but Oltner et al. (1985) and Broderick and Clayton (1997) suggested that MUN is more related to N and ME ratio (N/ME) and N concentrations rather than absolute N intake. Furthermore, Nousiainen et al. (2004) used RDP concentration in excess of predicted requirement as a single predictor of MUN, but it did not explain more of the variation in MUN than dietary N. Use and interpretation of MUN in relation to nutritional management is not easy, because some studies showed a positive relationship between MUN and milk production (Oltner et al., 1985), while others demonstrated a negative relationship or no relationship (Carroll et al., 1988; Baker et al., 1995). Despite this controversy, Frank and Swensson (2002) demonstrated that cows offered diets which resulted in highest NUE (up to about 43 %) had the lowest concentrations of MUN. Jonker et al. (1998) recommended producers should aim for a concentration between 100 to 160 mg/l. Bulk tank values above 160 mg/l indicate that either dietary N concentration is too high, rumen FME supply is too low, or a combination of the two. Bulk tank values below 100 mg/l indicate that N concentration is too low, the balance of RDP and UDP is incorrect, or rumen FME supply is too high.

The variations in measured MUN may be due to dietary factors including N intake, ME intake, types of N and energy from diet; animal factors including parity, milk production, days in milk and body weight; and other factors such as water intake and methodologies used for measuring. Reynolds and Kristensen (2008) suggested when animals are fed on high level of CP, the possible reason for MUN not proving to be a consistent indicator of N status was because MUN production reaches a maximum level when dietary CP concentration exceeds 20 %. Gustafsson and Palmquist (1993) reported that a lower proportion of total urea excretion in milk was observed in the morning sampling than afternoon sampling which is supported by Broderick and Clayton (1997). This is inconsistent with the finding of Cheng et al. (2010), possibly due to variation in feed composition at the different periods of the day. Regardless the diurnal variations in MUN concentration, the afternoon MUN was found to be well correlated with morning MUN (Geerts et al., 2004; Cheng et al., 2010). Subsequently, Broderick (2003) recommended that for evaluation of a herd's feeding program, samples should be collected at the similar time of the day to eliminate the diurnal variations. Furthermore, De Campeneere et al. (2006) mentioned that the consumption of water by dairy cows may contribute to the variation in concentration of urea in the body fluids.

Broderick (2003) compared different methods for analysing MUN (i.e., infrared scanning vs colorimetric essay vs urease hydrolysis). He concluded that there was a big difference in results and warned researchers to be consistent with analytical methods. Overall, Schepers and Meijer (1998) suggested that the fundamental problem in using MUN is that it provides no detail about actual rumen processes; it is only a reflector of overall N metabolism. In general, a high MUN indicates high N losses in the N metabolism and hence a low efficiency in N utilisation.

2.2.3. Prediction of urinary nitrogen output

Urinary N output (UN) is associated with a risk of environmental pollution (Pacheco and Waghorn, 2008). In general, UN contributes to nitrate leaching and greenhouse gas emissions, which may cause ground water contamination and global warming respectively (Di and Cameron, 2002). The formation of UN has been discussed in Section 2.2.2, by assuming constant blood urea clearance rate in a group of animal; UN prediction equations have been established using MUN and urinary excretion of creatinine (Broderick et al., 2003; Pacheco et al., 2007). Furthermore, more complex prediction equations using dietary factors (e.g., dry matter intake (DMI) and crude protein (CP) intake) and animal factors (e.g., milk yield and body weight) have been established by Fox et al. (2004) based on Cornell net carbohydrate and protein system. However, Carroll et al. (1988) and Tas (2006) found the relationship between MUN and UN was poor, because MUN is useful in indicating N loss from rumen fermentation but not absorbed N utilisation. In addition, Jonker et al. (1998) suggested the majority of unexplained prediction error of MUN is due to animal variation (e.g., blood urea clearance rate). Recent publications showed large variation in predicting UN from varies published equations in dairy cows with genetic difference and dietary difference (Cheng et al., 2012a and 2012b).

2.3. Use of nitrogen isotopic fractionation to indicate nitrogen partitioning

Current methods for measuring and predicting N partitioning (Section 2.2) either require large labour inputs or do not provide consistent results. Therefore, it is important to develop simpler approaches to evaluate N partitioning, based on sampling that could be conducted without the need to house animals in metabolism stalls. An alternative approach proposed is to characterise N partitioning based on N isotopic fractionation ($\delta^{15}N$).

2.3.1. The principle of nitrogen isotopic fractionation

Wattiaux and Reed (1995) stated that in a natural environment, ^{14}N and ^{15}N, known as stable isotopes, are the two components of air N. The ^{15}N content of atmospheric N is 0.3663 %, and the rest of N is ^{14}N (99.6337 %). The ^{15}N abundance is measured as described by Yoneyama et al. (1983) and expressed as $\delta^{15}N$ value by deviation relative to atmospheric N_2:

$$\delta^{15}N\ (‰) = [(^{15}N\ /^{14}N)\ \text{sample} - (^{15}N\ /^{14}N)\ \text{air}] / [^{15}N\ /^{14}N]\ \text{air} \times 1000$$

$\delta^{15}N$ is not an absolute measurement; it is only a relative measurement (i.e., proportion). A negative $\delta^{15}N$ value indicates depletion in ^{15}N and a positive $\delta^{15}N$ value indicates enrichment in ^{15}N, relative to the natural abundance of the atmospheric N.

The tiny mass difference between ^{15}N and ^{14}N cause the isotopes to behave differently in both physical and chemical reactions. In general, the lighter isotope (^{14}N) tends to form weaker bonds and to react faster than the heavier isotope (^{15}N). Macko et al. (1987) suggested as the reaction (Glutamic acid + Oxalacetic acid = α-Ketoglutaric acid + Aspartic acid) progressed, glutamic acid in the origin became enriched in $\delta^{15}N$ relative to aspartic acid in the product, due to the deposition of stronger bonded/slower reacting ^{15}N in the glutamic acid. As a consequence of these bond energy and reaction rate differences, the abundance of ^{15}N and ^{14}N vary between chemical processes. Therefore, over the last few decades, $\delta^{15}N$ has been widely used for studying plant and soil interaction (Wade et al., 1975); ecology (Hobson et al., 1993); human physiology (O'Connell and Hedges, 1999) and more recently, mammalian nutrition and N metabolism (Parker et al., 1995;

MacAvoy et al., 2005). Arguably this tiny mass difference (‰ or per thousands) may not be precisely measured to quantify the level of fractionation from various body organs. However, isotope ratio mass spectrometer is generally used for measuring $\delta^{15}N$ values of the sample. For a well homogenised sample, the reproducibility for $\delta^{15}N$ measurement is high and the standard deviation is within ±0.2 ‰. In most cases, either the depletion or enrichment of the measured biological samples compared with its feed is more than 0.5 ‰, which should provide sufficient level of fractionation for mass spectrometer to detect. Sample preparation is considered as one of the most important steps to ensure obtaining a representative tiny sample. The suitability of using dried or liquid sample is also to be determined prior to conduct each experiment.

2.3.2. Nitrogen isotopic fractionation in animal body

DeNiro and Epstein (1980) investigated the influence of diet on the distribution of N isotopes in animals (fly, snail, mouse, shrimp, nematode and weevil) grown in the laboratory. They discovered that the N isotopic composition of the diet was reflected in the N isotopic composition of an animal body parts. They also pointed out that in most cases; it is more practical to measure the $\delta^{15}N$ of a part of an animal rather than its whole body. Honbson and Clark (1992) published similar findings that $\delta^{15}N$ was elevated in body tissues compared with that in the diet, and it varied among tissues and biological compounds.

In general, the explanation for these differences in natural abundance of $\delta^{15}N$ in animal body is the kinetic isotope fractionation during biochemical reactions in the animal body (Macko et al., 1987). Studies with Ross' geese indicated that a primary source of N isotopic fractionation occurs during processes of deamination and transamination of amino acids. This resulted in amino acid enrichment during anabolism, and N waste products depleted in ^{15}N relative to diet. Sick et al. (1997) found as N intake increased (above minimum requirement intake for optimum growth), plasma protein and urea were more enriched and less depleted in $\delta^{15}N$ respectively compared with feed. It is believed that changes in $\delta^{15}N$ values in animal tissues could offer important information about N metabolism (Hobson et al., 1993). Moreover, Balter et al. (2006) suggested for a given body mass and diet ^{15}N, the isotopic fractionation is mainly controlled by the dietary N

intake and composition. Consequently, $\delta^{15}N$ was proposed to be used as a simple indicator of N partitioning of ruminants (Cheng et al., 2011).

2.3.3. Nitrogen isotopic fractionation in ruminant body

In general, faeces, milk and plasma are enriched in $\delta^{15}N$ compared with feed (Koyama et al., 1985). On the other hand, urine is depleted in $\delta^{15}N$ compared with feed (Sutoh et al., 1987; Figure 2.3.3). The majority of N exists as true protein in milk and plasma, which has been reported to be enriched in $\delta^{15}N$ (Cheng et al., 2010). Sponheimer et al. (2003) suggested that the main reason for the enrichment of $\delta^{15}N$ in faeces is the presence of enriched endogenous N. In contrast, urea is the main N source of urine and is reported to be heavily depleted in $\delta^{15}N$ (Steele and Daniel, 1978).

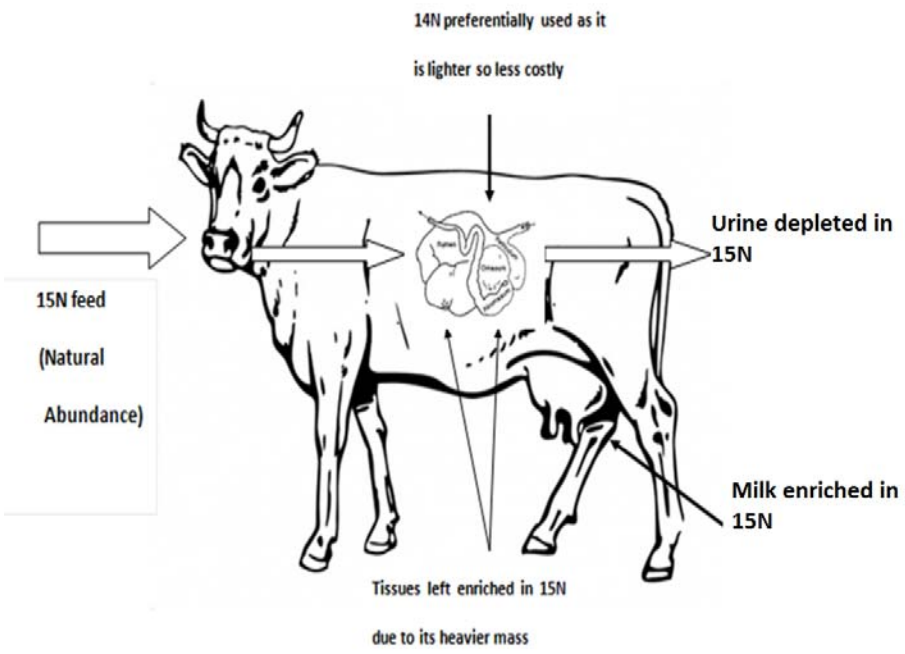

Figure 2.3.3. Nitrogen isotopic fractionation in ruminant body

The protein (from precursor amino acids) and urea (amino N mainly incorporated into urea) synthesised in the ruminant liver mainly lead to the separation of usable protein (e.g., milk, meat and wool) and waste N (e.g., urinary N) in terms of their $\delta^{15}N$ (Koyama et al., 1985; Sponheimer et al., 2003). As a result of synthesis, the ^{15}N content relative to

precursor amino acids was depleted in the urea, whilst it was enriched in the protein (Steele and Daniel, 1978; Cheng et al., 2010). It is believed that due to deamination and transamination processes in the liver; the more protein is synthesised relative to urea (e.g., higher milk N output/urinary N output), the less the protein is enriched while urea under these conditions is depleted more compared with the diet (Macko et al., 1986; Parker et al., 1995). Sponheimer et al. (2003) provided preliminary evidence of increased enrichment of ^{15}N in hair protein when ruminants were fed high protein diets. The situation is likely to be more complex in ruminants, since synthesis of protein by rumen micro-organisms is an additional potential site of isotopic fractionation (Sutoh et al., 1993). Dietary protein is synthesised into microbial protein in the rumen by the microbes, at the same time ammonia is produced. Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein resulted in a depletion of ^{15}N in microbial protein, which was not observed when microbial protein was synthesised from amino acids. In contrast, little is known about how UDP fractionates in the body and its impact on MP ^{15}N value.

2.3.4. Nitrogen isotopic fractionation in relation to nitrogen partitioning

According to Cheng et al. (2011), there are two major plausible sites (liver and rumen) of N isotopic fractionation that may be related to N partitioning in ruminant body; through major biochemical reactions, namely deamination/transamination and microbial protein synthesis. Plasma N pool is the combination of plasma amino acid pool and plasma urea pool. They are the precursors of body protein pool (e.g., milk nitrogen, muscle nitrogen) and urinary nitrogen pool respectively. Liver is a major site for plasma N metabolism. When transamination/deamination processes take place for plasma N in the liver, lighter mass ^{14}N reacts faster compared with heavier mass ^{15}N, therefore ^{14}N became abundant in plasma urea pool (i.e., ^{15}N depleted) and later the urinary nitrogen. On the other hand, when more protein is derived from amino acids pool in the liver (e.g., high in NUE) through deamination/transamination processes; the more similar is the isotope pattern of the protein to that of the precursor amino acids (i.e., less ^{15}N enrichment in the protein; Sick et al., 1997). Microbial protein synthesised in the rumen from ammonia results in a depletion of ^{15}N in microbial protein (Wattiaux and Reed, 1995). This implies that when ruminant is fed on high level of N (excess maintenance requirement), ammonia forms microbial protein which is depleted in ^{15}N and also the major source of milk protein.

Therefore a depleted ^{15}N in milk protein in turn is expected from increasing microbial protein synthesis.

CHAPTER 3

THE EFFECT OF DIETARY NITROGEN TO WATER SOLUBLE CARBOHYDRATE RATIO ON NITROGEN PARTITIONING AND ISOTOPIC FRACTIONATION OF NON-LACTATING SHEEP

3.1. Introduction

Ruminant production systems are based on grazing temperate pasture which generally provides nitrogen (N) in excess of animal requirements (Litherland and Lambert, 2007). This leads to a low efficiency of incorporating feed N into milk or meat N, and outputs of surplus N (mainly urine) to the environment (Di and Cameron, 2002). It has been proposed that production and N partitioning (i.e., the distribution of feed N to urine, faeces, milk and retains in the body) can be manipulated through changing the N to water soluble carbohydrate (WSC) ratio in the diet (Miller et al., 2001). More N is expected to be partitioned to milk and faeces rather than urine with a decrease of N/WSC. However, a meta-data analysis and an *in vitro* study demonstrated that there may be an optimum concentration of N and WSC that will optimise rumen fermentation and animal production regardless of changes in N/WSC (Pacheco et al., 2007; Burke et al., 2011).

The most widespread method used to measure N partitioning is an N balance study (NB; MacRae et al., 1993). The utilisation of N can be indicated by urea N concentration in blood or milk (Broderick and Clayton, 1997). However, NB is difficult to conduct, particularly with large numbers of grazing animals (MacRae et al., 1993) and urea N is subject to substantial diurnal variation (Gustafsson and Palmquist, 1993). Earlier studies identified that fractionation of N isotopes occurs during digestion and metabolism, notably during utilisation of N by ruminal bacteria fermenting carbohydrates (Wattiaux and Reed, 1995) and metabolism of amino acids in the liver (Sick et al., 1997). Therefore, Cheng et al. (2011) suggested that N isotopic fractionation could potentially be used to quantify N partitioning of ruminants. The first objective of this study was to examine the N partitioning of non-lactating sheep offered three levels of N/WSC in the diet, which should exclude milk as an extra N sink for N partitioning and fractionation. The second

objective was to evaluate the use of N isotopic fractionation as a simple indicator of N partitioning.

3.2. Material and methods

Six non-lactating sheep aged between 1.5 to 2 years old and weighing 65.0 (SD = 3.9), 61.8 (SD = 4.4) and 62.5 (SD = 3.5) kg at the start of the three collection periods were used. Three dietary treatments (N/WSC) were evaluated in a 3 × 3 Latin square design with three periods of 3 weeks (2 weeks dietary adaptation and 1 week animal measurement in each period). Two sheep were allocated to each treatment in each period.

Diets

All sheep grazed on ryegrass/white clover based pasture prior to the study. Lucerne pellets and straw were gradually introduced to the sheep 10 days before the study commenced. Four days prior to period 1, sheep were consuming a 100 % lucerne pellet diet and housed in metabolism crates individually. Throughout the study, fresh water was available *ad libitum.*

The diets were designed to provide three levels of N/WSC (Low, Medium and High), and expected to generate variations in both N partitioning and isotopic fractionation. The diets were produced by adding sucrose and urea to lucerne pellets (Winslow Ltd; New Zealand) (Table 3.1). Additional WSC and N were provided by sprinkling white cane sugar (Chelsea; New Zealand; 1700 KJ/100g) or spraying liquid urea (Biolab; Australia; 46.7 % N) onto the pellets. Due to the variation of chemical composition of purchased lucerne pellet, each batch of pellet was analysed for N and WSC concentrations pre experiment. The amount of sucrose and urea that were needed to achieve three levels of dietary treatments was calculated based on analytical results.

Animal measurements

This study was undertaken at the Johnstone Memorial Laboratory, Lincoln University, New Zealand, under the authority of Lincoln University Animal Ethics Committee (approval No. 339). Sheep were housed separately in metabolism crates with feed offered

once a day at 10.00 am. One sheep in period 2 (on the Medium N/WSC treatment) had to be taken out from the study due to metabolic disorders, at the start of period 2 adaptation. All sheep were weighed on the day before and after each NB period.

Each sheep was offered 1.5 kg fresh weight (aimed to deliver 14 MJME intake/day/head with 50-100 g of LWG/d) of pellets daily and feed refusals were recorded to calculate DMI. Feed samples from each treatment were collected for seven days. Faeces and urine output were measured and sampled during days 2 - 7 of each NB period (last six days). A plastic bucket with a layer of metal mesh was placed under the drainage channel of each metabolism crate to allow urine to drain through the mesh and faeces stay on top of the mesh. To keep urine pH between 2 and 3, 250 ml of sulphuric acid (10 % vol/vol) was added into each bucket daily prior to collection.

Blood samples were collected from the jugular vein into 10 ml Li-heparinised evacuated tubes at 11.30 am on days 3 and 6 of each NB period. Plasma was immediately harvested by centrifugation at 4 °C at 1200 ×g for 15 min. Gluteal muscle and liver biopsy samples (0.5 g) were taken from each animal at 9.00 am on the last day of each period (Miller-Graber et al., 1991). A 30 cm × 30 cm patch of wool from each sheep was shaved off at the beginning of period 1. The regrowth of wool from this patch was harvested 14 days after each NB period (Rogers and Schlink, 2010). All samples were stored at -20 °C and pooled per sheep per period prior to chemical analysis. Retained N (RN) was calculated using the equation of [RN (g/d) = N intake (g/d) - faecal N output (g/d) - urinary N output (g/d)].

Analytical methods

All samples were freeze dried and analysed for $\delta^{15}N$ follow the procedure described by Cheng et al. (2011). *In vitro* digestibility of organic matter on DM basis (DOMD) was measured follow the procedure described by Clarke et al. (1982). The ME content of feed was calculated using the equation of [MJME/kg DM = DOMD (g/kg DM) × 0.016]. DM digestibility was calculated using the equation of [DM digestibility (%) = 1 – (faecal output (g/d) / (DMI (g/d)) × 100]. The apparent N digestibility was calculated using the equation of [N digestibility (%) = 1 – (faecal N output (g/d) / N intake (g/d)) × 100]. The concentration of WSC in feed samples was analysed following the method described by

Rasmussen et al. (2007). Feed, urine and faeces were analysed for N concentration using a Variomax CN Analyser (Elementar Analysensysteme GmbH, Hanau, Germany). Blood urea N was analysed using an enzymatic kinetic method on a COBAS MIRA clinical analyser (Roche Diagnostics, Switzerland). Urinary excretion of purine derivative (PD) was analysed follow the method described by George et al. (2006) using high-performance liquid chromatography (Agilent 1100 series, Germany).

Statistical analysis

The Genstat statistical package (Version 13.3; VSN International Ltd) was used for ANOVA and linear regression analysis. All measured data were combined into single mean values for each animal per period. One sheep had metabolic disorders in period 2, was taken out from the study and treated as missing value in the statistical analysis. The significance of linear and quadratic effects was determined using ANOVA with N/WSC as treatment factor and period + sheep as block. P-value less than 0.05 was considered as statistically significant.

3.3. Results

Feed intake and composition

Table 3.1 shows DMI was not affected by treatment and ranged between 1.27 and 1.35 kg/d when N/WSC (g/g) ranged between 0.11 to 0.27. Both feed N concentration (% of DM) and apparent N digestibility (% of N) increased, while apparent DM digestibility (DMD) (%) and feed WSC concentration (% of DM) decreased with increased N/WSC. Feed ME ranged between 10.4 and 11.5 MJ/kg DM.

Nitrogen partitioning, blood urea nitrogen and purine derivative

Nitrogen intake (NI) increased from 30.9 to 42.3 g/d as N/WSC increased (Table 3.2). Dietary manipulation had a significant effect on urinary output (UO, kg/d), urinary N output (UN, g/d), faecal N concentration (%) and BUN (mM/l), but not on urinary N concentration (%), faecal output (g DM/d), faecal N output (FN, g/d) and PD (mM/d) (Table 3.2). On average, RN was 0.16 g/d; the range was -1.7 to 2.1 g/d. Both the proportion of UN per unit of NI and FN were not significantly different among dietary treatments. However, a significant treatment effect was detected for FN/NI ($P < 0.01$) (Table 3. 2).

Table 3.1. Feed intake and diet composition for the three diets varying in formulated N/WSC[1]

	Low	Medium	High	N	SED	L[2]	Q[3]
ME, MJ/kg DM	11.5	10.6	10.4	17	-	-	-
WSC concentration, % of DM	22.7	13.2	12.2	17	-	-	-
N concentration, % of DM	2.44	2.76	3.25	17	-	-	-
N/ME, g/MJ	2.12	2.60	3.12	17	-	-	-
N/WSC, g/g	0.11	0.22	0.27	17	-	-	-
DMI, kg/d	1.27	1.35	1.30	17	0.054	NS	NS
ME intake, MJ/d	14.6	14.4	13.5	17	0.58	NS	NS
Apparent DM digestibility, % of DM	61.6	60.8	57.8	17	0.91	**	NS
Apparent N digestibility, % of N	56.1	63.6	64.6	17	1.90	**	NS

[1] Diets based on: Low (0.11g N/1g WSC); Medium (0.22g N/1g WSC); High (0.27g N/1g WSC)

[2] Linear effects

[3] Quadratic effects

**$P < 0.01$; NS = non-significant

Table 3. 2. Nitrogen partitioning, blood urea N and urinary excretion of purine derivatives for sheep offered one of three diets varying in N/WSC[1]

	Low	Medium	High	N	SED	L[2]	Q[3]
N intake, g/d	30.9	37.4	42.3	17	1.55	***	NS
Urinary N output, g/d	18.9	21.7	27.2	17	1.06	***	NS
Urine output, kg/d	1.48	1.76	1.98	17	0.106	**	NS
Urinary N concentration, %	1.30	1.24	1.42	17	0.080	NS	NS
Faecal N output, g/d	13.7	13.6	15.0	17	1.09	NS	NS
Faecal output, g/d	488	531	549	17	30.1	NS	NS
Faecal N concentration, % of DM	2.78	2.56	2.71	17	0.088	NS	*
Retained N, g/d	-1.7	2.1	0.1	17	1.44	NS	*
Urinary N/faecal N, g/g	1.47	1.59	1.86	17	0.216	NS	NS
Urinary N/N intake, g/g	0.62	0.58	0.65	17	0.057	NS	NS
Faecal N/N intake, g/g	0.44	0.36	0.35	17	0.019	**	NS
Retained N/N intake, g/g	-0.06	0.06	0.00	17	0.042	NS	*
BUN, mM/L	6.60	7.95	9.24	17	0.410	***	NS
Urinary purine derivatives, mM/d	32.8	32.4	33.0	17	2.63	NS	NS

[1] Diets based on: Low (0.11g N/1g WSC); Medium (0.22g N/1g WSC); High (0.27g N/1g WSC)

[2] Linear effects

[3] Quadratic effects

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = non-significant

Nitrogen isotopic fractionation and its relationship with nitrogen partitioning

Dietary treatments had no significant effects on N isotopic fractionation apart from a quadratic effect that was detected in muscle $\delta^{15}N$ - feed $\delta^{15}N$ (Tables 3.3 and 3.4). Wool, muscle, liver, plasma and faeces were enriched in ^{15}N compared with feed; and urine was depleted in ^{15}N relative to feed (Table 3.3). Significant linear relationships were found between: muscle $\delta^{15}N$ - feed $\delta^{15}N$ and urine $\delta^{15}N$ - feed $\delta^{15}N$ ([Equation 1]); urine $\delta^{15}N$ - feed $\delta^{15}N$ and UN/NI ([Equation 2]); muscle $\delta^{15}N$ - feed $\delta^{15}N$ and RN/NI ([Equation 3]); and plasma $\delta^{15}N$ - feed $\delta^{15}N$ and RN/NI ([Equation 4]) by regression analysis.

Muscle $\delta^{15}N$ - feed $\delta^{15}N$ (‰) = 0.81 × (urine $\delta^{15}N$ - feed $\delta^{15}N$) (‰) + 6.92 [1]
(n = 17; r^2 = 0.44; *P* = 0.002; SE = 0.54)

Urine $\delta^{15}N$ - feed $\delta^{15}N$ (‰) = 4.03 × UN/NI (g/g) - 3.94 [2]
(n = 17; r^2 = 0.43; *P* = 0.002; SE = 0.17)

Muscle $\delta^{15}N$ - feed $\delta^{15}N$ (‰) = - 4.30 × RN/NI (g/g) + 5.73 [3]
(n = 17; r^2 = 0.43; *P* = 0.003; SE = 0.55).

Plasma $\delta^{15}N$ - feed $\delta^{15}N$ (‰) = - 1.92 × RN/NI (g/g) + 5.64 [4]
(n = 17; r^2 = 0.20; *P* = 0.04; SE = 0.39).

Table 3.3. N isotopic fractionation (‰) in sheep offered one of three diets varying in N/WSC[1]

	Low	Medium	High	n	SED	L[2]	Q[3]
Feed $\delta^{15}N$	0.00	0.13	0.01	17	0.077	NS	NS
Urine $\delta^{15}N$	-1.46	-1.36	-1.39	17	0.297	NS	NS
Faecal $\delta^{15}N$	3.47	3.04	3.13	17	0.274	NS	NS
Wool $\delta^{15}N$	3.94	3.78	3.91	17	0.385	NS	NS
Plasma $\delta^{15}N$	5.62	5.76	5.69	17	0.067	NS	NS
Liver $\delta^{15}N$	5.81	5.89	6.00	17	0.115	NS	NS
Muscle $\delta^{15}N$	5.87	5.54	5.86	17	0.205	NS	NS

[1] Diets based on: Low (0.11g N/1g WSC); Medium (0.22g N/1g WSC); High (0.27g N/1g WSC)

[2] Linear effects

[3] Quadratic effects

NS = non-significant

Table 3.4. N isotopic fractionation (‰) of different body N sinks for sheep offered one of three diets varying in N/WSC[1]

	Low	Medium	High	n	SED	L[2]	Q[3]
Urine $\delta^{15}N$ - feed $\delta^{15}N$	-1.46	-1.49	-1.40	17	0.288	NS	NS
Faecal $\delta^{15}N$ - feed $\delta^{15}N$	3.47	2.91	3.12	17	0.295	NS	NS
Plasma $\delta^{15}N$ - feed $\delta^{15}N$	5.62	5.63	5.68	17	0.094	NS	NS
Liver $\delta^{15}N$ - feed $\delta^{15}N$	5.81	5.76	6.04	17	0.137	NS	NS
Wool $\delta^{15}N$ - feed $\delta^{15}N$	3.95	3.65	3.90	17	0.430	NS	NS
Muscle $\delta^{15}N$ - feed $\delta^{15}N$	5.87	5.41	5.85	17	0.202	NS	*
Plasma $\delta^{15}N$ - urine $\delta^{15}N$	7.08	7.12	7.08	17	0.275	NS	NS
Liver $\delta^{15}N$ - urine $\delta^{15}N$	7.27	7.30	7.41	17	0.201	NS	NS
Plasma $\delta^{15}N$/urine $\delta^{15}N$	-4.75	-4.52	-4.95	17	0.934	NS	NS
Plasma $\delta^{15}N$ /faecal $\delta^{15}N$	1.72	1.97	1.85	17	0.144	NS	NS
Faecal $\delta^{15}N$/urine $\delta^{15}N$	-2.75	-2.32	-2.73	17	0.464	NS	NS

[1] Diets based on: Low (0.11g N/1g WSC); Medium (0.22g N/1g WSC); High (0.27g N/1g WSC)

[2] Linear effects

[3] Quadratic effects

*$P < 0.05$; NS = non-significant

3.4. Discussions

Nitrogen metabolism

A similar amount of DM was consumed by the sheep at all three levels of N/WSC, which eliminated variation in DMI as a factor influencing the dietary effect on N partitioning (Ellis et al., 2011). The changes in N partitioning and microbial protein synthesis are thus expected to be solely a result of the different diet composition. The proportion of NI excreted in urine (UN/NI) in the current study (0.58 to 0.65 g/g) was comparatively higher than that reported (55 %) by Brand et al. (1992). The dietary effect on UN/NI contrasted with Edwards et al. (2007) who concluded that a reduction in UN/NI of dairy cows should be expected when N/WSC (g/g) decreased from 0.27 to 0.12. It is reasoned that the low fermentable metabolisable energy (FME) relative to high ruminal degradable protein limited microbial protein synthesis in the rumen on current diets and resulted in a lack of treatment effect on N partitioning (van Vuuren et al., 1993). This argument is supported by PD results (Table 3.2). Dietary treatments had little effect on PD in the study (Table 3.2), which implies no differences in rumen microbial protein synthesis (Dewhurst et al., 1996; Cheng et al., 2011). Furthermore, the calculation of the yield of MCP per MJ of FME based on AFRC (1993); MCP/FME (g/MJ) = $7 + 6 \times [1 - e^{(-0.35L)}]$ suggests an average 9.15 g of MCP was synthesised from per MJ of FME (SED = 0.072; $P > 0.05$) irrespective of N/WSC ratio in the diets.

Partitioning surplus N away from urine to faeces may contribute to reduced nitrate leaching to the ground water, and ammonia volatilization to atmosphere as this is primarily related to urine (Varel et al., 1999). Similar to results reported from a study with dairy cows (Moorby et al., 2006). The current study showed UN/FN decreased by around 27 % when WSC was added to the diet (Table 3.2). This change of N excretion was achieved through decreasing NI, UN and UO rather than increasing rumen microbial protein synthesis as Miller et al. (2001) concluded. The minor change in FN was the net effect of opposite effects on apparent DM digestibility (decreased) and N digestibility (increased; Table 3.1 and 3. 2).

An increase in UN (g/d) and UO (kg/d) by 44 and 34 %, combined with no change in urinary N concentration (%) was observed as NI increased from treatment Low to High.

Calculations based on Brookes and Nicol (2007) showed the average NI of sheep was at least 1.8 times the maintenance N requirement (assuming 50 % of CP is metabolisable protein and that there was no significant RN). In general, UO is a function of dietary minerals (e.g., Na and K), with higher mineral intakes leading to higher UO (Nennich et al., 2006). Urea is a waste product of N metabolism; sheep excreted excessive N through higher UO for urea detoxification purpose (Cocimano and Leng, 1967).

Blood urea N (BUN) is used as an indicator of N utilisation in ruminants (Kohn et al., 2005). In this study, mean BUN increased significantly ($P < 0.001$) as N/WSC increased across the treatments. The results were within the range previously reported (Cocimano and Leng, 1967; Kohn et al., 2005). However, contrary to the findings of Oltner and Wiktorsson (1985), urea N concentration was not closely related to the ratio of dietary energy to protein. Regression analysis indicated only 19 % ($P < 0.05$) of the treatment (N/WSC) variation explained by changes in individual BUN. Moreover, the linear relationship between BUN and UN/NI was significant, but weak ($r^2 = 0.26$; $P < 0.05$). This poor relationship may be due to the diurnal variation of urea in the body (Broderick and Clayton, 1997; Cheng et al., 2010).

Nitrogen isotopic fractionation and its relationship with nitrogen partitioning

Nitrogen isotopic fractionation between different N sinks in the body is normally expressed by subtracting feed $\delta^{15}N$, as this is usually a function of dietary composition (Hobson et al., 1993; Sutoh et al., 1993). The average feed $\delta^{15}N$ in current study was 0.05 ‰ (Table 3.3), which is similar to the value reported for lucerne (0.00 ‰) by Steele et al. (1983). Faeces, wool, plasma, muscle and liver were on average enriched in $\delta^{15}N$ by 3.17, 3.83, 5.64, 5.71 and 7.33 ‰ compared with feed, and urine was depleted in $\delta^{15}N$ by 1.45 ‰ (Table 3.4). These findings agree with the results of others (Koyama et al., 1985; Sutoh et al., 1993; Sponheimer et al., 2003). In addition, the enrichment of wool in current study was comparable to that previously reported by Männel et al. (2007) with sheep grazing pure C_3 sward. Some ecology studies (Hobson et al. (1993) and Sponheimer et al. (2003)), suggested that body tissues with slower N turnover rates (e.g., muscle and wool) are less likely to reflect N metabolic changes; compared with active tissues (e.g., liver and plasma) when feeding studies have short periods. This finding is supported by the low correlation between $\delta^{15}N$ of wool and plasma ($r^2 = 0.22$; $P < 0.05$); but was not the case

for muscle $\delta^{15}N$ which was well correlated with plasma $\delta^{15}N$ ($r^2 = 0.70$; $P < 0.001$). This might due to the enzymatic reactions involved in capture N in wool discriminated differently from the enzymatic reactions involved in muscle synthesis.

Sick et al. (1997) demonstrated preliminary evidence in their study with rats that urea and protein from plasma were respectively less depleted and more enriched in $\delta^{15}N$ compared with feed when NI increased. In general, UN/NI is positively related to NI of the animal (Castillo et al., 2001; Cheng et al., 2011). Furthermore, body protein (e.g., plasma or muscle) and urinary N are mainly derived from plasma protein and plasma urea pools respectively (Young, 1970; Sick et al., 1997). Consequently, negative relationships between plasma enrichment (plasma $\delta^{15}N$ - feed $\delta^{15}N$) and RN/NI; and muscle enrichment (muscle $\delta^{15}N$ - feed $\delta^{15}N$) and RN/NI were participated. Similarly, it was predicted positive relationship between UN/NI and urine depletion (urine $\delta^{15}N$ - feed $\delta^{15}N$). The data provided support for these relationships (Equations 2 to 4), with all relationships being statistically significant. It is notable that these relationships occurred even with animals in negative NB (i.e., losing body N) ([Equations 3 and 4]). Retained N is the balance of protein synthesis and degradation within the body and N turnover is expected to be high even when animals lose weight (McCarthy et al., 1983). Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein results in depletion and enrichment of ^{15}N in microbial protein and ruminal ammonia respectively, which should lead to opposite relationships as described above (Cheng et al., 2011). Since both urinary PD excretion (Table 3.2) and calculations of the yield of microbial protein (AFRC, 1993) suggested no change in microbial protein synthesis across treatments, it was expected little changes in N isotopic fractionation in the rumen. However, possibility of N turnover leads to ruminal N isotopic fractionation without altering microbial protein synthesis cannot be excluded. The level of urea recycling from dietary N is expected to be low when NI is greater than requirements (Reynolds and Kristensen, 2008). Thus, under the condition of this study, the relationship between N isotopic fractionation and N partitioning were mainly driven by deamination or transamination in the liver, as suggested by other works (Macko et al., 1986; Parker et al., 1995), and similar to the fractionation reported by Sick et al. (1997) for rats fed on a range of protein sources.

3.5. Conclusion

Varying the N/WSC ratio of diets had only limited effects on N partitioning, and no significant effect on N isotopic fractionation. However, moderately strong relationships were found between N partitioning and N isotopic fractionation when considering between-animal variation. Urinary N output per unit of faecal N output increased by 27 % when N/WSC increased 2.5-fold. The BUN from spot sampling was poorly correlated with N utilisation. The linear correlations found in this study confirmed the potential to use N isotopic fractionation as an indicator of N partitioning in non-lactating sheep. However, the rate of N and ^{15}N turnover of different body N sinks needs to be determined in order to confirm the most appropriate tissue to use.

CHAPTER 4
THE EFFECT OF SUPPLEMENTING UREA ON NITROGEN-USE EFFICIENCY AND ISOTOPIC FRACTIONATION OF PASTURE-FED DAIRY COWS

4.1. Introduction

Chapter 3 provided some support on the use of N isotopic fractionation to indicate N utilisation (UN/NI) in non-lactating sheep without involving milk as a sink for N isotopic fractionation. Cheng et al. (2011) found little evidence of relationship between N partitioning and isotopic fractionation in lactating dairy cows offered a range of forages. It was suggested that the lack of relationship might be a result of widely different proportions and types of N in the diets, since the incorporation of ammonia-N into bacterial N resulted in the depletion of ^{15}N, whilst incorporation of amino acids did not (Wattiaux and Reed, 1995). Therefore, to confirm the above speculation, the current study was designed to evaluate the effects of supplying additional urea as a source of rumen degradable nitrogen (RDN) on N isotopic fractionation in pasture-fed lactating dairy cows. At the same time, the usefulness of using N isotopic fractionation to indicate N-use efficiency (milk N output/N intake) of lactating cows was also evaluated.

4.2. Materials and methods

Animals and their management

This study was undertaken at Lye Farm, Hamilton, New Zealand (37°46′S, 175°18′E) under the authority of Ruakura Animal Ethics Committee (No. 11896). The study used 15 multiparous, rumen-cannulated, Holstein-Friesian cows that were 15 to 18 weeks in lactation. Cows grazed Perennial ryegrass (*Lolium perenne L.*) dominant pasture prior to the experiment and were then housed in metabolism stalls and fed freshly cut pasture twice daily for five days during adaptation to the metabolism facility. A sample collection period was then commenced.

Diets and feeding

Fresh pasture was cut once-a-day in the morning. All 15 cows were randomly grouped into three dietary treatments (five cows per treatment). The dietary treatments were designed to allow the comparison of N utilisation from different levels of N intake by feeding cows on cut pasture twice a day (available between 9.00 am to 1.00 pm and 4.00 pm to 9.00 pm) and supplementing with one of 3 levels of urea given via the rumen cannulae (0 g/d (Control), 350 g/d (Low) and 700 g/d (High)). Equal amounts of urea were placed into the rumen and mixed with rumen digesta three times daily at 9.30 am, 1.00 pm and 4.30 pm timed to coincide with the major bouts of feeding.

Cows were gradually adapted to urea supplementation over 12 days prior to the metabolism stall adaptation period. Concerns regarding high levels of feed refusals for the Low and High urea treatments resulted in urea supplementation being reduced to 250 g/d and 336 g/d respectively, during the period between housing and the commencement of collections.

Animal measurements

Measurements of feed DMI, milk output, urine output and faecal output were made over the final five days of feeding for Control and Low cows, and over the final three days for cows on the High treatment. Feed DMI from each cow was calculated from daily amounts

offered and refused, with a target of 10 % refusals. Daily feed and urea samples were combined over the collection period for subsequent chemical analysis. Cows were milked twice daily and milk yields were recorded. Representative samples were retained for milk composition analysis.

Separate total collections of faeces and urine were made using the externally applied separators described by Meier et al. (2008). Faecal and urine collections were started one day after the measurement of intake. Five days before collection commenced, a separator mounting was attached around the vulva and anus with contact adhesive. On the following day, a urine collector fitted with 4 m of flexible hose was placed over the vulva and fastened to the separator mounting. Urine was retained in a 50 litre plastic container positioned in a drainage channel below the cow. Faeces were collected into container suspended in the drainage channel below the cow.

Blood samples from the jugular were collected (10 ml EDTA heparin tube) from individual cows at 11.30 am on each collection day. Plasma was harvested by centrifugation at 1200 $\times g$ for 12 minutes at 4 ^{o}C. At the end of the study, all samples (feed, faeces, milk, plasma and urine) were bulked by cow, sub-sampled and stored at -20 ^{o}C.

Analytical methods

Near-infrared spectrophotometry provided composition analysis of the pasture (Feed and Forage Analyser 500; Foss, Hillerød, Denmark). Acidified frozen urine and freeze-dried faeces were analysed for N concentration using a Variomax CN Analyser (Elementar Analysensysteme GmbH, Hanau, Germany). Milk samples were analysed for fat, protein and lactose concentrations using Fourier-transform infra-red spectroscopy (FT120, Foss, Hillerød, Denmark). Blood and milk urea nitrogen, and plasma ammonia were analysed using a kinetic UV assay and enzymatic kinetic assay, respectively (Modular P800 analyser; Roche Diagnostics, Mannheim, Germany).

Sample preparation and ^{15}N measurements

Bulked freeze-dried samples of urea, pasture, milk, faeces and plasma were ground

(ZM200 mill with 1 mm sieve; Retsch GmbH, Haan, Germany) prior to ^{15}N analysis. The isotope ratio mass spectrometer (IRMS) operates optimally when supplied with between 100-200 μg N. Therefore, sample weights were calculated for each sample based on the results of N analysis. Each freeze-dried sample was weighed using a 5-decimal place balance (XP205; Mettler Toledo, Columbus, Ohio, USA) onto 5 mm × 8 mm tin capsules. All the capsules were loaded onto an autosampler for ^{15}N analysis. Samples were initially combusted at 1000 ^{o}C in an oxygen atmosphere in an automated Dumas-style elemental analyser, converting all N in the sample to dinitrogen gas which was then carried to a continuous flow stable IRMS (PDZ Europa Ltd, Crewe, UK) for the ^{15}N test. All results are expressed in delta units ($\delta^{15}N$, ‰); that is, the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air).

Calculations

The $\delta^{15}N$ content of pasture and urea were analysed separately. Therefore total feed $\delta^{15}N$ was calculated using the formula (Cheng et al., 2011): Total feed $\delta^{15}N$ = [(N intake of pasture) × ($\delta^{15}N$ of pasture) + (N intake of urea) × ($\delta^{15}N$ of urea)] / [N intake of pasture + N intake of urea]

Statistical analysis

The Genstat package (Version 10.1; Lawes Agricultural Trust) was used for ANOVA and regression analysis. All N partitioning and N isotopic fractionation data were combined into a single mean value for each cow. The significance of treatment effects was determined using general ANOVA with level of urea supplementation as treatment factor. Urinary N output was measured, but values were much lower than expected (Castillo et al., 2000; Cheng et al., 2011), probably due to problems with collection or storage (e.g. volatilisation of ammonia). These data are, therefore, not presented.

4.3. Results

Pasture composition

Pasture composition was consistent across the five-day collection period. On average, the pasture contained (on a DM basis): 16.7 % CP (SD = 0.91), 23.5 % water-soluble carbohydrates (WSC) (SD = 1.98) and 40.9 % NDF (SD = 1.77). Estimated ME content of the pasture was 12.0 MJ/kg DM (SD = 0.12) and the estimated degradability of CP was 78.8 % (SD = 0.44).

Dry matter intake and milk production

Increasing urea intake reduced DMI, milk yield, fat yield, and the concentration and yield of lactose ($P < 0.05$). In contrast, there was no effect of dietary treatment on milk fat and N concentration (Table 4.1). Cows on the High treatment consumed 21 % less pasture DM than those on the Control treatment.

Table 4.1. Effects of dietary treatments[†] on dry matter intake, apparent nitrogen digestibility, milk yield, and milk composition

	Dietary treatment[†]				
	Control	Low	High	SED	P
Dry matter intake (kg/d)	19.1[b]	18.7[b]	15.1[a]	0.93	**
Digestibility of nitrogen (%)	73.0[a]	78.8[b]	80.8[b]	2.02	**
Milk Yield (kg/d)	24.7[b]	22.7[b]	16.6[a]	1.79	**
Milk fat concentration (%)	4.17	4.33	4.24	0.454	NS
Milk nitrogen concentration (%)	0.52	0.56	0.53	0.033	NS
Milk lactose concentration (%)	5.04[b]	4.94[b]	4.67[a]	0.117	*
Fat yield (g/d)	1024[b]	961[b]	705[a]	73.3	**
Lactose yield (g/d)	1248[b]	1119[b]	775[a]	92.9	***

[†]Diets based on: Control (0g urea/d); Low (250g urea/d); High (336g urea/d)

NS, not significant

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

[a–c]Means with different superscripts are significantly different

Nitrogen utilisation

Nitrogen intake was greatest for the Low treatment (616 g/d) and lowest for the Control treatment (510 g/d). The reduction in pasture DMI for the High treatment meant that N intake (543 g/d) was lower than anticipated for this treatment (Table 4.2). The apparent digestibility of dietary N increased with urea addition (Table 4.1). The high level of urea supplementation reduced milk N output and NUE (Table 4.2).

Urea supplementation increased MUN and BUN concentration ($P < 0.001$; Table 4.2). Regression analysis indicated a positive correlation between MUN and BUN concentration (Equation [1]).

MUN (mmol/l) = 0.70 (SE = 0.107) + 1.535 (SE = 0.578) × BUN (mmol/l)
(n = 15; $r^2 = 0.75$; $P < 0.001$) [1]

Moderate linear relationships were detected between NUE and MUN ($r^2 = 0.63$), and NUE and BUN ($r^2 = 0.31$).

Table 4.2. Effects of dietary treatments[†] on nitrogen intake, faecal nitrogen output, milk nitrogen output, nitrogen use efficiency (milk nitrogen/nitrogen intake), blood urea, milk urea and plasma ammonia

	Dietary treatment[†]				
	Control	Low	High	SED	P
Nitrogen intake (g/d)	510[a]	616[b]	543[a]	24.6	**
Faecal nitrogen output (g/d)	138[b]	131[ab]	103[a]	13.7	NS
Milk nitrogen output (g/d)	129[b]	126[b]	89[a]	7.0	***
Nitrogen use efficiency (%)	25.3[b]	20.1[b]	16.4[a]	0.99	***
Blood urea (mmol/l)	4.34[a]	5.95[b]	5.53[b]	0.295	***
Milk urea (mmol/l)	4.10[a]	5.74[b]	6.15[b]	0.333	***
Plasma ammonia (μmol/l)	59.5[a]	64.4[a]	101.1[b]	12.17	**

[†]Diets based on: Control (0g urea/d); Low (250g urea/d); High (336g urea/d)

NS, not significant

$^{**}P < 0.01$; $^{***}P < 0.001$

[a–c]Means with different superscripts are significantly different

Nitrogen isotopic fractionation

Pasture $\delta^{15}N$ was consistent across the collection period, within the range of 3.17 to 3.25 ‰. The $\delta^{15}N$ value of urea was 1.02 ‰. Dietary treatment had no effect on milk or plasma $\delta^{15}N$ (Table 4.3). However, there was a treatment effect ($P < 0.05$) on faecal $\delta^{15}N$ (Table 4.3).

When $\delta^{15}N$ results for milk and plasma were adjusted for feed $\delta^{15}N$ (Table 4.3); both (milk $\delta^{15}N$ -feed $\delta^{15}N$) and (plasma $\delta^{15}N$ -feed $\delta^{15}N$) were negatively associated with NUE (Figure 4.1; Equations [2] and [3]).

Milk $\delta^{15}N$ -feed $\delta^{15}N$ (‰) = -0.0827 × NUE (%) + 5.46

(n = 15; $r^2 = 0.83$; $P < 0.001$) [2]

Plasma $\delta^{15}N$ -feed $\delta^{15}N$ (‰) = -0.1001 × NUE (%) + 5.75

(n = 15; $r^2 = 0.85$; $P < 0.001$) [3]

Table 4.3. Effects of dietary treatments† on $\delta^{15}N$ contents of feed, faeces, plasma and milk, as well as the differences in $\delta^{15}N$ between milk and feed and plasma and feed

	Dietary treatment†				
	Control	Low	High	SED	P
Feed $\delta^{15}N$ (‰)	3.25^{c}	2.83^{b}	2.55^{a}	0.018	***
Milk $\delta^{15}N$ (‰)	6.59	6.63	6.68	0.095	NS
Faeces $\delta^{15}N$ (‰)	6.17^{b}	5.78^{a}	6.15^{b}	0.149	*
Plasma $\delta^{15}N$ (‰)	6.44	6.57	6.67	0.120	NS
Milk- Feed $\delta^{15}N$ (‰)	3.34^{a}	3.80^{b}	4.13^{c}	0.096	***
Plasma- Feed $\delta^{15}N$ (‰)	3.19^{a}	3.73^{b}	4.13^{c}	0.121	***

†Diets based on: Control (0g urea/d); Low (250g urea/d); High (336g urea/d)

NS, not significant

*$P < 0.05$; ***$P < 0.001$

[a–c]Means with different superscripts are significantly different

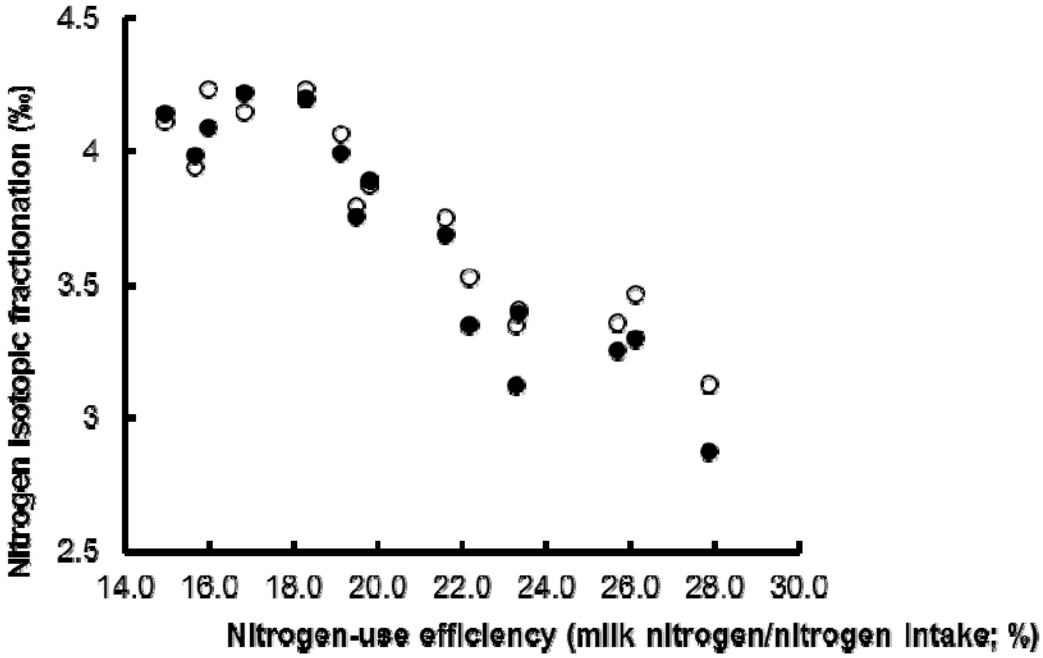

Figure 4.1. Correlations between nitrogen-use efficiency and isotopic fractionation in cows fed on pasture and supplemented with 3 levels of urea.• plasma $\delta^{15}N$ -feed $\delta^{15}N$, ◦ milk $\delta^{15}N$ -feed $\delta^{15}N$

4.4. Discussions

Dry matter intake and milk production

The WSC, NDF and CP content of the pasture is consistent with temporal patterns reported by Roche et al. (2009), suggesting that the pasture composition was typical of cool season grasses in a temperate/Mediterranean climate. The DMI declined unexpectedly in the High treatment (336 g/d). The reason for this decline in DMI is not clear. Results from earlier research did not predict an adverse effect of urea N supplementation on DMI up to 54 % of total N intake (379 g urea/d) (Mugerwa and Conrad, 1971). The possibility of ammonia toxicity was closely investigated, but plasma ammonia results (Table 4.2) did not indicate toxicity (Zhu et al., 2000). However, it is possible that sub clinic ammonia toxicity was developed in cows fed the High treatment, which could lead to low NUE from reduced DMI. The adverse effect of urea supplementation on DMI in the present experiment may be due to the additional high N intake from pasture (396 g/d) and the high degradability of pasture protein (NRC, 2001). Cows may have reached a physiological limit for metabolizing urea or renal excretion of urea, resulting in reduced N intake via lower DMI as a protective mechanism (Cocimano and Leng, 1967). The reduction in the yields of milk, milk fat and milk protein which directly reflected the depression in DMI, were similar to those reported by Brito and Broderick (2007) for cows supplemented with different proteins.

Nitrogen utilisation

The increase in apparent digestibility of N with urea supplementation is consistent with the high ruminal degradation of dietary urea. Correcting for urea digestion, the apparent digestion of the pasture N was unchanged by treatment and averaged 73 %. Since the digestibility of microbial protein is estimated to be 80 % (NRC, 2001), the implication is that urea supplementation had no effect on microbial protein synthesis, probably because pasture already supplied adequate RDN per MJ fermentable ME for maximum microbial growth (NRC, 2001).

Nitrogen intake has been reported as a major driver of NUE across a wide range of diets (Castillo et al., 2000). However, this relationship was not as clear in the current

experiment, suggesting that other factors, such as form of dietary N and energy supply, also affect NUE (Edwards et al., 2007). In this case, the low NUE for the High urea diet appears to be from the reduction in (energy) intake and the relatively high level of RDN in the diet (Castillo et al., 2000; NRC, 2001).

Nitrogen isotopic fractionation

Although infusion of a low $\delta^{15}N$ urea into the rumen reduced the total diet $\delta^{15}N$ from 3.25 to 2.55 ‰, supplementation with urea had little effect on the $\delta^{15}N$ content of body N pools (Table 4.3). On average, faeces, milk and plasma were enriched in $\delta^{15}N$ by 3.16, 3.76 and 3.88 ‰, respectively, compared with feed (Table 4.3). These findings are consistent with the literature, in which herbivore faeces, milk and plasma were consistently enriched in $\delta^{15}N$ compared with their diets (Table 4.4). Sponheimer et al. (2003) suggested that the main reason for the enrichment of $\delta^{15}N$ in faeces is the presence of enriched endogenous material. The majority of N in milk and plasma exists as true protein, which has been reported to be enriched in $\delta^{15}N$ (Sick et al., 1997; Cheng et al., 2010). In contrast, urea is the main N source of urine and is reported to be heavily depleted in $\delta^{15}N$ (Steele and Daniel, 1978); this is consistent with the low $\delta^{15}N$ in the supplementary urea in the current experiment relative to the pasture.

Table 4.4. Summary of $\delta^{15}N$ content (‰) of feed, milk, plasma, faeces and urine from literature studies with a range of herbivore species

Animal (Reference‡)	Feed	Milk	Plasma	Faeces	Urine
Jersey cows (1)	0.65 (0.60-0.70)	4.30 (4.20-4.30)	-	2.65 (2.30-3.10)	-1.75 (-2.40- -1.40)
Angus steer (1)	0.60 (0.60)	-	4.80 (4.70-4.90)	2.45 (2.10-2.70)	-1.58 (-2.80- -0.60)
Cows (2)	2.55 (1.65-3.05)	5.73 (5.15-6.15)	5.14 (4.75-5.55)	5.07 (4.95-5.15)	2.25 (1.85-2.55)
Dairy cows (3)	3.03 (2.70-3.30)	5.17 (4.90-5.50)	5.40 (5.00-5.80)	3.83 (3.50-4.10)	-1.77 (-2.40- -0.70)
Sheep (4)	0.76 (0.76)	-	-	3.76 (3.59-3.93)	0.64 (0.63-0.65)
Llamas (5)	0.40 (0.40)	-	-	3.30 (2.90-3.50)	0.10 (-0.20-0.60)
Dairy cows (6)	4.17 (1.82-8.38)	6.75 (5.24-9.21)	-	5.71 (4.20-8.76)	0.87 (-1.42-4.09)
Dairy cows (Current study)	2.88 (2.55-3.25)	6.63 (6.59-6.68)	6.56 (6.44-6.67)	6.03 (5.78-6.17)	-

‡ 1. Steele and Daniel, 1978; 2. Koyama et al., 1985; 3. Sutoh et al., 1987; 4. Sutoh et al., 1993; 5. Sponheimer et al., 2003; 6. Cheng et al., 2011.

Relationships between nitrogen-use efficiency and isotopic fractionation

A major observation from this study was the negative relationships between milk $\delta^{15}N$ - feed $\delta^{15}N$ and plasma $\delta^{15}N$ -feed $\delta^{15}N$ and NUE (Figure 4.1; Equations [2] and [3]). The first possible explanation for this effect relates to the lower ^{15}N content of urea compared with total dietary protein. The $\delta^{15}N$ of urea was 1.02 ‰ whilst the average $\delta^{15}N$ of milk and plasma were 6.64 and 6.56 ‰, respectively. Since urea N was only 2.2, 2.9 and 3.2 % of milk N for the Control, Low and High treatment, respectively, it could only explain a 7 % difference in $\delta^{15}N$ between the Control and High groups (i.e. 0.056 ‰). The possible contribution of urea to the effect on plasma is even less, since urea N makes up an even lower proportion of total plasma N. Therefore, these negative relationships must be primarily due to isotopic fractionation within the animal body (Parker et al., 1995; Sick et al., 1997; Balter et al., 2006).

There are two plausible sites (liver and rumen) of N isotopic fractionation that may be related to urea supplementation. Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein results in a depletion of ^{15}N in microbial protein, which was not observed when microbial protein was synthesised from amino acids. Since bacterial protein would be the major source of amino acids for milk protein synthesis with these diets, it is unlikely that there was an increase in microbial protein synthesis from ammonia when urea was added. The calculated level of urea recycling from dietary CP % (Reynolds and Kristensen, 2008) suggests that there was little variation across treatments (42, 34, and 39 % of total urea production that is returned to the gut via blood and saliva for Control, Low and High treatments, respectively). Furthermore, these are inversely related to N intake. It is, therefore, predicted that the absolute amount of urea recycling would be similar across treatments. Thus, it appears that the negative relationships between NUE and milk $\delta^{15}N$ -feed $\delta^{15}N$ and plasma $\delta^{15}N$ -feed $\delta^{15}N$ are mainly driven by deamination or transamination in liver (Macko et al., 1986; Parker et al., 1995), similar to the fractionation reported by Sick et al. (1997) for rats fed on a range of protein sources. It implies when more product protein (e.g., milk) is derived relative to urea from the liver amino acid pool (e.g., increased NUE), the less the fractionation occurs due to the isotope pattern of product protein is more similar to the precursor amino acid in the liver (e.g., the less enriched milk $\delta^{15}N$ - feed $\delta^{15}N$; Figure 4.1).

4.5. Conclusion

Results indicate effects of urea treatments on N utilisation and N isotopic fractionation. High level of urea supplementation (336 g/d) reduced DMI, milk yield, milk N output and NUE of dairy cows fed on pasture. Milk urea N was correlated with BUN. This experiment confirmed the potential to use the ^{15}N enrichment of milk or plasma compared with feed as an indicator of NUE in dairy cows on a high RDN diet. The site and the level of N isotopic fractionation and the effect of N source need further clarification. In addition, the urea toxicity level for pasture fed dairy cows should be further investigated.

CHAPTER 5
THE EFFECT OF DIETARY WATER SOLUBLE CARBOHYDRATE TO NITROGEN RATIO ON NITROGEN PARTITIONING AND ISOTOPIC FRACTIONATION OF LACTATING GOATS OFFERED A HIGH NITROGEN DIET

5.1. Introduction

As it has been discussed in Chapter 3 and 4, traditional techniques are not suitable for measuring N partitioning in grazing ruminants particularly when feed N concentration is high. In addition, Ellis et al. (2011) suggested that separating the effect of dietary components (e.g., WSC and N levels) on N partitioning is important, in order to determine the contributions of each dietary factor to animal performance. Followed with the successful establishment of relationships between N isotopic fractionation and N partitioning in non-lactating sheep and lactating cows; the two objectives of this study were to examine the N partitioning of lactating goats offered a diet with high but similar concentration of feed N, with no changes in intake but increasing levels of WSC; and further evaluate the use of N isotopic fractionation as a simple indicator of N partitioning.

5.2. Materials and methods

The study used eight multiparous goats weighing 51 kg (SD = 5.0) and 52 kg (SD = 4.5) at the start of two collection periods. Four dietary treatments were evaluated in an incomplete changeover design with two periods of three weeks (the first two weeks were used for dietary adaptation and the last week was used for measurement). Two goats were placed on each treatment in each period. One goat receiving the Medium Low treatment in period 1 was replaced due to a metabolic disorder with a spare goat the day before starting the NB of period 2.

Diets

Four dietary treatments were designed to provide increasing concentrations of WSC (Low, Medium Low, Medium High and High WSC/N); at a constantly high level of dietary N (4.5 %) and fixed DM allowance (2000 g/d). The main component of the diet was lucerne (*Medicago sativa*) pellets (Dunstan Horsefeeds; Camtech Nutrition Ltd; New Zealand). Additional WSC was provided from oligofructose powder containing 5 % glucose/fructose and 95 % sucrose (Orafti®P95; BENEO-Orafti S.A; Belgium). Nitrogen levels were equalized across treatments (Table 5.1) by adding acid casein (NZMP™; Fonterra Ltd; New Zealand) and non-protein-N as urea (N-rich™; Ballance Agri-Nutrients Ltd; New Zealand).

Feed for each treatment by period was prepared daily prior to feeding. In comparison to period 1, an extra quantity of lucerne pellet was included in the feed to avoid negative energy balance in period 2. Additionally, the amount of urea was reduced as a result of the high BUN measured in period 1 (Table 5.1).

Animal measurements

This study was undertaken at AgResearch Grasslands Research Centre, Palmerston North, New Zealand (Grasslands Animal Ethics Committee No. 11894). Goats were housed individually in metabolism crates and automatically fed two hourly. The measurement period consisted of two days of facilities adaptation, five days of NB study, followed by insertion of two jugular catheters for two days of infusion of stable isotope-labelled

compounds for 4 hour/day to determine the whole-body production rates of glucose, leucine, urea, plus the oxidation of leucine to bicarbonate (results will be published separately) (Table 5.2).

Feed, faeces, urine and milk samples were collected during the first five days of each measurement period. The goats were weighed on the day before and after the NB study. Blood was sampled into 10 ml EDTA tube from individual animals on the last day of each NB period. Plasma was harvested by centrifugation at 4 °C at 1300 rpm for 15 min.

Sampling of feed was done in the morning before feeding and samples were oven dried. Milk yield and milk samples were recorded and collected respectively twice per day throughout the whole study. Daily faeces production was recorded after each morning milking. Faeces were collected in a stainless steel tray under the metabolism crates and samples were oven dried. Urine collection was carried out continuously, weighed and sampled after each morning milking. To avoid contamination with faeces and to give quantitative urine collection, a separator was glued over the vulva with an attached hose directing the urine flow to a plastic bucket containing 50 ml of 6 M hydrochloric acid. pH was measured after each sampling to confirm that urine pH was below 4.5. All five days samples were stored at -20 °C and pooled per goat per period and subsampled prior to chemical analysis. Nitrogen retention was calculated as the difference between N intake and outputs (urine, milk and faeces).

Analytical methods

The concentration of feed WSC was analysed following the method described by Rasmussen et al. (2007). Oven dried feed, acidified urine and oven dried faeces were analysed for N concentration using an Elementar (Variomax CN Analyser, Germany). Milk samples were analysed for fat, protein and lactose concentrations using Fourier-Transform Infra-Red Spectroscopy (FT120, Foss Electric, Hillerød, Denmark). The MUN was analysed using a kinetic UV assay and enzymatic kinetic assay (Modular P800, Germany). Urinary purine derivatives were analysed using the method described by George et al. (2006). All samples were freeze dried and analysed for $\delta^{15}N$ follow the procedure described by Cheng et al. (2011).

Statistical analysis

The Genstat statistical package (Version 12.2; VSN international Ltd) was used for ANOVA and general linear regression analysis. Dietary treatment and periods effect on both N partitioning and isotopic fractionation, were examined by including treatment × period as treatment factor in the ANOVA. Infusion of labelled N (urea) isotope from period 1 effect on N isotopic fractionation was examined by including treatment × infusion as treatment factor in ANOVA.

5.3. Results

Feed formulation and composition

The feed formulation and chemical composition are shown in Table 5.1, separately for the two periods. Across treatments, on average Orafti®P95 content increased from 0 (Low treatment) to 454 and 473 g/d (High treatment) in period 1 and 2 respectively. The ratio of WSC to N (WSC/N) increased progressively from 4.3 to 7.5 from Low to High treatment (Table 5.1).

Table 5.1. Feed formulation (on a DM basis) and chemical composition for the dietary treatments

	Period 1				Period 2			
	L[‡]	ML	MH	H	L	ML	MH	H
Lucerne (g/d)	2401	2202	2001	1802	2501	2301	2117	1901
Casein (g/d)	36	87	129	163	38	92	134	171
Orafti®P95(g/d)	0	178	330	454	0	188	344	473
Urea (g/d)	19	19	19	19	9	8	9	10
N[#] (%)	4.5	4.3	4.3	4.1	4.6	4.5	4.6	4.5
WSC[+] (%)	18.7	25.4	30.0	32.4	20.1	27.2	29.7	31.8
WSC/N (g/g)	4.2	5.9	6.9	7.8	4.4	6.0	6.5	7.1

[‡] L = low; ML = medium low; MH = medium high; H = high

[+] Water soluble carbohydrate; [#] nitrogen

Table 5.2. Infusion rates (mmol/hr) of different isotopes, according to experimental period

	Period 1	Period 2
^{13}C-glucose	1.68	1.68
^{13}C-sodium bicarbonate	0.43	0.43
^{13}C-leucine	0.56	0.64
^{15}N-urea	1.25	1.04

DMI, milk yield, DM and nitrogen digestibility, MUN, urinary excretion of purine derivatives

Both dietary treatment and period had significant effects on DMI (g/d), DM digestibility (g/kg DM; DMD) and N digestibility (g/kg N; ND) ($P < 0.001$). The DMI changed little (±2 %) across dietary treatments. On average, DMD and ND increased by 12 and 4 % respectively from the Low to High treatment (Table 5.3). As WSC/N increased, milk yield (MY; $P < 0.05$) increased from 1833 (Low) to 2724 g/d (High). On average, MUN was 20 % higher in period 1 than period 2. No significant treatment effect was observed for urinary excretion of purine derivatives (mM/d; PD) (Table 5.3).

Table 5.3. Treatment and period effects on dry matter intake **(**DMI; g/d), milk yield (MY; g/d), DM digestibility (DMD; g/kg DM) and nitrogen digestibility (ND; g/kg N), milk urea nitrogen (MUN; g/l) and urinary excretion of purine derivatives (PD; mM/d)

	Period 1				Period 2						
	L[‡]	ML	MH	H	L	ML	MH	H	T[+]	P[#]	T×P
DMI	2113	2151	2155	2123	1969	2014	2024	2005	***	***	***
DMD	685	695	735	754	620	648	677	713	***	***	NS
ND	780	764	792	806	739	757	770	777	***	***	*
MY	1807	2434	2422	2885	2165	1860	2964	2563	*	NS	NS
MUN	0.42	0.38	0.42	0.45	0.32	0.34	0.38	0.34	NS	***	NS
PD	26.9	27.7	26.8	25.9	27.7	27.3	30.1	33.0	NS	NS	NS

[‡] L = low; ML = medium low; MH = medium high; H = high

[+] Treatment effect; [#] Period effect

*P < 0.05; ***P < 0.001; NS = non-significant

Nitrogen partitioning and weight changes

Mean N intake (NI) was 92.3 and 91.2 g/day across dietary treatments in period 1 and 2 (P < 0.001). Milk N output (MN) and urinary N output (UN) averaged 12.0 and 48.1 g/day respectively with no statistical significant effects for either treatment or period. In comparison, both dietary treatment (P < 0.001) and period (P < 0.001) had effects on faecal N output (FN). On average, FN was higher in period 2 than in period 1 (P < 0.001) and decreased from Low treatment (22.3 g/d) to High treatment (18.6 g/d) (P < 0.001). The range in MN/NI (0.11 to 0.15 g/g) and UN/NI (0.48 to 0.57 g/g) was small, no significant effect was observed from either dietary treatment or period. However, there was a difference in UN/FN between period 1 and 2 (Table 5.4). Overall, there was no change in BW, though individual BW change ranged between -110 and +140 g/d.

Nitrogen isotopic fractionation and infusion effects

Milk, plasma and faeces were enriched in ^{15}N compared with feed. On the other hand, urine was depleted in ^{15}N relative to feed (Table 5.5). No significant linear relationship was detected between N partitioning and isotopic fractionation. Goats infused with ^{15}N labelled urea in period 1 had slightly higher levels of ^{15}N in all of the body N sinks (i.e., urine, faeces, milk and plasma) compared with non-infused goats (Figure 5.1). In addition, reduced milk $\delta^{15}N$ - feed $\delta^{15}N$ and plasma $\delta^{15}N$ - feed $\delta^{15}N$ were observed in goats in period 1 (non-infused goats) compared to period 2 (infused goats) (Figure 5.1).

Table 5.4. Treatment and dietary effects on nitrogen intake (NI; g/d), milk nitrogen output (MN; g/d), urinary nitrogen output (UN; g/d), faecal nitrogen output (FN; g/d), retained nitrogen (RN; g/d), UN/FN (g/g), UN/NI (g/g) and MN/NI (g/g)

	Period 1				Period 2						
	L[‡]	ML	MH	H	L	ML	MH	H	T[+]	P[#]	T×P
NI	95.1	92.9	93.1	87.9	90.6	90.7	93.2	90.3	***	***	***
MN	10.0	12.7	12.0	13.3	12.7	9.7	13.4	11.9	NS	NS	*
UN	54.3	50.6	48.2	45.1	46.0	48.2	45.1	47.2	NS	NS	NS
FN	20.9	21.9	19.3	17.0	23.6	22.0	21.4	20.1	***	***	*
RN	9.9	7.8	13.6	12.4	8.3	10.7	13.3	11.0	NS	NS	NS
UN/FN	2.60	2.31	2.50	2.65	1.94	2.19	2.11	2.35	NS	***	NS
UN/NI	0.57	0.54	0.52	0.51	0.51	0.53	0.48	0.52	NS	NS	NS
MN/NI	0.11	0.14	0.13	0.15	0.14	0.11	0.14	0.13	NS	NS	*

[‡] L = low; ML = medium low; MH = medium high; H = high

[+] Treatment effect; [#] Period effect

*$P < 0.05$; ***$P < 0.001$; NS = non-significant

Table 5.5. Effects of dietary treatments on nitrogen isotopic fractionation of lactating goats (‰)

	L‡	ML	MH	H	SED	P
Feed $\delta^{15}N$	1.90	2.29	2.49	2.84	0.081	***
Milk $\delta^{15}N$	7.11	7.72	7.85	8.29	0.658	NS
Urine $\delta^{15}N$	1.22	1.68	2.20	2.09	0.217	**
Faecal $\delta^{15}N$	4.01	4.41	4.77	5.03	0.156	***
Plasma $\delta^{15}N$	6.92	7.99	7.97	8.01	0.614	NS
Milk $\delta^{15}N$ - feed $\delta^{15}N$	5.22	5.43	5.36	5.45	0.623	NS
Urine $\delta^{15}N$ - feed $\delta^{15}N$	-0.68	-0.60	-0.29	-0.75	-0.226	NS
Plasma $\delta^{15}N$ - feed $\delta^{15}N$	5.03	5.70	5.48	5.17	0.572	NS

‡ L = low; ML = medium low; MH = medium high; H = high

*P < 0.05; **P < 0.01; ***P < 0.001; NS = non-significant

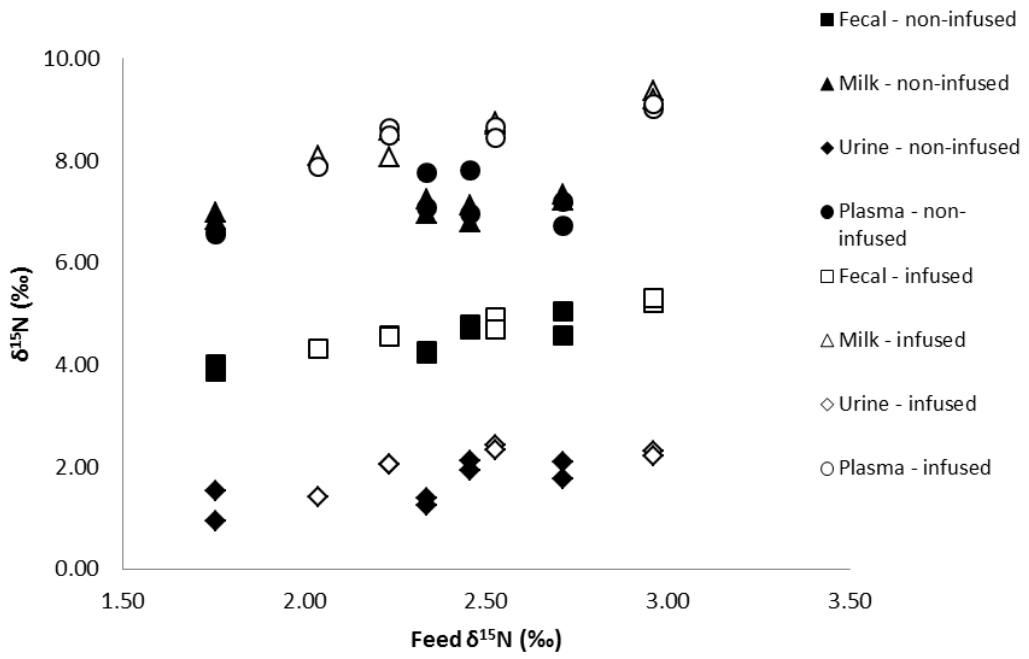

Figure 5.1. Relationship between feed $\delta^{15}N$ (‰) and $\delta^{15}N$ in milk, plasma, faeces and urine (‰) for individual observations of infused (period 2; n = 7) and non-infused (period 1; n = 8) goats

Regression analysis demonstrated that faecal $\delta^{15}N$ and urine $\delta^{15}N$ were linearly related to feed $\delta^{15}N$ ([Equations 1 and 2]).

Faecal $\delta^{15}N$ (‰) = 1.1 × feed $\delta^{15}N$ (‰) + 2.0 [1]
(n = 16; $r^2 = 0.80$; $P < 0.001$; SE = 0.20)

Urine $\delta^{15}N$ (‰) = 0.9 × feed $\delta^{15}N$ (‰) - 0.5 [2]
(n = 16; $r^2 = 0.48$; $P < 0.01$; SE = 0.35)

5.4. Discussion

Nitrogen metabolism

This study provided a good base to examine the change of WSC effect on N partitioning alone, with a relatively constant NI across treatments (Table 5.4). Although a statistical significant difference between treatments was observed for NI, the difference was numerically small (4 %) and unlikely to have influenced overall N partitioning. The average NI was 91.8 g N/d of which 13 %, 52 % and 23 % were excreted in milk (non-significant treatment effect), urine (non-significant treatment effect) and faeces ($P < 0.001$) respectively. The relatively low and non-significant dietary effect on NUE contrasted with results from cows fed a diet of around 3.2 % N with elevated WSC in the diet, which showed improvement in NUE when intake stayed the same (Miller et al., 2001). On the other hand, the proportion of NI excreted in urine (UN/NI) in this study was high (on average > 48 %; Table 5.4) compared with results from lactating dairy cows studies (Miller et al., 2001; Moorby et al., 2006) reflecting the very high N intake in this study. Results were also in contrast to the suggestion from the review of Edwards et al. (2007) that increased NUE and decreased UN/NI of dairy cows should be observed when WSC/N increased from 4.4 to 8.1 %. The high dietary N (on average 4.5 %) and high WSC (on average 26.9 %) concentrations may have been the main reasons for no response in N partitioning of UN/NI and NUE (Table 5.4). Pacheco et al. (2007) conducted a meta-data analysis (n = 129) and found no improvement in NUE when dietary N concentration was above 3.68 % of DM. Broderick and Clayton (1997) proposed this may be due to high dietary N concentration exceeding the limit for rumen microbes to capture N for microbial protein synthesis. On the other hand, an *in vitro* study demonstrated that there may be a concentration of WSC (24 %) that optimises rumen fermentation and animal production regardless of changes in WSC/N (Burke et al., 2011). Owens et al. (1998) suggested that the synthesis of microbial protein in the rumen may also be reduced by acidosis from feeding high level of WSC. This is partly evident from the low PD for Medium High and High treatments in period 1. Notably, these two treatments also received the highest WSC/N (i.e., 6.9 and 7.8 g/g). Overall, dietary treatment had little effect on PD in the study (Table 5.3), which implies no differences in rumen microbial protein synthesis (Dewhurst et al., 1996; Cheng et al., 2011). Furthermore, estimation of the yield of MCP per MJ of FME based on AFRC (1993);

MCP/FME (g/MJ) = 7 + 6 × [1 - $e^{(-0.35L)}$] suggested an average 10.9 g of MCP was synthesised from per MJ of FME (SED = 0.16; P > 0.05) irrespective of WSC/N in the diets.

A recent focus in ruminant research is to redirect UN to FN through feeding animals with diets containing high level of WSC. Results from feeding cows on 3.2 % dietary N (on DM basis) showed that it was possible to decrease UN/FN by around 27 % (Miller et al., 2011). This change of N excretion was achieved either through increasing FN, as a function of increasing DMI (Moorby et al., 2006) or decreasing UN, as a function of increasing rumen microbial protein synthesis (Miller et al., 2001). The goats in the current study were fed high dietary N (on average 4.5 %) and high WSC (on average 26.9 %), but there was no effect on UN/FN (Table 5.4). The most likely explanation is the lack of improvement in microbial protein synthesis (van Vuuren et al., 1993). In addition, decreased FN resulted from increasing DMD with inclusion of WSC in the diets.

On average, goats retained 11 g N/day from feed in this study. Converting 11 g N/day into protein by adopting a coefficient of 6.25 (i.e., assuming a body protein N content of 16 %), a protein gain of about 68 g/day was obtained and, as body protein associated with water in an average ratio of 1:3, gives 272 g/day of lean tissue gain during the study. This estimation was not realistic for goats fed on around 2000 g of DM/d, and also inconsistent with the weight change data (0 g/d on average). Spanghero and Kowalski (1997) suggested that overestimation of retained N is commonly observed in NB studies. The possible sources of errors for high N retention value include the incomplete collection of materials, volatile N losses from faeces and urine as well as scurf and dermal losses. It is not possible to quantify these potential errors in this study.

Milk urea nitrogen

The MUN was lower in period 2 than period 1 ($P < 0.001$), associated with a reduced urea intake in period 2 (Table 5.1). The MUN is widely used to indicate the efficiency of N utilisation of animals. Oltner et al. (1985) postulated that MUN was more closely related to the ratio of dietary energy to protein than the absolute protein intake. Even though the MUN valued for the goats were comparatively higher than previously reported (Bonanno et al., 2008), there was no significant dietary treatment effect on MUN (Table 5.3). This

may be caused by similar reasons to lack of dietary treatment effect on N partitioning (van Vuuren et al, 1993; Broderick and Clayton, 1997).

DMI, milk yield and DM digestibility

Miller et al. (2001) and Moorby et al. (2006) found there was a positive relationship between DMD and MY, but this was not supported in the current study ($r^2 = 0.20$, $P < 0.05$). Instead, dietary WSC concentration accounted for 36 % of the MY variation ($P < 0.001$), confirming that feed composition can induce changes in MY (Holmes et al., 1956). Alternative explanations for the increased MY from L to H treatment may be due to improved metabolisable protein utilisation through additional casein in the feed or an increased water intake as more WSC was added into the diet.

Nitrogen isotopic fractionation and infusion effects

On average, faeces, plasma and milk were relatively enriched in $\delta^{15}N$ by 2.18, 5.35 and 5.37 ‰ compared to the diet, whilst urine was depleted in $\delta^{15}N$ relative to the diet by 0.58 ‰ (Table 5.5). This is consistent with the previous sheep (Chapter 3) and cow (Chapter 4) studies, and Sponheimer et al. (2003) who suggested that the main reason for the enrichment of $\delta^{15}N$ in faeces is the presence of enriched endogenous material. The majority of N in milk and plasma exists as true protein, which has been reported to be enriched in $\delta^{15}N$ (Sick et al., 1997; Cheng et al., 2010). In contrast, urea is the main N source of urine and is reported to be depleted in $\delta^{15}N$ (Steele and Daniel, 1978).

The difference in apparent fractionation (e.g., milk $\delta^{15}N$ - feed $\delta^{15}N$) effects between periods 1 and 2 (Figure 5.1) may relate to the difference in basal diet, and/or a residual effect of ^{15}N infusion at the end of the first period. Diets for period 1 had almost twice as much as urea in the feed compared with period 2; this high level of RDP may have negative impact on ruminal microbe growth/population, which in turn would reduce deamination and N isotopic fractionation (Chen et al., 2008). However, the reductions in PD excretion for the Medium High and High treatments in period 1 were not statistically significant; which does not support any limitation on microbial protein synthesis. An alternative explanation is that a reduced milk $\delta^{15}N$ - feed $\delta^{15}N$ results when more

ammonia is incorporated into microbial protein (Wattiaux and Reed, 1995). This may be related to an increased proportion of microbial protein synthesise from ammonia-N and hyper-ammonia-producing bacteria with the high urea diet (Maeng and Baldwin, 1976; Cherdthong and Wanapat, 2010). Although the enrichment and depletion effects were consistent with literature, the levels of enrichment in body N sinks were generally higher than previously observed (Koyama, 1985; Sponheimer et al., 2003; Cheng et al., 2011). There was a carry-over effect from infusion ^{15}N enriched urea into blood at the end of period 1, which led to body N sinks in period 2 (excluding one replacement goat) being slightly enriched in ^{15}N compared with non-infused goats from period 1 (Figure 5.1). Statistical analysis confirmed the infusion effects between periods were significant for milk (SED = 0.20; $P < 0.001$), urine (SED = 0.20; $P < 0.05$), plasma (SED = 0.23; $P < 0.001$) and faeces (SED = 0.20; $P > 0.05$).

Regression analysis demonstrated that urine and faecal $\delta^{15}N$ were linearly related to feed $\delta^{15}N$ ([Equations 1 and 2]). In addition, feed ($r^2 = 0.80$; $P < 0.001$), urine ($r^2 = 0.49$; $P < 0.01$) and faecal $\delta^{15}N$ ($r^2 = 0.67$; $P < 0.001$) responded to increased WSC/N linearly (Table 5.5). Therefore, these observed relationships ([Equations 1 and 2]) would be related to isotopic fractionation during digestion and absorption of feed components (i.e., WSC and N) (Sutoh et al., 1993; Wattiaux and Reed, 1995) or a possible effect of the endogenous N contribution to urine and faeces from feed (Cheng et al., 2011).

Unlike Sick et al. (1997) who demonstrated in their study with plasma from rats, the data provided little support for a relationship between N partitioning and N isotopic fractionation. Several possible causes exist for the absence of the expected relationship. Firstly, differences in N partitioning between body N sinks were small (e.g., Milk N/N intake ranged between 0.11 to 0.15 g/g) and did not response to dietary treatment in this study (Table 5.4). Secondly, there was little effect of dietary treatments on microbial protein synthesis, as indicated by both PD and MCP/FME calculations. Thirdly, Balter et al. (2006) suggested that the biological value of N and level of N are the major sources of variation in N isotopic fractionation rather than energy supplies to the body.

5.5. Conclusion

There was little change in N partitioning in response to increasing WSC, when N content was consistent and high in the diet. There were no significant effects of dietary treatments on N isotopic fractionation. The absence of dietary effects on apparent microbial protein synthesis may be associated with high dietary N and WSC concentrations. Infusion of ^{15}N enriched urea in period 1 caused a carry-over effect on ^{15}N enrichment of goats in period 2. This study failed to confirm the potential use of N isotopic fractionation as an indicator of N partitioning in dairy goats. The association between N partitioning and isotopic fractionation at different WSC levels need further clarification, as current levels may be too high to result in correlations between N partitioning and isotopic fractionation.

CHAPTER 6
GENERAL DISCUSSION

It has been reported that temperate pasture fed to ruminant generally contains a high concentration of N relative to energy supply (Litherland and Lambert, 2007). This can lead to a low efficiency of incorporating feed N into usable N (e.g., milk, meat); and large outputs of surplus N to the environment as urine (Pacheco and Waghorn, 2008). Any investigation into factors affecting N partitioning, such as DMI, dietary composition, energy/N ratio in the feed, physiological status of the animal, individual genetic differences, requires a simple, cheap and non-invasive method of determining N partitioning between the various N sinks (milk, meat, urine and faeces).

The classical approach to establish N partitioning has been an N balance study. However, it is subject to large experimental errors and difficult to conduct with grazing animals (Spanghero and Kowalski, 1997; Cheng et al., 2011). Milk or blood urea N has also been widely used to indicate N partitioning. However, it is susceptible to diurnal variations and limited in its use when dietary N is high (Geerts et al., 2004). Therefore, a novel and simple method is needed to give a good indication of N partitioning from large groups of grazing animals. Wattiaux and Reed (1995) suggested that N isotopic fractionation could potentially be used to quantify N partitioning of ruminants. However, there is limited information on the utility of this technique and it is not known how dietary changes affect N isotopic fractionation. Therefore, the objective of this PhD study was to measure the N partitioning of a range of ruminants (lactating goats, lactating cows and non-lactating sheep) offered various levels of energy and N intake; and use these data sources to test the value of N isotopic fractionation as a simple indicator of N partitioning.

Section 6.1 will discuss the dietary effect on N metabolism and partitioning in ruminants. The discussion will then move onto looking at dietary interactions between diet, N isotopic fractionation and N partitioning in Section 6.2 and 6.3.

6.1. Altering nitrogen metabolism and partitioning in ruminants

Increasing dietary water soluble carbohydrate (WSC) to N ratio (WSC/N) can alter N metabolism within the ruminant body, which may improve N-use efficiency (NUE; milk N output/N intake), reduce urinary N output as a proportion of N intake (UN/NI) and also re-direct urinary N output to faecal N output (UN/FN). Studies of lactating cows (Chapter 4) and lactating goats (Chapter 5) explored the manipulation of WSC/N effect on NUE, whilst studies of non-lactating sheep (Chapter 3) and lactating goats (Chapter 5) explored the manipulation of WSC/N effect on UN/NI and UN/FN.

Nitrogen-use efficiency

The NUE of lactating cows (Chapter 4) was between 16-25 %, while a lower and narrower range of NUE (11-15 %) was obtained from lactating goats study (Figure 6.1.1). Castillo et al. (2000) reported NUE ranges between 15-35 % in a review involving almost 100 diets fed to 600 cows.

According to Edwards et al. (2007), an improvement in NUE should be expected when WSC/N is increased from 4.4 to 8.1. The diet of lactating goats (Chapter 5) ranged from 4.3 to 7.5 WSC/N, and that of lactating cows from 6.6 to 8.8 WSC/N. The increase in WSC/N increased NUE of lactating cows by 56 % through both increasing energy intake and milk N output (MN) and decreasing N intake; without changing microbial protein synthesis. On the other hand, an increase in NUE and MN were not shown in the lactating goats study. It is believed the lack of response was mainly due to the high dietary N concentration (on average 4.5 % of DM) and very high WSC concentration (on average 27 % of DM) in conjunction with unchanged DMI, compared with the lactating cows study. Pacheco et al. (2007) reported no improvement in NUE when dietary N concentration was above 3.7 % of DM. Broderick and Clayton (1997) proposed this may be because the high dietary N concentration exceeded the limit for rumen microbes to capture N for microbial protein synthesis; which is supported by the small changes in a commonly used MCP synthesis indicator (urinary excretion of purine derivatives; PD) and the calculated yield of MCP production per unit of FME supplied (MCP/FME) in the current studies. Furthermore, an *in vitro* study demonstrated that there may be a concentration of WSC (24 %) that optimises rumen fermentation and animal production

regardless of changes in WSC/N (Burke et al., 2011); possibility due to MCP synthesis in the rumen being reduced by acidosis from feeding high levels of WSC (Owens et al., 1998). The energy from feed that is used for maintenance and production is ME. If it is assumed the low WSC/N dietary treatment from lactating goats contained 11 MJ ME/kgDM and all goats were fed a fixed DMI (2 kg DM/d), the increase in dietary WSC concentration from 18.7 to 32.4 %; (WSC is 100 % digestable) from low to high dietary treatment could only increase ME intake by 7 % from 22.0 MJ ME/d to 23.6 MJ ME/d. This simple calculation clearly shows significant increase in WSC concentration in the diet may have little impact on changing ME intake, when DMI is fixed as in lactating goats study. Therefore it is believed that the small change in ME intake may also contributed to the lack of response in NUE in the lactating goats study.

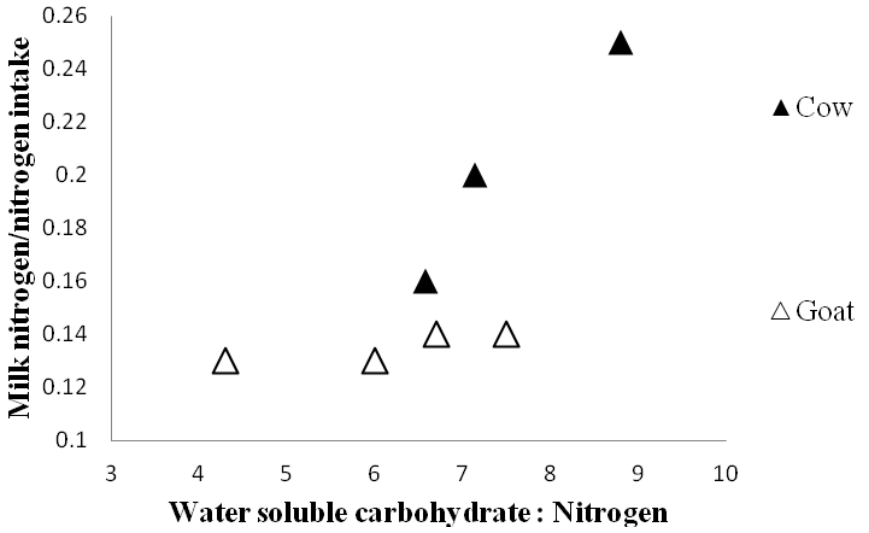

Figure 6.1.1. Effect of dietary manipulation of water soluble carbohydrate to nitrogen ratio on milk nitrogen:nitrogen intake ratio (NUE) in lactating goats and cows

Urinary nitrogen output as a proportion of nitrogen intake

The UN/NI is used as an indicator of how efficiently animals can utilise their dietary N. The higher the UN/NI, the greater the risk of environmental pollution (Edwards et al., 2007). Although more than 50 % of NI was excreted in the urine in both the non-lactating sheep and lactating goats studies, there was no effect on UN/NI from increasing WSC/N

(Figure 6.1.2). As was discussed above (nitrogen-use efficiency section), similar reasons i.e., high dietary N and WSC concentration and fixed DMI, may explain the lack of a dietary effect on UN/NI in the lactating goats study. In the non-lactating sheep study, the levels of dietary N (2.4-3.3 %) and WSC (12-23 %) were relatively low and changes in N partitioning was expected according to Edwards et al. (2007) and Pacheco et al. (2007). However, calculated MCP/FME and PD analysis suggested no improvement in MCP synthesis from the non-lactating sheep fed on increasing levels of WSC/N. This together with the non-significant change in ME intake across dietary treatments, indicated that little change in FME was achieved, therefore, no extra rumen degradable protein (RDP) was captured in the high WSC treatment as MCP production and excess N was lost in the urine (van Vuuren et al., 1993). Furthermore, calculations based on Brookes and Nicol (2007), showed the average NI of the sheep was at least 1.8 times the maintenance N requirement. Therefore, previously identified individual dietary component thresholds (WSC and N as % of DM) and UN/NI response range to WSC/N changes from dairy cows studies, may not be appropriate for assessing the N partitioning response from altering WSC/N in non-lactating sheep. Even if the WSC/N had increased MCP, there would have been no increase in N retention or reduction in UN, as MCP supplies have exceeded MCP demand.

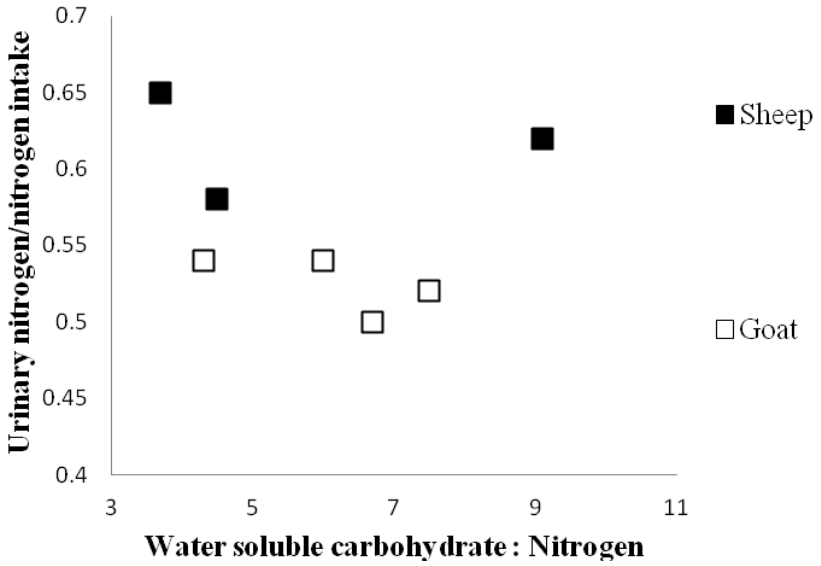

Figure 6.1.2. Dietary manipulation of water soluble carbohydrate to nitrogen ratio effect on urinary nitrogen:nitrogen intake ratio of lactating goats and non-lactating sheep

Redirecting urinary nitrogen output to faecal nitrogen output

To partition surplus N away from urine to faeces, may reduce nitrate leaching to the ground water and ammonia volatilisation to the atmosphere which cause environmental pollution (Varel et al., 1999). As WSC/N increased, decreased UN/FN can be achieved through either decreasing UN, as a function of increasing MCP production (Miller et al., 2001); or increasing FN by increasing DMI (Moorby et al., 2006). The non-significant UN/FN change in both lactating goats study and non-lactating sheep study (Figure 6.1.3) was the net effect of changes in FN and UN. The opposite effect from dry matter digestibility and N digestibility resulted in little changes in non-lactating sheep FN. On the other hand, lack of changes in MCP synthesis resulted in no changes in UN in lactating goats study.

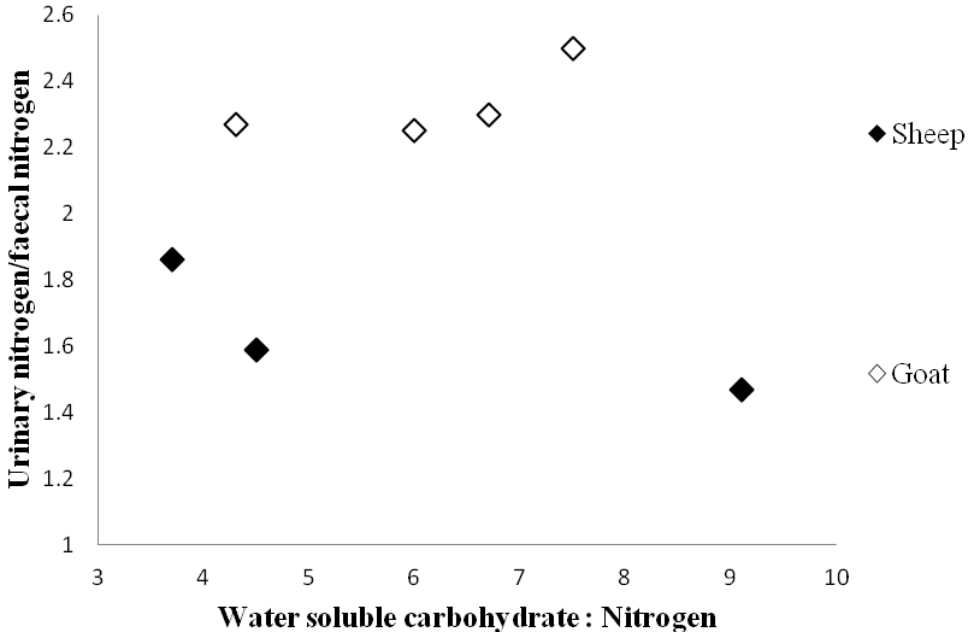

Figure 6.1.3. Dietary manipulation of water soluble carbohydrate to nitrogen ratio effect on urinary nitrogen:faecal nitrogen ratio in lactating goats and non-lactating sheep

6.2. Nitrogen isotopic fractionation

Relative enrichment and depletion of different nitrogen sinks

The range of feed $\delta^{15}N$ from the three studies is comparable to values reported previously (Steele et al., 1983; Cheng et al., 2011). Plasma and milk were enriched (about 5 ‰) in $\delta^{15}N$ compared with feed. Faeces were slightly enriched (about 3 ‰) and urine depleted in $\delta^{15}N$ (about 1 ‰) relative to feed (Table 6.2). These findings agree with the results of others (Koyama et al., 1985; Sutoh et al., 1993; Sponheimer et al., 2003). In addition, the enrichment of $\delta^{15}N$ in wool, liver and muscle from non-lactating sheep study were comparable to that previously reported (Hobson and Clark, 1992; Männel et al., 2007). Sponheimer et al. (2003) suggested that the main reason for the enrichment of $\delta^{15}N$ in faeces is the presence of enriched endogenous material. The majority of N in wool, liver, muscle, milk and plasma exists as true protein, which has been reported to be enriched in $\delta^{15}N$ (Sick et al., 1997; Cheng et al., 2010). In contrast, urea is the main N source of urine and is reported to be depleted in $\delta^{15}N$ (Steele and Daniel, 1978).

Regression analysis demonstrated that urine and faecal $\delta^{15}N$ were linearly related to feed $\delta^{15}N$ (Chapter 5). Milk $\delta^{15}N$ and plasma $\delta^{15}N$ were related to each other (Chapter 4), and muscle $\delta^{15}N$ - feed $\delta^{15}N$ was related to urine $\delta^{15}N$ - feed $\delta^{15}N$ (Chapter 3). It is believed that these observed relationships related to N isotopic fractionation during digestion and absorption of feed components (Sutoh et al., 1993; Wattiaux and Reed, 1995) or a possible common effect of the endogenous N contribution to urine and faeces from feed (Cheng et al., 2011). Although Hobson et al. (1993) and Sponheimer et al. (2003) suggested that body tissues with slower N turnover rates (e.g., muscle and wool) are less likely to reflect N isotope changes; compared with active tissues (e.g., liver and plasma) when a feeding study is short, findings from the non-lactating sheep study showed a low correlation between $\delta^{15}N$ of wool and plasma; but a high correlation between $\delta^{15}N$ of muscle and plasma. This might due to the time lag in wool growth or/and the enzymatic reactions involved in capture N in wool discriminated differently from the enzymatic reactions involved in muscle synthesis. In the lactating goats study, the levels of enrichment in body N sinks were generally higher than previously observed (Koyama, 1985; Cheng et al., 2011). It was confirmed that there was a carry-over effect from

infusion ^{15}N enriched urea into blood (Chapter 5); therefore, labelled ^{15}N infused animals should be avoided when examining natural N isotopic fractionation.

Table 6.2. Summary of $\delta^{15}N$ content (‰) of feed, milk, plasma, faeces and urine from literature and current studies with a range of herbivore species

Animal (Reference‡)	Feed	Milk	Plasma	Faeces	Urine
Jersey cows (1)	0.65 (0.60-0.70)	4.30 (4.20-4.30)	-	2.65 (2.30-3.10)	-1.75 (-2.40- -1.40)
Angus steer (1)	0.60 (0.60)	-	4.80 (4.70-4.90)	2.45 (2.10-2.70)	-1.58 (-2.80- -0.60)
Cows (2)	2.55 (1.65-3.05)	5.73 (5.15-6.15)	5.14 (4.75-5.55)	5.07 (4.95-5.15)	2.25 (1.85-2.55)
Dairy cows (3)	3.03 (2.70-3.30)	5.17 (4.90-5.50)	5.40 (5.00-5.80)	3.83 (3.50-4.10)	-1.77 (-2.40- -0.70)
Sheep (4)	0.76 (0.76)	-	-	3.76 (3.59-3.93)	0.64 (0.63-0.65)
Llamas (5)	0.40 (0.40)	-	-	3.30 (2.90-3.50)	0.10 (-0.20-0.60)
Dairy cows (6)	4.17 (1.82-8.38)	6.75 (5.24-9.21)	-	5.71 (4.20-8.76)	0.87 (-1.42-4.09)
Dairy cows (Chapter 4)	2.88 (2.55-3.25)	6.63 (6.59-6.68)	6.56 (6.44-6.67)	6.03 (5.78-6.17)	-
Sheep (Chapter 3)	0.05 (0.00-0.13)	- -	5.69 (5.62-5.76)	3.21 (3.04-3.47)	-1.40 (-1.46 - -1.36)
Goats (Chapter 5)	2.38 (1.90-2.84)	7.74 (7.11-8.29)	7.72 (6.92-8.01)	4.55 (4.01-5.03)	1.80 (1.22-2.20)

‡ 1. Steele and Daniel, 1978; 2. Koyama et al., 1985; 3. Sutoh et al., 1987; 4. Sutoh et al., 1993; 5. Sponheimer et al., 2003; 6. Cheng et al., 2011.

6.3. Nitrogen isotopic fractionation in relation to partitioning

Can nitrogen isotopic fractionation reflect nitrogen partitioning or nitrogen use efficiency?

Sick et al. (1997) suggested urea and protein from plasma are respectively less depleted and more enriched in $\delta^{15}N$ compared with feed when N intake increases. In general, NUE and UN/NI are negatively and positively related to N intake of the animal (Castillo et al., 2001; Cheng et al., 2011). Furthermore, body N (e.g., milk, plasma or muscle) and urinary N are mainly derived from plasma amino acids and plasma urea pools respectively (Young, 1970; Sick et al., 1997). Consequently, the data (Table 6.3) provided support for negative relationships between muscle enrichment (muscle $\delta^{15}N$ - feed $\delta^{15}N$) and retained N/N intake in non-lactating sheep study, (milk $\delta^{15}N$ - feed $\delta^{15}N$) and (plasma $\delta^{15}N$ - feed $\delta^{15}N$) and NUE in lactating cows study. In addition, a positive relationship between UN/NI and urine depletion (urine $\delta^{15}N$ - feed $\delta^{15}N$) in non-lactating sheep study. These relationships reflected an association between dietary N digestion/metabolism and N isotopic fractionation within ruminant body (Sutoh et al., 1993; Parker et al., 1995). Compared with the traditional N utilisation indicators (NI, BUN and MUN) measured in current lactating cow and non-lactating sheep study, $\delta^{15}N$ predicted NUE and UN/NI with a better accuracy in the same study. However, due to the narrow NUE range from lactating goats study, no such comparison was allowed to be made.

It is important to note that the accuracy (r^2) of using $\delta^{15}N$ to predict N partitioning for sheep and cow studies was varied, and no relationship was established for goats study. There are two possible reasons resulted in prediction variations. Firstly, the cause of fractionation was different, namely the fractionation in lactating cow study was due to dietary effect, whilst the non-lactating sheep study was due to possible animal variation in N metabolism. Secondly, the better prediction accuracy using $\delta^{15}N$ in lactating cow study may be achieved through a larger variation in NUE compared with a narrower range of UN/NI was generated from non-lactating sheep study. No relationship was established for lactating goats study was mainly due to narrow range of NUE being generated and no fractionation was likely occurred at rumen level when MCP was not significantly improved through adding WSC in the diet. Overall, current established prediction

equations should be extrapolated further with cautions, as different feeding system and animal variation may well change the prediction accuracy in different studies.

What drives these relationships?

There are two plausible sites (i.e., liver and rumen) of N isotopic fractionation that may be related to N partitioning. Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein results in a depletion of ^{15}N in microbial protein, which was not observed when microbial protein was synthesised from amino acids. Since bacterial protein would be the major source of amino acids for milk, plasma and muscle protein synthesis with the diets used in the current studies, it is unlikely that there was an increase in microbial protein synthesis when N intake was high in both lactating cows and non-lactating sheep studies. In addition, the possible complication from urea recycling is unlikely to important since the level of urea recycled from dietary N is expected to be low when N intake is greater than requirements (Reynolds and Kristensen, 2008). Thus, under the condition of these studies, it is likely that the relationships between N isotopic fractionation and N partitioning were mainly driven by deamination or transamination in the liver, as suggested by other workers (Macko et al., 1986; Parker et al., 1995) and similar to the fractionation reported by Sick et al. (1997) for rats fed on a range of protein sources.

Table 6.3. Simple linear relationship between nitrogen partitioning and nitrogen isotopic fractionation

Animal type	Equation	r^2	P
Lactating cows	Milk $\delta^{15}N$ -feed $\delta^{15}N$ (‰) = -0.0827 × NUE (%) + 5.46	0.83	< 0.001
Lactating cows	Plasma $\delta^{15}N$ -feed $\delta^{15}N$ (‰) = -0.1001 × NUE (%) + 5.75	0.85	< 0.001
Non-lactating sheep	Urinary nitrogen/nitrogen intake (g/g) = 0.25 × urine $\delta^{15}N$ - feed $\delta^{15}N$ (‰) + 0.98	0.43	< 0.05
Non-lactating sheep	Retained nitrogen/nitrogen intake (g/g) = - 0.23 × muscle $\delta^{15}N$ - feed $\delta^{15}N$ (‰) + 1.33	0.43	< 0.05

6.4. Lessons learnt

Manipulate dietary composition without changing $\delta^{15}N$ of feed

Keeping feed $\delta^{15}N$ similar across diets, but changing dietary composition is a useful model to test for a dietary effect on N isotopic fractionation which can be assessed by comparing the enrichment and depletion of different N sinks (e.g., milk, urine and faeces). However current PhD studies and other work (e.g., Sutoh et al., 1993; Wattiaux and Reed, 1995) showed that keeping feed $\delta^{15}N$ similar across diets is difficult particularly when changing dietary N levels. This is due to different forms of N in the diet have various $\delta^{15}N$ levels. For example, true protein generally contains a higher $\delta^{15}N$ compared with non-protein N (e.g., urea) (Steele and Daniel, 1978; Sick et al., 1997; Cheng et al., 2010). Legumes contain a lower $\delta^{15}N$ in comparison to grasses due to N fixation from ^{15}N depleted N_2 from atmosphere (Steele and Daniel, 1978). Because of the variation in feed $\delta^{15}N$, N isotopic fractionation between different N sinks in the body is normally expressed by subtracting feed $\delta^{15}N$ (Hobson et al., 1993; Sutoh et al., 1993). It is arguable that the ratio of $\delta^{15}N$ between sinks may be a better measurement of the relative enrichment and depletion between N sinks when feed $\delta^{15}N$ is unchanged across diets but the lactating goats study did not provide evidence for using the ratio of $\delta^{15}N$ from different N sinks.

Nitrogen isotopic fractionation in rumen

Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein resulted in a depletion of ^{15}N in microbial protein, which was not observed when microbial protein was synthesised from amino acids. The fact of no changes in ^{15}N from synthesising amino acids into microbial protein does not mean no fractionation happened; perhaps the fractionation went one way when amino acids are broken down to ammonia and then reversed when the ammonia was re-incorporated into microbial protein, lead to no apparent fractionation. Therefore the net effect on ^{15}N of varies N sinks is minimal and may due to the cancellation of anabolic and catabolic fractionation processes in the rumen.

Accuracy and robustness of using nitrogen fractionation to indicate partitioning

A representative sample for $\delta^{15}N$ measurement is important; this ensures the results is reproducible and with acceptable level of standard deviation (< 0.3 ‰). Dried and finely ground sample is found to produce more precise results than using liquid sample, possibly due to higher level of homogenisation. The depletion and enrichment of the measured samples compared with its feed is more than 0.5 ‰ (Table 6.2), which is considered as having sufficient level of fractionation for mass spectrometer to detect the difference. However, caution is needed to interpret data with low standard deviation and explanation is required to show the depletion is due to fractionation rather than measurement variation.

Although current studies illustrated the potential of using isotopic fractionation to indicate N partitioning, care is needed when extrapolating the established prediction equations further. The robustness of using the technique should be better determined across different feeding systems (e.g., high vs low N intake, source of N, TMR vs pasture based system), as well as including animal differences (type of animal, breed of animal and genetic potential of the animal).

Conclusions

Factors that may contributed to the lack of a dietary effect (WSC/N) on N partitioning when dietary N or WSC is high

- No change in dry matter intake and metabolisable energy intake. Such as at a constant ME (or FME intake) and excess RDP, MCP yield is not going to increase, therefore there will be no redistribution of extra N to MCP from amino acids and therefore no net change in the $\delta^{15}N$ absorbed to provide for milk or body tissue sinks with no change in microbial protein synthesis.

- Acidosis in the rumen/sub clinic ruminal acidosis with very high levels of WSC may reduce feed intake and thus no improvement in NUE can be expected (subject to further investigation).

Nitrogen isotopic fractionation indicated N partitioning and NUE

- N-use efficiency in dairy cows was predicted reasonable accurately by N isotopic fractionation mainly because DMI increased with increasing WSC/N ratios

- However, when DMI was fixed, as in the non-lactating sheep N isotopic fractionation was less effective in predicting N partitioning.

Future research on the accuracy of using N isotopic fractionation predicting N partitioning at different feeding system (preferably using autumn dairy pasture with graded levels of sugar added to generate difference in NUE, as autumn pasture is known to be low in WSC and high in RDP); animal variations contribute to changes in N isotopic fractionation; and contributions from different body parts (e.g., rumen vs liver vs intestines) are needed to provide full understanding of using N isotopic fractionation in various ruminant production.

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1. Cheng, L. Kim, E, J. Merry, R. J. and Dewhurst, R. J. (2011). Nitrogen partitioning and isotopic fractionation in dairy cows consuming diets based on a range of contrasting forages. J. Dairy. Sci. 94: 2031-2041.

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Nitrogen partitioning and isotopic fractionation in dairy cows consuming diets based on a range of contrasting forages

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ABSTRACT

Nine multiparous Holstein-Friesian cows (initially 97 d in milk), were used in a 3 × 3 lattice square design experiment with 4-wk periods. All cows received 4 kg/d concentrates and dietary treatments were based on silages offered ad libitum: perennial ryegrass (PRG); timothy (TIM); tall fescue (TF); red clover (RC); red clover/corn silage mixture [40/60 on a dry matter (DM) basis; RCC]; red clover/whole-crop oat silage mixture (40/60 on a DM basis; RCO); or red clover/whole-crop oat silage mixture (25/75 on a DM basis; ORC). The remaining treatments were based on RCO with feed intake restricted to the level of PRG (RCOr) or with a low protein concentrate (50/50 mixture of barley and molassed sugar beet pulp; RCOlp). Experiment objectives were to evaluate diet effects on N partitioning and N isotopic fractionation. Yields of milk and milk protein were consistently high for diets RC, RCC, and RCO and low for the diets based on poorly ensiled grass silages. Restriction of intake (RCOr) and inclusion of a higher proportion of whole-crop oat silage (ORC) and the low-protein concentrate (RCOlp) led to some loss of production. Diet had little effect on milk fat, protein, and lactose concentrations: low concentrations of milk protein and lactose reflect the restricted energy intakes for all treatments. The highest diet digestibilities were measured for RC and PRG, whereas increasing inclusion of the whole-crop oat silage (0, 60, and 75% of forage DM) led to a marked decrease in diet digestibility (0.717, 0.624, and 0.574 g/g, respectively). Urinary excretion of purine derivatives, an indicator for rumen microbial protein synthesis, was significantly higher for RCC than for TIM and TF. Nitrogen intake ranged between 359 and 626 g/d (treatment means). Partitioning of N intake to feces and urine was closely related to N intake, although urinary N losses were less than predicted from N intake for the 60/40 mixtures of cereal silage and red clover silage. The ^{15}N content of milk, urine, and feces were all influenced by diet ^{15}N content. Isotopic fractionation meant that feces and milk were enriched and urine was depleted in ^{15}N relative to the diet. Significant relationships were observed between the extent of enrichment of urine, feces, and milk, suggesting some commonality in fractionation pathways. The trend for the lowest ^{15}N enrichment in milk protein occurring in diets with low N-use efficiency (milk N/feed N) was contrary to expectations, possibly because of endogenous contributions to milk protein or fractionation when dietary ammonia was incorporated into microbial protein.

Key words: dairy cow, nitrogen partitioning, nitrogen-15, isotopic fractionation

INTRODUCTION

Adequate dietary N is required to maximize production and profitability of dairying (Pfeffer and Hristov, 2005). It is important to feed the correct level and type of N to maximize conversion to product N and avoid increasing levels of potential N pollutants (urinary N leads to nitrates in water and nitrous oxide losses to the atmosphere). Nitrogen-use efficiency (**NUE**) is the efficiency of converting feed N into product N (in this case, milk) and can be assessed using the N balance (**NB**) technique. However, the NB technique is difficult to run and tends to overestimate N retention (MacRae et al., 1993; Spanghero and Kowalski, 1997).

Many previous studies have evaluated N partitioning with a wide range of diets, and overall relationships, such as that between N intake and urinary N, have been described by assembling results from many studies (e.g., Kebreab et al., 2001; Huhtanen et al., 2008). Dewhurst et al. (2010) showed that these relationships might differ between diets based on ryegrass silage or mixtures of red clover and corn silages. The first objective of this study was a further evaluation of these relationships across diets based on grass silages from different species and mixtures of red clover silage with both corn silage and whole-crop oat silage.

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The second objective of this work was to develop simpler approaches to evaluate NUE, based on sampling that could be conducted without the need to house animals in metabolism stalls. Earlier work has used measurements of milk urea N for this purpose, and although problems with analytical methods have occurred (Broderick, 2003), strong correlations with urinary N (Nousiainen et al., 2004; Wattiaux and Karg, 2004) and NUE (Nousiainen et al., 2004) have been established. This work evaluated an alternative approach based on the phenomenon of N isotopic fractionation (isotopic discrimination), whereby some biochemical pathways discriminate between ^{14}N and ^{15}N as a result of the mass difference, resulting in differential enrichment of ^{15}N, with protein enriched and urine depleted relative to the diet (DeNiro and Epstein, 1981; Sutoh et al., 1987). Sick et al. (1997) demonstrated relationships between amino acid utilization and isotopic fractionation in the liver of rats fed different proteins. Sponheimer et al. (2003) provided preliminary evidence of increased enrichment of ^{15}N in hair protein when ruminants were fed high protein diets. However, the situation is likely to be more complex in ruminants, because synthesis of protein by rumen microorganisms is an additional potential site of isotopic fractionation (Wattiaux and Reed, 1995). The wide range of N intake and the diversity of diets used in this study provide a good basis to evaluate the range of N isotopic fractionation in ruminants.

MATERIALS AND METHODS

This study used 9 multiparous Holstein-Friesian cows that were, on average, 97 (SD = 6.8) DIM and producing 24.3 (SD = 4.87) kg/d of milk at the start of the experiment. Cows weighed 611 (SD = 57.1), 609 (SD = 51.7), and 597 (SD = 63.1) kg at the start of the 3 collection periods, respectively. Nine dietary treatments were evaluated in a 3 × 3 lattice square design (Cochran and Cox, 1950) experiment with 3 replicates; treatments were grouped 1 to 3, 4 to 6, and 7 to 9 for allocation to the squares. Animal measurements were made in the final wk of each 4-wk period.

Diets

Pure stands of grasses, red clover, and corn were grown at Trawsgoed Research Farm (52°25′N, 4°5′W), and winter oats were grown at Bow Street, Ceredigion (52°26′N, 4°1′W). Perennial ryegrass (cultivar 'AberDart'), timothy (cultivar 'Promesse'), and tall fescue (cultivar 'Excella') were sown on April 20, 2004, and primary growth was harvested on August 5, 2004 (vegetative growth stage). Primary growth from red clover (cultivar 'Milvus') established in 2003 was harvested on May 19, 2004 (early bud growth stage). Slurry was applied to the grass areas before cultivation and the stands received 250 kg/ha of 27-5-5 (N-P-K) fertilizer on June 17, 2004. The red clover stand received a winter application of 250 kg/ha of triple super phosphate and 250 kg/ha of muriate of potash, but no N fertilizer. The grasses and red clover were wilted for 24 h and ensiled as round bales using Powerstart microbial inoculant (Genus Breeding Ltd., Nantwich, UK) applied according to the manufacturer's instructions. Whole-crop winter oats (cultivar 'Gerald') at the early-dough stage was precision-chopped and ensiled in a bunker silo, using Powerstart additive according to the manufacturer's instructions, on July 13, 2004. Corn (cultivar 'Calypso') at the mid-dough stage was precision-chopped and ensiled in bunker silos without the use of an additive.

All cows were fed a flat rate of 4 kg/d of standard dairy concentrates, apart from 1 treatment that used a low-protein concentrate mix based on 2 kg of rolled barley and 2 kg of molassed sugar beet pulp. The dairy concentrate contained (g/kg on an air-dry basis): wheat (240), beans (200), rapeseed meal (175), corn distillers grains (150), soybean meal (50), palm kernel expeller (44), molasses (100), vegetable oil (21), and mineral/vitamin premix (20). All cows received 100 g/d of a mineral-vitamin mixture (Maxcare Phos Mag + Cuprex 5, Trouw (UK) Ltd., Northwich, UK) that contained calcium 13.5%, sodium 10%, magnesium 10%, phosphorus 8%, vitamin A 400,000 IU/kg, vitamin D_3 80,000 IU/kg, vitamin E 800 IU/kg, cobalt 160 mg/kg, manganese 4,000 mg/kg, copper 2,500 mg/kg, zinc 3,000 mg/kg, iodine 400 mg/kg, and selenium 35 mg/kg. The cows also had access to mineralized salt licks (Red Rockies, Rockies, Winsford, UK).

In addition to the concentrates, the dietary treatments were as follows: (1) perennial ryegrass silage ad libitum (**PRG**); (2) timothy silage ad libitum (**TIM**); (3) tall fescue silage ad libitum (**TF**); (4) red clover silage ad libitum (**RC**); (5) red clover silage/corn silage mixture (40% red clover on a DM basis) ad libitum (**RCC**); (6) red clover silage/whole-crop oat silage mixture (40% red clover on a DM basis) ad libitum (**RCO**); (7) red clover silage/whole-crop oat silage mixture (25% red clover on a DM basis) ad libitum (**ORC**); (8) red clover silage/whole-crop oat silage mixture (40% red clover on a DM basis), feed intake restricted to a level comparable to treatment 1 (**RCOr**); and (9) red clover silage/whole-crop oat silage mixture (40% red clover on

a DM basis) ad libitum, with 4 kg/d of the low-protein concentrate mixture instead of standard concentrates (**RCOlp**).

Animal Measurements

This work was carried out under the authority of licenses issued by the UK Home Office. Feed intakes were recorded throughout the collection week, with amounts of forage offered adjusted to ensure 10% refusals. Daily feed samples were composited over the collection week. Milk yields were recorded twice daily using mechanical milk meters (Tru-Test Ltd., Auckland, New Zealand) and samples for analysis taken from 4 consecutive milkings.

Separate total collections of feces and urine were made over 6 consecutive days using the externally applied urine separators described by Aston et al. (1998). Fecal and urine outputs lagged 1 d behind the measurement of intake. One day before collection commenced, a separator mounting (Velcro, Selectus Ltd., Stoke-on-Trent, UK) was attached around the vulva and anus with contact adhesive (Evo Stik, Evode Ltd., Stafford, UK). On the following day, a urine collector fitted with 1.5 m of flexible hose was placed over the vulva and fastened to the separator mounting. Urine was retained in a 25-L plastic container positioned in a drainage channel behind the cow. Urine was preserved during collection by adding 2 M H_2SO_4 into the container to maintain pH between 2 and 3 (approximately 2.8 L/cow per day, in 2 portions). To collect feces, a 350-mm-wide flexible plastic chute was secured by 4 elasticated straps attached to a girth band on the cow and supported with 2 straps fastened to a tubular framework at the rear of the stall. Fecal output was directed by the chute into a container suspended in the drainage channel. At the end of each collection period, bulked samples of feed, feces, and urine from individual cow were mixed, subsampled, and stored at –20°C. A further subsample of urine was diluted (1 volume urine plus 4 volumes of water) before freezing and used for analysis of purine derivatives.

Analytical Methods

Analytical methods used for feed have been described previously (Dewhurst et al., 2000). Urine was analyzed for N concentration using a Leco FP428 Nitrogen Analyzer (Leco Instruments (UK) Ltd., Stockport, UK), and feces were analyzed, without drying, using a Kjeldahl method. Milk samples were analyzed for fat, protein, and lactose concentrations using an infrared milk analyzer (NMR Central Laboratory, Somerset, UK). The diluted urine samples were analyzed for purine derivatives (**PD**; allantoin plus uric acid) using the HPLC method of Dewhurst et al. (1996).

Sample Preparation and ^{15}N Measurements

Samples of feeds, feces, and milk were freeze-dried and ground before N isotope measurements, and urine was analyzed in liquid form. Duplicates of each freeze-dried sample were weighed into 5 × 8 mm tin capsules; the sample size was calculated to supply 100 to 200 μg of nitrogen. Duplicate 10-μL aliquots of each urine sample were pipetted into 5 × 8 mm tin capsules containing an absorptive bed of Chromosorb W (Supelco, Bellefonte, PA). Nitrogen isotope measurements were made using a continuous-flow isotope ratio mass spectrometer (PDZ Europa Ltd., Crewe, UK) and results are expressed in delta units ($\boldsymbol{\delta^{15}N}$, ‰); that is, the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air). The ^{15}N content of digested N was calculated according to Yoneyama et al. (1983):

$$\text{Digested } \delta^{15}\text{N} = [(\text{N intake}) \times (\delta^{15}\text{N of feed}) - (\text{N output in feces}) \times (\delta^{15}\text{N of feces})]/[\text{N intake} - \text{N output in feces}].$$

Statistical Analysis

The Genstat statistical package (version 10; Lawes Agricultural Trust, Rothamsted, UK) was used for ANOVA and linear regression analysis. Data were combined into single mean values for each period and dietary treatment. The overall ANOVA used the REML (linear mixed model) directive with cow + period as the random model and dietary treatment as the fixed model. Treatment means were compared using a Student-Newman-Keuls multiple comparison test.

RESULTS

Feed Composition

Feed analysis is presented in Table 1. The mean CP content of sugar beet pulp and barley grain (117.8 g/kg of DM) was less than half the CP content of the standard dairy concentrate (251.2 g/kg of DM). The forages covered an extremely wide range of CP content (58 to 226 g/kg of DM), as well as fermentation quality, with the grass silages having poor fermentation quality and very high levels of ammonia-N.

Table 2 provides calculated chemical composition for the overall diets. This shows the wide range of CP (130 to 231 g/kg of DM) and starch (35 to 159 g/kg of DM) concentrations across this set of diets, as well as

Table 1. Chemical composition (g/kg of DM, unless stated otherwise) of the diet components used in this study (n = 3 for each feed)

	Diet component								
Item	Concentrates	Sugar beet pulp	Barley grain	Ryegrass silage	Timothy silage	Tall fescue silage	Red clover silage	Corn silage	Whole crop oat silage
DM (g/kg)	866	900	845	248	335	336	205	273	413
OM	919	869	976	867	891	878	888	949	960
CP	251	103	133	226	185	194	206	98.8	57.5
NDF	195	283	326	463	596	570	414	549	651
ADF	123	153	70.6	296	372	332	348	265	341
Water-soluble carbohydrates	121	227	120	28.7	10.1	15.2	9.2	4.3	23.0
Starch	178	ND[1]	611	ND	ND	ND	ND	258	154
Ether extract	ND	ND	ND	42.5	32.9	26.6	30.7	35.3	35.8
Acid hydrolysis ether extract	55.8	9.1	31.5	ND	ND	ND	ND	ND	ND
pH	ND	ND	ND	4.58	5.05	5.24	4.20	3.97	3.92
Ammonia-N (g/kg total-N)	ND	ND	ND	165	206	186	97	95	85
Lactic acid	ND	ND	ND	76.7	35.6	46.3	94.6	52.7	34.1
Acetic acid	ND	ND	ND	31.1	41.3	35.2	30.3	20.1	7.7
Butyric acid	ND	ND	ND	7.8	3.1	3.7	2.4	0.0	0.5
Ethanol	ND	ND	ND	6.6	3.5	2.8	6.2	9.8	12.5

[1]ND = not determined.

the high ammonia-N content of the grass silage-based diets.

Feed Intake and Milk Production

Feed intakes (Table 3) tended to be lower for TF and higher for the mixtures RCC and RCO. The yields of milk and milk protein (Table 3) were consistently high for diets RC, RCC, and RCO and low for the grass silage-based diets, particularly TIM and TF. Restriction of intake (RCOr) and inclusion of a higher proportion of whole-crop oat silage (ORC) led to some loss of production, whereas a greater loss of production occurred when the low-protein concentrate was offered (RCOlp). Diet had little effect on milk fat, protein and lactose concentrations.

Diet Digestibility and Urinary Excretion of PD

The highest diet digestibilities were measured for the PRG and RC diets, with values for TIM, TF, and RCC being only slightly lower (Table 4). Increasing inclusion of the whole-crop oat silage (0, 60, and 75% of forage DM) led to a marked decrease in diet digestibility (0.717, 0.624, and 0.574 g/g, respectively). Urinary excretion of PD was similar for most treatments, but significantly higher for RCC than for TIM and TF (Table 4). Allantoin made up a constant 0.9 (SD = 0.02) mol/mol of PD excretion.

Nitrogen Partitioning

Nitrogen intakes ranged from 359 to 626 g/d across the dietary treatments (Table 5) with a mean value of 495 g/d. The differences in N intake between treatments were strongly related to differences in dietary CP concentration (Table 2). Inclusion of an increasing proportion of whole-crop oat silage and the use of low protein concentrates both reduced N intake. Differences in fecal N (Table 5) closely mirrored differences in N intake. Numerically large differences in urinary N output were observed between the treatments (Table 5),

Table 2. Calculated chemical composition (g/kg of DM, unless stated otherwise) of the overall diets used in this study

	Diet[1]								
Item	PRG	TIM	TF	RC	RCC	RCO	RCOr	RCOlp	ORC
CP	231	198	207	214	169	159	160	130	134
NDF	406	516	483	372	468	489	484	534	504
Starch	37.7	35.3	41.4	33.9	158	85.5	86.7	127	116
Ether extract	45.3	37.5	33.4	35.5	36.6	36.6	36.8	40.3	29.8
Ammonia-N (% of N intake)	13.0	16.5	14.3	7.9	7.9	7.4	7.3	7.2	7.0

[1]Diets based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC).

Table 3. Effects of dietary treatments on feed intake, milk yield, and milk composition

	Dietary treatment[1]										
Item	PRG	TIM	TF	RC	RCC	RCO	RCOr	RCOlp	ORC	SED	Significance
Silage DM intake (kg/d)	12.9	13.9	11.4	14.7	15.7	15.5	14.4	13.7	13.6	1.24	†
Total DM intake (kg/d)	16.3	17.4	14.9	18.2	19.1	19.0	17.8	17.2	17.1	1.24	†
Milk yield (kg/d)	24.4^{bc}	22.9^{abc}	19.5^{a}	26.1^{c}	27.2^{c}	26.1^{c}	25.2^{bc}	20.5^{ab}	24.8^{bc}	1.58	**
Milk fat (%)	4.02	3.86	4.29	3.95	3.89	4.04	3.88	4.39	4.16	0.224	NS
Milk protein (%)	3.03	2.96	2.99	3.03	3.06	3.05	2.91	3.13	3.01	0.096	NS
Milk lactose (%)	4.61^{ab}	4.48^{a}	4.56^{ab}	4.67^{ab}	4.66^{ab}	4.75^{b}	4.66^{ab}	4.68^{ab}	4.67^{ab}	0.060	*
Milk fat (g/d)	988	854	829	1,013	1,050	1,064	970	882	1,009	71.8	*
Milk protein (g/d)	730^{bcd}	657^{abc}	577^{a}	775^{cd}	816^{d}	799^{d}	731^{bcd}	639^{ab}	739^{bcd}	38.9	**
Milk lactose (g/d)	$1,128^{bcd}$	$1,010^{abc}$	885^{a}	$1,217^{cd}$	$1,271^{d}$	$1,246^{cd}$	$1,176^{bcd}$	958^{ab}	$1,163^{bcd}$	78.0	**

[a–d]Means with different superscripts are significantly different at the 5% confidence level (Student-Newman-Keuls test).

[1]Diets based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC).

†$P < 0.1$; *$P < 0.05$; **$P < 0.01$; NS = not significant.

Table 4. Effects of dietary treatments on the digestibility of DM, OM, and N, as well as urinary excretion of purine derivatives

	Dietary treatment[1]										
Item	PRG	TIM	TF	RC	RCC	RCO	RCOr	RCOlp	ORC	SED	Significance
DM digestibility (g/g)	0.724^{c}	0.666^{cd}	0.682^{d}	0.717^{c}	0.687^{cd}	0.624^{b}	0.648^{bc}	0.637^{bc}	0.574^{a}	0.0104	***
OM digestibility (g/g)	0.744^{d}	0.681^{c}	0.689^{c}	0.730^{d}	0.695^{c}	0.627^{b}	0.651^{b}	0.639^{b}	0.579^{a}	0.0119	***
N digestibility (g/g)	0.706	0.701	0.703	0.691	0.674	0.692	0.672	0.619	0.613	0.0276	*
Urinary allantoin (mmol/d)	260^{ab}	201^{a}	200^{a}	259^{ab}	312^{b}	275^{ab}	254^{ab}	261^{ab}	276^{ab}	23.4	**
Urinary uric acid (mmol/d)	33.2	25.3	26.1	29.4	33.2	26.5	27.8	28.6	31.2	2.37	†
Urinary purine derivatives (mmol/d)	292^{ab}	227^{a}	226^{a}	290^{ab}	344^{b}	300^{ab}	282^{ab}	289^{ab}	308^{ab}	24.4	*

[a–c]Means with different superscripts are significantly different at the 5% confidence level (Student-Newman-Keuls test).

[1]Diets based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC).

†$P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ranging from values that were almost as low as milk N for diets RCOlp and ORC to values that were over 2.5 times milk N for the grass silage-based diets. On average, cows retained 21.8 g of N/d and this was not significantly different across treatments owing to the large variation in these estimates.

N-Isotopic Fractionation

Results of ^{15}N analysis in feed, feces, milk, and urine, as well as the calculated ^{15}N content in digested N are shown in Table 6. Highly significant differences were observed in feed ^{15}N and, although feces and milk were generally enriched and urine was depleted relative to the diet, the diet pattern was still evident in their ^{15}N content. Because of the diet differences, we have also presented deviations of milk and urine ^{15}N content from dietary (or digested N) values. These latter values facilitate comparison between diets of the amount of isotopic fractionation within our cows.

Weak but significant relationships were observed between ($\delta^{15}N$ urine – $\delta^{15}N$ feed) and ($\delta^{15}N$ fecal – $\delta^{15}N$ feed) ($r^2 = 0.26$; $P < 0.01$; n = 27) and between ($\delta^{15}N$ urine – $\delta^{15}N$ feed) and ($\delta^{15}N$ milk – $\delta^{15}N$ feed) ($r^2 = 0.29$; $P < 0.01$; n = 27). A much stronger relationship was observed between ($\delta^{15}N$ fecal – $\delta^{15}N$ feed) and ($\delta^{15}N$ milk – $\delta^{15}N$ feed) ($r^2 = 0.73$; $P < 0.001$; n = 27).

DISCUSSION

Feed Composition

The grass silages used in this study were wilted to 250 to 330 g of DM/kg, which partially restricted fermentation. Nonetheless, the fermentation quality of these silages was poor, with pH greater than 4.5, appreciable levels of acetic and butyric acids, as well as a particularly high level of protein breakdown (ammonia-N). The quality of the grass silages was poor considering that an inoculant providing high numbers of homofermentative lactic acid bacteria was used. The most likely explanation is a shortfall in the amount of water-soluble carbohydrate (**WSC**) available to fuel the rapid fermentation and pH decline required to stabilize the silages, as even inoculant lactic acid bacteria cannot function effectively when substrate is limiting. Low WSC values are common in timothy and fescue grasses (McDonald et al., 1991) and high N fertilizer use also leads to high N and low WSC contents (White, 1973). Unfortunately, the substrate limitation hypothesis cannot be confirmed because values for herbage WSC content are not available, although the high N content of the grass silages is consistent with this effect.

The red clover and corn silages were more typical of these materials in the United Kingdom, with pH and ammonia-N relatively low for the red clover silage (Dewhurst et al., 2003) and the corn silage starch content relatively high (Kirkland and Patterson, 2006). Whole-crop oat silage is not common in the United Kingdom; the material used for this study contained more NDF and starch and less CP than spring-sown oat silages used in Canada (Khorasani et al., 1993, 1997). The starch content of the current whole-crop oat silage was similar to the early dough-stage silage prepared by Wallsten et al. (2010).

Milk Production and Composition

The relatively low replication in this study meant that numerically large differences in DM intake were only evident as an overall trend ($P < 0.1$). Nonetheless, the lowest intake for TF silage is consistent with the high content of NDF and particularly poor fermentation characteristics of this silage. The high intakes for RCC and RCO were also consistent with the high quality of the component forages, as well as previous experience with mixtures of red clover and corn silage (Dewhurst et al., 2010). These differences between dietary treatments were more evident in yields of milk and milk components. The low yields for grass silage-based diets reflect the low feed intake rather than diet digestibility, which was generally high for these diets.

Milk protein and lactose contents were low for all treatments, particularly TIM and TF. This suggests a general lack of dietary energy (Sutton, 1989) because of the low level of concentrates offered, the low intakes of the grass silages, and the low digestibility of the whole-crop oat silage.

Diet Digestibility and Urinary Excretion of PD

The forage proportion of DM intake only ranged from 0.767 (TF) to 0.819 (RCC), despite the use of a flat rate of concentrates and ad libitum forages. Consequently, the diet digestibilities largely reflect differences between the forages. Digestibilities were highest for the grass and red clover silage, reflecting their comparative immaturity as evidenced by relatively high protein and low NDF contents (Dewhurst et al., 2003). The digestibility of red clover-based diets was reduced slightly by inclusion of corn silage, and substantially by the inclusion of whole-crop oat silage. Despite containing 154 g of starch/kg of DM, this silage was relatively mature with 651 g of NDF/kg of DM; the digestibility of NDF in mature whole-crop oats is low (Wallsten et al., 2010).

Table 5. Effects of dietary treatments on nitrogen intake and outputs (g/d)

	Dietary treatment[1]										
Item	PRG	TIM	TF	RC	RCC	RCO	RCOr	RCOlp	ORC	SED	Significance
N intake	605^{c}	547^{bc}	494^{b}	626^{c}	512^{b}	473^{b}	464^{b}	379^{a}	359^{a}	36.4	***
Fecal N	175	163	151	193	166	148	149	141	139	15.6	†
Milk N	115^{bcd}	103^{abc}	90.5^{a}	121^{cd}	128^{d}	125^{d}	115^{bcd}	100^{ab}	116^{bcd}	6.10	**
Urine N	302^{c}	259^{c}	256^{c}	275^{c}	181^{b}	148^{ab}	162^{ab}	117^{a}	122^{a}	18.8	***
Retained N	21	16	13	35	44	51	39	17	−21	29.8	NS

$^{a-d}$Means with different superscripts are significantly different at the 5% confidence level (Student-Newman-Keuls test).

[1]Diets based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC).

†$P < 0.1$; **$P < 0.01$; ***$P < 0.001$; NS = not significant.

Tas and Susenbeth (2007) confirmed the strong relationship between urinary PD excretion and the duodenal flow of purine bases, which are mainly derived from rumen microorganisms. Urinary excretion of PD (mmol/d) was high for diet RCC and low for TIM and TF. It seems likely that variation in the level and composition of feed intake affects rumen microbial yield through the availability of substrate for rumen fermentation. However, variation also existed in PD excretion per kilogram of DM intake, indicative of variation in microbial energetic efficiency. The low values for TIM, TF, and RCOr may be related to low rumen passage rates increasing rumen residence time and so the proportion of energy used for microbial maintenance (Agricultural and Food Research Council, 1992).

Nitrogen Partitioning

Although the mean N retention in this study (21.8 g/d) was close to the corrected mean value (20.6 g/d) reported by Spanghero and Kowalski (1997), it implies lean tissue growth around 0.5 kg/d, which seems unlikely for cows in early to mid lactation. This discrepancy illustrates the difficulties of the NB technique and its tendency to overestimate N retention, even when well conducted.

This study generated a very wide range of N intakes across divergent diets and so is useful to evaluate relationships between N intake and N outputs. Taking individual values, no significant relationship was observed between N intake and milk N output. A highly significant increase was observed in fecal N in response to increasing N intake, with approximately 21% of additional N partitioned to feces (equation [1]):

$$\text{fecal N (g/d)} = 54.3\ (\text{SE} = 13.6) + 0.21\ (\text{SE} = 0.0270)\ \text{N intake (g/d)} \quad [1]$$

$$(n = 27;\ r^2 = 0.70;\ P < 0.001).$$

Approximately 60% of additional N intake was partitioned to urine (equation [2]):

$$\text{urinary N (g/d)} = -91.4\ (\text{SE} = 38.0) + 0.59\ (\text{SE} = 0.0753)\ \text{N intake (g/d)} \quad [2]$$

$$(n = 27;\ r^2 = 0.70;\ P < 0.001).$$

The relationship between N intake and urinary N was explored further by comparing treatment means with the relationships established by Kebreab et al. (2001) and Huhtanen et al. (2008). Most of the studies in these meta-analyses used grass silage-based diets. Kebreab et al. (2001) suggested some curvilinearity in the relationship, with a slightly higher proportion of N apportioned to urine at higher N intakes. These relationships and the current results were in good overall agreement (Figure 1).

Urinary N was greater than predicted for TF, suggesting that low energy intake, low microbial protein synthesis, or both led to some inefficiency with this diet. Urinary N was less than predicted for RC and the diets based on 40/60 mixtures of red clover silage with corn silage or whole-crop oat silage. This is consistent with earlier results with red clover silage (Dewhurst et al., 2003) or mixtures of red clover silage and corn silage (Dewhurst et al., 2010). Moving N excretion from urine to feces is valuable because urine N is more likely to lead to ammonia volatilization and nitrate leaching (Castillo et al., 2000). Interestingly, the reduced urinary N relative to prediction was not evident for the low protein diets ORC and RCOlp where urea recycling would be a more important component of N utilization.

Nitrogen Isotopic Fractionation

The $\delta^{15}N$ of grass silage-based diets was higher than that of the diets based on red clover silage (mean 5‰

Table 6. Effects of dietary treatments on $\delta^{15}N$ contents of feed, feces, urine, and milk, as well as the differences in $\delta^{15}N$ content of these fractions[1]

	Dietary treatment[2]										
Item	PRG	TM	TF	RC	RCC	RCO	RCOr	RCOlp	ORC	SED	Significance
Feed $\delta^{15}N$	6.59^{f}	8.38^{d}	7.15^{e}	2.27^{a}	4.03^{b}	2.37^{a}	2.29^{a}	2.59^{a}	1.82^{a}	0.348	***
Fecal $\delta^{15}N$	7.95^{g}	8.76^{e}	7.04^{d}	4.27^{a}	5.75^{c}	4.20^{b}	4.62^{b}	4.53^{b}	4.27^{ab}	0.369	***
Milk $\delta^{15}N$	8.67^{h}	9.21^{h}	8.54^{h}	5.84^{a}	6.57^{bc}	5.24^{a}	5.75^{b}	5.65^{b}	5.33^{a}	0.385	***
Urine $\delta^{15}N$	4.00^{g}	3.91^{d}	2.62^{e}	1.01^{b}	0.71^{b}	−1.13^{a}	−0.99^{a}	−1.06^{a}	−1.42^{a}	0.584	***
Digested $\delta^{15}N$	6.05^{g}	8.25^{f}	7.19^{g}	1.53^{b}	3.26^{c}	1.41^{b}	1.49^{b}	1.81^{b}	0.45^{a}	0.419	***
Fecal $\delta^{15}N$ − feed $\delta^{15}N$	1.51^{b}	0.151^{a}	−0.03^{a}	1.69^{b}	1.65^{b}	2.06^{b}	2.40^{bcd}	1.85^{b}	2.61^{d}	0.211	***
Milk $\delta^{15}N$ − feed $\delta^{15}N$	2.06^{bc}	0.81^{a}	1.32^{ab}	3.31^{d}	2.33^{bc}	3.18^{d}	3.40^{d}	3.15^{d}	3.63^{e}	0.391	***
Urine $\delta^{15}N$ − feed $\delta^{15}N$	−2.48^{b}	−4.43^{a}	−4.58^{a}	−1.19^{c}	−3.30^{ab}	−3.43^{ab}	−3.35^{ab}	−3.73^{ab}	−3.12^{ab}	0.496	***
Milk $\delta^{15}N$ − urine $\delta^{15}N$	4.57^{e}	5.32^{ab}	5.79^{b}	4.57^{e}	5.56^{b}	6.82^{c}	6.46^{c}	6.95^{c}	6.81^{c}	0.328	***
Urine $\delta^{15}N$ − digested $\delta^{15}N$	−1.99^{bc}	−4.30^{a}	−4.65^{a}	−0.35^{c}	−2.53^{b}	−2.57^{b}	−2.54^{b}	−2.85^{b}	−1.91^{bc}	0.525	***
Milk $\delta^{15}N$ − digested $\delta^{15}N$	2.68^{b}	0.89^{a}	1.29^{a}	3.99^{g}	3.05^{b}	4.24^{b}	4.49^{b}	4.31^{b}	5.33^{b}	0.397	***

$^{a-h}$Means with different superscripts are significantly different at the 5% confidence level (Student-Newman-Keuls test).

[1]Delta units ($\delta^{15}N$) describe the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air) and are expressed per mil (‰).

[2]Diets based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC).

*** $P < 0.001$.

difference). Although a small N isotopic fractionation was associated with N fixation by legumes (Ledgard, 1989), considerable variation exists in ^{15}N content of feeds, particularly between farms, that is not yet explained (Schwertl et al., 2005). These differences mean that it is most useful to evaluate the ^{15}N content of milk, feces, and urine using deviations from feed values. In agreement with the earlier study with cattle (Sutoh et al., 1987), feces (Sutoh: 0.8‰; this study: 1.5‰) and milk (Sutoh: 2.1‰; this study: 2.9‰) were enriched in ^{15}N relative to the diet, whereas urine was depleted (Sutoh: −4.8‰; this study −3.3‰).

Highly significant ($P < 0.001$) relationships were observed between $\delta^{15}N$ in feed and feces (Figure 2), as well as between $\delta^{15}N$ in feed and digested N (Figure 3). The intercepts for these relationships were significantly different ($P < 0.001$) from zero and slopes were significantly different from 1 ($P < 0.001$). This effect may be related to isotopic fractionation during digestion and absorption or an effect of the endogenous contribution to feces. If the endogenous material has a high $\delta^{15}N$, it would exert a greater effect on the overall $\delta^{15}N$ of feces from the red clover silage-based diets, which had a lower $\delta^{15}N$. It is interesting that the effect was similar for diets RC and RCO, despite the latter containing 25% less CP. If this effect is related to endogenous fecal N, the implication is that endogenous fecal N must have been higher for diet RC than for RCO. This would be contrary to the general observation that endogenous N increases with increasing DM intake (Tamminga et al., 1995).

After correcting for differences in feed values, significant correlations still existed between the ^{15}N content of milk, feces, and urine, although the correlations between milk and urine and feces and urine were only modest ($r^2 = 0.29$ and 0.26, respectively), suggesting that different sources of isotopic fractionation may have been operating. A much stronger relationship between ($\delta^{15}N$ fecal − $\delta^{15}N$ feed) and ($\delta^{15}N$ milk − $\delta^{15}N$ feed) was observed ($r^2 = 0.73$; Figure 4). The most likely cause of this relationship is the common effect of ^{15}N from body reserves—the endogenous component of feces (Ouellet et al., 2002) and the contribution of body protein to milk protein (Wilson et al., 1988).

The second major objective of this work was to evaluate the relationships between N isotope fractionation and NUE. It is expected that fractionation of N isotopes during transamination and deamination reactions leads to increased ^{15}N enrichment in protein when NUE is low (Sick et al., 1997). Sponheimer et al. (2003) provided preliminary evidence for enrichment of ^{15}N in protein (in that case, hair) when ruminants were offered a high protein diet, which would be expected to result in lower NUE. However, we observed an opposite

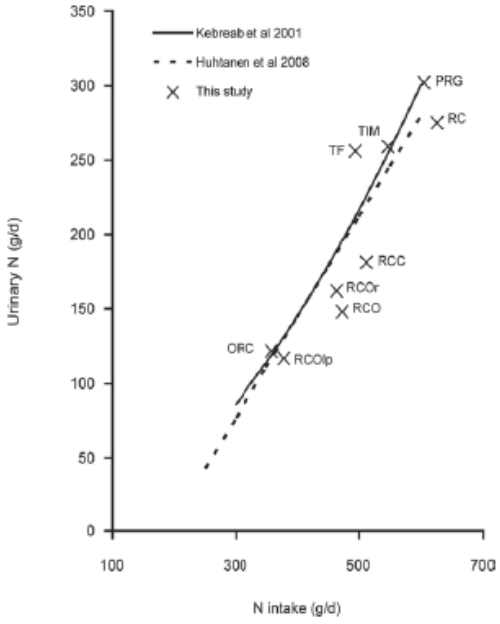

Figure 1. Relationship between N intake (g/d) and urinary N output (g/d) in this experiment and relationships established by previous reviews of the literature (Kebreab et al., 2001; Huhtanen et al., 2008). Values are adjusted treatment means for dietary treatments from this study: PRG = perennial ryegrass silage; TIM = timothy silage; TF = tall fescue silage; RC = red clover silage; RCC = mixture of red clover and corn silages (40/60 on a DM basis); RCO = mixture of red clover and whole-crop oat silages (40/60 on a DM basis); RCOr = RCO with restriction on forage intake; RCOlp = RCO with low protein concentrate; ORC = mixture of red clover and whole-crop oat silages (25/75 on a DM basis).

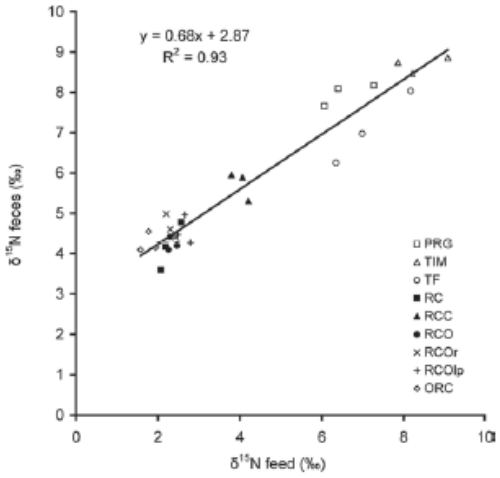

Figure 2. Relationship between $\delta^{15}N$ in feed (‰) and $\delta^{15}N$ in feces (‰) for individual observations. Diets were based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC). Delta units ($\delta^{15}N$) describe the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air) and are expressed per mil (‰).

trend in this study, with the lowest ^{15}N enrichment in milk protein for diets with low NUE (TIM and TF).

Several possible causes exist for the absence of the expected relationship between N isotope fractionation and NUE. It is anticipated that there would be proportionately more synthesis of microbial protein from ammonia, as opposed to amino acids, for the grass silage-based diets because of their high content of ammonia and low starch content (Bryant, 1973). Incorporation of ammonia into rumen bacteria results in depletion of ^{15}N in bacteria (Wattiaux and Reed, 1995), which leads in turn to lower ^{15}N in milk protein. The particularly low ^{15}N enrichment of milk from TF and TIM groups appears difficult to explain given that the urinary excretion of purine derivatives suggests low microbial protein synthesis for these diets. However, these findings are not necessarily inconsistent because a reduction in microbial growth yield (microbial protein produced per mole of ATP) has been associated with increased synthesis from ammonia (Maeng and Baldwin, 1976).

A second potential issue is the effect of $\delta^{15}N$ in mobilized body reserves contributing to milk protein (Wilson et al., 1988); the wide range in the $\delta^{15}N$ of feeds used in this study could easily have biased $\delta^{15}N$ for milk and urine. Differences in the use of N fertilizer between crops may have affected the distribution of N isotopes within diet components (DeNiro and Epstein, 1981), which may further complicate the interpretation of differences in N-isotope fractionation between diets.

CONCLUSIONS

This study confirmed the high intake and milk production potential of diets based on red clover silage, as well as mixtures of red clover silage and cereal silage. A wide range of N intake was recorded across this study, and around 21 and 60% of incremental N was excreted in feces and urine, respectively. Inclusion of 60% cereal

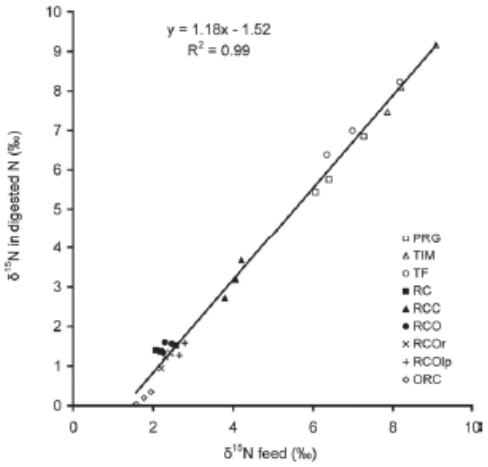

Figure 3. Relationship between $\delta^{15}N$ in feed (‰) and $\delta^{15}N$ in digested N (‰) for individual observations. Diets were based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC). Delta units ($\delta^{15}N$) describe the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air) and are expressed per mil (‰).

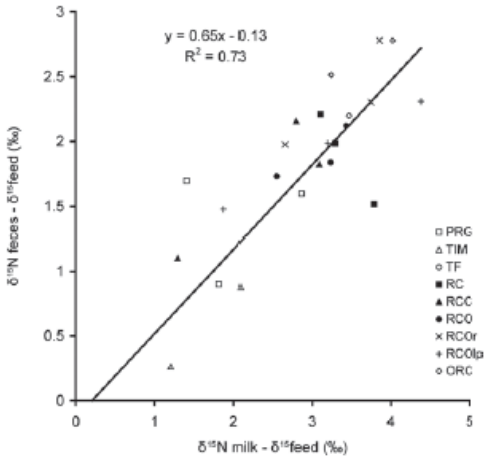

Figure 4. Relationship between $\delta^{15}N$ feces − $\delta^{15}N$ feed (‰) and $\delta^{15}N$ milk − $\delta^{15}N$ feed (‰) for individual observations. Diets were based on perennial ryegrass silage (PRG); timothy silage (TIM); tall fescue silage (TF); red clover silage (RC); mixture of red clover and corn silages (40/60 on a DM basis; RCC); mixture of red clover and whole-crop oat silages (40/60 on a DM basis; RCO); RCO with restriction on forage intake (RCOr); RCO with low protein concentrate (RCOlp); or a mixture of red clover and whole-crop oat silages (25/75 on a DM basis; ORC). Delta units ($\delta^{15}N$) describe the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air) and are expressed per mil (‰).

silage in a forage mixture was confirmed as an effective way to obtain the production benefits of red clover silage without excessive urinary N losses. Urinary N output was close to predictions from N intake based on literature reviews, although lower levels were recorded for the red clover silage-based diet as well as for the 40/60 red clover silage/cereal silage-based diets. This is an important effect because urine N is more likely to cause environmental pollution than fecal N. We were not able to confirm a relationship between N-isotopic fractionation and NUE for these diets. The N-isotopic fractionation approach appears unsuited for comparisons of NUE in short-term changeover designs where the contribution of ^{15}N from body reserves may complicate relationships, or for diets containing different amounts of ammonia-N.

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BRIEF COMMUNICATION: Investigation of N isotopic fractionation in dairy cows using milk samples collected at the morning and afternoon milkings

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Keywords: nitrogen-15; stable isotope; milk; nitrogen use efficiency; nitrogen partitioning.

INTRODUCTION

Conversion of feed protein into milk protein is a basis of sustainable dairy production systems from both economic and environmental perspectives. This project addresses the need for rapid screening tools to support nutritional and breeding strategies to improve the efficiency of converting feed into milk protein. The approach is based on the phenomenon of N isotopic fractionation, whereby N sinks become relatively enriched or depleted in ^{15}N, as a result of differential incorporation during various biochemical reactions. Macko *et al*. (1986) showed that N isotopic fractionation was related to the deamination and transamination of amino acids. Sick *et al*. (1997) conducted a feeding experiment with rats and found that plasma protein was enriched and plasma urea was depleted in ^{15}N relative to the diet. Furthermore, Robbins *et al*. (2005) suggested that dietary protein quality had significant effects on nitrogen isotopic discrimination in mammals and birds. More recent studies showed that N isotopic fractionation occurs between urine and milk, and is indicative with N partitioning of dairy cows (Cheng, 2008; Cheng and Dewhurst, 2009). However, this approach still requires separate collection of milk and urine. Therefore, this study investigated the potential of using N isotopic fractionation of protein and non-protein fractions of milk samples only, which are easily collected, as an indicator of N partitioning.

MATERIALS AND METHODS

Milk samples were collected from ten Holstein-Friesian cows (mean 140 days in milk), that were rotationally grazing summer ryegrass/white clover pasture and receiving no supplementary feed on Ballydague Farm in Ireland. Fresh pasture was allocated every 48 hours. Cows were milked twice daily and separate samples of morning and afternoon milk were collected, preserved with a lactab (Thompson and Capper Ltd., Runcorn, UK) and stored frozen at -20°C. Milk samples were then thawed, defatted by centrifugation at 11,500 rpm for 20 minutes at 4°C and protein precipitated using acetone (4:1 acetone: milk (vol/vol)); left to stand for 2 hours, then centrifuged at 14,000 rpm for 10 minutes at 4°C; before washing with acetone. Samples of whole milk, the protein pellet and the supernatant, being the non-protein-N fraction, were freeze-dried and prepared for ^{15}N and N concentration analysis using a continuous flow isotope ratio mass spectrometer (PDZ Europa, Crewe, UK). Nitrogen-^{15} content (^{15}N) is conventionally presented in delta units ($\delta^{15}N$) by expressing the $^{15}N/^{14}N$ ratio of the sample relative to the $^{15}N/^{14}N$ ratio of air, on a per-thousand basis (‰).Comparisons of the ^{15}N content in the different milk fractions and between morning and evening samples were conducted using one-way analysis of variance and linear regression using GenStat, Version 10 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK).

RESULTS AND DISCUSSION

The ^{15}N content ($\delta^{15}N$, ‰) of whole milk ranged from 6.30 to 7.24. The mean ^{15}N content in the protein fraction was 7.05 (Standard deviation (SD) = 0.25), which was significantly ($P < 0.001$) higher than in whole milk (6.81; SD = 0.265), whilst the ^{15}N content in the non-protein-N fraction was significantly ($P < 0.001$) lower (1.61; SD = 0.448). There were significant differences between morning and evening samples in content of ^{15}N in protein, non-protein-N and whole milk, values for morning milk being higher for milk protein and lower for milk non-protein-N (Table 1). There were significant relationships between individual morning and evening values for all three variables (Table 2).

The enrichment of ^{15}N in milk protein and depletion of ^{15}N in milk non-protein-N, relative to whole milk, is comparable with the earlier observations of enrichment of whole milk and depletion of urine relative to feed (Cheng & Dewhurst, 2009). The numerical difference in $\delta^{15}N$ between protein pellet and milk non-protein-N (Mean = 5.4‰; Range: 4.6 to 6.4‰) was similar to the difference in $\delta^{15}N$ between milk and urine (Mean = 6.2‰; Range: 4.3 to 7.6‰) for treatment

TABLE 1: Effect of morning or afternoon sampling on the relative ^{15}N content ($\delta^{15}N$, ‰) of whole milk, milk protein and milk non-protein-N fractions isolated from milk samples obtained from ten grazing dairy cows. SED = Standard error of difference.

Milk fraction	Time of milking		SED	P value
	Morning	Afternoon		
Whole milk $\delta^{15}N$	6.90	6.72	0.04	0.002
Milk protein $\delta^{15}N$	7.13	6.98	0.05	0.009
Milk non-protein-N $\delta^{15}N$	1.36	1.87	0.05	<0.001

TABLE 2: Linear regression equations relating ^{15}N content ($\delta^{15}N$, ‰) in morning samples (Y) to ^{15}N content ($\delta^{15}N$, ‰) in afternoon samples (X). Milk samples were obtained from ten grazing dairy cows. SE = Standard error.

Milk fraction	Equation	R^2	SE (Observation)	P value
Whole milk	Y = 0.951X + 0.51	0.74	0.14	<0.001
Milk protein	Y = 1.007X + 0.11	0.64	0.16	0.003
Milk non-protein N	Y = 1.019X – 0.55	0.81	0.17	<0.001

means in the study of Cheng and Dewhurst (2009). The $\delta^{15}N$ of whole milk was close to that in milk protein, whilst the $\delta^{15}N$ of the non-protein N fraction was much lower. The relative contents of ^{15}N in protein, non-protein N and whole milk suggest that the milk protein fraction made up on average 4.4% of milk N, which is very close to the measured value of 4.3%. The small, but highly significant, difference in fractionation between morning and evening samples may be related to diurnal variation in the rate or composition of herbage intake (Cosgrove *et al.*, 2009). Nonetheless, the highly significant relationships between individual morning and evening values demonstrate the consistent and strong cow effect and suggest that this low-cost approach will be useful for studies to understand genetic control of N-use efficiency.

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Approaches to measure nitrogen use efficiency of dairy cows

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ABSTRACT

Nitrogen use efficiency (NUE) is defined as the conversion of feed N into milk N and it is an important component of sustainable and profitable dairy farming. NUE can be increased through feeding animals well balanced diets, breeding improved grasses and cow genetic selection. However, such work is limited by the fact that NUE is difficult to measure, particularly in large numbers of animals. The traditional nitrogen balance technique for measuring NUE is difficult, particularly with grazing animals. Milk urea nitrogen concentration has been widely used to indicate NUE of dairy cows, however, inconsistent relationships have been described when cows are fed high-protein diets. Novel and simple methodology is needed to give a good indication of NUE from large groups of grazing animals. Initial results from a new approach based on stable N isotopic fractionation among different N sinks indicate that milk and urine were relatively enriched and depleted respectively in ^{15}N relative to feed. Isotopic N fractionation either as the ratio difference of N outputs and inputs (milk $\delta^{15}N$-feed $\delta^{15}N$) or as a concentration (milk non-protein-N $\delta^{15}N$) may prove useful as an indicator of NUE.

Keywords: nitrogen-15; nitrogen balance; nitrogen partitioning, sustainable farming.

INTRODUCTION

Nitrogen use efficiency (NUE) is defined as the amount of milk nitrogen that is produced per unit of nitrogen intake. It is a key issue for profitable and sustainable dairy production in Australasia. Current approaches to measure this efficiency are either subject to large experimental errors (Spanghero & Kowalski, 1997) or provide no detail about actual rumen processes (Schepers & Meijer, 1998). An alternative approach of measuring the fractionation of stable N isotopes in N sinks to indicate NUE has been investigated using cows offered on both silage-based diets and free grazing pasture (Cheng, 2008; Cheng & Dewhurst, 2009).

CURRENT TECHNIQUES FOR ESTIMATING NUE

The most established technique for measuring NUE is a nitrogen balance (NB) trial. A dairy cow NB involves measuring the partitioning of total N intake to N outputs. Large requirement for labour, overestimating of N intake and underestimating of N outputs are the main limitations encountered with the NB method (Spanghero & Kowalski, 1997).

Milk urea nitrogen concentration (MUN) analysis is becoming the most widespread means of evaluating NUE for milk production. However, Baker *et al.* (1995) and Tas (2006) have suggested that this relationship was poor and not significant when feeding high levels of CP in the diet.

A NEW APPROACH: STABLE NITROGEN ISOTOPIC FRACTIONATION

What is $\delta^{15}N$?

Both ^{14}N and ^{15}N are stable N isotopes with different mass and they naturally exist in the environment. Wattiaux & Reed (1995) pointed out that ^{14}N and ^{15}N are both components of air N, with the ^{15}N content of atmospheric N is 0.3663%, and the rest of N is ^{14}N (99.6337%). The ^{15}N abundance is expressed as $\delta^{15}N$ value, the deviation in the 15 and 14 ratio in any other N source relative to atmospheric N (Yoneyama *et al.*, 1983):

$\delta^{15}N$ (‰) = [(^{15}N /^{14}N) sample – (^{15}N /^{14}N) air] / [^{15}N /^{14}N] air×1000. In general, 10 microlitres of liquid sample or 5 milligrams of dried sample is required for analysis through using a continuous flow isotope ratio mass spectrometer, which is widely available in NZ research institutions.

Studies on stable nitrogen isotopic fractionation

The small mass difference between ^{14}N and ^{15}N causes the isotopes to behave differently in both physical and chemical processes (fractionation). The analysis of naturally occurring stable N isotopes has been used to provide insights into a variety of aspects of biology. DeNiro & Epstein (1980) investigated the influence of diet on the distribution of N isotopes in animals. They discovered that the N isotopic composition of the diet could reflect the isotopic compositions of N in an animal. Later, Sick *et al.* (1997) identified the relationships between protein utilisation and isotopic discrimination of N

TABLE 1: Effects of nine silage treatments on N partitioning and N isotopic fractionation of Holstein-Friesian cows.

	PRG	TIM	TF	RC	RCMZ	RCWCO	RCWCOr	RCWCOlp	RC25WCO	s.e.d.	Sig
N intake (g/day)	605^{c}	547^{bc}	494^{b}	626^{c}	512^{b}	473^{b}	464^{b}	379^{a}	359^{a}	36.4	<0.001
Milk N (g/day)	$115b^{cd}$	103^{abc}	90.5^{a}	121^{cd}	128^{d}	125^{d}	115^{bcd}	100^{ab}	116^{bcd}	6.10	0.002
Feed $\delta^{15}N$ (‰)	6.59^{c}	8.38^{d}	7.15^{c}	2.27^{a}	4.03^{b}	2.37^{a}	2.29^{a}	2.59^{a}	1.82^{a}	0.348	<0.001
Milk $\delta^{15}N$ (‰)	8.67^{b}	9.21^{b}	8.54^{b}	5.84^{a}	6.57^{a}	5.24^{a}	5.73^{a}	5.65^{a}	5.33^{a}	0.385	<0.001
Urine $\delta^{15}N$ (‰)	4.09^{e}	3.91^{d}	2.62^{c}	1.01^{b}	0.71^{b}	-1.13^{a}	-0.92^{a}	-1.06^{a}	-1.42^{a}	0.584	<0.001

^{a,b,c,d} superscripts that differ are significant, P <0.05. PRG=Perennial ryegrass, TIM=Timothy, TF=Tall Fescue, RC=Red clover, RCMZ=Red clover/maize mixture (40/60), RCWCO=Red clover/whole-crop oat mixture (40/60), RC25WCO=Red clover/whole-crop oat mixture (25/75), RCWCOr=RCWCO with feed intake restricted, RCWCOlp=RCWCO with low protein concentrate.

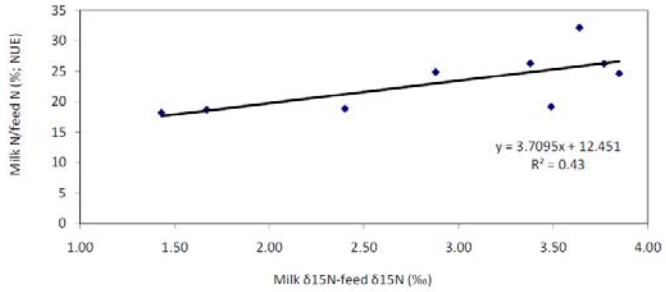

FIGURE 1: The relationship between milk $\delta^{15}N$ -feed $\delta^{15}N$ and NUE of Holstein-Friesian cows offered with nine silage treatments.

in rat liver. They suggested that the quality and quantity of dietary protein ingested by the animal could be indicated by analysis of isotopic discrimination in the product of N metabolism.

Work has also been done on large ruminants. Samples from a NB study from the UK (Cheng & Dewhurst, 2009) were analysed for $\delta^{15}N$ in New Zealand using a continuous flow isotope ratio mass spectrometer (PDZ Europa, Crewe, UK). Nine multiparous Holstein-Friesian cows with average daily milk production of 24 ± 5kg, were used in an incomplete changeover design (3 cows per dietary treatment), in which nine different silages were offered. Table 1 shows that dietary treatment had highly significant effects ($P < 0.001$) on N isotopic discrimination. Milk was enriched in ^{15}N compared with feed by 1.62 times on average; on the other hand, urine was depleted in ^{15}N relative to feed by 4.79 times on average. Milk $\delta^{15}N$ - feed $\delta^{15}N$ was positively correlated to milkN/feedN (NUE) with $R^2 = 0.43$; SE (obs) = 0.75 (Figure 1).

Another dairy cow study (Cheng *et al*., 2010), investigated the potential use of the N fractionation among milk components to assess NUE. Morning and afternoon milk samples were collected from nine grazing Holstein-Friesian cows. Milk samples were defatted and protein was precipitated using acetone. Samples of whole milk, the protein pellets (PP) and the supernatant (non-protein-N fraction; NPN) were freeze-dried and analysed for ^{15}N. MUN (mg/dl) was also measured. The difference in $\delta^{15}N$ between protein pellets and milk non-protein-N (mean = 5.4‰; range: 4.8 to 6.0‰) was similar to the difference in $\delta^{15}N$ between milk and urine (mean = 6.2‰; range: 4.3 to 7.6‰) for treatment means in the study of Cheng & Dewhurst (2009). The correlation between am and pm values was weaker for MUN ($P < 0.05$) than for $\delta^{15}N$ in milk fractions ($P < 0.001$). There was a positive linear relationship between MUN and $\delta^{15}N$ in NPN ($r^2 = 0.35$, $P = 0.05$). The small, but highly significant, difference in fractionation between am and pm samples may be related to diurnal variation in the rate or composition of herbage intake (Cosgrove *et al*., 2009). Nonetheless, the highly significant relationship between MUN and NPN $\delta^{15}N$ suggest that NPN $\delta^{15}N$ could be a useful substitute for MUN. With further understanding of N isotopic fractionation within dairy cows, N isotopic fractionation technique will be a useful approach to address environmental issues in ruminant systems that includes plant breeding, animal breeding and animal nutrition.

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