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Incorporating grass-clover and lucerne silages into UK dairy systems: forage agronomy, silage analysis accuracy and lucerne feeding strategy

Doctor of Philosophy

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DECLARATION OF ORIGINAL AUTHORSHIP

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Red clover (*trifolium pratense*), white clover (*trifolium repens*) and lucerne (*medicago sativa*) are currently the most viable forage legumes for european farming systems. Knowing the agronomy and feeding strategies representing 'best practice' for these forages in livestock production systems is key as the industry turns to low input forages to meet sustainability targets. The research question addressed in this thesis was whether these forage legumes could be grown and fed more efficiently, with a focus on their use in the UK dairy sector. Limitations to their utilisation are discussed and potential practical solutions were assessed. This included investigations into the effects of sowing timing and plant maturity on yield and feeding value of lucerne, and the effect of chop length and inclusion rate of lucerne silage in a total mixed ration with maize silage on dry matter intake, milk yield, total tract digestibility, and rumen functionality in both 'normal' and 'challenging' rumen environments of dairy cattle. Furthermore, to ensure precise diet rationing, the method by which most legume-containing silage samples are analysed for nutritional content in the UK (Near Infra-Red Reflectance Spectroscopy) was tested for accuracy for both grass-clover and lucerne silages. Improved grass-clover prediction equations were calibrated from the data collated in the study, which will be adapted for commercial implementation in the future. Key findings from these studies which can be used to enhance best practice guidelines for farmers included: (i) it was advantageous to sow lucerne in spring rather than autumn for greater yield and reduced weed burden, (ii) including lucerne silage in a TMR diet with maize silage at 25% of forage DM and at a short chop length was beneficial to dry matter digestibility, and therefore metabolisable energy supply, relative to inclusion at 75% of forage DM and a long lucerne chop length, and (iii) a high inclusion rate of lucerne silage in the diet can mitigate against sub-acute rumen acidosis risk resulting from short-term feed deprivation followed by refeeding.

TABLE OF CONTENTS

Chapter 1

General Introduction

GENERAL INTRODUCTION

There is a need to improve the sustainability of dairy systems in line with United Kingdom (**UK**) government recommendations to reduce greenhouse gas emissions and protect rural biodiversity (Foresight, 2011). One of the largest variable costs to the dairy industry is feed which must provide sufficient protein and energy to support the maintenance needs of the cow and allow for a high yield of milk to be obtained. At least half of the diet of a modern UK dairy cow is comprised of forage, most commonly conserved grass and maize silages, however, grass requires a high input of inorganic nitrogen fertiliser to achieve high crude protein concentration and biomass yield. Therefore, there is rising interest in alternative forages for dairy cattle, with the most promising option being the use of forage legumes as they can fix nitrogen from the atmosphere and so do not need extra application of nitrogen fertiliser. A reduction in inorganic fertiliser usage combined with an increase on-farm biodiversity make forage legumes viable from both an economic and an environmental perspective. Additionally, feeding forage legumes to dairy cattle is thought to result in increased diet quality through greater provision of crude protein, increased intake due to faster rate of digestion and passage through the gut, and improved rumen functionality by increasing effective fibre concentration in the diet. However relatively few studies have tested these effects within UK diets which are already forage-rich and low in starch in comparison to those fed in the United States of America (**USA**) where use of forage legumes (predominantly lucerne, *medicago sativa*) has been practiced and researched in greater detail.

Despite the benefits attributed to the inclusion of forage legumes in farming systems, most species remain niche crops within UK agriculture which suggests there are significant barriers affecting their uptake. This poses the research question: can forage legumes be utilised more extensively and efficiently in UK dairy systems? Efficiency in

this context meaning increased forage biomass yield, silage nutritional quality, and milk yield in the cow, whilst decreasing wastage of nutrients in cow urine and faeces. Compared to traditional grass species, there is less information appropriate for UK farmers available that offers advice on growing alternative legume crops, and, as a result, many farmers encounter difficulties when trying to adapt to their more complex management needs, particularly if they are sown in mixtures. Furthermore, practical issues exist, such as a lack of appropriate nutritional analysis options for legume silages in the UK, which may be causing imbalances in metabolisable protein and energy when fed, or dissuading farmers from including forage legumes in precise ration formulations completely. Other characteristics of forage legumes also constrain their feeding value such as potentially low digestibility and rapid degradation of rumen available protein leading to nitrogen wastage in faeces and urine. Due to these drawbacks, which are examined in detail in this thesis, it is likely that forage legumes are not being utilised to their full potential at present. Therefore, the overall aim of this project was to identify and investigate solutions to factors limiting efficient legume utilisation and uptake into dairy systems in the UK. A key objective of the study was to contribute information needed for growing and feeding guidelines for forage legume crops that were applicable at farm level. To this end, a series of studies were conducted at the University of Reading, each with a focus on a different limiting factor from agronomy, ensiling and silage analysis to feeding, digestion and milk production. Whilst there is a vast range of forage legume species that could be utilised in UK agriculture, the scope of the project described in this thesis was limited to silage analysis of red and white clover (*trifolium repens* and *trifolium pratense*) and growing and feeding of lucerne, as the three legumes which show the greatest potential for inclusion in the temperate dairy farming systems of the UK based on their current popularity and economic viability.

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The research carried out for this thesis includes three experiments funded by the dairy division of the Agriculture and Horticulture Development Board (**AHDB**) as part of the 'Soil, Forage and Grasslands Research Partnership' and in association with the Forage Analytical Assurance group (**FAA**). Experiments were carried out at two University of Reading research facilities: Centre for Dairy Research (**CEDAR**), Arborfield, and the Crops Research Unit, Sonning. Some additional analysis using near infra-red reflectance spectroscopy (**NIRS**) was also carried out at the Agri-Food and Biosciences Institute (**AFBI**), Northern Ireland, as they were the FAA 'masterlab' for forage NIRS equations in the UK. Additionally, elements of the project required samples to be sourced and collected from many working farms across the country with the kind cooperation of relevant farm owners and managers.

Chapter 2

A Review:
Can forage legumes be utilised more efficiently in UK Dairy systems?

The role of forage in dairy cow nutrition and rumen function

Mechanisms of digestion

Forage performs a vital role in the stabilisation and health of the dairy cow's digestive system. Furthermore, maximising milk yield from forage is closely tied to good economic performance, as forages are cheaper to obtain than bought-in concentrate products which form the remainder of the diet and are generally the largest input cost to the dairy farmer. Delivery of the forage and concentrates to the cow can either be in the form of grazing (or zero-grazing/cut and carry feeding) with supplemental feeding of concentrates, or alternatively, combined in the form of a total mixed ration (**TMR**) using forage which has been ensiled or dried for conservation. The TMR approach is common in housed herds where grazing land is insufficient, and in intensive systems as nutrient and energy supply can be finely balanced for optimised conversion of feed to milk. A recent survey of British farmers indicated a trend for increased indoor housing during the summer moving away from traditional summer grazing routines (March *et al.*, 2014). Feeding in the form of a TMR can also benefit digestive health by providing a constant plane of nutrition, whereas systems in which concentrates are offered separately can lead to a large amount of material that is rapidly digested and has a high concentration of energy reaching the rumen of the cow in a short time period. For these reasons the focus of this project is the inclusion of forages in TMR based diets.

When feed is ingested by the cow, a bolus of feed is chewed and mixed with saliva in the mouth which is then delivered to the rumen upon swallowing. The function of the rumen is to provide a large area in which feed particles can be broken down, predominantly through the action of a highly specialised community of commensal gut bacteria and protozoa. At this stage the long particles of forage within the diet form a solid, buoyant mat which floats within a slightly acidic liquid pool (rumen liquor)

comprised of water, saliva, microbiota, very small particles and dissolved nutrients including short chain volatile fatty acids (**VFA**s), long chain fatty acids, amino acids, minerals, and ammonia. Material is unable to pass onwards through the omasum and abomasum until particle length is shortened beyond a critical point and specific gravity is increased (Maulfair *et al.*, 2011; Kornfelt *et al*., 2013). To facilitate microbial attachment to particles the rumen undergoes constant contraction during which the liquid pool is repeatedly flushed through the solid mat. Intermittently, rumination occurs in which a bolus of rumen contents is regurgitated and re-chewed in the mouth to further reduce particle size and increase surface area. While these basic mechanisms are well studied, some aspects of rumen function remain mysterious. One example being the high level of efficiency at which a cow can turn feed into basic nutrients in comparison with an anaerobic digester despite the similarity of the process (Mason and Stuckey, 2016). Other areas that are only recently being explored in greater depth include mitigating the production of methane as a waste product of ruminal digestion (Reynolds *et al.*, 2011), and the relationship between the species of bacterium present in the microbial community of the cow and feed efficiency, as well as how these may be affected by animal genetics (Drackley *et al.*, 2006; Khiaosa-ard and Zebeli, 2014).

Protein and energy supply

Metabolisable protein (**MP**) is the portion of true protein reaching the small intestine that the animal can make use of. There are two sources of metabolisable protein, microbial protein (synthesized from rumen degradable feed, endogenous protein and non-protein N) and bypass protein (protein which has not degraded in the rumen that is digested and absorbed in the small intestine). The key principle of formulating TMRs is to optimise the ratio of metabolisable protein and metabolisable energy (**ME**) in the diet.

Additionally, the rate of ruminal degradation of each should be matched so that sufficient ME to drive microbial capture of available MP is constantly supplied (Lykos *et al.*, 1997). An oversupply of MP in relation to ME, or an oversupply of rumen degradable protein relative to rumen fermentable energy, leads to wastage of nitrogen that is excreted as urea in the urine, and lower efficiency of nitrogen capture in milk, which is both economically and environmentally damaging (Colmenero and Broderick, 2006). Protecting protein and lipid from ruminal degradation or biohydrogenation respectively, and allowing them to be broken down in the lower gastrointestinal tract for optimal absorption is one strategy that can improve nutrient utilisation (Vagnoni and Broderick, 1997). Utilisation and partitioning of nitrogen from forages varies considerably both within and between species (Hoffman *et al.*, 1993; Cheng *et al.*, 2011). Often combinations of forages are used together where their unique properties complement each other. A common example of this being to combine a starch-rich crop such as maize (*zea mays*) with a protein-rich crop such as ryegrass or a legume in which protein can degrade quickly and be easily lost before capture if ME supply is insufficient (Givens and Rulquin, 2004). Increasing the total tract digestibility of a feed is key to overall nutrient use efficiency and directly related to ME supply and therefore forages with very high concentrations of indigestible fibre (often the result of forage over-maturity) are undesirable (Nousiainen *et al.*, 2009). Any nutrient which is indigestible to the cow is excreted in faeces, therefore the study of nutrient partitioning between urine and faeces is often used to evaluate mechanisms of feed breakdown and nutrient utilisation. Nitrogen excreted in urine and faeces contributes to environmental loading through production of harmful gases (ammonia and nitrous oxide) and leaching into soils as nitrate which eventually enters watercourses (Edouard *et al.*, 2016). Currently the efficiency of nitrogen capture from feed into milk is approximately 25%, however evidence suggests a theoretical potential of 43% efficiency

(Dijkstra *et al.*, 2013) is possible, and there has been a wide range of recent studies conducted to investigate nitrogen metabolism and maintenance of milk yield in low protein diets which are beyond the scope of this review e.g. Calsamiglia *et al.* (2010).

Dietary fibre and rumen pH regulation

Reducing fibre concentration by reducing forage inclusion rate can often benefit production through increased feed intake and greater provision of nutrients in the concentrate portion of the diet. For example, in one study a reduction in neutral detergent fibre (**NDF**) concentration from 360 to 280 g/kg of total diet dry matter (**DM**) improved capture of MP through increased non-structural carbohydrate provision in the diet (Broderick, 2003). However, too drastic a reduction, such as an inclusion rate of <40% DM forage (Farmer *et al.*, 2014), can reduce milk yield and, over prolonged periods, would likely result in increased risk of ill health due to an overly acidic rumen environment (sub-acute rumen acidosis, **SARA**).

Recently feeding systems have focussed around utilising physically effective fibre (**peNDF**) as a better tool to match provision of forage fibre with rumen degradable starch and prevent acidosis while maintaining optimal production levels. In this context, peNDF was originally defined as the NDF within forage particles greater than 1.18 mm in length which was considered to be the length at which particles could pass from the rumen (Mertens, 1997). However, new estimates suggest 4 mm to be a more accurate threshold length (Maulfair and Heinrichs, 2012) considering research has shown that between 12 - 28% of faecal dry matter in a dairy cow can be retained on a 1 mm sieve (Oshita *et al.*, 2004) suggesting particles were longer than 1 mm when passing from the rumen. Physically effective fibre contributes to the formation of the rumen mat and stimulates rumination, in part through the physical action of stems and other fibres

rubbing the rumen wall. These mechanisms aid in the regulation of rumen pH by reducing passage rate of particles through the rumen (Dixon and Milligan, 1981), therefore spreading the production of VFA over time, and through increased addition of sodium bicarbonate and phosphate to the rumen in the form of saliva which acts as a buffer and also facilitates VFA absorption (Dijkstra *et al.*, 2012). There remain some conflicting views in the literature over the relative importance of differing particle lengths, for example, while 4 mm is now generally accepted as the peNDF threshold, some state that only particles greater than 8 mm in length contribute to the rumen mat, coupled with a concern that too high a concentration of very long particles (>27 mm) may actually have a detrimental effect due to increased risk of diet sorting (Leonardi and Armentano, 2003) and very slow ruminal degradation rate which could then limit intake through excessive rumen fill (Zebeli *et al.*, 2012).

The way in which different forages breakdown during ingestion and rumination leads to variation in physical effectiveness between species (Mtengeti *et al.*, 1996), for example, plants with highly lignified stems are likely to contain a greater concentration of peNDF than those mostly comprised of leaves (Poppi *et al.*, 1985). Furthermore, forages have varying physical and chemical properties that impact on their behaviour during digestion. Buffering capacity (or cation exchange capacity) is one example of a characteristic which is highly variable between different forage species. A high buffering capacity is a desirable attribute of forages to prevent accumulation of acidic ions in the rumen liquor (McBurney *et al.*, 1983; Dewhurst *et al.*, 2003b). Some limited research has been conducted into further defining physical properties of forages such as buoyancy, specific gravity, and compression (Yansari *et al.*, 2004), however, greater testing of these techniques is required to determine their relationship to tangible production factors, and not all are well adapted for ease of utilisation on farm.

Physically effective fibre cannot be considered alone when formulating rations as the requirement for fibre is strongly influenced by the rumen degradable organic matter (**RDOM**) contained in forage plant material which is largely comprised of carbohydrate including sugars and starch in the cell contents, and pectin, cellulose and hemi-cellulose in the plant walls (Allen, 1997; Krause and Combs, 2003; Zebeli *et al.*, 2010). When these are broken down by microbes in the rumen these result in three main VFAs: acetic, propionic and butyric acids which are absorbed through the epithelium of the rumen into portal blood and used to meet energy requirements. Regulation of pH is the combined effect of maintaining VFA flux so that levels do not become too high and the addition of buffers to the rumen to neutralise acidic conditions (Aschenbach *et al.*, 2011). Over time, the dairy industry has also increased use of non-forage carbohydrates in diets to increase the energy density of the feed and support high milk yields, but with a negative side-effect of creating an increasingly challenging rumen environment. There is some conflict within the literature over the effect of prolonged sub-acute low rumen pH, with some studies linking SARA to a wide range of negative effects including milk loss, milk fat depression, and health conditions (Enemark, 2008) including lameness (Nocek, 1997), but it is difficult to directly link some of these health effects to SARA due to a delay between low pH and the onset of such conditions. Moreover, other studies have shown an ability of animals to adapt to low rumen pH over time, lessening its detrimental effects (Russell, 1998; Kmicikewycz and Heinrichs, 2015). However, despite some lack of consensus on the negative effects of low pH, there is sufficient evidence to support the need to balance peNDF and RDOM in the diet, with an emphasis on strategies in which the effectiveness of forage is optimised without increasing the overall concentration of forage in the diet to maintain digestive function while maximising milk production.

Utilisation of forage legumes within UK dairy systems

The need for alternative forages

In moist, temperate, livestock systems, ryegrass has traditionally been the forage of choice as it has a high yield and good nutritional content (crude protein and ME averaging 133 g/kg DM and 10.3MJ/kg DM; Yan and Agnew (2004)), but it also has a high requirement for inorganic nitrogen fertilisation. In recent years, the rising price of inorganic fertiliser combined with increased incidence of summer drought due to rising global temperatures has led to a search for alternative perennial forages which offer increased economic and environmental sustainability in comparison to ryegrasses while maintaining or improving on feed value. Forage legumes have the potential to provide a suitable solution to this problem (Luescher *et al.*, 2014). There are many species of forage legume which have been successfully tested in temperate European climates and proved suitable for use as cattle fodder either as grazing with notable examples being red clover, white clover, lucerne, sainfoin and birdsfoot trefoil.

Forage legumes vs. Ryegrasses?

Environmental sustainability. Forage legumes provide clear benefits over ryegrasses from an environmental perspective. The key benefit of a legume crop is that there is little or no need for inorganic nitrogen fertilisation because of their nitrogen fixing ability through the colonisation of roots with *rhizobium* bacteria. Producing and applying inorganic fertiliser releases harmful greenhouse gas emissions and can increase nitrate levels in watercourses where excess nitrogen leaches from soils. The level of nitrogen fixation provided by a mixed ley containing at least one third legume is likely to have a soil nitrogen value 57% higher than grass alone (Suter *et al.*, 2015). This can equate to between 180-380 kg of nitrogen fixed per hectare per year (Peyraud *et al.*, 2009) much

of which will be retained in the soil after the crop is ploughed in at the end of the growth period (typically 3-5 years depending on the species) which can be utilised to fulfil the requirement for fertilsation in the following crop. To make the best use of fixed nitrogen, legumes can be sown as part of binary or multi-species sward mixtures, so that non-legume species (grasses and herbs) can utilise nitrogen as it is fixed providing a contstant source of nutrients during their growth and resulting in a higher total biomass than can be produced by a fertilised monoculture grass ley (Ledgard, 2001; Fustec *et al.*, 2010; Nyfeler *et al.*, 2011). Optimising species evenness can often produce an effect known as 'transgressive overyeilding' where a multispecies mixture is able to yield more than any of its components sowed in monoculture due to their complementary niche functions (Kirwan *et al.*, 2007; Finn *et al.*, 2013). The most common example of species mixtures in practice is the simple combination of white or red clover with grass. Multispecies mixtures increase the biodiversity of an area and have been shown to support a wide range of small mammals and insects, in comparison to monoculture grass swards (Sheridan *et al.*, 2008; Piotrowska *et al.*, 2013). There are, however, some potential drawbacks of nitrogen fixation by legumes, the first being that leaching risk could be increased where fixed nitrogen is not recycled back into plant material effectively or excess fertiliser is applied unneccesarily (Sulas *et al.*, 2012). Secondly, complex management is needed to maintain an even distribution of legumes in mixtures which becomes increasingly difficult if more than two species are included in the sward as there will be temporal changes in relative species dominance throughout the year (Laidlaw *et al.*, 1992; Sharp *et al.*, 2013).

The yield of legume species can be variable in comparison to fertilised ryegrass which is more straight forward in terms of management, however it has been demonstrated that, with good management, annual yields of red clover and lucerne can

be as high as 16 and 18 T/ha DM respectively with no application of nitrogen (Frame and Harkess, 1987). Different species of legumes can have specific requirements in terms of soil and weather conditions needed for establishment and growth and therefore, may be restricted in terms of where they can be grown in the UK (Sainfoin, *Onobrychis viciifolia*, is one example of this, requiring very alkaline and well drained soils) (Liu *et al.*, 2008). However, there are many different forage legume species available and farmers should be able to identify at least one species or species mixture suited to their region. Current trends in global climate change are increasingly improving the productivity of legumes in comparison to ryegrasses as they are more resilient to extreme weather. Most species of forage legume prefer warm dry soils (Frankow-Lindberg *et al.*, 2009) and therefore thrive in late summer in the UK when grasses may wilt due to drought. Lucerne is especially suited to these conditions with a long tap root which enables it to grow comfortably in drought-prone areas and semi-arid land, although it will struggle on shallow, compacted or waterlogged soils. In addition to the increasing resilience and lengthening of the foraging season, deep rooting species also help to increase the structure and organic matter (**OM**) content of soils providing ecological benefits both above and below ground (Autret *et al.*, 2016). Choice of forage legume species is also determined by feed system, as many forage legumes grow upright and some have a fragile crown, making them susceptible to grazing damage. White clover has a lower growth profile than most legumes so is best suited to grazing, whilst other forage legumes should be reserved for silage or hay-making.

Feeding value. While forage legumes are clearly the more sustainable choice over ryegrass in the field, the difference is less clear cut when it comes to ensiling and feeding value. Feeding value can be defined in different ways, however, broadly speaking,

desirable properties include a high crude protein (**CP**) content with an effective degradability of protein (**EDN**) no greater than 70% (Coblentz and Grabber, 2013), and a high total tract digestibility which is generally correlated with a low content of acid detergent fibre (**ADF**). In addition to these factors, a high water soluble carbohydrate (**WSC**) concentration increases ease of ensiling by providing accessable substrate to drive anearobic fermentation in the silo, resulting in a high concentration of lactic acid to swiftly reduce pH and lesser concentrations of acetic acid, propionic acid and alcohols in the resulting silage (Duniere *et al.*, 2013; Davies and Orosz, 2014). Any residual WSC also acts as an energy source in the diet. Neither grass or forage legumes contain reserves of starch and are therefore often combined with starch-rich forage maize silage in the diet to ensure sufficient energy supply for microbial protein synthesis in the rumen. Ten studies (Table 1) in which comparisons have been drawn between the chemmical composition of silages made from either grass or common legume species (red clover, white clover, lucerne, sainfoin, or birdsfoot trefoil) have been combined to give an indication of the nutritional value of these species in Table 2.

***Table 1**: A summary of ten studies which reported the chemical composition of at least two silages made from either grass or a common legume species.*

	Ensiled forage species					
Paper	Grass	Red Clover	White clover	Lucerne	Sainfoin	Birdsfoot Trefoil
Al-Mabruk et al. (2004)	X	X				
Bertilsson and Murphy (2003)	X	X	X			
Broderick et al. (2000)		X		X		
Coblentz and Grabber (2013)				X		X
Dewhurst et al. (2003b)	X	X	X	X		
Girard et al. (2016)	X			X	X	X
Huyen et al. (2016)	X				X	
Hymes-Fecht et al. (2013)		X		X		X
Sinclair et al. (2014)	X			X		
Wang et al. (2006)				X	X	

***Table 2**: The mean (± SE) chemical composition (in g/kg DM) of silage made from six forages (grass or one of five legumes) based on data from ten studies in the literature.*

Item	Ensiled forage species					
	Grass	Red Clover	White clover	Lucerne	Sainfoin	Birdsfoot Trefoil
CP	153 ± 14	190 ± 5	244 ± 9	207 ± 8	178 ± 26	198 ± 4
NDF	518 ± 18	421 ± 23	277 ± 9	413 ± 15	419 ± 45	397 ± 52
ADF	312 ± 9	322 ± 13	271 ± 9	334 ±16	376 ± 43	315 ± 39
WSC[1]	27 ± 16	7 ± 8	12 ± 12	4 ± 4	-	-

CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate.

[1] Not all papers reported WSC, the number of studies included in each reported mean was 4, 3, 2, and 2 for grass, red clover, white clover and lucerne, respectively. No studies reported the WSC concentration of sainfoin or birdsfoot trefoil.

Table 2 indicates that forage legumes have favourable chemical composition as CP levels are often equal to, if not higher than, that of grass silage which would have been fertilised. It is worth noting however that maturity at harvest would strongly influence the chemical composition of the resulting crop. White clover in particular is shown to have a high crude protein value and low fibre concentration however only two of the ten studies reported data for white clover silage, so results should be interpretted with caution. Grass silages had a high NDF concentration but a lower ADF concentration indicating a high hemi-cellulose concentration relative to the forage legumes, and also higher WSC content although standard error for WSC was high, likely reflecting that variance was introduced by fermentation quality and also the smaller number of studies that reported these data.

Although the chemical composition of legumes silages from Table 1 appears promising, when forage legumes are fed, the portion of protein that is available in the rumen is often degraded very quickly (Dewhurst *et al.*, 2003a) and in some species EDN will be greater than the target level of 70% above which excess nitrogen is less likely to be captured effectively by microbial action. Excess nitrogen is instead converted to ammonia and urea which can be recycled between the blood and the gut (Reynolds and

Kristensen, 2007) but is predominantly transported out of the body in the milk and urine (Broderick, 1995). A fast rate of soluble protein release can also lead to an increased risk of bloat in grazed animals (Dewhurst, 2013; Hancock *et al.*, 2014) again highlighting that most forage legumes are better suited to ensiling which lessens bloat-risk. Some legumes contain secondary metabolites which can protect a portion of nitrogen from ruminal degradation – the compound polyphenol oxidase (**PPO**) found in red clover is one example and condensed tannins have also been reported to have similar properties in some studies (Albrecht and Muck, 1991; Reed, 1995). Tannin-containing legumes (sainfoin and birsfoot trefoil, *lotus corniculatus*, being two examples) have also been shown to have other beneficial characteristics such as anthelmintic properties in sheep and potential for reducing methane emissions (Min *et al.*, 2003). However, one study in which red clover and sainfoin containing silages were compared, indicated that red clover had the greater nutrient use efficiency which would suggest that PPO is more effective than condensed tannin at inhibitting excess proteolysis in the rumen (Copani *et al.*, 2016). Furthermore, high tannin concentrations have been shown to reduce intake and therefore productivity in diary cattle (Min *et al.*, 2003) which could be explained by observed reductions in fibre digestibility linked to suppression of some bacterial populations when condensed tannins have been tested *in vitro* (Jayanegara *et al.*, 2015). Additionally, a number of tannin-containing legumes show poor persistency within a mixed sward, birdsfoot trefoil in particular is prone to being outcompeted by other species (Storkey *et al.*, 2015; Gierus *et al.*, 2012), and therefore, despite the promising properties of these plants, they are not particularly suited to the dairy industry, making red clover the more viable choice of legume for increased protein efficiency.

With regards the fibre concentration and OM digestibility of legumes, forage legumes and ryegrasses can have similar total tract digestibility provided the harvest is

taken at a suitable maturity stage. White and red clovers have been repeatedly shown to increase intake in comparison with grass, particularly in grazed ruminants, enabling higher milk yields to be obtained likely as a result of rapid degradation of available OM in the rumen reducing rumen fill (Dewhurst *et al.*, 2003b; Kammes and Allen, 2012; Dewhurst, 2013). In a meta-analysis in which grass, lucerne, and red and white clover silages were assessed, the average increase in both DM intake and milk yield was 1.6 kg/d when legumes replaced grass silage in the diet of dairy cows (Steinshamn, 2010) over 20 separate studies. Not all species follow this pattern however, for instance, species where the stem is naturally high in lignin (e.g. lucerne), or any legume species that is harvested in late flower (an over-mature stage) can have reduced digestibility (and therefore ME) as a result of reduced leaf:stem ratio (Alstrup *et al.*, 2016; Kammes *et al.*, 2012) which leads to reduced intake compared with less mature material. High stem lignification can also reduce the ease with which these crops can be ensiled as carbohydrate is less available to lactic acid producing bacteria during anaerobic fermentation.

Economic performance. In 2004 it was calculated that a potential saving of €1300 million would be possible across the EU if 10% of grassland were converted to forage legume production due to reductions in inorganic fertiliser usage (Doyle and Topp, 2004). Positive economic effects of legume swards have since been confirmed more recently in other studies (Reckling *et al.*, 2016). Some recent works have shown that there is less economic variation between legume swards and fertilised grass when fertiliser price is low (Humphreys *et al.*, 2012; Phelan *et al.*, 2015). This helps to explain why some studies show little or no variation between these forage types (Butler *et al.*, 2012), and additionally, the parameters of the swards compared can vary considerably. For example,

in the study of Butler *et al.* (2012) where no difference in economic performance was observed between a fertilised ryegrass and a legume sward, fertiliser was applied to the grass sward at a low rate (110kg/ha N per year) and fertiliser costs were relatively low during the years of the study duration. Care must also be taken to assess whether economic studies use a systems approach including the performance of animals utilising the swards, or simply measure production at a botanical yield level.

The economic viability of forage legumes could be improved further by better exploiting their genetic potential as there are relatively few varieties of legume species available at present. In comparison, grass varieties have greatly benefitted from targetted breeding programmes to improve dry matter yield (McDonagh *et al.*, 2016) and feeding value, resulting in the development of high sugar varieties for optimum ensiling performance and energy supply, and hybrid varieties (such as Italian-perennial ryegrass crosses) which can be both resilient and high yielding. Initial attempts at implementing a UK-based red clover breeding programme have already led to improvements in persistency and yield (Marshall *et al.*, 2012) and natural genetic variation within populations of other forage legumes (Huyghe and Tabel, 2009) indicate the potential gain that could be achieved by widening such breeding programmes to include other common legume species.

Past and present forage legume utilisation

Legume forages are utilised to great effect globally, lucerne being the most widely grown of the legume species at 300M T/yr with notable success in the semi-arid climates of the United States of America (**USA**), Australia, the Middle East and parts of Europe. However, in the UK, lucerne currently covers just 6000 ha (Germinal, 2016), which is less than 0.1% of the total argicultural land area. There are very few UK-specific data

tracking the land use by different legumes over time, although, there is evidence to suggest a decline within the European Union (**EU**) since a peak in the 1980s. In the 1990's 4 million hectares of forage legumes were replaced with other crops or grasses due to the European agricultural policy favouring intensive high inorganic nitrogen fertiliser use during this time making legumes unprofitable (Rochon *et al.*, 2004). In the late 1990's two symposia were held in the UK with a focus on increasing forage legume utilisation (Younie, 1996; Lane and Wilkinson, 1998) however these seem to have had limitted impact based on the lack of popularity for most forage legumes at present (grass-clover swards being the only notable exception), perhaps because of the low fertiliser price at the time of the symposia, or due to farmers failing to see the benefits of growing forage legumes because of the limitations on feeding value, ease of establishment, or complexity of sward management discussed previosuly. Additionally, increasing use of contractors to perform field operations as farm sizes increase make it more difficult to precisely time applications and harvest to meet the complex management needs of a legume crop and this may also have negatively impacted their uptake.

However, there has been a recent renewal of interest in forage legumes created by the recent emphasis placed on sustainable farming methods. Results from DEFRA's Farm Practices Survey in 2015 indicate that clover (either red or white) is currently sown in over 50% of UK farmed grassland and is most popular in the dairy sector where over 70% of respondents had at least one clover-containing sward (DEFRA, 2015b). Recent reforms to the Common Agricultural Policy (**CAP**) have also highlighted the importance of legume forages by designating them as Ecological Focus Areas (DEFRA, 2014). Farmers with more than 15 ha of arable land are required to show a portion of their land falls in this designation as part of new 'greening' measures, which is likely to cause an increase in forage legume production. The Farm Practices Survey 2015 reported that 26%

of holdings surveyed already grew nitrogen fixing crops and a further 25% planned to add them to their system to comply with the new requirements (DEFRA, 2015a). A pure legume stand is required to satisfy the criteria for an ecological focus area, and therefore legumes typically grown in monoculture such as lucerne and sainfoin are the most likely to be adopted as a result of the new measures. Other than red clover, white clover, lucerne, and to a lesser extent sainfoin and birdsfoot trefoil, there is currently minimal utilsation of other legume species in the UK (examples of other viable species include black medick, vetches, lupins and meadow pea), as seeds of these species are generally only sold as parts of herbal ley formulae or as green manure.

Future prospects for legume utilisation

The price of oil and fertiliser often follow similar trends, at present the cost of oil and fertiliser is relatively low following a sharp fall in cost during 2014, however, forecasts predict a slow recovery over time (WorldBank, 2016). Rising fertiliser prices coupled with the increasing requirement for greater sustainability of farming systems mean that the economic and environmental benefits of forage legumes provide a strong basis to conclude that forage legumes should be utilised in place of fertilised grass as a forage source for dairy cow diets wherever possible. However, there are clearly areas, particularly elements of feeding value, where efficiency of legume utilisation could be improved which has guided the direction of the present study. Optimising feeding value not only requires information for ration formulation but also a combined approach of correct agronomy, ensiling, and analysis of nutritional composition. As the range of potential forage legumes species is vast, the scope of the present study was limited to considering how these factors might be improved for red and white clovers (in grass mixtures) and lucerne which are the most popular forage legumes within the UK dairy

industry at present. The following section reviews limiting factors that hinder the efficient utilisation of these species, and studies to date which have contributed to our knowledge of defining and addressing these limitations.

Limitations to the efficient utilisation of clovers and lucerne

Agronomy and ensiling

Agronomic challenges of lucerne. Due to the relatively small land area used for lucerne, few studies into lucerne agronomy have been conducted that consider the weather and soil types found within the UK where rainfall is higher, and temperatures colder than other areas where lucerne is commonly grown. Therefore, one of the greatest challenges to successfully utilising lucerne is achieving good establishment. During the establishment phase, lucerne preferentially partitions nutrient resources to below ground organs for tap root development, however in doing so, above ground organs grow slowly at first and are susceptible to being outcompeted by weeds in monocultures, or other species in multispecies swards and thus, lucerne is rarely included in mixed leys. Moreover, since lucerne is a broadleaved species, few herbicides are available that target weeds without harming the crop which poses a difficult challenge for farmers. Timing sowing to encourage lucerne persistence and reduce weed burden can have a lasting effect on yield, and feed value over the lifetime of a sward, as shown by a study from New Zealand in which later sowing dates reduced yield in the second-year yield by 1.3 T/ha DM (Wigley *et al.*, 2012). The beneficial effect of spring sowing has also been repeated in other studies from New Zealand (Sim *et al.*, 2015) and France (Justes *et al.*, 2002) with poorer autumn-sown performance attributed to low winter soil temperatures and wet conditions inhibiting bacterial and enzymatic activity which slows root development and lengthens the phase in which above ground organs receive fewer nutrients. Additionally,

nodulation of the roots with rhizobium is reduced in lower temperatures preventing nitrogen fixation (Phelan *et al.,* 2015). Use of an inoculant to promote rhizobium colonisation is typically advised. High levels of root nodulation are beneficially linked with greater above ground yield and tend to negatively correlate with nitrogen concentration in the soil suggesting a negative feedback mechanism through which atmospheric nitrogen fixation is reduced if soil nitrogen concentration is in excess of requirements (Suter *et al.*, 2015; Chmelikova *et al.*, 2015). This highlights the importance of not applying unnecessary inorganic fertilisation to lucerne swards.

Another promising method of non-chemical weed control in lucerne is utilising a cover crop. In the US, Wiersma *et al.* (1999) investigated oat, oat and pea, ryegrass and festuloium cover crops for lucerne and found they increased yield relative to no cover crop, with the grass species producing a higher CP concentration at harvest than oat or oat and pea. However use of ryegrass as a cover crop in wetter UK conditions is likely to lead to grass outcompeting lucerne. More recently, in Poland, barley and vetch cover crops were used with success in a study by Sowinski (2014), where a 53-83% reduction in broadleaved weed invasion was recorded relative to no cover crop for lucerne. Cover cropping also comes with an added benefit that the first harvest can be made into a nutritious wholecrop silage to improve returns from the land in the establishment year. Using a cereal such as barley also allows the possibility of taking a grain harvest from the cover crop, although Tan and Serin (2004) showed that increasing the seed rate of a barley cover crop also suppressed the concentration of lucerne plants, but despite this, there was still a beneficial effect on lucerne establishment which resulted in increased lucerne yeild relative to no cover crop over two growth years. From these studies, a combination of spring sowing and the use of a cereal cover crop show promise as tools

to aid in reducing weed burden at establishment, which require testing under UK conditions for efficacy at increasing yield and crop quality.

Although not a primary focus of this thesis, it should also be noted that lucerne can be challenging to protect from pests and diseases as it is not always possible to enter the crop with machinery unless tramlines (parallel wheel tracks left unsown to allow access) are utilised and there are no effective control measures available for some pests. Pests that can affect lucerne crops include slugs, aphids, eelworm, weevil larvae and leatherjackets while the predominant disease risk is *verticillium* wilt for which resistant varieties are available on the market (McConnell and Genever, 2015).

The agronomy of lucerne also has a direct impact on it's feeding value at harvest. Low digestibility and therefore ME value in comparison to other forages is another limitation on efficient forage legume utilisation, which could be influenced by choice of harvest maturity. In a comparison of different forage species Dewhurst et al. (2003b) reported that lucerne was the least digestible silage with 64% DM digestibility when fed to dairy cows (with a concentrate supplement) compared with red and white clover, and their combinations with ryegrass where DM digestibility ranged from 65-72% and ryegrass silage alone which had the highest digestibility at 72%. Altering harvest maturity affects the concentration of fibre in forage, particularly ADF, which strongly correlates with digestibility (Nousiainen *et al.*, 2009). A high proportion of fibre within lucerne is contained within the ADF fraction, of which literature values suggest 23% is comprised of lignin and the remainder is cellulose (NRC, 2001). Over-maturity at cutting not only increases ADF concentration through lignification reducing digetsibility (Iwaasa *et al.*, 1996; Homolka *et al.*, 2012) but also reduces the percentage of leaf material on the plant correlating with lower crude protein content (Tyrolova and Vyborna, 2008). Current recomendations suggest that the correct timing of cut is when 10% of stems are in bloom.

However, according to the study of Tyrolova and Vyborna (2008) in which leaf biomass and crude protein concentration of freshly harvested material was observed at four different growth stages from early bud to post-bloom, the proportion of biomass in leaves and also the CP concentration of the herbage is greatest if a cut was taken when small buds and no flowers were present which is consistent with previous findings (Fick and Mueller, 1989). Further confirmation of this came from the work of Hakl et al. (2016) where the effect of early and late cut timing on fractionation of nitrogen within leaves and stems of lucerne was assessed. Results from this study showed that slowly degradable nitrogen (fractions B1 and B3 of the Cornell Net Carbohydrate and Protein System, **CNCPS**) were decreased and indigestible nitrogen (fraction C) was increased in more mature plants. This suggests that feeding value may be improved in lucerne silages if maturity at cutting occured at the bud stage, prior to infloresce, however, these studies did not assess the impact of taking an early cut on regrowth and subsequent harvest quality which must be addressed in more detail. There is some evidence to suggest that repeatedly cutting lucerne at an early growth stage reduces survival rate and overall yield (Ventroni *et al.*, 2010) although it is not yet clear whether alternating cut timing from early to late over different cuts may overcome this barrier and still allow for a higher quality of silage to be acheived in some cuts, the first cut being of greatest importance. It should also be remembered that for optimal lucerne persistence over several years the stand should be allowed to produce seed once a year, perhaps late in the year when the crop has less value as silage, and following this the stand should be defoliated in good time prior to winter frost to reduce the biomass maintained by the plant over the dormant period.

Successfully ensiling lucerne is a third major barrier to improving its utilisation within intensive dairy systems. Many legumes have a low concentration of WSC in

comparison to grass and therefore can lack sufficient reserves to fuel anaerobic fermentation. In mixed swards the presence of ryegrass which is richer in WSC can overcome this problem, however, a farmer may desire to ensile lucerne alone (to increase silage homogeniety and enable precise feed rationing) and in this case the use of additive containing lactic-acid producting bacterium (**LAB**) such as *lactobacillus plantarum* would be required. Bacterial additives are effective in producing a correct fermentation profile for stable conservation but are not a substitute for adequate sources of carbohydrate in the crop, and therefore more research is required into the effect of varying harvesting conditions including dry matter concentration, cut, and harvest maturity, on the WSC concentration, and fermentation profile of lucerne silages. Poor fermentation profiles are typically characterised by high concentrations of acetic or propionic acids in place of lactic acid. Whilst low levels of acetic and propionic acid have been shown to beneficial affect aerobic stability at feedout (Wambacq *et al.*, 2013), very high concentrations can reduce intake in dairy cattle as observed in the study of Daniel et al. (2013) in which acetic acid and ethanol at a rate of 50 g/kg DM reduced intake. A lack of lactic acid in silage also reduces the rate at which pH drops upon establishment of anearobic conditions which can also lead to increased growth of yeasts and protein deamination in the silo (Duniere *et al.*, 2013). There are now increasing numbers of additives available with novel active ingredients such as fibrolytic enzymes to unlock greater carbohydrate resources within plant cells and these may be of greater use in lucerne silages than bacterial innoculants with further testing.

Agronomic challenges of clover-grass mixtures. While the establishment, maintenance and ensiling of clover-grass mixtures is relatively well understood in the UK in comparison to lucerne, the balancing of species evenness within a mixture remains a

significant challenge. Growers should aim for clover to form 30-60% of a grass-clover sward as this has been shown to be optimal in terms of evenness of species and symbiotic N fixation (Nyfeler *et al.,* 2011; Luescher *et al.,* 2014). Similarly, in more complex mixtures a roughly even distribution of species often brings the best results in terms of balancing the niche complementarity of each component (Cardinale *et al.,* 2007). However, in practical terms, the ability of the grower to influence botanical composition in an established ley is limitted, and furthermore, this challenge is compounded by seasonal and annual changes in relative species dominance over the lifetime of the sward. In grass-clover mixtures, cooler and wetter conditions initially favour grass growth in spring, but drier and warmer temperatures during summer then favour clover (Chen *et al*., 2016). Additionally, clover tends to become less competetive in each subsequent year post establishment leading to a three-year life span for most grass-clover mixed leys. Some methods of influencing clover proportion in an established ley through management have been investigated in the literature. According to Laidlaw et al. (1992) defoliating in winter or early spring using sheep (or topping) improved clover percentage relative to grass in the following summer, however, grazing with cattle rather than sheep over the winter negatively impacted clover yield in the subsequent year, likely due to stolon damage. A study by Phelan *et al.* (2014) contributed further advice with regards summer defoliation management for grass-clover swards, showing that a 42d regrowth interval and a defoliation height of 2.7-3.5 cm improved the yield of clover within the sward whereas both shorter and longer regrowth intervals and a higher defoliation height (6 cm) negatively impacted clover yield. One further management strategy to control clover concentration is the tactical use of nitrogen fertiliser. Although a key driver for the economic viability of legume swards is the reduction in inorganic fertiliser use (as discussed previously), the application of fertiliser to a mixed grass-clover sward has been

shown in multiple studies to favour the production of grass over clover and therefore could be used at low application rates (such as 50 kg/ha) to reduce clover content in cases where the clover is becoming very dominant in late summer (Laidlaw *et al.*, 1992; Yarrow and Penning, 1994; Schils and Snijders, 2004).

While these studies on clover management provide some simple guidelines by whch farmers may adjust management to favour or disadvantage clover depending on clover persistance within their sward, one key factor which is not considered within these studies is the management of mixed swards in which there is an uneven spatial distribution of clover. Many factors can influence the establishment of clover within a mixed sward in a single field, including changes in soil type, soil nutrient status, exposure to light, slope gradient and height of the water table to name just a few, and as a result clover concentration is rarely uniform within a field which negatively affects its yield and feeding value (Sharp *et al.*, 2014). This introduces a further challenge to correct management because a strategy to suit one portion of the field may have an opposite effect in others. In fact, it is likely that, in such a case, it would not be possible for a farmer to micro-manage the field for optimum clover concentration, especially considering that no simple tools exist that allow the farmer to measure clover concentration accross a field (which should be considered a pre-requisite for proper management). In the future, such micro-management may be achieved through the application of precision technologies to forage crops. Examples of innovations which may be tailored to suit mixed forages to better facilitate management for species evenness include global positioning system (GPS) navigation for precision inputs as are currently used in arable systems, and tractor mounted NIRS machines capable of real-time detection of chemical or botanical composition. However, such developments will take significant time and financial input to reach market and therefore, in the short term,

research into simple methods that allow farmers to measure clover concentration within a sward, and understanding whether farmers currently alter management depending on clover presence, will underpin intial improvements in clover-grass agronomy.

Improving silage analysis for legume species

Utilising near infra-red reflectance spectroscopy (NIRS). A barrier to the commercial uptake of legume silages, is the lack of a bespoke NIRS equation which is sufficiently accurate for forage legumes. This is particularly relevant to the UK where most silages are analysed undried and unmilled in comparison to europe where dry analysis is more common. Dry analysis requires more sample preparation but has the benefit of increased precision of NIRS prediction, partly explained by the increased stability of the feedstuff after the removal of water (Sorensen, 2004). Milling samples also improves homogeniety and should reduce analytical error (Fernandez-Cabanas *et al.*, 2006). Many European studies have developed NIRS equations to predict the chemical composition of legume species, but due to these differences sample preparation between europe and the UK these equations cannot be used without further testing. Therefore at present, the range of commercial forage-based equations available to UK growers are limitted to grass (Givens *et al.*, 1989), maize (Rymer and Humphries, 2013) and wholecrop (Deaville *et al.*, 2009) silage.

Analysis of clover-grass silages. A preliminary study has shown that the current NIRS analysis available for use on grass silages in the UK cannot always accurately analyse some variables of chemical composition when used on grass-clover mixtures (Davies *et al.*, 2012). In particular, CP was shown to be under-predicted possibly due to high concentrations of CP falling outside of the grass-based calibration dataset. Where mixed

rations are formulated on a CP basis, this may lead to legume silages being under-valued as protein sources, causing farmers to add an oversupply of supplemental protein sources in the diet which is likely to result in poor nitrogen efficiency resulting from losses in the faeces and urine. However, many farmers now use a combination of CP and EDN to estimate effective rumen degradable protein and formulate rations on a MP basis using the Feed Into Milk system (FiM consortium, 2004). This approach also involves the use of NIRS predicted microbial dry matter (**MDM**) synthesis per unit of expected DMI, which is based on the rumen effective degradability of dry matter, to calculate ME supply. Finally, digestible organic matter in total dry matter (**DOMD**) is also predicted by NIRS to allow the rationing system to account for the proportion of nutrients that are digestible but non rumen-degradable. As the study of Davies *et al.* (2012) did not investigate the prediction accuracy of digestibility and degradability parameters, such as DOMD, MDM and EDN, further evidence is required to understand the current impact of prediction bias on ration formualtion accuracy when using a system such as Feed into Milk.

If further investigation proves that the grass equation is unsuitable for use to analyse CP or other nutrients in clover-containing silages, one solution would be to create a new bespoke calibration equation for clover-grass silages. However, this also presents a challenge due to these silages being mixtures of two species, grass and clover, meaning that any resulting equation must be able to deal with a broad spectrum of sample composition. Examples of NIRS equations designed for use on grass-clover mixed silages exist in the literature however their focus is on measuring botanical composition rather than chemical composition (Wachendorf *et al.*, 1999; Cougnon *et al.*, 2014) and all use dried and milled samples. Collecting a sample set which is sufficiently robust from which to produce prediction equations is time consuming due to the need to collect real-world samples to generate sufficient variation (Cougnon *et al.*, 2014) and therefore,

investigating ways in which the scope of existing grass equations might be broadened so that they could be applied accurately to other forages is of interest in order to increase the capability of UK NIRS analysis in a timely fashion. One potential solution to this which requires further investigation, is to use the clover concentration of the silage as a modifier to the grass equation if bias is significantly correlated with clover concentration. However, currently, the accurate determination of botanical composition in silage mixtures is limited to manual aspeciation; a labour-intensive and subjective method. Another method which has shown some success is to use n-alkane concentration as a proxy for clover concentration however further validation of this method would first be required (Jurado *et al.*, 2015). As mentioned previously, NIRS equations exist which have been able to robustly predict clover concentration in grass-clover mixtures, and the development of such an equation appropriate for use on undried and unmilled samples would be a practical solution to determination of clover concentration for use as a correction factor.

Analysis of lucerne silages. There is also a lack of a bespoke UK analysis for pure legume silages such as lucerne, equations that have been developed in Europe and the US primarily involve the analysis of dried samples (Brogna *et al.*, 2009) or freshly harvested un-ensiled samples (Marten *et al.*, 1984) and these are not necessarily appropriate for use on silages. The nature of calibration models suggests that prediction accuracy will be good where the concentration of the desired chemical falls within the same range as was present within the calibration set, and, that concentrations falling outside the original calibration space require extrapolation which is likely to reduce prediction accuracy (Estienne *et al.*, 2001). It is therefore reasonable to hypothesise that some nutritional components of lucerne could be well predicted by a calibration equation developed on

another legume species although no such testing of this concept has been carried out on silages previously, other than that already mentioned in relation to using grass equations for grass-clover samples. It is not yet clear to what extent differing physical characteristics between species may also reduce accuracy in such a situations.

Feeding strategy for lucerne silages

Nutritional characteristics. A final limitation to the increased utilisation of forage legumes that affects lucerne to a greater extent than clovers is feeding value. As discussed previously, lucerne contains a higher proportion of ADF relative to NDF than most forages due to indigestible cell wall (high lignin concentration) comprising a large proportion of cell carbohydrate which can result in a low DM digestibility. For this reason lucerne is very commonly fed in combination with maize to provide fermentable energy and ensure sufficient capture of degradable nitrogen, and because both are suited to growing in semi-arid climates. However, the swift degradation rate of the diestible portions of the lucerne cell still poses a challenge for efficient nutrient capture (Homolka *et al.*, 2008). Lucerne contains no PPO, unlike red clover, or condensed tannin, unlike sainfoin, which both help to protect protein from ruminal degradation, and since lucerne can contain a high CP concentration, an oversupply of rumen degradable nitrogen with insufficient energy to drive rumen microbial nitrogen capture is typical of lucerne-based diets leading to low nitrogen efficiency. Initial work to reduce the nitrogen degadbility of lucerne in the rumen began as early as the 1970's where the use of formaldehyde and other acid salts as silage and hay additives were investigated and shown to greatly improve the supply of digestible but non-rumen degradable nitrogen to the small intestine (Waldo, 1973; Tyrrell *et al.*, 1992). However this came with a negative side-effect of favouring clostridial fermentation in the silo reducing silage fermentation quality which

perhaps explains why the use of such additives has not gained particular popularity. More recently, some genetic attempts to reduce nitrogen degradability in lucerne have been made, two interesting examples being the successful engineering of lucerne plants to produce PPO (Sullivan *et al.*, 2004; Sullivan *et al.*, 2008) and, in a different study, condensed tannins (Hancock *et al.*, 2012) however such varieties are not available to UK farmers. Tannins can be added to silage as an additive, although they have not been found to be as efficacious as formaldehyde at protecting protein from ruminal degradation (Salawu *et al.*, 1999). In 2014 conventional breeding methods in the USA produced a new lucerne variety with a longer harvest window (Hi-Gest 360, Alforex Seeds, CA, USA) to help reduce lignin concentration and improve digestibility however this variety is still in early stages of implementation and is not available to the UK market at present. Therefore, practical solutions are required to target (i) improved digestibility to provide greater ME value and (ii) improved ruminal nitrogen capture or ruminal protection of protein, in order for the feeding value of lucerne to be increased. There is also increased interest in the use of lucerne to improve rumen functionality through raised rumen pH as it has a higher buffering capacity than grass and most other forage legumes (McBurney *et al.*, 1983) and may stimulate more rumination through provision of a high concentration of peNDF (Kowsar *et al.*, 2008). However, evidence that lucerne has such a beneficial function is controversial as some studies have reported that replacing other forages with lucerne in the diet had no effect on rumen pH (Wattiaux and Karg, 2004; Brito and Broderick, 2006). In another example, Larsen and Kristensen (2010) theorised that feeding lucerne silage might improve the health status of freshly calved cows through provision of effective fibre in the first week post-parturition however no such benefits were proven in the study and subsequent intake in weeks 2-4 of lactation was reduced by feeding lucerne at a high rate in the diet. Due to these conflicting data, further research is

required to determine whether effective fibre provided by lucerne is of benefit to the cow, particularly within forage-rich European diets as many studies involving rumen pH used very challenging diets, rich in non-forage carbohydrate, or containing a high rumen degradable starch:peNDF ratio.

Inclusion rate within a total mixed ration. The optimum inclusion rate for different forage species within a mixed diet varies depending on its feeding value (i.e. ME and MP concentration) and the diet formulation. In the case of lucerne and maize forages, several studies have been conducted into the effect of differing inclusion rates of lucerne silage or hay replacing maize silage to discern the optimum inclusion rate for DMI and milk yield. Achieving the correct ratio should result in increased milk yield and increased capture of MP leading to higher milk protein, lower milk urea, and lower urinary nitrogen excretion. A summary of eleven studies from the literature in which lucerne replaced maize at varying concentrations without confounding from other dietary treatments and the effect of doing so on DMI and milk yield is shown in Figure 1. The inclusion rates tested within these studies ranged from 70-220 g/kg diet DM as the lower inclusion rate and 210-410 g/kg diet DM as the higher inclusion rate with the average low rate being 130 g/kg DM and the average high rate being 320 g/kg DM. Maize silage was the forage replaced by lucerne silage or hay in every instance.

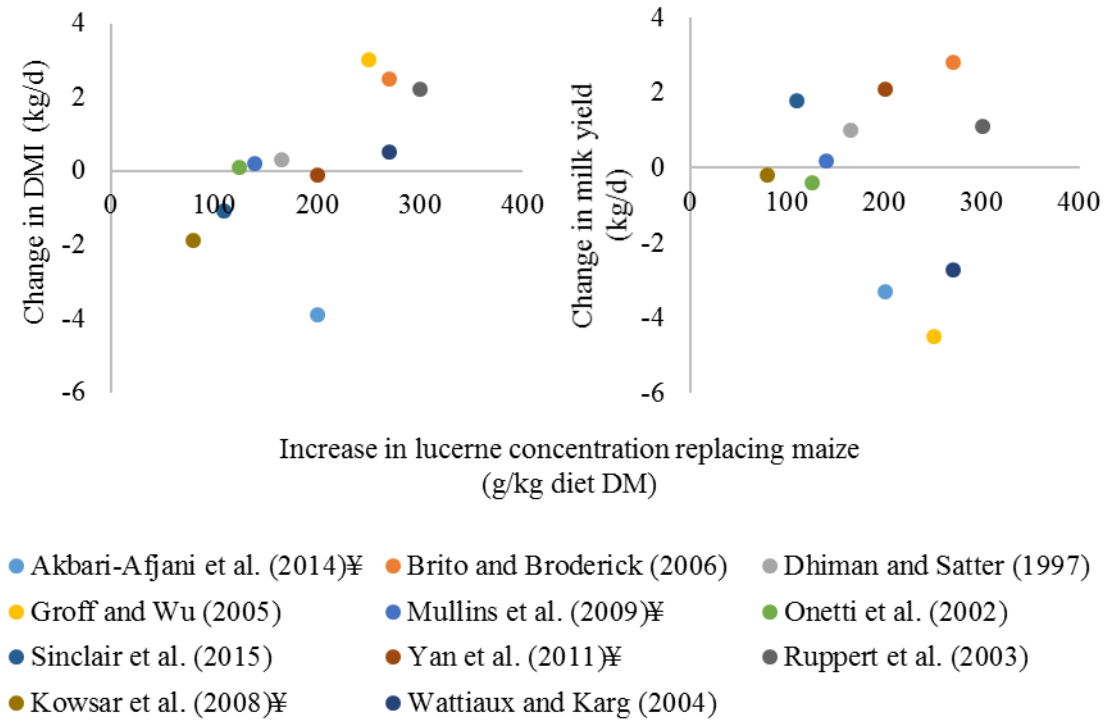

Figure 1: A summary of results from eleven studies which tested the effect of replacing maize silage with lucerne silage or hay (¥) on DMI and milk yield in dairy cattle.

For DMI changes in inclusion rate where the increase in lucerne concentration was 200 g/kg diet DM either did not affect, or reduced, DMI including one recent UK study (Sinclair *et al.*, 2014). In a minority of three studies where large quantities of mazie were replaced with lucerne, significant increases in DMI were observed (Ruppert *et al.*, 2003; Groff and Wu, 2005; Brito and Broderick, 2006). The passage rate of particles within the rumen has been shown to increase in TMRs containing a higher inclusion rate of lucerne silage relative to maize silage which can be explained by a rapid rate of degradability of rumen-available material and therefore the effect of high inclusion rates of lucerne improving DMI in these three studies likely related to reduced rumen fill. Alternatively a reduction in ME supply may have increased appetite in animals fed higher rates of lucerne. The degree to which rumen fill limits intake can vary from silage to silage (or hay to hay) depending on fibre degradability. The study of Akbari-Afjani et al. (2014) which utilised lucerne hay resulted in an unusually large reduction in intake in comparison to the other studies, even those others that also utilised lucerne hay (Kowsar

et al., 2008; Mullins *et al.*, 2009; Yan *et al.*, 2011). In this study, the lucerne hay was very finely chopped whereas corn silage was fed at a long chop length (30 mm) resulting in the high lucerne diet containing less peNDF than the high maize diet which perhaps impaired digestive functionality through a lack of material to form the rumen matt and stimulate rumination, leading to reduced buffer provision to the rumen combined with a rapid rate of VFA production.

Milk yield responses to changes in lucerne inclusion rate were much more varied than DMI and this may reflect differences in the digestibility of each individual silage or hay used, differences in ration formulation criteria in each study (for example, the studies of Dhiman and Satter (1997), Wattiaux and Karg (2004), and Yan et al. (2011) were not formulated on an isonitrogenous basis whereas, in all other studies, diets contained similar levels of CP), or differences in the stage of lactation of the enrolled animals affecting the magnitude off responses to changes in diet composition. In the studies of Wattiaux and Karg (2004) and Groff and Wu (2005), increasing lucerne inclusion rate reduced feed conversion efficiency considerably as intakes were increased at higher inclusion rates whereas milk yield was decreased which is quite likely a response to the lower ME value provided by the lucerne silage. There is a need to better characterise lucerne silages for their potential to maintain milk yield when replacing maize silage in the diet.

Silage chop length and physical characteristics. The interaction between the inclusion rate of lucerne within the diet and the chop length of lucerne has rarely been tested to date. Many studies report effects of lucerne inclusion rate (as summarised previously) or particle size (summarised in Figure 2) alone on intake and milk yield, however, Rode and Satter (1987) showed that significant interactions between the two occur for their effects

on intake and total tract nitrogen digestibility although in this study lucerne hay was replaced by grain in the diet rather than another forage. There is a need to better understand this link in relation to lucerne and maize silage based diets so that the two factors can be combined for optimum milk production and to examine whether their interaction significantly affects other variables such as rumen functionality.

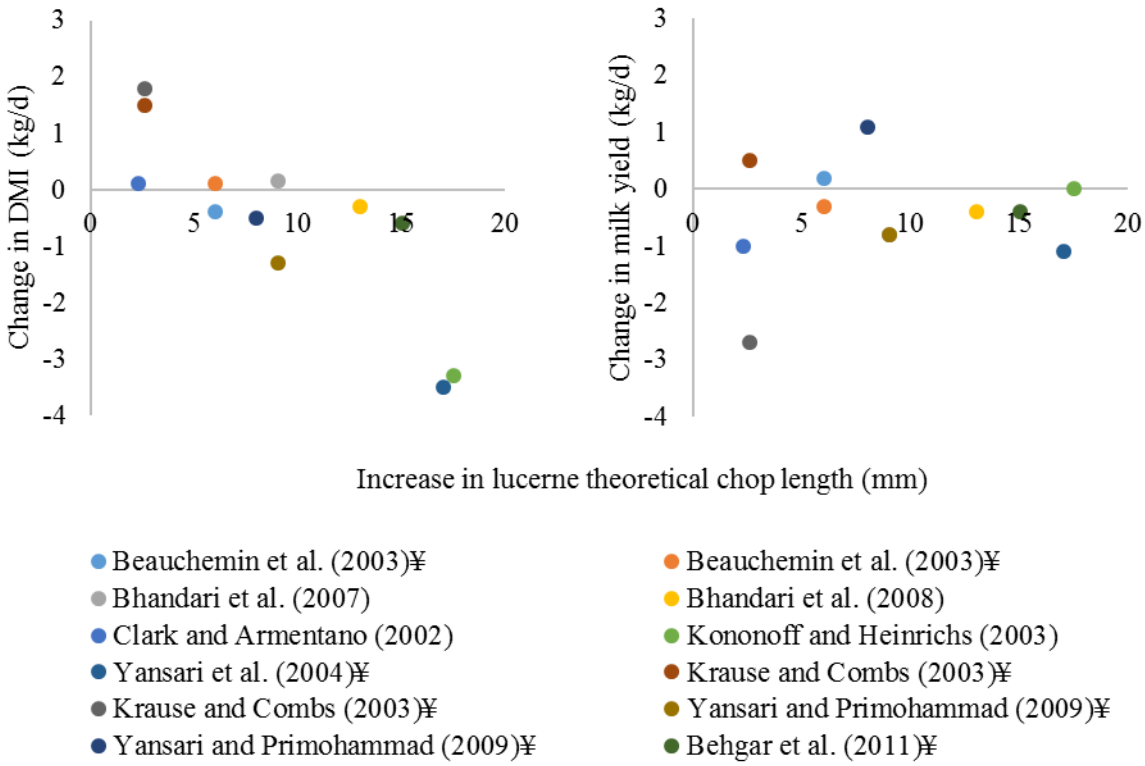

Figure 2: A Summary of results from ten studies which tested the effect of increasing the chop length of lucerne silage or hay (¥) within a TMR on DMI and milk yield in dairy cattle. Two studies in which there were more than one pair of comparable treatments are included twice each.

Examining the main effect of increasing the theoretical chop length of lucerne alone, a summary of ten studies from the literature that specifically utilised lucerne as a forage source are presented in Figure 2. The average theoretical chop length difference between two comparable treatments in these studies was 9 mm, although, it is also important to consider that theoretical chop length and the geometric mean particles size can differ greatly and the theoretical desired difference was not always achieved in every study. The trend shown in Figure 2(a) suggests that larger increases in lucerne chop length

correlates with decreases in DMI relative to smaller increases in chop length. Very small increases in chop length sometimes resulted in increased intake, most notably in the study of Krause and Combs (2003), however in this study the geometric mean particle length of the shortest lucerne silage was just 2.7 mm. This suggests that a large portion of the short chopped lucerne was too fine to be physically effective and as low daily mean ruminal pH was also observed in the study, indicating a chellenging rumen environment. Acheiving such a fine chop required rechopping with specialist equipment and such a forage would be unlikely to be fed on-farm in the UK. This highlights a need for more chop length studies in which particle size differences are relevant and achievable under realistic conditions and with standard machinery.

With regards to particle size effects on milk yield, the majority of studies showed a small response with more studies reporting a negative effect of increasing chop length than those that reported positive effects although results were varied and no clear trend was observed.

Conclusions

From the evidence considered in this review it may be concluded that forage legumes could be utilised more efficiency in UK dairy systems if limitations to their succesful agronomy, conservation and feeding value could be addressed. In the case of grass-clover silages, key limitations include managing the proportion of clover within the ley for optimum feeding value and nitrogen fixation, and also the lack of an NIRS analysis which is specifically calibrated for use on clover containing samples. Whereas, for lucerne silages, key concerns include difficulty in achieving sucessful establishment, a need for improved feed value in the first year, lack of a bespoke NIRS analysis, and the possibility of reducing diet digestibility and nitrogen use efficiency when utilised as part of a TMR.

It should be noted that these are not the only limitations known to effect the uptake of these forages however these were considerred some of the most important limitations that could reasonably be addressed within the scope of the present study.

AIMS OF THE PROJECT

The overall goal of the present study was to investigate and remove key factors that currently limit the efficiency of clover and lucerne utilisation in the UK dairy industry. The aims listed below were set out to ensure a wide range of limitting factors were addressed, and in such a way that data would be created that could inform best practice advice for farmers. Possible limitations to consider were identified anecdotally initially through interaction with the UK levy board, AHDB Dairy, and the use of a workshop which was attended by agronomists, nutritionists and farmers. Where applicable, hypotheses have also been devised based on existing knowledge from previous research in the literature on forage legumes and are listed beneath each aim. The list of aims is presented in three sections: agronomy, silage analysis, and feeding strategy (for lucerne silages).

Agronomy

1. To investigate the effect of varying the timing of lucerne establishment on yield, weed burden and resulting silage quality over one establishment year and one subsequent year by comparing 8 spring-sown plots and 4 autumn-sown plots (where autumn is established after spring in the same establishment year).
    i. Spring establishment will increase yield, reduce weed burden and improve the feed value of harvested lucerne relative to autumn establihsment.
2. To investigate the effect of using a cover crop for spring establishment on yield, weed burden and resulting silage quality over one establishment year and one further year by sowing 4 spring sown plots with a barley cover crop and another 4 without.

   i. The use of a cover crop will increase yield, reduce weed burden and improve the feed value of harvested lucerne relative to establishing without a cover crop.

3. To investigate the effect of harvesting lucerne at an early growth stage (early bud stage) in comparison to harvesting at 10 % flower stage (currently recomended), for a first and a second cut, on DM yield and feeding value.
   i. Harvesting material at an early growth stage will increase CP concentration and reduce ADF concentration relative to the recommended harvest timing at both a first and a second cut.
   ii. Harvesting material at an early growth stage will reduce yield relative to the recommended harvest timing at both a first and a second cut.
4. To increase understanding of common agronomy practices employed by growers of grass-clover mixed swards to provide a basis for targetting future research efforts into aiding in the management of mixed herbage.

Silage analysis

1. To assess an existing grass-based NIRS prediction equation (developped for use on undried and unmilled samples) for accuracy when utilised to predict the nutrient composition of clover-grass silages containing varying concentrations of clover.
   i. There will be a difference between predicted and observed values for some nutritional variables, including crude protein concentration.
   ii. For variables where there is a difference between predicted and observed values, the bias will increase as clover concentration increases.

2. If needed, to utilise measured data from the clover-grass silage sample set to calibrate new, bespoke, NIRS equations for predicting nutritional composition of clover-grass silages and validate their performance.
    i. Bespoke clover-grass-based equations will improve the prediction accuracy of nutritional variables relative to the grass-based equation.
3. To assess existing forage-based NIRS equations (the grass-based equation and any equations developed as a result of work on clover-grass equations) for accuracy when utilised on undried and unmilled lucerne silages.
    i. Equations developed for clover-grass silages will show greater accuracy when used to predict the nutritional composition of lucerne silages than the grass-based equation.

Feeding strategy for lucerne silages

1. To investigate the effect of varying inclusion rate of lucerne silage relative to maize silage within a TMR on dry matter intake, milk yield and milk composition of lactating dairy cows.
    i. A higher rate of lucerne silage inclusion within the diet will decrease intake and milk yield, and therefore yield of milk solids, relative to a low inclusion rate.
2. To investigate the effect of varying inclusion rate of lucerne silage relative to maize silage within a TMR on rumen function and response to a ‘feed withholding and refeeding’ rumen challenge in lactating dairy cows.
    i. A higher rate of lucerne silage inclusion within the diet will increase mean daily rumen pH and rumination relative to a low inclusion rate under normal conditions.

   ii. The rumen pH of cows fed a high inclusion rate of lucerne silage during a rumen challenge will be more resilient to the effects of the challenge than that of cows fed a low inclusion rate.

3. To investigate the effect of of varying lucerne silage chop length within a TMR with maize silage on dry matter intake, milk yield and milk composition of lactating dairy cows.
   i. Feeding a longer chop length of lucerne silage within the diet will decrease intake and milk yield, and therefore yield of milk solids, relative to a shorter chop length.
4. To investigate the effect of of varying lucerne chop length within a TMR with maize silage on rumen function and response to a 'feed withholding and refeeding' rumen challenge in lactating dairy cows.
   i. Feeding a longer chop length of lucerne silage will increase mean daily rumen pH and rumination relative to a short chop length under normal conditions.
   ii. The rumen pH of cows fed a longer chop length of lucerne silage during a rumen challenge will be more resilient to the effects of the challenge than that of cows fed a short chop length.
5. To investigate whether there are interactions between chop length and inclusion rate of lucerne silage within a TMR with maize silage on intake, milk production, rumen function and response to a rumen challenge in lactating dairy cows.
   i. There will be a greater effect of varying lucerne silage chop length when lucerne silage inclusion rate is high than when inclusion rate is low on dry matter intake, milk yield and parameters of rumen function.

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Chapter 3

Improving the accuracy of Near Infra-Red Reflectance Spectroscopy analysis for fresh grass-clover mixture silages

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ABSTRACT

The purpose of the present study was to ascertain whether Near Infra-Red Reflectance Spectroscopy (NIRS) prediction equations calibrated on predominantly pure grass silage samples, could accurately predict the chemical composition of mixed grass-clover silage samples. To this end, a diverse set of 94 grass-clover silages (ranging in clover content from 4 to 1000 g/kg as fed) were analysed for chemical composition using reference laboratory techniques, *in vivo* digestible organic matter digestibility in the dry matter (DOMD, in sheep), *in situ* degradability of dry matter, organic matter, and protein (in cows), and by NIRS (at AFBI, Northern Ireland). Botanic composition of each sample was determined by manual aspeciation. Predicted and observed results were compared using the ratio of standard error of prediction to the standard deviation of the measured sample set (RPD) with values greater than 2.0 denoting good agreement, amongst other measures of correlation.

Of 15 chemical components that were tested for prediction accuracy, only volatile corrected dry matter and nitrogen were well predicted (RPD values of 4.9 and 2.4 respectively). Neutral detergent fibre and DOMD did not meet the RPD threshold of 2.0, however the predicted and observed datasets had no significant bias between them and also had low root mean square errors of prediction (RMSEP), therefore these equations were also considered as fit for purpose. Components with a significant bias between predicted and observed datasets including crude protein, ash, ammonia nitrogen, acid detergent fibre, ether extract, microbial dry matter yield and the effective degradability of nitrogen, were not considered suitably accurate. In the majority of these cases, bias could be attributed at least in part to clover concentration and changes in the fractionation of nutrients present. For some chemical components such as crude protein and acid

detergent fibre, grass-based equations were sufficiently accurate at low clover concentrations but became increasing inaccurate as clover concentration increased.

Two solutions to inaccuracy of prediction of certain nutrients are suggested: (i) the calibration of bespoke grass-clover prediction equations based on the spectra obtained in the present study and (ii) the adoption of a correction factor for clover concentration. The former of these solutions was tested for viability and the resulting grass-clover-based equations outperformed their grass-based counterparts although many chemical components still fell below the RPD threshold of 2.0 possibly due to the relatively small size of the current sample set.

HIGHLIGHTS

- NIRS equations calibrated on grass silages were tested on clover-containing silages
- There was adequate prediction accuracy for some components, including digestibility
- Crude protein, and nitrogen degradability showed poorer prediction accuracy
- Crude protein and nitrogen degradability bias increased with clover concentration
- Creating bespoke grass-clover calibration equations increased prediction accuracy

INTRODUCTION

Near Infra-Red Reflectance Spectroscopy (**NIRS**) is a relatively rapid and inexpensive technique, routinely used to provide nutritional analysis of silage and other livestock feeds in the dairy and beef industries. However, obtaining accurate results requires robust prediction equations. This is particularly relevant for the UK where most silages are analysed ‘fresh’ (i.e. undried and unmilled) for high through-put in comparison to continental Europe where analysis of dried, ground samples is more common. Dry analysis requires longer sample preparation but has the benefit of increased precision of

NIRS prediction, partly explained by the increased homogeneity of ground samples as well as the stability of the feedstuff after the removal of water (Sorensen, 2004). Currently UK laboratories do not offer bespoke NIRS equations for grass-legume mixtures and prediction equations with a grass-based calibration equations are used for a number of different grass and legume-based forages.

The present study focusses on NIRS analysis for grass-clover silages, since clover is thought to be present within grass swards on up to 70% of UK dairy farms, and therefore is likely to be the most widely-grown forage legume in the UK (DEFRA, 2015). Furthermore, clover-containing forages are thought to be a promising feed to increase sustainability on farms due to reduced inorganic fertilser required for growth in comparison to ryegrasses (Elgersma *et al.*, 2000), while maintaining high yields of milk or meat due to a fast rate of passage promoting intake (Dewhurst *et al.*, 2009; Copani *et al.*, 2016). A preliminary study has shown that the current NIRS analysis available for use on grass silages in the UK has poor prediction accuracy of crude protein, pH and lactic acid when used on mixtures containing both clover and grass (Davies *et al.*, 2012). However, Davies *et al.* (2012) did not evaluate the degradability of dry matter (**DM**), nitrogen (**N**), or the apparent total tract digestibility of organic matter (**OM**; from which metabolisable energy (**ME**) is calculated) for prediction accuracy, despite these nutrient fractions being very important for diet formulation when balancing the ratio of metabolisable protein to metabolisable energy supply. Imbalances in the degradable protein to fermentable energy ratio will result in poor N use efficiency. Creating calibration equations for grass-clover silages poses a challenge because these silages are a mixture of two (or more) forage species, meaning that any resulting equation must be able to deal with a broad spectrum of sample composition. To date, the majority of forage based NIRS calibrations have focussed on predicting the nutritional composition of just

one species, and moreover, in a few instances where mixtures were analysed using NIRS, typically the focus of the study was on predicting botanical composition rather than chemical composition (Wachendorf *et al.*, 1999; Cougnon *et al.*, 2014; Karayilanli *et al.*, 2016).

The objective of the present study was primarily to assess the adequacy of grass-based prediction equations, commonly used in the UK for predicting chemical composition, when they are applied to grass samples that contain clover in varying concentrations. A secondary objective was to investigate whether using grass-clover based prediction equations could improve accuracy of predicted chemical composition.

MATERIAL AND METHODS

Experimental design

In total, 94 clover/grass silages were sourced from commercial farms and transported to the Centre for Dairy Research (**CEDAR**; Arborfield, Reading, UK) for processing. Samples were acquired from a diverse range of UK farms to ensure maximum variation within the sample set in line with the recommendations of Cougnon *et al.* (2014) for sourcing robust calibration data. Silage was collected over three consecutive years (2012/13, 2013/14, and 2014/15). The clover content range of greatest importance was deemed to be 300 - 600 g/kg DM as a more even distribution of grass-clover within a ley has been shown to create the most advantageous conditions for growth and promote symbiotic N fixation (Nyfeler *et al.*, 2011; Luescher *et al.*, 2014); although samples containing < 300 and > 600 g/kg DM clover were also included to provide sufficient range for statistical analysis and equation evaluation. A number of silages with 0 -50 g/kg DM clover inclusion rate were included as a control group in which prediction accuracy should be comparable to that of pure grass silages. Wrapped (baled) and clamp silages

were included in roughly equal numbers and the set also comprised a mixture of clover varieties to ensure adequate testing of prediction accuracy for silages containing both red (*trifolium pratense*) and white (*trifolium repens*) clover.

Sample processing

Once a sample arrived at CEDAR, if it was in the form of an unchopped bale silage, it was mixed and chopped in a feeder wagon (Hi-Spec Mix Max, Hi-Spec Engineering, Co. Carlow, Ireland) for 45 minutes. Clamp silages that were already chopped, were mixed in a DataRanger diet mixer that did not contain knives (American Calan, Northwood, NH, USA). The DM content of the silage was estimated from the loss in weight of a subsample after it has been repeatedly placed in a microwave oven (Belling 384TC, 850 Watts) until a constant weight was achieved. From this determination, the amount of silage (fresh weight) required for feeding an individual sheep for one day was calculated. This amount was then weighed into a series of polythene bags, the air was removed under vacuum, and the bag was sealed and stored frozen (-20°C) until required. Frozen subsamples of each silage were stored separately for future analysis by aspeciation, NIRS and for chemical composition.

Nutritional analysis

A 2kg frozen subsample of each silage was sent to the Agri-Food and Biosciences Institute (**AFBI**; Hillsborough, Northern Ireland) where the reference chemical composition of the silages was determined using UKAS accredited methods and NIRS spectra were obtained through scanning the samples using NIR wavelengths 1100-2498nm with a Foss 6500 machine (Foss, Hillerød, Denmark). Dry matter was calculated after the removal of volatile compounds using toluene and reported as volatile-corrected

oven dry matter (**VCODM**). Ash was measured through combustion in a muffle oven at 500°C for 18h. Lactic acid (**LA**) and other volatile compound measurements: total volatile fatty acids (**TVFA;** the sum of lactic, acetic, propionic, butyric, and valeric acids) and total volatile content (**TVC**; TVFA plus ethanol and propanol), were determined using gas chromatography following extraction of representative samples in distilled water (Erwin *et al.*, 1961; Givens *et al.*, 2009). Nitrogen (**N**) was measured using the macro Kjeldahl method 954.01 (AOAC, 2000) and Ammonia-N (**NH^3-N**) was determined in the same way following extraction of ammonia by soaking a 50 g silage sample in sulphuric acid for a minimum of 24 h. Both ether extract (**EE**) and water soluble carbohydrate (**WSC**) were measured on dried and ground samples, EE according to AOAC method 920.29 (AOAC, 1990), and WSC as described previously (Fuller, 1967). Dried and ground samples were subsequently passed on to Trouw Nutrition (Ashbourne, Derbyshire) who performed analyses for neutral detergent fibre (**aNDF**) and acid detergent fibre (**aADF**) both inclusive of residual ash using Fibrecap equipment (Foss, Hillerod, Denmark) (Robertson and Van Soest, 1981; Kitcherside *et al.*, 2000; Mertens *et al.*, 2002). A further 200 g of silage was manually aspeciated at CEDAR into clover, grass and other species as a means of determining the clover concentration (**CC**) of the silage. Resulting fractions were then dried to determine species composition on a DM basis. In vivo reference methods were performed at CEDAR to determine silage digestibility and degradability.

In vivo digestibility. Silage digestibility was measured using Mule x Texel wether sheep in a series of 3 x 3 Latin square design experiments such that the final digestibility values comprised the mean of measurements from three animals. Each wether was fed a silage sample *ad libitum* (with 10% refusals) for 16 d adaption followed by a 5 d sampling

period during which sheep were placed in a metabolism crate for faeces and urine collection as described previously (Bratzler, 1951; Givens *et al.*, 1989). All *in vivo* procedures were licensed and monitored by the UK government Home Office under the Animal (Scientific Procedures) Act 1986.

Initially 18 wether were enrolled on the study when they reached adult weight (>30 kg) and were replaced if they became too large to comfortably fit within metabolism crates (in total 50 animals were used over the duration of the study). Sheep were allowed two days to adapt to the metabolism crate before sampling began. The diet was supplemented with 20 g/d of a general purpose vitamin/mineral mixture for sheep (Countrywide, Evesham, Worcestershire, UK). The weight of feed offered and refused was recorded each day during the collection period. A subsample of feed was taken and analysed for DM and ash to calculate OM concentration. Refused feed was analysed for DM concentration. For all digestibility and degradability parameters, DM was not corrected for volatile loss. Weights of the sheep were recorded at the start and end of each sampling week. Out of the 94 samples, 4 were excluded from *in vivo* analysis due to being exceptionally low in clover content and there being insufficient material for the 9 week feeding schedule but were still used for all other analyses. Complete collections of faeces were taken for each sheep and refrigerated until the end of the 5 d collection period after which all faecal material was bulked together, thoroughly mixed and three 200 g subsamples were taken. These subsamples were placed in a forced air oven at 60°C for 72 h to determine DM concentration. Dried samples were then bulked, ground and a further subsample was placed in a muffle oven for combustion at 500°C for 16 h for determination of ash concentration. Digestibility results have been presented as digestible organic matter in total dry matter (**DOMD**, g/kg DM).

In situ degradability. Degradability values were obtained using an *in situ* method with rumen cannulated Holstein dairy cattle (Ørskov and McDonald, 1979). These cattle were kept together in a dedicated metabolism unit, fed a commercial grass-maize based total mixed ration (**TMR**) diet once daily and milked twice daily at 0600 h and 1600 h approximately. Samples of each silage were placed in porous (40-45 µm pore size) nylon bags that were then sequentially incubated in the rumen for six time intervals (3, 6, 12, 24, 48, and 72 h) using a complete exchange method (i.e. bags for each timepoint were incubated one after the other rather than simultaneously). All bags were kept frozen until required and thawed 24 h prior to incubation. Replicates were obtained by using three different animals, with each animal incubating two bags for each time period per silage making six replicates per time-point in total. After incubation bags were immediately washed in cold water and placed in a freezer to ensure no further bacterial degradation took place. Additionally, to quantify '0' h washing loss, three further bags per silage were thawed, placed in a tub of cold tap water, and swirled for 5 minutes before being refrozen alongside the bags that were incubated in the rumen. After a minimum of 24 h in the freezer all bags were removed and left to thaw, then rinsed in cold water and washed in a washing machine (53 minute cold wash cycle; Zanussi SuperLuxe, Electrolux PLC, Luton, UK). Bags were subsequently dried (60°C) and residues analysed for the determination of DM, ash and N concentrations (as described previously). The solubility (**S**) of DM and N was determined by adding 1g of DM to 30ml of water and stirring for 5 minutes every half hour for a period of 2 h, the remaining material was then filtered (Whatman filter paper grade 4, Sigma-Aldrich, MO, USA). The filter paper and substrate was then dried and weighed to determine DM solubility by difference and residual N was measured as described previously.

The percentage of material degraded at each time-point was used to plot a degradation curve. Degradability fractions termed 'a', 'b' and 'c' were obtained from the curve. Fraction 'a' contains soluble material that is degraded almost immediately upon ingestion and 'b' contains the remaining non-soluble but degradable material with 'c' being the fractional rate of degradation of 'b' (Ørskov and McDonald, 1979). Degradation curves were calculated for each cow (3 replicates per sample). Two different approaches were used to calculate effective degradabilitiy (**ED**) based on the above fractions. To ensure the best comparison with predicted data, the ED of protein (**EDN_{FIM}**) and of dry matter (**$EDDM_{FIM}$**) were calculated using the 'Feed into Milk' (**FiM**) rationing software equations that corrected the 'a' fraction for S as recommended by Hvelplund and Weisbjerg (2000) shown in equation 1. In these equations the outflow rate of small (k_{liq}) and large (k_f) particles was standardised at 0.075 and 0.045 respectively to fairly compare against predicted data. $EDDM_{FIM}$ was converted to microbial dry matter (**MDM_{FIM}**, g/kg DM) using standard equations to convert EDDM into ATP supply as described previously (FiM consortium, 2004).

Equation 1. $$ED_{FIM} = (0.9s/(0.9+k_{liq}))+(b_D\, c/(c+k_{liq}))+(bc/(c+k_f))$$

Where s is the soluble proportion, k_{liq} is the fractional outflow rate of the liquid pool (0.075), b_D is the degradable small particle proportion, b is the degradable large particle proportion, c is the fractional degradation rate of b, and k_f is the fractional outflow rate of the large particle pool (0.045).

A second, simpler, approach was also tested simultaneously using equation 2 to calculate the ED of N and DM using 0.08 as the standard outflow rate (k) of all particles ($EDN_{0.08}$, and $EDDM_{0.08}$) (Ørskov and McDonald, 1979).

Equation 2. $ED = a+bc/(c+k)$

Where a is the soluble, b is the non-soluble but degradable proportion, c is the fractional rate of degradation of b, and k is the fractional outflow rate of material (0.08).

Statistical analysis

Tests of relationships and trends within the measured dataset. Statistical analysis was conducted using Genstat 16th Edition (VSNI, Hemel Hempstead, UK). The measured dataset has been presented as maximum, minimum, mean and coefficient of variation (**CV**, %) values for each measured variable. The effect of clover concentration on each of the other chemical components was tested by grouping samples into minimal, low, medium and high groups (that were equal quartiles of the dataset; representing 0 - 65, 66 - 239, 240 - 500 and > 500 g/kg DM CC respectively) that were compared using analysis of variance (ANOVA). A post hock Tukey test was performed to determine whether there was significant differences between the means of the 4 groups. In addition a regression analysis was performed to examine linear, quadratic and cubic effects of CC on the measured chemical components. Crude protein (**CP**) was not directly measured or predicted but calculated using either measured or predicted N and VCODM values (6.25 x Total N on a DM basis). For all dry matter values throughout the present study (other than for digestibility and degradability parameters), VCODM has been used rather than DM, in accordance with the industry standard used by UK laboratories.

Tests of prediction accuracy during validation. The difference between laboratory assays and NIRS predicted values has been calculated using measured minus predicted values and is henceforth termed 'bias'. The means of the observed and NIRS predicted datasets were compared using a student's t-test to determine significance. Bias was also assessed using a regression analysis to examine the effect (linear, quadratic or cubic) of CC on

bias. Other techniques employed to test the predictive accuracy of equations through a blind validation test were relative root mean square standard error of prediction (**RMSEP** as a percentage of the measured mean), ratio of the standard error of prediction to the standard deviation of the measured dataset (**RPD**) as recommended by Williams (2014), and the r-squared value of the relationship between observed and predicted data ($\mathbf{r^2}$). The extent to which clover concentration correlated with any bias between the two analyses was examined using a linear regression.

Data pre-treatment and production of new NIRS equations Spectral data were pre-treated by taking the first derivative, smoothing, and applying standard normal variate detrending (**SNVD**) scatter correction (Barnes *et al.*, 1989) and a repeatability file (a file containing multiple spectra from the same sample measured under different conditions, designed to reduce the variability caused by differing environmental conditions and instruments). New equations developed were compared by standard errors of calibration (**SEC**) and cross-validation (**SECV**; expressed as a precentage of the observed mean) using a 'leave one out' method (i.e. data for one sample were removed and predicted using all remaining samples to calibrate the prediction equation, a process repeated for all samples in the set). For the purposes of a blind validation test, 10 samples were removed from the dataset and tested using the remaining equation.

RESULTS

Sample chemical composition

The set of 94 silage samples consisted of 58 bales and 36 samples from clamps that were collected from 50 different locations across the UK. Of the samples where the clover variety was known (n = 65) 66 % were red clover, 20 % were white clover and 14 % were

a mixture of both. Different cuts were also represented within the set with 36 first, 20 second, 16 third and 4 fourth cut silages (22 samples unknown). The range of clover concentration represented within the sample set covered a full distribution from 4-1000 g/kg DM CC, with a mean concentration of 310 g/kg DM (Table 1). Twenty-three of the 94 samples contained between 0 and 65 g/kg DM CC and were considered as a control group. The measured concentration of weed species within samples (any species other than grass or clover) ranged from 0-380 g/kg DM with a mean of 50 g/kg DM. The silages also contained a wide range of chemical composition with LA, WSC and TVFA being the nutritional characteristics with the greatest variance (highest CV) of those measured. Volatile corrected dry matter of the silages was normally distributed with a mean of 395 g/kg. Measured CP concentration (calculated from N and VCODM) ranged from 57 to 215 g/kg DM and with a mean of 138 g/kg DM.

With the exception of ash, NDF, and WSC, all other nutritional components showed significant linear relationships with CC and a smaller number also showed quadratic and cubic relationships. VCODM and N were significantly increased in the high clover group (>500 g/kg DM CC) relative to the other three groups (both $P < 0.001$) reflected by cubic relationships with CC (both $P < 0.04$), whereas for crude protein (calculated from these two components) the relationship was only linear with stepwise increases between groups ($P < 0.001$). Degradability parameters calculated using the Ørskov and McDonald, (1979) model and DOMD were lowest in the high clover group (all $P < 0.04$) and numerically highest in the low clover group (60-240 g/kg DM CC). As a result for both $EDN_{0.08}$ and EDN_{FIM} a quadratic relationship with CC was observed *(*$P < 0.02$). When degradability parameters were calculated using FiM equations, differences between clover groups were non-significant but there were still linearly related to CC ($P < 0.03$). Fermentation end products (LA, TFVA and TVC) decreased in concentration

sequentially as clover concentration increased (*P* LIN < 0.009) while pH was similar for minimal, low and medium groups and higher for the high clover group (*P* < 0.001). NH_3-N was also highest in the high clover group in comparison to the minimal clover group while the other two groups contained intermediate concentrations of NH_3-N (*P* < 0.02).

Blind validation of grass-based NIRS equations

Using data from the grass-clover sample set in a blind validation of the current grass-based equations, a wide range of prediction accuracy was observed depending on the chemical component tested (Table 3). Volatile corrected DM and N showed good prediction accuracy with RPD values of 4.92 and 2.35 respectively, and no significant difference between observed and predicted means. Furthermore, the relationship between the observed and predicted data for both these chemical components closely followed a line of parity (Figure 1) especially at low concentrations. However, all other chemical components led to RPD values that were < 2 denoting inadequate performance. Digestible organic matter in total dry matter, and NDF, had low relative RMSEP (both < 10 % of the observed mean) and no significant difference between the observed and predicted means that could be considered acceptable despite having an RPD value < 2. For these components the slope of the relationship between observed and predicted data followed a line of parity however there was greater variability in the relationship than was seen for VCODM and N (Figure 1). Crude protein prediction showed a relatively high RPD value (1.58) and good correlation between predicted and observed data ($r^2 = 0.75$) however the slope of the relationship did not follow a line of parity (Figure 1) leading to a significant bias (*P* < 0.005) for under-prediction at higher concentrations with the average under-prediction being 12.4 g/kg DM.

Fermentation characteristics (LA, pH, TVC and TVFA) all showed intermediate prediction accuracy with RPD values ranging from 1.15 to 1.22. Of these chemical components, LA in particular had a very high relative RMSEP at 71% of the observed mean as a result of high variability in prediction accuracy where concentration was low (Figure 1). For both TVC and TVFA there was a significant bias towards over-prediction (both $P < 0.001$). Poor prediction accuracy (RPD value <1) was observed for NH_3-N, ADF, EE, EDN_{FIM}, and MDM_{FIM} all of which showed a significant bias between the predicted and observed means (all $P < 0.001$). Of special note, EDN_{FIM} and MDM_{FIM} showed the least prediction accuracy of all the chemical components tested with a significant over-prediction for EDN_{FIM} of 139 g/kg N and an under-prediction for MDM_{FIM} of 17 g/kg DM. Moreover, predicted and observed data for these chemical components showed little correlation (Figure 1) indicated by r^2 values of 0.01.

Where prediction bias was significantly correlated with sample CC, regression analysis results are reported in Table 4. Significant linear relationships between bias and CC were observed for many of the components tested including VCODM, N, ADF, NDF and degradability parameters. For VCODM, EDN_{FIM} and MDM_{FIM} significant quadratic relationships reflected reduced bias in the low and medium CC groups compared to the two extreme groups: minimal and high. For ADF, MDM_{FIM}, EE, NDF and NH_3-N a cubic relationship between bias and CC was observed, reflecting a greater rate of bias increase at the high range of CC than at the low range. The degree of variation and the magnitude of bias in relation to sample CC is further illustrated in Figure 2 using CP and EDN_{FIM} as examples that are important for diet formulation. In the case of CP, prediction bias in samples containing 800-1000 g/kg DM CC is greater than 30 g/kg DM (based on the distance between lines of best fit as shown in Figure 2a), and similarly for EDN_{FIM}, a prediction bias greater than 200 g/kg N was observed in this very high CC range (Figure

2b). Meanwhile, bias was comparatively lower in the low clover control group (0-60 g/kg DM CC) at 6 g/kg DM for CP and 103 g/kg N for EDN_{FIM} reflecting the degree of bias that might be expected for a pure grass sample.

Validation of new grass-clover equations

Following production of new equations using the NIRS spectra from the grass-clover silages in the sample set, a cross validation test indicated 12 out of 22 new equations had a relative SECV of 10% of the observed mean, suggesting a good calibration was achieved for these nutritional components (Table 5). VCODM, pH, NDF, ADF, and $EDDM_{0.08}$ were amongst the strongest calibrations according to cross validation while TVC, WSC, TVFA, Alcohol and LA were the least robust. For ash, EE, WSC, ADF, and NDF (chemical components where the measured concentration is produced from a dry sample) equations were produced that predicted concentrations on both a fresh and a DM basis. For ash, EE and WSC the fresh equation improved relative SECV in comparison to the DM equation whereas the opposite was true for ADF and NDF.

A blind validation test was also applied to the new grass-clover prediction equations through random removal of 10 samples from the calibration data-set that were then predicted using the equation based on the remaining calibration samples (Table 6). Seven components gave an RPD value > 2 denoting good accuracy including VCODM, ADF, NDF, EDN and N. Additionally the RPD score of all values were improved relative to prediction accuracy using the grass-based equations, which was reflected in greatly reduced bias, for example, new equations reduced crude protein mean bias from -12.4 to -0.82 g/kg DM and EDN mean bias improved from 139 to 12 g/kg N on average. The new alcohol and EE (DM basis) equations gave a low RPD value (> 1) suggesting these equations are unlikely to be suitable for use without further improvement.

DISCUSSION

Chemical composition and clover concentration

The wide range of nutrient concentrations collected in the present study provided a robust test for the current grass-based prediction equations. The sample set was dominated by samples containing predominantly grass with only a quarter of the samples obtained containing > 500 g/kg DM CC. Roughly half the total number of samples obtained were below the minimum CC of 300 g/kg DM suggested for optimal niche complementarity by Nyfeler *et al*. (2011). This may be due to the sample set comprising a greater number of first cut silage samples than second, third or fourth cuts in which clover concentration would have been greater due to warmer and drier conditions in the latter half of the year favouring legume growth (Chmelikova *et al.*, 2015).

Crude protein concentration (ranging from 57 to 215 g/kg DM with a mean concentration of 138g/kg DM) indicated that, although some of the samples contained very high levels of crude protein, mean concentration was similar to that expected for well fertilised modern grass silages that have been reported to have CP concentrations ranging from 120-270 g/kg DM (Burns *et al.*, 2015) although they are more expensive and less environmentally friendly to grow (McAuliffe *et al.*, 2016). This mean is also significantly lower than those reported in published feed composition tables for crude protein concentration of grass-clover silages e.g. 173 g/kg DM; AFRC (1993). Crude protein concentration did increase linearly with increasing clover concentration, however, the samples that contained minimal clover should not be taken as representative of the chemical composition of monoculture grass swards as variety choice and management would vary. This suggests that the greatest benefit of sowing grass-clover silages, often termed as ‘home-grown protein’ crops, is not in protein concentration alone

but predominantly in the ability to produce protein without the need for inorganic nitrogen fertilisation.

The rate of protein degradation (i.e. EDN) is another important factor in ruminant diet formulation. High concentrations (> 700 g/kg N) of diet N in degradable protein are wasteful as there is insufficient bacterial N capture, which is often a characteristic of legume silages (Coblentz and Grabber, 2013; Dewhurst, 2013). In the present study, average EDN_{FIM} (623 g/kg N) was within the suggested optimal range and lower than values cited in other studies. Average EDN of grass-clover silages was 880 g/kg N at an assumed passage rate of 0.05/hr (Hvelplund and Weisbjerg, 2000). The lower concentration of EDN in the present study may be due to clover varieties in this sample set being predominantly comprised of red clovers containing the enzyme poly-phenol oxidase that is thought to reduce proteolysis in the rumen (Lee *et al.*, 2009). Both $EDN_{0.08}$ and EDN_{FIM} showed a quadratic relationship with CC where the low clover range 66 - 239 g/kg DM gave the optimal value compared with the other clover quartiles. Poor digestibility and degradability was observed in the high group that could relate to an increased maturity of clover and grass with higher lignification within samples in that range (Nousiainen *et al.*, 2009). Increasing ratios of ADF:NDF in samples with a higher CC indicates the differing fractions of fibre present in legumes in comparison to grasses, especially red clover that is largely comprised of stem where ADF concentration is higher than in leaves (Alstrup *et al.*, 2016). There was a linear decrease in volatiles content (LA, TVC and TVFA) and a linear increase in pH with CC. This suggests samples with a very high CC silages were more difficult to ensile, perhaps due to reduced availability of sugar in clover relative to grass to fuel bacterial activity. These data suggest that levels of clover > 500 g/kg DM were beneficial in terms of increased nitrogen concentration, however where such a high inclusion rate is the case, farmers must be careful to avoid either clover

or grass becoming over-mature that is likely to negatively impact digestibility, degradability, and ensiling quality. Using a factor of 0.0157 of DOMD, mean ME within the sample set was predicted as 9.9 MJ/kg DM that is considerably lower than recently measured values for modern monoculture grass silages that ranged from 12-13 MJ/kg DM (Burns *et al.*, 2015). Some of this discrepancy could be attributed to practical difficulties (i.e. weather and machinery availability) preventing farmers from harvesting at optimal maturity that can be more tightly controlled in experimental work. This also provides evidence to suggest that grass-clover silages are not being utilised to their full potential at present and may be disadvantageous to include in the dairy cow diets in comparison to a well-managed ryegrass silage that could provide more energy.

Using grass-based NIRS equations for clover containing samples

The key objective of this project was to determine whether the current grass NIRS equations used in the UK could be applied to grass-clover samples and predict nutritional characteristics with good accuracy. Although there is not a standardised threshold value denoting whether or not a calibration is adequate, a minimum RPD of 2.0 is often used as a threshold for good performance (Williams, 2014). Variates that were considered most important for correct diet formation included CP, EDN, MDM and DOMD as these are the nutritional components involved in balancing rumen degradable protein and energy supply. The prediction accuracy of DOMD and some other components (including VCODM, N and NDF) could be considered suitably accurate. It should be noted that DOMD can be affected by level of feed intake and in the present study the decision to feed *ad libitum* rather than at maintenance, as is recommended for this technique, was made to minimise psychological stress considering the long duration of the study, however this does limit conclusions that can be drawn about the prediction accuracy

shown for this variable. Feeding *ad libitum* can cause a reduction in observed digestibility, for example, in one recent study using grass-based diets, OM digestibility was reduced by 30 g/kg OM in texel wether sheep fed *ad libitum* versus at maintenance, however the difference reduced as forage maturity increased (Andueza *et al.*, 2013). In the present study, measured DOMD was lower than predicted DOMD by 13 g/kg DM and therefore some of this difference could be attributed to choice of feeding level. However, overall, DOMD was still one of the better predicted variables and so it could be concluded that the discrepancy in feeding level had relatively little impact on our findings.

Crude protein, MDM, and EDN, were amongst the chemical components with an RPD value less than 2.0 combined with a significant bias. Similar results were seen in a smaller preliminary study using 58 grass-clover silages that tested the same equations, in which crude protein was significantly under-predicted by 22 g/kg DM on average (Davies *et al.*, 2012). The consequence of this bias in mixed rations formulated for specific concentrations of protein would be excess N supply in the ration, which would lead to reduced N use efficiency in cattle with higher levels of N excretion in urine and faeces contributing to environmental loading (Kebreab *et al.*, 2002). Under-prediction of CP in silage samples could result in farmers under-valuing grass-clover silages as protein sources, and compensating through an unnecessary inclusion of expensive bought-in protein within the concentrate portion of the diet. For CP, MDM, and EDN, increasing bias correlated with increasing CC. The effect of CC might be explained by samples containing a high concentration of grass being chemically similar to the calibration samples used to create the current grass-based equations whereas samples containing predominantly clover, that has different concentrations of nutrients, would be less akin to the original calibration samples possibly causing a need for extrapolation of the

prediction model model if a chemical was present in a concentration higher or lower than any included in the calibration sample set. Also, bias for N fractions be due to different N fractionation within clover compared to grass, with some fractions present that are absent (or present in different concentrations) in grass, such as the concentration of non-protein N (Chrenkova *et al.*, 2014). According to evidence generated in the present study the current grass equation was adequate for prediction of CP where there was less than 200 g/kg DM CC in the sample, but beyond this point bias becomes too great and therefore this could be considered a cut-off point for suitability of this equation as it currently stands. Additionally, It is worth noting that for nutritional components with particularly poor prediction accuracy, such as EDN and MDM, even within the samples with the lowest CC there was still substantial levels of bias that warrants further investigation. When considerring the impact of the observed inaccuracies on diet formulation it is estimated metabolisable protein, and not CP, that is often the protein fraction by that diets are formulated. Crude protein multiplied by EDN (as a proportion of total N) is used to calculate effective rumen degradable protein (**eRDP**) that is one of the factors that determines metabolisable protein (alongside digestable undegraded true protein, **DUP**) in diet formulation software. The effect of CC on calculated eRDP bias is shown in Figure 3. The opposing bias in EDN and CP cancel each other out to some extent at low CC however the overall effect is an over-prediction of eRDP that increases slightly with CC. This may lead to an undersupply of degradable N relative to fermentable energy, creating an imbalance that could reduce the efficiency of dietary nutrient utilisation. This would only be further compounded by the inaccuracy seen in MDM prediction that is used to determine fermentable energy.

Performance of the new calibration equations

Comparing the performance of new bespoke grass-clover equations with the grass-based equations for use on clover-containing samples, and using relative SECV as a measure of potential performance of the calibration (Table 5), some of the grass-clover equations produced in the present study are likely to perform well (including important variates such as VCODM, N, EDN, and DOMD) whereas others have very high errors (particularly the volatile compounds) and would require further development. Many chemical components still fell below the threshold RPD value of 2.0, possibly due to size of the sample set being too small to create robust equations for these components. The accuracy of prediction for volatile compounds (LA, TVC, and TVFA) is notable in all equations tested (both grass and grass-clover based) for producing poor reliability, one possible reason for this being that the range of volatile concentrations showed some of the greatest variation within the measured sample set that may be too great for one equation to predict robustly. For variates where the measured value was calculated on a dry sample (ash, EE, NDF, ADF and WSC) equations have been calibrated to give both a fresh and a DM basis value. In most instances, the calibration for the fresh value was more robust, however, because presenting information on a DM basis is widely practiced, transforming fresh values using predicted VCODM introduces further error. Overall however, in a small blind validation test, new equations were better able to predict all chemical components when compared to the accuracy of grass-based prediction equations. The prediction of $EDN_{0.08}$ and $EDDM_{0.08}$ showed marked improvement over the previous prediction accuracy for EDN_{FIM} and MDM_{FIM} using the grass-based equations, perhaps due to the greater complexitiy of calculating these components from measured data using the FiM model. For example, calculating reference values for MDM_{FIM} from measured degradability at different timepoints *in vivo* is a multi-step

process involving many different variables (such as corrections for solubility, fatty acid content and crude protein concentration) and therefore it may not be feasable to predict such a value based on NIRS spectra alone.

A key drawback of practically implementing such equations is the extra infrastructure that would need to be provided to introduce a separate analysis option into practice, such as ensuring nutritionists, feed company representatives, and farmers are aware of the new option, and ensuring samples are correctly identified as containing clover. Additionally, grass-clover mixtures are just one example of alternative forages that are currently gaining popularity, and it is unlikely that bespoke equations can be created and implemented for all of them due to the time needed to collect a sufficiently large group of calibration samples. Therefore, it would be more convenient if one equation (such as the current grass-based equation or alternatively a seperate general 'legume' equation) could be adapted to analyse many different grass and legume based forages. Another solution would be to use a two step process in which the CC of the sample is predicted using NIRS, and then used to apply a correction to nutritional predictions based on the best fitting model of CC against bias for relevant components. A number of previous studies have used NIRS to determine the botanical composition of a mixed sample (containing two species) with success for both grass-clover (Cougnon *et al.*, 2014) and lucerne-grass silages (Karayilanli *et al.*, 2016) however in all instances the calibration was performed on dry samples and therefore further work is required to create an analysis for species composition of fresh samples that would be appropriate for use in the UK.

CONCLUSIONS

For some nutritional components, notably VCODM, N, DOMD and NDF, current UK grass-based equations were able to be applied to clover-containing samples with adequate accuracy. However, overall it was concluded that the NIRS calibration equations developed for use on grass silages, could not predict the concentration of a number of key chemical components (including CP and EDN) in grass-clover mixtures with sufficient accuracy. Therefore, we suggest two potential approaches that would be appropriate for laboratories using NIRS on fresh samples, as occurs in the UK: (i) the introduction of new bespoke grass-clover prediction equations calibrated initially using the sample set obtained for the present study or (ii) the use of a correction factor that could be applied based on the clover concentration of the sample. Furthermore, in a wider sense, the present study provides evidence that caution should be used whenever NIRS equations are applied to forage mixtures where only one botancial component of the mixture was represented within the calibration set for the chosen equation. Where possible, using an equation based on a calibration set that is specific for the material requiring analysis will produce more accurate predictions.

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Table 1 The means, ranges and variation coefficients (CV) of chemical components and parameters of digestibility and degradability measured in a set of 94 diverse grass-clover silages from UK farms (in g/kg DM unless otherwise stated).

Item	Min	Max	Mean	Median	CV, %
ADF	229	513	335	337	10.2
Ash	58	158	97	95	20.6
CC	4	1000	310	239	91.3
CP	57	215	138	139	24.7
Degradability					
$EDDM_{0.08}$	217	626	472	486	16.4
$EDN_{0.08}$, g/kg N	55	821	625	644	18.0
MDM_{FIM}	60	274	146	132	34.8
EDN_{FIM}, g/kg N	297	811	623	637	14.3
DOMD	400	766	632	641	10.6
EE	14.6	42.9	26.6	26.4	26.0
LA, g/kg	0.0	64.4	13.4	10.4	91.5
pH	3.6	6.7	4.6	4.5	13.5
N, g/kg	3.6	17.7	8.8	7.7	42.2
NDF	299	585	447	444	10.0
NH_3-N, g/kg DM*100	17.5	203	62.5	55.1	42.2
TVC, g/kg	2.3	76.1	23.6	22.6	57.8
TVFA, g/kg	1.1	74.3	19.7	18.6	66.0
VCODM, g/kg	182	793	395	371	33.4
WSC	3.9	164	41.4	30.4	86.3

CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradability of protein; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

Table 2 Differences in chemical components and parameters of digestibility and degradability in 94 grass-clover silages grouped into four quartiles (Minimal (Min), Low (L), Medium (M) and High (H)) according to their clover concentration (in g/kg DM unless otherwise stated) and the results of a regression between measured clover concentration and each nutritional component.

	Clover concentration quartiles[1]					P value[2]		
Item	Min	L	M	H	SED	LIN	QUAD	CUB
CC	34[a]	145[b]	335[c]	743[d]	28.9	-	-	-
n	23	24	24	23				
Chemical components								
ADF	311[a]	329[ab]	345[b]	356[b]	12.5	0.001	0.951	0.038
Ash	91.2	94.9	103.4	96.8	5.84	0.455	0.014	0.613
CP	122[a]	130[a]	143[ab]	158[b]	9.3	0.001	0.096	0.595
Degradability								
$EDDM_{0.08}$	470[ab]	501[b]	478[ab]	436[a]	21.7	0.016	0.100	0.400
$EDN_{0.08}$, g/kg N	643[b]	682[b]	640[b]	531[a]	28.6	0.001	0.011	0.504
MDM_{FIM}	130	135	127	122	6.3	0.027	0.179	0.618
EDN_{FIM}, g/kg N	627	645	629	589	25.9	0.008	0.015	0.792
DOMD	647[b]	668[b]	631[b]	581[a]	18.5	0.001	0.357	0.210
EE	26.7[ab]	28.7[b]	27.4[ab]	23.2[a]	1.99	0.008	0.138	0.279
LA, g/kg	17.6[b]	16.2[b]	14.0[ab]	5.6[a]	3.42	0.001	0.620	0.503
pH	4.45[a]	4.41[a]	4.44[a]	5.23[b]	0.155	0.001	0.030	0.101
N, g/kg	7.8[a]	7.3[a]	7.9[a]	12.3[b]	0.93	0.001	0.143	0.021
NDF	465	452	443	432	16.5	0.058	0.070	0.542
NH_3-N, g/kg DM*100	48.3[a]	56.3[ab]	68.0[ab]	77.5[b]	9.71	0.002	0.446	0.726
TVC, g/kg	28.4[b]	26.8[b]	24.0[ab]	14.3[a]	3.74	0.001	0.715	0.368
TVFA, g/kg	23.3[b]	22.4[b]	20.8[ab]	11.6[a]	3.65	0.001	0.578	0.327
VCODM, g/kg	397[a]	350[a]	347[a]	498[b]	35.1	0.001	0.004	0.039
WSC	56.6	39.6	32.9	38.6	10.37	0.242	0.163	0.095

CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradability of protein; DOMD = digestible organic matter in total dry matter; EE = ether extract; FIM = Feed into Milk; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; SED = standard error of the difference between means; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

[1] The 94 samples were sorted by ascending clover concentration and divided into four equal quartiles of the dataset; representing 0 – 65 (Min), 66 – 239 (L), 240 – 500 (M) and > 500 (H) g/kg DM clover concentration.

[2] The probability of a significant linear (LIN), quadratic (QUAD), or cubic (CUB) relationship between measured clover concentration using a regression for each chemical component.

[a,b] Quartile mean values within a row with different superscripts differ significantly at $P<0.05$ tested using ANOVA and a post-hock Tukey test.

Table 3 The results of a blind validation in which 94 grass-clover silages were used to test the prediction accuracy of grass-based near infra-red reflectance spectroscopy (NIRS) equations for chemical composition when used on clover-containing samples (in g/kg DM unless otherwise stated).

Item	Measured mean	Predicted mean	Bias[1]	P value[2]	r^2	Relative RMSEP, %[3]	RPD
VCODM, g/kg	397	409	-12.0	0.558	0.98	6.6	4.92
N, g/kg	8.8	8.1	0.7	0.187	0.86	19.4	2.35
CP	138	126	12.4	0.005	0.75	17.1	1.58
DOMD	632	645	-13.0	0.195	0.64	6.7	1.56
NDF	448	438	9.65	0.209	0.56	8.9	1.45
Ash	96.6	91.6	5.0	0.033	0.52	16.5	1.32
WSC	41.8	48.8	-7.0	0.113	0.40	58.4	1.25
LA, g/kg	13.4	14.3	-0.9	0.622	0.48	70.6	1.22
pH	4.6	4.8	-0.1	0.122	0.48	10.8	1.21
TVFA, g/kg	19.6	25.6	-5.9	0.001	0.51	43.5	1.17
TVC, g/kg	23.4	30.2	-6.8	0.001	0.52	39.3	1.15
NH_3-N, g/kgDM*100	62.5	85.2	-22.6	0.001	0.34	45.0	0.89
EE	26.5	30.1	-3.6	0.001	0.25	25.9	0.89
ADF	336	292	43.0	0.001	0.61	17.6	0.87
EDN_{FIM}, g/kg N	623	762	139	0.001	0.01	24.5	0.48
MDM_{FIM}	129	146	-17	0.003	0.01	38.1	0.39

DOMD = digestible organic matter in total dry matter; EDN = effective degradability of protein; EE = ether extract; FIM = Feed into Milk; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; RMSEP = root mean standard error of prediction; RPD = ratio of standard deviation of the measured population to the standard error of prediction; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

[1] Bias was the measured mean minus the predicted mean, therefore minus values indicate over-prediction and positive values indicate under-prediction of the equation.
[2] The probability of there being no significant difference between the measured mean and the predicted mean analysed using student's t-test.
[3] Root mean square error of prediction presented as a percentage of the measured mean for standardisation

Table 4 Differences in near infra-red reflectance spectroscopy (NIRS) prediction bias of chemical components in 94 grass-clover silages grouped into four quartiles (Minimal (Min), Low (L), Medium (M) and High (H)) according to their clover concentration (in g/kg DM unless otherwise stated) and the results of a regression between measured clover concentration bias and each nutritional component. Only chemical components where there was a significant ($P < 0.05$) relationship or quartile means difference have been presented. Units are g/kg DM unless otherwise stated.

	Bias within CC quartiles[1]					P value[2]		
Item[3]	Min	L	M	H	SED	LIN	QUAD	CUB
ADF	21.6^{a}	43.1^{b}	51.7^{b}	55.3^{b}	7.64	0.001	0.440	0.001
CP	6.1^{a}	6.2^{a}	15.7^{ab}	21.6^{b}	4.95	0.001	0.214	0.560
Degradability								
MDM_{FIM}	-24.3^{ab}	1.9^{b}	5.6^{b}	-54.2^{a}	14.22	0.002	0.003	0.015
EDN_{FIM}, g/kg N	-156^{ab}	-107^{b}	-94^{b}	-204^{a}	35.0	0.010	0.002	0.140
EE	-3.63^{ab}	-1.97^{b}	-2.16^{b}	-6.71^{a}	1.705	0.012	0.221	0.045
LA, g/kg	-0.98^{ab}	-4.81^{a}	-2.60^{a}	4.94^{b}	2.830	0.003	0081	0.213
N, g/kg	0.22	0.29	0.84	1.29	0.411	0.014	0.092	0.155
NDF	30.3^{b}	24.7^{b}	8.7^{b}	-25.7^{a}	9.34	0.001	0.194	0.047
NH_3-N, g/kg DM*100	-22.9^{ab}	-19.7^{ab}	-10.4^{a}	-38.2^{b}	8.82	0.071	0.148	0.037
VCODM, g/kg	-11.7	-6.0	-7.2	-23.5	6.94	0.006	0.004	0.345
WSC	4.3^{b}	-2.0^{b}	-7.9^{ab}	-22.7^{a}	7.76	0.001	0.917	0.914

EDN = effective degradability of protein; EE = ether extract; FIM = Feed into Milk; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; SED = standard error of the difference between means; WSC = water soluble carbohydrates.

[1] The 94 samples were sorted by ascending clover concentration and divided into four equal quartiles of the dataset; representing 0 – 65 (Min), 66 – 239 (L), 240 – 500 (M) and > 500 (H) g/kg DM clover concentration.

[2] The probability of a significant linear (LIN), quadratic (QUAD), or cubic (CUB) relationship between measured clover concentration and bias observed uing a regression for each nutritional component.

[3] The bias of ash, digestible organic matter in total dry matter, pH, total volatile fatty acids and total volatile content showed no significant relationships to clover concentration and no significant differences between quartile means and therefore are not shown. Bias was the measured mean minus the predicted mean, therefore minus values indicate over-prediction and positive values indicate under-prediction of the equation.

[a,b] Quartile mean values within a row with different superscripts differ significantly at $P<0.05$ tested using ANOVA and a post-hock Tukey test.

Table 5 Indicators of calibration strength and prediction accuracy using a 'leave one out' cross-validation method for a range of new near infra-red reflectance spectroscopy (NIRS) equations calibrated on spectra from 95 diverse grass-clover silages.

Item[1]	n[2]	SEC	r^2	Relative SECV[3], %
VCODM	181	7.17	1.00	2.10
pH	180	0.16	0.93	4.18
ADF (DM)	183	13.4	0.90	4.49
NDF (DM)	183	18.5	0.89	4.80
NDF (Fresh)	182	7.79	0.98	5.26
$EDDM_{0.08}$	174	2.15	0.88	5.28
DOMD	172	3.10	0.83	5.47
ADF (Fresh)	181	6.22	0.98	5.71
$EDN_{0.08}$	174	3.93	0.79	7.03
N	180	0.65	0.97	8.33
EE (Fresh)	179	0.94	0.90	10.8
Ash (Fresh)	179	3.30	0.91	11.1
EE (DM)	180	2.67	0.83	11.2
Ash (DM)	185	10.4	0.70	12.5
NH_3-N	176	0.01	0.88	18.8
TVC	185	5.39	0.82	27.9
WSC (Fresh)	181	4.62	0.93	29.6
WSC (DM)	180	10.1	0.92	31.4
TVFA	183	5.17	0.81	31.8
Alcohol[4]	178	1.08	0.83	37.1
LA	173	4.76	0.81	41.5

CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradability of protein; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; NH_3-N = ammonia nitrogen; SEC = standard error of calibration; SECV = standard error of cross-validation; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

[1] For components that are measured on a dry sample (Ash, ADF, NDF and WSC) two equations were produced, one predicting on a fresh basis and one on a DM basis.
[2] The number of spectra that were included in the prediction equation.
[3] Standard error of cross validation presented as a percentage of the measured mean for standardisation
[4] Alcohol is the sum of ethanol and propanol

Table 6 The results of a blind validation in which 10 grass-clover silages were used to test the prediction accuracy of new clover/grass-based near infra-red reflectance spectroscopy (NIRS) equations generated from the spectra of 85 other grass-clover silages (in g/kg DM unless otherwise stated).

Item	Measured mean	Predicted mean	Bias[1]	r^2	Relative RMSEP, %[2]	RPD
VCODM, g/kg	451	448	2.87	0.99	2.46	14.2
ADF, g/kg	162	159	1.93	0.99	6.17	8.66
NDF, g/kg	212	215	-2.54	0.98	5.81	7.87
N, g/kg	8.9	9.0	-0.02	0.92	13.4	3.76
$EDN_{0.08}$, g/kg N	600	588	11.9	0.92	6.74	3.43
ADF	343	352	-9.28	0.93	7.31	2.94
NDF	459	479	-20.6	0.85	8.02	2.15
Ash, g/kg	39.2	40.6	-1.38	0.73	18.8	1.82
TVC, g/kg	22.9	21.7	1.20	0.76	33.5	1.85
CP	120	120	-0.82	0.74	12.3	1.92
WSC	51.7	58.7	-7.01	0.69	35.3	1.76
LA, g/kg	15.1	12.7	2.41	0.72	49.0	1.74
TVFA, g/kg	19.5	18.4	1.03	0.73	37.0	1.86
pH	4.7	4.6	0.06	0.70	10.8	1.86
DOMD	637	637	-0.2	0.71	9.18	1.76
NH3-N, g/kg DM*100	104	112	-8.01	0.64	25.0	1.64
WSC, g/kg	22.4	27.2	-4.84	0.69	44.4	1.57
$EDDM_{0.08}$	452	438	14.7	0.68	16.3	1.58
EE, g/kg	9.9	9.8	0.15	0.67	16.9	1.82
Ash	88.5	87.0	1.49	0.26	16.5	1.19
Alcohol[3], g/kg	3.5	4.9	-1.41	0.19	75.7	0.93
EE	22.8	21.6	1.22	0.46	23.2	0.95

DOMD = digestible organic matter in total dry matter; EDN = effective degradability of protein; EE = ether extract; LA = lactic acid; NH_3-N = ammonia nitrogen; RMSEP = root mean standard error of prediction; RPD = ratio of standard deviation of the measured population to the standard error of prediction; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

[1] Bias was the measured mean minus the predicted mean, therefore minus values indicate over-prediction and positive values indicate under-prediction of the equation.
[2] Root mean square error of prediction presented as a percentage of the measured mean for standardisation
[3] Alcohol is the sum of ethanol and propanol

Figure captions

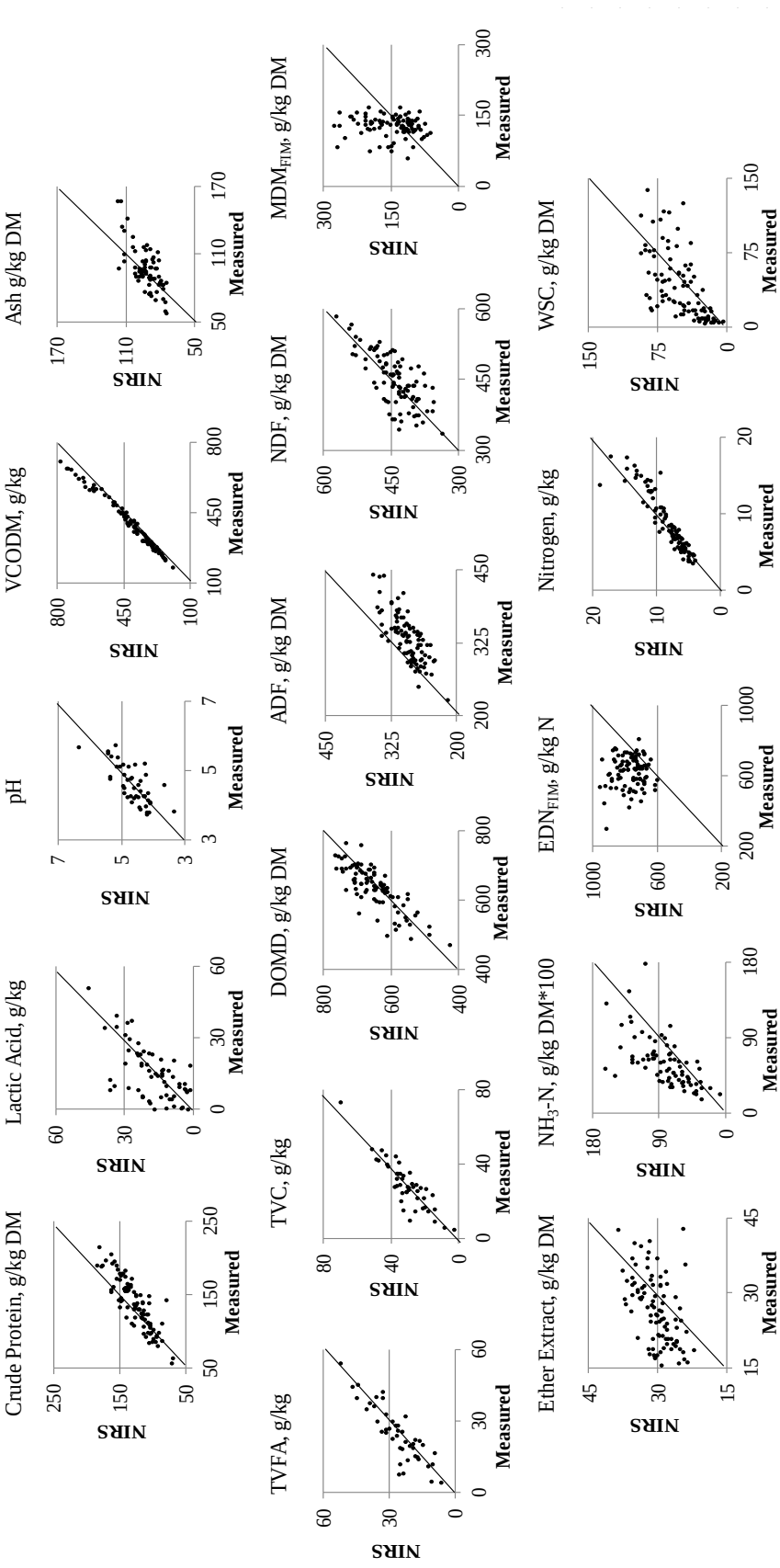

Figure 1 The relationship between predicted and measured values in a blind validation test where 94 grass-clover silages were used to assess prediction accuracy of grass-based near infra-red reflectance spectroscopy (NIRS) equations for 16 chemical components when used on clover-containing samples. Lines of parity are shown. VCODM = volatile corrected oven dry matter; TVFA = total volatile fatty acids; TVC = total volatile content; DOMD = digestible organic matter in total dry matter; ADF = acid detergent fibre; NDF neutral detergent fibre; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; EDN = effective degradable nitrogen; WSC = water soluble carbohydrate

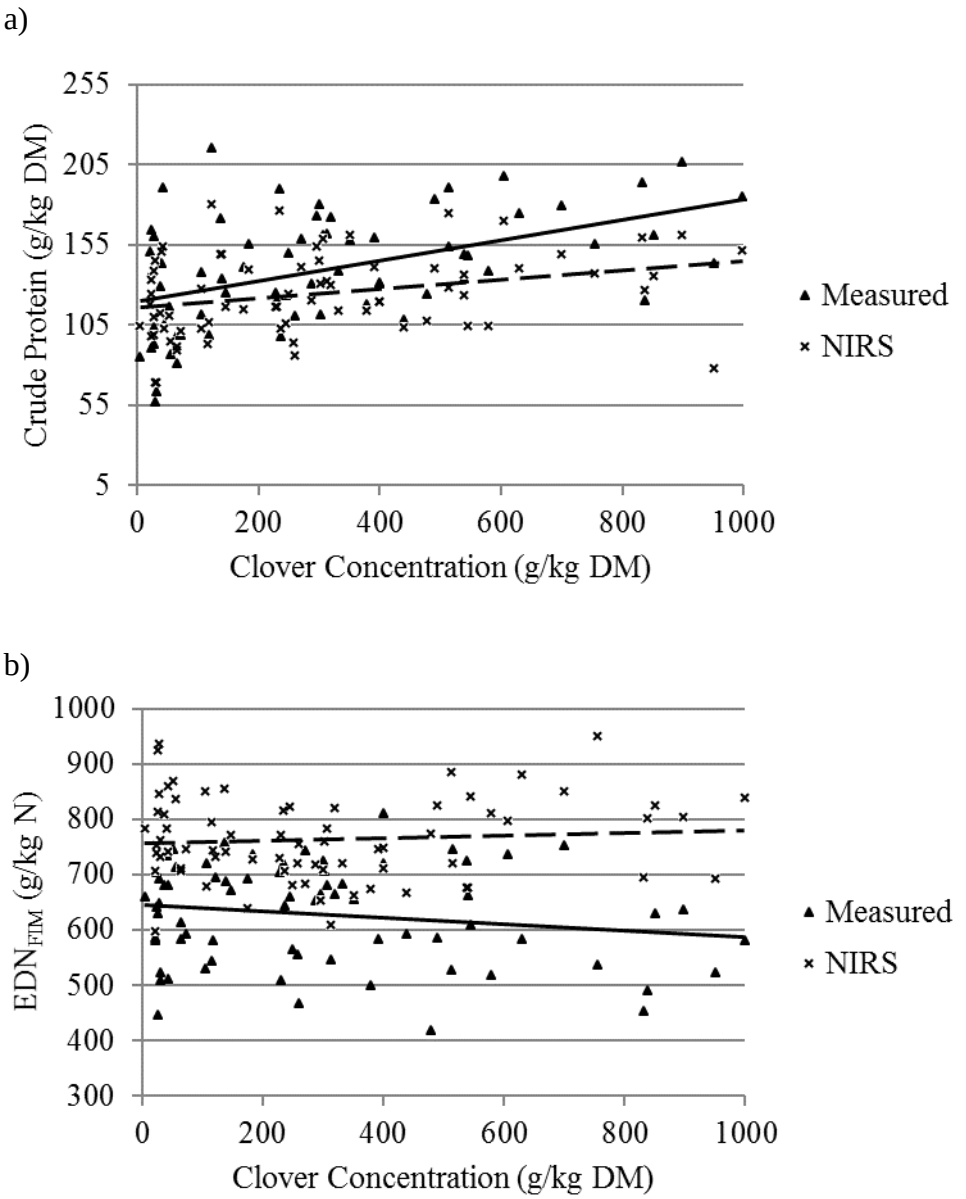

Figure 2 The relationship between bias and sample clover concentration in a blind validation test where 94 grass-clover silages were utilised to assess prediction accuracy of grass-based near infra-red reflectance spectroscopy (NIRS) equations for a) crude protein and b) The effective degradability of protein (EDN_{FIM}) when used on clover-containing samples. Linear lines of best fit are shown for measured (——) and NIRS predicted (— —) data.

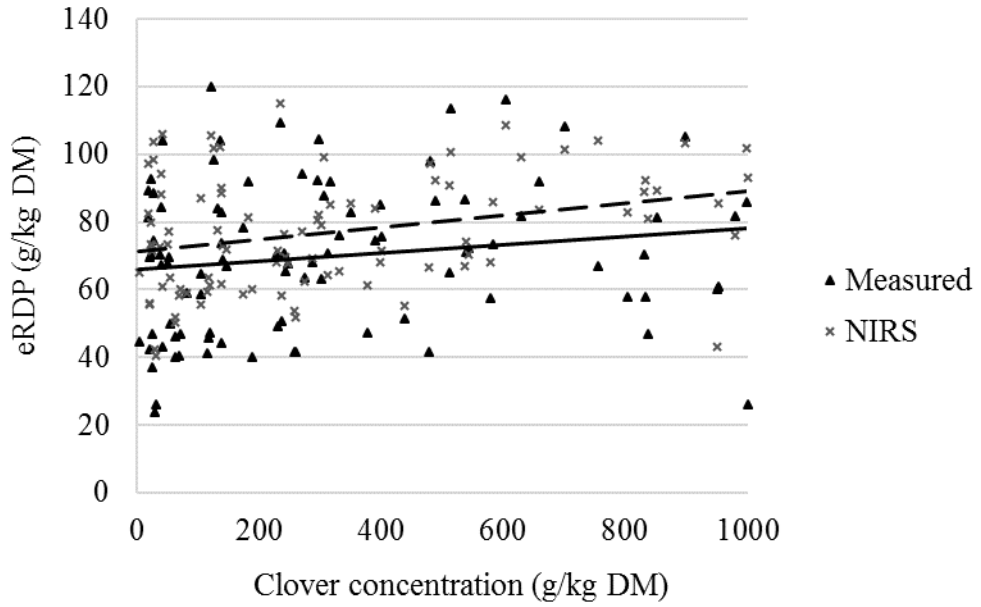

Figure 3 The effect of clover concentration bias when grass-based near infra-red reflectance spectroscopy (NIRS) predictions for the nutritional composition of 94 grass-clover silages are used to calculate effective rumen degradable protein (eRPD) concentration (eRDP = CP * (0.8 * EDN_{FIM}) in comparison with the same calculation based on measured nutritional composition. Linear lines of best fit are shown for measured (——) and NIRS predicted (— —) data.

Chapter 4

Using Near Infra-Red Reflectance Spectroscopy to predict clover concentration within grass-clover mixed silages

Intended for submission to Journal of Agricultural Science

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SUMMARY

The purpose of the present study was firstly to examine current practice for the agronomy of grass-clover mixed swards used for silage making in the UK, and secondly, to evaluate the potential use of a Near Infra-Red Reflectance Spectroscopy (NIRS) equation capable of predicting the botanical composition of grass-clover silage samples. A calibration set of 94 perennial ryegrass-clover (white, *trifolium repens*, and red, *trifolium pratense*) mixture silage samples were sourced from UK farms and an accompanying questionnaire was used to obtain information on the sward agronomy used to produce each sample. Reference botanical composition data were generated by hand separation and a new NIRS equation was generated and assessed by both cross validation and blind validation. Questionnaire data highlighted that (i) reducing the use of fertiliser inputs (ii) increasing uptake of new varieties, and (iii) increasing the farmer's ability to measure botanical composition as potential strategies for improving the utilisation of clover in ryegrass swards. The NIRS equation developed to predict clover concentration had a relative standard error of cross validation of 36.4%, and, in an initial blind validation test, the ratio of standard error of prediction to the standard deviation of the reference dataset (RPD) was 2.65. The equation could be improved by increasing accuracy at high clover concentrations but showed promise as a simple tool to assist growers in sward management decisions and may have further use in analytical labs as a correction factor where predictive analyses are calibrated for pure grass samples (such as for NIRS nutritional composition predictions).

IINTRODUCTION

The use of mixed grass-clover swards for both grazing and silage production is now relatively wide-spread across temperate European agricultural systems and particularly in the UK where 70% of grass swards on dairy farms are thought to contain clover (DEFRA, 2015). Grass-legume swards offer a sustainable approach to reduce fertiliser input into grasslands, as the atmospheric nitrogen (**N**) fixed by clover can be utilised by grass, an example of niche complementarity between two species (Nyfeler *et al.*, 2011; Phelan *et al.*, 2015). Utilising niche complementarity in this way is becoming an area of increasing interest for both binary and complex (3+ species) sward mixtures. The benefits of combining different functional groups are manifold, not simply increasing productivity and minimising the need for inputs, but also in supply different beneficial nutrients, minerals, and secondary plant compounds to livestock (Provenza *et al.*, 2007). However a key determinant of the success of such swards is determining and maintaining the correct concentration ratio of species or functional groups (e.g. grass:legume ratio) such that the species work in harmony. Previous research has shown that, in general, the best results can be achieved by an even distribution of species within a sward, without one species becoming over-dominant (Finn *et al.*, 2013). Where one species is over-dominant the other cannot reach its production potential or fulfil its niche function, and the productivity of the whole sward could be reduced (Kirwan *et al.*, 2007; Luescher *et al.*, 2014). Therefore, there is a need for increased development of practical methods by which growers can manage species evenness within a sward, beginning with simple binary mixtures. The first step to improved management is the ability to measure the botanical composition of a sward with ease. Near Infra-Red Spectroscopy (**NIRS**) analysis offers a quick and inexpensive method, already routinely used for silage analysis, by which the composition of a mixed sample might be determined. Prediction equations

for NIRS analysis of clover in a mixed grass-clover silage sample have been successfully reported previously using dried samples for calibration (Wachendorf *et al.*, 1999; Cougnon *et al.*, 2014) however no prediction equations currently exist which are appropriate for the UK where silage analysis is performed on un-dried (fresh) and un-milled samples. Once the botanical composition of a sward is known, inputs may be adjusted to suit one species or another, for example, in grass-clover swards the addition of nitrogen as fertiliser favours grass production whereas removing fertiliser inputs favours clover production. In this way, selective fertilisation depending on sward botanical composition can be then used as a management strategy (Schils and Snijders, 2004). Other management factors that have been shown to affect species composition include cutting height, cutting frequency, and grazing intensity (Yarrow and Penning, 1994; Phelan *et al.*, 2014). Any management strategies are made particularly difficult by the transient nature of sward composition over time, as changes in climate throughout the growing season favour one species or another.

The objectives of the present study were therefore to develop an NIRS equation to measure the botanical composition of fresh grass-clover silages appropriate for uptake by laboratories in the UK, and secondly, to assess current management practices of grass-clover swards to better understand where further research into management of botanical composition is required.

MATERIALS AND METHODS

Experimental design

In total, 94 clover/grass silages were sourced from commercial farms and brought to the University of Reading's Centre for Dairy Research (**CEDAR**; Arborfield, Reading, UK) for processing. A further 95th sample was created by combining one of the original 94

samples with additional grass silage to create a new sample. The samples were obtained to evaluate the use of NIRS analysis for nutrient concentrations as described previously (Thomson *et al.*, 2017). The quantity of silage collected was either one un-opened large bale or the equivalent in clamp silage (~500 kg fresh weight). Samples were sourced from a diverse range of UK farms to ensure maximum variation within the sample set in line with the recommendations of Cougnon *et al.* (2014) for sourcing robust NIRS calibration data. Silage was collected over three consecutive years (2012/13, 2013/14, and 2014/15). The original set of 94 silage samples consisted of 58 bales and 36 samples from clamps which were collected from 50 different locations distributed across the UK. Of the samples where the clover species was known (n=65) 66% were red clover (*trifolium pratense*), 20% were white clover (*trifolium repens*) and 14% were a mixture of both. Many of the grass species present were perennial ryegrass varieties. Different cuts were also represented within the set with 36 first, 20 second, 16 third and 4 fourth cut silages (22 samples unknown).

Sample processing is described in detail by Thomson et al. (2017), however, in brief, once a sample arrived at CEDAR, it was chopped in a feeder wagon (Hi-Spec Mix Max, Hi Spec Engineering, Co. Carlow, Ireland) for 45 minutes if the sample was an unchopped bale silage. For clamp silages that were already chopped, the silage was mixed in a DataRanger feed mixer that did not contain knives (American Calan, Northwood, NH, USA). After mixing, representative subsamples of each silage sample were stored separately at -20ºC for future analysis by manual aspeciation and NIRS.

Silage Questionnaire

A questionnaire (Table 1) was given to each farmer who donated a silage sample to the study. The questionnaire comprised 17 questions relating to the timing of establishment,

fertilizer applications, and harvesting; the composition of seed mixtures used; and ensiling practices. To assess seed mixture composition, the variety sown was recorded for ryegrasses and clovers, whilst for any other components, only species was recorded because the variety was rarely provided. In addition farmers were asked to estimate the percentage of clover in the sward (Question 9, Table 1). Farms were permitted to contribute more than one silage sample to the study provided the samples originated from differing cuts, years, or swards. Separate questionnaires were completed for each of the samples. Questionnaire forms were returned for 64 of the 94 samples (68% response rate) however not all questions were answered on all returned questionnaires and in some instances answers were insufficiently detailed to be included. These 64 completed questionaires originated from 36 individual farms, reflecting that a number of farms returned more than one questionnaire, each relating to a different crop of silage.

Sample analysis

Approximately 200 g of silage was manually aspeciated into clover, ryegrass and other species as a means of determining the clover content of the silage. Resulting fractions were then oven dried at 60ºC for 72 h to determine clover concentration (**CC**) on a DM basis. A second 2 kg subsample of frozen material was sent to the Agri-Food and Biosciences Institute (**AFBI**; Hillsborough, Northern Ireland) where NIRS spectra were obtained through scanning the samples using NIR wavelengths 1100-2498nm with a Foss 6500 machine (Foss, Hillerød, Denmark). Samples were analysed fresh (undried) with no further sample preparation.

Data Analysis

Statistical analysis. Genstat 16th Edition (VSN International, Hemel Hempsted, UK) was used to perform statistical analyses on questionnaire data. Linear regression analysis was used to test the effect of pasture age (in years) on CC, and ANOVA followed by a Tukey test was used to assess the effect of cut number in the year (1^{st} to 4^{th}) on CC.

Data pre-treatment and production of new NIRS equations. Spectral data for the 95 samples were pre-treated by taking the first derivative, smoothing, and applying standard normal variate detrending (**SNVD**) scatter correction (1,4,4,1) and a repeatability file (a file containing multiple spectra from the same sample measured under different conditions, designed to reduce the variability caused by differing environmental conditions and instruments). New equations developed were compared by standard errors of calibration (**SEC**) and cross-validation (**SECV**, expressed as a precentage of the observed mean; determined using a 'leave one out' method i.e. the spectra from each sample was sequentially removed from the calibration set and predicted using the equation derived from the remaining spectra). Additionally, for the purposes of a blind validation test, 10 samples were removed from the dataset and tested using the remaining equation. A second blind validation test using spectra from a completely independant set of 30 grass-red clover silage combinations (where clover was included at 5 set levels: 0, 150, 450, 600 and 1000 g/kg of DM) was also applied to the equations. In the blind validation tests the adequacy of equations was assessed using the following metrics: relative root mean square standard error of prediction (**RMSEP** as a percentage of the measured mean (Shenk and Westerhaus, 1993)), ratio of RMSEP to the standard deviation of the measured dataset (**RPD**) as recommended by Williams (2014), and the R-squared value of the linear relationship between observed and predicted data ($\mathbf{r^2}$).

RESULTS

Questionnaire results

The final set of 95 samples was found to have a wide range of botanical composition (Figure 1) with the mean concentration of clover, grass and other species being 310, 640 and 50 g/kg DM, respectively (median CC = 280 g/kg DM). There was a larger number of samples with low CC than there was those with a high CC. Either ploughing or subsoiling was the most common form of cultivation prior to establishment followed by discs, tines or harrowing. There were very few examples of minimum or zero tillage (3 and 4 out of 56 responses respectively). In 15 instances a sward was established by under-sowing into a cereal crop. The timing of crop establishment (where known) was evenly split with 23 swards established in spring (March – June) and 24 in autumn (July - September).

All farms reported sowing more than one variety of grass and many sowed more than one variety of clover. On average, clover seeds made up 21% of the sown mixture by weight. In total 38 different grass varieties including examples of timothy, cocksfoot, fescues, and festuloliums were sown, but predominantly ryegrasses were sown in combination with 19 different varieties of clover. In a small number of instances clover was combined in a multispecies sward with either lucerne, chicory, or birdsfoot trefoil in addition to grass. The four most common grass and clover varieties found are illustrated in Figure 2. The two most popular grasses: Solid and Tetragraze are both examples of Italian x Perennial ryegrass varieties, however the majority (23 out of 38) of the total number of varieties sown were perennial ryegrasses. Relatively few (3 out of 38) examples of Italian ryegrasses were included in the silages obtained for the present study. Of the four most common clover varieties, all were red clovers, suggesting that swards were sown predominantly for the purpose of silage-making, although this was not known

for certain as no data were gathered on grazing patterns. Merviot was the red clover variety sown most frequently.

Information relating to applications prior to cutting was received for 46 of the silages. Of the 46 silages, 23 silages had slurry applied prior to harvesting and 12 had been fertilised using an inorganic fertiliser containing N. A further 5 had an application of farm yard manure. Only 5 silages had been reported as having no fertiliser applied prior to cutting. The average age of the sward at the point the silage harvested was 3 years, however there was a wide range from first year post establishment to permanent pasture established over 20 years. Fifty-six out of 61 silages for which data on years established were given were all harvested within the first three years of sward establishment. There was a significant correlation between increased age of pasture and reduced clover concentration ($P < 0.04$) tested using a linear regression ($y = 41.4 - 3.3\ x$, where x = age of sward in years; standard error = 5.30 and 1.51 for the intercept and slope respectively). However pasture age only explained a small proportion of the variation within the dataset ($r^2 = 0.059$) indicating many other factors also influencing clover concentration. The most common months for taking silage cuts were May, July and September for 1st, 2nd, and 3rd cuts respectively; however, the majority of questionnaires would not usually have come from successive cuts on the same farm so it is not possible to draw conclusions about the regrowth period allowed in each system surveyed. Stepwise increases in CC were seen in 1st to 3rd cuts ($P < 0.001$) as shown in Figure 3, while CC in 4th cuts was not significantly different from that of 2nd or 3rd cuts but greater than 1st cuts.

The use of silage additives was relatively common with 13 different brands of additive reported. Of these 13 additives used, 10 were bacterial inoculants and the

remainder contained either enzymes or salt as the active ingredient. Of 29 total responses to the question on the use of additives, 5 specified that no additive had been used.

One question required farmers to estimate the CC of the sample based on their knowledge of the sward. Fifty-five estimations were received and compared against values from the aspeciation assessment of that silage (Figure 4). The majority of farmers estimated a value between 300 to 700 g/kg DM clover reflecting a desire to choose a mid-range value. As a result samples containing less than 400 g/kg DM CC were generally over-estimated while samples containing greater than 400 g/kg DM were under-estimated. The greatest errors in farmer estimation were seen where the sample contained a very low (<100 g/kg DM) or very high (>800 g/kg DM) clover concentration. The number of estimates that successfully predicted clover concentration to within ±100 and ±200 g/kg DM were 15 and 31 out of 55 respectively.

Validation of an NIRS equation to predict clover concentration

An NIRS prediction equation for CC was produced using 182 spectra based on a calibration set of duplicate spectra from each of 95 grass-clover silage samples. The standard error of calibration was 8.99 and the relative SECV was 36.8. An initial self-predicition test of the equation indicated good accuracy with an RPD score of 2.49 (Table 2; above the threshold of 2.0 specified by Williams (2014) for good accuracy). However a relative RMSEP of 36.4% suggested a high level of variability in accuracy which was further investigated through plotting of the residuals for each prediction (Figure 6). The line of best fit through the residual plot did not follow a line of parity reflecting a tendency for under-prediction of CC where the CC was measured at 400 g/kg DM or higher and a tendancy for over-prediction at very low CC (<100 g/kg DM). From this test the bias seen

at high clover concentrations was an under-prediction of 100 g/kg DM clover concentration.

A second test was performed in which ten samples were removed at random from the calibration set and used to independantly validate the new prediction equation. The measured mean CC of this validation set was 354 g/kg DM which was similar to that of the original dataset (favouring low clover silages). In this test r^2 and RPD values were higher than in the self prediction test and RMSEP was slightly lower, indicating good accuracy of prediction for these samples. Accross these ten validation samples, the average prediction bias was an underprediction of 60 g/kg DM clover concentration.

In a final test of the new equations, spectra from 30 independant red clover and grass mixture samples of known CC were used to test the equations produced from the full calibration set. The measured mean CC within this independent set of samples was 440 g/kg DM, which was greater than that of the calibration set or the validation set used previously. Prediction accuracy for this dataset was reduced in comparison to previous tests showing a very high relative RMSEP (52.2%) and an RPD value of 1.56 which was lower than the threshold of 2.0. Plotting measured against predicted clover concentration in this test indicated similar trends to that of the self prediction test with over-prediction at low CC and under-prediction at high CC with the average bias being towards under-prediction (Figure 7). For samples that contained 1000 g/kg clover by DM, the equation underpredicted CC by 300-400 g/kg DM. A group of samples with 15 g/kg DM CC showed the best prediction accuracy. As prediction for samples with the same botanical composition often differed by 150-400 g/kg DM CC (Figure 7), repeatability of the equation was also thought to be poor.

DISCUSSION

Common grass-clover sward management practices

Questionnaire data indicated some adoption of strategies for successful grass-clover management as defined by the UK's Agriculture and Horticulture Development Board, however, there was evidence to suggest that best practice is not followed consistently, for example, there was a high incidence of N fertiliser use on grass-clover swards (AHDB, 2013). All farmers used seed that contained mixed grass varieties, and the majority also sowed more than one variety of clover within the sward. Sowing for increased varietal richness is a common strategy employed to mitigate the risk of any one variety performing poorly (Surault *et al.*, 2010). The greater total number of grass varieties represented in comparison to clovers was likely reflective of the wider range of grass varieties available on the market. Most of the silages from which data were gathered were from swards aged between 1 and 3 years, suggesting that most swards (other than permanent pastures) were reseeded after 3 years in line with recommendations on current variety persistence (Hejduk and Knot, 2010). Merviot and Milvus, the clover varieties sown with the greatest frequency, are both older varieties (for example, Merviot was first introduced to the UK recommended list of varieties in 1980) perhaps indicating a need for greater adoption of newer varieties to take advantage of genetic gains (Frick *et al.*, 2008). For example, in recent UK research, out of 12 clover varieties sown, ten new varieties which were developed in recent years showed increased persistency within a 3-year ley relative to Merviot and Milvus (Marshall *et al.*, 2012). Furthermore, resources now exist to allow development of genomic approaches that can be applied to breeding legumes (Annicchiarico *et al.*, 2015; Abberton *et al.*, 2008) and use of genomic approaches in breeding strategies may accelerate gains in yield, feeding quality (enhanced digestibility) and soil nutrient use efficiency in forage legumes so that they

can best suit the needs of the farmer in the drive towards sustainable intensification. Choice of grass variety is equally important as that of clover variety. Most farmers sowed clover with a perennial ryegrass or a hybrid instead of an Italian ryegrass which was likely due to the need to prevent clovers being outcompeted and increase sward persistency and resilience if grazed (Cougnon *et al.*, 2012). Adoption of new high sugar grass varieties was shown to be good with AberDart and AberStar the joint 5th most popular grass varieties, possibly as a result of research indicating increased use of high-sugar grasses in combination with red clover promotes a favourable balance of metabolisable protein and fermentable energy in the ruminant diet in comparison to feeding red clover alone (Merry *et al.*, 2006).

A majority of questionnaire respondents reported the use of a fertiliser (either inorganic or slurry) prior to harvest. Farmers were not required to state the timing of the application; therefore, many of the applications may have been early in the year or during establishment for first year silages, however, even out-of-season N applications have been shown to negatively impact CC over the summer months (Laidlaw *et al.*, 1992). The incidence of fertilisation seems high considering that there should be little to no need to apply expensive inorganic fertilisation in an established sward due to atmospheric N fixed by the clover (Ledgard, 2001). Advice will differ depending on CC within the sward, for example, fertilisation could be used as a management strategy to enhance grass growth if clover has become too dominant (Schils and Snijders, 2004). Very high CC may reduce digestibility due to increased lignin contained in clover stems, depending on maturity, and is therefore undesirable (Alstrup *et al.*, 2016). However, strategic use of fertilisation in this way should most likely occur towards the end of the season when clover tends to thrive due to warm dry conditions disadvantaging grass (as was true in the present study). Considering the greater number of 1st and 2nd cut silages included in the present study, it

is unlikely that the fertiliser applied was a management response to high clover concentrations. A high proportion of farmers applying slurry prior to harvest likely reflects a need to dispose of slurry regardless of the status of the crop. Where extra applications of N are applied, clover adapts by reducing the rate of fixation, and is more likely to be outcompeted by grass (Nyfeler *et al.*, 2011). The distribution of clover concentration within the sample set confirms this with a high number of samples falling below the minimum 300 g/kg DM threshold identified by Nyfeler *et al.* (2011) at which niche complementarity between clover and grass is optimised. Assuming that the silage samples obtained are an average representation of sward composition, this evidence suggests that few farmers are fully utilising the beneficial N-fixation properties of clover and possibly seeing reduced economic performance as a result, particularly where expensive inorganic N is applied to clover-containing swards. Furthermore, the addition of fertiliser N at a greater rate than is required by the sward leads to an increased rate of N leaching, contributing to N loading of the environment (Ledgard, 2001).

Methods of measuring botanical composition

Very few farmers were able to estimate the clover concentration of the sample to within ±100 g/kg DM, based on prior knowledge of the sward. One possible explanation for this would be poor uniformity within the sward meaning that the sample taken was not representative of the general crop (Marriott *et al.*, 1997), although this explanation is more valid for baled samples as opposed to those ensiled in a clamp where mixing is performed in the forage harvester. Uneven distribution of clover concentration in a sward poses a great challenge to crop management. In the future use of precision technology such as variable rate inputs to tailor management depending on botanical composition might assist in improving efficient use of mixtures. The inability of many farmers to

accurately estimate the concentration of clover within their forage highlights the need for tools to be developed which automate this process; one option being the use of NIRS on resulting silage which has been explored in the present study. Another option which has been explored previously is to determine n-alkane concentration which differs distinctively between species (Jurado *et al.*, 2015), however, the use of a laboratory assay of this kind is expensive and time consuming. While such a technique may be well suited to describe forages used in experimental work, it would not be recommended routinely in an applied context without further development. Ultimately using tractor-mounted NIRS machines to measure botanical composition and other variables in real-time would allow for the highest level of automation (van Maarschalkerweerd and Husted, 2015) however, whilst such technology does exist, it is not yet optimised for use in forage crops.

The NIRS equation developed in the present study showed promise, especially in determining low CC, however above 400 g/kg DM an increasing trend for under-prediction reduced accuracy. This explains why the equation showed the best prediction accuracy in the self-prediction and initial validation tests where the sample sets were predominantly comprised of low clover samples, but was less accurate when applied to an independent validation set where the mean CC was higher. Furthermore, the repeatability of the prediction was poor and would need to be improved before such an equation could be used commercially. Other studies in which prediction equations for clover concentration have been produced have shown more robust prediction accuracy than the present study, for example Cougnon *et al.* (2014) produced an equation with an RPD of 3.8 using just 42 silage samples as a calibration set. In another study Wachendorf *et al.* (1999) produced separate equations for freshly cut grass-red clover and grass-white clover mixtures with large calibration sets (n=282 and 183 respectively) where the relative SECV were 29.9 and 23.5% respectively, which again shows an improvement

over equations in the present study where relative SECV was 36.4%. A major difference between the calibration samples used in the present study and those used by Cougnon *et al*. (2014) and Wachendorf *et al*. (1999) is that no sample preparation such as drying or milling was used in line with UK recommendations whereas, in the previous studies samples were dried and milled. The drying and milling process firstly reduced heterogeneity within the sample (which is particularly important to representatively subsample mixtures for analysis) and also removes peaks produced by water molecules from the resulting NIRS spectra, reducing noise, and improving interpretation and repeatability (Sorensen, 2004). The reference technique used to measure clover concentration (manual separation prior to drying and milling) was the same in the present study and in the studies of Cougnon *et al.* (2014) and Wachendorf *et al.* (1999), however, the drawback of this technique is that results can be subjective, particularly on chopped samples as were used in our study, due to the lack of species-defining characteristics on some particles. Using a reference technique prone to human error such as this reduces the likelihood of producing a robust prediction equation.

While the equation produced in the present study was less robust than in previous trials, potential was shown for the equation to be used particularly at lower clover concentrations (< 400 g/kg DM). A secondary use of the equations, beyond assisting farmers with sward management, would be to generate a clover-based correction factor for UK NIRS predictions of the chemical composition of grass-clover samples which has recently been shown to be negatively impacted by the proportion of clover within a sample for some components, and notably crude protein (Davies *et al.*, 2012; Thomson *et al.*, 2016). Further development of the equation produced in the present study using increased numbers of samples to better represent higher clover concentrations within the calibration sample set may improve predictive ability, ensuring it is sufficiently robust

for such a use. In the future, further work on improving the capability of NIRS to predict botanical composition both in the laboratory and in the field with portable machines could enable the adoption of precision forage equipment with the goal of automating mixed sward management to better cope with spatial and temporal changes in botanical composition.

CONCLUSIONS

We conclude that improvements can be made to management practices currently employed by UK farmers for grass-clover swards intended for silage-making. Reducing or being more strategic in the use of fertiliser inputs, increasing uptake of newer varieties, and improved assessment of sward and silage botanical composition will allow better exploitation of the benefits of grass-clover swards and optimise niche complementarity between species. An equation suitable for predicting the concentration of clover within a mixed silage was developed and calibrated on un-dried and un-milled silage samples. Although the resulting equation proved less robust than similar equations produced using dried and milled samples (which likely improved homogeneity and stability), the equation showed promise, particularly for samples containing less than 400 g/kg DM clover. Further development of the NIRS approach for assessing botanical composition of mixed swards is warranted and could contribute to precision forage management and also could be used to provide a correction factor for silage chemical composition analysis based on grass silage NIRS predictions that are negatively affected by clover inclusion.

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Table 1 A list of questions that were asked within a questionnaire given to farmers that offered a grass-clover silage sample for inclusion in a dataset to test and improve near infra-red reflectance spectroscopy equations in the UK. Questions related to agronomy and ensiling practices used on the sward/crop from which the sample originated.

Question number	Question	Number of respondents
1	Establishment date	59
2	Establishment method	59
3	Age of pasture	61
4	Grass varieties sown (and rate)	49(44)
5	Clover varieties sown (and rate)	46(43)
6	Applications prior to cutting (including FYM, slurry and any chemicals)	45
7	Cutting date	62
8	Silage cut (i.e. 1st, 2nd)	59
9	Estimated clover percentage in sward	55
10	Weed problems	48
11	Time wilted (in hours)	60
12	Chop length (in cm, where applicable)	33
13	Clamp type (where applicable)	34
14	Bale wrap layers (where applicable)	43
15	Dry matter of silage	26
16	Additives used	30
17	Silage analysis (if known)	
17(a)	Metabolisable energy	18
17(b)	Protein	19
17(c)	Fibre	15
17(d)	Ash	17
17(e)	D value	17
17(f)	NCGD	0

Table 2 The results of three validation tests in which a new prediction equation for the concentration of clover within a grass-clover silage sample (in g/kg DM) was assessed for accuracy using a self-prediction test, a blind validation (by removing 10 samples from the calibration set), and an independent blind validation (using a separate set of 30 grass and red clover mixture samples).

Item[1]	Measured mean	Predicted mean	Bias[2]	r^2	Relative RMSEP, %[3]	RPD
Self-prediction	312	316	-4.2	0.84	36.4	2.49
Blind validation	354	292	61.9	0.88	35.5	2.65
Independent blind validation	440	323	117	0.78	52.2	1.56

RMSEP = root mean standard error of prediction; RPD = ratio of RMSEP to the standard deviation of the measured population;

[1] The number of samples tested in each instance: cross validation n=95; blind validation n=10; independent blind validation n=30.
[2] Bias is the measured mean minus the predicted mean
[3] Root mean square error of prediction presented as a percentage of the measured mean for standardisation

Figure Captions

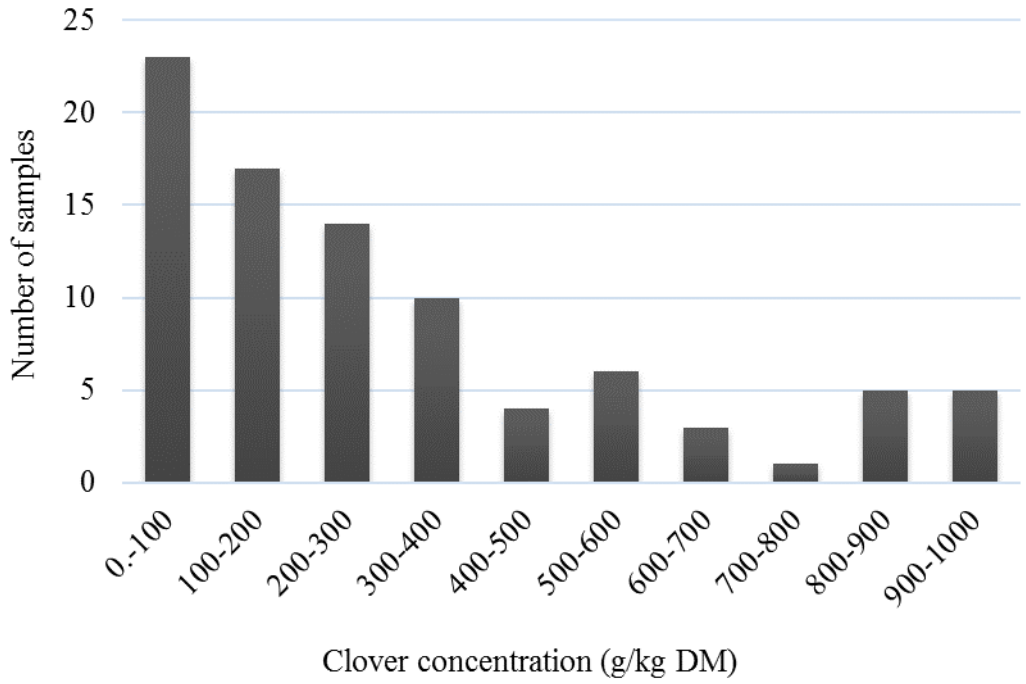

Figure 1 The distribution of clover concentration within a set of 94 grass-clover silages sourced from working farms across the UK over various cuts (1st - 4th) and years (2012-2015).

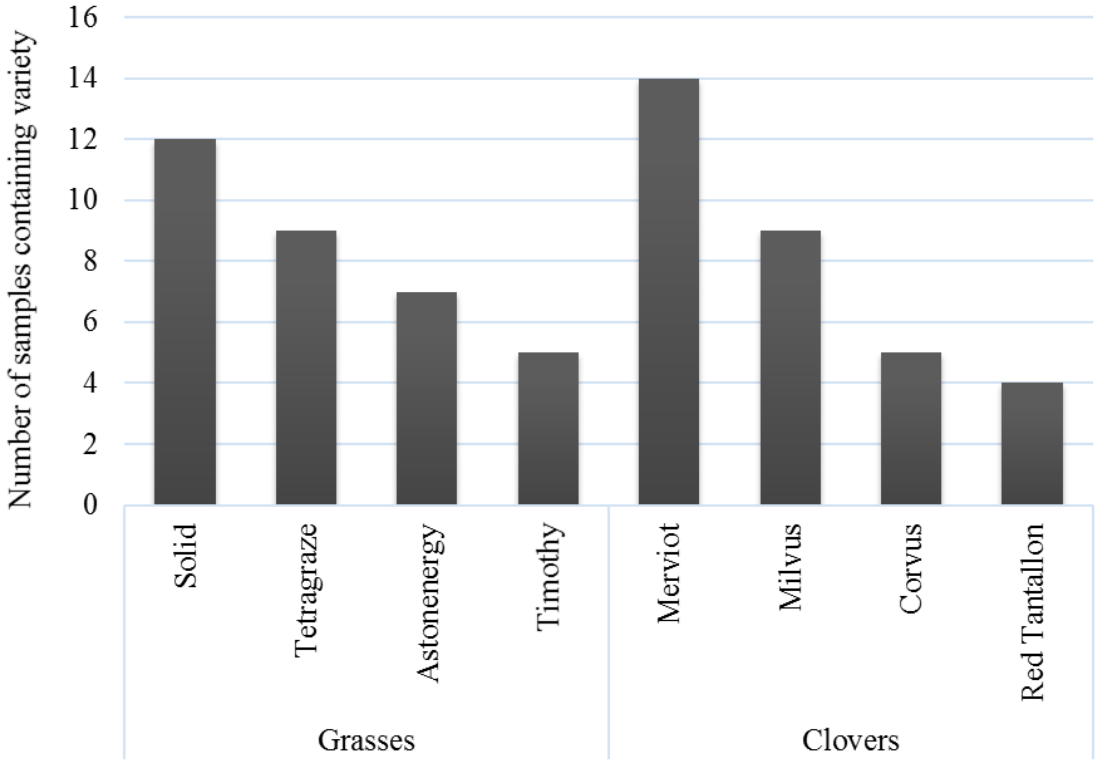

Figure 2 The top four most commonly sown grass and clover varieties and the number of grass-clover UK silage samples in which they occurred, out of a possible 49 samples for which information on varieties sown was provided by the grower.

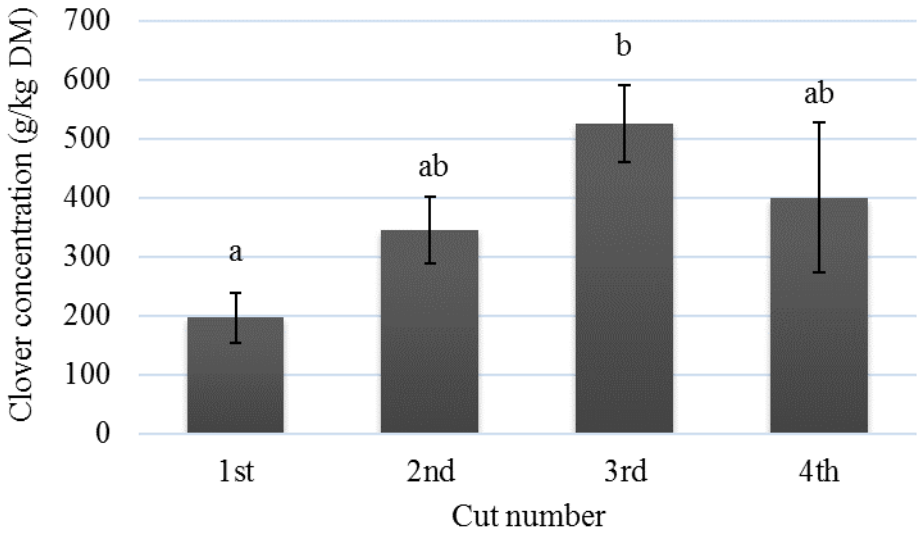

Figure 3 The effect of cut (1^{st} – 4^{th}; n= 36, 20, 16, and 4 respectively) on the concentration of clover within 72 grass-clover silage samples sourced from UK farms over several years (2012-2015).

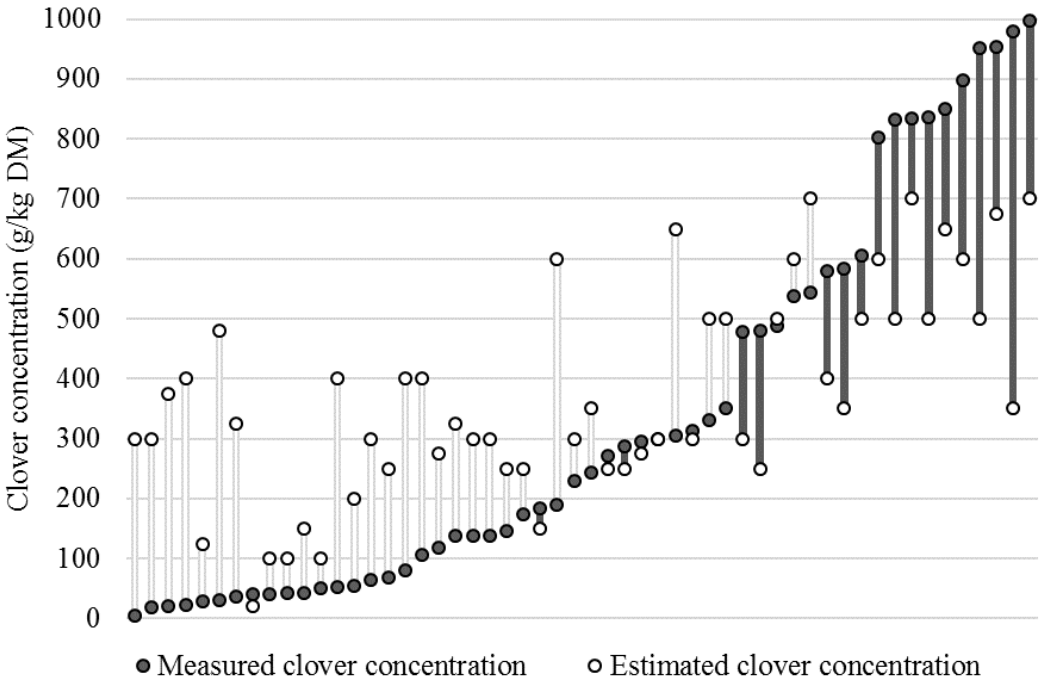

Figure 4 The relationship between actual clover concentration (●) and the grower's prediction of clover concentration (○) in a range of 54 grass-clover silage samples sourced from working UK farms over several years (2012-2015). White drop lines indicate over-prediction and dark drop lines indicate under-prediction of clover concentration.

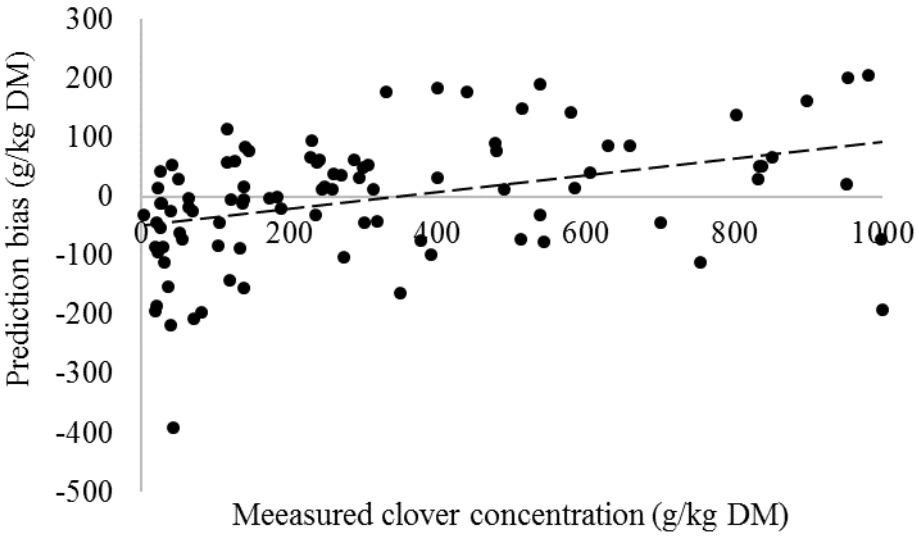

Figure 6 The effect of clover concentration (as measured using manual aspeciation) on clover concentration prediction bias (measured minus predicted data) according to a self-prediction validation of a new calibration equation developed for near infra-red reflectance spectroscopy (NIRS) analysis which was created from the spectra of 95 diverse un-dried and un-milled grass-clover silages from farms across the UK.

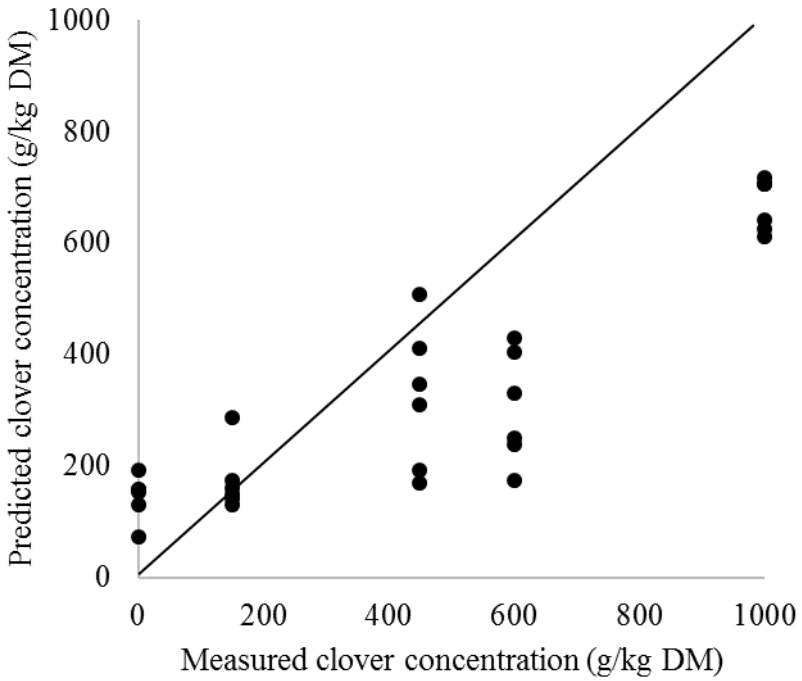

Figure 7 The results of a blind validation of a near infra-red reflectance spectroscopy (NIRS) prediction equation for clover concentration calibrated using a set of 94 diverse fresh grass-clover silage samples which were manually aspeciated to produce reference values and validated using an independent set of 30 grass-red clover silage samples of 6 known clover concentrations (0, 150, 450, 600, and 1000 g/kg dry matter).

Chapter 5

The effect of varying proportion and chop length of lucerne silage in a maize silage-based total mixed ration on diet digestibility and milk yield in dairy cattle

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ABSTRACT

The objective was to assess the effects of inclusion rate and chop length of lucerne silage, when fed in a total mixed ration (TMR), on milk yield, dry matter (DM) intake (DMI) and digestion in dairy cows. Diets were formulated to contain a 50:50 forage:concentrate ratio (DM basis) and to be isonitrogenous (170 g/kgCP). The forage portion of the offered diets was comprised of maize and lucerne silage in proportions (DM basis) of either 25:75 (HL) or 75:25 (LL). Lucerne was harvested and conserved as silage at either a long (L) or short (S) chop length. These variables were combined in a 2x2 factorial arrangement to give four treatments (HLL, HLS, LLL, LLS) which were fed in a Latin square design study to Holstein dairy cows in two separate experiments. Sixteen and 8 multiparous, mid-lactation, cows were used in experiments 1 and 2, respectively. To ensure sufficient silage for both experiments, different cuts of lucerne silage (taken from the same sward) were used for each experiment: first cut for experiment 1 (which was of poorer quality) and second cut for experiment 2 (good quality). Dry matter intake, milk yield and milk composition where measured in both experiments, and total tract digestibility and nitrogen (N) balance were assessed using four cows in experiment 2. In experiment 1 cows fed LL had increased DMI (+3.2 kg/day), compared with those fed HL. In contrast, there was no difference in DMI due to lucerne silage inclusion rate in experiment 2. A reduction in milk yield was observed with the HL treatment in both experiment 1 and 2 (-3.0 and -2.9 kg/day, respectively). The HL diet had reduced digestibility of DM and organic matter (OM) (-3 and -4%, respectively), and also reduced the efficiency of intake N conversion into milk N (-4%). The S chop length increased total tract digestibility of DM and OM (both +4%), regardless of inclusion rate. Inclusion of lucerne silage at 25% of forage dry matter increased milk yield relative to 75%

inclusion, but a S chop length partially mitigated adverse effects of HL on DMI and milk yield in experiment 1 and on DM digestibility in experiment 2.

IMPLICATIONS

A high inclusion rate of lucerne at 75% of forage dry matter (DM) within a total mixed ration (TMR) negatively affected diet digestibility and milk yield relative to a low inclusion rate. However, a short chop length increased diet digestibility at both lucerne inclusion rates, and therefore could be used to partly mitigate the negative effects of high lucerne silage inclusion in diets.

INTRODUCTION

Lucerne (*medicago sativa*) is widely utilised as a forage legume in dairy cow diets in semi-arid environments including parts of the US, Eastern Europe and Australia. Reduced requirement for inorganic N fertilisation may make it more economical to grow than well fertilised grasses depending on fertiliser price (Phelan *et al*., 2015) and therefore shows potential for greater use in intensive Northern European dairy systems. Establishing guidelines for the feeding of lucerne in such systems is critical for efficient utilisation.

Lucerne and maize silages in the diet are complementary to each other with the former providing rumen degradable protein and the latter providing fermentable energy from starch to drive microbial protein synthesis using ammonia and amino acids from lucerne protein degradation. Previous work has shown that the milk yield obtained from lucerne-maize forage combinations can equal that of grass-maize combinations (Sinclair *et al.*, 2015). However, the optimum inclusion rate of each is not certain. In one study where inclusion rates of chopped lucerne hay to maize silage were varied between 25%

and 75% lucerne inclusion within forage dry matter (DM), milk production decreased by 3.3 kg/d with the high rate of lucerne hay inclusion (Akbari-Afjani *et al.,* 2014).

Lucerne is also a source of physically effective neutral detergent fibre (peNDF) in diets for lactating dairy cows as it has a highly lignified stem that can encourage rumination. Physically effective NDF has been defined as the NDF present in longer particles within a feed (Mertens, 1997), typically considered to be particles greater than 4 mm using the Penn State Particle Separator (PSPS) system (Maulfair and Heinrichs, 2012). Previous research has shown short chop lengths (5 mm theoretical length) of lucerne haylage (Kononoff and Heinrichs, 2003) and silage (Beauchemin *et al.,* 1994) can increase DM Intake (DMI) and improve energy balance relative to long chop lengths of 22 and 10 mm respectively. Therefore, the objective of our study was to examine the effects of lucerne silage chop length on diet DMI and milk yield. A second objective was to investigate how chop length may interact with the inclusion rate of lucerne silage when substituted for maize silage in a TMR. We hypothesised that a lower inclusion rate of lucerne silage and a shorter chop length will increase intake and milk yield in line with previous studies discussed above and that these effects will relate to increased digestibility.

MATERIAL AND METHODS

Forage harvesting and clamp sampling

This study involved two separate experiments carried out simultaneously at the Centre for Dairy Research (CEDAR), University of Reading, between June and September 2015. The lucerne silage for both experiments was made on-site in the year prior to the start of the trial and conserved in concrete-walled clamps sheeted with a layer of oxygen-barrier film, two layers of plastic sheeting and a weighted top sheet. Experiment 1 utilised a first

cut, which was ensiled on 31 May 2014 (estimated 10% bloom). The harvested material was windrowed and wilted for 24 h. Alternate swaths originating from the same field area were used to create the two chop lengths, long (L) and short (S), by altering the knife arrangement of the precision chop forage harvester (Claas Jaguar 840 model, Claas Group, Harsewinkel, Germany) from a theoretical chop length of 14 mm (shortest setting) to 19 mm (longest setting). The long chopped material was collected from the field first followed by short chopped material and each were placed in identical adjacent clamps. The resulting silage was ensiled using Axphast Gold additive containing *Lactobacillus Plantarum* (Biotal, Cardiff) for low DM silages. The silage produced for Experiment 2 was created on 11 July 2014 in the same way, from the same sward, at second cut (also at an estimated 10% bloom). A longer wilting period of 48 h was allowed, and Axcool Gold additive containing *Lactobacillus Buchneri* (Biotal, Cardiff, UK) for high DM silages was applied. Following fermentation, core samples for all cuts were taken for chemical composition analyses (Sciantec Analytical Services, Cawood, UK). Maize silage for the study was taken from a commercial crop of mixed varieties harvested in autumn 2014 and ensiled in a concrete-walled clamp with no additive and sheeted as described for the lucerne clamps. The average particle size for the maize silage was determined to be 10mm using a PSPS.

Diets

A TMR with 50:50 ratio of forage:concentrate on a DM basis was fed. The forage was comprised of maize and lucerne silage in proportions (DM basis) of either 25:75 (high lucerne; HL) or 75:25 (low lucerne; LL), respectively. The two inclusion rates and the two chop lengths (L or S) were combined in a 2x2 factorial design to give four treatments (HLL, HLS, LLL, LLS). Diets were formulated to be isonitrogenous (170g CP/kg DM)

and contain similar levels of NDF (330 and 320 g/kg DM for Experiments 1 and 2 respectively) based on an analysis of core samples from the silage clamps used. Maize meal was included at higher rates in the HL diet to partly offset the reduction in maize starch associated with lower maize silage inclusion in these diets (Table 1), however there was still a significant difference between starch concentration in the resulting TMRs (Table 2).

Animals

For Experiment 1, 16 multiparous Holstein-Friesian dairy cows in mid lactation (144 d in milk, s.e.m. ± 4.3) weighing 701 kg and in fourth parity on average, were blocked (4 cows per block) according to milk yield and randomly assigned to one of four initial treatments within each block in a replicated 4x4 Latin square design experiment with three week periods. Cows were housed in a cubicle yard, bedded on sand and individually fed using CALAN gates (American Calan, Northwood, NH, USA). Continual access to water was given. Fresh feed was offered for *ad libitum* intakes (10% refusals per day) once daily at 1000 h. Refusals were removed on Mondays, Wednesday and Fridays.

For Experiment 2, eight multiparous Holstein-Friesian dairy cows in mid lactation (141 d in milk, s.e.m. ± 13.4) weighing 704 kg and in fourth parity on average, in two blocks (of which one block contained four cows fitted in a previous lactation with Bar Diamond rumen cannula (Parma, Idaho, USA)) were randomly assigned within each block to one of four initial treatments according to a 4x4 Latin square design with three week periods as in experiment 1. The block of four non-fistulated cows were used for measurements of total tract diet digestion. All procedures carried out in experiment 2 were licensed and monitored by the UK government Home Office under the Animal (Scientific Procedures) Act 1986. Animals were housed in a cubicle yard and individually

fed once daily for *ad libitum* intake through Insentec RIC feeders (Insentec B.V., Marknesse, The Netherlands) during weeks one and two of each period. Cubicles were bedded with wood shavings and continuous access to water was provided. In the final week of each period animals were housed and milked in individual tie stalls situated adjacent to the cubicle yard to facilitate sampling. Animals were given two days to acclimatise to the stalls before sampling began. While in tie stalls, animals were fed twice daily at 1000 and 1600 h for *ad libitum* intake (10% refusals). Refusals were taken daily at 0930 h.

Experimental routine

Intake and diet analysis. Weights of feed offered and refused were taken during the final week of each period. For the four animals used for digestibility measurements (experiment 2) only measurements from five days were statistically analysed. The DM of the feed offered and refused was measured in a forced air oven at 100°C for 24 h. Bulked daily grab samples of the TMR and diet components were also taken and frozen at -20°C until analysed. Samples of the constituents of the TMR were analysed for DM, N (using the macro kjeldahl method), ash (by combustion at 500ºC for 16 h), NDF (assayed with heat-stable amylase, inclusive of residual ash), ADF (inclusive of residual ash), starch, and water soluble carbohydrates (WSC) as described previously (Reynolds *et al*., 2014). Starch was converted to glucose by treatment of the hot water extract with amyloglucosidase followed by acid hydrolysis (Macrae and Armstrong, 1968). Total reducing sugars were measured calorimetrically and the result was corrected for cold water soluble reducing sugars (Fuller, 1967). Crude protein (CP) concentration was calculated by multiplying N (g/kg DM) by 6.25. Concentrations (g/kg DM) of CP, NDF, ADF, ash, starch and WSC in each TMR were calculated based on constituent inclusion

rates. Furthermore, TMR and diet components were analysed in triplicate for particle size distribution using a PSPS with holes measuring 4 mm, 8 mm and 19 mm in diameter and a bottom pan. Material from each sieve was collected and dried (at 60ºC for 72 h) to give a DM correction. Average particle size of the sample was calculated as described previously (Heinrichs, 2013).

Degradability of DM and N in each forage was measured using an *in situ* method with rumen cannulated lactating Holstein dairy cattle (Ørskov and McDonald, 1979). These cattle were housed in cubicles, in a dedicated metabolism unit, fed a commercial grass-maize based TMR diet once daily. Samples (not dried or further chopped) of each silage were placed in polyester bags (40 μm pore size) that were then incubated sequentially in the rumen of three different animals for six time intervals (3, 6, 12, 24, 48, and 72h). Three replicate '0' h bags were soaked in a tub of cold tap water with agitation for 5 minutes before being refrozen alongside the bags that were incubated in the rumen. Residue was subsequently analysed for DM and N concentration as described above.

Milk yield and composition. Cows were milked twice daily at 0630h and 1630h. In experiment 1 separate milk samples were taken during each of the last four consecutive milkings in each period and analysed for fat, crude protein, casein, lactose, urea, and somatic cell count (SCC) by mid infra-red spectroscopy on a CombiFoss machine (National Milk Laboratories, Chippenham, Wiltshire, UK). In Experiment 2 milk samples obtained throughout the third week of each period were analysed as for experiment 1.

Diet apparent digestibility and N balance. Beginning at 1000 h (prior to morning feeding) on day 17 of each period cows used for digestion trials were fitted with a harness and chute allowing total collection of faeces and urine for five consecutive 24 h periods

(Reynolds *et al*., 2014). Urine was collected into containers containing 1 L of 5 molar sulphuric acid. In addition, 200ml spot urine samples were collected twice daily in each of the five consecutive 24h periods, immediately acidified using 10ml of 5 molar sulphuric acid, and bulked. At the end of the collection period a representative subsample of the bulked spot samples was obtained and stored frozen until analysed for N. The bulked spot samples were used to determine urinary N concentration to account for any volatilised N losses. At the end of each 24 h period the total faeces and urine collected were weighed. Faeces were mixed, and subsampled as a fixed proportion of total volume to produce a representative bulk sample and stored at -20ºC for subsequent analysis. Faecal and feed samples were analysed for DM, N, OM, Starch, NDF, and ADF concentration and urine samples analysed for N concentration as described above for feed samples (Reynolds *et al*., 2014).

Statistical analysis

For silage degradability, an exponential curve fitted to percentage degradation at each time point was used to obtain fractions termed ‘a’, ‘b’ and ‘c’ as described previously (Ørskov and McDonald, 1979). Rumen outflow rate (k) was assumed to be 0.05 hr^{-1}. Feed efficiency was calculated as estimated milk energy yield (Tyrrell and Reid, 1965) divided by DMI. Data from each experiment were analysed separately. Experiment 1 was analysed as four simultaneous Latin Squares. Averages for each cow and treatment combination were analysed to determine fixed effects of square, period, lucerne inclusion rate (IR), chop length (CL), and their interaction (IRxCL) and random effects of cow within square using mixed models procedures of SAS (version 9.1). For experiment 2, data obtained within two simultaneous Latin Squares were analysed in the same way. For each variable the covariance structure giving the best fit was selected. Data from one cow

(not one used for the digestion trial) in experiment 2 in period four were removed as her DMI and milk yield did not fully recover following mastitis that occurred during the adaptation period.

RESULTS

Forage quality

The first cut silage used for experiment 1 had lower DM (-354 g/kg), and a higher pH (+1.1), than the second cut silage used for experiment 2 (Table 3). Higher DM (second vs first cut) and shorter chop length were associated with lower pH and greater lactic acid concentration but reduced acetic, butyric and propionic acid concentrations. Crude protein concentrations were similar for the first and second cut silages (174 g/kg DM). Of particular note, NDF and ADF were higher in the first cut silages then the second cut, suggesting greater maturity in the first cut silages.

The degradability fraction a was smaller in the experiment 1 lucerne silages than in the experiment 2 silages (-13%) and there was also reduced effective degradability and total degradation of DM (fractions a + b = 64% vs 75% for experiment 1 and experiment 2 silages, respectively). Degradation profiles for N showed that the lucerne silages had a higher EPD than that of maize. The rate of degradation of N (c) in the rumen was faster for the short chopped silages for both cuts but the difference was greater within the experiment 2 silages (0.04/h for L and 0.09/h for S; $P < 0.001$).

Forage and diet particle size

The average silage particle length was 12.6 mm and 9.4 mm in first cut silages ($P < 0.006$), and 14.3 mm and 9 mm in second cut silages ($P = 0.001$) for the long and short chop silages respectively. For both experiments the long chop increased particles retained

on the 8 mm sieve and reduced particles on the 4 mm sieve and the bottom pan relative to the short chop silages ($P < 0.01$; Figure 1). The long chop length increased the proportion of particles on the 19 mm sieve for the lucerne silage used in experiment 2 ($P < 0.001$), but not the lucerne silage used for experiment 1 (Figure 1).

In both experiments, average particle size of the diets fed (Table 4) increased with both greater lucerne inclusion ($P < 0.001$) and chop length ($P < 0.05$, < 0.001 in experiments 1 and 2 respectively). In experiment 1, the proportion of particles retained on the 19 mm screen increased ($P < 0.02$) with increased chop length. The proportion of particles retained on the 4 mm screen in experiment 1 was decreased ($P < 0.03$) by increased chop length for the HL, but not the LL diet (inclusion rate by chop length interaction, $P = 0.03$). In experiment 2, there were greater effects of chop length on particle distribution on the 19 and 4 mm screens for the HL than the LL diets (inclusion rate by chop length interaction, $P < 0.01$) and a greater difference on the 8 mm screen for the LL than the HL diet (inclusion rate by chop length interaction, $P < 0.05$).

Intake, milk yield and milk composition

The effect of lucerne silage inclusion rate on DMI varied between experiments with a DMI reduction of 3.2 kg/d where HL diets were fed in experiment 1 ($P < 0.001$), whereas there was no difference in DMI between treatments in experiment 2 ($P > 0.22$). In both experiments feeding the HL diets decreased milk yield (-3.0 and -2.9 kg/d in Experiments 1 and 2 respectively; $P < 0.02$; Table 4). In experiment 1, a longer chop length decreased milk yield relative to using a shorter chop length by -1.6 kg/d ($P < 0.001$), although this effect was not observed in experiment 2. As a result, the estimated conversion efficiency of feed DM into milk energy also differed between experiments, with HL diets tending

to produce greater conversion efficiency in experiment 1 ($P < 0.08$) and LL diets increasing feed efficiency in experiment 2 ($P = 0.001$).

Milk fat concentration was not affected by treatment in either experiment (Table 5), however, in experiment 1, milk fat yield was greater ($P < 0.017$) when LL diets were fed. In experiment 1, milk protein concentration was increased by 0.7 g/kg ($P < 0.001$) when HL diets were fed, although, due to increased milk yield, milk protein yield was highest ($P < 0.001$) when LL diets were fed. In experiment 2, feeding HL diets led to a decrease in milk protein concentration of 1.0 g/kg ($P < 0.04$) although there were no differences in total protein yield between treatments. Milk protein yield in experiment 1 was reduced by chop length, where a 45 g/d reduction with longer chop length ($P < 0.003$) was observed. Milk urea concentration was higher ($P < 0.001$) when HL diets were fed in experiment 1.

Apparent digestibility and N balance

Increasing lucerne inclusion rate decreased DM and starch intake and increased ADF intake of cows used for measurements of digestibility and N-balance ($P < 0.04$, < 0.003, and < 0.006 respectively, Table 6). Digestibility of DM was lower for the HL diets by 3.6% relative to the LL diets ($P < 0.05$). Increasing chop length also reduced DM digestibility by 4.3% ($P < 0.02$). Greater inclusion rate of lucerne and longer chop length both decreased the digestibility of organic matter by 3.7% and 3.2% ($P < 0.03$ and $P < 0.006$, respectively). There were no differences in the digestibility of starch, NDF or ADF between HL and LL diets, although NDF digestibility tended ($P < 0.10$) to be lower for longer lucerne chop length diets.

Intakes of N were greater for LL diets ($P < 0.01$) as a result of higher DMI (Table 7). There was a tendency for increased faecal N concentration ($P < 0.06$) when HL diets

were fed. Faecal N also tended to increase when the chop length was increased ($P < 0.07$). There was greater partitioning of intake N into the milk for the LL diets with an increase in N use efficiency of 3.3% ($P < 0.009$) and N digestibility was also greater ($P < 0.02$).

DISCUSSION

Forage quality and particle size

The nutritive value of the four lucerne silages used in the study was variable. Although crude protein levels were similar at 172 g/kg, the second cut (experiment 2) silages were lower in NDF and ADF than the first cut (experiment 1) silages, suggesting increased maturity in the first cut relative to the second cut forage. In the experiment 1 silage, high acetic and butyric acid and low lactic acid concentrations indicated very poor fermentation, although pH reduction was adequate, the quality of this silage was lower than would typically be advised for feeding lactating dairy cows. This may have contributed to the reduced intake observed in cows fed this silage at the high IR relative to the low IR. High levels of WSC in the experiment 2 silage may indicate that increased time spent wilting this crop (48h vs. 24h for the first cut) resulting in a higher DM reduced fermentation activity, or that the original concentration of sugar in this crop was higher than for the first cut crop. These results collectively indicate increased silage quality in the second cut silage with higher DM concentration. The effective degradation of DM and protein in the lucerne silages ranged from 37.8-56.7% and 72.6-78.6% respectively which are similar to previously published figures (56% for EDMD and 72% EPD for mid-bloom fresh lucerne (Hoffman *et al*., 1993)).

Variation between the long and short chop silages within each experiment was observed despite care being taken at harvest to control variables other than chop length. Notably, pH and acetic, propionic and butyric acids were reduced for both short cut

silages relative to long cut silages while lactic acid was increased. This may be explained by increased silage density through better compaction achieved with the short chop which helps to create the necessary anaerobic environment in the silo. Short cut silage was also collected from the field after long chopped silage leading to a small increase in wilting time which may also have increased the concentration of sugars available for fermentation.

The differences in physical structure achieved by varying chop length of the silages were similar for both experiments. The theoretical difference between average particle size according to the settings of the forage harvester was 5 mm which was relatively close to the achieved differences of 3.6 mm and 5.3 mm for experiments 1 and 2 respectively. The differences in mean particle lengths achieved by varying chop length in this study are similar to those used in previous research (e.g. 5mm, Beauchemin *et al.* (1994); and 7mm, Bhandari *et al*., (2007)). Although the difference in mean particle length is small, there were larger differences in the relative quantities of particle size fractions measured using a PSPS.

Intake, milk yield and nutrient digestibility

Effects of lucerne inclusion rate. The effects of a higher dietary inclusion rate of lucerne on DMI differed in the two experiments. In experiment 1, feeding the first cut silage at the higher inclusion rate decreased DMI, and milk yield. In contrast, feeding the second cut silage at the same rate to a smaller number of cows had no effect on DMI, but a reduction in milk yield was still observed. In a similar UK study where grass silage was replaced with lucerne silage in the diet a reduction in intake was also seen when 60% of forage DM was comprised of lucerne silage (Sinclair *et al*., 2015). Rumen fill can be limiting factor on DMI depending on the the extent to which the diet is comprised of

forage (Beauchemin *et al*., 2003). In this case the second cut silages showed 11% greater total DM degradability (a+b) over 72 h than the first cut silage indicating that the first cut silage would have contained a greater mass of forage dry matter within the rumen during this time. The differences in forage DM degradability might explain conflicting effects on DMI seen between experiments. Furthermore, the silage used in experiment 1 had a high concentration of acetic acid which has been linked with reduced intake in some studies where dietary concentrations were 25-50 g/kgDM (Daniel *et al.*, 2013), and this may have also contributed to lower intakes on HL diets in this instance.

Feeding HL diets led to a reduction in the digestibility of DM, OM and N. This indicates that the lucerne silages used in this study were less digestible than the maize silage, reflecting greater ADF concentration. Decreases in milk yield observed for the HL diets in both experiments could be related to this reduction in DM and OM digestibility, and therefore ME, and also the lower starch concentration of the HL diets (Table 2). Furthermore, there would have been a greater imbalance between supply of metabolisable protein to ME in HL diets which would contribute to the reduction in milk yield observed. These findings align with previous studies which show that lucerne typically has a lower ME content than many other forage legumes or grasses (Steinshamn, 2010). Efficiency of N utilisation was also reduced when HL diets were fed, which is partly attributable to lower milk yield seen on HL diets. Also, since diets were not balanced for rumen degradable N supply in this study, there was a surplus of rapidly degradable N for the HL diets which would have contributed to reduced N utilisation and high milk urea values. There was greater partitioning of N into faeces than urine in this study which was contrary to initial expectations. This likely reflects low N digestibility, particularly on HL diets, and may also indicate some loss of urinary N during the collection process perhaps due

to the spot sampling method adopted in this study failing to fully account for diurnal changes in urine concentration which should be borne in mind when considering results.

Effects of lucerne chop length. In experiment 1, DMI increased when a short chop silage was fed which was also observed with lucerne haylage (Kononoff and Heinrichs, 2003) but was contrary to the results of experiment 2 and a numerous previous studies in which reducing lucerne chop length did not affect DMI (Beauchemin *et al*., 1994 and 2003; Bhandari *et al*., 2007). Increased DMI with shorter chop length might suggest increased speed of particle breakdown and/or rumen outflow and lower rumen fill allowing higher rates of intake relative to the longer chop length. Some studies found this to be the case where short and long chop lucerne lengths were compared (using mean particle lengths of 1 and 8 mm; Yansari *et al*., 2004) although others noted no change in passage rate even when an effect on DMI was observed (Kononoff and Heinrichs, 2003). The short chop length also increased DM digestibility, and the magnitude of the effect was greater than that of inclusion rate. This may be explained by smaller particles exhibiting a greater surface area for microbial attachment, although this is only one of many factors that govern the rate of cellulolysis in the rumen (Mason and Stuckey, 2016). The increase in digestibility might also explain why the short chop mitigated some of the negative effect of the HL diet on milk yield (+1.6 kg/d milk produced on LL diets; Table 6).

Using shorter chop lengths with high lucerne silage diets shows potential as a strategy to partly mitigate reduced nutrient use efficiency when lucerne is included at higher rates in the diet. Further research into lucerne agronomy and variety development for delayed plant lignification to increase the acceptable harvest window is an approach which may improve prospects for feeding lucerne in the future.

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Table 1 Ingredients used to create experimental total mixed rations in two separate experiments.

	Diet			
	Experiment 1		Experiment 2	
Item	LL	HL	LL	HL
Ingredients, g/kg DM				
Lucerne silage	125	375	125	375
Maize silage	375	125	375	125
Concentrate blend				
Cracked Wheat	80	80	80	80
Maize Meal	61	70	54	97
Unmolassed Sugar Beet Feed	40	40	40	40
Soy Hulls	79	88	82	108
Soybean Meal	98	89	100	65
Rapeseed Meal	98	89	100	65
Molasses	10	10	10	10
Dicalcium phosphate	5	5	5	5
Salt	5	5	5	5
Dairy Mineral	10	10	10	10
Megalac[1]	15	15	15	15

LL = low lucerne diet; HL = high lucerne diet;

[1] Megalac rumen protected fat supplement (Volac International ltd., Royston, UK)

Table 2 The chemical composition of four total mixed rations containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length fed in two separate experiments.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Experiment 1, g/kg DM								
DM[1], g/kg	467	424	358	334	8.8	0.001	0.011	0.366
Ash	65	66	86	86	0.8	0.001	0.844	0.844
CP	181	179	163	171	3.4	0.138	0.367	0.343
NDF	334	337	329	348	6.0	0.689	0.164	0.280
ADF	269	244	227	222	4.4	0.096	0.163	0.283
Starch	256	256	165	155	7.9	0.044	0.589	0.609
WSC	37	36	27	27	2.5	0.009	0.993	0.896
n	4	4	4	4				
Experiment 2, g/kg DM								
DM[1], g/kg	553	572	611	635	3.3	0.001	0.002	0.386
Ash	61	62	77	78	0.4	0.001	0.350	0.946
CP	170	170	171	174	2.5	0.115	0.435	0.535
NDF	318	321	327	338	2.8	0.002	0.026	0.206
ADF	204[a]	208[a]	234[b]	236[c]	1.7	0.001	0.004	0.042
Starch	242	242	162	164	2.1	0.001	0.195	0.597
WSC	37	36	34	32	0.4	0.001	0.001	0.105
n	7	8	8	8				

IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; DM = dry matter; OM = organic matter; WSC = water soluble carbohydrate.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.
[1] Dry matter determined in a forced air oven and not corrected for volatile loss.

Table 3 Analysis of the chemical composition and degradability characteristics of four lucerne silages harvested at first cut (used in experiment 1) or second cut (used in experiment 2) at either a long (L) or short (S) chop length.

		Lucerne silage					
	Maize	Exp. 1		Exp. 2		SEM	*p* value
Item	silage	L	S	L	S		
Chemical composition[1], g/kgDM							
DM, g/kg	384[a]	218[b]	225[b]	587[c]	559[c]	10.0	0.001
CP	73[a]	176[b]	175[b]	170[b]	175[b]	6.4	0.001
OM	965[a]	874[b]	875[b]	892[c]	893[c]	2.1	0.001
NDF	368[a]	513[b]	498[c]	408[d]	390[ad]	7.6	0.001
ADF	215[a]	441[b]	418[c]	355[d]	328[e]	4.7	0.001
Starch	376	-	-	-	-	-	-
WSC	3[a]	1[a]	1[a]	10[b]	16[b]	1.1	0.001
n	4	4	4	4	4		
Fermentation characteristics[2]							
pH	-	6.2	5.6	4.9	4.7	-	-
Ethanol, g/kgDM	-	22.8	6.13	0.05	1.36	-	-
Lactic acid, g/kgDM	-	<5.0	7.29	27.1	43.1	-	-
Acetic acid, g/kgDM	-	56.9	40.7	7.77	0.97	-	-
Propionic acid, g/kgDM	-	8.07	4.80	0.51	0.39	-	-
Butyric acid, g/kgDM	-	41.3	15.6	1.02	0.72	-	-
Degradability parameters[3]							
DM degradability							
a, %	44.1[a]	26.5[b]	16.5[c]	32.7[d]	34.6[d]	0.43	0.001
b, %	37.6[a]	38.2[a]	46.5[b]	42.8[ab]	40.4[a]	1.76	0.037
c, %/h	3.26[a]	4.34[a]	4.24[a]	3.82[a]	6.02[b]	0.495	0.037
EDMD, %	58.6[a]	44.2[b]	37.7[c]	51.3[d]	56.5[e]	0.72	0.001
Protein degradability							
a, %	62.3[a]	67.2[b]	63.0[a]	59.0[c]	61.9[ac]	0.91	0.003
b, %	24.2	24.2	21.2	30.6	26.4	4.04	0.593
c, %/h	1.95[a]	2.95[ab]	4.19[b]	3.97[b]	8.63[c]	0.614	0.001
EPD, %	67.4[a]	74.8[b]	72.6[c]	72.5[c]	78.6[d]	0.76	0.001
n	3	3	3	3	3		

DM = dry matter; OM = organic matter; WSC = water soluble carbohydrates; ME = metabolisable energy; EDMD = effective dry matter degradability; EPD = effective protein degradability.

[1] Average chemical composition from analyses of bulk samples taken in each period of the study analysed using mixed models with fixed effect of silage and period. Dry matter was not corrected for volatile loss.
[2] The analysis from clamp core samples taken at 3 separate points in the clamp and bulked.
[3] Degradability parameters determined by *in sacco* incubation in the rumen, using the model of (Ørskov and McDonald, 1979) where a = rapidly soluble material; b = non-soluble but degradable material; c = rate f degradation of b; effective degradability = a+b[c/(c+k)] where k = an assumed outflow rate of 0.05/hr. Mean values from each of 3 cows were analysed using mixed models with fixed effects of silage and random effects of cow.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 4 The distribution of particle size (DM basis) in four total mixed rations containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length in two separate experiments.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Experiment 1								
Material retained, %DM								
19mm	8.4	9.6	21.7	30.0	1.67	0.001	0.018	0.104
8mm	39.9	32.8	41.3	39.9	0.60	0.001	0.421	0.226
4mm	17.3[a]	17.2[a]	21.4[b]	16.2[a]	0.86	0.148	0.025	0.030
Bottom pan	35.4	31.1	24.7	20.6	1.40	0.074	0.189	0.965
Mean particle size, cm[1]	0.62	0.67	0.82	0.97	0.415	0.001	0.046	0.230
n	4	4	4	4				
Experiment 2								
Material retained, %DM								
19mm	3.2[a]	5.0[a]	5.3[a]	12.1[b]	0.75	0.001	0.001	0.007
8mm	36.4[a]	41.9[b]	37.4[ac]	39.1[c]	0.50	0.129	0.012	0.026
4mm	16.5[a]	13.5[b]	18.7[c]	12.6[b]	0.24	0.033	0.001	0.004
Bottom pan	43.8	39.8	37.9	36.3	0.50	0.001	0.010	0.094
Mean particle size, cm	0.50	0.56	0.54	0.65	0.014	0.001	0.001	0.099
n	3	4	4	4				

IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; DM = dry matter

[1] Mean particle size was determined using the recommended equation of Penn State University (Heinrichs, 2013).

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 5 Dry matter intake, milk yield, milk composition and feed conversion efficiency (FCE) of lactating dairy cows fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length in two separate experiments.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Experiment 1								
DMI, kg/d	26.4	26.0	23.7	22.3	0.74	0.001	0.017	0.172
Milk yield, kg/d	35.2	33.9	32.5	30.6	1.04	0.001	0.001	0.449
Est. Milk energy, MJ/d [1]	101.4	100.0	93.6	89.7	4.12	0.001	0.073	0.379
FCE, MJ/kg[2]	3.84	3.85	3.99	4.00	0.119	0.079	0.926	0.970
Milk composition								
Milk fat, g/kg	36.9	37.7	37.6	38.3	1.37	0.263	0.242	0.705
Milk protein, g/kg	30.2	30.5	31.2	30.9	0.68	0.001	0.962	0.066
Milk urea, mg/kg	292	311	424	432	14.4	0.001	0.088	0.469
Fat yield, kg/d	1.29	1.28	1.21	1.21	0.065	0.017	0.844	0.954
Protein yield, kg/d	1.10	1.06	1.00	0.95	0.036	0.001	0.003	0.706
n	16	16	16	16				
Experiment 2								
DMI, kg/d	23.0	23.0	23.8	23.7	0.75	0.227	0.994	0.916
Milk yield, kg/d	31.5[ab]	33.7[b]	30.8[a]	28.7[a]	2.21	0.013	0.953	0.043
Est. Milk energy, MJ/d[1]	85.5[ab]	91.9[a]	86.1[ab]	82.1[b]	5.95	0.074	0.636	0.047
FCE, MJ/kg[2]	3.73	3.95	3.59	3.54	0.249	0.002	0.409	0.068
Milk composition								
Milk fat, g/kg	35.0	33.9	35.1	35.9	1.53	0.357	0.907	0.378
Milk protein, g/kg	30.1	30.6	29.7	29.0	0.62	0.034	0.768	0.146
Milk urea, mg/kg	291	306	324	333	23.0	0.105	0.508	0.862
Fat yield, kg/d	1.03	1.08	1.09	1.12	0.831	0.104	0.216	0.795
Protein yield, kg/d	0.92	0.95	0.92	0.90	0.667	0.261	0.842	0.387
n	7	8	8	8				

IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; DMI = dry matter intake; FCE = feed conversion efficiency

[1] Estimated milk energy = Milk yield, kg*((fat concentration, g/kg *0.0384+protein concentration, g/kg *0.0223+lactose concentration, g/kg *0.0199)-0.108)

[2] Feed conversion efficiency calculated as Estimated milk energy in MJ/d divided by DMI in kg

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 6 The apparent DM, OM, NDF, ADF and starch digestibility of four total mixed rations containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length when fed to lactating dairy cows (in experiment 2).

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Dry matter								
DMI, kg/d	23.8	25.0	23.1	22.1	0.57	0.040	0.862	0.116
Faecal DM, kg/d	6.70	7.90	7.70	8.89	0.445	0.022	0.010	0.992
DM digestibility, %	70.4	67.2	67.9	62.5	1.40	0.043	0.015	0.424
Organic Matter								
OM intake, kg /d	20.9	22.7	22.0	22.0	0.91	0.846	0.376	0.368
Faecal OM, kg/d	5.66	6.69	6.38	7.42	0.378	0.029	0.007	0.984
OM digestibility,%	73.1	70.5	71.0	66.2	1.22	0.021	0.006	0.292
Starch								
Starch intake, kg/d	5.28	5.73	3.98	4.00	0.257	0.002	0.407	0.438
Faecal starch, kg/d	0.16	0.24	0.14	0.16	0.034	0.015	0.019	0.081
Starch digestibility, %	96.7	95.8	96.9	96.3	0.78	0.668	0.250	0.858
Fibre								
NDF intake, kg/d	6.99	7.69	7.85	8.07	0.313	0.107	0.208	0.492
Faecal NDF, kg/d	3.05	3.56	3.43	3.77	0.200	0.095	0.032	0.564
NDF digestibility, %	56.0	52.8	56.9	53.8	2.04	0.572	0.095	1.000
ADF Intake, kg/d	4.51	5.01	5.59	5.86	0.207	0.005	0.135	0.628
Faecal ADF, kg/d	2.30	2.69	2.56	2.93	0.355	0.616	0.490	0.985
ADF digetsibility, %	50.4	47.3	52.3	50.0	2.67	0.381	0.309	0.863
n	4	4	4	4				

IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; DM = dry matter; DMI = dry matter intake; OM = organic matter.

Table 7 The apparent digestibility of N and N balance in lactating dairy cows fed total mixed rations containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length (in experiment 2).

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
N intake, g/d	674	692	654	635	10.7	0.010	0.953	0.067
Faecal N, g/d (a)	209	241	242	278	19.2	0.054	0.061	0.921
N digested, g/d	389[ab]	453[ab]	465[b]	380[a]	10.4	0.820	0.263	0.041
N digestibility, %	70.3	65.6	62.3	56.4	2.37	0.019	0.053	0.706
Urinary N, g/d (b)	157	168	187	166	12.5	0.171	0.551	0.127
Excreted N, g/d (a+b)	397	407	416	427	19.3	0.270	0.497	0.988
Milk N, g/d [1]	160	170	150	144	5.8	0.028	0.773	0.247
N use efficiency, % [2]	25.7	25.1	22.3	21.9	0.78	0.008	0.572	0.886
n	4	4	4	4				

IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; N = Nitrogen.

[1] Milk N = milk protein yield / 6.25
[2] N use efficiency calculated as the percentage of ingested N found as milk protein N.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$

Figure captions

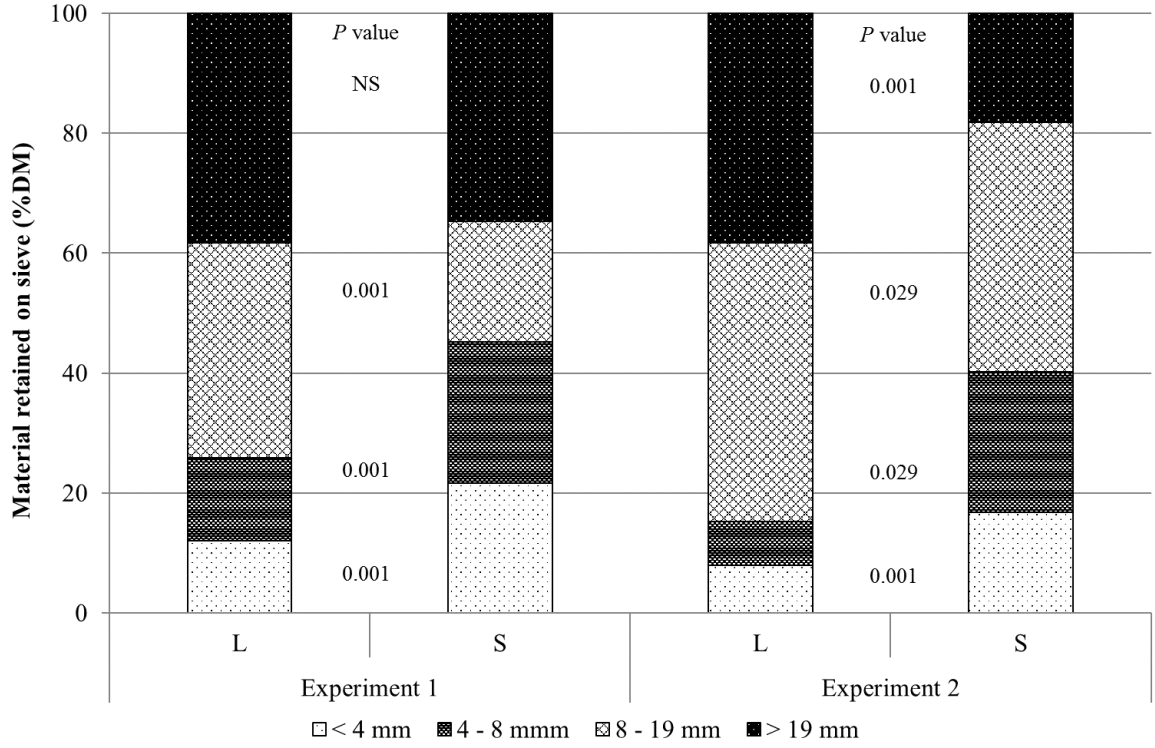

Figure 1 The effect of Short (S) or Long (L) chop length of lucerne silage on the distribution of particles (dry matter corrected) across the sieves of a Penn State Particle Separator for first cut silage (experiment 1) and second cut lucerne silage (experiment 2). Values are the means of measurements taken in each period (n=4).

Chapter 6

Effects of replacing maize silage with lucerne silage and lucerne silage chop length on rumen function and milk fatty acid composition

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INTERPRETIVE SUMMARY

Including a longer chop length lucerne silage in dairy cow diets had positive effects on rumination time per unit feed intake and daily rumination time was highest when longer chop lucerne silage was fed at higher inclusion rates. Longer chopped lucerne silage may be beneficial for diets where low rumen pH is a concern. In addition, higher lucerne levels in cow diets improved milk fatty acid profile in terms of human health, potentially increasing its value for human consumption.

ABSTRACT

The objective of this study was to investigate whether higher lucerne (*medicago sativa;* alfalfa) silage inclusion rate and longer lucerne chop length improves rumen function through increased provision of physically effective fiber, when included in a maize and lucerne silage-based total mixed ration. Diets were formulated to contain a 50:50 forage:concentrate ratio (dry matter [DM] basis) and be isonitrogenous and contain equal levels of neutral detergent fiber (320 g/kg). The forage portion of the offered diets was comprised of maize and lucerne silage DM in proportions (w/w) of either 25:75 (high lucerne; HL) or 75:25 (low lucerne; LL). Second cut lucerne was harvested and conserved as silage at either a long (L) or a short (S) chop length (theoretical chop lengths of 19 and 14 mm respectively). These variables were combined in a 2x2 factorial arrangement to give four treatments (HLL, HLS, LLL, LLS) which were fed in a 4 x 4 Latin square design study to four rumen-cannulated, multiparous, Holstein dairy cows in mid-lactation. Effects on dry matter intake (DMI), chewing behaviour, rumen volatile fatty acid (VFA) concentration, rumen pH, rumen and fecal particle size, milk production and milk fatty acid (FA) profile were measured. Longer chop length increased rumination times/kg DMI (+2.8 min/kg) relative to the S chop length, with HLL diets resulting in the

most rumination chews. Rumen concentrations of total VFA, acetate, and n-valerate were higher for the HLS diet than the other three diets, while rumen propionate concentration was lowest for the HLL diet. Physically effective fiber (particles >4 mm) percentage in the rumen mat was increased when L chop length was fed regardless of lucerne inclusion rate. No effect of treatment was observed for milk yield although milk protein concentration was increased by L for the LL diet (+1.6 g/kg) and decreased by L for the HLL diet (-1.4 g/kg). Milk fat concentrations of total *cis*-18:1 (+3.7 g/100g FA) and 18:3 n-3 (+0.2 g/100g FA) were greater with HL. In conclusion, longer lucerne silage chop length increased time spent ruminating per kg DMI, but had no effect on rumen pH in the present study. Increasing dietary lucerne inclusion rate had no effects on rumination activity or rumen pH, but increased lucerne silage inclusion rate decreased the ratio of n-6:n-3 polyunsaturated fatty acid concentrations in milk fat.

INTRODUCTION

The physical form of a total mixed ration (**TMR**) can affect rumen function and the efficiency of digestion in lactating dairy cows (Allen, 1997). Lucerne silage is thought to promote rumen health as it contains high NDF and ADF concentrations as well as having a higher natural buffering capacity (based on cation exchange capacity) than silages such as maize or ryegrass (McBurney *et al.*, 1983). Factors that are considered markers of rumen health include pH, volatile fatty acid (**VFA**) profile, time spent ruminating (increasing saliva production), and consistency of the rumen mat (Weidner and Grant, 1994; Plaizier *et al.*, 2008; Zebeli *et al.*, 2012). For optimal rumen health, highly fermentable concentrate feedstuffs must be adequately balanced by forage physically effective fiber (**peNDF**) in TMR.

Physically effective fiber is defined as the NDF present within the long forage particles (Mertens, 1997) and can be increased by lengthening forage particle size. However, relationships between particle size and the rumen environment are complex and different particle sizes can play different roles, such as rumen mat formation and stimulation of rumination, although there are conflicting views within the literature on the relative effectiveness of different particle sizes. For example, Zebeli *et al.* (2012) suggested that all particles greater than 1.18 mm are effective at stimulating rumination whereas only particles greater than 8 mm form the structure of the rumen mat; whereas Heinrichs (2013) suggested that only particles greater than 4 mm should be considered physically effective. Furthermore, an oversupply of long particles has been shown, in some instances, to reduce DMI and milk yield, possibly through excessive rumen fill (Kononoff and Heinrichs, 2003) and reduced surface area for bacterial attachment and thus digestibility (Zebeli *et al.*, 2008). Therefore, the optimum dietary inclusion rate (**IR**) of individual forages may vary depending on their chop lengths (**CL**). To this end, the main objective of this study was to evaluate the effect of two IRs of lucerne silage within a maize and lucerne silage-based TMR with two different lucerne CLs on parameters associated with rumen health and function. A secondary objective was to examine whether any changes in diet composition and rumen fermentation were associated with changes in milk yield and composition.

MATERIALS AND METHODS

Forage Harvesting and Clamp Sampling

The present study formed part of a larger trial reported previously (Thomson et al., 2017) utilizing the same dietary treatments and a larger cohort of cows. In brief, the lucerne silage used was a second cut crop, harvested in the calendar year before the present study

at an estimated 10 % bloom, windrowed, and wilted for 48 h to produce a high DM concentration (576 g/kg) silage. Alternate swaths originating from the same field area were used to create two silages with differing chop length (CL), long (**L**) and short (**S**) by altering the knife arrangement of a precision chop forage harvester (Claas Jaguar, Claas Group, Harsewinkel, Germany) from a theoretical chop length of 14 mm (shortest setting) to 19 mm (longest setting). The L and S chopped material was ensiled separately in identical adjacent clamps. Maize silage for the study was taken from a crop of mixed varieties harvested in the year before the present study and ensiled in a concrete-walled clamp with no additive. The average particle size for the maize silage was determined to be 10 mm using a Penn State Particle Separator (**PSPS**) (Heinrichs, 2013).

Diets

A TMR with 50:50 ratio of forage:concentrate (DM basis) was fed. The forage was comprised of maize and lucerne silage at IRs (DM basis) of either 25:75 (high lucerne; **HL**) or 75:25 (low lucerne; **LL**), respectively. The two IRs (LL or HL) and the two CL (**L** or **S**) were combined in a 2 x 2 factorial arrangement to give four treatments (**HLL, HLS, LLL, LLS**). Diets were formulated to be isonitrogenous (170g CP/kg DM) and contain similar levels of NDF (320 g/kg DM) through variation in the inclusion rates of soy hulls and rapeseed meal, based on preliminary analysis of core silage samples and reference values for other components. Maize meal was included at higher rates in the HL diet to offset the reduction in maize silage starch inclusion (Table 1).

Animals

Four multiparous Holstein-Friesian dairy cows in mid lactation (161 d in milk, s.e.m. ± 23.1), producing 39.7 L/d milk yield (s.e.m ± 6.2 L), in 6^{th} parity (s.e.m ± 0.3) on average

at the start of the study were used. Cows were fitted in a previous lactation with Bar Diamond rumen cannula (Parma, Idaho, USA). Animals were randomly assigned to one of four initial treatments according to a 4x4 Latin square design balanced for carryover effects with 21 day periods. All regulated animal procedures used were licensed and monitored by the UK Government Home Office under the Animal (Scientific Procedures) Act 1986. Animals were housed in a cubicle yard and individually fed once daily for *ad libitum* intake through Insentec RIC feeders (Insentec B.V., Marknesse, The Netherlands) during weeks 1 and 2 of each period. Cubicles were bedded with wood shavings and continuous access to water was provided. In the final week of each period animals were housed and milked in individual tie stalls situated adjacent to the cubicle yard to facilitate sampling. Animals were given 3 days to acclimatise to the stalls before sampling began. While in tie stalls, animals were fed twice daily at 1000 and 1600 h for *ad libitum* intake (10 % refusals). Refusals were taken daily at 0930 h.

Experimental Routine

Intake and Diet analysis. Weights of feed offered and refused were taken during days 14 – 17 of each period and the DM of both determined by oven drying at 100°C for 24 h. Bulked daily grab samples of the TMR and diet components fed were frozen at -20 °C until analysed. Diet components were analysed for DM, nitrogen (**N**), ash, NDF and ADF, starch, and water soluble carbohydrates (**WSC**) as described previously (Kliem *et al.*, 2008) and concentrations for each TMR were calculated based on constituent inclusion rates. Crude protein concentration was calculated by multiplying N (g/kg DM) by 6.25. The fatty acid (**FA**) profile of the TMR was determined using dried and ground TMR samples from each cow in each period. A one step extraction-transesterification procedure was performed as described previously (Kliem *et al.*, 2008) using methyl

heneicosanoate in toluene as the internal standard. A sample of each TMR was analysed in triplicate for particle size distribution using a PSPS (fresh weight basis). The PSPS used had sieves with holes measuring 4 mm, 8 mm and 19 mm in diameter and a bottom pan. Material from each sieve was collected, bulked over each of the triplicate sub-samples, and oven-dried at 60ºC for 72 h to give a DM correction. Average particle size of the sample was calculated as described previously (Heinrichs, 2013). During the sample week of each period, each cow was fitted with a chew-monitoring headcollar and supporting analytical software (Rumiwatch, ITIN+HOCH GmbH, Fütterungstechnik, Liestal, Switzerland) capable of detecting jaw movements through pressure on the noseband and categorising them as either eating, ruminating or drinking (Ruuska *et al.*, 2016).

Milk Yield and Composition. Cows were milked twice daily at 0630h and 1630h. Representative 30 ml milk samples, preserved using potassium dichromate, were taken at six consecutive milkings between days 15 and 18 of each period and analysed for fat, protein, casein, lactose, urea, and somatic cell count (SCC) by mid infra-red spectroscopy on a CombiFoss machine (National Milk Laboratories, Chippenham, Wiltshire, UK). On day 18 a further sample was taken at each milking and stored at -20ºC before being thawed, pooled according to milk yield, and analysed for FA profile. Lipid was extracted from 1 ml of milk using ethanol, hexane and diethylether and transesterified to FA methyl esters (**FAME**) using methanolic sodium methoxide with subsequent FAME separation using a gas chromatograph (**GC**; 3400, Varian Inc., Palo Alto, CA, USA) equipped with a flame-ionisation detector as described previously (Kliem *et al.*, 2008). Concentrations of FA are presented as g/100g total FA. Apparent recovery rates for 18:3 n-3 and 18:2 n-6 were calculated as the daily yield of these FA in milk as a percentage of the daily

amount ingested in feed based on mean DMI, milk yield and milk composition data for each cow in each period.

Rumen and Fecal Sampling. On day 15 of the treatment period 100ml samples of rumen liquor were taken at just prior to feeding and then at 0.5, 1.0, 1.5, 2.0 h post feeding and at each subsequent hour until 2200 h making a total of 15 samples. Rumen samples were collected by aspiration using a 50 ml catheter tip syringe through a coarse filtered sample tube as described previously (Dittmann *et al.*, 2016). The fluid was mixed immediately and the pH was measured (HANNA instruments, Woonsocket) after which a subsample was acidified to pH <2 using concentrated H_2SO_4 and stored at -20 ºC prior to analysis for ammonia (NH_3) using a segmented flow analyzer as described previously (Sutton *et al.*, 2000). A further non-acidified subsample was immediately placed in a freezer (-20 ºC) until analysed for VFA concentration using GC (3400, Varian Inc., Palo Alto, CA, USA) procedures as described previously (Erwin *et al.*, 1961).

On day 15 and 16 of each period spot samples (approx. 500 g) of feces voided were collected and bulked, until sufficient material (approx. 3 kg daily) was obtained. Up to six samples were obtained per day providing that the feces were uncontaminated by bedding. Furthermore, on day 17, a grab sample of the rumen mat (approx. 3kg) was obtained at 4 h post feeding. The sample was taken by vertically removing handfuls of material until the liquid phase in the ventral rumen was reached, with each handful immediately placed into a collection bucket. Bulked samples of rumen and fecal material were mixed and a subsample of each was oven-dried at 60 ºC for 72 h. Subsamples were then sieved using an adaptation of the wet sieving procedure described by Kononoff and Heinrichs (2003) using three sieves of 1, 2 and 4 mm diameter. Sieves were manually shaken while held under a cold water tap at a fixed flow rate for 30 seconds. Any material

passing through the 1 mm sieve could not be retained and was assumed to be very small or soluble – a value for this was obtained as the difference between the starting dry weight and the combined dry weight of the other three fractions. A minimum of four replicates per sample were sieved with resulting material on each sieve being collected and bulked across replicates. Material from each sieve was analyzed for DM and NDF concentration by oven drying each fraction at 60 ºC for 72 h followed by subsequent determination of NDF concentration as described for feed samples.

Rumen pH. A weighted (300g) indwelling pH meter (Sentix 41-3 probe, WTW Trifthof, Weilheim, Upper Bavaria) inserted through the bung of the rumen cannula and anchored to 50cm of nylon cord was placed within the rumen of each animal for 24 h beginning just prior to feeding on day 15 of each period until refusals were removed at 0930 h on day 16. The probe was calibrated before every insertion and checked for drift after use through immersion in standard solutions of pH 4 and 7. The pH probe was attached to a datalogger (ph340i, WTW, Trifthof, Weilheim, Upper Bavaria) with readings recorded every 10 minutes. Readings were further averaged over each hour for analysis.

Data Analysis

Feed conversion efficiency (**FCE**) was calculated as estimated milk energy yield (Tyrrell and Reid, 1965) divided by DMI. Dietary peNDF was calculated as the percentage of particles greater than either 4, 8 or 19 mm (measured using a PSPS) multiplied by total dietary NDF for each TMR in each period on a DM basis. All measured variables were averaged for each cow and treatment combination and analysed using mixed models procedures of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) to determine fixed effects of period, lucerne IR, lucerne CL, and IR and CL interaction, and random effects

of cow. For rumen VFA, NH_3 and pH measurements the effect of time (T) was included as a repeated effect and tested for interactions with IR and CL (TxIR, TxCL, TxIRxCL) with the 'SLICE' option used to test for treatment effects at each time point. For each variable the covariance structure giving the best fit for repeated effects of time was selected (compound symmetry, heterogeneous compound symmetry, auto-regressive, heterogeneous autoregressive, unstructured, or spatial power covariance). Data from one cow in period 4 were removed as her DMI and milk yield did not fully recover following mastitis that occurred during the adaptation period.

RESULTS

Diet Composition

Concentrations of DM, OM and ADF (Table 2) were higher in HL diets than LL diets (all $P < 0.03$) while starch and water soluble carbohydrate concentrations were lower (both $P < 0.04$) despite inclusion of maize meal in the HL diets. The HL diets also had lower concentrations of *cis*-9 18:1 ($P < 0.03$) and 18:2 n-6 FA ($P < 0.003$), while the concentration of 18:3 n-3 was higher ($P < 0.02$). Overall the HL diets contained less total FA than LL diets ($P < 0.04$). The HLL diet contained more than double the concentration of very long particles (>19mm) relative to the other diets, an effect mirrored by concentrations of $peNDF_{>19mm}$ (IR x CL interaction, $P < 0.009$). The concentration of $peNDF_{>8mm}$ was mainly influenced by CL, with L diets containing 3.5% more $peNDF_{>8mm}$ than S diets ($P < 0.03$), while HL and L both increased $peNDF_{>4mm}$ ($P < 0.004$). A longer lucerne CL also increased concentration of ADF ($P < 0.007$) and decreased WSC concentration ($P < 0.02$) relative to a shorter CL.

Rumination Patterns and Rumen Parameters

Cows fed the L diets spent more time ruminating per unit DMI ($P < 0.04$) and also tended to chew a greater number of times during rumination ($P < 0.09$) than when fed S diets. The greatest number of rumination chews per day was observed for the HLL diet (CL x IR interaction, $P < 0.05$), whereas the greatest eating chews per day was observed for the LLS diet (IR x CL interaction, $P < 0.05$), which contained the least physically effectively fiber.

Over 12 h post feeding, HL diets increased the concentration of rumen NH_3 relative to LL diets ($P < 0.001$; Table 4). Additionally, both CL and IR affected the rumen VFA profile in this time period, with LL diets increasing concentration of propionate ($P < 0.01$) and reducing acetate:propionate ratio ($P < 0.001$), and iso-butyrate concentration ($P < 0.007$), while total VFA concentration and n-butyrate concentration was greater in S diets than L diets (both $P < 0.03$). The HLS diet resulted in higher concentrations of total VFA, acetate and n-valerate concentrations than the other three diets as indicated by CL x IR interactions (all $P < 0.009$; Table 4). In the case of propionate, LLS, LLL and HLS diets showed similar concentrations with an average of 25 mM whereas feeding the HLL diet resulted in a lower propionate concentration of 20 mM (CL x IR interaction, $P < 0.004$).

Rumen propionate concentration was consistently lower throughout the 12 h time period with the HLL diet compared with the other diets with significant differences recorded at 1300, 2000, 2100, and 2200 h ($P < 0.05$, figure 1a). The HLS diet resulted in a higher rumen concentration of both acetate and total VFA at certain time points, but the effect was inconsistent (Figure 1b, 1c). Despite these effects on VFA profile, there were no significant effect of treatments on average, minimum, or maximum rumen pH measured over the same 24 h period, although, the mean pH during just 12 h post-feeding

did show a tendency for HLL diets to have an elevated pH in comparison to the means of the other three diets (IR x CL interaction, $P < 0.06$; Figure 1d).

For samples of rumen mat, feeding L increased the DM percentage of large particles (>4mm) by 14 % units ($P < 0.002$; Table 5) and decreased the DM percentage of medium particles (2-4 mm) by 3.8 % units ($P < 0.002$). The percentage of medium length particles in the mat was greatest when the HLS diet was fed (IR x CL interaction, $P < 0.008$ on a DM basis and < 0.001 on an NDF basis). On an NDF basis, feeding HL diets led to more small particles (1-2 mm) retained within the rumen mat than LL diets ($P < 0.05$). Fecal particle structure was largely unaffected by treatment diets, except for an increase in NDF retained on the 1mm sieve when HL versus LL diets were fed ($P < 0.03$). There was also a tendency for cows fed HL diets to void feces with a higher DM concentration ($P < 0.06$).

Intake, Milk Yield and Composition

There was no consistent effect of CL or IR on DMI although LLL resulted in a lower DMI relative to LLS (IR x CL interaction, P < 0.03; Table 6). Milk yield and the yield of milk solids was not affected by diet, although milk protein yield tended to be greater for LL versus HL diets (P < 0.063). Milk protein concentration was increased by longer CL with the LL diets and decreased by longer CL with the HL diets (CL x IR interaction, P < 0.001), whilst overall milk protein concentration was higher for LL than HL diets (P < 0.033). Lucerne silage IR affected concentrations of some milk FA (Table 7). Milk concentrations of total *cis*-18:1 isomers (mainly comprised of *cis*-9 18:1) and 18:3 n-3 were both higher for diets containing more lucerne silage (both $P < 0.006$). Cows fed HL diets also produced milk with higher 4:0 and lower 10:0 concentration relative to cows fed LL (both $P < 0.04$). The apparent recovery of 18:2 n-6 was increased by 3.8 % where

HL diets were fed ($P < 0.04$). Total MUFA concentration was higher in the HLL diet than in the LLL or HLS diet (IR x CL interaction $P < 0.04$). A longer CL of lucerne tended to increase 18:1 and decrease 18:3 n-3 concentrations in comparison to the shorter CL (both $P < 0.07$). In addition, longer CL increased n-6:n-3 PUFA concentration ratio in milk fat ($P < 0.018$).

DISCUSSION

Diet Physical Properties and Rumen Function

Particle size distribution in the diet was affected by both lucerne IR and CL (Table 2). Heinrichs (2013) suggested that all dietary particles greater than 4 mm contribute to formation of the rumen mat however the model of Zebeli *et al.* (2012) proposes that only particles greater than 8 mm in length promote increased rumination. In this study, both HL and L increased diet peNDF measured in all particles sizes. The longer lucerne CL, but not lucerne IR, tended to increase both rumination chews per unit DMI and the concentration of particles >4 mm within the rumen mat which suggests agreement with the proposed model of Zebeli *et al.* (2012) and is consistent with findings of a number of studies that have examined the effect of CL of the diet (Beauchemin *et al.*, 1994; Clark and Armentano, 2002; Teimouri Yansari *et al.*, 2004). From these data it could be concluded that although HL inclusion did increase effective fiber concentration in the diet relative to LL, the effect of CL on rumination was greater than the effect of IR. This may be due in part to the lack of effect of IR on particles >4 mm in the rumen mat. In the case of rumination, numbers of chews and time spent ruminating were highest for the HLL diet (although the effect did not always reach statistical significance). However, rumination activity did not always correlate with peNDF concentration as might be expected. For example, cows fed the LLS diet chewed more when eating than cows fed

the other diets and also chewed more when ruminating relative to both LLL and HLS diets. Differences might be partly attributed to increased uniformity of the diet altering particle prehension or preventing cows sorting against the longer particles as has been observed previously in CL studies (Kmicikewycz and Heinrichs, 2015). Regardless of the cause, the higher chewing activity in the LLS diet could have led to a higher saliva production, which could explain why the daily mean pH of cows fed this diet were comparatively high.

Rumen propionate concentrations in cows fed the HLL diet were consistently lower than in cows fed the other diets over a 12 h period after morning feeding. The reduction in propionate concentration might be attributed to reduced starch intake combined with longer CL reducing the rate of production or that the increased rumination seen in cows fed HLL diets led to higher levels of saliva production thus increasing the provision of bicarbonate, which is involved in the removal of VFAs from the rumen by epithelial absorption (Dijkstra *et al.*, 2012). In contrast cows fed the HLS diet had the highest total VFA concentration and also the highest acetate concentration. The HLS diet, with a higher concentration of short particles, may have facilitated a greater rate of fermentation in the rumen leading to a more rapid supply of volatile fatty acids (Allen, 1997). The observation that the rumen mat of cows fed HLS diets had a greater proportion of 2-4mm particles and a lower concentration of particles >4 mm than other diets at 4 h after feeding may support this explanation. Particles >4 mm within the rumen mat are thought to play a role in trapping smaller particles within the rumen for longer, allowing increased digestion of nutrients (Zebeli *et al.*, 2012).

Positive changes in rumination, physically effective fiber concentration, and rumen mat structure are often associated with a rise in rumen pH (Zebeli *et al.*, 2006). However, in the present study, no effects of lucerne silage IR or CL on daily mean rumen

pH were observed although there was a tendency for increased pH over the first 12 h post-feeding in the HLL diet. This contrasts with numerous studies investigating CL that have reported decreased rumen pH when average particle size is decreased (Kononoff and Heinrichs, 2003; Bhandari *et al.*, 2008) although the effect is not always seen (Beauchemin and Yang, 2005; Suarez-Mena *et al.*, 2013). Similar to our study, altered ratios of maize silage to lucerne hay in the diet had no effect on rumen pH in lactating dairy cows in a study by Akbari-Afjani *et al.* (2014). Differences in the response of rumen pH to lucerne silage IR and CL may be influenced by the extent to which the basal diet represents a rumen pH challenge. For instance, in this study daily mean rumen pH was above 6.3 and minimum values were above 5.9, indicating that the threshold for SARA was never reached, even though the LLS, LLL and HLS diets contained less than 18% $peNDF_{>8mm}$ which is proposed to be the threshold below which SARA risk increases (Zebeli *et al.*, 2010). Zebeli *et al.* (2010) proposes that SARA risk can be avoided even in high starch diets by balancing rumen degradable starch (RDS) concentration with an equal or greater concentration of peNDF. In this study the ratio of $peNDF_{>4mm}$ to estimated total RDS supply was 0.96, 1.11, 1.54 and 1.60 for the LLS, LLL, HLS and HLL diets, respectively, which may explain why there was little effect of treatment diets on rumen pH. However, the range from minimum to maximum pH was greatest in the LLS diet (0.87 pH units) with a smaller range observed in HL diets (0.67 pH units on average), which could be a consequence of the higher buffering capacity of the lucerne silage.

Effect on Milk Yield and Composition

Diets used in this study had little effect on milk or milk constituent yield. The decrease in milk protein concentration for the HLL versus HLS diets was observed when these diets were fed to a larger group of cows (Thomson et al, 2017) and reflected the lower

starch concentration provided by diets with high concentration of lucerne silage. Additionally, digestibility can be reduced in lucerne-based diets leading to a reduction of fermentable energy to drive microbial protein synthesis (Sinclair *et al.*, 2015).

Substituting legumes for other forages such as grass has been shown to alter the FA profile of milk in ways which can be advantageous to milk quality (Dewhurst *et al.*, 2006) by improving the concentration of PUFA which is thought to benefit human health, particularly by providing essential FA that can only be obtained by humans through the diet, and possibly also reducing SFA. Lucerne silage contains a higher concentration of 18:3 n-3 than maize silage (Onetti *et al.*, 2002; Benchaar *et al.*, 2007), which was consistent with the present study. The higher concentration of 18:3 n-3 FA in HL diets was probably the main reason for the higher concentration of 18:3 n-3 in milk fat from cows fed HL diets (Khiaosa-ard *et al.*, 2015), which led to a reduced n-6:n-3 FA ratio. Increased concentrations of n-3 FAs relative to n-6 is thought to be desirable as n-3 FAs are thought to reduce platelet aggregation in the blood whereas n-6 FA have the opposite effect (Yamada *et al.*, 1996). The reduction in the n-6:n-3 FA ratio in milk fat observed in the present study is consistent with previous reports of effects of increased dietary lucerne IR (Dhiman *et al.*, 1999; Sinclair *et al.*, 2015). For instance, in the study of Sinclair *et al.* (2015), the concentration of 18:3 n-3 increased by 0.6 g/kg FA when lucerne silage IR was increased from 40 to 60% of offered forage DM; which is in line with the present study where a larger increase in lucerne concentration (50% units of forage DM) resulted in a 1.6 g/kg FA increase in 18:3 n-3 concentration in milk fat. In the case of 18:2 n-6, HL diets supplied a lower concentration than LL diets, however due to a greater recovery rate in these diets, 18:2 n-6 concentration was numerically greater in the milk from cows fed HL diets. Khiaosa-ard *et al.* (2015) proposed that recovery rate of 18:2 n-6 is exponentially increased where a low rate of this FA (<10 g/kg of diet DM)

is supplied by the diet. As HL diets were below the 10g/kg 18:2 n-6 threshold this might explain increased efficiency of dietary 18:2 n-6 transfer to milk fat observed in the present study. High lucerne silage diets also increased milk fat *cis*-9 18:1 concentrations despite containing less *cis*-9 18:1 per kg DM. Around 50% of *cis*-9 18:1 in milk fat is derived from the action of mammary Δ^9 desaturase on 18:0 from the circulation (Enjalbert *et al.*, 1998), which arises following complete biohydrogenation of dietary unsaturated 18-carbon FA. This is reflected in the numerically higher concentration of 18:0 in milk fat from cows fed HL diets. As HL diets contained less total unsaturated 18-carbon FA than LL diets, the difference in *cis*-9 18:1 is probably due to the rumen environment promoted by the HL diets, which likely favoured complete biohydrogenation. There was relatively little effect of CL on the milk fatty acid profile. The ratio of n-6:n-3 PUFA concentrations in milk fat was decreased for S compared to L diets. In addition, there were tendencies for lower concentration of 18:3 n-3 and higher concentration of 18:1 *c*9 when L was fed relative to S. This may suggest that L diets create a rumen environment that leads to more complete biohydrogenation of dietary PUFA, perhaps through a reduction of rumen passage rate. Similarly, Dhiman *et al.* (1999) observed a small increase in total 18:1 isomer concentration (+2.1 g/kg FA) when coarse alfalfa hay was fed in place of finely ground alfalfa hay, which is similar to the present study where total 18:1 concentration increased by 2.8g/kg FA when L was fed in place of S.

CONCLUSION

In the present study, feeding a higher lucerne silage IR and longer lucerne silage CL increased the dietary concentration of peNDF. Longer lucerne silage CL, but not greater IR, increased $peNDF_{>4mm}$ in the rumen mat and rumination activity. However, there were no effects of dietary treatments on rumen pH, despite LL diets being higher in starch and

lower in physically effective fiber. Whilst lucerne silage IR had no effects on rumination activity or rumen pH in the present study, greater IR decreased the ratio of n-6:n-3 PUFA concentrations in milk fat.

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Table 1 Ingredients used to create experimental total mixed rations.

	Diet[1]	
Item	LL	HL
Ingredients, g/kg DM		
Lucerne silage	125	375
Maize silage	375	125
Concentrate blend		
Cracked Wheat	80	80
Maize Meal	54	97
Unmolassed Sugar Beet Feed	40	40
Soy Hulls	82	108
Soybean Meal	100	65
Rapeseed Meal	100	65
Molasses	10	10
Dicalcium phosphate	5	5
Salt	5	5
Dairy Mineral	10	10
Megalac[2]	15	15

[1] LL = low lucerne diet; HL = high lucerne diet;
[2] Megalac rumen protected fat supplement (Volac International ltd., Royston, UK)

Table 2 The chemical and physical composition of four total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Chemical composition, g/kg DM								
DM, g/kg	555	571	610	632	5.0	0.022	0.065	0.364
OM	62	63	78	77	0.6	0.001	0.471	0.070
CP	164	163	168	167	3.5	0.200	0.710	0.945
NDF	311	322	335	340	4.8	0.115	0.221	0.510
ADF	202	208	237	245	1.5	0.004	0.007	0.322
Starch	234	235	164	168	7.0	0.039	0.680	0.780
WSC[2]	37	35	35	32	0.7	0.006	0.020	0.371
Fatty acid profile, g/kg DM								
16:0	8.82^{a}	7.13^{b}	7.21^{b}	7.51^{b}	0.391	0.089	0.061	0.014
18:0	1.03	1.03	0.98	0.86	0.091	0.247	0.453	0.494
18:1 *c*9	8.43	8.75	6.76	4.82	0.828	0.023	0.302	0.201
18:2 n-6	10.9	10.6	8.9	7.0	0.74	0.003	0.077	0.182
18:3 n-3	1.51^{c}	1.73^{c}	2.40^{a}	2.07^{b}	0.034	0.012	0.336	0.027
Total fatty acids	33.3	33.3	29.4	23.5	2.42	0.038	0.218	0.248
Particle size distribution[3]								
Material retained, %DM								
19mm	3.2^{b}	5.0^{b}	5.3^{b}	12.1^{a}	0.75	0.001	0.001	0.007
8mm	36.4^{c}	41.9^{a}	37.4^{bc}	39.1^{b}	0.50	0.129	0.012	0.026
4mm	16.5^{b}	13.5^{c}	18.7^{a}	12.6^{c}	0.24	0.033	0.001	0.004
Bottom pan	43.8	39.8	37.9	36.3	0.50	0.001	0.010	0.094
Mean particle size[4], cm	0.50	0.56	0.54	0.65	0.014	0.001	0.001	0.099
peNDF[5], %DM								
$peNDF_{>19mm}$	1.03^{b}	1.64^{b}	1.74	4.04^{a}	0.268	0.001	0.001	0.009
$peNDF_{>8mm}$	12.3	14.8	13.8	18.2	0.27	0.056	0.030	0.137
$peNDF_{>4mm}$	17.2	19.9	20.5	21.3	0.38	0.003	0.004	0.051
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;

[2] WSC = water soluble carbohydrate.

[3] Particle size distribution measured using a Penn State Particle Separator with three sieves: 19, 8 and 4mm diameter.

[4] Mean particle size was determined as described by Heinrichs (2013).

[5] peNDF determined as the proportion of particles (DM basis) greater than the threshold length (specified in subscript) multiplied by NDF concentration.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 3 Rumination activity and eating patterns of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Time spend								
Ruminating, min/d	447	453	445	566	41.9	0.106	0.077	0.097
Eating, min/d	290	223	239	221	34.8	0.362	0.205	0.441
Ruminating, min/kg DMI	19.4	20.7	19.0	23.4	1.18	0.341	0.035	0.140
Eating, min/kg DMI	12.9	10.2	10.0	9.5	1.89	0.132	0.208	0.375
Number of chews								
Ruminating, ‘000/d	27.9^{a}	26.9^{b}	25.6^{c}	34.9^{a}	2.69	0.192	0.081	0.043
Eating, ‘000/d	19.0^{a}	12.3^{b}	12.6^{b}	13.1^{ab}	2.37	0.093	0.081	0.050
Ruminating, ‘000/kgDMI	1.27	1.24	1.09	1.40	0.090	0.882	0.147	0.097
Eating, ‘000/kgDMI	0.84^{a}	0.55^{b}	0.54^{b}	0.58^{b}	0.123	0.052	0.087	0.042
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 4 Rumen pH, volatile fatty acid profile and ammonia concentration of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Manual samples[2]								
Ammonia, mg/L	90	96	156	141	14.1	0.001	0.664	0.331
Rumen pH	6.25	6.24	6.22	6.38	0.141	0.186	0.103	0.058
VFA Profile, mM								
Total VFA	118^{b}	121^{b}	130^{a}	114^{b}	7.11	0.296	0.030	0.002
Acetate	72.3^{b}	74.4^{b}	81.6^{a}	74.2^{b}	3.39	0.013	0.132	0.009
Propionate	24.1^{b}	25.9^{b}	24.5^{b}	20.2^{a}	2.58	0.009	0.181	0.004
n-Butyrate	16.4	15.1	17.8	15.0	1.52	0.253	0.002	0.185
iso-Butyrate	0.70	0.77	1.03	0.87	0.064	0.006	0.526	0.097
n-Valerate	1.82^{b}	2.07^{b}	2.55^{a}	1.91^{b}	0.201	0.009	0.062	0.001
Iso-Valerate	1.35	1.41	1.65	1.19	0.143	0.737	0.136	0.054
Caproate	0.71	1.00	0.95	0.74	0.161	0.927	0.760	0.067
Acetate:Propionate	3.10	3.05	3.37	3.74	0.257	0.001	0.155	0.079
n	3	4	4	4				
24h pH measurements[3],								
Average pH	6.52	6.38	6.31	6.43	0.281	0.764	0.973	0.622
Maximum pH	6.84	6.70	6.68	6.81	0.148	0.702	0.916	0.142
Minimum pH	5.97	5.88	6.02	6.14	0.127	0.254	0.378	0.686
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; VFA = volatile fatty acids.
[2] The least squares mean of measurements taken at -0.5, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12h post morning feeding in each cow in each period.
[3] pH measurements were taken every 10 minutes over a 24h period with an indwelling pH meter with data averaged every hour for analysis.
[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

Table 5 Rumen and fecal particle size distribution of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Rumen particle profile								
Total DM, g/kg	182	149	166	159	10.3	0.789	0.116	0.250
Total NDF, g/kg DM	583	579	584	550	14.2	0.385	0.183	0.259
Material retained, %DM								
<1mm or soluble[2]	36.1	28.6	30.0	28.5	3.45	0.297	0.148	0.311
1mm – 2mm	14.5	15.0	19.3	15.7	2.02	0.400	0.593	0.500
2mm – 4mm	14.8[b]	12.3[c]	17.1[a]	12.0[c]	0.46	0.021	0.002	0.008
>4mm	34.6	44.8	33.8	43.3	2.27	0.482	0.002	0.840
Material retained, %NDF								
<1mm or soluble[2]	19.9	9.2	10.5	7.7	3.63	0.129	0.072	0.259
1mm – 2mm	19.2	17.8	24.3	21.8	1.95	0.045	0.280	0.764
2mm – 4mm	17.7[b]	15.5[c]	22.0[a]	14.9[c]	0.61	0.003	0.001	0.001
>4mm	43.4	57.4	42.7	56.2	2.80	0.662	0.001	0.901
Fecal particle profile								
Total DM, g/kg	147	145	156	157	0.68	0.060	0.834	0.752
Total NDF, g/kg DM	474	488	442	433	1.57	0.168	0.855	0.486
Material retained, %DM								
<1mm or soluble[2]	51.3	51.7	54.1	53.1	2.51	0.323	0.878	0.760
1mm – 2mm	18.5	21.4	21.6	22.7	1.45	0.075	0.118	0.425
2mm – 4mm	11.5	13.3	14.1	11.4	1.72	0.845	0.733	0.216
>4mm	14.6	13.1	12.7	12.9	0.85	0.143	0.213	0.111
Material retained, %NDF								
<1mm or soluble[2]	31.7	33.6	22.6	25.8	5.09	0.152	0.637	0.893
1mm – 2mm	30.7	33.7	39.9	40.4	2.76	0.024	0.484	0.624
2mm – 4mm	18.8	17.6	22.4	20.1	1.37	0.058	0.187	0.587
>4mm	14.8	13.4	16.0	15.2	6.96	0.344	0.416	0.817
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL.
[2] >1mm or soluble material calculated as the starting amount minus material retained on each of the three sieves.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at P<0.05.

Table 6 Intake, milk yield, milk composition and feed conversion efficiency of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
DMI, kg/d	25.1[a]	22.4[b]	23.1[ab]	24.4[ab]	1.08	0.991	0.152	0.027
Milk yield, kg/d	29.3	29.4	29.1	28.4	3.93	0.519	0.704	0.646
Milk composition, g/kg								
Fat	34.4	34.9	35.7	34.6	1.35	0.648	0.798	0.472
Protein	30.0[c]	31.6[a]	31.2[b]	29.8[c]	0.64	0.033	0.560	0.001
Lactose	45.0	44.6	45.5	44.6	0.60	0.572	0.190	0.642
Casein	22.8[b]	24.4[a]	23.9[a]	22.2[b]	0.88	0.051	0.763	0.019
Urea, mg/kg	276	257	270	281	40.6	0.591	0.799	0.393
Component yield, kg/d								
Fat	1.02	1.02	1.00	0.98	0.139	0.215	0.519	0.745
Protein	0.92	0.93	0.86	0.83	0.120	0.063	0.850	0.520
Lactose	1.32	1.37	1.29	1.22	0.184	0.172	0.859	0.401
Casein	0.70	0.71	0.66	0.64	0.095	0.096	0.773	0.570
n	3	4	4	4				

[1]IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;
[2] FCE = feed conversion efficiency.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 7 Milk fatty acid profile of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Fatty acid profile, g/100g FA								
4:0	2.49	2.49	2.75	2.76	0.134	0.001	0.894	0.871
6:0	1.71	1.69	1.73	1.69	0.054	0.670	0.894	0.765
8:0	1.11	1.07	1.02	0.99	0.054	0.168	0.426	0.987
10:0	2.73	2.63	2.44	2.27	0.194	0.034	0.300	0.750
12:0	3.36	3.28	2.19	2.70	0.656	0.159	0.700	0.603
14:0	11.3	11.2	10.4	10.7	0.30	0.084	0.631	0.307
16:0	32.0	32.9	31.7	32.4	1.17	0.461	0.161	0.807
18:0	8.88	8.19	9.05	9.11	0.628	0.266	0.501	0.427
18:1 *c*9	17.1	17.6	20.3	21.6	0.51	0.008	0.099	0.253
18:1 total cis	18.5	19.0	21.8	23.0	0.47	0.006	0.065	0.172
18:1 total trans	3.49	3.36	3.43	3.61	0.258	0.621	0.884	0.405
18:2 n-6	2.46	2.40	2.67	2.69	0.176	0.141	0.869	0.780
18:2 total excluding CLA	2.94	2.87	3.25	3.17	0.203	0.127	0.681	0.967
18:2 total CLA	0.77	0.76	0.74	0.78	0.077	0.957	0.838	0.577
18:3 n-3	0.37	0.35	0.57	0.47	0.018	0.002	0.066	0.197
20:0	0.14	0.14	0.14	0.16	0.018	0.400	0.649	0.560
20:1 total cis	0.08	0.09	0.10	0.13	0.027	0.238	0.392	0.773
20:2 n-6	0.04	0.03	0.04	0.04	0.006	0.425	0.519	0.276
20:3 n-6	0.07	0.07	0.06	0.10	0.030	0.794	0.392	0.373
20:4 n-6	0.14	0.15	0.22	0.13	0.029	0.338	0.126	0.096
20:5 n-3	0.03	0.03	0.05	0.04	0.003	0.012	0.349	0.630
22:0	0.14	0.13	0.46	0.11	0.030	0.885	0.177	0.497
22:4 n-6	0.06	0.06	0.05	0.05	0.008	0.332	0.743	1.000
22:5 n-3	0.07	0.08	0.08	0.06	0.015	0.675	0.759	0.380
Summary, g/100g FA[2]								
Total SFA	66.5	67.5	67.5	65.2	0.83	0.385	0.377	0.170
Total MUFA	28.7[ab]	28.0[b]	27.7[b]	29.8[a]	0.62	0.250	0.103	0.036
Total cis MUFA	23.8	23.9	25.3	24.7	0.98	0.196	0.780	0.653
Total trans MUFA	4.07	3.93	4.06	4.18	0.277	0.568	0.967	0.509
Total PUFA	4.53	4.53	5.06	4.96	0.303	0.093	0.826	0.833
Total n-3 PUFA	0.65	0.65	0.86	0.76	0.090	0.106	0.464	0.560
Total n-6 PUFA	2.89	2.84	3.11	3.12	0.195	0.177	0.901	0.854
Ratio n-6:n-3 PUFA	4.72	5.00	3.31	3.77	0.413	0.002	0.018	0.438
Total unsaturates	33.6	32.6	32.4	34.8	0.82	0.409	0.326	0.145
Total trans-fats excluding CLA	4.54	4.41	4.64	4.67	0.297	0.434	0.801	0.717
Recovery rates, %								
Apparent recovery 18:2 n-6	10.3	10.4	12.3	16.0	1.63	0.034	0.212	0.250

Apparent recovery 18:3 n-3	10.3	10.2	10.1	9.3	1.33	0.576	0.682	0.725
n	3	4	4	4				

[1]IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;

[2]FA = fatty acid.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Figure captions

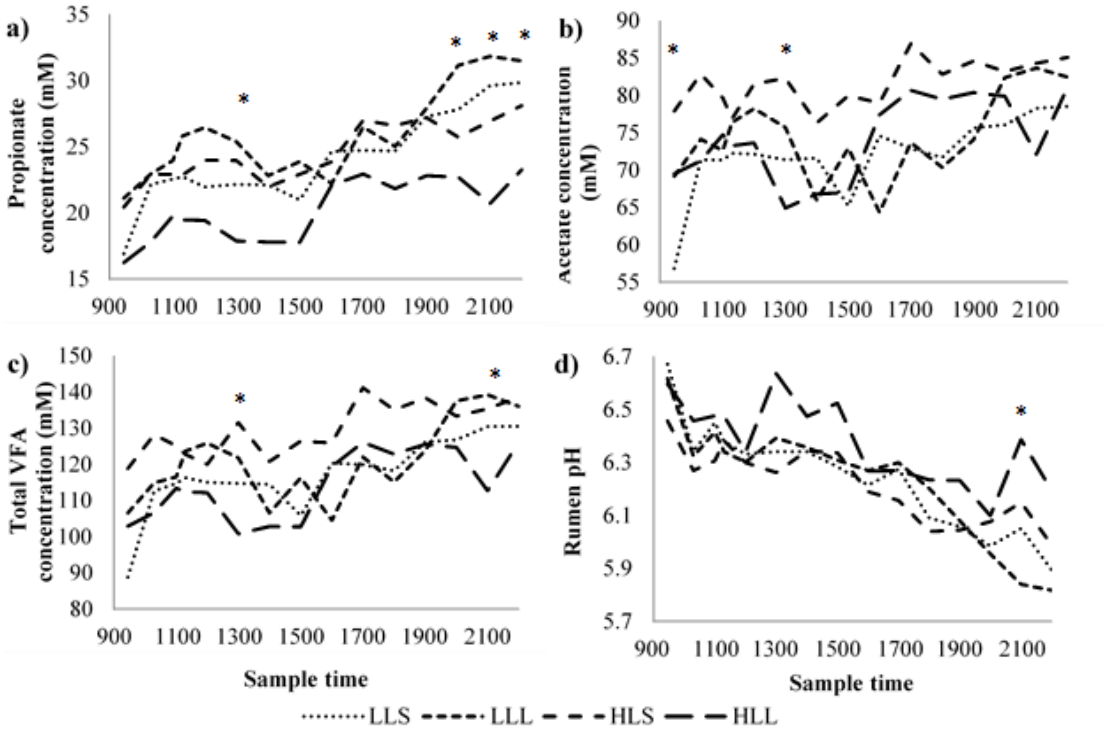

Figure 1 The rumen concentrations of (a) acetate, (b) propionate (c) total volatile fatty acids and (d) pH of lactating dairy cows just prior to, and until 12 h post morning feeding when fed total mixed rations containing a high (3:1 ratio with maize silage, DM basis; HL) or low (1:3 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length. Significant effects of time ($P < 0.05$) are marked (*).

Chapter 7

The effect of lucerne silage chop length and inclusion rate within a total mixed ration on the ability of lactating dairy cows to cope with a short-term feed withholding and refeeding challenge

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COVERING LETTER

This study investigated the effect of short term feed witholding, followed by refeeding, in dairy cattle adapted to near-continuous access to a total mixed ration (TMR) diet. Such scenarios may be relatively common in modern dairy systems, however there is limitted research quantifying the effect of such a challenge on rumen health, feeding patterns and milk production. Feeding lucerne silage which has a high natural buffering capacity, and longer forage chop lengths to stimulate rumination and saliva production, have been suggested as mitigation strategies to prevent sub acute rumen acidosis (SARA) as a result of a feed witholding and refeeding challenge. Two different chop lengths and inclusion rates of lucerne silage within a total mixed ration were tested with a high inclusion rate (375 g/kg diet dry matter) and a short chop length (14mm theoretical length) providing the greatest level of acidosis mitigation. Poor performance in animals fed a long lucerne silage chop length of 19 mm (especially combined with a low lucerne inclusion rate of 125 g/kg diet dry matter) raised questions over the effect of long forage particles in the diet during a feed witholding and refeeding rumen challenge. Short term milk loss was observed in cows fed low lucerne diets on the day of the challenge, which would represent a significant reduction in farm profit if the scenario were to occur regularly. This research highlights the importance of maintaining feeding routines and ensuring adequate feed access throughout the day in TMR-based dairy systems.

ABSTRACT

The objective of this study was to quantify the acidosis mitigation potential of lucerne silage in the diet of lactating dairy cows at two inclusion rates (high and low; HL or LL) and two chop lengths (long and short; L or S) during a rumen challenge instigated by feed witholding followed by refeeding. Dietary treatments LLS, LLL, HLS or HLL were

offered to four mid-lactation Holstein dairy cattle in a 4 x 4 Latin square design study with 21 day periods. Feed was withheld for 6 h followed by *ad libitum* refeeding on d 18 of each period. Measurements of dry matter intake, milk yield and composition, rumen pH, and eating and rumination behaviour were taken on a baseline day, the challenge day and two further recovery days. After refeeding, rumen pH was significantly reduced in cows fed LL diets but not for HL diets. Milk yield on the challenge day was reduced in animals fed LL diets by 14.3% in comparison to baseline. Cows fed L chop lengths had a greater pH reduction than those fed S, with LLL experiencing the greatest pH reduction (-0.2 and -0.4 pH units lower than baseline on challenge and recovery day 1 respectively). This study highlights the detrimental effect short-term feed deprivation can have on rumen health and milk production in dairy cattle normally fed *ad libitum*, and demonstrates replacing maize silage with lucerne silage can mitigate acidosis risk due to interrupted feed supply.

INTRODUCTION

Lactating dairy cow diets are increasingly formulated to include high concentrations of rapidly fermented non-forage carbohydrate (**NFC**) as sources of energy to support milk production. However, such diets also decrease rumen pH through greater production of volatile fatty acids (**VFA**s) (Allen, 1997). In circumstances where pH remains below 5.8 for 3 consecutive hours, a dairy cow is purported to suffer from Sub-Acute Rumen Acidosis (**SARA**), a condition that can reduce milk yield and milk fat concentration (Plaizier *et al.*, 2008). Dietary strategies to increase the resilience of dairy cattle to SARA include feeding forages with high buffering capacities (e.g. Lucerne, *medicago sativa*) or increasing the physical effectiveness (**peNDF**) of the diet through lengthening forage chop length (McBurney *et al.*, 1983; Zebeli *et al.*, 2006). Physically effective fibre being

defined as the fibre contained within particles which are longer than the critical particle size for rumen escape (which recent research suggests is 4 mm although was previously defined as 1.18 mm (Oshita *et al.*, 2004; Maulfair and Heinrichs, 2012)) and therefore can contribute to the rumen mat (Mertens, 2000). A lower rumen pH has also been linked with changes in cow feeding behaviour and the adoption of natural coping mechanisms, including showing preferences for long particles in the diet (Maulfair *et al.*, 2013; DeVries *et al.*, 2008) or for supplementary hay (Kmicikewycz and Heinrichs, 2015).

Experimentally, the stability of rumen pH can be tested by induction of a rumen fermentation challenge. This is typically achieved through the addition of a large quantity of a rapidly degradable carbohydrate to the diet such as cereal grains or lucerne pellets (Colman *et al.*, 2013). However, it is unclear whether such a method accurately replicates conditions that cause SARA, or to what extent dietary treatment effects may be obscured by the SARA-inducing component. An alternative approach to instigate a rumen challenge is deprivation of feed for a period of several hours (Oetzel, 2007). A period of fasting is then followed by a period of overeating when access to feed is returned, termed 're-feeding'. Periods of feed deprivation lasting up to 6 h may be relatively common in a commercial setting, for instance, where there is insufficient feed or pasture allocation, feeding equipment failure, or feed removal for routine processes such as milking or health checks. However, relatively little is known about the severity of the effect of such events on rumen function and milk production. Studies in the literature have examined the effect of longer periods of fasting such as 12 to 48 h (Oetzel, 2007; Chelikani *et al.*, 2004; Toerien and Cant, 2007) that generally result in high levels of temporary milk yield loss, however, we are not aware of any studies that have examined the effects of shorter fasts in dairy cattle that would be more representative of commercial practice. Therefore, the aims of the present study were i) to test whether a 6 h fast followed by refeeding induces

an acidosis challenge ii) to examine the transient patterns of rumen pH and eating behaviour just prior to, during, and after a fast and refeeding challenge and iii) to examine the effect of varying inclusion rate and chop length of lucerne silage, replacing maize silage in a total mixed ration (**TMR**), on resilience to a refeeding challenge.

MATERIALS AND METHODS

Forage Harvesting and Clamp Sampling

The present study formed part of a larger research trial that utilised the same dietary treatments and observed their effects on milk yield, dry matter intake, diet digestibility, and rumen function under non-challenging conditions, in a larger cohort of cows and over a longer time period, as reported previously (Thomson *et al.*, 2017a; Thomson *et al.*, 2017b). In brief, lucerne silage was harvested as a second cut crop at an estimated 10% bloom in July 2014 and conserved in concrete-walled clamp. The crop was wilted prior to ensiling for 48 h, producing a high dry matter (**DM**; 576 g/kg) silage. Two CLs (long; **L** and short; **S**) were created from material collected in alternate swaths by altering the knife arrangement of the precision chop forage harvester (Claas Jaguar, Claas Group, Harsewinkel, Germany) from a theoretical chop length of 14 mm (shortest setting) to 19 mm (longest setting). An additive was applied (Axcool Gold containing *L. Buchneri*; 2 L/Tonne; Biotal, Cardiff, UK) to prevent heating in the clamp. Samples for chemical composition analysis (Sciantec Analytical Services, Cawood, UK) were obtained using a clamp corer. Maize (*Zea Mays*) silage for the study was taken from a crop of mixed varieties harvested in autumn 2014 and ensiled as described for the lucerne clamps (geometric mean particle length of 10 mm).

Diets

Diets comprised a TMR with 50:50 ratio of forage:concentrate on a DM basis (Thomson *et al.*, 2017a), in which the forage portion consisted of maize and lucerne silage at IRs (DM basis) of either 25:75 (high lucerne; **HL**) or 75:25 (low lucerne; **LL**), respectively. These treatments were combined with the two lucerne silage CLs in a 2 x 2 factorial arrangement to give four treatments (**HLL, HLS, LLL, LLS**). which were formulated to be isonitrogenous (170g CP/kg DM) and contain similar levels of NDF (320 g/kg DM). The reduction in maize starch associated with lower maize silage inclusion in HL diets was partially offset by increasing the concentration of maize meal (Table 1), however for the experimental diets fed, starch concentration was lower in the HL diets (Table 2).

Animals

Four multiparous Holstein dairy cows, previously prepared with rumen fistulae (Bar Diamond rumen cannula; Parma, Idaho, USA), in mid lactation (161 d in milk, SE ± 23.1) weighing 739 kg (SE ± 13.9), 7-9 years of age, were randomly assigned to one of four initial treatments according to a 4x4 Latin square design balanced for carryover effects with three week periods. All procedures were licensed and monitored by the UK Government's Home Office under the Animal (Scientific Procedures) Act 1986. During adaptation weeks (weeks 1 and 2 of each period) animals were housed in a cubicle yard and individually fed once daily for *ad libitum* intake (10% refusals) through Insentec RIC feeders (Insentec B.V., Marknesse, The Netherlands). Continuous access to water was provided. From d 12 of each period animals were housed and milked in individual tie stalls to facilitate sampling. Animals were allowed to acclimatise to the stalls for 3 days prior to sampling beginning on d 15. While in tie stalls, animals were offered their daily feed allocation in two halves at 1000 h and 1600 h. Refusals were taken daily at 0930 h.

Between d 15 – 18 measurements of rumen function under non0challenging conditions were performed including rumen VFA and ammonia concentrations, rumen pH, rumen mat particle distribution and faecal particle distribution which have been reported previously (Thomson *et al.*, 2017b). The feeding routine differed on d 18 of each period when a refeeding challenge was simulated (described below). While in ties stalls, each cow was also fitted with a rumination headcollar (ITIN+HOCH GmbH, Fütterungstechnik, Liestal, Switzerland) to measure eating and rumination behaviour as described previously (Ruuska *et al.*, 2016).

Experimental Routine

The refeeding challenge. Baseline measurements of all variables were taken on d 16 of each period (other than rumen pH, which was measured on d 15). On d 18 of each period, refusals from the previous day were removed from the cows one hour early (0830 h) to begin a period of fasting. Feed was withheld for 6 h until 1430 h when half the daily diet allocation was offered followed by the second half two hours later at 1630 h. On d 19 refusal and feeding routine was returned to that of d 17.

Intake and diet analysis. The weight and dry matter concentration of feed offered and refused were measured during d 14 – 21 of each period, although, only data from days 16 (baseline), 18 (challenge), 19 (recovery day 1) and 20 (recovery day 2) were included in the statistical analysis. Dry matter concentration of feed was determined by oven drying at 100 ºC for 24 h. Samples of the TMR constituents were stored frozen at -20 ºC until analysed for DM, nitrogen (**N**; using the macro kjeldahl method; 954.01 (AOAC, 2000)), ash (by combustion at 500 ºC for 16 h), aNDF and ADF (Mertens *et al.*, 2002; Robertson and Van Soest, 1981), starch (Fuller, 1967; Macrae and Armstrong, 1968), and water

soluble carbohydrates (**WSC**) as described previously (Reynolds *et al.*, 2014; Kliem *et al.*, 2016). Concentrations (g/kg DM) of CP, NDF, ADF, ash, starch and WSC in each TMR were calculated based on constituent inclusion rates. A sample of each TMR was analysed for particle size distribution using a Penn State Particle Separator (sieve apertures measuring 4 mm, 8 mm and 19 mm in diameter and a bottom pan). A dry matter correction for material retained on each sieve was obtained. Average particle size of the sample was calculated as described previously (Heinrichs, 2013) and peNDF was calculated as the proportion of particles (DM corrected) greater than the threshold length (4, 8, or 19 mm; shown in subscript) multiplied by the NDF concentration of the diet. The chemical and physical composition of the diets is shown in Table 2.

Milk Yield and Composition. Cows were milked twice daily at 0630h and 1630h and milk samples, preserved using potassium dichromate, were analysed for fat, protein, casein, lactose, urea, and somatic cell count (SCC) by mid infra-red spectroscopy on a CombiFoss machine (National Milk Laboratories, Chippenham, Wiltshire, UK). Only data from days 16 (baseline), 18 (challenge), 19 (recovery day 1) and 20 (recovery day 2) were statistically analysed.

Rumen pH. An indwelling pH meter (Sentix 41-3 probe, WTW Trifthof, Weilheim, Upper Bavaria) attached to a weight (200 g) and connected to the rumen cannula using nylon cord (50 cm) was placed within the rumen of each animal for 24 h beginning just prior to feeding (0930 h) on day 15 of each period until refusals were removed at 0930 h on day 16 to establish baseline patterns of rumen pH, and inserted again at 0830 h on day 18 (challenge day), remaining within the rumen until 0930 h on day 21. The probe was calibrated before every insertion and checked for drift after use. No drift was observed

during the present study. The pH probe was attached to a datalogger (ph340i, WTW, Trifthof, Weilheim, Upper Bavaria) with readings recorded every 10 minutes. Readings were further averaged over each hour for analysis, beginning on the hour for Baseline, and Recovery days 1 and 2, and at the half hour mark for challenge day to coincide with feeding times. Any measurements within the first hour of insertion (0830 to 0930 h) were not included in statistical analysis due to differences in the start time of each cow.

Statistical analysis

Average daily data starting at morning feeding, 1000 h, were calculated for 4 phases (days) of the challenge: Baseline (d 16), Challenge (d 18), Recovery 1 (d 19), and Recovery 2 (d 20). Averages for each cow, treatment, and day (D) combination were analysed to determine fixed effects of period, lucerne IR, lucerne CL, D and their interactions (IR x CL, IR x D, CL x D and IR x CL x D), and random effects of cow using mixed models procedures of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). The 'SLICE' option was used to show IRxCL interactions for each day. Least squares means (LSM) for each treatment, and effects of IR, CL and IR x CL interactions within each day, are presented separately. Challenge or recovery days where the LSM within a treatment significantly differed from the baseline value for that treatment are highlighted. For measurements of eating time and relative rumen pH within each D, the same model was used except day was replaced with hour (H) and each day was analysed separately.

For rumen pH a baseline value for each hour of a 24 h period (starting at morning feeding, d15, 1000 h) was taken for each cow on each treatment that was then subtracted from the hourly mean at the same time point for each subsequent phase to analyse and present each hourly value relative to baseline. The data were transformed in this way to ensure the magnitude of any effects could be compared between animals with differing

baseline rumen pH levels. A mean of relative pH for each D was also analysed (with the challenge day subdivided into 'fast' and 'refeeding') to determine fixed effects of period, lucerne IR, lucerne CL, and IR x CL interaction, and random effects of cow using mixed models procedures with each day and sub-phase tested separately.

RESULTS

Baseline treatment effects

The effect of treatment on diet chemical composition in the present study (Table 2) have been reported previously (Thomson *et al.*, 2017b) however to summarise briefly, the concentration of starch was greater in LL diets than in HL diets ($P < 0.04$), whereas ADF concentration was greater in HL diets ($P < 0.01$). Increasing CL from S to L increased the proportion of particles retained on both the 8 and 19 mm sieves of the PSPS while reducing the proportion that was retained on the 4mm sieve and in the bottom pan (all $P < 0.02$). Both greater IR and greater CL of lucerne increased or tended to increase peNDF concentrations using 4, 8 and 19mm threshold lengths ($P < 0.06$) relative to a low IR and a short CL.

Dry matter and water intake, milk yield, milk composition and the yield of milk solids showed no effect of treatment during the baseline phase (Table 3). There was no effect of diet on mean daily rumen pH (6.36 on average; Table 4), nor were there any time points during the baseline day in which there was an effect of treatment on rumen pH (Figure 1a). Following feeding, rumen pH showed a downwards trend reaching a nadir between 9 and 13 h post morning feeding followed by a return to pre-feeding levels between 15 and 22 h post feeding. Baseline eating patterns, shown in figure 1b, show an increase in time spent eating (20-40 min/h) in the first hour after fresh feed was offered (at both 1000 and 1600 h), followed by a reduction in time spent eating in the second

hour post feeding to roughly 10 min/h, a rate that was sustained throughout the daytime hours. Between 13 and 19 h post feeding <5 min/h eating occurred that corresponded to the rise in rumen pH shown in figure 1a. Both daily time spent eating (Table 5) and transient eating patterns were similar for all dietary treatments at baseline. Cows fed HLL diets had more rumination chews and spent more time ruminating than cows fed either LLL or HLS, while cows fed LLS had an intermediate number of ruminating chews (IRxCL; $P < 0.04$), and cows fed HLL diets also showed a tendency to spend the greatest time ruminating per day compared to other dietary treatments (IRxCL; $P < 0.07$).

Challenge effect on rumen pH and eating patterns

Cows spent a greater proportion of time eating in the 3 h following refeeding than during the same period after the initial feed was offered at baseline (57 % vs 29 %, respectively; Figure 2b). At 4 h post refeeding eating intensity reduced for cows fed all diets, although at 6 h post refeeding cows fed the LLS diet again spent a high proportion of time eating in comparison to cows fed other diets ($P < 0.01$). Following this, cows on all diets continued to eat at a steady rate of between 0-20 min/h. Throughout the observation period, eating patterns fluctuated between diets creating significant differences at 13, 22, 28, 35, 43, 47, 49, 51, 54, 62 and 63 h post refeeding, however, the differences were small and not sustained. Cows fed LLS diets spent more time eating at morning feed times than cows fed other diets, although the time-point was only significant on recovery day 2, which correlates with cows fed this diet having numerically the highest DMI and eating chews per day.

Relative rumen pH increased steadily during the fast period for all diets (figure 2a). There was no significant effect of treatment on the mean relative pH (Table 4) nor at any individual time-points over the fast phase. At the peak of the fast phase, mean rumen

pH across the treatments ranged from 6.8 to 7.2. A steep fall in rumen pH on all treatments occurred with the refeeding event. In the first hour post re-feeding, relative rumen pH in cows fed the LLL diet decreased immediately to the baseline level in comparison to the other three diets ($P < 0.03$) where relative pH remained elevated above baseline levels until 2 h post refeeding that coincided with the second offering of feed. At 8-12 h post refeeding, rumen pH of cows fed LL diets fell significantly lower than HL relative to their baseline values (all $P < 0.04$). Cows fed HLS diets maintained a rumen pH that was close to baseline pH throughout the refeeding period: 0.04 pH units higher than baseline over the entire refeeding phase. Cows fed LL diets had a rumen pH 0.16 pH units lower on average over the refeeding phase then HL diets relative to their own baseline values ($P < 0.008$).

On recovery day 1 cows on all diets recovered close to baseline levels prior to morning feeding. However, post feeding, the rumen pH of cows fed LL diets again decreased relative to their baseline values leading to significant treatment differences at 20, 25 and 27 h post refeeding. At 31 h post refeeding cows fed LLS diets returned closer to their respective baseline values compared to cows fed LLL diets that continued to have a low relative rumen pH that did not begin to return to baseline values until 36 h post refeeding (LLL significantly lower than the other three diets at 31, 34, 35 and 36 h post refeeding, all $P < 0.04$). Similar to the challenge day, cows fed HLS diets showed a rumen pH pattern close to baseline while cows fed HLL diets were marginally lower than baseline values. Mean relative rumen pH for the recovery day 1 phase demonstrated that cows fed LL and L diets had reduced relative pH in comparison to HL and S diets (effect of IR $P < 0.001$; effect of CL $P < 0.03$). On recovery day 2 there were no significant differences in relative rumen pH between treatments or any hours in which treatment differences occurred although the relative rumen pH of cows fed LLS diets were the

lowest of the four treatments and on average 0.17 pH units below baseline values for that diet.

Taking the definition of SARA to be a period of 3 consecutive hours where rumen pH is less than 5.8, then 6 bouts of SARA were observed within the dataset of which 2 bouts were in the same cow when fed the LLS diet and the remaining 4 were in 3 cows when fed the LLL diet (with 1 cow experiencing 2 separate bouts on this diet). Of these 6 bouts of SARA, 2 occurred on the day of the challenge and 4 occurred on recovery day 1. No episodes of SARA were observed in cows fed HL diets.

Challenge effect on intake and milk production

On the day of the challenge, DMI was similar to that eaten on baseline day (Table 4) as was daily mean time spent eating and ruminating (Table 5) despite the pattern of eating during the day being altered as described earlier. Intake was numerically reduced for cows fed LLS, LLL and HLL diets relative to baseline on subsequent days, however, these effects did not reach significance, while intakes of cows fed HLS remained close to baseline. On recovery day 2 animals fed L diets had reduced DMI relative to those fed S diets (-2.7 kg/d; $P < 0.05$). Cows fed L diets also tended to consume less water on recovery day 1 than S diets (Table 4; $P < 0.07$).

Milk yield was significantly reduced in cows fed LLS and LLL diets on challenge day relative to milk yield at baseline ($P < 0.05$), by 4.5kg and 4.3kg respectively, although yield was not significantly lower than that of cows fed the HL diets on the challenge day. The reduction in milk yield on LL diets on this day, also led to significant reductions in milk protein yield compared to baseline for these treatments. On recovery day 2 milk yield for all treatments was not different ($P > 0.05$) from baseline levels. Concentrations of milk protein were unaffected by treatment and day. The milk fat yield for LLS and

HLL diets on recovery day 2 was significantly higher than baseline, and furthermore the protein yield for HLL cows on that day was greater than that of any other dietary treatment (IRxCL; $P < 0.04$).

DISCUSSION

The effect of a refeeding challenge on eating patterns and rumen pH

During the fasting phase, prior to re-feeding, rumen pH rose for all animals, likely as a result of rumen VFAs being absorbed and not replaced, and perhaps as an effect of salivation while the animals were waiting for feed to be offered. Following refeeding, animals exhibited a 3 h period in which a high proportion of time was spent eating across all treatments in comparison to the baseline day. An increase in eating intensity following feed deprivation is consistent with the findings of other studies (Patterson *et al.*, 1998; Oetzel, 2007) and has been linked with low rumen fill prior to refeeding (Gregorini *et al.*, 2007). This over-eating episode resulted in a rapid decrease in rumen pH such that 3 h after refeeding animals had reached the same rumen pH as was observed 7 h after feeding on the baseline day. This accelerated decline in rumen pH may be due to the pH of the ingested feed and from VFAs produced from fermentation. Total VFA concentration in the rumen is dependent on the rate at which VFA is produced in comparison to the rate at which VFA can be absorbed through the ruminal epithelium. There are various absorption mechanisms that facilitate VFA removal from the rumen however the most predominant are bicarbonate-dependant transport (Aschenbach *et al.*, 2011) and passive diffusion (Penner, 2014). For the latter, a low VFA concentration in the rumen, such as that created by short-term feed deprivation, would reduce VFA removal rate initially until a sufficient diffusion gradient was established. Contrarily, recent research suggests that such conditions are likely to simultaneously favour increased production rate of VFA by

microbes that benefit from a diffusion gradient that swiftly removes VFA from their boundary layer (Russell *et al.*, 2009; Mason and Stuckey, 2016). Furthermore, high feed intake in a short time-period would have increased the supply of rapidly degraded starch and sugars to the microbial population, especially those from cereal grains within the concentrates fed. Therefore the swift decline in rumen pH observed is likely to be a combined effect of increased microbial productivity combined with reduced ability to remove VFA from the rumen through absorption. Other longer term studies have also noted a reduction in epithelial absorption rate during and after feed restriction which has been attributed to reduced blood flow due to feed deprivation e.g. 5 day feed restriction followed by refeeding (Zhang *et al.*, 2013); however this is unlikely to be case in the present study where feed was only withheld for 6 h. There are few previous studies in which withholding and refeeding TMR have been examined. Studies have examined effects in grazing animals (Chilibroste *et al.*, 2007), but there is still a lack of data on rumen kinetics to explain the mechanisms underpinning responses to such a challenge and further work is required to fully understand responses in TMR-fed animals.

Despite the reduced window of time when animals were allowed access to feed on the challenge day (18.5 h), there was no difference in the quantity of feed consumed in comparison to baseline days, again highlighting the increased eating rate over the shorter time period. Milk yield was reduced on the day of the challenge for all diets, but significantly for LL diets, and this might indicate that there was a carryover effect of the fasting period for these diets, or that the increased rate of feed consumption after refeeding reduced the efficiency of energy capture from the diet, which would also support the theory of impaired epithelial VFA absorption in the rumen. Concentrations of fat and protein within the milk were largely unaffected, other than an unexpected rise

in milk fat concentration seen on recovery day 2 in both LLS and HLL diets, which may in part be due a numerical decrease in milk yield.

The acidosis mitigation potential of the dietary treatments

In this study, cows fed diets comprising a high IR of lucerne silage were less affected by the rumen challenge than those with a low IR, despite there being no difference in rumen pH profile between the diets at baseline. Lucerne silage provided more effective fibre (Table 2) to the diet than the maize silage and has also been reported previously to have a higher cation exchange capacity than maize (McBurney *et al.*, 1983) and therefore a combination of these two factors could explain the increased ability of the cows to buffer against low rumen pH. Furthermore, lucerne often contains a higher proportion of indigestible, lignified, stem in comparison to other forages that may reduce rumen passage rate and therefore maintain a slow rate of VFA production in the rumen during a period of feed deprivation. This may have helped prevent disruption to epithelial function. The LL diets also contained a higher concentration of starch that would have contributed to reduced rumen pH. No incidence of SARA was observed in cows fed HL diets confirming that feeding lucerne at the higher IR of 375 g/kg diet DM, and consequently feeding less maize silage and starch, was successful at mitigating acidosis risk in comparison to the lower inclusion rate. Milk loss in cows fed LL diets on the day of the challenge (4.4 kg/d) was a decrease of 14.3% compared to baseline yield, represents cost to the farmer if animals were regularly fasted for similar periods (6 h total) as part of a commercial system, particularly as there is evidence to suggest the severity of acidosis can increase where challenges are repeated in quick succession (Dohme *et al.*, 2008).

Evidence from jaw movement monitors gathered in this study confirmed that the long chop length increased rumination activity as would be expected, however, animals fed diets containing L chop lucerne silage had lower ruminal pH on average on recovery day 1 than animals fed S, with those fed LLL diets having the greatest and most prolonged reduction in ruminal pH in comparison to the other diets. This contrasts with previously published work suggesting a positive correlation between rumen pH and peNDF concentration (Zebeli *et al.*, 2006) attributed to increased rumination supplying more saliva to the rumen, although these relationships were generated from studies where no feed withholding/refeeding challenge was applied. Lengthening chop length can negatively affect diet uniformity and allow increased sorting against longer particles, which would contain the most peNDF (Leonardi and Armentano, 2003), however, this is unlikely to explain the lower rumen pH of cows on L diets on recovery day 1, as animals have previously been shown to increase selection of longer particles in response to a rumen challenge (DeVries *et al.*, 2008). The beneficial effect of peNDF is thought to be the result of increased stimulation of rumination producing saliva to buffer the rumen, and, in line with this, HLL diets did increase rumination however the same effect was not seen in the other diets, including LLL, where the concentration of peNDF was lower. Longer particles would have required rumination to aid digestion after ingestion, however rumination is often delayed after a refeeding event while eating is prioritised (Chilibroste *et al.*, 2007), meanwhile smaller forage particles and concentrates can be broken down without the need for further rumination chewing. This delay in rumination due to overeating may have reduced the ability of animals fed LLL diets to digest the forage portion of the diet. It is also possible that fibre digestion was impaired as a result of the low pH conditions affecting microbial populations (Grant and Mertens, 1992). Reductions in DMI in animals fed the long CL diets on both recovery day 1 and 2 relative

to those fed short CL diets (a difference that was not observed at baseline) also supports this explanation as reducing fibre digestibility of dietary lucerne has previously been linked to reduced appetite (Getachew *et al.*, 2011; Fustini *et al.*, 2017) likely due to increased feeling of satiety. Based on the negative effect of increasing peNDF provision through increased chop length, it is likely the mitigation effect of high lucerne IR was attributable to the buffering capacity of lucerne, and reduced diet starch concentration, rather than any effect of peNDF.

In the LLL diet, effects of the challenge continued throughout recovery day 1 despite a return to baseline feeding patterns, with DMI also reduced for this diet on recovery day 2. The timeline is similar to that observed by Oetzel (2007) where a cow faced with a 12 h fast followed by a refeeding challenge took 60 h for rumen pH to return to pre-fast levels. The extended number of days over which significant effects were seen despite no further challenges being applied highlights the need for rumen pH to be observed over several days when investigating induced SARA experimentally.

CONCLUSIONS

We conclude that a relatively short fast (6 h) followed by a refeeding event, in which a day's allocation of feed equal to the pre-fast level was offered *ad libitum*, was sufficient to induce SARA in some cows fed diets with low lucerne IR. However, the risk was mitigated by a high lucerne IR within the diet, which was particularly effective when chopped to a short CL. We attribute this mitigation effect to the buffering capacity provided by the lucerne, as well as reduced diet starch concentration, rather than increased effective fibre provision, as a long CL led to greater reductions in rumen pH after refeeding than a short CL. This may be due to a delayed rumination response affecting the animal's ability to digest longer forage particles in hours directly after

refeeding. The effect was particularly pronounced in the LLL diet in which reductions in rumen pH relative to baseline were observed in both the day of challenge and the following day. Milk lost from cows on the low IR diets would represent a significant financial loss if such a refeeding challenge were to occur regularly, highlighting the need to ensure uniformity of feeding routines in *ad libitum* TMR feeding systems for dairy cows on a day to day basis.

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Table 1 Ingredients used in diet formulation

	Diet	
Item	LL	HL
Ingredients, g/kg DM		
Lucerne silage	125	375
Maize silage	375	125
Concentrate blend		
Cracked Wheat	80	80
Maize Meal	54	97
Unmolassed Sugar Beet Feed	40	40
Soy Hulls	82	108
Soybean Meal	100	65
Rapeseed Meal	100	65
Molasses	10	10
Dicalcium phosphate	5	5
Salt	5	5
Dairy Mineral	10	10
Megalac[1]	15	15

LL, low lucerne diet; HL, high lucerne diet;

[1] Megalac rumen protected fat supplement (Volac International ltd., Royston, UK)

Table 2 The chemical and physical composition of four total mixed rations containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length.

	Diet					*P* value		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Chemical composition								
DM, g/kg	555	571	610	632	5.0	0.022	0.065	0.364
OM, g/kg DM	62	63	78	77	0.6	0.001	0.471	0.070
CP, g/kg DM	164	163	168	167	3.5	0.200	0.710	0.945
NDF, g/kg DM	311	322	335	340	4.8	0.115	0.221	0.510
ADF, g/kg DM	202	208	237	245	1.5	0.004	0.007	0.322
Starch, g/kg DM	234	235	164	168	7.0	0.039	0.680	0.780
WSC, g/kg DM	37	35	35	32	0.7	0.006	0.020	0.371
Particle size distribution[1]								
Material retained, %DM								
19mm	3.2[a]	5.0[a]	5.3[a]	12.1[b]	0.75	0.001	0.001	0.007
8mm	36.4[a]	41.9[b]	37.4[ac]	39.1[c]	0.50	0.129	0.012	0.026
4mm	16.5[a]	13.5[b]	18.7[c]	12.6[b]	0.24	0.033	0.001	0.004
Bottom pan	43.8	39.8	37.9	36.3	0.50	0.001	0.010	0.094
Mean particle size[2], cm	0.50	0.56	0.54	0.65	0.014	0.001	0.001	0.099
peNDF[3], %DM								
$peNDF_{>19mm}$	1.03[a]	1.64[a]	1.74[a]	4.04[b]	0.268	0.001	0.001	0.009
$peNDF_{>8mm}$	12.3	14.8	13.8	18.2	0.27	0.056	0.030	0.137
$peNDF_{>4mm}$	17.2	19.9	20.5	21.3	0.38	0.003	0.004	0.051

IR, Inclusion rate; CL, chop length; IRxCL, interaction between IR and CL; SEM, standard error of the mean; WSC, water soluble carbohydrate.

[1] Particle size distribution measured using a Penn State Particle Separator with three sieves: 19, 8 and 4mm diameter.
[2] Mean particle size was determined using the recommended equation of Penn State University (Heinrichs, 2013).
[3] peNDF determined as the proportion of particles in the TMR greater than the threshold length (specified in subscript) multiplied by the NDF concentration of the TMR.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 3 Mean relative rumen pH of lactating dairy cows fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length prior to, during, and following a rumen challenge that involved a 6 h fast followed by a refeeding challenge.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IrxCL
Baseline daily rumen pH	6.30	6.38	6.31	6.43	0.130	0.785	0.396	0.828
Relative rumen pH[1]								
Challenge day								
Fast[2]	0.43	0.42	0.46	0.38	0.081	0.905	0.517	0.592
Refeeding[3]	-0.15	-0.21	0.04	-0.08	0.063	0.007	0.115	0.643
Recovery day 1	-0.20	-0.41	-0.01	-0.11	0.071	0.001	0.023	0.443
Recovery day 2	-0.01	-0.17	0.02	0.01	0.098	0.241	0.365	0.428

IR, Inclusion rate; CL, chop length; IRxCL, interaction between IR and CL; DMI, dry matter intake; SEM, standard error of the mean.

[1] Relative rumen pH calculated as hourly rumen pH measurements minus the baseline measurement (Thomson *et al.*, 2017b) at the same hour of the day for each cow on each treatment. Measurements used for each day begin at 1000h (normal morning feeding time).
[2] The fast period combines measurements from 0930h until 1430h on the day of the challenge during which time animals were not allowed to access feed (note, the start of the feed withdrawal was 0830h however the time taken to insert rumen pH probes meant that data for this hour were incomplete so was not included in the analysis).
[3] The refeeding period combines measurements from 1430h on the day of the challenge until 0930h the following morning. After which the subsequent two 24 h periods are termed recovery day 1 and recovery day 2 which both begin at 1000h.

Table 4 Daily mean intake, milk production and milk composition of lactating dairy cows fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length prior to, during, and following a 6 h fast followed by a refeeding challenge.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Dry Matter Intake, kg/d								
Baseline day	25.5	21.3	22.5	24.4	1.44	0.998	0.359	0.133
Challenge day	25.2	23.7	24.5	23.6		0.764	0.352	0.810
Recovery day 1	25.3	21.5	23.1	22.2		0.548	0.085	0.251
Recovery day 2	23.1	19.6	23.7	21.8		0.289	0.049	0.130
Milk Yield, kg/d								
Baseline day	31.7	30.1	27.8	29.2	5.89	0.654	0.992	0.938
Challenge day	27.2*	25.8*	25.9	27.3		0.985	0.994	0.994
Recovery day 1	31.9	29.5	29.0	30.8		0.879	0.954	0.978
Recovery day 2	30.9	28.2	29.0	27.0		0.775	0.664	0.962
Milk fat, g/kg								
Baseline day	32.6	35.2	35.3	33.2	3.04	0.766	0.832	0.305
Challenge day	38.7	38.4	34.0	34.8		0.218	0.948	0.638
Recovery day 1	34.7	35.3	35.8	35.8		0.588	0.817	0.939
Recovery day 2	37.0*[a]	36.5[a]	35.3[a]	40.3*[b]		0.292	0.046	0.033
Milk fat yield, kg/d								
Baseline day	1.07	1.04	0.94	1.03	0.208	0.701	0.897	0.963
Challenge day	1.06	1.00	0.84	0.95		0.485	0.886	0.864
Recovery day 1	1.11	1.05	1.04	1.09		0.946	0.996	0.991
Recovery day 2	1.12	1.01	1.01	1.07		0.892	0.898	0.968
Milk protein, g/kg								
Baseline day	31.3	31.5	30.9	30.9	1.16	0.602	0.923	0.952
Challenge day	31.4	31.0	30.2	29.5		0.209	0.583	0.585
Recovery day 1	30.3	30.8	30.2	29.1		0.364	0.764	0.637
Recovery day 2	30.6	30.4	30.5	29.1		0.546	0.490	0.755
Milk protein yield, kg/d								
Baseline day	0.99	0.95	0.84	0.87	0.173	0.440	0.996	0.869
Challenge day	0.85*	0.80*	0.77	0.80		0.808	0.954	0.990
Recovery day 1	0.96	0.91	0.87	0.89		0.740	0.920	0.982
Recovery day 2	0.94	0.87	0.88	0.75		0.571	0.549	0.857

IR, Inclusion rate; CL, chop length; IRxCL, interaction between IR and CL; SEM, standard error of the mean.

[1] Baseline data were collected on d16 and the challenge day was d18 (starting at 1000 h) of each period, during which animals spent 4.5 h of the day fasting (post a 1.5 h period during which refusals were removed early to make a total fast of 6 h) and a 17.5h period in which feed was offered ad libitum. Recovery days 1 and 2 were the subsequent 24 h periods (d 19 and d 20 respectively both beginning 1000 h).

* Where a value differs significantly ($P<0.05$) from a baseline value (Table 3) for that treatment.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 5 Eating and rumination behaviour of lactating dairy cows fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length prior to, during, and following a 6 h fast followed by a refeeding challenge.

	Diet					*P* value		
Item[1]	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Eating chews '000/d								
Baseline day	17.8	12.1	12.8	14.0	2.56	0.509	0.351	0.392
Challenge day	16.2	12.0	15.4	14.5		0.707	0.283	0.626
Recovery day 1	15.4	9.8	13.5	11.4		0.950	0.120	0.410
Recovery day 2	14.4	11.1	11.9	15.0		0.791	0.961	0.605
Eating time, min/d								
Baseline day	268	225	339	239	35.0	0.795	0.490	0.817
Challenge day	250	217	267	227		0.681	0.271	0.665
Recovery day 1	241	177	249	189*		0.768	0.073	0.305
Recovery day 2	229	184	217	222		0.703	0.551	0.808
Ruminating chews '000/d								
Baseline day	27.6[ab]	27.5[a]	24.2[a]	35.4[b]	3.01	0.414	0.052	0.038
Challenge day	28.7	26.2	26.1	34.2		0.336	0.319	0.124
Recovery day 1	31.0	29.0	27.8	32.0		0.958	0.691	0.673
Recovery day 2	30.1	26.1	28.1	32.7		0.459	0.934	0.479
Ruminating time, min/d								
Baseline day	442	460	421	574	47.1	0.281	0.056	0.065
Challenge day	464	432	439	548		0.303	0.389	0.198
Recovery day 1	499	484	469	520		0.944	0.686	0.835
Recovery day 2	494	438	478	533		0.414	0.991	0.591

IR, Inclusion rate; CL, chop length; IRxCL, interaction between IR and CL; **S**EM, standard error of the mean

[1] Baseline data were collected on d16 and the challenge day was d18 (starting at 1000 h) of each period, during which animals spent 4.5 h of the day fasting (post a 1.5 h period during which refusals were removed early to make a total fast of 6 h) and a 17.5h period in which feed was offered ad libitum. Recovery days 1 and 2 were the subsequent 24 h periods (d 19 and d 20 respectively both beginning 1000 h).

* Where a value differs significantly (P<0.05) from a baseline value (Table 3) for that treatment.

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at P<0.05

Figure captions

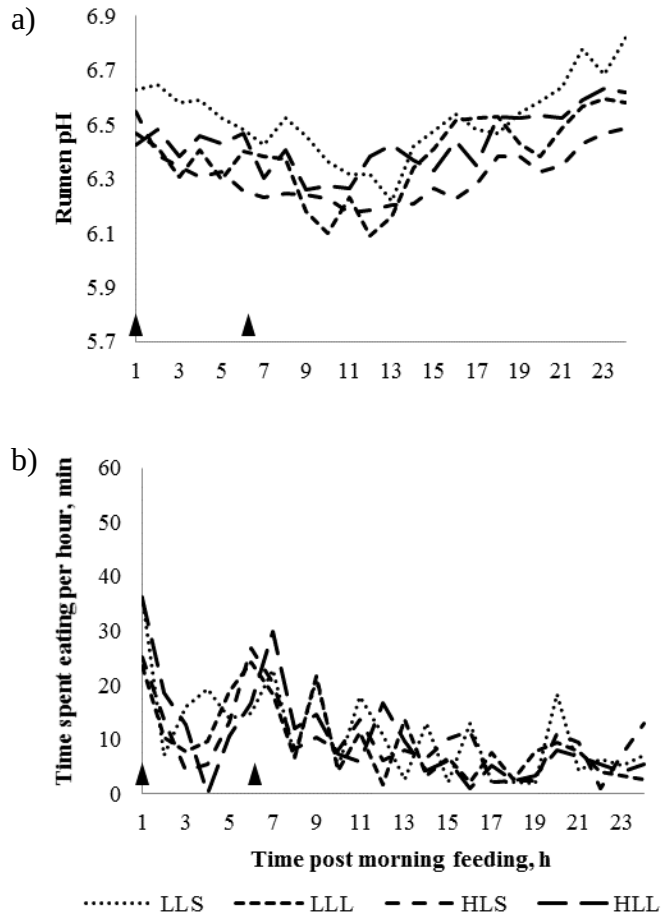

Figure 1 Patterns of (a) rumen pH measured using an indwelling pH probe and (b) time spent eating measured using a rumination headcollar of lactating dairy cows, fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length, over a 24 h baseline period beginning at 1000h (hour 1). Baseline values were measured two (for eating pattern) or three (for rumen pH) days prior to a feed deprivation/refeeding challenge being administered. Black triangles indicate time points at which half a daily allowance of feed was offered. Hours at which there was a significant effect of treatment are marked (*).

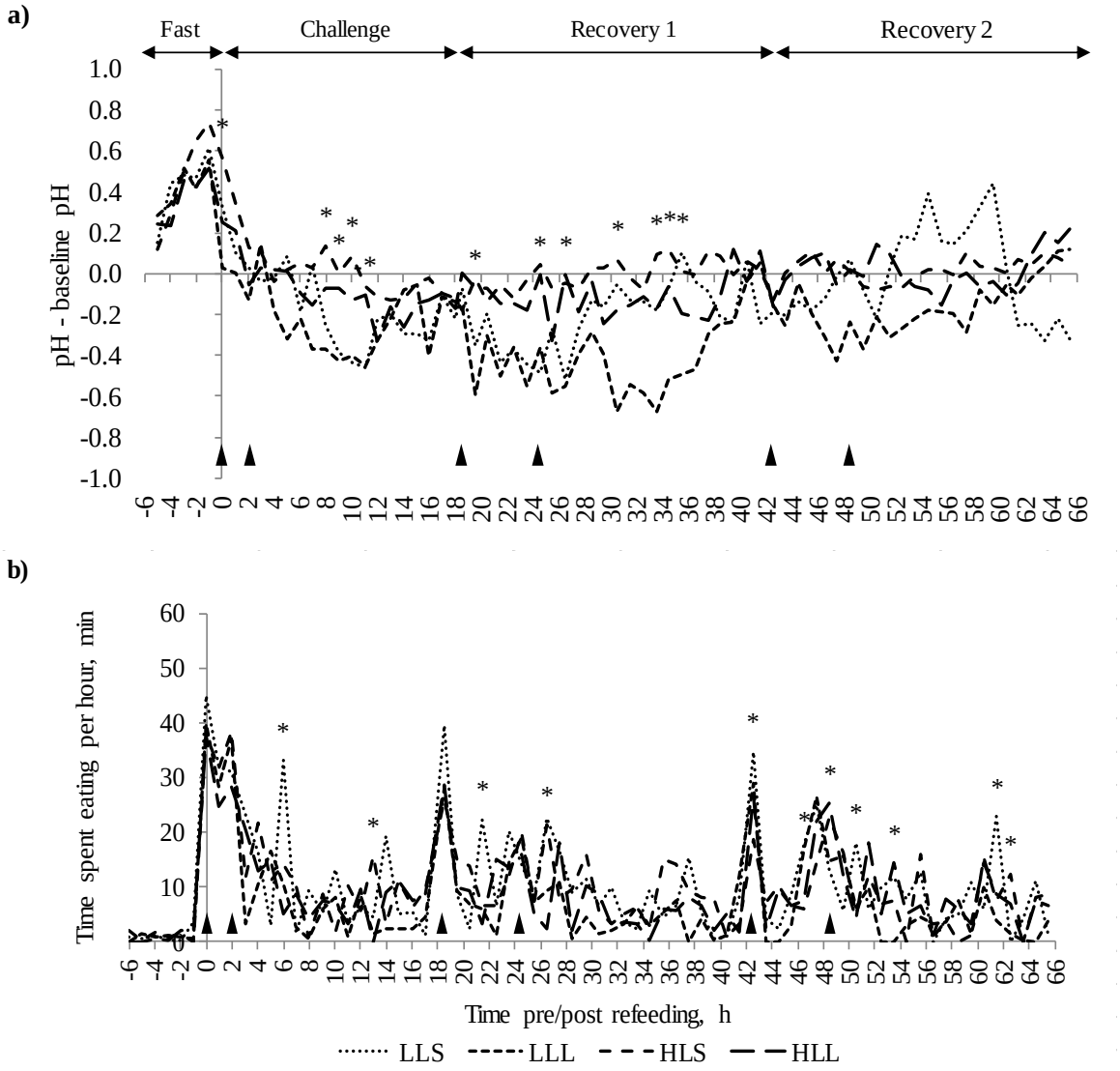

Figure 2 Patterns of (a) relative rumen pH and (b) time spent eating of lactating dairy cows, fed a total mixed ration containing a high (HL) or low (LL) concentration of lucerne silage at a long (L) or short (S) chop length, over a 72 h period beginning at 0830 h on day 18 of the period, when feed was withheld for 6 h followed by a refeeding challenge at 1430 h. The hour beginning 1430 is represented by 0 on the *x* axis. Black triangles indicate time points at which half a daily allowance of feed was offered. Hours at which there was a significant effect of treatment are marked (*).

Chapter 8

The effect of establishment method and maturity at harvest on yield and chemical composition of ensiled lucerne

Including the prediction accuracy of Near Infra-Red Reflectance Spectroscopy analysis for lucerne silages

Intended for submission to Grass and Forage Science

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ABSTRACT

The aim of this study was, firstly, to test whether timing of establishing and harvesting lucerne (*medicago sativa*) affected dry matter yield and silage chemical composition over two years, and secondly, to use the resulting silages to investigate whether existing grass and grass-clover silage-based near infra-red reflectance spectroscopy (NIRS) equations were suitable for application to lucerne samples in the UK. Twelve lucerne trial plots were established in either spring (with or without barley cover crop) or autumn (without barley) in year 1 and then, in the following year, each plot was subdivided to test the effect of harvesting at either bud or flower stage at a 1st and a 2nd cut, using a 2 x 2 factorial arrangement of treatments for the 2nd cut. Silages were created in mini silos and subsequently analysed for chemical composition. Results indicated that sowing in spring improved yield and silage chemical composition through reduced weed concentration and likely increased length of time for root development prior to winter relative to autumn sowing. At first cut (Year 2), harvesting at an early growth stage increased crude protein concentration but yield was halved in comparison to harvesting at flower and fermentation profile was also poorer with lower concentrations of lactic acid and higher concentrations of acetic acid. It was therefore concluded that current guidelines to cut at 10% flower should not be changed in favour of earlier harvest maturity as marginal improvements to crude protein concentration at first cut were outweighed by lower yield and poorer silage fermentation. Furthermore, an NIRS equation developed for grass was shown to accurately predict dry matter and nitrogen concentrations in the resulting silages increasing the viability of including lucerne in precise rations in the UK.

INTRODUCTION

Lucerne (*medicago sativa*) is the most popular legume forage crop grown globally with over 300 M tonnes produced each year (FAO, 2013). The crop is predominantly utilised in semi-arid regions where a long tap root allows the plant to thrive in challenging conditions, however high yields and good nutritional value have led to the crop being utilised extensively in temperate regions. In the UK, the crop has been shown to produce in excess of 18 T of dry matter (**DM**) per hectare annually for up to 5 years, a much greater yield potential than that of monoculture ryegrass or ryegrass-clover leys (Frame and Harkess, 1987), however such targets are rarely reached on farm due to poor establishment in the 1st year. Lucerne is also able to fix high concentrations of N in the soil, not only negating the need for inorganic N fertilisation during the lucerne growth period but also providing as much as 800 kg/ha N in the soil for subsequent crops to use (Gault *et al.*, 1995). Following previous interest in the crop for the UK market in the 1980s, the economic viability of legumes was reduced due to improved ratio of milk price to fertiliser price which resulted in lucerne remaining a relatively under-used crop (Rochon *et al.*, 2004; Phelan *et al.*, 2015). Current estimates suggest that around 6000 ha of UK land is used for lucerne production (Germinal, 2016). Recently interest in forage legumes has been growing due to higher fertiliser prices and increasing emphasis on the sustainability of farming systems, resulting in a need for a modern assessment of best practice for growing lucerne under UK conditions. Challenges presented by lucerne for growers include (i) poor persistence against weeds at establishment (Wigley *et al.*, 2012) (ii) selecting the correct maturity at harvest (Tyrolova and Vyborna, 2008) and (iii) difficulty in ensiling the harvested material due to high buffering capacity (McBurney *et al.*, 1983). A further issue is the lack of an appropriate and quick method of analysing chemical composition of the resulting silage for ration formulation. The most common

method for routine grass silage analysis in the UK is to use Near Infra-Red Reflectance Spectroscopy (**NIRS**) on un-dried and un-milled samples. This varies from European samples where drying and milling prior to analysis is more common to improve sample stability and homogeneity (Sorensen, 2004). In the UK, equations are available for grass silages and more recently, grass-clover silage equations have also been developed (Thomson *et al.*, 2016), although these are still undergoing further improvement prior to commercial implementation. However, no equations exist for lucerne which is a significant barrier to its effective use in precision feeding systems, incorporating lucerne into mixed diets.

Therefore, the aims of the current study were twofold: (i) to investigate the effect of establishment methods and maturity at harvest on yield and chemical composition of resulting lucerne silage and (ii) to test the accuracy of existing UK grass-based NIRS equations when applied to lucerne silage samples.

MATERIAL AND METHODS

Experimental design

Twelve lucerne trial plots were established in 2014 at the University of Reading Crops Research Unit, Sonning, (average annual rainfall 2013/2014: 701 mm) and were monitorred over a total of five cuts over the course of the establishment year and subsequent year. Furthermore, the first two cuts of the second year (2015) were used for silage-making in mini-silos. Differing establishment methods (Autumn, **A**; Spring, **S**; or Spring+Barley, **S+B**) were tested on the trial plots in 2014, and in the following year tests of maturity at harvest (Bud, **B**; or Flower, **F**; combined in all possible combinations to make the following regimes over two cuts: B,B; B,F; F,B; F,F, as per Figure 1) were carried out. Each plot was subdivided into four sub-plots (termed A-D) with one sub-plot

being used to test each of the four maturity combinations. As harvest timing was dependant upon weather conditions, the timing of the B and F treatments were targetted to be between 'early' and 'late' bud stage, and 'early' and 'late' flower stage respectively according to previously defined growth stages (Kalu and Fick, 1981). Material at F being broadly representative of the recommended cutting timing for lucerne and B stage being more vegetative and less mature at harvest than currently recommended. The regrowth interval between 1st and 2nd cut of each treatment varied in order to allow harvests to be taken at the correct maturity stages: regrowth intervals were 56, 64, 34 and 42 d for the BB, BF, FB and FF treatments respectively.

Establishment and harvesting

Twelve plots (dimensions 2m x 10m) were arranged in two rows of six separated by 2 m wide grass buffer strips. The land was prepared using light cultivation followed by drilling and rolling. Soil samples were taken and assessed prior to establishment to ensure nutrient and pH requirements of the crop were met. The lucerne variety used for all treatments was inoculated 'Daisy' which was drilled at a seed rate of 20 kg/ha. The Spring Barley variety used was 'Westminster' which was intercropped at 150 seeds/m^2 in alternative rows. Initially, establishment of A and S sown plots was planned for August 2013 and March 2014, however A sown plots failed to establish successfully and were reseeded in August 2014. Therefore, the A treatment was removed from analyses in Year 1 (2014) whereas S sown plots grew sufficiently for two cuts to be taken in August and October 2014. In year 2 (post-establishment year; 2015) plots from all three establishment treatments were cut three times with the 1st cuts being used to test for the effects of differing maturity regimes as described previously (cuts were taken in June, July/August, and September for 1st, 2nd and 3rd cuts respectively). At 1st cut the difference between bud

and flower cuts was 21 days, whereas at 2nd cut maturity advanced more quickly leading to just a seven-day difference between bud and flower cutting dates. Other than for the two cuts in which differing maturities were tested, the target cutting maturity was 10% flower. In the 1st year, yield was determined by measuring the mean weight of material cut from three 50 x 50 cm quadrats taken at random from within each plot, immediately prior to plots being cut using a tractor-mounted topper. Material within each quadrat was cut to a height of 10 cm to prevent crown damage. Dry matter concentration was determined by oven drying at 60ºC for 72 h. A separate subsample was manually separated into lucerne, barley and other species (i.e. weeds) to determine botanical composition. The lucerne fraction was further separated to determine the proportion of stems that were in the vegetative, bud, flower, or seed stages of maturity before being recombined. Aspeciated fractions were then dried at 60ºC for 72h to determine DM concentrations. In the second year, material was harvested using a power scythe (Model 615L, BCS, Walsall, UK), to allow for precise separation of material from each subplot. The total weight of all cut material in each sub-plot was weighed immediately and subsamples were obtained for analysis of DM content and botanical composition and growth stage analysis as described previously. Subsequently, all remaining material from each subplot was piled into individual windrows and left to wilt for 24 h before undergoing ensiling. At third cut, each plot was cut as one whole rather than as sub-plots to allow a total yield for the year for each establishment method to be calculated. All material was weighed, subsampled for DM analysis, and discarded.

Ensiling in Mini Silos. At 1st and 2nd harvest in year 2, following wilting, material from each sub-plot x establishment combination (four sub-plots and three establishment methods therefore 12 per cut) were combined, mixed, and chopped using an electric

shredder (Model AXT25, Bosch, Uxbridge, UK). Two replicate mini-silos were created out of the resulting bulked, chopped, material. Therefore, a total of 48 mini silos were created and stored for 12 months. No additive was applied prior to ensiling so that natural lucerne fermentation could be examined. Mini silos were comprised of a plastic drainpipe tube (10 cm diameter and 50 cm length) with an airtight seal (Bailey brothers, Birmingham, UK) at each end which was densely packed using a 2.5 kg weight. Upon opening, one mini silo contained excessive spoilage and was discounted from subsequent analyses.

Silage analyses. Laboratory analysis for neutral and acid detergent fibre (**NDF** and **ADF**, respectively) were performed on a dried, ground (1 mm screen) subsample using heat-treated amylase and expressed inclusive of residual ash (aNDF), using fibrecap (Foss, Hillerod, Denmark) equipment (Robertson and Van Soest, 1981; Kitcherside *et al.*, 2000; Mertens *et al.*, 2002). Volatile corrected oven dry matter (**VCODM**) was measured on silages using toluene as described by Steen (1989) and dried at 60ºC for a minimum of 48 h. Total nitrogen (**N**) was determined using the macro kjeldahl method, as was ammonia N (**NH_3N**) following extraction of ammonium ions by soaking a subsample in sulphuric acid (Method 954.01, (AOAC, 2000)). Water soluble carbohydrate (**WSC**) concentration was measured as described previously (Fuller, 1967) on a dried sample. Ash concentration was measured by placing a dried sample in a muffle oven at 500ºC for 18 h. Fermentation end products, including lactic (**LA**), acetic (**AA**), propionic (**PA**), butyric and valeric acids plus ethanol and propanol, were determined using gas chromatography (**GC**) as described previously (Erwin *et al.*, 1961; Givens *et al.*, 2009). Scanning of samples using NIRS was also carried out by AFBI (Hillsborough, NI) using a Foss 6500 NIRS machine (1100-2498nm wavelengths; Foss, Hillerod, Denmark), and

predictions of chemical composition were generated from the resulting spectra using two existing equations: the UK's grass equation (used in practice by the Forage Analysis Assurance [FAA] group laboratories since 2013) and a new grass-clover equation (developed in 2016 but not yet used in practice). Reference and predicted values for crude protein (**CP**) were obtained by multiplying total N (on a DM basis using VCODM) by 6.25. Predicted values for NDF, ADF, Ash, NH_3N and WSC were also converted to a DM basis using predicted VCODM for comparison with the reference data on a DM basis.

Statistical analysis

All data were analysed using Mixed Models procedure of SAS (version 9.4) with data from each cut in each year analysed separately. In Year 1 data from each plot were tested for the fixed effect of establishment method and the random effect of plot. This model was also used to analyse total combined yield per plot across all three cuts in year 2 (with means of each of the four subplots used as the total plot yield for 1st and 2nd cut). In year 2, for cut 1, data from each subplot were analysed for fixed effects of 1st cut maturity (B or F), establishment method (A, S or S+B) and their interaction, and random effects of plot (unless for silage chemical composition where the random effect of plot was removed by bulking multiple plots within treatments). In year 2, for cut 2, subplot data were analysed for fixed effects of 2nd cut maturity (B or F), previous maturity at 1st cut (B or F), and establishment (A, S or S+B) and their interactions, and random effects of plot (unless for a silage variable, as previously stated). This model was also used to analyse the combined DM yield from cuts 1 and 2 for each subplot. In each Mixed models analysis, contrasts were used to separate the effects of establishment timing and use of a

cover crop by comparing A vs S and S vs S+B. Data are presented as least square means with the main effects of establishment and maturity reported separately.

For NIRS data, the difference between laboratory assays and NIRS predicted values were calculated using measured minus predicted values and is henceforth termed 'bias'. Techniques employed to test the predictive accuracy of equations through a blind validation test were relative root mean square standard error of prediction (**RMSEP** as a percentage of the measured mean) (Shenk and Westerhaus, 1993), ratio of the standard error of prediction to the standard deviation of the measured dataset (**RPD**) as recommended by Williams (2014), and the R-squared value of the linear relationship between observed and predicted data ($\mathbf{r^2}$). Prediction accuracy was compared twice, first using all data-points and then using a subset of data-points with those samples where lucerne concentration within the sample was < 500 g/kg DM (termed 'weedy') removed.

RESULTS

Establishing lucerne

Plots established using the S and S+B methods gave a similar yield within the first year at 2.8 T/ha DM and 1.4 T/ha DM in the 1st and 2nd cut respectively (Table 1). Using the S+B undersowing method tended to reduce weed burden within the sward relative to the S method alone with a 248 g/kg DM reduction in weed concentration ($P < 0.06$). There was no effect of establishment method in the 2nd cut on either yield or botanical composition however the weed burden was greatly diminished in all treatments in this cut relative to the 1st cut with lucerne comprising 957 g/kg DM within the plot on average: an increase of 592 g/kg DM over the 1st cut material.

In the following growth year, by which time A sown plots were successfully established in addition to S sown plots, the DM yield of A plots was lower in comparison

to both S sown treatments over both 1st and 2nd cuts combined (Table 2; $P < 0.001$) by 7.4 T/ha DM. This coincided with a large weed burden within A sown plots of 890 g/kg DM in the 1st cut which improved in the 2nd cut to 353 g/kg DM. However, the DM yield for the 1st cut of the A sown plots in June (Table 2) was more comparable to the 1st cut of the S and S-B sown plots in August of the previous year (Table 1). Taking into account the 3rd cut in year 2, the total DM yield for the year was 4.6, 11.2 and 10.3 T/ha for A, S and S+B respectively (A vs S, $P < 0.001$; S vs S+B, non-significant).

Concentrations of NDF and ADF were reduced in A sown plot silages relative to S sown plot silages by 35 and 30 g/kg DM at 1st cut (both $P < 0.05$) and 30 and 29 g/kg DM at 2nd cut (both $P < 0.02$) respectively. At 1st cut the A silages were lower in VCODM, CP and NH_3N (all $P < 0.001$) and higher in ash and WSC (both $P < 0.01$) than S silages. At 2nd cut, A silages continued to contain less CP and NH_3N (both $P < 0.01$), and more ash and WSC (both $P < 0.02$) than S silages, however in this cut VCODM of A silages tended to be higher than S silages by 10 g/kg ($P < 0.06$). Fermentation profiles were also affected by establishment method with silages from A plots having or tending to have a lower AA concentration but a higher concentration of LA (both $P < 0.08$ and < 0.01 at 1st and 2nd cut respectively), than S silages resulting in A silages having a lower pH ($P < 0.001$ and < 0.09 at 1st and 2nd cut respectively). Other than a small reduction in ash concentration at 2nd cut ($P < 0.008$), the chemical composition of S silages did not differ from that of S+B silages in either cut.

Cutting maturity of lucerne

The differentiation between maturity stages of the stands at 1st and 2nd cut in year 2 is shown in Figure 2. At 1st cut a clear differentiation was seen with the stand at 20% bud for the B treatment and 40% flower for the F treatment. However, at 2nd cut the variation

in stem maturities within the same stand was much greater leading to less differentiation between treatments. In both 2nd cut F treatments a proportion of stems had proceeded to set seed whereas no stems set seed in the 2nd cut B treatments (24 and 0.5% of stems proceeding to seed stage for those cut previously at bud and flower respectively). There was a strong effect of 1st cut treatment on 2nd cut maturity with plots which were previously cut at B being more mature (although regrowth interval was longer) with a larger proportion of stems at flower and a smaller proportion of stems remaining vegetative than those previously cut at flower.

In the 1st cut, stands cut at F yielded 3.4 T/ha more DM than those cut at B (Table 3; $P < 0.001$). Silage CP concentration was increased by 21 g/kg DM when taking an earlier harvest at B in comparison with F ($P < 0.01$), and the concentration of ADF was also reduced by 34 g/kg DM at B ($P < 0.03$) although a similar difference (33 g/kg DM) in NDF concentration was not different ($P > 0.13$). Volatile corrected oven dry matter was greatest in F silage ($P < 0.001$) while B silage had higher concentrations of NH_3N, Ash and WSC (all $P < 0.01$). The fermentation profile of F silages was favourable in comparison to that of B silages, with a tendency for lower pH ($P < 0.09$) and greater LA concentration ($P < 0.05$) than B silages in which AA concentration was higher ($P < 0.03$).

At 2nd cut the effect of the previous maturity treatment at 1st cut influenced both yield and chemical composition of resulting silages (Table 4). Cutting at F in the 2nd cut gave a small yield increase of 0.7 T/ha DM in comparison to B, independent of 1st cut treatment; however, the main effect of 1st cut treatment was greater as subplots previously cut at B yielded 1.4 T/ha more DM than those previously cut at F. Overall however, Cutting at F rather than B in the 1st cut resulted in the greater total yield over the two cuts due to the beneficial effect on yield at 1st cut whereas maturity at 2nd cut did not significantly affect the combined yield. Lucerne concentration within the stand was also

improved at 2nd cut by previously cutting at F rather than at B ($P < 0.04$) regardless of maturity at the time of the 2nd cutting. The chemical composition of 2nd cut silages was improved by cutting at F rather than B at 1st cut by increasing CP concentration ($P < 0.001$) and reducing NDF and ADF concentration (both $P < 0.01$).

Harvest maturity at 2nd cut did have some effect on resulting silage chemical composition independently of previous cutting choices, including higher VCODM ($P < 0.001$), and WSC concentration ($P < 0.001$) and lower ash content ($P < 0.001$) for silages cut at F rather than B. Additionally the fermentation profile was strongly effected by maturity at 2nd cut with F treatments tending to contain increased LA ($P < 0.07$) but decreased AA ($P < 0.001$) and propanol ($P < 0.01$). There were significant interactions between 1st cut and 2nd cut maturity for AA and PA where in each case the FB treatment produced a higher concentration than the other treatments. The pH of the silages at 2nd cut was unaffected by treatment with an overall high average of 5.31.

NIRS for lucerne silages

Taking all silages produced in the study and combining them into one set for the purposes of NIRS validation allowed for a robust test of equations, with a broad range of chemical composition achieved. The silages were all relatively low in DM concentration (190-397 g/kg) with a mean VCODM concentration of 274 g/kg. The concentration of CP showed a wide range from 67-215 g/kg DM however all samples in which CP was less than 100 g/kg DM were from plots established by A sowing which led to poorer establishment and increased presence of weeds. The mean CP concentration was 157 g/kg DM. The variable showing the greatest variation was LA with a coefficient of variation (**CV**) of 84%. The 5.2 average pH of the silages indicates good fermentation in the majority of mini-silos although a maximum value of 6.4 highlights that fermentation was limited in some

instances. On average, silages contained 728 g/kg DM lucerne with the remaining proportion comprising weeds. The average concentration of barley remaining within under sown crops in year 2 31 g/kg DM at 1st cut and thereafter 0 g/kg DM. Ten samples in which the proportion of lucerne present was less than 50% of total DM (termed 'weedy') all originated from A sown plots.

The results of utilising lucerne spectra as an independent validation set to the existing grass and grass-clover equations are shown in Table 2, with plots of predicted against measured data depicted in Figure 3. The RPD value is used as the main determinant of suitability for commercial use with values greater than 2.0 denoting good accuracy (Williams, 2014). It was hypothesised that an equation developed for use on grass-clover samples would be more suitable for analysis of lucerne samples than an equation developed for use on monoculture grass silages due to the similarity of chemical components within legume species. However, the findings presented in Table 2 suggest that this was not the case as the grass equation showed increased prediction accuracy for most chemical composition variables other than NDF, ADF, and NH_3-N relative to the grass-clover equation. The grass equation showed very high accuracy when predicting N and VCODM, sufficient to be utilised commercially for lucerne samples, however other variables were below threshold. Acid detergent fibre was greatly under-predicted for both equations. Fermentation end products were also poorly predicted, as was WSC. When using the grass-clover equation CP was over-predicted by 19 g/kg DM on average although there was little bias in prediction of N and therefore the inaccuracy originated mainly from an under-prediction of VCODM concentration used to convert N to a DM basis. Low N silages (also all A sown samples) stood out as being poorly predicted in Figure 3 in comparison to higher N concentrations (within the 6-10 g/kg range). Removing ten of the silages that were predominantly weeds improved prediction

accuracy of N, Ash and WSC in both equations. Crude protein, NDF, ADF, and LA prediction by the grass-clover equation were also improved by the removal of weedy samples. However, for other variables, removal of these ten samples decreased prediction accuracy, partly due to the smaller sample size. This was re-iterated by contrasting the effects of A vs. S treatments on prediction bias, as reported in Figure 6. There were many significant negative effects of A sowing on prediction accuracy but in some instances bias was reduced by A sowing relative to S sowing, for example, NH_3N was better predicted in A sown samples than in S sown samples using the grass equation for both cuts and the grass-clover equation for 2nd cut. Under-sowing rarely affected bias.

The main effects of maturity treatments in each of the two silage cuts on NIRS prediction bias is further explored in Table 7. Effects of harvest maturity on bias were inconsistent in both equations with NH_3N and NDF showing the most significant effects between equations and cuts alongside WSC suggesting the prediction accuracy of these equations may be influenced by physical properties of the plant.

DISCUSSION

Timing of lucerne establishment

In this study, establishing lucerne using an S sown method (March establishment) either with or without a cover crop was more reliable (with one failed A establishment) and resulted in a considerable improvement in yield and persistence in the following year in comparison to the A sown method (August establishment). The carryover effect of establishment on productivity in the following year (Justes *et al.*, 2002; Sim *et al.*, 2015) and on regrowth vigour (Thiebeau *et al.*, 2011) has been documented previously. In the study by Justes *et al.* (2002) July and August establishment dates were compared, and, in a similar result to our findings, the earlier date increased yield in the following year

(Justes *et al.*, 2002) with the difference attributed to increased time allowed for root growth and root N storage prior to winter which was correlated with improved growth the following spring, (providing soil moisture reserves were not limiting in the summer of the establishment year). Sim *et al.* (2015) investigated the importance of root development in more detail, finding that lucerne crops in which the taproot and crown combined biomass failed to reach 3 T/ha DM at the end of the first season partitioned 20-25% greater resources to root development in the following season than plants reaching this biomass threshold in the first year, to the detriment of stem and leaf production. This partly explains the reduced performance of A sown plots in Year 2 of this study, however the yield difference of 57% between A and S crops likely indicates other factors also contributed to the poor performance of A sown plots in Year 2. One likely factor being the high weed burden experienced by these crops. The overall yield achieved by the S sown crops of 4.1 T/ha DM in Year 1 and 10.8 T/ha DM in year 2 were similar to previously published figures e.g. 8-12 T/ha DM (Chmelikova *et al.*, 2015) although lower than those reported in the study of Frame and Harkess (1987) who demonstrated that an annual yield 18 T/ha DM is achievable in the UK. The establishment effects seen in the present study for lucerne were similar to UK studies on optimal Sainfoin establishment (Liu *et al.*, 2008), where S sowing also improved yield and weed resistance, perhaps suggesting that this establishment strategy may also apply more broadly to a range of legume forage crops when grown in UK conditions. Studies in which S sown legumes failed to establish generally relate to conditions where summer drought is a particular problem (Norton and Koetz, 2014).

Use of barley as a cover crop

In this study there was little difference between the S and S+B treatments in each measurement year on either yield or chemical composition other than a tendency for S+B to reduce weed burden at the 1st cut in the establishment year. This is in line with previous studies which suggest that, in conditions where soil moisture is plentiful, the addition of a cover crop does no harm and can improve lucerne persistence through improving resilience of the stand to weed invasion (Tan and Serin, 2004; Norton and Koetz, 2014; Sowinski, 2014). In the present study, barley comprised 12% of harvested DM at 1st cut (0.37 T/ha DM) in year one and concentration was negligible thereafter. This was a lower rate than is seen in other studies such as Sowinski et al. (2014) where barley comprised 5 T/ha DM at 1st cut and reduced yield of lucerne relative to a pure lucerne control as a result. Care must therefore be taken when deciding on the seed rate of cover crops, although, this will depend on whether the cover crop is intended to be harvested alone for grain or combined into a whole crop silage.

Optimising harvest maturity

Lucerne has a relatively indigestible stem that is rich in lignin (Jung and Lamb, 2003). This can provide a source of effective fibre that can benefit rumen digestive health, but also decreases digestibility of the forage (therefore also lowering metabolisable energy concentration; (Sinclair *et al.*, 2015). As a result, feeding high lucerne diets can have an undesirable effect on diet digestibility and decrease production due to increased gut fill and reduced feed intake, particularly where longer lucerne chop lengths are offered (Kononoff and Heinrichs, 2003). Harvesting lucerne at an earlier stage, prior to inflorescence, is one way in which the feeding value (in terms of digestibility and ME concentration) of the crop can be improved (Iwaasa *et al.*, 1996; Lamb *et al.*, 2014). In

the present study, at 1st cut, material harvested and ensiled at bud had increased CP and reduced ADF concentrations relative to cutting at flower (the recommended timing) in agreement with the findings of a number of other studies (Yari *et al.*, 2012; Iwaasa *et al.*, 1996), although DM yield was halved as a result. Some yield was recovered in the following cut where plots previously cut at bud yielded more than those cut at flower: however, the total yield over the two cuts was still greater for plots that were cut initially at flower. The effects of cutting at bud in the 2nd cut were inconsistent with that of the 1st cut, with no effect on CP concentration and, surprisingly, reduced ADF concentration in F cut material compared to B cut material which could be due to varying proportions of stems that remained in a vegetative state. The regrowth length required by plots that were initially cut at bud was longer (+21 d) than those previously cut at flower suggesting slower regrowth in these stems although overall, by harvest, those plots initially cut at bud were then slightly more mature than those originally cut at flower. The added maturity may explain why 1st cut maturity affected chemical composition at 2nd cut particularly in the case of CP where plots previously cut at bud contained much lower concentrations than those previously cut at flower. Crude protein concentration is optimised with greater leaf:stem ratio (Hakl *et al.*, 2016) and is normally greatest during bud and flower stage, but can be lower in immature (vegetative) and over-mature (seed) material (Yari *et al.*, 2012). Furthermore plots cut initially at bud contained a higher proportion of weeds than those cut previously at flower which has also been shown to reduce CP concentration in early cut material (Moyer *et al.*, 1999).

Varying maturity also affected fermentation profile in the ensiled herbage . At both cuts material harvested at the flower stage had greater production of LA during fermentation whereas material cut at bud was higher in AA concentration. Fermentations favouring AA rather than LA are often low DM and low WSC silages (Muck and Hintz,

2003) and are generally considered undesirable due to slower pH reduction in the silo which can lead to increased spoilage (Duniere *et al.*, 2013; Davies and Orosz, 2014). While AA can be useful to promote thermal stability in silages at feedout, due to yeasts being unable to breakdown AA to fuel growth (Kleinschmit and Kung, 2006), levels as high as 50 g/kg DM have been shown to reduce DM intake in cattle (Daniel *et al.*, 2013). This, combined with other yield reducing effects observed limits the viability of cutting at bud as a strategy to improve lucerne utilisation.

Applying existing NIRS equations to lucerne

While it is best practice to create bespoke calibrations for NIRS based on the species for which analysis is required, the time and resources needed to collect a robust set of calibration samples limits the creation of new equations for chemical composition on alternative forages. Other studies have shown that it is possible to obtain accurate predictions from broad multi-forage equations although typically calibration sets comprised some spectra from the material in question (Shenk and Westerhaus, 1993). In this study, encouraging results were seen for predicting VCODM and N concentrations where lucerne was analysed using the equation calibrated on grass samples. This challenges the necessity of including spectra from the material desired for analysis within the calibration set, so long as the calibration set is comprised of samples which share a similar chemical composition. The grass-based equations used in the present study struggled to predict ADF and WSC concentrations; however, this can be explained by the vast differences in the concentrations of these nutrients in lucerne compared with those typical of grass (Dewhurst *et al.*, 2003). For these variables, using the grass-clover equation showed a small improvement in prediction accuracy, which is likely due to similarities in the chemical composition and morphology of clover and lucerne.

Although, surprisingly, for CP and numerous other variables, the grass-clover equation reduced prediction accuracy in comparison with the grass equation.

Prediction bias was significantly affected by the establishment timing and harvesting maturity treatments although effects were inconsistent between chemical components tested and between cuts and therefore it is not possible to conclude which establishment method or harvest maturity is most suitable for accurate analysis. The variable bias due to forage source observed (Tables 6 and 7) could be linked to varying weed concentrations within the plots which caused extreme concentrations of some chemical components. Extreme data of this kind pose a challenge in predictive analytics as calibration models are generally only effective within the concentration range on which they were calibrated. Any material within a validation set that falls outside of the calibration range requires the model to extrapolate in order to formulate a prediction and this increases the likelihood of bias occurring (Estienne *et al.*, 2001) as was likely the case for some variables in the A plots such as CP. This also explains variation in the effect of removing predominantly-weed samples from the validation dataset on different chemical variables, as prediction accuracy of some variables increased while others decreased. The evidence provided in this experiment emphasizes that care should be taken when submitting forage with a high weed burden for nutritional analysis.

CONCLUSIONS

Optimal establishment of lucerne in the UK was achieved by sowing in spring rather than autumn. The use of barley as a cover crop at a low rate did not negatively or positively influence either yield or chemical composition of lucerne harvested within the establishment year or the following year and tended to reduce weed presence at the 1st cut after establishment. Autumn sown plots were particularly poor, likely due to slow

root development continuing to require plant resources even in the second year. When harvesting silage in the second year, taking an early cut at bud stage slightly improved nutrient concentrations (greater CP and less ADF) relative to cutting at flower at 1st cut but at a cost of considerably lower yield and with poorer fermentation characteristics. Furthermore, a longer regrowth interval was required to reach harvest maturity for 2nd cut. Plots initially cut at flower produced a silage containing higher crude protein and lower ADF at 2nd cut likely relating to slightly reduced maturity relative to those previously cut at bud. Manipulating maturity at 2nd cut posed challenges due to a greater spread of maturity present within the same stand and therefore maturity choices at this cut had a lower impact than at 1st cut. We concluded that, despite benefits to feeding value by cutting at bud stage in 1st cut, on balance, cutting at flower was the better strategy for total yield and chemical composition over two cuts.

The grass-based equation used in the present study provided robust predictions of DM and N content of lucerne silages however predictions for other variables were not sufficiently accurate to be of future commercial use. Nevertheless, offering growers the ability to predict the DM and N concentration of lucerne silages is of value in precision feeding systems and provides a basis for future improvements in the analysis of lucerne silages by NIRS in the UK.

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Table 1 Effect of establishing lucerne in or spring (S) with a barley cover crop (+B) or alone on the yield, and botanical composition of two cuts taken in August and October of the establishment year (2014).

	Establishment[1]			
Item	S	S+B	SEM	*P*-value
Year 1: 1st cut				
DM Yield, T/ha	2.62	2.92	0.307	0.519
Botanical composition, g/kg DM				
Lucerne	302	428	66.7	0.233
Barley	-	122	35.7	-
Weeds	698	450	75.2	0.059
Botanical yield, T/ha DM				
Lucerne	0.82	1.26	0.225	0.212
Barley	-	0.37	0.121	-
Weeds	1.80	1.30	0.250	0.202
Year 1: 2nd cut				
DM Yield, T/ha	1.32	1.43	0.120	0.533
Botanical composition, g/kg DM				
Lucerne	937	975	18.0	0.188
Barley	-	0	0.0	-
Weeds	63.3	25.5	17.96	0.188
Botanical yield, T/ha DM				
Lucerne	1.24	1.39	0.118	0.382
Barley	-	0	0.0	-
Weeds	0.08	0.04	0.024	0.238

DM = dry matter; SEM = standard error of the mean.

[1] Plots sown in Autumn 2013 failed to successfully establish over the 2014 season and were reseeded successfully in August 2014 however there was insufficient growth at these measurement stages to take a cut and therefore the autumn treatment was removed from year 1 analyses.

Table 2 The main effect of establishing lucerne in autumn (A) or spring (S) with a barley cover crop (+B) or alone on dry matter yield and the botanical and chemical composition of resulting silages at the first and second cuts in the second year of growth (2015).

	Establishment[1]				P Value[2]		
Item	A	S	S+B	SEM	ES	A v S	S v S+B
Year 2: 1st cut							
DM Yield, T/ha	2.34	6.23	6.13	0.327	0.001	0.001	0.844
Ensiled material, g/kg DM							
VCODM, g/kg	242	317	317	8.7	0.002	0.001	0.953
Crude protein	90	163	156	4.6	0.001	0.001	0.353
Ammonia-N, g/kg DM*100	20.4	72.4	60.5	4.62	0.001	0.001	0.117
NDF	410	447	443	11.0	0.105	0.042	0.801
ADF	365	403	387	9.3	0.072	0.038	0.279
Ash	110	95	93	1.9	0.002	0.001	0.516
WSC	5.71	4.12	4.08	0.329	0.021	0.008	0.924
Fermentation characteristics							
pH	4.48	5.31	5.24	0.085	0.001	0.001	0.608
LA	35.9	19.1	11.3	7.70	0.148	0.071	0.501
AA	23.0	31.9	30.6	3.12	0.172	0.073	0.791
PA	5.50	8.74	5.14	1.07	0.103	0.316	0.056
Ethanol	15.7	10.3	10.8	1.53	0.044	0.013	0.814
Propanol	2.19	0.53	0.45	0.53	0.100	0.039	0.913
Botanical composition[3]							
Lucerne concentration	190	807	769	51.5	0.001	0.001	0.619
Year 2: 2nd cut							
DM Yield, T/ha	0.95	4.41	4.62	0.255	0.001	0.001	0.570
Ensiled material, g/kg DM							
VCODM, g/kg	293	288	278	4.3	0.043	0.051	0.103
Crude protein	172	181	185	2.8	0.016	0.007	0.345
Ammonia-N, g/kg DM*100	55.7	73.8	76.7	3.22	0.001	0.001	0.520
NDF	406	438	433	9.6	0.056	0.019	0.699
ADF	376	405	404	6.8	0.011	0.003	0.899
Ash	100	91	93	0.5	0.001	0.001	0.008
WSC	3.57	3.17	3.13	0.126	0.037	0.013	0.823
Fermentation characteristics							
pH	5.04	5.42	5.46	0.142	0.079	0.089	0.868
LA	32.6	13.2	16.5	4.91	0.025	0.008	0.627
AA	35.3	45.3	40.2	1.83	0.006	0.004	0.060
PA	3.34	9.70	8.63	0.387	0.001	0.001	0.063
Ethanol	6.41	6.10	7.54	1.092	0.584	0.741	0.347
Propanol	0.59	0.66	0.71	0.151	0.824	0.565	0.872
Botanical composition[3]							
Lucerne concentration	647	981	972	79.2	0.024	0.008	0.939

SEM = standard error of the mean; VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic aicd; AA = acetic acid; PA = propionic acid;

[1] Spring sown plots were established first in May 2014 followed by autumn sown plots in August 2014 due to an initial failed autumn sowing in 2013.
[2] Data were analysed to determine the fixed effects of establishment, 1st cut maturity, and their interaction and random effects of plot. Effects of 1st cut maturity are presented separately.
[3] Data from manual separation performed on a subsample of material prior to ensiling

Table 3 The main effects of cutting at either bud or flower stage on dry matter yield and the chemical composition of the resulting silages in the first cut of the second year of growth (2015).

	Maturity			
Item	Bud	Flower	SEM	*P* value[1]
Yield, T DM/ha	3.19	6.61	0.241	0.001
Ensiled material, g/kgDM				
VCODM, g/kg	245	339	7.1	0.001
Crude protein	147	126	3.8	0.007
Ammonia-N, g/kgDM*100	65.3	36.9	3.78	0.002
NDF	422	445	9.0	0.130
ADF	368	402	7.6	0.021
Ash	109	90	1.6	0.001
WSC	5.38	3.90	0.269	0.008
Fermentation characteristics				
pH	5.11	4.91	0.069	0.086
LA	11.1	33.1	6.29	0.049
AA	33.9	23.1	2.55	0.025
PA	7.41	5.51	0.877	0.177
Ethanol	12.0	12.5	1.25	0.763
Propanol	0.86	1.25	0.43	0.546
Botanical composition[2]				
Lucerne concentration	518	659	38.3	0.006

SEM = standard error of the mean; VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic aicd; AA = acetic acid; PA = propionic acid;

[1] Data were analysed to determine the fixed effects of establishment, 1st cut maturity, and their interaction and random effects of plot. Effects of establishment are presented separately.
[2] Data from manual separation performed on a subsample of material prior to ensiling

Table 4 The effect of maturity at second cut (Bud or Flower), previous maturity at first cut (Bud or Flower), and their interaction on second cut yield and resulting silage chemical composition of lucerne grown in trial plots in the second year of growth (2015).

Item	Harvesting Regime				SEM	*P* value[1]		
	1st cut bud		1st cut flower					
	2nd cut Bud	2nd cut Flower	2nd cut Bud	2nd cut Flower		Effect of 1st cut maturity	Effect of 2nd cut maturity	1st cut x 2nd cut maturity interaction
DM Yield, T/ha	3.67	4.35	2.27	3	0.291	0.001	0.023	0.921
Total DM yield, T/ha[2]	6.8	7.64	9.18	9.31	0.487	0.001	0.312	0.452
Ensiled material, g/kgDM								
VCODM, g/kg	291	323	245	287	5.11	0.001	0.001	0.317
Crude protein	167	169	195	185	3.3	0.001	0.250	0.077
Ammonia-N, g/kgDM*100	56.8	62.8	82.1	73.2	3.84	0.001	0.687	0.055
NDF	455	438	412	398	11.4	0.002	0.158	0.882
ADF	416	405	393	367	8.2	0.002	0.030	0.299
Ash	90^{c}	87^{d}	104^{a}	98^{b}	0.6	0.001	0.001	0.005
WSC	2.71	3.52	3.08	3.87	0.15	0.024	0.001	0.92
Fermentation characteristics								
pH	5.14	5.57	5.23	5.3	0.17	0.554	0.135	0.272
LA	17.6	20.9	13.2	31.4	5.85	0.580	0.067	0.186
AA	35.3^{b}	26.8^{c}	60.1^{a}	38.9^{b}	2.18	0.001	0.001	0.008
PA	4.36^{c}	5.52^{c}	11.7^{a}	7.29^{b}	0.461	0.001	0.003	0.001
Ethanol	8.53	3.47	7.25	7.46	1.312	0.272	0.063	0.467
Propanol	1.07	0.25	0.95	0.36	0.181	0.977	0.002	0.508
Botanical composition[3]								
Lucerne concentration	789	869	894	915	54.6	0.037	0.154	0.401

SEM = standard error of the mean; VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic acid; AA = acetic acid; PA = propionic acid;

[1] Data were analysed to determine the fixed effects of 1st cut maturity, 2nd cut maturity, establishment and their interactions and random effects of plot. Effects of establishment are presented separately.

[2] The yield from cut 1 and cut 2 combined.

[3] Data from manual separation performed on a subsample of material prior to ensiling

[a,b,c,d] Values with differing superscript letters within the same row differ significantly($P<0.05$)

Table 5 An independent validation test of two NIRS equations developped for use on UK silages containing either grass or Grass-Clover when applied to a set of 47 lucerne silages of varying maturity and chemical composition either with or without 10 samples in which weeds comprised greater than half of sample dry matter.

	Grass equation[1]				Grass-Clover equation[1]			
Item	Bias[2]	RMSEP[3]	r^2	RPD	Bias[2]	RMSEP[3]	r^2	RPD
All data-points								
VCODM, g/kg	0.97	3.85	0.96	4.42	28.2	11.7	0.95	1.61
Nitrogen, g/kg	-0.46	10.6	0.93	2.27	-0.01	12.6	0.92	2.03
Crude protein, g/kg DM	-11.3	12.5	0.81	1.69	-19.0	16.8	0.62	0.62
Ammonia-N, g/kgDM*100	-68.4	56.7	0.49	0.36	-51.2	49.6	0.57	0.47
NDF, g/kg DM	14.0	10.1	0.10	0.80	-18.1	9.20	0.06	0.82
ADF, g/kg DM	111	41.6	0.07	0.26	91.8	32.8	0.03	0.30
Ash, g/kg DM	1.80	8.48	0.63	1.42	6.46	12.1	0.51	1.04
WSC, g/kg DM	-18.0	85.8	0.03	0.07	-34.0	94.4	0.05	0.03
pH	0.08	7.40	0.30	1.16	0.22	7.86	0.48	1.12
LA, g/kg DM	-3.86	59.0	0.37	0.89	6.85	126	0.40	0.54
TVC[4], g/kg DM	-12.8	38.3	0.16	0.39	-12.6	38.6	0.07	0.39
TVFA[5], g/kg DM	-9.48	36.2	0.07	0.44	-10.9	38.9	0.05	0.39
Weedy samples removed[6]								
VCODM, g/kg	0.75	3.28	0.96	4.78	29.2	12.2	0.96	1.51
Nitrogen, g/kg	-0.38	9.42	0.92	2.36	0.12	11.1	0.86	2.14
Crude protein, g/kg DM	-9.74	12.1	0.74	1.58	-17.7	16.1	0.51	1.13
Ammonia-N, g/kgDM*100	-66.5	54.4	0.42	0.35	-50.4	47.5	0.48	0.46
NDF, g/kg DM	18.2	11.0	0.08	0.79	17.4	9.60	0.04	0.84
ADF, g/kg DM	111	42.5	0.07	0.26	90.9	33.1	0.03	0.31
Ash, g/kg DM	0.44	7.39	0.69	1.57	5.53	11.3	0.53	1.10
WSC, g/kg DM	-17.4	85.4	0.05	0.08	-31.3	93.2	0.10	0.04
pH	0.09	6.79	0.32	1.13	0.20	7.04	0.49	1.11
LA, g/kg	-4.15	63.6	0.41	0.83	-7.23	65.0	0.45	0.62
TVC[4], g/kg	-12.8	38.9	0.12	0.37	-12.9	39.2	0.10	0.37
TVFA[5], g/kg	-9.40	36.8	0.06	0.44	-11.0	39.4	0.08	0.39

RMSEP = relative root mean square error of prediction; RPD = ratio of standard deviation of the measured population to the standard error of prediction, VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic acid, TVC = total volatile content, TVFA = total volatile fatty acids

[1] Equations were provided by the Forages Analytical Assurance group (FAA), and are designed for commercial use on un-dried and un-milled silage samples. The Grass-Clover equation was formulated using the same reference data as the grass equation with the addition of 95 extra data points originating from diverse grass-clover silages.

[2] Bias is the measured mean minus the predicted mean, therefore minus values indicate over-prediction and positive values indicate under-prediction of the equation.

[3] RMSEP is presented as a percentage of the measured mean for standardisation

[4] TVC is the sum of lactic, acetic, propionic, butyric and valeric acids plus ethanol and propanol.

[5] TVFA is calculated as for TVC but excluding ethanol and propanol.

[6] Sample in which weed concentration was greater than 500g/kg DM (n=10) were removed from the validation dataset leaving 37 samples.

Table 6 The effect of varying establishment timing (autumn, A or spring, S) and use of a cover crop (Barley, +B) on the bias between chemical components of lucerne silage analysed using near infra-red reflectance spectroscopy (NIRS) or a reference laboratory technique. Two different equations are tested, one developped for pure grass silages and one developped for grass-clover silages. (+) indicates an increase in bias using the first method vs. second method and (-) indicates decreased bias using the first vs. the second method. Only significant ($P < 0.05$) effects are shown.

	Grass equation[1]				Grass-Clover equation[1]			
	1st Cut		2nd Cut		1st Cut		2nd Cut	
Item	A vs S	S vs S+B	A vs S	S vs S+B	A vs S	S vs S+B	A vs S	S vs S+B
VCODM, g/kg					-			
Nitrogen, g/kg					+			
Crude protein, g/kg DM					+			
Ammonia-N, g/kgDM*100	+		+		-	-	+	
NDF, g/kg DM	-		-		+			
ADF, g/kg DM	-		-		-		-	
Ash, g/kg DM			+				-	
WSC, g/kg DM								
pH	-		-					
LA, g/kg	-		-				-	
TVC[2], g/kg								
TVFA[3], g/kg								

VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic acid, TVC = total volatile content, TVFA = total volatile fatty acids

[1] Equations were provided by the Forages Analytical Assurance group (FAA), and are designed for commercial use on un-dried and un-milled silage samples. The Grass-Clover equation was formulated using the same reference data as the grass equation with the addition of 95 extra data points originating from diverse grass-clover silages.

[2] TVC is the sum of lactic, acetic, propionic, butyric and valeric acids plus ethanol and propanol.

[3] TVFA is calculated as for TVC but excluding ethanol and propanol.

Table 7 The effect of varying harvest maturity (bud or flower) over two cuts on the bias between chemical components of lucerne silage analysed using near infra-red reflectance spectroscopy (NIRS) or a reference laboratory technique. Two different equations are tested, one developped for pure grass silages and one developped for grass-clover silages. (+) indicates an increase in bias cutting at bud vs. flower and (-) indicates decreased bias cutting at bud vs. flower. Only significant ($P < 0.05$) effects are shown.

Item	Grass Equation			Grass-Clover equation		
	1st Cut	2nd Cut	Effect of 1st cut on 2nd cut	1st Cut	2nd Cut	Effect of 1st cut on 2nd cut
VCODM, g/kg		+	+			
Nitrogen, g/kg						
Crude protein, g/kg DM						
Ammonia-N, g/kgDM*100	+		-	+		-
NDF, g/kg DM	-	-			-	
ADF, g/kg DM			-			-
Ash, g/kg DM			+			+
WSC, g/kg DM	+	+		+		-
pH						
LA, g/kg						
TVC[2], g/kg			-			-
TVFA[3], g/kg						-

VCODM = volatile corrected oven dry matter; N = nitrogen, NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water soluble carbohydrate; LA = lactic acid, TVC = total volatile content, TVFA = total volatile fatty acids

[1] Equations were provided by the Forages Analytical Assurance group (FAA), and are designed for commercial use on un-dried and un-milled silage samples. The Grass-Clover equation was formulated using the same reference data as the grass equation with the addition of 95 extra data points originating from diverse grass-clover silages.

[2] TVC is the sum of lactic, acetic, propionic, butyric and valeric acids plus ethanol and propanol.

[3] TVFA is calculated as for TVC but excluding ethanol and propanol.

Figure captions

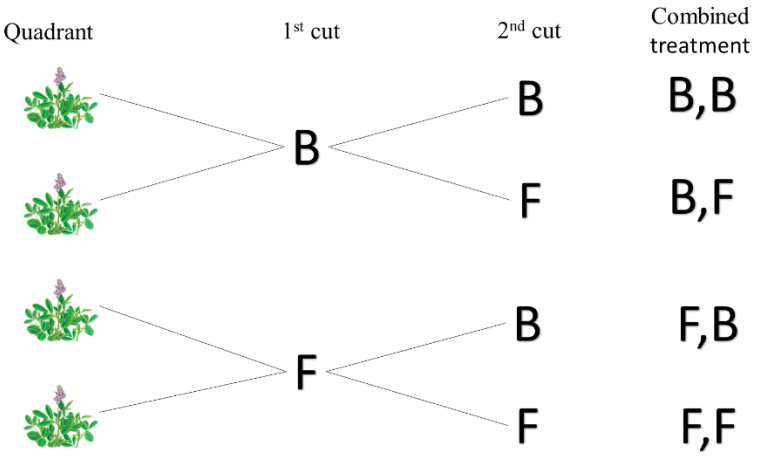

Figure 1 The experimental design of a test of harvest maturity in which 12 lucerne trial plots (previously established using one of three differing methods) were subdivided into four quadrants A-D, with each subplot used to test one of four harvest regimes comprising two harvest maturities (bud; B or flower; F) and two cuts (1st or 2nd).

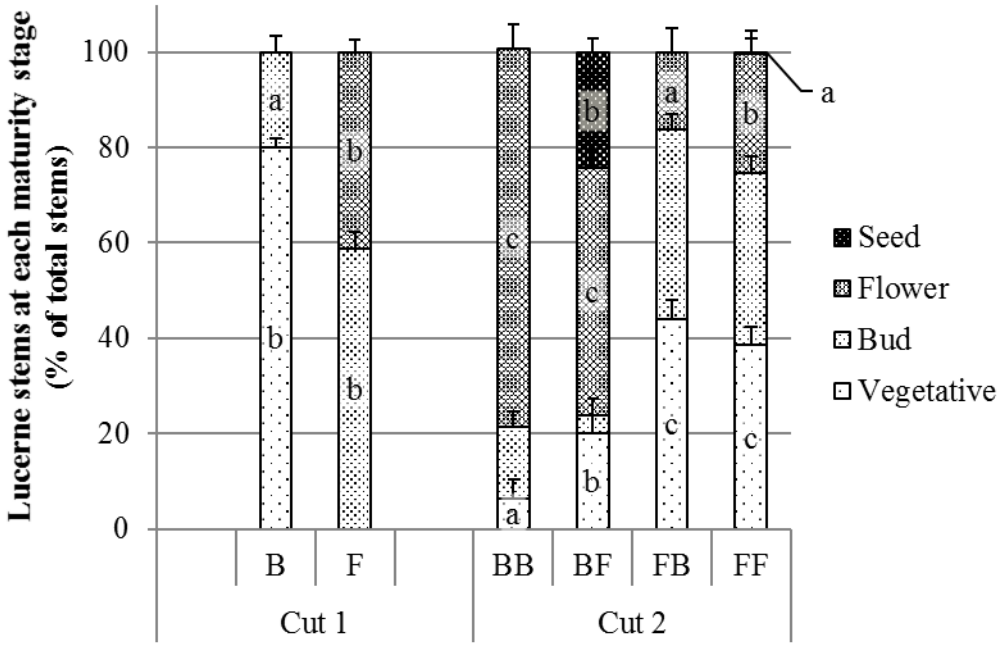

Figure 2 The maturity of lucerne cut according to one of four different harvesting regimes (1st cut: bud, 2nd cut bud; BB; 1st cut: bud, 2nd cut: flower; BF; 1st cut: flower, 2nd cut: bud; FB; 1st cut: flower 2nd cut: flower; FF) spanning two cuts (1st and 2nd cut in 2015). Bars with differing letters within the same maturity stage and cut differ significantly ($P < 0.05$). Letters are not shown where there is a bar equal to 0 but should be assumed to be 'a'. Data were analysed using mixed models to test fixed effects of 1st cut maturity, 2nd cut maturity (where applicable), establishment and their interactions (at 2nd cut) and random effects of plot.

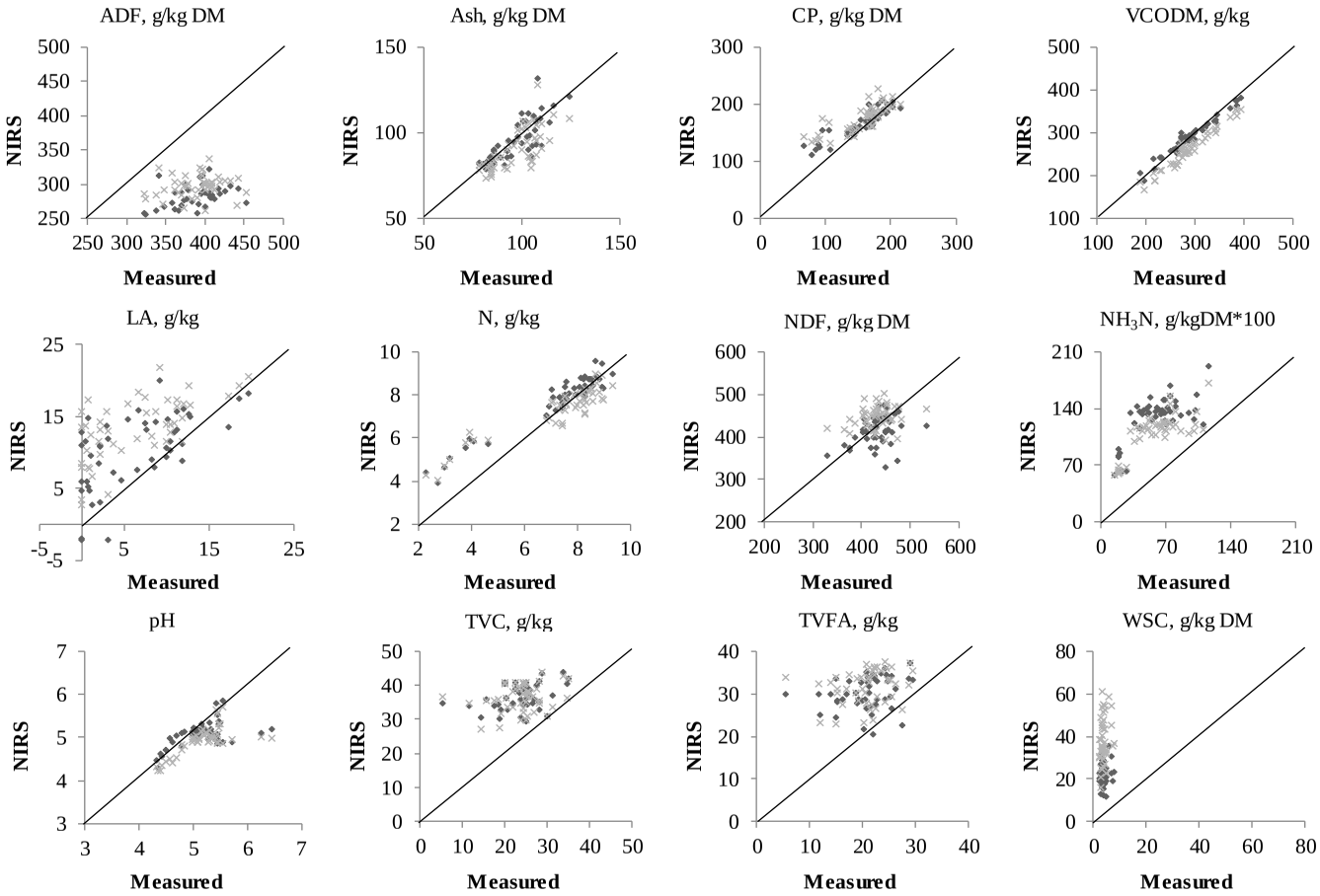

Figure 3 Relationships between predicted and observed data for chemical composition variables of lucerne silages analysed by either a grass-based equation (◊) or a Grass-Clover based equation (×) using spectra obtained by near infra-red spectroscopy (NIRS). ADF = acid detergent fibre; CP= crude protein; VCODM = volatile corrected oven dry matter; LA = lactic acid; N = Nitrogen; NDF = neutral detergent fibre; NH_3N = ammonia nitrogen;TVC = total volatile content; TVFA = total volatile fatty acids; WSC = water soluble carbohydrates

Chapter 9

General Discussion

GENERAL DISCUSSION

Advice for farmers

The overarching research question addressed in this thesis was: can the efficiency of red clover, white clover and lucerne utilisation be improved in the UK dairy industry? Utilisation efficiency in this instance being defined as increasing forage dry matter yield, silage nutritional quality, and resulting milk yield from the cow, whilst reducing wastage of nutrients in cow faeces and urine. Furthermore, a key objective for the research work undertaken was to produce practical advice applicable to farmers to assist them in making improvements to legume forage utilisation in their production systems. To answer the research question posed, the experiments described in this thesis have highlighted several areas in which the utilisation of clover-grass mixtures and lucerne could be made more efficient.

Growing and feeding lucerne. Advice generated from these studies begins with improvements in agronomy for lucerne including sowing in spring rather than autumn and using barley as a cover crop. Topping the crop was shown to be an effective tool for weed control with 53% weed reduction observed following the first cut in spring sown crops. Increased understanding of the growth pattern of lucerne and the importance of taking an initial cut to reduce weed burden may help new growers who might otherwise assume the crop had failed to establish due to initial weed concentrations. Good second year yield from spring crops was achieved in this experiment (10-11 T/ha DM), with no chemical inputs, highlighting the economic viability of lucerne, although yields were not as high as 18 T/ha DM achieved by Frame and Harkess (1987) in initial UK experimental trials that may have been performed on a more favourable soil type. When harvesting lucerne, evidence from the present study suggested that current guidelines to cut at 10 %

flower (early flower stage; Kalu and Fick (1981)) are correct for optimum yield. Harvesting earlier at late vegetative/early bud growth stages (particularly at first cut) could result in small improvements in feed quality (higher crude protein (**CP**) and lower acid detergent fibre concentrations) at first cut, but at the cost of reduced yield and slower regrowth so is not advised. Cutting at the early flower stage was also beneficial in terms of fermentation profile during ensiling with greater concentrations of lactate being produced in silages harvested at this stage. Strategies for improving feeding value of lucerne by increasing its metabolisable energy value within the diet should therefore focus on breeding for reduced lignin concentration within stems and improved leaf to stem ratio to increase neutral detergent fibre digestibility and reduce ruminal retention time allowing for higher dry matter (**DM**) intakes to be achieved (Getachew *et al.*, 2011; Fustini *et al.*, 2017).

Based on the findings of this project alone, when feeding lucerne, farmers should be advised to incorporate lucerne at a lower inclusion rate (25 % of forage DM in the present experiment) and at a short chop length (theoretical chop length of 14 mm) within a maize-based total mixed ration (**TMR**) diet to achieve a greater level of nutrient use efficiency than incorporating lucerne at a high inclusion rate (75 % of forage DM) or with a long chop length (theoretical chop length of 19mm). It must be remembered, however, that farmers may experience very different results when incorporating lucerne into diets depending on the characteristics of the individual silages they feed and only two specific silages were tested in this project. To assess how these findings fit with previously published data, the results from the studies presented in this thesis have been combined with those that were previously compared in Chapter 2 and are presented in Figures 1 and 2. For chop length (Figure 2), results from this project in which longer chop lengths decreased DM intake (**DMI**) are consistent with other findings creating a strong

relationship between increased chop length and a reduction in DMI ($r^2 = 0.60$ when tested using a linear regression). The effect of increasing lucerne inclusion rate is less consistent between studies (Figure 1). Whilst results from Experiment 2 (Chapter 5, good quality silage) fitted well with previous results that showed an upwards trend in DMI in response to higher lucerne inclusion rates, the finding from Experiment 1 (Chapter 5, poor quality silage) corresponded more closely with the study of Akbari-Afjani et al. (2014) that was a notable outlier to the other studies with a large reduction in DMI resulting from higher lucerne inclusion in the diet. If the study of Akbari-Afjani et al. (2014) and Experiment 1 (Chapter 5) were removed from the analyses the resulting relationship between increasing lucerne inclusion rate and increasing DMI would be strong ($r^2 = 0.72$ when tested using a linear regression). With regards to the overall effects of both inclusion rate and chop length on milk yield there remains no clear trends in either case. One possible explanation for this would be that the cows fed higher lucerne diets increased DMI as a response to lower metabolisable energy contained in lucerne and therefore milk yield did not change because energy intake was similar. Furthermore, other variables such as days in milk will have a strong influence on milk yield response. Therefore, it is reasonable to conclude that it is not possible to predict how milk yield will be affected by changes to chop length and inclusion rate of lucerne silage replacing maize silage in the diet based on these two variables alone

It should also be noted that in the present study, only the effect of replacing maize silage was assessed, however in practice, farmers may also consider replacing grass silage with lucerne silage in the diet and in such a scenario, different results could be expected. For example, the study of Sinclair et al. (2015) provided evidence that 110g/kg diet DM of high quality grass silage can be replaced by a lucerne silage without affecting DMI, milk yield, milk composition or diet DM digestibility.

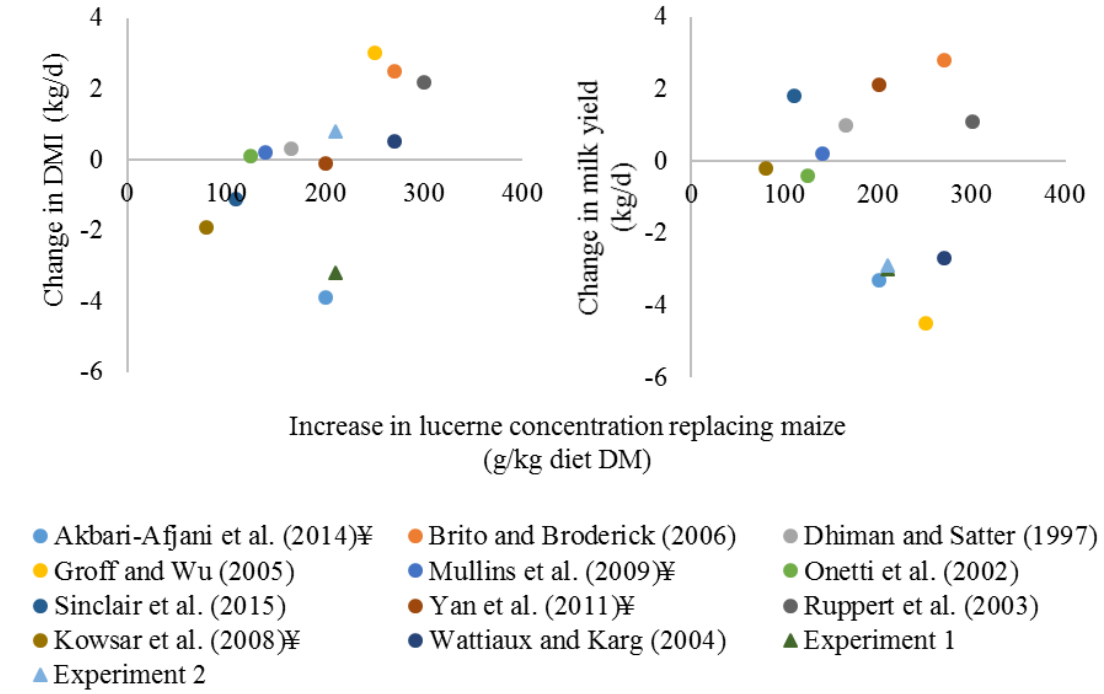

Figure 3: A summary of results from eleven studies that tested the effect of replacing maize silage with lucerne silage or hay (¥) in a total mixed ration (TMR) on dry matter intake (DMI) and milk yield in dairy cattle combined with results from the experimental silages tested in this thesis as described in chapter 5 (▲; a mean of the two chop lengths has been taken).

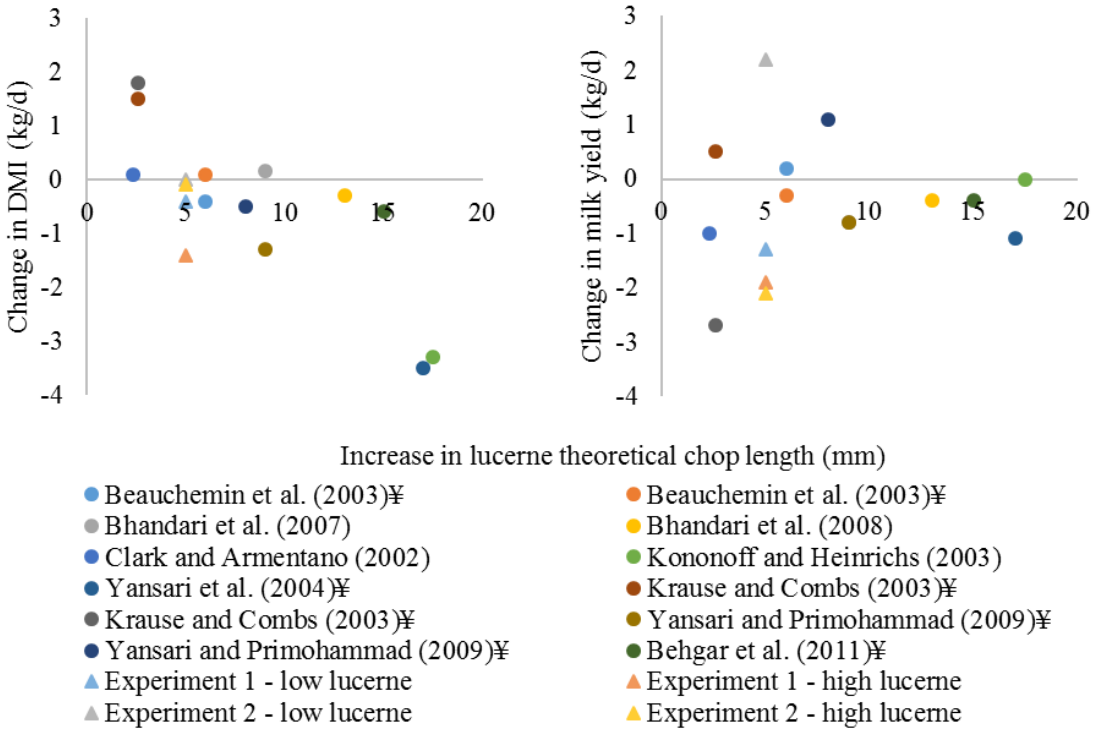

Figure 4: A Summary of results from ten studies (●) that tested the effect of increasing the chop length of lucerne silage or hay (¥) within a total mixed ration (TMR) on dry matter intake (DMI) and milk yield in dairy cattle combined with results from the four experimental silages tested in this thesis as described in chapter 5 (▲). Two studies from the literature in which there were more than one pair of comparable treatments are included twice each.

At present, farmers might choose a high inclusion rate of lucerne combined with a long chop length because it is purported to improve rumen mat formation, stimulate rumination and therefore prevent prolonged episodes of low rumen pH through provision of physically effective fibre and greater buffering capacity of lucerne, however, the present study indicated no benefit to rumen pH of feeding a long chop length or a high inclusion rate in the non-challenging diets tested, and furthermore, DM digestibility was reduced relative to using a short chop length and a low lucerne inclusion rate. Only under challenging rumen conditions was there a positive effect of feeding a high lucerne inclusion rate on increasing rumen pH and preventing milk yield loss, which was attributed to increased buffering capacity, however such conditions are unlikely to be prevalent in UK systems and furthermore, utilising a long chop length reduced rumen pH during the rumen challenge relative to a short chop perhaps due to reduced capacity for fibre digestion preventing digestion of the long particles. The feed withholding and refeeding method of rumen challenge utilised in the present study highlighted the importance of maintaining near-constant access to feed where cows are adapted to *ad libitum* feeding systems and the cost of milk loss associated with short term feed deprivation. This is an area that relates to indoor TMR-fed systems for which there is little recent information in the literature. In summary, the lower DM and nitrogen digestibility but high ruminal protein degradability associated with a high inclusion rate of lucerne in the diet will continue to limit lucerne utilisation efficiency, and grass-red clover mixtures that can have a lower rate of ruminal protein degradation, due to presence of poly-phenol oxidase (Albrecht and Muck, 1991), and a higher DM digestibility, if harvested at the correct growth stage (Alstrup *et al.*, 2016), may therefore be the more viable option in terms of obtaining the highest milk yield from a legume forage. However, for those farmers that find lucerne suits their systems environmentally and economically,

utilising a lower inclusion rate and a shorter chop length will help to increase milk production and nutrient utilisation from lucerne in the future.

Silage Analysis for grass-clover and lucerne silages. With regards grass-clover silages, the work undertaken in the present study has highlighted the importance of updating Near Infra-Red Reflectance Spectroscopy (**NIRS**) silage analysis procedures available in the UK in order to improve accuracy. The dataset generated within the project has enabled the forage analytical assurance (**FAA**) group (responsible for maintaining and improving UK NIRS analysis equations for forages) to begin working on such an update. This will build upon previous datasets that have been used to improve the capability of UK NIRS analysis for various forages (Givens *et al.*, 1989; Deaville *et al.*, 2009; Rymer and Humphries, 2013). As of March 2017, the FAA group have agreed to incorporate both the grass-clover silage spectra and the lucerne silage spectra of samples obtained in this project into commercial equations with the goal of implementing these equations across all partner UK laboratories by July 2017. The goal of the FAA is to combine all these spectra and the previous spectra used for grass calibration to create broader equations than are available at present, similar to those produced and successfully tested by Shenk and Westerhaus (1993) and more recently by Andueza et. al. (2011), although these will require further testing before implementation. It is notable that equations developed in the present study on spectra from un-dried and un-milled samples were not as robust (based on measures such as standard error of calibration) as similar European equations developed on spectra from dried and ground samples e.g. Andueza *et al.* (2011). While UK laboratories currently favour the option of analysing silage samples 'as received' to improve response time to clients, some are now considering introducing an alternative for samples to be dried and ground prior to analysis that could also improve accuracy, and

may allow for the adoption of existing European silage analysis equations that are calibrated on dried and milled samples.

Where grass-clover silages are incorporated into rations on a CP basis, correction of the current under-prediction would result in less protein supplements (e.g. soya-bean meal) being included in diets. Table 1 gives a basic estimation of the value of such a reduction to farmers per cow fed per lactation dependent on the concentration of clover in their silage, based on a case study diet including soya-bean meal. For those farmers who formulate rations using Feed into Milk rationing software, it is also likely that improved balance between metabolisable protein and fermentable energy supply can be achieved through improved prediction accuracy of digestibility and degradability parameters that will improve nutrient use efficiency and may improve other factors such as milk yield and milk solids yield, although the complexity of this rationing system makes it difficult to conclude exactly what the net effect to dietary input costs, if any, will be. Reducing wastage of nitrogen (**N**) in urine and therefore reduced environmental loading of N may be the main benefit.

Table 1. An estimation of the saving that could be achieved by farmers formulating total mixed rations (TMRs) based on an NIRS assessment of crude protein concentration in grass-clover silages if a commercial update to current NIRS equations were to be implemented that corrected current under-predictions that increase with clover concentration.

	Clover concentration in silage (g/kg DM)		
	200	450	700
Hi-pro soya in current diet[1] *(g/kg of TMR DM)*	130	122	115
Revised hi-pro soya level *(g/kg of TMR DM)*	115	99	85
Annual saving per head per lactation[2] *(£)*	21.20	32.10	42.40

DM = dry matter;
[1] Assuming a case study diet that contained a 50:50 forage:concentrate ratio in which the forage portion was comprised of three parts grass-clover silage to one part maize silage and formulated to contain 17% crude protein. Hi-pro soya bean meal price correct as of February 2016.
[2] Assuming a 305 d lactation length eating 25 kg dry matter per day.

The inclusion of lucerne samples into analysis options will also allow more precise formulation of diets containing lucerne when laboratory analysis of lucerne composition is based on NIRS. Furthermore, with additional development, the equation for prediction of clover concentration in grass-clover mixture silages, tested in chapter 4, will also be considered for inclusion in analyses alongside the main update as an extra tool for farmers. Having a measure of clover concentration within swards will be a first step towards allowing farmers to manipulate clover concentration so that it remains closer to the optimum of 30 – 60 % over the life-time of the ley. A preliminary survey conducted in the present study to assess agronomy and ensiling practices for grass-clover swards in the UK also highlighted areas where further practical research is required to aid farmers in better utilising these leys. Many farmers involved within the project reported applying nitrogen (either as inorganic fertiliser of slurry) to grass-clover swards and this suggests an increased need for advice to be made available on fertiliser input requirements for legume-containing leys.

Feeding value of grass-clover and lucerne silages

The assembly of a large sample set of diverse clover-grass and lucerne silages for the present study afforded an opportunity to assess whether published feed tables accurately represent the feed value of leguminous forages produced in the UK. Feed tables are used for a variety of reasons, primarily to provide standard data for rationing software but also as a tool to assist farmers when choosing which forages might best suit their system, and therefore it is important that the data contained within them are current and realistic. Considering that forage legumes are often colloquially referred to as ‘home grown protein’ crops, the accuracy of published CP values are of great importance. Farmers and nutritionists looking to reduce the concentration of high-protein soybean meal in the diet

may rely on these when choosing whether to start growing a forage legume. Comparisons between CP values produced from silages in the present study and those reported in three common feed tables (MAFF, 1990; AFRC, 1993; FiM consortium, 2004) are presented in Table 2.

Table 2. A comparison between the measured crude protein concentration of different ensiled species reported in the present study and in feed tables published in the literature.

Silage species	Average crude protein concentration, g/kg DM			
	Thomson *et al.*[1]	MAFF (1990)	AFRC (1993)	FiM Consortium (2004)
Grass	119	168	142	140
Grass-Clover	145	-	174	-
Clover[2]	151	234	-	245
Lucerne	173	194	194	190

[1] The grass, grass-clover, and clover categories are comprised of silages from Chapter 3 where the clover concentrations were <10% (n=26), 10-90% (n=63) or >90% (n=5) DM respectively. The lucerne category, is comprised of silages produced in Chapter 8 that contained a minimum of 75% (n=27) lucerne by DM.
[2] Literature values were measured on pure clover samples containing either red clover only (FiM consortium, 2004) or mixed clover varieties (MAFF, 1990).

For all categories for which a comparison could be drawn, the mean CP concentration of silages used in the present study was lower than that published in the literature. One possible explanation for this is that the data used in feed tables originated from controlled experimental trials in which optimised conditions created unrealistic CP concentrations. Equally, the low CP value of samples collected may indicate a need for improved silage-making practices. Silages predominantly comprised of clover (> 90 % DM) showed the greatest discrepancy although the number of such silages represented within the present sample set was low (n = 5). Predominantly grass silages (< 10 % clover) had a lower CP concentration than the silages that were legume mixtures or legume monocultures, as might be expected, although the grass species sown in these swards that were intended to contain clover may not be as rich in CP, or as well fertilised, as grass silages obtained from monoculture grass silage leys that were not represented within the current study.

The results from the present study indicate that caution should be taken when choosing a forage legume species purely based on an expectation for higher protein provision as it is possible that the CP concentration may be no higher (or lower) than a well-fertilised grass silage. Updating feed tables to ensure they accurately represent the feed value of UK silages may aid farmers and nutritionists in formulating precise rations in the future. Furthermore, if increased provision of CP in the diet cannot be guaranteed from forage legumes, the main drivers for their adoption in systems must therefore be either government policy favouring sustainable forage crops, or reduction in fertiliser inputs providing an economic benefit. The importance of the latter will vary from farm to farm depending on whether slurry or inorganic N fertiliser is used. While the current single farm payment system will undergo further reforms over the process of the UK leaving the EU, it remains likely that sustainability and the environment will continue to play a role, perhaps a greater role, in any resulting farm payment scheme based on the global need for climate change mitigation.

Areas for further research

While some simple advice has been generated from this project on incorporating grass-clover and lucerne silages successfully into dairy systems, there are still greater challenges to be addressed. Their persistence in the field (both at establishment and long-term) and their feeding value remain limitations to their utilisation in the UK and this creates opportunities for research into precision agronomy and targeted breeding that has been used to great success in the arable sector but is not as often applied to forage crops, other than ryegrasses. Based on the findings of these studies, increasing automation of field operations, for example, using real-time imaging, or remote sensing technologies to better maintain botanical composition and assist in achieving optimal crop maturity at

harvest, would be a logical next step in helping farmers to meet the complex agronomic requirements of forage legumes and their mixtures. Hand-held and mixer wagon mounted NIRS may also be of use in rapid determination of silage and TMR chemical composition providing prediction accuracy is sufficient, which is a subject of ongoing study. Related to the use of precision technologies, is the need to continue improving UK laboratory NIRS analysis by adding more spectra to calibration sets particularly for alternative crops such as lucerne and sainfoin. The prediction accuracy of some variables (e.g. volatile compounds) was shown to be consistently poor over several different validation tests and comparing other spectroscopy techniques (e.g. fourier transform infra-red spectroscopy) with NIRS may highlight a technique with overall greater accuracy for the future. With regards feeding value, it has been shown in the present study that high lucerne inclusion rate maintains rumen function under challenging conditions, and this might be utilised by enabling farmers to increase the provision of energy in the concentrate portion of the diet without negative digestive side-effects to support high milk yields. Further research into the ability of high lucerne inclusion rates to buffer rumen pH in diets varying in non-forage carbohydrate concentration may shed more light on the best way to incorporate lucerne into dairy cow diets.

GENERAL CONCLUSIONS

Grass-clover and lucerne silages are two of the most viable forage legumes for temperate dairy systems as they reduce the need for inorganic nitrogen fertilisation in the field, can be high yielding, and contribute to meeting sustainability targets through reduced environmental footprint. Economic analyses have shown that these crops are cheaper to grow than traditional monoculture grasses, particularly when nitrogen fertiliser price is high. For these reasons, the uptake of such crops can be expected to rise. However, there exist some barriers to their efficient utilisation, and these may explain why certain forage legumes have remained niche crops within UK agriculture to date. High weed burdens during establishment, difficulty in ensiling, and low nitrogen use efficiency in the cow when fed are some of the limitations that may have affected lucerne uptake and/or its efficient utilisation. Utilising evidence gathered during the present project, it is possible to conclude that, by making some practical changes to the way lucerne silages are grown, ensiled, and fed, their efficiency of utilisation could be improved by increasing yield of forage dry matter, silage nutritional quality, and resulting milk yield from the cow, whilst reducing wastage of nutrients in cow faeces and urine. This includes sowing in spring and using a barley cover crop, topping to control weeds, cutting at early flower stage, and chopping to a short chop length when fed. Moreover, a lack of a proven low cost, rapid nutritional analysis option has affected both lucerne and grass-clover utilisation in precision feeding systems. To address this, the present project has afforded an opportunity to improve the Near Infra-red Reflectance Spectroscopy nutritional analysis available for both grass-clover and lucerne silages, with updates likely to be taken up by commercial UK laboratories. In the future, further research into the application of precision technologies and targeted breeding programmes for forage legumes may further improve prospects for their utilisation within dairy systems.

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Appendix

ABOUT THE AUTHOR

Anna graduated from the Royal Agricultural University with a Bsc (Hons) in Agricultural Science in 2013. She subsequently began her PhD at the Centre for Dairy Research (CEDAR), University of Reading, where she also worked as a research technician. Following successful completion of her PhD, Anna hopes to continue to work at Reading in a post-doctoral, dairy science, role. The focus of her research to date, including her PhD research, which was funded by AHDB Dairy as part of the Grasslands, Forage and Soils research partnership, has been improving the efficiency of utilisation of sustainable legume forages (specifically clovers and lucerne) and multi-species swards for the dairy industry. Sourcing a large number of silage samples from commercial farms was an integral part of her PhD study which allowed her to visit many varied farming systems throughout the UK over a three-year period: an invaluable experience! The outcomes of these studies have received positive responses from industry, particularly within the field of forage analytics. In addition to her postgraduate studies, Anna has been responsible for completing a number of dairy, sheep and poultry studies at CEDAR which have fostered collaborations both within the University of Reading and between institutions and companies in the UK and further afield. Furthermore, Anna has been an active member of the British Society of Animal Science's (BSAS) student committee between 2015-17 and led the committee from the chair-persons position from April 2016-17. In this role, she organised a number of successful events on behalf of BSAS. In April 2017 Anna was elected a council member of BSAS, beginning a three-year term.

LIST OF AUTHOR'S PUBLICATIONS

Journal Articles

Thomson, A. L., Humphries, D. J., Jones, A. K., and Reynolds, C. K. (2017) The effect of varying proportion and chop length of lucerne silage in a maize silage-based total mixed ration on diet digestibility and milk yield in dairy cattle. Animal [in press]

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Thomson A. L., Humphries, D. J., Rymer, C., Archer, J., Grant, N., and Reynolds, C. K. Improving the accuracy of near infra-red spectroscopy analysis for fresh grass-clover mixture silages [Animal Feed Science and Technology]

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Near Infra-Red Spectroscopy for Grass-Clover Silages in the UK. 2016. Final Project Report for AHDB Dairy Grasslands, Forage and Soil Research Partnership.