The role of higher protein forages and home grown protein sources within Northern Ireland dairy systems

End of Project Report for AgriSearch in Relation to Projects D-66-14 and D-70-15

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Research team:
David Johnston, Debbie Hynes, Scott Laidlaw, Alan Gordon, Andrew Dale and Conrad Ferris

Report prepared by:
David Johnston, Scott Laidlaw, Debbie Hynes and Conrad Ferris

Agri-Food and Biosciences Institute, Agriculture Branch,
Hillsborough, County Down, Northern Ireland BT26 6DR

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EXECUTIVE SUMMARY

- In order to sustain milk production, dairy cows must have an adequate intake of dietary protein. Within higher output systems cows must be offered additional protein in the form of concentrate supplements. However, these protein supplements are expensive, and are subject to price volatility. In addition, the long term supply of non-genetically modified protein into Europe cannot be guaranteed, while the import of these ingredients has a negative effect on the carbon footprint of local dairy systems. These factors have an adverse effect on the performance of the local dairy sector within the global market place.

- For these reasons there is increasing interest in the potential of locally grown 'protein crops' within dairy systems. However, there is little evidence that these crops really offer potential to replace imported protein in the diets of dairy cows. In addition, while protein crops have many advantages in that they can be high yielding, have a high nutritive value and fix atmospheric nitrogen (thus reducing the reliance on imported fertiliser nitrogen), these crops can be difficult to establish and grow in Northern Ireland (NI). For example, previous experience at AFBI suggests that the performance of crops such as lucerne and lupins can be unreliable under NI conditions.

- Nevertheless, a small number of farmers are successfully growing other protein crops. This project was designed to provide the Northern Ireland dairy sector with information on the potential of home grown protein crops to improve performance in the market place.

- A total of seven studies were conducted within this project. Three of these involved feeding field beans (*Vicia Faba*) to dairy cows (Section 1, Experiments 1, 2 and 3). Four further studies were conducted with red clover (*Trifolium pratense* L.). Two of these were dairy cow feeding studies (Section 2, Experiments 4 and 5) and two of these involved wilting and ensiling studies with red clover (Section 2, Experiments 6 and 7).

- Experiment 1: <u>The impact of field bean inclusion level in dairy cow diets on cow performance and nutrient utilisation</u>. This study was designed to examine the impact of field bean inclusion level in dairy cow diets on performance and nutrient utilisation. Sixty mid-lactation dairy cows were used in a four treatment continuous design (ten weeks duration) experiment. All cows were given *ad libitum* access to grass silage, and were supplemented with 10.0 kg concentrate/cow/day. Concentrates offered contained 0, 166, 333 or 473 g FB/kg concentrate (treatments FB0, FB1.7, FB3.3 and FB4.7, respectively), with the FB partially replacing soya bean meal and rapeseed meal. On

completion of the 10 week experiment, ration digestibility was measured using four cows from each treatment.

While there was a trend for milk protein content (linear: P = 0.081) to decrease with increasing FB inclusion levels, FB inclusion had no effect on silage dry matter (DM) intake, total DM intake, milk yield, milk fat content, milk fat yield, milk protein yield, milk fat + protein yield, live-weight change and body condition score change. This suggests that the anti-nutritional factors in filed beans has no effect on performance. Similarly, FB inclusion had no effect on DM, organic matter and gross energy digestibility coefficients, although there was a linear increase in nitrogen (N) digestibility (P < 0.041) with increasing FB inclusion levels. Faecal N/N intake (P < 0.042) and milk N/N intake (P < 0.047) decreased with FB inclusion, while energy utilisation was unaffected. Field bean inclusion had no effect on any of the methane production parameters measured. The results of this study demonstrate that FB can be included in dairy cow diets at levels up to 4.7 kg/day with few negative effects on cow performance, and as such may have potential to replace imported protein feeds in dairy cow diets. However, total diet crude protein levels were relatively high in this study, and different responses might have been observed if diet crude protein levels had been lower.

- Experiment 2: <u>The impact of field bean inclusion in the diet of early lactation dairy cows on performance and nutrient utilisation</u>. The current study followed on from Experiment 1, and was designed to examine the effects on cow performance and nutrient utilisation of including high levels of field beans (up to 8.4 kg/cow per d) in the diet of early lactation dairy cows. This is important as field bens contain phyto-estrogens, which can have negative effects on fertility. Seventy dairy cows were used in a 3 treatment continuous design (from calving until week 20 of lactation) experiment. All cows were given *ad libitum* access to a mixed ration comprising grass silage and concentrates (45:55 on a DM basis). Concentrates offered contained either 0, 349, or 698 g FB/kg concentrate (treatments FB0, FB-Low and FB-High, respectively), with the FB completely replacing soya bean meal, rapeseed meal, maize gluten and wheat in the concentrate with the FB-High treatment. Following completion of the 20 week experiment, ration digestibility, nutrient utilisation and methane (CH_4) production were measured using 4 cows from each treatment.

  None of silage DM intake, total DM intake or milk yield were affected by treatment, which again highlights that the anti-nutritional factors in beans had no adverse effects on intakes. Cows on FB0 had a higher milk fat content than those on FB-High, whilst cows on FB0 and FB-Low had a higher milk protein content than those on FB-High. Field bean inclusion increased the degree of saturation of milk fat produced. Milk fat yield, milk protein yield and milk fat + protein yield were higher with FB0 than with either of FB-Low or FB-High.

Treatment had no effect on the digestibility of DM, organic matter, nitrogen (N), gross energy, and neutral detergent fibre, whilst digestibility of acid detergent fibre was higher with FB0 than FB-High. Neither the efficiency of gross energy or N utilisation, nor any of the CH_4 production parameters examined, were affected by treatment. Similarly, none of the fertility or health parameters examined were affected by treatment. The reduction in milk fat observed may have been due to the higher starch content of the FB-High diet, while the reduction in milk protein may have been due to a deficit of specific amino acids in the diet. It is likely that these issues could be overcome by changes in ration formulation, thus allowing FB to be included at higher levels in dairy cow diets.

- Experiment 3: <u>The effect of post-harvest treatment of field beans on dairy cow performance and nutrient utilisation</u>. Experiments 1 and 2 demonstrated that field beans could be included in dairy cow diets at up to approximately 4.5 kg/cow/day. In the more northerly parts of the UK beans are frequently harvested with a moisture content in excess of 16%, and as such must be treated to prevent mould growth. While drying is the practice most commonly used, there is little information available on the impact of the degree of milling of dried beans on subsequent performance, an important issue given the high starch content of beans. In addition, acid treatment of moist beans has been conducted on some farms, although the impact of this on subsequent performance, is unknown. This study was designed to examine the impact of moist preservation of field beans using propionic acid, and the extent of physical treatment of dried field beans, on dairy cow performance and nutrient utilisation. Eighteen mid-lactation Holstein-Friesian dairy cows were used in a three period (each of four weeks duration) change over design experiment. The field bean crop used in the experiment (*Var. Boxer*) was harvested at a moisture content of approximately 25%. Three treatments, each comprising a different post-harvest treatment of field beans, were examined. Following harvest, approximately 2/3 of the bean crop was dried at 80^{o}C for four hours to achieve a moisture content of 16%, before being left to cool. Beans were then either coarsely rolled (Dry-CR) or finely milled (Dry-FM). The remaining 1/3 of the bean crop was coarsely rolled and the beans then treated with propionic acid at a rate of 20 litres/ton fresh beans (Moist-P). Cows on all three treatments were offered a mixed ration comprising grass silage and concentrates (forage : concentrate ratio of 60: 40 on a dry matter basis). The concentrate component of the diet with treatments CR and FG comprised a common ‘pre-mix’ (600 g/1000 g concentrate) with the remaining 400 g per 1000 g concentrate comprising field beans (CR or FM treated beans, respectively). With treatment ACID the same concentrate pre-mix was used (600 g/1117 g concentrate), while the moist field beans were incorporated at 517 g/1117 g concentrate, the higher inclusion reflecting their lower DM content. The experimental concentrates were designed to achieve an intake of field beans of approximately 3.5 kg per day with each treatment.

Neither degree of processing of dried field bean (coarsely rolled or finely milled), or treatment of moist rolled field bean with propionic acid had any effect on any of the cow performance parameters measured. Treatment had some effect on a number of milk fatty acids, which is likely to reflect changes in rumen pH between treatments.

- Experiment 4: Performance and nutrient utilisation of dairy cows offered silages produced from three successive harvests of either a red clover-perennial ryegrass sward or a perennial ryegrass sward. This 13 week study examined the performance of 28 dairy cows offered silages produced from three successive harvests (H) of either a pure grass sward (GS) receiving 315 kg N/ha/annum or a red clover-perennial ryegrass sward (RCGS) receiving 22 kg N/ha/annum. The crops for H1, H2 and H3 were wilted for 48, 72 and 72 hours, respectively. Silages from H1, H2 and H3 were offered for 5, 5 and 3 weeks, respectively, with cows supplemented with 8.0 kg concentrate per day throughout the experiment.

  Silage DM intakes were higher in RCGS than GS in H1 and H2, with no differences in H3. Milk yield was higher in RCGS than GS in H3 with no differences in H1 and H2. Milk fat and milk protein contents were lower with RCGS than GS in H3, but did not differ in H1 and H2. Faecal N/N intake was higher in RCGS than GS in H1, with no differences in H2 and H3. Gross energy digestibility was lower in RCGS than GS in H2. Although cow performance was higher for RCGS treatments, responses were variable between harvests largely reflecting the changing proportion of RC in the swards as the season progressed. The study has clearly demonstrated the very different cow performance responses to red clover inclusion which can arise between individual harvests within a season, especially during the first full season following establishment, when the relative proportions of the two species changed considerably over the season. The variability in silage composition between harvests also creates very practical challenges. For example, the very different forage crude protein levels with the grass/red clover mix creates real practical difficulties in balancing the protein content of the diets offered. The total yield of DM over the three harvests was 10.4 t/ha with the grass sward and 9.9 t/ha with the grass/red clover sward. Using these herbage yields (in-silo losses assumed as 10% of DM ensiled), and the mean silage intakes across the experimental period, one hectare of the grass sward was able to produce sufficient silage for 985 ‘cow feeding days’, while one hectare of the grass/red clover sward was able to provide sufficient silage for 803 ‘cow feeding days’. Given that the value of milk produced per cow/day was £7.49 with the grass silage and £7.69 with the grass/red clover silage (milk at 30 pence per litre) the total value of milk per hectare was £7380 and £6170 with the grass silage and grass/red clover silage, respectively. Thus, even considering the saving in

fertiliser use with the grass/red clover silage system (approximately £750/ha), this did not compensate for the loss in the value of milk produced. Furthermore, limits to red clover persistence also need to be taken into account, with reseeding of red clover swards normally required ever 3 – 4 years. In addition, the saving in fertiliser nitrogen creates another potential dilemma, in that red clover swards still have a requirement for phosphorus and potassium. Thus if these nutrients are to be supplied from slurry, the crop will likely be oversupplied in nitrogen, and the nitrogen fixing potential of the red clover will not be realised.

- Experiment 5: <u>Cow performance, nutrient utilisation and the 'concentrate sparing effects' arising from the partial replacement of grass silage by red clover silage</u> This four-period partially balanced change-over (28 cows: period duration four weeks) study was designed to investigate the potential concentrate sparing effects arising when red clover (RC) silage partially replaced grass silage in the diet of dairy cows. Seven treatments were examined. In four of these treatments grass silage diets were supplemented with four levels of concentrate (8.5, 11.0, 13.5 and 16.0 kg/cow/day; GS8.5, GS11.0, GS13.5 and GS 16.0, respectively), to provide a response curve to concentrate supplementation. The remaining three treatments involved mixing grass and red clover silages so that the mixtures contained 30%, 50% and 70% red clover (RC30, RC50 and RC70, respectively) on a DM basis. Each of these diets were supplemented with 12.5 kg concentrate/cow/day. On completion of this part of the study, 16 cows were subject to nutrient utilisation measurements, with cows offered one of four diets (RC30, RC50 and RC70, or grass silage supplemented 12.5 kg concentrate/cow/day). All diets were designed to be iso-nitrogenous (target, 170 g crude protein (CP)/kg DM) which was achieved by varying the proportions of two concentrates (a high CP concentrate containing 214 g CP/kg DM, and low CP concentrate containing 175g CP/kg DM) in the diet.

  With the GS treatments, total DM intake, milk yield, energy corrected milk yield and yield of each of fat and protein increased with increasing concentrate inclusion level, while milk fat content decreased ($P<0.001$). Although total silage DMI increased with increasing red clover (RC) content in the diet ($P<0.001$), the three RC inclusion treatments did not differ in milk yield, energy corrected milk (ECM), milk fat and protein content and fat yield, fat + protein yield, lactose content and yield, energy content and yield. Concentrations of saturated fatty acids decreased with increasing concentrate inclusion, while concentrations of monounsaturated fatty acids increased, however neither parameter differed across the three RC treatments. With regards the expected concentrate 'sparing' effects of red clover, the results were disappointing. For milk yield and ECM yield, there was no 'sparing'. Indeed by interpolation from the response curve developed from the response to supplementing a grass silage only diet,

on average an additional 1.02 and 1.18 kg concentrate would have been required daily with the red clover treatments (mean of the three treatments) in order to achieve the same level of milk yield and energy corrected milk yield as was achieved when grass silage was supplemented with 12.25 kg concentrate/day. Milk fat content on the other hand was associated with a positive concentrate saving with red clover inclusion. Digestibility of all DM constituents measured declined with increase in RC content in silage. RC silages had or tended to have a higher output of N and GE in faeces and less in urine than had GS12.25. The results of this study suggested increased intakes when red clover was included in the diet, but few cow performance benefits.

- Experiment 6: Effect of management strategy on wilting of monocultures and mixture of red clover and perennial ryegrass. Limitations to the adoption of red clover as a silage crop include its low dry matter (DM) at cutting and a perception that it loses moisture slowly during wilting. The dynamics of fresh weight (FW) loss over 46 h wilting periods was examined in a 3 × 3 factorial study over two harvests (H1 and H2) using three conditioned mown forages i.e. red clover (RC), perennial ryegrass (*Lolium perenne* L: PRG) and a PRG/RC mixture (210 and 580 g RC per kg DM at H1 and H2, respectively) and three management regimes i.e. Undisturbed, occupying 75% of total area; Tedded over same area as undisturbed and Tedded over whole area (swathe weight per unit area reduced by 25%) followed by tedding a second time in Day 2. Following mowing swathes were reconstructed in mesh trays to measure FW loss (6 replicates per treatment).

  At both harvests RC had a lower DM content than PRG, RC had a slightly but significantly faster rate of wilting than PRG, and wilting of both forages was 2 to 3 times faster on the first than second day. Rate of wilting of the mixtures was generally between the rate of the two monocultures. Despite RC having a slightly faster rate of wilting than PRG, it still had a lower DM content than PRG after two days. Reducing weight per unit area of tedded swathe followed by tedding a second time (irrespective of forage type), significantly enhanced wilting of conditioned herbage compared with the 'tedding only' treatment. Thus spreading the swathe during tedding will quicken drying rate.

- Experiment 7: The effect of increasing the proportion of red clover in a grass clover mixture on ensiling characteristics and in-silo losses. Herbage mixtures varying in red clover (RC):perennial ryegrass (PRG) ratios, namely: 0:100, 25:75, 50:50, 75:25 and 0:100 by fresh weight, were ensiled in plastic pipe mini-silos containing 6 kg fresh chopped herbage. Silos were destructively sampled after 3, 6, 12, 24, 48 and 96 days of ensilage. Treatments were replicated three times (i.e. 90 mini-silos) in a split plot design with treatments as main plots and sampling dates as subplots. The experiment was conducted over two harvests

(July, H1, and September, H2) with herbage from pure swards of perennial ryegrass and red clover harvested with a precision chop forage harvester. Aerobic stability was determined on the silage sampled on day 96, by exposing to air for 20 days in a constant environment.

Grass ensiled consistently had a higher dry matter and NDF content and lower nitrogen content than RC; water soluble carbohydrate content of red clover was particularly low. Losses, measured at H2, due to effluent were higher in red clover than grass (32% and 1.3% of original weight, respectively). Rate of decline of pH and WSC during ensilage was faster in PRG and low RC silages than high RC silages at both harvests. Lactic acid content was higher at H1 than H2 and was significantly higher in 50 and 75% RC silages than some of the other treatments, lactic acid as a proportion of total fermentation products following this pattern. Acetic acid content was higher and ethanol content was lower in RC silages than PRG silages. Ammonia N was slightly lower in PRG than RC silage at H2. Silages with pure or a high content of RC were more stable than those with high PRG content e.g. slower to reach a maximum temperature after exposure to air. Some characteristics of mixtures can be predicted from knowing the characteristics of pure red clover and perennial ryegrass and the proportion of each in the mixtures. However, particularly in mixtures with a high red clover content, pH and acetic acid were lower and lactic acid and lactic acid as a proportion of total fermentation products higher than the content of red clover in the mixture would suggest.

SECTION 1

STUDIES ON THE INCLUSION OF LOCALLY GROWN FIELD BEANS ON DAIRY COW DIETS

Experiment 1

The impact of field bean inclusion level in dairy cow diets on cow performance and nutrient utilisation

Introduction

Genetic selection for milk production during the last few decades has resulted in an increase in annual milk production per cow in most European countries (Oltenacu and Broom, 2010). In order to meet the greater nutrient requirements of these higher yielding cows, concentrate feed levels have increased. This increase in concentrate use has led to an increased demand for quality 'protein' ingredients such as soya bean meal and rapeseed meal. As some protein ingredients, especially soya bean meal, are often imported from countries outside the European Union (EU), this has left the dairy sector vulnerable to instability of supply, price volatility, and the unavailability of non-genetically modified protein crops. For these reasons, there is interest in the potential of locally grown protein grain crops to replace imported protein feeds (Tufarelli et al., 2012).

Field bean *(Vicia Faba)* (FB), a grain legume, may provide an alternative to imported protein feeds. It can be successfully grown in some of the wetter and more Northerly regions of Europe (Crépon et al., 2010), with grain yields of between 5 and 6 tons/ha possible for winter and spring FB varieties in the UK (PGRO, 2017). Although the crude protein content of FB is lower (280-290 g/kg DM) than for soya bean meal (470 g/kg DM), its starch content (400 g/kg DM) is considerably higher than that of soya bean meal (45 g/kg DM) (Ewing, 1997). Field bean and soya bean meal also differ in their amino acid profile, with the former having a lower total lysine content, and a substantially lower methionine content (Ewing, 1997). Field bean is also known to contain a number of anti-nutritional factors (ANF), including tannins, trypsin inhibitors, protease inhibitors, lectins, gallic acid and phytoestrogen compounds, and these can have negative effects on animal performance (Dvořák et al., 2006).

In view of the potential negative impact of ANF, caution is often adopted when including FB in dairy cow diets. Nevertheless, evidence concerning the impact of FB inclusion levels on the performance of dairy cows is both limited and somewhat contradictory. For example, FB inclusion levels (up to 4.5 kg/cow/day) reduced the protein content of milk from 31.3 to 30.7 g/kg (Trommenschlager et al., 2003). Similarly, Puhakka et al. (2016) found both milk yield and milk protein content to be reduced when rapeseed meal was replaced with FB in dairy cow diets at up to 3.7 kg/cow/day. In contrast, the inclusion of FB at up to 30% of the concentrate (representing a total FB intake of 3.5 kg per day) had no impact on either milk

production or milk composition (Brunschwig and Lamy, 2002); cited by Crépon et al. (2010). While Mogensen et al. (2010) offered approximately 5 kg FB DM/cow/day, the highest feed level identified in the literature, this study did not involve a zero FB treatment for comparison purposes. In addition, given concerns about the environmental impact of dairying, the impact of FB inclusion on methane (CH_4) emissions needs to be considered. In one of the few studies that have examined this issue, Ramin et al. (2017) found that CH_4 production was unaffected when rapeseed meal was replaced by FB.

The single FB inclusion level adopted in most studies makes it difficult to identify an optimum inclusion level for dairy cows. In addition, many of these studies involved non-grass silage based diets, and this may have influenced the responses obtained. Given the inconsistent outcomes of the studies highlighted, the current study was designed to examine the impact on cow performance and nutrient utilisation of partially replacing 'conventional protein' ingredients in dairy cow concentrates with FB, the latter included at rates of up to up to 4.7 kg/cow/day. The conventional protein ingredients that were partially replaced in this experiment were primarily soya bean meal and rapeseed meal, with these being two commonly used 'protein' ingredients within the UK. It was hypothesised that FB could partially replace conventional protein ingredients in dairy cow concentrates without loss of cow performance, and with no adverse effects on CH_4 production.

Materials and methods

This study was conducted at the Agri-Food and Biosciences Institute, Hillsborough, Northern Ireland. All experimental procedures were conducted under an experimental licence granted by the Department of Health, Social Services & Public Safety for Northern Ireland in accordance with the Animals (Scientific Procedures) Act 1986.

Animals and housing

Sixty Holstein Friesian dairy cows (mean lactation number, 3.3 (s.d., 1.49) were used in a continuous design 10-week experiment. Cows were a mean of 134 (s.d., 28.7) days-in-milk at the start of the experiment, and had a mean pre-experimental milk yield of 34 (s.d., 3.71) kg per day.

Throughout the 10 week experimental period cows were housed in a single group in a free-stall house with concrete flooring, and had access to individual cubicles that

were fitted with rubber mats and bedded with sawdust. The cubicle-to-cow ratio was ≥1:1 at all times, thus meeting the recommendations of FAWC (1997). The floor area was cleaned every 3 hours using an automated slurry scraper system.

Treatments

During a two-week pre-experimental period cows were offered grass silage plus 6.0 kg of a non-experimental concentrate via in-parlour feeders (3.0 kg at each milking), and 6.0 kg of the same concentrate via an out-of-parlour feeding system.

Cows were allocated to one of four experimental treatments on the final day of the pre-experimental period, with cows in each treatment group balanced for parity (2, 3 and ≥4), days since calving, and for milk yield, milk fat and milk protein content, live-weight and body condition score (BCS), as recorded during the week prior to the start of the study.

Treatments examined (FB0, FB1.7, FB3.3 and FB4.7) differed in concentrate type offered, with the experimental concentrates differing in FB (*Vicia Faba. Var. Fuego*) inclusion level, namely zero, 166, 333 and 473 g/kg (fresh basis), respectively. The beans used within this study were spring sown, harvested on 15th September 2015, and dried for approximately 2 hours at 80^{o}C to achieve a target dry matter (DM) content of 870 g/kg. Concentrates were offered via three out-of-parlour feeding stations. The ingredient composition of the concentrates offered with FB0 and FB4.7 are presented in Table 1. The concentrate for treatments FB1.7 was produced by offering the FB0 and FB4.7 concentrate in a two-thirds : one-third ratio, respectively, while that for FB3.3 was produced by offering the FB0 and FB4.7 concentrate in a one-third : two-thirds ratio, respectively. All concentrates were designed to be isonitrogenous and to have similar starch contents. Cows on each treatment were offered 10.0 kg concentrate per day, split between four time intervals across each 24-h period. No concentrates were offered in the milking parlour during milking.

All cows were offered a common grass silage throughout the experiment, which was produced from secondary re-growth herbage (harvested on 12 September) from a perennial ryegrass (*Lolium Perenne*) dominated sward. Fresh silage was offered daily at approximately 09.30 h (at 1.07 of the previous days intake), with uneaten silage removed the following day at approximately 08.00 h. Silage for cows on all treatments was mixed in a feeder wagon (Vari-cut 12: Redrock, Armagh, Northern Ireland) for

approximately six minutes prior to being deposited in a series of feed boxes mounted on weigh scales. Cows accessed the silage in these feed boxes via Calan gates (American Calan Inc., Northwood, NH, USA) linked to an electronic identification system, thus enabling individual cow intakes to be recorded daily. Cows had free access to fresh water at all times.

Animal measurements

All cows were milked twice daily (between 06.00 and 08.00 h and between 15.00 and 17.00 h) throughout the experiment using a 50-point rotary milking parlour (Boumatic, Madison, USA), with milk yields automatically recorded at each milking, and a total daily milk yield for each cow for each 24-hour period calculated. Milk samples were taken during two consecutive milkings each week and analysed for fat, protein, and lactose using an infrared milk analyser (Milkoscan Combifoss^{TM}7; Foss Electric, Hillerød, Denmark), and a weighted concentration of each constituent determined for the 24-hour sampling period. Each sample was treated with a preservative tablet (lactab Mark III, Thompson and Cooper Ltd., Runcorn, UK), and stored at 4°C until analysed.

Live-weight was recorded twice daily (immediately after each milking) using an automated weighbridge, and a mean weekly live-weight for each cow determined. The BCS of each cow was estimated fortnightly by a trained technician according to Edmondson *et al.* (1989). At the end of weeks 6 and 10 of the experiment blood samples were collected from the coccygeal vein of each cow prior to feeding, and centrifuged (3000 rpm for 15 minutes) to isolate the serum. Serum urea concentrations were determined using the Kinetic UV method (Roche Diagnostics Ltd., Burgess Hill, UK) using an Olympus AU640 analyser (Olympus, Center Valley, PA), and a mean value (for the two sampling weeks) calculated for each cow.

Nutrient utilisation

On completion of the 10-week feeding study, four cows from each treatment were selected for use in a nutrient utilisation study, with the four cows selected from each treatment group balanced for daily milk yield and live weight. Cows were transferred in pairs (8 pairs in total) from the main experimental group into a Digestibility Unit (at approximately 09.00 h) on two days each week (Monday and Thursday) over a four week period. Cows were tied by the neck in individual stalls, with their lying area

comprising a rubber mat, while continuing to access their experimental rations from feed boxes at the front of each stall. Grass silage was offered *ad libitum* daily at 09:00 h, at proportionally 1.1 of the previous day's intake, while uneaten silage was removed the following day at 08:00 h. The experimental concentrates (10.0 kg/day) were offered in four equal meals each day (2.5 kg/meal) at 07:00, 11:00, 15:00 and 19:00 h, to simulate the out-of-parlour feeding regime used during the main experimental period. Concentrates were offered in plastic feed buckets which were placed within the feed boxes, with these removed after all the concentrates had been consumed at each feeding time. Cows had access to fresh water at all times via a drinker located within each stall.

Measurements of nutrient utilisation commenced 24 h after cows were moved to the Digestibility Unit, and comprised a 6-day feeding period, followed 48 hours later by a 6-day total faeces and urine collection period. Faeces were collected in a plastic collection tray (96 cm × 108 cm × 36 cm deep) placed behind each cow. Urine was collected into a 25 litre plastic container via a flexible plastic tube which was attached to a urine separation system. This was held in position over the vulva by attaching it using Velcro fasteners to a 'patch' glued (Bostik, France) either side of the cow's tail head. Approximately 300 ml of 50% sulphuric acid was added to each urine collection container daily to minimise nitrogen (N) losses as ammonia. The total weight of faeces and urine produced during each 24 h collection period was recorded, and a sample of each (0.05 by weight) stored in a fridge (approximately 4^{o}C) until the final day of the collection period, when the six daily faecal samples and six daily urine samples from each cow were bulked for subsequent analysis. Fresh faeces and urine were analysed for N content, while freeze dried urine samples were analysed for gross energy (GE) content. Faeces samples were dried at 85^{o}C for 100 hours to determine oven DM (ODM) content, with the dried samples subsequently milled and analysed for acid detergent fibre (ADF), neutral detergent fibre (NDF), ash and GE concentrations. Milk samples were taken at each milking, bulked in proportion to yield for days 1-3 and days 4-6, and subsequently analysed for GE and N concentrations. Energy corrected milk yield (ECM) was determined as described by Chen and Yan (2015).

After faeces and urine had been collected on the third occasion within each ration digestibility study, each pair of cows was transferred into two indirect open-circuit respiration chambers for a 72 h period, with each chamber used twice with each

treatment to remove any possible chamber effects. Chambers were made of insulated fenestrated panels mounted on a profiled floor, incorporating airlocks for entry and feeding. Each chamber had a total volume of 22 m^3, and was ventilated by suction pumps, with a flow rate of 75 m^3/h, thus giving approximately 3.5 air changes/h. Temperature and humidity were controlled using air-conditioning units with targets of 15°C (±1°C) and 65% (±10%) relative humidity, respectively. Chambers were operated under negative pressure (50 N/m^2) and exhaust air extracted at three positions for volume measurement and gas analysis. Total air flow was measured by in-line turbine flow meters (GH Flow Automation Ltd., Andover, UK). Methane (CH_4) concentrations were measured by differential Luft analyzers (Analytical Development Co. Ltd., Hoddesdon, UK). Temperature, humidity, and atmospheric pressure were measured using Vaisala HUMICAP sensor probes and a Vaisala PTA 427 digital barometer (Delta-T Devices, Cambridge, UK), respectively. Results were recorded via a 16-bit analogue-digital converter (Strawberry Tree Model ACPC-16; Adept Scientific Micro System Ltd., Letchworth, UK). Air samples from each chamber, and samples of ambient air, were collected and analysed for 75 second periods, and the average values of the last 60 seconds logged for post-run analysis. Sixteen measurements were conducted per chamber each hour, while the ambient air samples were analysed hourly, with the analysis train designed to run automatically. Prior to, and at the end of the experimental period, the chamber function was tested by comparing the amount of carbon dioxide (CO_2) and CH_4 recorded by the gas analyser three hours after the release of a known quantity of pure CO_2 and CH_4. A mean CH_4 recovery rate of 93.9% was recorded. While cows remained in the chamber for 72 hours, measurements of CH_4 and CO_2 production recorded during the final 48 hour period were used in the subsequent analysis. Cows were weighed prior to and on completion of each six day nutrient utilisation period, with average live weight used in the subsequent calculations.

Feed analysis

A sample of each concentrate type (FB0 and FB4.7) was taken weekly, and dried at 85°C for 24 hours to determine ODM content. An additional weekly sample was dried at 60°C for 48 hours, bulked for each 14-day period, milled through a 1.0 mm sieve, and subsequently analysed for N, NDF, ADF, ash, GE and starch concentrations. Concentrates offered during the nutrient utilisation study were sampled daily, bulked

for each 6-day nutrient utilisation measurement period, and analysed for ODM, GE, NDF, ADF, N and ash concentrations. Samples of the grass silage offered were taken daily throughout the experiment, and dried at 85°C for 18 hours to determine ODM content. Sub-samples of the dried silages were taken twice weekly and bulked for each 14 day period, with the bulked sample milled and analysed for NDF, ADF and ash concentrations. Each week a further fresh silage sample was analysed using near infrared reflectance spectroscopy (NIRS) for pH, ammonia N (% of total N), N, and metabolisable (ME) concentration according to Park *et al.* (1998). During the nutrient utilisation study, silages were sampled daily for ODM determination, with the daily dried samples bulked for each measurement period, and subsequently analysed for ADF, NDF and ash concentrations. A fresh silage sample was taken daily throughout each nutrient utilisation measurement period and analysed for GE and N content.

Concentrations of NDF and ADF in feeds and faeces were determined using a Fibertec analyser using the method of Van Soest (1976), and ash concentrations were determined following combustion in a muffle furnace (Vecstar, UK) at 550°C for approximately 10 hours. Concentrate starch concentrations were determined using a Megazyme Kit (Megazyme International Ireland, Bray, Ireland: McCleary et al., 1994). Nitrogen concentrations of dried concentrates were analysed using the Dumas method (Elementar, Vario Max CN), while N concentrations of urine, milk, fresh faeces and fresh silage were determined using the Kjeldahl method (Tecator Kjeltec Auto 2400/2460 Analyser/Sampler System). GE concentrations of dried concentrates and faeces, fresh silage and freeze dried milk and urine samples were determined using a bomb calorimeter (Parr 6400 Bomb Calorimeter).

Statistical analysis

Daily DM intake and milk yield data, weekly milk composition and live-weight data, and fortnightly BCS data, were averaged over the 10 week experimental period, and mean data analysed. Similarly, nutrient utilisation data was averaged for the six day measurement period, while methane production data was averaged for the two day measurement, with mean data analysed. Data was analysed using ANOVA, with the model including the following terms as fixed effects: Covariate + Treatment. In addition, polynomial contrasts of order two were fitted to the treatment effects to test the data for linear and quadratic effects associated with increasing FB inclusion levels. Within the analysis the following pre-experimental measures (recorded during the

week period prior to the start of the experiment) were included in the model as co-variates when analysing the corresponding dependant variables: pre-experimental DMI, milk yield, milk fat content, milk protein content, milk lactose content, milk fat yield, milk protein yield, milk fat-plus-protein yield, live-weight and BCS. Data were analysed using GenStat Version 16.2 (VSN International, Oxford, UK).

Results

The silage offered had a DM content of 297 g/kg and a crude protein content of 144 g/kg DM, and was well fermented (Table 2). As planned, the FB0 and FB4.7 concentrates had similar crude protein (217 and 219 g/kg DM, respectively) contents and relatively similar starch (300 and 326 g/kg DM, respectively) contents (Table 2).

Field bean inclusion had no effect (neither linear nor quadratic: $P > 0.05$) on either silage DM intake or total DM intake (Table 3). Similarly, none of milk yield, milk fat content, milk lactose content, milk fat yield, milk protein yield or milk fat + protein yield were affected by FB inclusion ($P > 0.05$). However, there was a trend (linear: $P = 0.081$) for milk protein content to decrease with increasing level of FB inclusion. Average live-weight over the experimental period decreased with increasing FB inclusion level (linear; $P < 0.01$) while live-weight change tended to follow a quadratic trend ($P = 0.076$). Average BCS was unaffected by FB inclusion level, while BCS change also followed a quadratic trend ($P = 0.076$). Mean plasma urea concentrations tended to increase (linear; $P = 0.078$) with increasing levels of FB inclusion.

Field bean inclusion had no effect (either linear or quadratic: $P > 0.05$) on either milk yield or DM intake recorded during the nutrient utilisation study, or on the digestibility coefficients for DM, organic matter, GE, ADF or NDF (Table 4). However, there was a linear decrease in N digestibility ($P < 0.041$) with increasing FB inclusion levels.

Faecal N output tended ($P = 0.067$) to decrease with FB inclusion (Table 5), with this reflected in the decrease in faeces N/N intake ($P < 0.042$). There was a quadratic effect ($P < 0.021$) on milk N/N intake with increasing FB inclusion. None of the other N utilisation parameters were affected by FB inclusion level in the diet.

There was a quadratic relationship ($P < 0.037$) between urinary energy excretion and FB inclusion (Table 6). None of the energy utilisation coefficients presented (DE/GE, ME/GE, heat production/ME, Milk energy/ME, retained energy/ME) were affected by FB inclusion level in the diet ($P > 0.05$).

Total CH_4 production (g/day) was unaffected by FB inclusion level ($P > 0.05$; Table 7). Similarly, none of CH_4 production as a proportion of nutrient intake or milk production, or CH_4 energy as a proportion of GEI, DEI and MEI, were affected by FB inclusion ($P > 0.05$).

Discussion

The objective of this study was to examine the impact on cow performance and nutrient utilisation of partially replacing conventional 'protein' ingredients in dairy cow concentrates with FB. The FB4.7 concentrate contained 473 g FB per kg concentrate, with FB replacing approximately 74% of the soya bean meal, 53% of the rapeseed meal and 62% of the maize gluten in the concentrate. In addition, wheat and maize meal inclusion levels in the FB4.7 concentrate were reduced by approximately 69% and 38% respectively, compared to the FB0 concentrate. The FB0 and FB4.7 concentrates were formulated to have similar crude protein contents, with this achieved (crude protein of 217 and 219 g/kg DM. While it was not possible to fully balance the starch contents with the two concentrates, these were relatively similar (300 and 326 g/kg DM, respectively). Concentrate feed levels with all treatments were 10.0 kg/cow/day, with this representing FB intakes of 0, 1.7, 3.1 and 4.7 kg/day with treatments FB0, FB1.7, FB3.1 and FB4.7, respectively. With the exception of Ingalls et al. (1974), which involved a FB intake of 4.3 kg DM/cow/day, no other dairy cow study appears to have examined the impact of FB inclusion at levels as high as adopted in the current study.

Cow performance

Field beans are known to contain a large number of anti-nutritional factors, some of which (for example tannins and trypsin inhibitors) are known to have a negative impact on intakes (Dvořák et al., 2006). For this reason, caution is often adopted when including FB in dairy cows diets. However, while relatively few studies have examined the impact of FB on dairy cows intakes, in the majority of these (Ingalls et al., 1974; Brunschwig and Lamy, 2002; Trommenschlager et al., 2003; Tufarelli et al., 2012; Ramin et al., 2017) intakes were unaffected by FB inclusion, in agreement with the results of the current experiment. In contrast, Puhakka et al. (2016) observed a linear reduction in silage DM intake when the rapeseed meal component of the concentrate was either partially or completely replaced by FB. For example, at a FB DM intake of

3.4 kg/day, silage DM intakes were reduced by 2.2 kg/day compared to diets without FB inclusion (mean data for primiparous and multiparous cows). However, this reduction was not attributed to the presence of anti-nutritional factors in FB, but rather a consequence of an improved supply of essential amino acids with the rapeseed meal treatments (driving milk yield, and as a consequence intakes) and a lower NDF digestibility with the FB treatments..

The effect of FB inclusion in dairy cow diets on milk production and composition has been examined in a series of on-farm studies in Italy (Mordenti et al., 2007; Comellini et al., 2009; Volpelli et al., 2010; Tufarelli et al., 2012), with FB levels in these studies not normally exceeding 10% of the concentrate component of the diet. In the majority of these studies (with the exception of Mordenti et al., 2007), milk yield was unaffected by FB inclusion in the diet. However, Puhakka et al. (2016) and Ramin et al. (2017) observed a reduction in milk yield when replacing rapeseed meal with FB, with this associated with lower intakes in the former study. In contrast, Ingalls et al. (1974) and Brunschwig and Lamy (2002), in studies involving maximum FB intakes of approximately 4.3 and 3.1 kg DM/cow/day, respectively, found milk yield to be unaffected by FB inclusion, in agreement with the outcomes of the current study. However, it is important that the results of the current study are considered within the context of the total diet crude protein levels used (approximately 174 g/kg DM), and the fact that the FB4.7 concentrate did contain some high quality protein. Thus, given the stage of lactation of the cows, it is possible that these diets provided an oversupply of metabolisable protein, and as such decreased the sensitivity to detect relatively small changes in milk production. Nevertheless, Puhakka et al. (2016) did examine the response to replacing rapeseed meal with FB at two diet crude protein levels (namely 15.4 and 19.0 % of DM), and found no interaction between total diet protein levels and FB inclusion level in the diet.

The effect of FB inclusion on milk composition is inconsistent within the literature. For example, milk fat content was unaffected by FB inclusion in a number of studies (Comellini et al., 2009; Volpeli et al., 2010; Volpelli et al., 2012; Puhakka et al., 2016; Ramin, et al., 2017), in agreement with the outcomes of the current study. However, FB inclusion has been found to increase milk fat in some studies (Ingalls et al., 1974; Mordenti et al., 2007) while tending to reduce it in another ($P = 0.09$: Tufarelli et al., 2012). The increased milk fat content observed by Ingalls et al. (1974) occurred when

FB levels increased from 2.18 to 4.34 kg/day, with these authors suggesting that this may have been due to the increased diet fibre contents with FB inclusion, as reflected in an increase in the acetate: propionate ratio in the rumen fluid. While milk protein content was not affected by FB inclusion in a number of studies (Ingalls et al., 1974; Comellini et al., 2009; Tufarelli et al., 2012; Volpelli et al., 2012), there was a trend for milk protein content to decrease in the current study, in agreement with the reduction in milk protein contents observed by Puhakka et al. (2016). While the reason for the inconsistency of the milk protein response to FB inclusion across studies is unclear, it does not appear to be linked to FB inclusion level, but rather may be due to the composition of the basal diet, perhaps reflecting an undersupply of amino acids such as methionine, which FB is low in.

There were trends for quadratic relationships for both live weight change and BCS change, with increasing FB inclusion, although there were no obvious reasons for these effects. Few studies examining the impact of FB inclusion on cow performance have presented data on body tissue reserves. Both Ingalls et al. (1974) and Tufarelli et al. (2012) found live weight change to be unaffected, while plasma non-esterified fatty acids concentrations, an indicator of body tissue breakdown, was unaffected by FB inclusion in either the latter study, or the study by Puhakka et al. (2016). The magnitude of the changes in the current study were relatively small.

Nutrient utilisation

While Melicharova et al. (2009) stated that the anti-nutritional substances present in FB can negatively influence digestion of individual nutrients; there is relatively little information available on the impact of FB inclusion in the diet on ration digestibility and nutrient utilisation. In two on-farm studies Volpelli et al. (2010; 2012) used faecal grab samples as an ‘empirical indicator of digestibility’, and found that neither faecal scores or the undigested faecal fractions differed between cows offered diets either with or without FB. However, maximum FB levels in these studies were limited to 10% of the concentrate component of the diet. Puhakka et al. (2016) replaced rapeseed meal with FB in the concentrate component of the diet, and found that the apparent digestibility of DM, OM, N and starch increased ($P<0.1$), while NDF digestibility decreased. These authors attributed the increasing digestibility of DM, OM, N and starch to the lower proportion of silage in the diets of cows offered FB. They also suggested that the fall in NDF digestibility may have been due to the fermentation products arising from FB

carbohydrates having a negative effect on cellulolytic bacteria activity. Within the current study, with the exception of N, none of the other digestibility coefficients were affected by FB inclusion level. Thus the current study provided little evidence that increasing FB level had a detrimental effect on ration digestibility. Indeed, there is little *in vivo* evidence that nutrient utilisation is impaired by ANF in FB. This was highlighted in a study in which cow performance was unaffected by FB variety, even though the FB varieties offered contained either high or low levels of ANF (Melicharova et al., 2009). Nevertheless, Larsen et al. (2009) observed that the digestibility of legume starch in both the rumen and small intestine of lactating dairy cows, was lower than for barley or wheat starch, and suggested that this was likely due to the different types of starch granules present, and their differing susceptibility to amylase hydrolysis. Similarly, while FB starch is highly degradable (Van Straalen and Tamminga, 1990), it is in general less degradable than barley or wheat starch (Korczynski, 1997; Offner et al., 2003).

In common with Puhakka et al. (2016), apparent N digestibility increased and faecal N/N intake decreased with FB inclusion in the current study. However, in contrast to Ramin et al. (2017) and Puhakka et al. (2016), N use efficiency (milk N/N intake) in the current study decreased with FB inclusion, with this reflected in a trend for retained N/N intake to increase with FB inclusion. These effects were reflected in a trend for plasma urea levels to increase with FB inclusion. However, within the literature the effects of including FB in dairy cow diets on urea concentrations in blood and milk have been inconsistent. For example, Puhakka et al. (2016) found milk urea to increase when the rapeseed component of the diet was replaced by FB, while Volpelli et al. (2012) found a similar effect for blood urea when FB partially replaced soya bean meal. In contract, Tufarelli et al. (2012) found cows offered diets containing FB to have lower blood and milk urea concentrations compared to those containing soya bean meal, and attributed this to the lower degradability FB protein. Similar effects on milk urea (Volpelli et al., 2010), and milk and blood urea (Comellini et al., 2009) have been observed, although these studies involved heat processed (flaked) FB. The protein fraction in FB consists largely of albumins and globulins (Van Straalen and Tamminga, 1990) with FB protein having a higher effective degradability in the rumen than protein from soya bean meal and rapeseed meal (Ewing, 1997). While heat treatments of beans can increase the proportion of protein that by-passes digestion in the rumen

(Yu et al., 2004), drying the FB at 80°C to remove excessive moisture, as occurred in this experiment, is unlikely to have impacted on degradability. Thus in general, although not always, when isonitrogenous diets are offered, blood and milk urea level increase when FB replaced a less degradable protein source.

While none of the energy utilisation parameters were affected by FB inclusion, given the global concerns around climate change, and the significant contribution of agriculture to greenhouse gas (GHG) emissions, the impact of any change in ruminant diets on CH_4 emissions needs to be considered. There is evidence that the presence of tannins in feeds can restrict CH_4 production, and indeed the use of legumes have been advocated as a means of mitigating CH_4 production (Benchaar et al., 2001). However, previous research has demonstrated that CH_4 emissions from cows offered diets containing different protein sources, either soya bean meal or rapeseed meal, did not differ (Gidlund et al., 2015). While the fermentation characteristics of FB and soya bean meal based diets have been compared using *in vitro* gas production techniques, with FB based diets having a higher CH_4 production (Guglielmelli et al., 2010), few studies have examined the impact of FB inclusion in the diet on CH_4 production *in vivo*. In one exception involving lactating dairy cows, CH_4 production per g DMI was unaffected when either barley or rapeseed expeller based concentrates were replaced by concentrates containing FB (Ramin et al., 2017). Thus the results of the current study are consistent with those of Ramin et al. (2017), with no evidence that CH_4 emission parameters were affected by FB inclusion level. While it is expected that CH_4 emissions will be reduced when carbohydrate is replaced by protein (Bannink et al., 2006), in the current study both protein and carbohydrate sources were replaced by FB.

Conclusions

The results of this study indicate that field beans (at inclusion rates of up to 4.73 kg/cow per day) can partially replace conventional protein ingredients such as soya bean meal and rapeseed meal in the diets of dairy cows. However, increasing FB inclusions were associated with a trend for a decrease in milk protein concentration, lower mean live-weights, a trend for a lower milk N/N intake, and associated higher blood urea nitrogen levels. As these trends were observed with diets containing relatively high protein levels, caution is required when adopting high FB levels, especially with low protein diets.

References

Bannink, A., Kogut, J., Dijkstra, J., France, J., Kebreab, E., Van Vuuren, A. & Tamminga, S. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. *Journal of Theoretical Biology,* 238**,** 36-51.

Benchaar, C., Pomar, C. & Chiquette, J. 2001. Evaluation of dietary strategies to reduce methane production in ruminants: a modelling approach. *Canadian Journal of Animal Science,* 81**,** 563-574.

Brunschwig, P. & Lamy, J. 2002. Utilisation de féverole ou de tourteau de tournesol comme sources protéiques dans l'alimentation des vaches laitières. *Renc. Rech. Ruminants,* 9**,** 316.

Chen, X.J and Yan, T. 2015. Development of enteric methane emission factors for Holstein-Friesian and Norwegian first lactation cows. *Acta Agriculturae Scandinavica, Section A - Animal Science,* 65, 127 - 130.

Comellini, M., Antonella Volpelli, L., Pietro Lo Fiego, D. & Scipioni, R. 2009. Faba bean in dairy cow diet: effect on milk production and quality. *Italian Journal of Animal Science,* 8**,** 396-398.

Crépon, K., Marget, P., Peyronnet, C., Carrouée, B., Arese, P. & Duc, G. 2010. Nutritional value of faba bean (Vicia faba L.) seeds for feed and food. *Field Crops Research,* 115**,** 329-339.

DvořÁk, R., Pechová, A., Pavlata, L., Klejdus, B., KovařČík, K., Dostálová, J., Culková, J., Filípek, J., Svajdlenka, E. & Capkova, V. 2006 Reduction in the content of antinutritional substances in Fava beans (Vicia faba) by different treatments. Slovenian Veterinary Research, 43, 174-179

Edmonson, A., Lean, I., Weaver, L., Farver, T. & Webster, G. 1989. A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science,* 72**,** 68-78.

Ewing, W.N. 1997. *The Feeds Directory. Volume 1: Commodity Products Guide*, Context Publications, Leicestershire, England.

Gidlund, H., Hetta, M., Krizsan, S., Lemosquet, S. & Huhtanen, P. 2015. Effects of soybean meal or canola meal on milk production and methane emissions in lactating dairy cows fed grass silage-based diets. *Journal of Dairy Science,* 98**,** 8093-8106.

Guglielmelli, A., Calabrò, S., Cutrignelli, M., Gonzalez, O., Infascelli, F., Tudisco, R. & Piccolo, V. 2010. In vitro fermentation and methane production of fava and soy beans. In: Energy and protein metabolism and nutrition, 3rd EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Parma, Italy, 6-10 September 2010. EAAP Scientific Series, Volume 127. Pp 457-460

Ingalls, J. & Mckirdy, J. 1974. Faba bean as a substitute for soybean meal or rapeseed meal in rations for lactating cows. *Canadian Journal of Animal Science,* 54**,** 87-89.

Korczynski, W. 1997. Rumen degradability of concentrate protein, amino acids and starch, and their digestibility in the small intestine of cows. *Journal of Animal and Feed Sciences,* 6**,** 451-470.

Larsen, M., Lund, P., Weisbjerg, M. R. & Hvelplund, T. 2009. Digestion site of starch from cereals and legumes in lactating dairy cows. *Animal Feed Science and Technology,* 153**,** 236-248.

McCleary, B.V., Solah, V. and Gibson, T.S. 1994. Quantitative measurement of total starch in cereal flours and products. Journal of Cereal Science, 20: 51 - 58.

Melicharová, V., Pechová, A., Dvořák, R. & Pavlata, L. 2009. Performance and Metabolism of Dairy Cows Fed Bean Seeds (Vicia faba) with Different Levels of Anti-Nutritional Substances. *Acta Veterinaria Brno,* 78**,** 57-66.

Mogensen, L., Vestergaard, J. S., Frette, X., Lund, P., Weisbjerg, M. R. & Kristensen, T. 2010. Effect of toasting field beans and of grass-clover: Maize silage ratio on milk production, milk composition and sensory quality of milk. *Livestock Science,* 128**,** 123-132.

Mordenti, A., Merendi, F., Fustini, M. & Formigoni, A. 2007. Effects of different protein plants in cows diet on milk for Parmigiano Reggiano production. *Italian Journal of Animal Science,* 6**,** 463-465.

Offner, A., Bach, A. & Sauvant, D. 2003. Quantitative review of in situ starch degradation in the rumen. *Animal Feed Science and Technology,* 106**,** 81-93.

Oltenacu, P. A. & Broom, D. M. 2010. The impact of genetic selection for increased milk yield on the welfare of dairy cows. *Animal welfare,* 19**,** 39-49.

PGRO. 2017. *2017 Pulse, Recommended Lists, Combinig peas, Spring and Winter Beans* [Online]. Available: http://www.pgro.org/images/site/2017-RL/rec-list-2017a.pdf [Accessed 09/01/2017 2017].

Puhakka, L., Jaakkola, S., Simpura, I., Kokkonen, T. & Vanhatalo, A. 2016. Effects of replacing rapeseed meal with fava bean at 2 concentrate crude protein levels on feed intake, nutrient digestion, and milk production in cows fed grass silage–based diets. *Journal of Dairy Science,* 99**,** 7993-8006.

Ramin, M., Höjer, A., Fogelberg, F., Hetta, M. 2017. The effects of legume seeds on the lactation performance of dairy cow fed grass silage-based diets. Agricultural and Food Science, 26: 129 - 137.

Trommenschlager, J., Thénard, V., Faurié, F. & Dupont, D. 2003. Effets de différentes sources de complémentation azotée sur les performances de vaches laitières Holstein et Montbéliardes et les aptitudes à la coagulation des laits Effects of various sources of nitrogenous supplementation on the performances of Holstein and Montbéliarde dairy cows and the coagulation properties of milk. *Renc. Rech. Ruminants,* 10**,** 382.

Tufarelli, V., Khan, R. U. & Laudadio, V. 2012. Evaluating the suitability of field beans as a substitute for soybean meal in early-lactating dairy cow: Production and metabolic responses. *Animal Science Journal,* 83**,** 136-140.

Van Straalen, W. & Tamminga, S. 1990. In (Wiseman. J.; Cole. D. J. A, eds *Protein degradability in ruminant diets*. *Feed evaluation.* Butterworth/Heinemann., Butterworths, University of Nottingham, Guildford, UK

Volpelli, L. A., Comellini, M., Masoero, F., Moschini, M., Fiego, D. P. L. & Scipioni, R. 2010. Faba beans (Vicia faba) in dairy cow diet: effect on milk production and quality. *Italian Journal of Animal Science,* 9**,** e27.

Volpelli, L.A., Comellini, M., Gozzi, M., Masoero, F. and Moschini, M., 2012. Pea (Pisum sativum) and faba beans (Vicia faba) in dairy cow diet: effect on milk production and quality. *Italian Journal of Animal Science*, *11*(2), p.e40.

Yu, P., Tamminga, S., Egan, A. & Christensen, D. 2004. Probing equivocal effects of heat processing of legume seeds on performance of ruminants: a review. *Asian-australasian journal of animal sciences,* 17**,** 869-876.

Table 1 Ingredient composition (g/kg, fresh weight) of the concentrates offered with FB0 and FB4.7

	Concentrate type	
	FB0	FB4.7
Field beans	0	473
Soya bean meal	181	48
Rapeseed meal	102	48
Maize gluten feed	71	24
Soya hulls (toasted)	142	118
Maize meal	250	155
Wheat	175	55
Molaferm[1]	40	40
Palm oil	10	10
Limestone ($CaCO_3$)	10	10
Salt	8	8
Calcined magnesite	7	7
Vitamin and mineral mix[2]	4	4

[1]United Molasses, Belfast, UK, [2]Superdairy, Trouw Nutrition, Belfast UK

Table 2 Chemical composition of the grass silage and the FB0 and FB4.7 concentrate offered during the 10-week experimental period

			Concentrate			
	Grass silage	(s.d)	FB0	(s.d)	FB4.7	(s.d)
Dry matter (g/kg)	297	38.6	888	7.2	876	8.2
Crude protein (g/kg DM)	144	7.2	217	1.8	219	1.3
Ash (g/kg DM)	97	2.6	72	0.7	67	0.9
Acid detergent fibre (g/kg DM)	295	8.5	138	4.9	143	2.2
Neutral detergent fibre (g/kg DM)	520	16.8	254	8.8	243	2.4
Starch (g/kg DM)			300	8.9	326	5.7
pH	3.95	0.15				
Lactic acid (g/kg DM)	101	11.2				
Ammonia N (g/kg total N)	95	13.7				
Metabolisable energy (MJ/kg DM)	11.23	0.35				

Table 3 Effects of field bean inclusion level in the concentrate component of the diet on mean cow performance during the 10-week experimental period

	Treatment					P-Value	
	FB0	FB1.7	FB3.3	FB4.7	S.E.M	Linear	Quadratic
Silage DMI (kg/day)	12.8	12.9	13.1	12.7	0.24	0.98	0.24
Concentrate DMI (kg/day)	8.9	8.9	8.8	8.8			
Total DMI (kg/day)	21.7	21.8	21.8	21.5	0.25	0.62	0.29
Milk yield (kg/day)	28.0	29.0	27.4	28.0	0.63	0.57	0.78
Milk fat (g/kg)	43.5	44.6	45.1	44.5	0.93	0.38	0.38
Milk protein (g/kg)	34.9	35.2	34.9	34.1	0.35	0.08	0.12
Milk lactose (g/kg)	46.2	46.2	46.7	46.4	0.21	0.21	0.60
Fat yield (kg/day)	1.12	1.23	1.22	1.21	0.049	0.24	0.24
Protein yield (kg/day)	0.96	1.00	0.94	0.93	0.024	0.21	0.35
Fat + protein yield (kg/day)	2.10	2.25	2.14	2.12	0.063	0.86	0.18
Average live-weight (kg)	647	647	644	631	4.1	0.010	0.11
Live-weight change (kg) [1]	21	37	38	31	6.7	0.31	0.08
Average body condition score	2.44	2.44	2.43	2.43	0.020	0.62	0.87
Body condition score change [1]	-0.03	0.12	0.06	-0.02	0.061	0.90	0.07
Plasma urea (mmol/L) [2]	2.97	3.31	3.23	3.42	0.157	0.078	0.61

[1] Mean change over the entire experimental period
[2] Mean composition of blood samples collected at weeks 6 and 10 of the experiment

Table 4 Effects of field bean inclusion level in the concentrate component of the diet on dry matter intake and milk yield during the nutrient utilisation study, and on total ration digestibility coefficients

	Treatment					P value	
	FB0	FB1.7	FB3.3	FB4.7	S.E.M	Linear	Quadratic
Silage DM intake (kg/day)	11.1	11.7	10.8	12.0	0.62	0.57	0.67
Total DM intake (kg/day)	20.0	20.5	19.6	20.6	0.62	0.69	0.67
Milk yield (kg/day)	25.7	25.6	25.0	24.9	0.98	0.53	0.99
Digestibility coefficients (g/g)							
Dry matter	0.800	0.781	0.813	0.796	0.0073	0.55	0.82
Organic matter	0.820	0.798	0.827	0.811	0.0073	0.96	0.64
Nitrogen	0.719	0.694	0.746	0.732	0.0091	0.041	0.52
Gross energy	0.789	0.765	0.799	0.780	0.0079	0.81	0.76
ADF	0.715	0.659	0.712	0.687	0.0131	0.60	0.26
NDF	0.734	0.691	0.736	0.714	0.0121	0.79	0.40

ADF, Acid detergent fibre: NDF, Neutral detergent fibre

Table 5 Effect of field bean inclusion level in the concentrate component of the diet on N intake, N output and N utilisation

	Treatment					P-Value	
	FB0	FB1.7	FB3.3	FB4.7	S.E.M	Linear	Quadratic
N intake and output (g/day)							
Total N intake	550	561	539	563	13.9	0.80	0.64
Digestible N intake	395	390	402	413	12.6	0.29	0.54
Faeces N	155	171	137	151	5.2	0.067	0.79
Urine N	200	172	192	198	11.6	0.76	0.17
Manure N	354	343	329	349	14.0	0.64	0.90
Milk N	140	151	140	133	4.8	0.16	0.10
N utilisation (g/g)							
Faeces N/N intake	0.281	0.306	0.254	0.267	0.0091	0.042	0.54
Urine N/N intake	0.363	0.306	0.356	0.353	0.0152	0.79	0.10
Manure N/N intake	0.644	0.611	0.609	0.620	0.0153	0.30	0.18
Milk N/N intake	0.256	0.269	0.260	0.236	0.0070	0.047	0.021
Faeces N/Manure N	0.437	0.501	0.419	0.432	0.0153	0.18	0.12
Urine N/Manure N	0.563	0.499	0.581	0.568	0.0153	0.18	0.12

Table 6 Effect of field bean inclusion level in the concentrate component of the diet on energy intake, energy output and energy utilisation

	Treatment					P value	
	FB0	FB1.7	FB3.3	FB4.7	S.E.M	Linear	Quadratic
Energy intake and output (MJ/day)							
GE intake	360	368	351	372	10.9	0.72	0.56
Faecal Energy	76	86	70	82	3.0	0.93	0.79
DE intake	284	282	281	290	9.9	0.71	0.57
CH_4 energy	29	27	26	28	1.3	0.56	0.31
Urinary energy	16	14	14	16	0.8	0.61	0.037
Milk energy	90	90	85	85	3.6	0.30	0.99
ME intake	243	243	242	248	8.4	0.72	0.70
Heat production	159	162	160	160	8.5	0.99	0.80
Retained energy	-5	-10	-3	3	8.0	0.41	0.50
Energy utilisation (MJ/MJ)							
DE/GE	0.789	0.766	0.800	0.781	0.0080	0.80	0.79
ME/GE	0.676	0.660	0.689	0.668	0.0067	0.84	0.73
Heat production/ME	0.652	0.673	0.658	0.642	0.0260	0.71	0.50
Milk energy/ME	0.369	0.372	0.350	0.344	0.0162	0.21	0.77
Retained energy/ME	-0.021	-0.045	-0.008	0.014	0.0340	0.38	0.51

GE, gross energy; DE, digestible energy; ME, metabolisable energy

Table 7 Effect of field bean inclusion level in the concentrate component of the diets on methane production

	Treatment					P value	
	FB0	FB1.7	FB3.3	FB4.7	S.E.M	Linear	Quadratic
CH_4 (g/day)	516	492	476	501	23.2	0.56	0.31
CH_4/feed intake or milk yield (g/kg)							
CH_4/DMI	26.0	24.0	24.4	24.4	0.76	0.21	0.23
CH_4/OM intake	28.2	26.1	26.4	26.5	0.80	0.19	0.20
CH_4/milk yield	20.2	19.3	19.0	20.3	1.25	0.98	0.40
CH_4/ECM yield	17.9	17.0	17.4	18.3	0.92	0.72	0.36
CH_4-E/energy intake (MJ/MJ)							
CH_4-E/GEI	0.079	0.074	0.075	0.075	0.0023	0.23	0.26
CH_4-E/MEI	0.117	0.112	0.108	0.112	0.0029	0.14	0.16

DMI, dry matter intake; OM, organic matter; ECM, energy corrected milk; GEI, gross energy intake; DEI digestible energy intake; MEI, metabolisable energy intake

Experiment 2

The impact of field bean inclusion in the diet of early lactation dairy cows on performance and nutrient utilisation

Introduction

The increasing milk yield potential of dairy cows within many European countries has led to a requirement for more nutrient dense diets. Increased nutrient density has often been achieved through the adoption of higher concentrate feed levels, and this in turn has increased the demand for high quality protein ingredients. However, European agriculture has a significant deficit of high quality protein feedstuffs (Watson et al., 2018). This is a particular problem within the United Kingdom **(UK)** where there is considerable interest in making increased use of locally grown protein ingredients (Wilkins et al., 2000). One protein crop of particular interest, especially within the cooler wetter regions of the UK, is field bean (FB: *Vicia Faba)*.

While FB have a moderate crude protein **(CP)** content (280 g/kg DM), they have a relatively high starch content (400 g/kg DM) (Ewing, 1997), and as such might appear to be ideal in ruminant diets. However, the inclusion of FB in ruminant diets is normally limited due to the perceived risk associated with anti-nutritional substances, including trypsin inhibitors, tannins, lectins, and protease inhibitors, which can reduce DM intakes and animal performance (Dvořák et al., 2006). In addition, FB contain a number of phytoestrogens which are known to have negative effects on reproductive performance (Zdunczyk et al., 2005), and concerns about their presence have also contributed to the limited inclusion of FB in dairy cow diets.

The use of FB in dairy cow diets has been examined in a number of studies, with the findings sometimes conflicting. For example, while neither DM intake nor milk yield were affected by FB inclusion in studies by Ingalls et al. (1974) and Johnston et al. (2019), Ramin et al. (2017) observed a reduction in milk yield with FB inclusion. In contrast, Puhakka et al. (2016) found both DM intake and milk yield to be reduced when rapeseed meal was replaced by FB.

While these studies have demonstrated that in some circumstances FB can be included in dairy cow diets at levels between 4.7 and 5.0 kg/d (Johnston et al., 2019: Ingalls et al., 1974) with no adverse effects on DM intakes and milk production, higher inclusion levels do not appear to have been examined previously. Given the high starch content of FB, which suggests it could replace both conventional protein and energy components of the diet, the impact of higher FB inclusion levels needs to be examined. In addition, as highlighted by Tufarelli et al. (2012), there is limited

information on the impact of FB inclusion in the diet of early lactation dairy cows, the period which encompasses the breeding season. This is important given concerns about the presence of estrogenic compounds in FB. Consequently, the current study was designed to examine the effects on cow performance, nutrient utilisation efficiency and fertility when early lactation dairy cows were offered diets containing higher levels of FB than examined previously. It was hypothesised that these parameters would be unaffected by these higher levels of FB inclusion.

Methodology

This experiment was conducted at the Agri-Food and Biosciences Institute **(AFBI),** Hillsborough, Northern Ireland **(NI).** All experimental procedures were conducted under an experimental licence granted by the Department of Health, Social Services & Public Safety for NI in accordance with the Animals (Scientific Procedures) Act 1986.

Animals and housing

This continuous design 20-wk experiment involved 72 Holstein-Friesian dairy cows, 33 primiparous and 39 multiparous; mean parity, 2.07 (s.d., 1.19). During the three wk period prior to calving, cows had *ad libitum* access to grass silage, while a dry-cow mineral/vitamin mix and calcined magnesite (Trouw Nutrition, Cheshire, UK) were mixed with the silage to achieve a target intake of 100 g/cow per d and 50 g/cow per d, respectively. Cows were moved to a straw bedded maternity pen approximately 24 - 48 h prior to calving, based on behavioural observations. Following calving (normally within 24 h) cows were transferred to an experimental free-stall house with solid concrete floors where they had access to cubicles fitted with rubber mats, which were bedded with sawdust twice daily. The floor was scraped every three h using an automated slurry scraper system.

Treatments

Cows were randomly allocated to one of three treatments at calving (FB0, FB-Low, FB-High), with primiparous and multiparous cows allocated separately. A check was made to ensure each treatment group remained balanced for parity, BW and BCS at drying off, and in the case of multiparous cows, for previous lactation 305 d milk yield, and milk fat and protein content.

Treatments differed in concentrate type offered (ingredient composition in Table 1). The concentrate offered with FB0 contained no FB, while concentrates offered with FB-Low and FB-High contained 349 and 698 g FB/kg (fresh basis), respectively. The FB used (*Vicia Faba. Var. Fuego*) were obtained from a spring sown crop grown in NI, which was harvested on 28th September 2016, and then dried at 80^{o}C for approximately 2 h to achieve a moisture content of approximately 14%. The experimental concentrates (in the form of a meal) were mixed with grass silage (forage : concentrate ratio of 45 : 55 on a DM basis), together with 320 g/cow per d of straw and 50 g/cow per d of a rumen buffer (Celtic sea minerals, Cork, Ireland), and offered in the form of a mixed ration. The grass silage component of the diet was produced from a primary growth (approximately 75% of cow feeding days **(CFD)**) and a primary regrowth (approximately 25% of CFD) of perennial ryegrass (Lolium Perenne) based swards. The rations were prepared and offered daily at approximately 09.00 h (at proportionally 1.07 of the previous day's intake), with uneaten ration removed the following day at approximately 08.00 h. Rations were prepared using a feeder wagon (Vari-Cut 12, Redrock, Armagh, NI). The total silage requirement for all three treatments was initially mixed for approximately 4 min and then deposited on a clean silo floor. Silage for each individual treatment was then removed from this 'pile', placed back in the feeder wagon, and the appropriate quantities of concentrate, straw and buffer added to the mix, and mixing continued for another 6 min. The rations were then transferred from the wagon to a series of feed boxes mounted on weigh scales, with cows accessing feed in these boxes via an electronic identification system, thus enabling individual cow intakes to be recorded daily (Bio-Control Feeding System, Bio-Control, Rakkestad, Norway). Cows had free access to fresh water at all times. In addition, all cows were offered 1.0 kg per d of soya hulls via an in-parlour feeder during milking (0.5 kg at each milking). Cows remained on the experiment until d 140 of lactation.

Cow measurements

During the experiment cows were milked twice daily (between 05.00 and 07.00 h, and between 15.00 and 17.00 h) using a 50-point rotary milking parlour (Boumatic, Madison, Wisconsin, USA), with milk yields recorded automatically at each milking, and a total daily milk yield for each cow for each 24-h period calculated. Milk samples

were taken during two consecutive milkings each wk and analysed for fat, protein, and lactose using an infrared milk analyser (Milkoscan Model FT+; Foss Electric, Hillerød, Denmark), and a weighted concentration of each constituent determined for the 24-h sampling period. A single milk sample was collected for progesterone analysis from each cow on two occasions each wk (Monday and Thursday pm) until 60 d postpartum. Samples were preserved and stored as above (for a maximum of 3 wk) with progesterone concentrations subsequently determined using a competitive enzyme-linked immuno-sorbent assay (**ELISA**) kit (Ridgeway Science Ltd, Gloucestershire, UK). In addition, at 10 and 20 wk post calving (± 1 wk) a milk sample was taken in proportion to yield during two successive milkings (am and pm), the two samples bulked, and the bulked sample frozen at -20ºC. These samples were subsequently analysed for milk fatty acids **(FA)**, as follows: milk fat was extracted from 1.0 ml of homogenised milk using a chloroform methanol extraction method, and FA as methyl esters **(FAME)** prepared, as described by Bligh and Dyer (1959). The FA composition was determined using gas-liquid chromatography, with an aliquot (1.0 ul) of the FAME extract injected onto a CP Sil88 capillary column (100 meters x 0.25 mm id x 0.2µm film thickness) in a Agilent 7800 gas chromatograph (both Agilent Technologies, Santa Clara, USA), equipped with a temperature programmable injector operated in the split mode and a flame ionisation detector. The oven was initially held at 50ºC for 4 min then ramped at 8ºC/min to 110ºC, then 5ºC/min to 170ºC (hold time 10 min) and finally ramped at 2ºC/min to 225 ºC (hold time 30 min). Fatty acids were identified by their retention time with reference to commercially available fatty acid standards (37 Supelco FAME mix) and individual standards for those not in the mix (SigmaAldrich Co. Ltd., Gillingham, UK), and were quantified using C13 FAME as an internal standard.

Bodyweight was recorded twice daily (immediately after each milking) using an automated weighbridge, and a mean weekly BW for each cow determined. The BCS of each cow was estimated fortnightly according to Edmondson *et al.* (1989) by a trained technician. Faecal scores were assessed at 6 and 12 wks post calving (+/- 1 wk) using a 1 to 5 scale, as described by Ireland-Perry and Stallings (1993). Blood samples were collected prior to feeding from the tail of each cow at 2, 4, 6, 8, 10 and 14 wks (+/- 3 d) post calving. Samples were collected into evacuated tubes (BD, Oxford, UK), which were either coated with a clot activator or fluoride oxalate. The

blood samples were centrifuged (1,800 × *g* at 17°C for 30 min) to obtain serum (tubes with a clot activator) or plasma (fluoride oxalate tubes), which were separated and stored at −20°C until analyzed. Plasma was analyzed for glucose concentrations, whereas serum was analyzed for BHB, non-esterified fatty acids **(NEFA)** and urea concentrations. Plasma glucose concentrations were determined using the hexokinase method (Roche Diagnostics Ltd., Burgess Hill, UK), while serum biochemistry analysis was carried out on a Randox Imola chemistry analyzer system (Randox, County Antrim, UK), using Randox reagent kits (Randox).

A clinical examination of vagina mucus was conducted at 2, 4 and 6 wks postpartum. The cow's vulva was thoroughly cleaned using a disinfected paper towel and then dried. A clean lubricated gloved hand was then inserted through the vulva into the vaginal area. In each cow the lateral, dorsal and ventral walls of the vagina and the external cervical os were palpated, and the mucus contents of the vagina withdrawn manually for examination. The vaginal mucus was assessed and scored by a single operator throughout the course of the study for colour, proportion and volume of pus, and a character score assigned as follows: (0) clear or translucent mucus; (1) mucus containing flecks of white or off-white pus; (2) <50 mL exudate containing ≤50% white or off-white mucopurulent material; and (3) >50 mL exudate containing purulent material, usually white or yellow, but occasionally sanguineous. The vaginal mucus was also assessed for odour, and given a score 0 for normal odour or a score of 1 if a fetid odour was detected. If a cow had a mucus score of 3 (or an elevated body temperature alongside a mucus score of 2), and an odour assessment of 1, intra-uterine antibiotics were administered (Metricure, Intervet/Schering-Plough Animal Health, Walton Manor, Walton, Milton Keynes, MK7 7AJ). Vaginal mucus scores were translated into one integer (0 = 0, 0; 1 = 1, 0; 2 = 2, 0; 3 = 3, 0; 4 = 2, 1; 5 = 3, 1) and were grouped into 3 categories for analysis (0, 1, ≥2).

Nutrient utilisation

On completion of the 20-wk feeding study, four cows from each treatment were selected for use in a nutrient utilisation study, with the cows selected from each treatment group balanced for daily milk yield and BW. Cows were transferred in pairs (pair 1 comprising cows from treatments FB0 and FB-Low, and pair 2 comprising cows from treatments FB-High and FB0, with this pattern repeated for all six pairs) from the

main experimental group into a digestibility unit, with pairs transferred at approximately 09.00 h on Monday or Thursday of each wk. Cows were tied by the neck in individual stalls fitted with a rubber mat, and continued to be offered their experimental rations via a feed box located at the front of each stall. Rations were offered *ad libitum* daily at 09:00 h, at proportionally 1.07 of the previous d intake, while uneaten ration was removed the following day at 08:00 h. Soya hulls was offered (0.5 kg at each milking) via a plastic feed bucket which was placed within the feed boxes during milking (at 06:30 and 16:30 h). Cows had access to fresh water at all times via a drinker located within each stall.

Measurements of nutrient utilisation commenced 24 h after cows were placed in this experimental byre, and comprised a 6-d feeding period, followed 48 h later by a 6-d total faeces and urine collection period. Following collection of faeces and urine on the third d of the six d collection period, each pair of cows was transferred into two indirect open-circuit respiration chambers for measurement of CH_4 production, with each chamber used twice with each treatment to remove any possible chamber effects. Cows remained in the chambers for 72 h, with measurements of CH_4 and CO_2 production recorded during the final 48 h period used in the subsequent analysis. The milking routine, milk sampling, milk analysis, faeces and urine collection methodologies, sample management throughout the 6 d collection period, a description of the respiration chambers, and details of measurements of CH_4 production, as well as a description of the laboratory analysis of all feed, milk, urine and faeces, have been described by Johnston et al. (2019). A mean CH_4 recovery rate of 98.5% was recorded.

Feed Analysis

Samples of the grass silage were taken daily throughout the experiment and dried at 85°C for 24 h to determine oven DM **(ODM)** content. Sub-samples of the dried milled silages were taken three times weekly and bulked for every 14 d, with the bulked sample analysed for NDF, ADF and ash concentrations. A fresh sample of the grass silage was taken weekly and analysed for concentrations of nitrogen **(N),** ammonia-N, lactic acid, acetic acid, ethanol, propanol and gross energy **(GE),** and for pH, while ME content was predicted using near-infrared reflectance spectroscopy. A sample of each experimental concentrate offered (FB0, FB-Low and FB-High) was taken weekly,

bulked for each 2 wk period, and subsequently dried at 85°C for 24 h to determine ODM content. Additional samples of each experimental concentrate were taken weekly, bulked for each 2 wk period and dried at 60°C for 48 h, and analysed for N, NDF, ADF, ash, GE and starch concentrations. Straw and soya hulls offered were sampled weekly, the samples bulked every 4 wk, and the bulked samples dried at 85°C for 24 h to determine ODM content, and dried samples subsequently analysed for N, GE, ADF, NDF and ash content. The laboratory analysis of all feed samples were undertaken as described previously by Purcell et al. (2016).

Statistical analysis

Two primiparous cows from treatment FB-Low did not complete the study due to lameness and very low milk yields, respectively. Mean weekly data for dry matter intake **(DMI)**, milk production, milk composition, milk constituent yields and BW, over the 20 wk experimental period, were analysed using REML repeated measures analysis (autoregressive order 1). The mixed model used included the following terms as fixed effects: Parity (1, 2, 3, >3) + Wk (1 – 20, as the repeated-measures time factor) + FB inclusion level (FB0, FB-Low, FB-High) + Wk × FB inclusion level, while cow was fitted as a random effect. Locomotion score and BCS data were analysed using the same model, except that fortnightly data were used as the repeated-measures time factor. Similarly, milk FA and blood metabolites were analysed using a similar model, except that for the former sampling wk (10 and 20 post calving) was used as the repeated-measure time factor, while with the latter sampling wk (2, 4, 6, 8, 10 and 14 post calving) was used as the repeated-measures time factor. Continuous BW, BCS and fertility data were analysed by ANOVA, with parity included as a covariate. Binomial fertility and health data were analysed using generalised linear model regression analysis using the binomial distribution with a logit link function. The model included treatment as a term, while parity was included as a covariate in the case of mastitis incidence. Mucus scores of 2 and 3 were combined into a single category (2-3), thus giving categories of 0, 1 and 2-3 for analysis. These categorical data were analysed using a Generalised Linear Mixed Model (ordinal logistic regression) with random effects (proportional odds model). The factorial arrangement of Treatment (FB0, FB-Low and FB-High) and sampling wk (wk 6 and 12 for faeces scores, and wk 2, 4 and 6 for mucus scores) were fitted as fixed effects, and cow as a random effect. Significance was identified using chi-squared. Mean nutrient utilisation data over the

6 d measurement period, and mean CH_4 production data over the 2 d measurement period were analysed using ANOVA. All data were analysed using GenStat (Release 18.1; VSN International Limited, Oxford, UK).

Results

The silage offered had an ODM, crude protein and ME content of 293 g/kg, 145 g/kg DM and 11.0 MJ/kg DM, respectively (Table 2). The three experimental concentrates had similar crude protein contents (mean, 223 g/kg DM), while the starch contents of FB0, FB-Low and FB-High concentrates were 291, 313 and 338 g/kg DM, respectively. The straw offered had a crude protein, NDF, ADF and ash content of 46 (s.d., 11.2), 501 (s.d., 9.6), 866 (s.d., 20.7) and 57 (s.d., 13.2) g/kg DM, and a GE content of 18.5 (s.d., 0.23) MJ/kg DM. The respective values for the soya hulls offered were 111 (s.d., 4.3), 492 (s.d., 11.3), 685 (s.d., 29.4), 53 (s.d., 1.1) g/kg DM, and 17.5 (s.d., 0.06) MJ/kg DM, with a starch content of 13.9 (s.d., 7.84) g/kg DM.

None of silage DMI, total DMI, concentrate DMI or milk yield were affected by treatment ($P > 0.05$) (Table 3). Cows on FB0 had a higher milk fat content than those on FB-High ($P = 0.031$), whilst cows on FB0 and FB-Low had a higher milk protein content than those on FB-High ($P < 0.001$). Milk lactose content was not affected by treatment ($P > 0.05$). Milk fat yield ($P < 0.001$), milk protein yield ($P = 0.035$) and milk fat + protein yield ($P = 0.007$) was higher with FB0 than with either of FB-Low or FB-High. All of these DMI and milk production parameters varied with time post calving ($P < 0.001$) (Figure 1 and Figure 2, for DMI and milk yield, respectively), while there was no treatment × time interaction for any of these parameters. The total concentration of SFA in milk fat was lower with FB0 than with either of FB-Low or FB-High ($P = 0.028$), while the reverse was true for the total concentration of MUFA ($P = 0.016$) (Table 3). Treatment had no effect on the total PUFA content of milk fat ($P > 0.05$). Concentrations of C18:0 ($P = 0.002$), C18:1 *cis*-9 ($P = 0.022$) and C18:2 cis-9, trans-11 ($P < 0.001$) decreased with increasing FB inclusion, while concentrations of C16:0 ($P < 0.001$) and C18:3 *n*-3 ($P < 0.006$) increased. Concentrations of C14:0 and C18:2 were not affected by treatment ($P > 0.05$). While total PUFA concentrations ($P < 0.001$), and concentrations of C16:0 ($P = 0.002$), C18:0 ($P < 0.001$), C18:2 ($P < 0.001$) and C18:3 *n*-3 differed between the two sampling periods (time), with the exception of

the latter ($P < 0.001$), there were no treatment × time interactions for any of the FA recorded ($P > 0.05$).

While average BCS was unaffected by treatment ($P < 0.05$), end of study BCS tended to increase with increasing levels of FB inclusion ($P = 0.054$) (Table 4). None of the BW parameters examined (average BW, end of study BW, nadir BW, BW loss to nadir and days to nadir BW), were affected by treatment ($P > 0.05$). While both BCS ($P = 0.046$) and BW ($P < 0.001$) varied with time (Figures 3 and 4, respectively), there were no treatment × time interactions for either ($P < 0.05$). Plasma glucose and BHB concentrations were unaffected by treatment ($P > 0.05$), while both increased with time post calving (Figure 5a, $P = 0.010$: Figure 5b, $P = 0.002$, respectively). Plasma NEFA concentrations were significantly higher with FB0 than FB-High ($P = 0.022$), while plasma urea concentrations followed the reverse trend ($P < 0.001$). While plasma NEFA concentrations decreased with time post calving (Figure 5c: $P < 0.001$), plasma urea concentrations increased (Figure 5d: $P < 0.001$). There were no treatment × time interactions for any of the blood metabolites examined ($P > 0.05$).

While faecal scores differed between wk 6 and 12 post calving (overall SEM = 0.048, $P = 0.012$), treatment had no effect on mean faecal scores (probability of having a score of 1, 2 or $\geq$3; 0.08, 0.47 and 0.45 for FB0; 0.05, 0.42 and 0.53 for FB-Low; 0.07, 0.45 and 0.48 for FB-High: overall SEM = 0.061, $P = 0.878$). There was no interaction between treatment and measurement period (SEM = 0.084, $P = 0.783$). The digestibility of ADF decreased ($P = 0.035$) from FB0 to FB-High, while none of the other digestibility coefficients were affected by FB inclusion level (Table 5). None of total N intake, digestible N intake, or N output in faeces, urine, manure or milk, or any of the N utilisation coefficients examined were affected by treatment ($P > 0.05$: Table 6). Treatment had no effect ($P > 0.05$) on GE intake, digestible energy (DE) intake, ME intake, or on energy output in faeces, urine, CH_4, heat or milk, or on any of the energy utilisation coefficients examined (Table 7). Total CH_4 production, CH_4 as a proportion of DMI, OMI and milk production were unaffected by treatment ($P > 0.05$) (Table 7). Similarly, CH_4-E, as a proportion of GE or ME intake, was unaffected by treatment ($P > 0.05$).

While vaginal mucus scores decreased with wk (2, 4 and 6) post calving (overall SEM = 0.042, $P < 0.001$), treatment had no effect on mean mucus scores (probability of

having a score of 0, 1 or 2-3; 0.70, 0.13 and 0.17 for FB0; 0.71, 0.13 and 0.16 for FB-Low; 0.67, 0.16 and 0.17 for FB-High: overall SEM = 0.047, P = 0.97). There was no interaction between treatment and measurement wk (SEM = 0.072, P = 0.476). Treatment had no effect on the proportion of cows showing commencement of luteal activity **(CLA)** pre d-42 post calving, d to CLA, and peak progesterone content at CLA (P > 0.05) (Table 8). None of conception to first service, to first and second service, d to conception or the proportion of cows pregnant at the end of the breeding season, were affected by treatment (P > 0.05). The proportion of cows with at least one incident of digestive upset, mastitis and lameness, as well as mean locomotion score, were unaffected by treatment (Table 8: P > 0.05).

Discussion

Average daily FB intakes with FB-Low and FB-High were 4.1 and 8.4 kg/cow, with the latter substantially higher than used in any previous study. At the maximum inclusion level adopted (FB-High: 698 g FB/kg concentrate), FB replaced all of the soya bean meal, rapeseed meal, maize gluten feed, and wheat in the concentrate, compared to the control treatment (FB0). While it was not possible to achieve a common starch level with each of the concentrates (291, 309 and 338 g/kg DM for FB0, FB-Low and FB-High concentrates, respectively), all had a similar CP content, resulting in a total diet CP content of 180 g/kg DM across the three diets.

Cow performance

The potential negative impact of anti-nutritional factors within FB on DM intake is often cited by nutritionists as a reason for low inclusion levels in dairy cow diets. In addition, preference trials (Hutson and van Mourik, 1981) have suggested that FB may be unpalatable. However, the current study clearly demonstrated that FB intakes of up to 8.4 kg/cow per d had no negative effects on DM intakes. This is in agreement with most published studies (Ingalls, et al., 1974: Tufarelli et al., 2012; Ramin et al., 2017; Johnston et al., 2019), albeit the maximum daily FB intake in these studies was 5.0 kg/cow per d. In contrast, Puhakka et al. (2016) found intakes to be reduced when FB replaced rapeseed meal in dairy cow diets. These authors suggested that this may have been due a reduction in NDF digestibility, a poorer amino acid profile with FB, and a ‘pull effect’ caused by the higher milk yield with the rapeseed meal treatment.

Although ADF digestibility was reduced with FB inclusion in the current study, this had no effect on DMI.

In common with DMI, milk yield was unaffected by FB inclusion. However, the reduction in both milk fat and milk protein concentrations with FB inclusion meant that yields of fat, protein and fat + protein, were lower with both FB-Low and FB-High, compared to the FB0 treatment. Literature evidence concerning the impact of FB inclusion on milk production and milk composition is inconsistent. For example, both Puhakka et al. (2016) and Ramin et al. (2017), in studies involving maximum FB intakes of 3.7 and 2.6 kg DM/d, respectively, observed a reduction in milk yield when rapeseed meal was replaced by FB. While milk protein content was reduced in the former study, milk fat content was unaffected by FB inclusion in either study. In contrast, at a FB intake of 4.7 kg/d (Johnston et al., 2019), neither milk yield nor milk fat content were affected by FB inclusion level, while milk protein content tended to decrease (P = 0.08). In a number of on-farm studies conducted in Italy (Comellini et al., 2009; Volpelli et al., 2010: Tufarelli et al., 2012; Volpelli et al., 2012) the inclusion of FB in dairy cow diets had no effect on either milk yield or milk fat and protein content, although in a further study (Mordenti et al., 2007) milk yield was reduced but milk fat content was increased with FB inclusion. However, FB inclusion levels in these studies were low, normally less than 2.0 kg/cow per d.

While energy intake is a key driver of milk protein content, the reduction in milk protein content with the FB-Low treatment is unlikely to be driven by energy supply given the similar intakes across all treatments, and the absence of treatment effects on any of the BW or BCS score parameters. Indeed, plasma NEFA concentrations decreased with FB inclusion, while there was a trend for end-of-study BCS to increase with FB inclusion, suggesting an improved energy balance with FB-High. Rather, it is more likely that this reduction was caused by a deficit of specific amino acids. For example, FB is lower in both lysine and methionine than soya bean meal and rapeseed meal (Ewing, 1997), with both of these amino acids important for milk protein synthesis (Schwab et al., 1976). When Puhakka et al. (2016) replaced rapeseed meal with FB, plasma concentrations of lysine and methionine, and total concentrations of essential amino acids were reduced, with these authors suggesting that this was likely partly responsible for the reduction in milk protein content and milk protein yield that they observed.

In the current study field beans replaced, or partially replaced, both maize starch (less degradable than FB starch) and wheat starch (more degradable than FB starch) in the concentrate (Offner et al., 2003), and this likely had an effect on rumen fermentation patterns, and the associated reduction in milk fat content with the FB-High treatment. While this reduction was in contrast to most previous studies, it is proposed that with the FB-High treatment, a reduction in rumen acetate production occurred due to the higher concentrate starch and slightly lower fibre content, and that this was responsible for the observed reduction in milk fat. This is supported to some extent by the lower ADF digestibility observed with this treatment. In addition, there is also some evidence that dietary methionine promotes milk fat synthesis (Hao et al., 2018), and as such the likely deficit of methionine in the diet may have contributed to the reduction in milk fat concentrations.

While the impact of FB inclusion on the fatty acid profile of milk does not appear to have been examined previously, FB inclusion in the current study increased the degree of saturation of the milk produced. This appears to have been largely driven by increasing concentrations of C16:0 in milk, with FB particularly high in C16:0 fatty acids (Grela and Gunter, 1995). Similarly, decreasing concentrations of C18:1 with FB inclusion may reflect the higher concentration of C18:1 in soya bean compared to FB (Grela and Gunter, 1995).

It is possible that the reduction in cow performance observed with FB inclusion could be avoided by supplementing the diet with specific limiting amino acids. Nevertheless, the cow performance data needs to be considered within the context of the total diet CP in the study (180 g/kg DM), a level not unusual with early lactation dairy cows offered grass silage based diets within the UK and Ireland due to the often variable composition of grass silage on offer. However, it is possible that greater differences between treatments may have been observed with lower protein diets, as in general responses to protein source and quality tend to be greater at lower diet protein levels. Nevertheless, Puhakka et al. (2016) observed no interaction between diet protein level (154 vs 190 g/kg DM) and milk production, when partially replacing rapeseed meal with FB.

Nutrient utilisation

Field beans contain a number of anti-nutritional substances, some of which are known to have negative effects on rumen function and digestibility (Newton and Hill, 1983; Dixon and Hosking, 1992). However, faecal scores, a simple proxy for rumen function, was unaffected by treatment. In addition, with the exception of ADF, digestibility coefficients for none of the other parameters examined were affected by FB inclusion. While Johnston et al. (2019) found fibre digestibility to be unaffected with a FB intake similar to that used with the FB-Low treatment, the reduction in ADF digestibility with the highest FB treatment in the current study is similar to the reduction in NDF digestibility observed by Puhakka et al. (2016). These authors attributed this to the readily fermentable FB carbohydrate, and associated fermentation products, decreasing rumen pH and having a negative effect on cellulolytic bacteria activity, rather than the presence of anti-nutritional factors. Puhakka et al. (2016) also found the apparent digestibility of DM, OM, N and starch to increase with FB inclusion, but attributed this to the lower proportion of silage in the diets of cows offered FB, rather than FB inclusion per se. Thus it appears that any effects of FB inclusion on digestibility is likely to be caused by 'other' dietary factors, rather than the presence of anti-nutritional factors. This is supported by the findings of Melicharova et al. (2009), who found cow performance to be unaffected when FB varieties containing either high or low levels of ANF were offered.

While Johnston et al. (2019) observed a reduction in milk N/N intake with FB inclusion, a similar effect was not observed in the nutrient utilisation part of the current study, in agreement with previous findings (Puhakka et al., 2016: Ramin et al., 2017). Nevertheless, the nutrient utilisation phase in the current study involved only four cows per treatment and was undertaken at the end of the 20 wk feeding study. When N use efficiency over the entire study period is examined using treatment mean intake and milk production data (with milk N calculated as milk protein/6.38), milk N/N intake was 0.30, 0.28 and 0.27 for FB-0, FB-Low and FB-High, respectively. The decreasing N use efficiency with increasing FB inclusion levels was reflected in an increase in plasma urea N levels, in agreement with Volpelli et al. (2012), the trend ($P = 0.078$) observed by Johnston et al. (2019), and the increase in milk urea concentrations observed by Puhakka et al. (2016) when the rapeseed component of the diet was replaced by FB. In contrast, other studies (Comellini et al., 2009; Volpelli et al., 2010; Tufarelli et al., 2012) have found both blood and milk urea N concentrations to be

either reduced or unchanged with FB inclusion. Protein in FB has a higher degradability in the rumen than protein from soya bean meal and rapeseed meal (Ewing, 1997), and in general blood and milk urea level increase when FB replaced a less degradable protein source.

None of the energy utilisation parameters examined were affected by FB inclusion level, which was largely in agreement with Johnston et al. (2019), except that these authors inexplicably found a quadratic effect of FB inclusion on urine E output. Similarly, that none of the CH_4 production parameters examined were affected by FB inclusion agrees with Johnston et al. (2019). However, while Ramin et al. (2017) found CH_4/DMI and CH_4/ECM to be unaffected when rapeseed meal was replaced by FB, total CH_4 production tended to be reduced, which they attributed in part to the higher fat content of rape seed meal. While there is evidence that CH_4 production may be reduced when ruminants are offered legume forages (Lüscher et al., 2004), there appears to be little evidence that this is the case with grain legumes, in line with the findings of the current study. While the results demonstrate enteric CH_4 emissions to be unaffected by FB inclusion, the impact of replacing 'imported' protein ingredients with locally grown FB, on the lifecycle of milk, needs to be examined.

Fertility and health

While negative energy balance can have a detrimental effect on reproductive performance, (Beam and Butler, 1999; Butler, 2003), BCS and BW data suggest that energy balance differed little between treatments. However, the presence of phytoestrogens in FB (for example, isoflavones, daidzein and genistein; Kaufman et al., 1997) often cause concern in relation to dairy cow fertility. Phytoestrogens are compounds that are produced naturally in many plant species, including legumes, and which may have adverse effects on ovarian function; inhibited luteinizing hormone surge, altered development of or lack of dominant follicles, abnormal follicular waves, early embryonic death and repeat breeding (Mostrom and Evans, 2018). Whilst these compounds have been shown to suppress the secretion of lutenizing hormone (Zdunczyk et al., 2005), and to lower progesterone concentrations (Piotrowska et al., 2006), neither the time to onset of cyclicity, the proportion of cows cycling within 42 d of calving, nor the peak progesterone concentrations during the first estrus cycle, was affected by diet. While actual conception rates within the study must be considered

within the context of the number of cows on the study, there was no evidence that reproductive outcomes were affected by FB inclusion level. Similar vaginal mucous scores suggest no difference in immune function across treatments. In addition, none of the health parameters examined were affected by FB inclusion in the diet, which again suggests that immune function was unaffected by treatment.

Conclusions

Including FB in dairy cow diets at up to 8.4 kg/cow per d had no detrimental effects on feed intakes, milk production, body tissue reserves, fertility performance, cow health and diet digestibility. The reduction in milk fat content with the highest FB treatment was likely due to the impact on rumen function of the high starch levels with this treatment, while the reduction in milk protein levels with FB inclusion was likely due to a deficit in specific limiting amino acids. Methane production was unaffected by FB inclusion.

References

Beam, S. and W. Butler. 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J. Reprod. Fertil. Suppl. 54:411-424.

Bligh, E.G. and W.J Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*:911-917. https://doi.org/10.1139/o59-099

Butler, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest. Prod. Sci.83**:** 211-218. https://doi.org/10.1016/S0301-6226(03)00112-X

Comellini, M., L. Antonella Volpelli, D. Pietro Lo Fiego, and R. Scipioni. 2009. Faba bean in dairy cow diet: effect on milk production and quality. Ital. J. Anim. Sci. 8**:** 396-398. https://doi.org/10.4081/ijas.2009.s2.396

Dixon, R.M. and B. J. Hosking. 1992. Nutritional value of grain legumes for ruminants. Nutr. Res. Rev, 5 (Suppl. 1):19-43. https://doi.org/10.1079/NRR19920005

Dvořák, R., A. Pechová, L. Pavlata, B. Klejdus, K. KovařČík, J. Dostálová, J. Culková, J. Filípek, E. Svajdlenka, and V. Capkova. 2006. Reduction in the content of antinutritional substances in Fava beans (Vicia faba) by different treatments. Slov. Vet. Zb. (Suppl. 10):174-179.

Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for holstein dairy cows. J. Dairy Sci. 72:68–78. https://doi.org/10.3168/jds.S0022-0302(89)79081-0

Ewing, W.N. 1997. The Feeds Directory. Volume 1: Commodity Products Guide, Context Publications, Leicestershire, England.

Grela, E.R., and K. D. Gunter. 1995. Fatty acid composition and tocopherol content of some legume seeds. Anim. Feed. Sci. Technol. 52: 325 – 331. https://doi.org/10.1016/0377-8401(94)00733-P

Gupta, R.C., M. S Mostrom, and T.J Evans. 2018. Zearalenone. Vet. Toxicol. 3:1055-1063. https://doi.org/10.1016/B978-0-12-811410-0.00076-3

Hao, Q., M. Chunyu, J. Xin, L. Xueying, L. Ping, and G. Xuejun. 2018. Methionine promotes milk protein synthesis and fat synthesis and cell proliferation via the SNAT2-P13K signalling pathway in bovine milk. J. Agric. Food. Chem. 66:11027 – 11033. https://doi.org/10.1021/acs.jafc.8b04241

Hutson, G.D. and S. Mourik. 1981. Food preferences of sheep. Aust. J. Exp. Agric. 21:575-582. https://doi.org/10.1071/EA9810575

Ingalls, J. and J. Mckirdy. 1974. Faba bean as a substitute for soybean meal or rapeseed meal in rations for lactating cows. Can. J. Anim. Sci. 54:87-89. https://doi.org/10.4141/cjas74-013

Ireland-Perry, R. and C. Stallings. 1993. Fecal consistency as related to dietary composition in lactating Holstein cows. J. Dairy Sci. 76:1074-1082. https://doi.org/10.3168/jds.S0022-0302(93)77436-6

Johnston, D. J., K. Theodoridou, and C. P. Ferris. 2019. The impact of field bean inclusion level in dairy cow diets on cow performance and nutrient utilisation. Livest. Sci. 220:166-172. https://doi.org/10.1016/j.livsci.2018.12.015

Kaufman, P. B., J. A. Duke, H. Brielmann, J. Boik, and J. E. Hoyt. 1997. A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health. J. Altern. Complement. Med. 3:7-12. https://doi.org/10.1089/acm.1997.3.7

Little, M. W., N. E O'Connell, and C. P Ferris. 2016. A comparison of individual cow versus group concentrate allocation strategies on dry matter intake, milk production, tissue changes, and fertility of Holstein-Friesian cows offered a grass silage diet. J. Dairy Sci. 99:4360-4373. https://doi.org/10.3168/jds.2015-10441

Little, M. W., N. E O'Connell, M. D. Welsh, F. J. Mulligan, and C. P. Ferris. 2017. Concentrate supplementation of a diet based on medium-quality grass silage for 4 weeks prepartum: Effects on cow performance, health, metabolic status, and immune function. J. Dairy Sci. 100:4457-4474. https://doi.org/10.3168/jds.2016-11806

Lüscher, A., L. Mueller-Harvey, J. F. Soussana, R. M. Rees, and J. L Peyraud. 2014. Potential of legume-based grassland–livestock systems in Europe: a review. Grass Forage Sci, 69:206-228. https://doi.org/10.1111/gfs.12124|

McMurray, C.H., W. J. Blanchflower, and D. A. Rice. 1984. Automated kinetic method for D-3-hydroxybutyrate in plasma or serum. Clin. Chem. 30:421-425.

Melicharová, V., A. Pechová, R. Dvořák, and L. Pavlata. 2009. Performance and metabolism of dairy cows fed bean seeds (Vicia faba) with different levels of anti-nutritional substances. Acta. Veterinaria. Brno. 78:57-66. https://doi.org/10.2754/avb200978010057

Mordenti, A., F. Merendi, M. Fustini, and A. Formigoni. 2007. Effects of different protein plants in cows diet on milk for Parmigiano Reggiano production. Ital. J. Anim. Sci. 6:463-465. https://doi.org/10.4081/ijas.2007.1s.463

Newton, S.D. and G. D. Hill. 1983. The composition and nutritive value of field beans. *Nutr. Abstr. Rev*. Series B. 53:99-115.

Offner, A., A. Bach, and D. Sauvant. 2003. Quantitative review of in situ starch degradation in the rumen. Anim. Feed Sci. Technol. 106:81-93. https://doi.org/10.1016/S0377-8401(03)00038-5

Piotrowska, K. K., L. Woclawek-Potocka, M. M. Bah, M. K. Piskula, W. Pilawski, A. Bober, and D. J. Skarzynski. 2005. Phytoestrogens and their metabolites inhibit the sensitivity of the bovine corpus luteum to luteotropic factors. J. Reprod. Dev. 52:33-41. https://doi.org/10.1262/jrd.17054

Puhakka, L., S. Jaakkola, L. Simpura, T. Kokkonen, and A. Vanhatalo. 2016. Effects of replacing rapeseed meal with fava bean at 2 concentrate crude protein levels on feed intake, nutrient digestion, and milk production in cows fed grass silage–based diets. J. Dairy Sci. 99:7993-8006. https://doi.org/10.3168/jds.2016-10925

Purcell, P.J., R. A. Law, A. W. Gordon, S. A. McGettrick, and C. P. Ferris. 2016. Effect of concentrate feeding method on the performance of dairy cows in early to mid lactation. J. Dairy Sci. 99:2811-2824. https://doi.org/10.3168/jds.2015-9988.

Ramin, M., A. Höjer, F. Fogelberg, M. Hetta. 2017. The effects of legume seeds on the lactation performance of dairy cow fed grass silage-based diets. Agr. Food. Sci. 26: 129 -137. https://doi.org/10.23986/afsci.64417

Reckling, M., T. F. Döring, G. Bergkvist, F. L. Stoddard, C. A. Watson, S. Seddig, F. M. Chmielewski, and J. Bachinger. 2018. Grain legume yields are as stable as other spring crops in long-term experiments across northern Europe. Agron. Sustain. Dev. 38:63. https://doi.org/10.1007/s13593-018-0541-3

Schwab, C., L. Satter, and B. Clay. 1976. Response of lactating dairy cows to abomasal infusion of amino acids. J. Dairy Sci. 59:1254-1270. https://doi.org/10.3168/jds.S0022-0302(76)84354-8
.
Tufarelli, V., R. U. Khan, and V. Laudadio. 2012. Evaluating the suitability of field beans as a substitute for soybean meal in early lactating dairy cow: Production and metabolic responses. Animal. Sci. J. 83:136-140. https://doi.org/10.1111/j.1740-0929.2011.00934.x

Van Soest, P.V., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy. Sci. 74:3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Volpelli, L. A., M. Comellini, F. Masoero, M. Moschini, D. P. L. Fiego, and R. Scipioni. 2010. Faba beans (Vicia faba) in dairy cow diet: effect on milk production and quality. Ital. J. Anim. Sci. 9:138-144. https://doi.org/10.4081/ijas.2010.e27

Volpelli, L.A., M. Comellini, M. Gozzi, F. Masoero, and M. Moschini. 2012. Pea (Pisum sativum) and faba beans (Vicia faba) in dairy cow diet: effect on milk production and quality. Ital. J. Anim. Sci. 11:217-222. https://doi.org/10.4081/ijas.2012.e40

Watson, C., M. Reckling, S. Preissel, J. Bachinger, G. Bergkvist, T. Kuhlman, K. Lindstrom, T. Nemecek, C. F . E. Topp, A. Vanhatalo, P. Zander, D. Murphy-Bokern, and F. L. Stoddard. 2017. Grain legume production and use in European Agricultural systems. Advances in Agronomy.144:235-303. https://doi.org/10.1016/bs.agron.2017.03.003

Wilkins, R.J. and R. Jones. 2000. Alternative home-grown protein sources for ruminants in the United Kingdom. Anim. Feed Sci. Tech. 85:23-32. https://doi.org/10.1016/S0377-8401(00)00140-1

Zdunczyk, S., M. Piskula, T. Janowski, W. Baranski, and M. Ras. 2005. Concentrations of isoflavones in blood plasma of dairy cows with different incidence of silent heat. Bull Vet Inst Pulawy, 49:189-191.

Table 1 Ingredient composition (g/kg) of concentrates offered during the experiment

(g/kg)	Treatment		
	FB0	FB-Low	FB-High
Field beans	0	349	698
Soya bean meal	170	85	0
Rapeseed meal	150	75	0
Maize gluten feed	74	37	0
Maize meal	245	178	110
Wheat	174	87	0
Soya hulls (toasted)	140	139	140
Molaferm[1]	25	25	25
Calcined magnesite	6	6	6
Limestone ($CaCO_3$)	7	6	5
Dicalcium phosphate	0	5	10
Salt	6	4	2
Mineral and vitamin premix[2]	4	4	4

[1]United Molasses, Belfast, UK
[2]Superdairy, Trouw Nutrition, Belfast UK

Table 2 Chemical composition of the grass silage and experimental concentrates offered during the experiment

			Concentrate					
	Grass silage	(s.d)	FB0	(s.d)	FB-Low	(s.d)	FB-High	(s.d)
Oven dry matter (g/kg)	293	61.5	892	5.2	889	5.5	882	10.4
Volatile corrected oven dry matter (g/kg)	311	61.8						
Crude protein (g/kg DM)	145	22.1	224	5.8	222	10.1	223	9.2
Ash (g/kg DM)	87	6.1	68	5.7	64	4.6	59	4.0
Acid detergent fibre (g/kg DM)	294	23.6	159	21.9	161	29.0	164	24.0
Neutral detergent fibre (g/kg DM)	480	42.0	279	42.5	267	41.5	266	31.9
Gross energy (MJ/kg DM)	18.9	1.47	18.3	0.12	18.1	0.13	18.0	0.10
Starch (g/kg DM)			291	25.3	313	38.8	338	27.2
Metabolisable energy (MJ/kg DM)	11.0	0.55						
Ammonia N (g/kg total N)	76	8.0						
pH	3.87	0.216						
Lactic acid (g/kg DM)	114	56.8						
Acetic acid (g/kg DM)	23	10.6						
Ethanol (g/kg DM)	14	10.2						
Propanol (g/kg DM)	0.9	0.94						

Table 3 Effects of field bean inclusion level in dairy cow concentrates on mean DM intakes and milk production parameters over the 20 wk experimental period, and on mean milk fatty acid concentrations (mean of two sampling occasions)

	Treatment				P-Value		
	FB0	FB-Low	FB-High	S.E.M	Treatment	Time	Treatment × Time
Silage DMI (kg/d)	9.7	9.6	9.8	0.22	0.497	<0.001	0.722
Concentrate DMI (kg/d)	11.8	11.7	12.0	0.27	0.497	<0.001	0.722
Total DMI (kg/d)	21.9	21.6	22.1	0.50	0.497	<0.001	0.722
Milk yield (kg/d)	35.7	33.2	33.9	0.89	0.272	<0.001	0.723
Milk fat (g/kg)	42.8^{a}	42.5^{ab}	41.3^{b}	0.42	0.031	<0.001	0.297
Milk protein (g/kg)	33.8^{a}	33.6^{a}	32.2^{b}	0.33	<0.001	<0.001	0.460
Milk lactose (g/kg)	48.3	48.2	47.9	0.112	0.113	<0.001	0.883
Milk fat yield (kg/d)	1.52^{a}	1.39^{b}	1.39^{b}	0.025	<0.001	<0.001	0.248
Milk protein yield (kg/d)	1.20^{a}	1.11^{b}	1.09^{b}	0.026	0.035	<0.001	0.712
Milk fat + protein yield (kg/d)	2.71^{a}	2.49^{b}	2.47^{b}	0.050	0.007	<0.001	0.196
Milk fatty acids (g/kg total fatty acids identified)							
Total saturated FA[1]	73.9^{a}	75.4^{b}	75.2^{b}	0.42	0.028	0.908	0.448
Total monosaturated FA[2]	22.8^{b}	21.4^{a}	21.5^{a}	0.37	0.016	0.197	0.551
Total polysaturated FA[3]	3.3	3.2	3.3	0.07	0.495	<0.001	0.240
C14:0	12.9	12.8	12.6	0.18	0.328	0.060	0.144
C16:0	35.8^{a}	38.4^{b}	39.4^{b}	0.57	<0.001	0.002	0.571
C18:0	9.8^{b}	9.2^{ab}	8.4^{a}	0.27	0.002	<0.001	0.835
C18:1 *cis*-9	18.2^{b}	17.0^{a}	17.1^{a}	0.35	0.022	0.912	0.512
C18:2	1.77	1.72	1.81	0.04	0.292	<0.001	0.387
C18:2 cis-9, trans-11 (CLA)	0.54^{b}	0.45^{a}	0.40^{a}	0.019	<0.001	0.133	0.751
C18:3 *n*-3	0.49^{a}	0.52^{b}	0.56^{c}	0.015	0.006	<0.001	<0.001

Means with the same superscript within a row do not differ significantly (P>0.05)

Table 4 Effects of field bean inclusion level in dairy cow concentrates on body condition score and bodyweight, and on plasma metabolite concentrations

	Treatment				P-Value		
	FB0	FB-Low	FB-High	S.E.M	Treatment	Time	Treatment × Time
Average body condition score	2.49	2.53	2.51	0.024	0.259	0.046	0.248
End of study body condition score	2.42	2.53	2.57	0.047	0.054		
Average bodyweight (kg)	598	600	599	8.0	0.899	<0.001	0.760
End of study bodyweight (kg)	614	621	617	9.6	0.870		
Nadir bodyweight (kg)	570	565	569	8.1	0.902		
Loss to nadir bodyweight (kg)	23	20	30	4.8	0.276		
Days to nadir bodyweight	50	35	46	11.2	0.608		
Plasma metabolites							
Glucose (mmol/L)	3.81	3.73	3.71	0.036	0.153	0.010	0.954
Beta-hydroxybutyrate (mmol/L)	0.51	0.51	0.48	0.018	0.419	0.002	0.123
NEFA (mEq/L)	0.43^{a}	0.39^{ab}	0.36^{b}	0.020	0.022	<0.001	0.633
Urea (mmol/L)	4.63^{a}	5.28^{b}	5.79^{c}	0.103	<0.001	<0.001	0.567

Means with the same superscript within a row do not differ significantly (P>0.05)

Table 5 Effects of field bean inclusion level in the concentrate component of the diet on dry matter intake and milk yield during the nutrient utilisation study, and on total ration digestibility coefficients

	Treatment				
	FB0	FB-Low	FB-High	S.E.M	P value
Silage DM intake (kg/d)	9.9	10.9	9.9	0.85	0.658
Concentrate DM intake (kg/d)	11.3	12.5	10.8	0.94	0.460
Total DM intake (kg/d)	21.2	23.4	20.7	1.73	0.537
Milk yield (kg/d)	30.2	31.0	31.2	3.00	0.967
Digestibility coefficients (g/g)					
Dry matter	0.784	0.775	0.771	0.0094	0.609
Organic matter	0.800	0.791	0.787	0.0098	0.602
Nitrogen	0.716	0.694	0.708	0.0186	0.517
Gross energy	0.777	0.767	0.764	0.0089	0.548
Acid detergent fibre	0.732^{a}	0.671^{ab}	0.644^{b}	0.0286	0.035
Neutral detergent fibre	0.699	0.659	0.666	0.0273	0.340

Means with the same superscript within a row do not differ significantly (P>0.05)

Table 6 Effect of field bean inclusion level in dairy cow concentrates on nitrogen utilisation parameters

	Treatment				
	FB0	FB-Low	FB-High	S.E.M	P-Value
N intake and output (g/d)					
Total N intake	582	642	569	49.7	0.560
Digestible N intake	420	444	405	34.7	0.729
Faeces N	163	198	164	17.9	0.332
Urine N	199	182	185	23.8	0.868
Manure N	362	380	349	34.43	0.817
Milk N	139	163	140	16.0	0.505
N utilisation (g/g)					
Faeces N/N intake	0.284	0.306	0.292	0.0132	0.517
Urine N/N intake	0.339	0.287	0.332	0.0316	0.477
Manure N/N intake	0.623	0.593	0.624	0.0276	0.673
Milk N/N intake	0.241	0.255	0.245	0.0175	0.843
Faeces N/Manure N	0.464	0.516	0.470	0.0285	0.403
Urine N/Manure N	0.536	0.484	0.530	0.0285	0.403

Table 7 Effects of field bean inclusion level in dairy cow concentrates on energy utilisation parameters, and on methane production

	Treatment				
	FB0	FB-Low	FB-High	S.E.M	P-Value
Energy intake and output (MJ/d)					
GE intake	392	425	375	31.7	0.548
Faecal Energy	87	99	88	8.1	0.542
DE intake	305	327	287	24.6	0.546
Methane energy	28	29	30	3.0	0.890
Urinary energy	13	13	13	1.0	0.889
Milk energy	102	105	102	9.6	0.964
ME intake	264	284	243	21.4	0.446
Heat production	151	158	165	15.1	0.803
Retained energy	11	21	-23	18.0	0.240
Energy utilisation (MJ/MJ)					
DE/GE	0.770	0.768	0.765	0.0092	0.518
ME/GE	0.675	0.667	0.650	0.0088	0.169
Heat production/ME	0.564	0.573	0.679	0.0495	0.243
Milk energy/ME	0.384	0.372	0.419	0.0171	0.188
Retained energy/ME	0.052	0.055	-0.097	0.0621	0.195
CH_4 production					
CH_4 (g/d)	512	527	549	54.4	0.890
CH_4/feed intake or milk yield (g/kg)					
CH_4/DM intake	23.8	22.8	26.4	1.51	0.270
CH_4/OM intake	25.7	24.5	28.4	1.63	0.281

CH_4/milk yield	16.8	17.2	17.8	1.21	0.850
CH_4/ECM yield	15.5	15.6	16.7	0.823	0.506
CH_4-E/energy intake (MJ/MJ)					
CH_4-E/GE intake	0.071	0.069	0.081	0.0067	0.241
CH_4-E/ME intake	0.106	0.104	0.124	0.0103	0.150

GE, gross energy; DE, digestible energy; ME, metabolisable energy, CH_4, methane; OM, organic matter; DM, dry matter; GE, gross energy; ME, metabolisable energy; ECM, energy corrected milk; CH_4-E, methane energy

Table 8 Effects of field bean inclusion level on fertility parameters and dairy cow health

	Treatment				
	FB0	FB-Low	FB-High	SEM	P-Value
Pre-d 42					
Proportion of cows showing luteal activity	0.75 (0.54-0.88)	0.67 (0.44-0.83)	0.79 (0.58-0.91)		0.632
Days to CLA	22.1	21.6	22.1	2.25	0.980
Peak progesterone content at CLA (ng/ml)	31.3	26.9	32.5	2.74	0.353
Conception to first service (proportion)	0.45 (0.27-0.65)	0.33 (0.16-0.55)	0.30 (0.15-0.51)		0.511
Conception to first and second service (proportion)	0.62 (0.42-0.79)	0.57 (0.36-0.75)	0.50 (0.31-0.69)		0.681
Days to conception	84	102	90	9.5	0.412
Cows pregnant at end of breeding season (proportion)	0.91 (0.70-0.98)	0.80 (0.57-0.92)	0.74 (0.53-0.88)		0.307
Proportion of cows with at least one incidence of:					
Digestive upset	0.17 (0.06-0.44)	0.35 (0.17-0.70)	0.08 (0.02-0.32)		0.117
Mastitis	0.10 (0.03-0.32)	0.07 (0.02-0.27)	0.08 (0.02-0.29)		0.889
Lameness	0.29 (0.14-0.61)	0.35 (0.17-0.69)	0.37 (0.19-0.72)		0.876
Mean locomotion score	2.5	2.5	2.4	7.30	0.593

CLA = Commencement of luteal activity

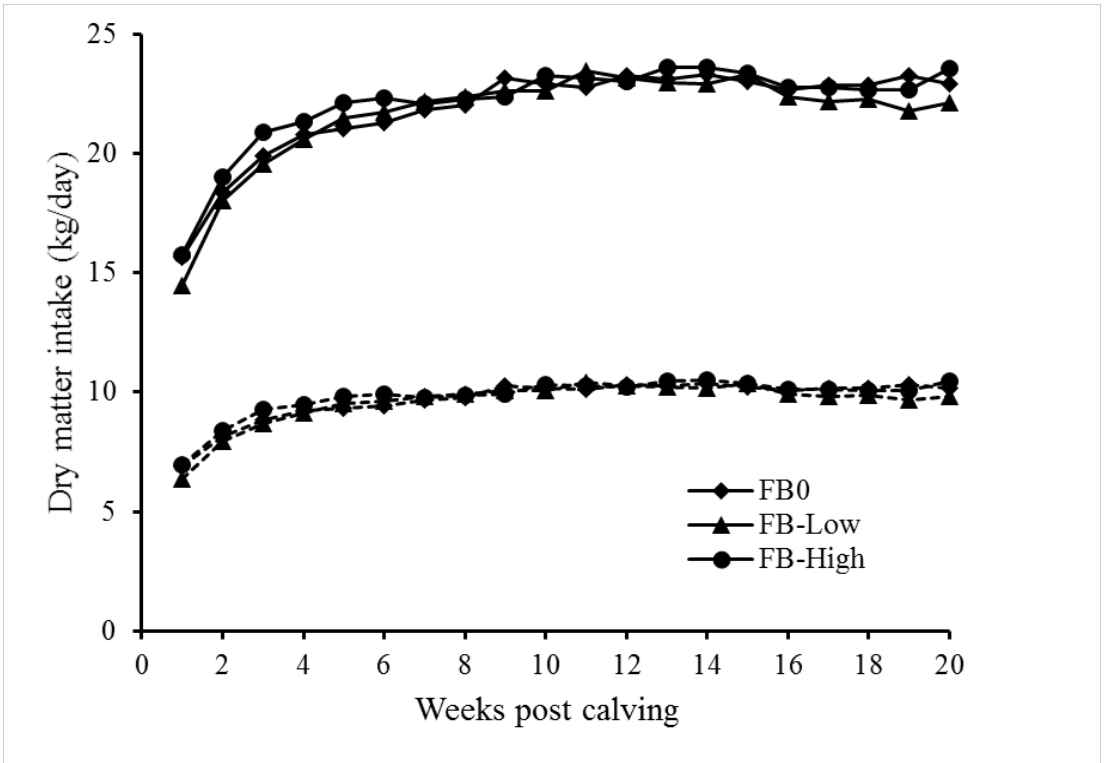

Figure 1 Silage DM (dashed lines) and total DM (solid lines) intake responses of dairy cows over the first 20 wk of lactation, to concentrates containing a range of field bean inclusion levels

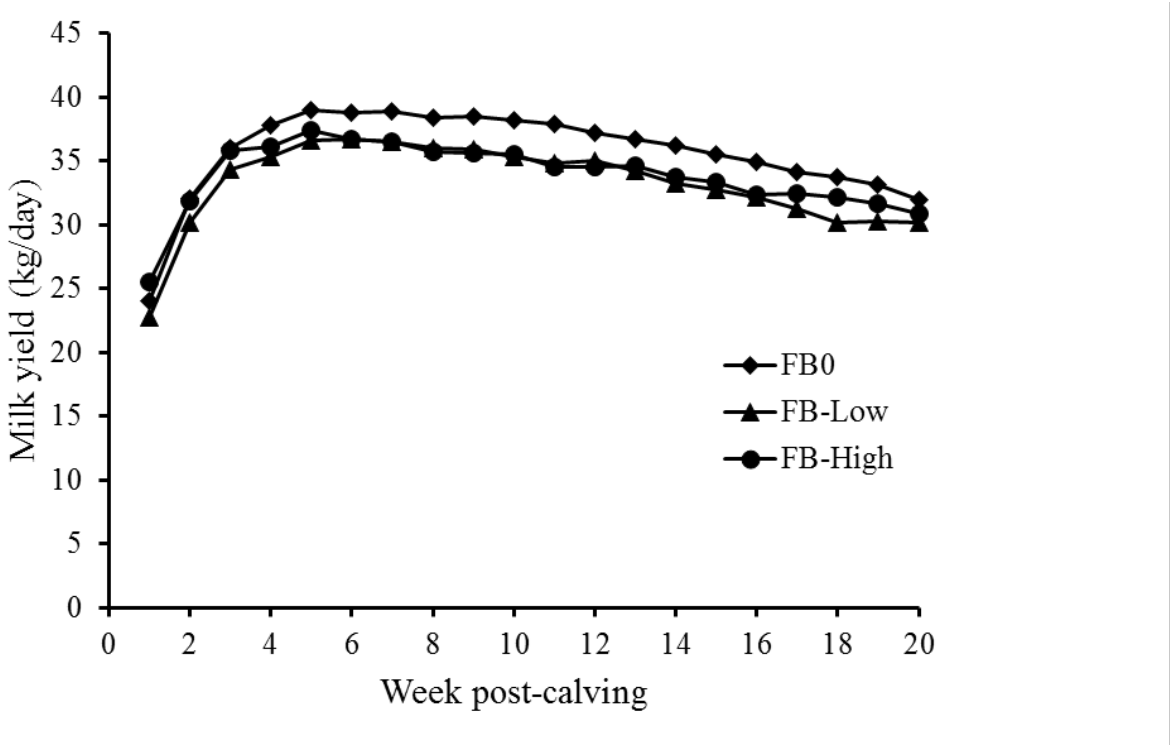

Figure 2 Milk yield response of dairy cows to concentrates containing a range of field bean inclusion levels over the first 20 wk of lactation

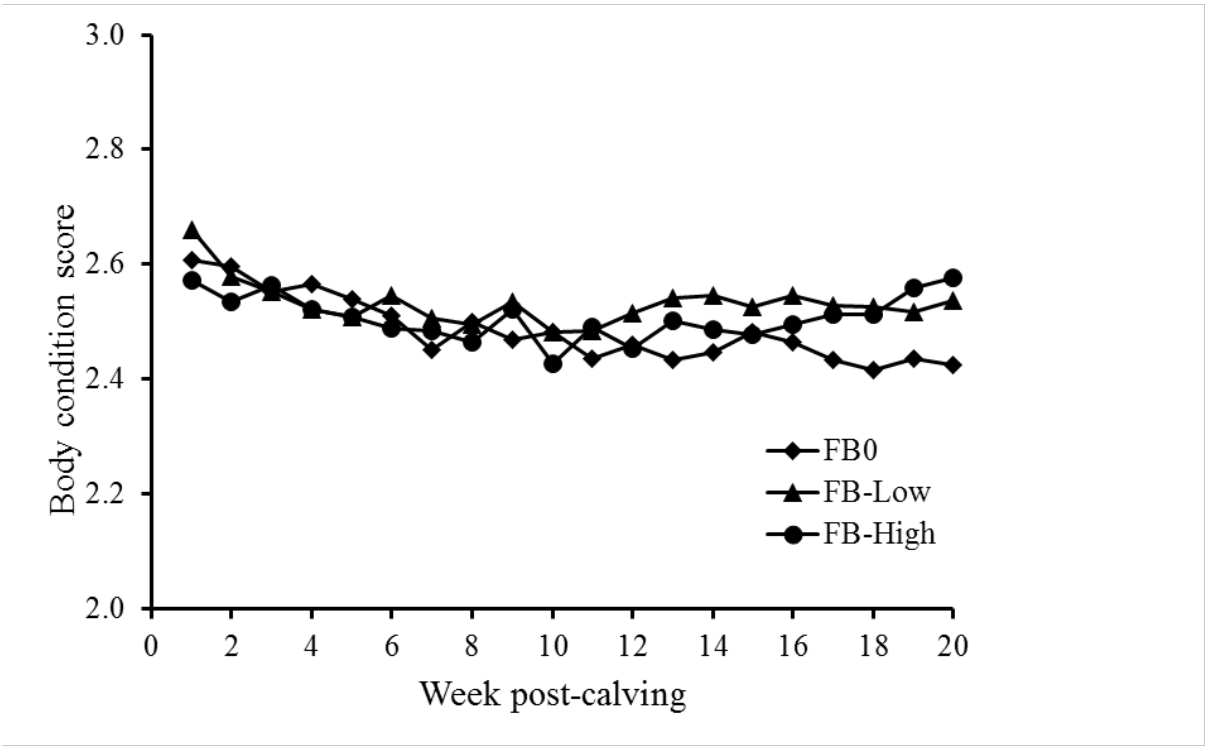

Figure 3 Body condition score response of dairy cows to concentrates containing a range of field bean inclusion levels over the first 20 wk of lactation

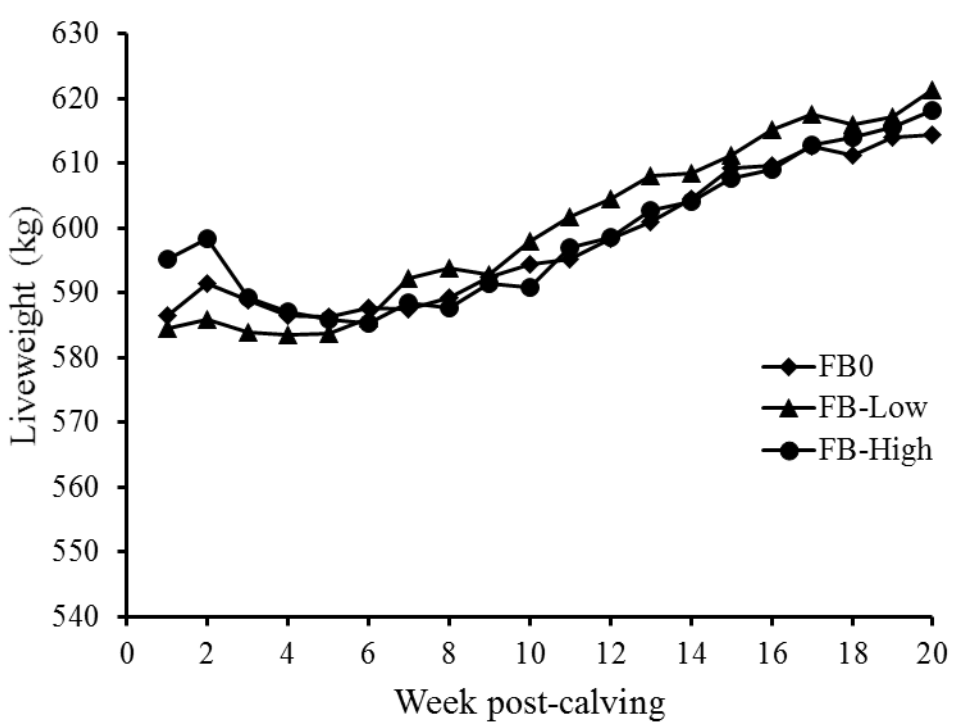

Figure 4 Bodyweight response of dairy cows to concentrates containing a range of field bean inclusion levels over the first 20 wk of lactation

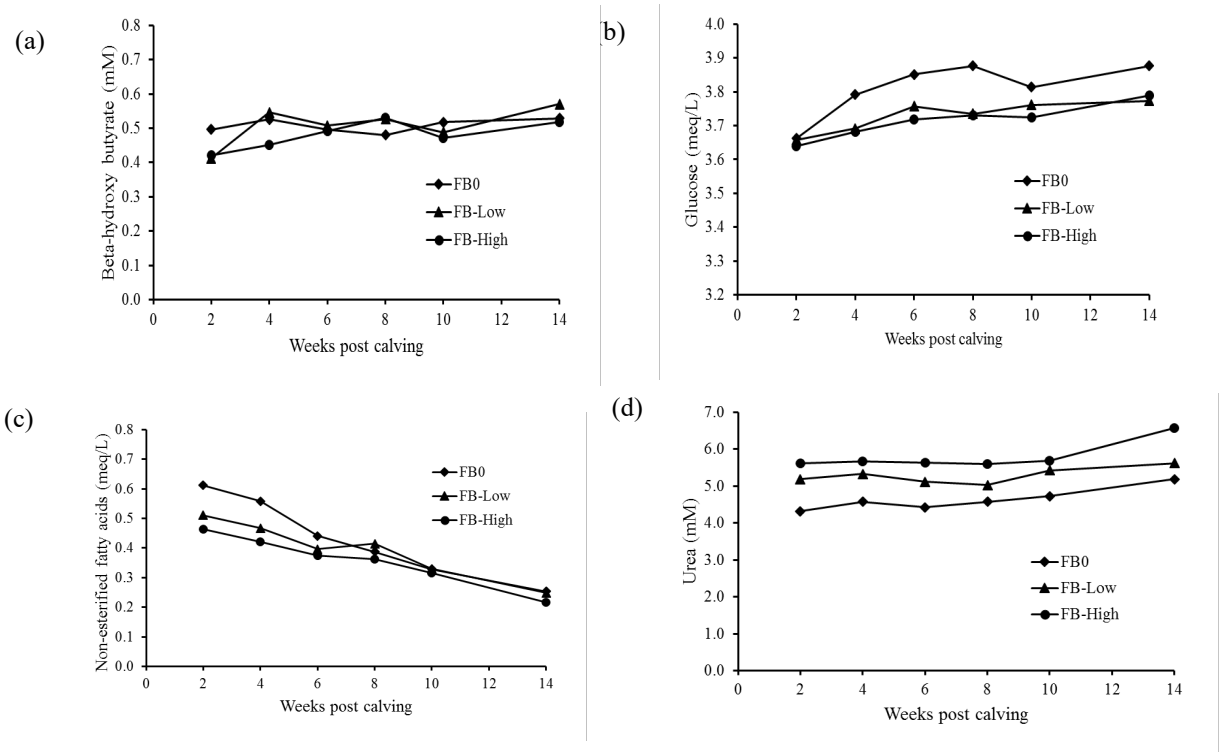

Figure 5 Effect of field bean inclusion level in the diet on plasma concentrations of (a) beta-hydroxybutyrate, (b) glucose, (c) non-esterified fatty acids, and (d) urea

Experiment 3

The effect of post-harvest treatment of field beans on dairy cow performance and nutrient utilisation

Introduction

The need to increase the supply of locally grown protein feeds for use by the ruminant sector within the United Kingdom (UK), highlighted by Wilkins and Jones (2000) almost two decades ago, and more recently by Watson et al. (2017), remains very real. This is driven by intensification within the dairy sector, together with volatility in supply and associated price fluctuations of imported protein sources such as soya-bean meal. In recognition of these issues, the potential of including a number of 'protein crops' in ruminant diets have been examined in recent years within the UK. For example, studies have examined the potential of forage legumes such as red clover (Dewhurst et al, 2003) and lucerne (Castillo et al, 2000), and protein grain crops such as peas, lupins (Masoero et al, 2010), and field beans (FB: Vicia Faba) (Johnston et al., 2019). There is particular interest in the latter, as FB can be grown relatively easily in the more Northerly and Westerly regions of the UK.

With regards FB, perceived limitations to their inclusion in dairy cows diets include their relatively high rumen protein degradability and the presence of anti-nutritional factors (ANF) which can have negative effects on feed intakes and animal performance (Dixon and Hosking, 1992). A number of studies have examined treatment strategies to overcome some of these limitations. For example, extrusion and de-hulling have been shown to reduce the condensed tannins content (contained in the outer skin of the bean), whilst also reducing the content of anti-nutritional factors (Alonso et al., 2000). Furthermore, Goelema et al. (1998) observed that combining high pressure and high temperature treatments (micro-ionisation and toasting) reduce rumen protein degradation. More recently, Mogensen et al. (2010) found that toasting FB at 140^{o}C resulted in a 145 g/kg reduction in total effective rumen protein degradability without reducing total tract protein digestibility. However, these treatments can be expensive, often require specialised facilities, and tend not to be undertaken on a large scale. In addition, Johnston et al. (2019) found that cow performance was unaffected by the inclusion of 'untreated' FB in the diet of mid-lactation dairy cows at up to 4.75 kg/day, a higher inclusion level than normally used in practice.

While expensive 'processing' treatments of FB may not be necessary, FB grown within the northern and western parts of the UK are frequently harvested at a moisture

content in excess of 16%, thus requiring either drying or moist preservation to ensure that the crop does not deteriorate. While drying is the approach most often adopted, drying normally requires that FB are removed to an off-farm drying plant. However, a number of grain crops can be preserved moist 'on-farm' using additives such as propionic acid (Goering et al, 1973). While the use of propionic acid as an effective mould inhibitor in moist cereal grain has been well documented (Christensen, 1973; Nelson *et al.,* 1973; Sauer and Burroughs, 1974), there appears to be little research on the preservation of FB using this approach. Alternatively, when FB is dried (as was the case in the study by Johnston et al., 2019), the dried FB must be either rolled or milled to break down the physical structure of the beans before inclusion in rations. However, there is evidence that the degree of physical processing may impact on the nutritive value of feedstuffs. For example, Larsen et al. (2009) found that apparent ruminal starch digestibility was higher for ground than rolled legumes (808 vs 706 g/kg starch intake).

Given the increased interest in the use of locally grown FB in livestock diets, this study was designed to examine the impact of moist preservation of FB using propionic acid, and the extent of physical treatment of dried FB, on dairy cow performance and nutrient utilisation.

Methodology

This study was conducted at the Agri-Food and Biosciences Institute, Hillsborough, Northern Ireland (NI). All experimental procedures were conducted under an experimental licence granted by the Department of Health, Social Services & Public Safety for Northern Ireland in accordance with the Animals (Scientific Procedures) Act 1986.

Animals and housing

Eighteen mid-lactation (mean of 150 (s.d., 4.2) days calved) Holstein-Friesian dairy cows (mean lactation number, 2.8 (s.d., 1.6)) were used in this three treatment experiment. Cows had a mean pre-experimental milk yield and bodyweight (BW) of 28.0 (s.d., 6.6) kg per day and 654 (s.d., 78.5) kg, respectively.

Throughout the main experimental period cows were housed in a free-stall house with concrete flooring, and had access to individual cubicles that were fitted with rubber

mats and bedded with sawdust. The cubicle-to-cow ratio was ≥1:1 at all times, thus meeting the recommendations of FAWC (1997). The floor area was scraped every 3 hours using an automated system. During a two-week pre-experimental period, cows were offered grass silage plus a non-experimental concentrate in a total mixed ration (60:40 DM ratio).

Treatments and ration preparation

Cows were blocked (n = 6) according to milk yields during the week prior to the start of the experiment, with primiparous and multiparous cows blocked separately. Cows within each block were randomly allocated to one of the three experimental treatments. Experimental treatments comprised different post-harvest processing treatments of FB, with these examined using a three-period (each of four weeks duration) completely balanced change-over design layout.

The FB crop used in this study (*Vicia Faba. Var. Boxer*) was grown in NI, and was harvested at a moisture content of approximately 25%, and then passed through a forced air system to remove dust and chaff. Two thirds of the FB were then dried at 80^{o}C for four hours (until their temperature reached approximately 65^{o}C) to achieve a moisture content of 16%, before being left to cool. Half of the dry FB were then subject to 'coarse' rolling (Dry-CR) using a grain crimper (Korte 1400, Murska, Ylivieska, Finland) fitted with fluted rollers (1.1 mm spacing). The remaining dry beans were finely milled (Dry-FM) using a hammer mill fitted with a 3 mm screen (Hammer mill Vertica DFZK-1, Bühler, London, UK). The undried FB were coarsely rolled using the grain crimper described above (roller spacing of 1.1 mm) and the rolled FB sprayed with propionic acid (99% propionic acid) at a rate of 20 litres/ton fresh FB (Treatment, Moist-P: moist, propionic acid).

Cows on all treatments were offered a mixed ration comprising grass silage and concentrates (forage : concentrate ratio of 60: 40 on a dry matter (DM) basis). The grass silage component of the diet was produced from a perennial ryegrass (Lolium Perenne) based sward which was harvested between 12th - 15th May 2016. The concentrate component of the diet with treatments Dry-CR and Dry-FM comprised a common 'pre-mix' (ingredient composition (g per 600 g): maize meal, 177; wheat, 78; soya-bean meal, 68; rapeseed meal, 68; soya hulls, 130; maize gluten feed, 34; lime flour, 14.7; salt, 7.6; calcined magnesite, 6.2; mineral/vitamin mix (Super dairy, Trouw

Nutrition, Belfast), 4.0; molaferm, 12.5), with the remaining 400 g per 1000 g total concentrate comprising dried coarsely-rolled and dry finely-milled FB, respectively. The same concentrate pre-mix was used with treatment Moist-P, while undried FB were incorporated at 517 g/1117 g total concentrate, reflecting their lower DM content. The objective was to achieve a daily intake of approximately 3.5 kg FB per cow with each treatment.

The rations were prepared and offered daily at approximately 09.00 h (at 1.07 of the previous days intake), with uneaten ration removed the following day at approximately 08.00 h. Rations were prepared using a paddle mixer-feeder (Vari-Cut 12, Redrock, Armagh, Northern Ireland). The total silage required for all three treatments was initially mixed for approximately five minutes and then deposited on a clean silo floor. The quantity of silage required for each individual treatment was then removed from this 'pile' in turn, placed back in the mixer-feeder, and the appropriate quantity of the concentrate 'pre-mix' and treated FB added, and mixing continued for another five minutes. The rations were then transferred from the mixer-feeder to a series of feed boxes mounted on weigh scales, with cows accessing feed in these feed boxes via an electronic identification system, thus enabling individual cow intakes to be recorded daily (Bio-Control Feeding System, Bio-Control, Rakkestad, Norway). Cows had free access to fresh water at all times. In addition, 1.0 kg per day of a common non-experimental commercial concentrate was offered using an in-parlour concentrate feeder during milking (0.5 kg at each milking).

Animal measurements

All cows were milked twice daily (between 06 00 and 08 00 h and between 15 00 and 17 00 h) throughout the experiment using a 50-point rotary milking parlour (Boumatic, Madison, USA), with milk yields automatically recorded at each milking, and a total daily milk yield for each cow for each 24-hour period calculated. Milk samples were taken during six consecutive milkings at the end of week four during each period, and analysed for fat, protein and lactose contents using an infrared milk analyser (Milkoscan Combifoss^{TM}7; Foss Electric, Hillerød, Denmark), and a weighted concentration of each constituent determined for each 24-hour sampling period, and a mean composition over the three day sampling period subsequently calculated for each cow. In addition, during the final week of each period, additional milk samples

were taken in proportion to yield during two successive milkings (am and pm), the two samples bulked, and the bulked sample frozen at -20^{o}C. These samples were subsequently analysed for milk fatty acids, as described by Johnston et al. (2019).

Cow BW was recorded twice daily during the final week of each period (immediately after each milking) using an automated weighbridge, and a mean BE for each cow determined. The body condition score (BCS) of each cow was estimated during the final week of each period according to Edmondson *et al.* (1989) by a trained technician. Blood samples were collected prior to feeding from the coccygeal vein of each cow at the end of each period. Samples were collected into evacuated tubes (BD, Oxford, UK) and centrifuged (3000 rpm for 15 minutes) to obtain serum which was separated and stored at -20^{o}C until analysed for beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and urea concentrations. Serum biochemistry analysis was carried out on a dry chemistry analyser system (Sapphire 800, Glenbio, UK), using Olympus kits (Olympus Life Science Research Europa, Munich, Germany).

Nutrient utilisation

On completion of the 12-week feeding study, four cows from each treatment were selected for use in a nutrient utilisation study, with the cows selected from each treatment group balanced for daily milk yield and BW. Cows were tied by the neck in individual stalls, with their lying area comprising a rubber mat. Cows continued to access their experimental rations from feed boxes at the front of each stall. Experimental rations were offered *ad libitum* daily at 0900 hours, at proportionally 1.07 of the previous day's intake, while uneaten silage was removed the following day at 0800 hours. The milking concentrate was offered via plastic feed buckets which were placed within the feed boxes during milking, at 06 30 and 16 30 hours, with these buckets removed after the concentrates had been consumed. Cows had access to fresh water at all times via a drinker located within each stall.

Measurements of nutrient utilisation commenced 24 h after cows were placed in this experimental byre, and was conducted over a 7-day period (6-day feeding period commencing two days before the first collection of faeces and urine) and a 6-day total faeces and urine collection period. Faeces were collected in a plastic collection tray (96 cm x 108 cm x 36 cm) placed behind each cow. Urine was collected into a 25 litre plastic container via a flexible plastic tube which was attached to a urine separation

system. This was held in position over the vulva by attaching it using Velcro fasteners to a 'patch' glued (Bostik, France) either side of the cow's tail head. Approximately 300 ml of 50% sulphuric acid was added to each urine collection container daily to reduce ammonia losses. The total weight of faeces and urine produced during each 24 h collection period was recorded, and a sample of each (0.05 by weight) retained for subsequent analysis. Faeces and urine samples were stored in a fridge (4 – 6^{o}C) until the final day of the collection period, when the six daily faeces samples and six daily urine samples from each cow were bulked into a single sample.

During the nutrient utilisation study cows were milked twice daily in an experimental parlour located within the experimental cow byre. During this time milk samples were taken at each milking, bulked in proportion to yield for days 1-6, and subsequently analysed for gross energy (GE) and nitrogen (N) concentrations. Cows were weighed prior to, and on completion of each six-day measurement period, with the average BW used in energy utilisation calculations. Bulked urine and milk samples were analysed for N concentrations, while a further urine sample was freeze dried (Heto Lyolab 3000, Fisher Scientific, UK) and analysed for GE concentrations using a bomb calorimeter (Parr 6400 Bomb Calorimeter). Similarly, a sample of the bulked faeces samples for each cow was analysed for N concentrations (fresh basis), while a subsample was dried at 85°C for 72 hours, and the dry sample analysed for acid detergent fibre (ADF), ash and GE concentrations.

Feed analysis

Samples of the grass silage offered were taken daily throughout the experiment and dried at 85°C for 18 hours to determine oven DM content. Sub-samples of the dried milled silages were taken three times weekly and bulked for each 14 day period, with the bulked sample milled through a sieve with 1.0 mm aperture, and analysed for NDF, acid detergent fibre (ADF) and ash concentrations. Each week a fresh silage sample was analysed using near infrared reflectance spectroscopy (NIRS) for ME concentrations according to Park *et al.* (1998). A further fresh silage sample was taken weekly and analysed for GE, N, pH and volatile components. A sample of each concentrate was taken daily and bulked for each two week period and dried at 85°C for 24 hours to determine DM content. An additional weekly sample was dried at 60°C for 48 hours, bulked for each 14 day period, milled through a 1.0 mm sieve, and

subsequently analysed for N, NDF, ADF, ash, GE and starch concentrations. During the nutrient digestibility study, silages were sampled daily for oven DM determination, with the daily dried samples bulked for the 6 day period, and subsequently analysed for ADF, NDF and ash concentrations. A fresh silage sample was taken daily throughout and analysed for GE, N, and pH concentrations as described above. Concentrates offered were sampled daily, bulked for each 6-day nutrient utilisation period, and analysed for ODM, GE, NDF, ADF, N, starch, and ash concentrations. Concentrations of NDF and ADF were determined using a Fibertec analyser based on the method of Van Soest (1991), and ash concentrations were determined following combustion in a muffle furnace (Vecstar, UK) at 550°C for approximately 10 hours. Starch concentrations were determined using a Megazyme Kit (Megazyme, Ireland). Nitrogen concentrations of dried concentrates were analysed using the Dumas method (Elementar, Vario Max CN). Gross energy concentrations of the dried concentrates were determined using a bomb calorimeter (Parr 6400 Bomb Calorimeter). Fresh urine samples were freeze dried and analysed for gross energy using a bomb calorimeter (Parr 6400 Bomb Calorimeter), while N concentrations of urine and fresh faeces were determined using the Kjeldahl method (Tecator Kjeltec Auto 2400/2460 Analyser/Sampler System). Gross energy concentrations in faeces were determined on a dried basis using a bomb calorimeter (Parr 6400 Bomb Calorimeter). The chemical composition of the feedstuffs offered is detailed in Table 1.

Statistical analysis

Data relating to DMI, milk yield, milk composition, milk fatty acids, BW, BCS and blood metabolites, recorded during the final week of each period, were analysed using REML variate components analysis, with constant + treatments as the fixed model, and block + block × cow + block × period as the random model. For variables where significant treatment effect were identified ($P<0.05$), differences between the individual treatments were tested using Fisher's protected least significant difference test. Nutrient utilisation data were analysed by ANOVA. All data were analysed using GenStat (Release 18.1; VSN International Limited, Oxford, UK).

Results

The grass silage offered (Table 1) had a volatile corrected oven DM and crude protein of 290 g/kg and 166 g/kg DM, respectively, was well fermented, and had a high ME

concentration (11.8 MJ/kg DM). The Moist-P FB had a DM content of 685 g/kg, compared to 862 and 863 g/kg for the Dry-CR and Dry-FM FB, respectively.

Animal performance

Neither silage DMI nor total DMI were affected by treatment (P>0.05), whilst concentrate DMI was significantly lower with the Moist-P treatments (P<0.001) (Table 2). Neither milk yield, nor milk fat, protein or lactose content were affected by treatment (P>0.05), and nor were the yields of any of the milk constituents (fat, protein, fat plus protein: P>0.05).

The total concentration of saturated fatty acids (SFA) was higher with Dry-CR than with either of Dry-FM and Moist-P (P=0.021), with the reverse effect (P=0.018) observed with total monounsaturated fatty acid concentrations (MUFA) (Table 2). The C16:0 content was significantly higher with Dry-CR than with either of Dry-FM and Moist-P (P = 0.015), with the reverse effect observed with C18:1 *cis*-9 content (P=0.013). None of total polyunsaturated FA (PUFA), C14:0, C18:0, C18:2, C18:2 *cis*-9, *trans*-11, nor C18:3 *n*-3 concentrations were affected by treatments (P>0.05).

While none of BCS, BW, serum NEFA or serum urea concentrations were affected by treatment (P>0.05), serum BHB concentrations were significantly higher in Dry-CR than Dry-FM (P=0.013).

Ration digestibility and nutrient utilisation

Neither DM intakes nor milk yields differed between treatments within the sub-group of cows used in the nutrient utilisation study, while none of the digestibility coefficients examined were affected by treatment (P>0.05: Table 3).

None of total N intake, N output in urine, N output in milk or N output in manure were affected by treatment (P>0.05), although N excreted in faeces was lower with Dry-CR than with Moist-P (P=0.035: Table 4). With the exception of milk N/N intake, which was higher with Moist-P, than with either of Dry-CR or Dry-FM (P=0.002), none of the other N use coefficients were affected by treatment (P>0.05).

Neither GE intake, nor energy output in faeces, urine nor milk were affected by treatment (P>0.05: Table 5). While milk energy/GE intake was higher with Moist-P,

than either of Dry-CR or Dry-FM (P=0.010), both faeces/GEI, and urine E/GEI, were unaffected by treatment (P>0.05).

Discussion

While many studies have examined the impact of post harvest processing techniques on the degradadability and digestibility of FB (for example, extrusion, de-hulling, high pressure and high temperature treatments, including micro-ionisation and toasting), few have examined the impact of degree of physical processing. In one exception, Larsen et al. (2009) compared the impact of grinding vs rolling on the site of starch digestion. No studies have been identified which examine the impact of degree of physical processing, or moist preservation using proprionic acid, on dairy cow performance. Thus the current study was designed to investigate the effects of post-harvest treatments of FB (dry coarsely rolled, dry finely rolled and moist-coarsely rolled followed by preservation with propionic acid) on dairy cow performance and nutrient utilisation. All diets were designed to be iso-nitrogenous and to have similar starch contents.

Effects of physical processing

The processing treatments adopted resulted in large differences in FB particle sizes. For example, the majority of particles from Dry-CR were retained within the three sieves (75%), while with Dry-FM, over 80% of particles passed through the 1.6 mm sieve. As cereal grains are more extensively processed, and become more rapidly fermentable, feed intakes can be supressed due to increased acid production in the rumen (Fulton et al., 1979: Mutsvanga et al., 2012). However, Scott et al. (1991) and Dhiman et al. (1997) found that DMI was unaffected when cows were offered soyabeans differing in degree of physical processing, although soyabeans contain relatively small amounts of starch compared to FB. Nevertheless, intakes did not differ between Dry-CR and Dry-FM, indicating that any impact on rumen fermentation patterns were insufficient to impact on intakes.

While degree of physical processing of FB had no effect on DM intakes, differences in particle size could impact on milk yield and milk composition through effects on starch and protein degradability in the rumen and overall digestive efficiency. In general, particle size reduction will increase nutrient availability to rumen microbes and

digestive enzymes (Offner et al., 2003). For example, in a review by Theurer et al. (1986), increasing the degree of processing of maize grains reduced the proportion of maize starch which excaped the rumen, and increased total tract digestibility. This is important as it is also recognised that the efficiency of ME utilisation from starch is greater when the starch is digested in the small intestine, and absorbed as glucose, rather than when starch is fermented in the rumen to volatile fatty acids, and the propionate fraction then converted back to glucose in the liver (Reynolds, 2006). In one of the few studies examining the impact of physical processing on FB, Larsen et al. (2009) compared the digestion site of starch from ground and rolled legume grains (including FB) and found that rolled legume seeds had a lower starch digestion in the rumen than ground legumes, and that small intestine starch digestion was also reduced with rolling, resulting in a lower total tract digestibility. This same study demonstrated that the digestibility of legume starch in lactating dairy cows was lower both in the rumen and small intestine compared to starch from cereals, which these authors suggested may be due to different type of starch in cereals and legumes, the association of the starch granules with the cell wall in legumes, and the possibe effect of pancreatic amylase inhibitors in legumes, which may inhibit bacterial amylase activity. Similarly, Crepon et al. (2010) summarised the results from a number of unpublished studies that showed that when FB were ground through a 0.8 mm mesh rather than a 3 mm mesh, the energy value for milk production (UFL), the theoretical degradability of N and the degradability of organic matter increased, while digestible protein in the intestine decreased. However, within the current study, none of the apparent digestibility coefficients or indices of nitrogen or energy utilisation were affected by degree of physical processing of FB.

No studies have been identified that examined the impact of physical processing of FB on cow performance, although a number have examined the impact of processing on soya-beans. For example, Scott et al. (1991) found no difference in performance when cows were offered either rolled or ground soyabean, with this largely in agreement with the findings of Tice et al. (1993). However, Dhiman et al. (1997) observed a fall in milk yield (but no effect on composition) as the degree of processing of soyabean increased. In the current study, none of the milk yield or milk composition parameters were impacted on by degree of physical processing of FB, which agrees with the outcomes of the nutrient utilisation data. Information on body tissue reserves

suggest that cows on all, three treatments had similar energy status, which is as expected based on the similar total DMI and yields of fat + protein across the treatments. This is supported by the absence of an effect of treatment on serum NEFA concentrations.

While milk fat content was unaffected by treatment, there were a number of changes in the fatty acid composition of the milk, most noticeably an increase in total SFA content with Dry-CR, and a corresponding decrease in MUFA content. These difference were largely driven by an increase in C16:0 concentrations with Dry-CR, and a reduction in C18:1 *cis-9*. Similarly, Mutsvangwa et al. (2012) observed a trend ($P \leq 0.1$) for SFA to decrease and MUFA to increase with increasing degree of processing of barley, although no such effect was observed by Mohammed et al. (2010). It is known that degree of biohydrogenation is influenced by rumen pH (Troegeler-Meynadier et al., 2003), and it is possible that rumen pH differed with the two treatments, a consequence of differences in rumen fermentation rates

In addition, the lower serum BHB concentrations with Dry-FM, compared to Dry-CR might explain some of the differences in milk fatty acids. Beta-hydroxybutyrate is produced in the rumen epithelium from absorbed butyrate, and provides about half of the first four carbons (primer) of de novo synthesised fatty acids (Bauman and Griinari, 2003). The C16:0 fatty acid is known to originate in part from de novo synthesis (Mote et al., 2007), and a higher BHB may contribute to higher C16:0 concentrations.

Acid preservation

With Moist-P, over 99% of particles were retained within the 4.6 mm sieve, a reflection of the fact that while the beans were ‘rolled’, they remained largely intact. Visible inspection of the FB offered with Moist-P over the course of the study suggested that propionic acid effectively preserved the high moisture (685 g/kg) FB used. Previous research has demonstrated that propionic acid is more effective at reducing mould and preserving grains than acetic acid, formic acid, and n-butyric acid (Sauer et al., 1973; Drysdale et al., 2016). The use of acids to preserve moist grain is not new, and was reviewed over 40 years ago by Jones et al. (1974).

While total DM intake was unaffected by processing treatment, concentrate DMI was lower with Moist-P, and as a consequence the intake of FB was also lower with this

treatment (3.4, 3.5 and 3.2 kg DM/day for Dry-CR, Dry-FM and Moist-P, respectively: SEM, 0.03, P<0.001). In the studies reviewed by Jones et al. (1974), there was little evidence that acid treatment had an adverse impact on intakes. In addition, the 'flattened' but largely intact grain in this treatment (Moist-P) is likely to have been more slowly fermented that for either of Dry-CR and Dry-FM. Thus the lower concentrate intake with Moist-P appears unlikely to be due to processing treatment per se, but rather due to the numerically lower total DM intake with this treatment, combined with a slight difference in forage : concentrate ratios between treatments, with concentrates representing 0.42, 0.42 and 0.40 of total diet DM with Dry-CR, Dry-FM and Moist-P, respectively.

Although treatment of moist FB with propionic acid has been advocated by a number of commercial companies, no published studies examining the effects of acid treatment of moist FB on dairy cow performance have been identified. The results of the current study agree largely with studies reviewed by Jones et al. (1974) (albeit those studies involved high moisture cereal grains), in that moist preservation of FB had no effect on milk production, milk fat content or protein content. Milk fatty acid concentrations with Moist-P were similar to those for Dry-FM, although concentrations of SFA with these two treatments were lower than for Dry-CR, while the reverse was observed for concentrations of MUFA. Supporting the absence of effects on cow performance in this study, acid treatment had no effect on ration digestibility, in agreement with the findings of Ingalls et al. (1974) who treated high moisture grain with a mixture of acids, including proprionic acid. However, nutrient utilisation measures within the current study indicated that both milk N/N intake and milk E/GE intake were higher with Moist-P than either of the other two treatments. Although limited in scope, an early study involving sheep (Sharma and Nicholson, 1975) provided some evidence of protection of protein from rumen degradation when FB were treated with a mixture of acetic acid and propionic acid. For example, in that study rumen ammonia concentrations 1 h post feeding and nitrogen excreted in urine, were either lower or tended to be lower, following acid treatment, while abomasal N flow tended to be higher. However, the improvements in apparent efficiencies in the current study may simply be due to the numerically lower total DM intake with the Moist-P treatment during the digestibility study.

References

Aldrich, J., Muller, L., Varga, G. & Griel, L. 1993. Nonstructural carbohydrate and protein effects on rumen fermentation, nutrient flow, and performance of dairy cows. *Journal of dairy science,* 76**,** 1091-1105.

Alonso, R., Aguirre, A. & Marzo, F. 2000. Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry,* 68**,** 159-165.

Beauchemin, K.A., McAllister, T.A., Dong, Y., Farr, B.I. and Cheng, K.J., 1994. Effects of mastication on digestion of whole cereal grains by cattle. *Journal of Animal Science*, *72*(1), pp.236-246.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *Journal of animal science*, *73*(9), pp.2804-2819.

Briggs, M. A., Petersen, K. S. & Kris-Etherton, P. M. Saturated fatty acids and cardiovascular disease: replacements for saturated fat to reduce cardiovascular risk. Healthcare, 2017. Multidisciplinary Digital Publishing Institute, 29.

Castillo, A. R., Kebreab, E., Beever, D. E. & France, J. 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *Journal of Animal and Feed Sciences,* 9**,** 1-32.

Crocker, L., Depeters, E., Fadel, J., Perez-Monti, H., Taylor, S., Wyckoff, J. & Zinn, R. 1998. Influence of processed corn grain in diets of dairy cows on digestion of nutrients and milk composition. *Journal of Dairy Science,* 81**,** 2394-2407.

Do Hyung Kim, S. H. C., Park, S. K., Lee, S. S. & Choi, C. W. 2018. Effect of corn grain particle size on ruminal fermentation and blood metabolites of Holstein steers fed total mixed ration. *Asian-Australasian journal of animal sciences,* 31**,** 80.

Drysdale, A.D., 2016. Chemical control of crop conservation. *Antibiotics and Antibiosis in Agriculture*, p.265.

Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T. and Webster, G., 1989. A body condition scoring chart for Holstein dairy cows. *Journal of dairy science*, *72*(1), pp.68-78.

Fulton, W.R., Klopfenstein, T.J. and Britton, R.A., 1979. Adaptation to high concentrate diets by beef cattle. I. Adaptation to corn and wheat diets. *Journal of Animal Science*, *49*(3), pp.775-784.

Goelema, J., Spreeuwenberg, M., Hof, G., Van Der Poel, A. & Tamminga, S. 1998. Effect of pressure toasting on the rumen degradability and intestinal digestibility of whole and broken peas, lupins and faba beans and a mixture of these feedstuffs. *Animal Feed Science and Technology,* 76**,** 35-50.

Goelema, J., Smits, A., Vaessen, L. & Wemmers, A. 1999. Effects of pressure toasting, expander treatment and pelleting on in vitro and in situ parameters of protein and starch in a mixture of broken peas, lupins and faba beans. *Animal Feed Science and Technology,* 78**,** 109-126.

Grum, D.E., Drackley, J.K., Younker, R.S., LaCount, D.W. and Veenhuizen, J.J., 1996. Nutrition During the Dry Period and Hepatic Lipid Metabolism of Periparturient Dairy Cows. *Journal of Dairy Science*, *79*(10), pp.1850-1864.

Grummer, R. R. 1991. Effect of feed on the composition of milk fat. *Journal of Dairy Science,* 74**,** 3244-3257.

Hironaka, R., Beauchemin, K.A. and Lysyk, T.J., 1992. The effect of thickness of steam-rolled barley on its utilization by beef cattle. *Canadian Journal of Animal Science*, *72*(2), pp.279-286.

Hurrell, R., Carpenter, K., Sinclair, W., Otterburn, M. & Asquith, R. 1976. Mechanisms of heat damage in proteins. *British Journal of Nutrition,* 35**,** 383-395.

Ingalls, J.R., Clarke, K.W. and Sharma, H.R. (1974) Acid-treated high moisture barley for dairy cows. Canadian Journal of Animal Science, 54: 205 0 209.

Johnston, D.J., Theodoridou, K. and Ferris, C.P., 2019. The impact of field bean inclusion level in dairy cow diets on cow performance and nutrient utilisation. *Livestock Science*, *220*, pp.166-172.

Jones, G., Donefer, E. & Elliot, J. 1970. Feeding value for dairy cattle and pigs of high moisture corn preserved with propionic acid. *Canadian Journal of Animal Science,* 50**,** 483-489.

Larsen, M., Lund, P., Weisbjerg, M. R. & Hvelplund, T. 2009. Digestion site of starch from cereals and legumes in lactating dairy cows. *Animal Feed Science and Technology,* 153**,** 236-248.

Lykos, T. & Varga, G. 1995. Effects of processing method on degradation characteristics of protein and carbohydrate sources in situ. *Journal of Dairy Science,* 78**,** 1789-1801.

Mathison, G.W., 1996. Effects of processing on the utilization of grain by cattle. *Animal Feed Science and Technology*, *58*(1-2), pp.113-125.

McMurray, C.H., Blanchflower, W.J. and Rice, D.A., 1984. Automated kinetic method for D-3-hydroxybutyrate in plasma or serum. *Clinical chemistry*, *30*(3), pp.421-425.

Moate, P.J., W. Chalupa, R.C.Boston, and I.J.Lean (2008) Milk fatty acids II: Prediction of the production of individual fatty acids in bovine milk. J. Dairy Sci. 91:1175-1188.

Mogensen, L., Vestergaard, J. S., Frette, X., Lund, P., Weisbjerg, M. R. & Kristensen, T. 2010. Effect of toasting field beans and of grass-clover: Maize silage ratio on milk production, milk composition and sensory quality of milk. *Livestock Science,* 128**,** 123-132.

Offner, A., Bach, A. and Sauvant, D. (2003) Quantative review of in situ starch degradation in the rumen. Animal Feed Science and Technology, 106: 81 – 93.

Owens, F.N., Secrist, D.S., Hill, W.J. and Gill, D.R., 1997. The effect of grain source and grain processing on performance of feedlot cattle: A review. *Journal of Animal Science*, *75*(3), pp.868-879.

Park, R. S., Agnew, R. E., Gordon, F. J. and Steen, R. W. J. 1998. The use of near infrared reflectance spectroscopy (NIRS) on undried samples of grass silage to predict chemical composition and digestibility parameters. Animal Feed Science and Technology 72: 155-167

Remond, D., Cabrera-Estrada, J., Champion, M., Chauveau, B., Coudure, R. & Poncet, C. J. J. O. D. S. 2004. Effect of corn particle size on site and extent of starch digestion in lactating dairy cows. 87**,** 1389-1399.

Reynolds, C. K., Crompton, L. A. & Mills, J. A. 2011. Improving the efficiency of energy utilisation in cattle. *Animal Production Science,* 51**,** 6-12.

Reynolds, C.K. 2006. Production and metabolic effects of site of starch digestion in dairy cattle. Animal Feed Science and Technology, 130: 78 – 94.

Robinson, P., Fredeen, A., Chalupa, W., Julien, W., Sato, H., Fujieda, T. & Suzuki, H. 1995. Ruminally protected lysine and methionine for lactating dairy cows fed a diet designed to meet requirements for microbial and postruminal protein. *Journal of dairy science,* 78**,** 582-594.

Safaei, K. & Yang, W. 2017. Effects of Grain Processing with Focus on Grinding and Steam-Flaking on Dairy Cow Performance. *Herbivores.* InTech.

Scott, T.A., Combs, D.K. and Grummer, R.R., 1991. Effects of roasting, extrusion, and particle size on the feeding value of soybeans for dairy cows. *Journal of dairy science*, *74*(8), pp.2555-2562.

Shingfield, K. J. & Griinari, J. M. 2007. Role of biohydrogenation intermediates in milk fat depression. *European Journal of Lipid Science and Technology,* 109**,** 799-816.

Tice, E.M., Eastridge, M.L. and Firkins, J.L., 1993. Raw Soybeans and Roasted Soybeans of Different Particle Sizes. 1. Digestibility and Utilization by Lactating Cows1, 2. *Journal of Dairy Science*, *76*(1), pp.224-235.

Van Soest, P.V., Robertson, J.B. and Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of dairy science*, *74*(10), pp.3583-3597.

Williams, C. M. 2000 Dietary fatty acids and human health. Annales de Zootechnie, EDP Sciences, 165-180.

Wilson, G.F., Davey, A.W.F. and Dolby, R.M., 1967. Milk composition as affected by intra-ruminal infusion of volatile fatty acids to cows on a restricted ration. *New Zealand Journal of Agricultural Research*, *10*(2), pp.215-225.

Yang, W., Beauchemin, K. & Rode, L. 2000. Effects of barley grain processing on extent of digestion and milk production of lactating cows. *Journal of dairy science,* 83**,** 554-568.

Table 1 Chemical composition of the grass silage, concentrate pre-mix and field beans offered during the 12 week experimental period

					Field beans					
	Grass Silage	(s.d)	Concentrate pre-mix	(s.d)	Dry-CR	(s.d)	Dry-FM	(s.d)	Moist-P	(s.d)
Oven dry matter (g/kg)	290	47.4	882	5.6	862	12.1	863	9.4	685	2.2
VCODM (g/kg)	319	39.6								
Crude protein (g/kg DM)	166	29.4	190	4.6	296	12.6	287	6.8	287	5.7
Ash (g/kg DM)	90	4.3	92	8.4	41	1.6	41	0.8	41	1.1
Acid detergent fibre (g/kg DM)	274	27.9	181	22.7	144	36.2	150	27.5	174	30.1
Neutral detergent fibre (g/kg DM)	459	39.3	330	29.3	207	48.9	286	74.9	351	29.9
pH	3.9	0.11								
Lactic acid (g/kg DM)	137	45.7								
Acetic acid (g/kg DM)	13.8	4.19								
Ethanol (g/kg DM)	7.9	3.49								
Ammonia (g/kg total N)	6.9	0.85								
Starch (g/kg DM)			252	29.0	333	20.4	349	4.3	335	6.3
Gross energy (MJ/kg DM)	18.8	0.47	17.4	0.23	18.5	0.05	18.5	0.11	18.8	0.17
Metabolisable energy (MJ/kg DM)	11.8	0.64								
Particle size distribution, proportion (fresh basis):										
- retained within 4.8 mm sieve					0.098	0.0261	0.000	0.0000	0.996	0.0042
- retained within 3.2 mm sieve					0.314	0.0664	0.002	0.0024	0.004	0.0042
- retained within 1.6 mm sieve					0.339	0.0247	0.169	0.0246	0.000	0.0000
- passing through the 1.6 mm sieve					0.248	0.1104	0.829	0.0257	0.000	0.0000

VCODM: Volatile corrected oven dry matter

Table 2 Effects of post-harvest treatment of field beans on the feed intake, milk production and composition, the fatty acid content of milk, body tissue reserves and serum metabolites, measured during final week of each period

	Treatment				
	Dry-CR	Dry-FM	Moist-P	S.E.M	P-Value
Silage DMI (kg/day)	13.1	13.3	13.3	0.15	0.474
Concentrate DMI (kg/day)[1]	9.6[a]	9.7[a]	8.9[b]	0.09	<0.001
Total DMI (kg/day)	22.7	23.0	22.2	0.24	0.096
Milk yield (kg/day)	33.5	33.3	32.0	0.62	0192
Milk fat (g/kg)	41.9	41.5	42.2	0.86	0.858
Milk protein (g/kg)	33.7	33.9	33.9	0.22	0.779
Milk lactose (g/kg)	46.9	47.2	47.3	0.26	0.604
Milk fat yield (kg/day)	1.39	1.36	1.30	0.044	0.349
Milk protein yield (kg/day)	1.12	1.11	1.04	0.034	0.200
Milk fat + protein yield (kg/day)	2.50	2.47	2.33	0.073	0.232
Milk fatty acid concentrations (g/100 g total fatty acids measured)					
Total SFA[2]	76.1[a]	74.7[b]	74.4[b]	0.432	0.021
Total MUFA[3]	21.0[a]	22.3[b]	22.6[b]	0.389	0.018
Total PUFA[4]	2.8	2.9	2.9	0.054	0.284
C14:0	12.8	12.7	12.6	0.140	0.403
C16:0	39.5[a]	37.8[b]	38.1[b]	0.420	0.015
C18:0	8.5	8.6	8.7	0.200	0.733
C18:1 *cis*-9	15.9[a]	17.0[b]	17.3[b]	0.327	0.013
C18:2	1.48	1.52	1.47	0.027	0.332
C18:2 *cis* 9, *trans* 11	0.44	0.46	0.48	0.018	0.232
C18:3 *n*-3	0.51	0.54	0.55	0.014	0.065
Body condition score (1-5)	2.58	2.58	2.55	0.022	0.490
Body-weight (kg)	658	658	653	3.06	0.444
Beta-hydroxybutyrate (mmol/L)	0.50[a]	0.39[b]	0.42[ab]	0.026	0.013
Non-esterified fatty acids (m Eq/L)	0.13	0.14	0.13	0.010	0.689
Urea (mmol/L)	5.02	4.58	4.75	0.177	0.247

Means with the same superscript within a row do not differ significantly (P>0.05)
[1]Includes FB component at 3.4, 3.5 and 3.2 kg DM/cow/day for Dry-CR, Dry-FM and Moist-P respectively (SEM, 0.08, P-value 0.055)

Table 3 Effect of post-harvest treatment of the field beans component of the concentrate on dry matter intake and milk yield during the nutrient utilisation study, and on total ration digestibility coefficients

	Treatment				
	Dry-CR	Dry-FM	Moist-P	S.E.M	P-Value
Silage DM intake (kg/day)	13.2	13.8	13.05	0.949	0.855
Total DM intake (kg/day)	22.2	23.1	20.84	1.522	0.597
Milk yield (kg/day)	29.0	29.9	30.1	1.16	0.932
Digestibility coefficients (g/g)					
Dry matter	0.75	0.76	0.76	0.008	0.431
Organic matter	0.76	0.77	0.77	0.006	0.640
Nitrogen	0.65	0.68	0.66	0.007	0.315
Gross energy	0.74	0.75	0.74	0.004	0.479
ADF	0.64	0.67	0.73	0.030	0.169
NDF	0.68	0.67	0.71	0.060	0.343

ADF, acid detergent fibre; NDF, Neutral detergent fibre

Table 4 Effect of post-harvest treatment of field beans on N intake, N output and N utilisation of dairy cows

	Treatment				
	Dry-CR	Dry-FM	Moist-P	S.E.M	P-Value
N intake/output (g/d)					
Total N intake	624	612	562	27.6	0.289
Faeces N	216[a]	196[ab]	188[b]	6.4	0.035
Urine N	188	216	202	7.1	0.065
Manure N	404	412	390	5.7	0.073
Milk N	149	152	159	8.4	0.746
N utilisation (g/g)					
Faeces N/N intake	0.35	0.32	0.34	0.012	0.315
Urine N/N intake	0.31	0.35	0.38	0.026	0.188
Manure N/N intake	0.66	0.67	0.71	0.033	0.466
Milk N/N intake	0.24[a]	0.25[a]	0.29[b]	0.006	0.002
Faeces N/Manure N	0.54	0.48	0.48	0.016	0.056
Urine N/Manure N	0.46	0.52	0.52	0.016	0.056

Means with the same superscript within a row do not differ significantly (P>0.05)

Table 5 Effect of post-harvest treatment of field beans on energy intake, energy output and energy utilisation

	Treatment				
	Dry-CR	Dry-FM	Moist-P	S.E.M	P-Value
Energy intake and output (MJ/day)					
Gross energy intake	410	410	381	18.40	0.478
Faecal energy	107	102	97	3.94	0.271
Urinary energy	14	14	14	0.59	0.930
Milk energy	97	95	108	5.35	0.231
Energy utilisation (MJ/MJ)					
Faeces E/GEI	0.26	0.25	0.26	0.007	0.414
Urine E/GEI	0.03	0.03	0.04	0.003	0.467
Milk E/GEI	0.24[a]	0.23[a]	0.29[b]	0.010	0.010

Means with the same superscript within a row do not differ significantly (P>0.05)

SECTION 2

STUDIES ON ENSILING AND FEEDING RED CLOVER TO DAIRY COWS

Experiment 4

Performance and nutrient utilisation of dairy cows offered silages produced from three successive harvests of either a red clover-perennial ryegrass sward or a perennial ryegrass sward.

Introduction

Within the United Kingdom (UK) and Ireland the protein requirement of higher yielding dairy cows is normally met in part through the use of imported protein feeds, including soybean meal and rapeseed meal. However, supply and price volatility, as well as restrictions on the use of genetically modified feeds, have created renewed interest in the use of forage legumes. In addition to their potential to produce high annual yields of dry matter (DM) and crude protein (CP), their ability to fix atmospheric nitrogen (N) reduces the need for N fertiliser, and this can contribute to legume-based systems having a lower carbon footprint (Peyraud *et al*., 2009).

Red clover is a forage legume of particular interest within the UK and Ireland (Wilkins and Jones, 2000), where it has the potential to produce high annual DM yields (Dale *et al*., 2014; Clavin *et al*., 2017). In addition, red clover silage normally has a higher protein content than grass silage (Dewhurst *et al*., 2003a). While the protein in red clover may degrade relatively quickly (Dewhurst *et al.,* 2009), the variable presence of polyphenol oxidase (PPO) in red clover can contribute to a lower rate of ruminal protein degradation compared to protein in other forage legumes, such as lucerne (Lee *et al*., 2004). However, due to its high protein content, and the associated reduction in nitrogen use efficiency (Dewhurst *et al*., 2003a; Moorby *et al*., 2009), red clover silage is often offered in mixtures with grass silage.

In general, when mixtures of red clover silage and grass silage are offered to dairy cows, relative to grass silage DMI increases resulting usually, but not always, in an increase in milk yield, milk fat and protein content are usually either not affected or decline (Dewhurst, 2013). However, in the majority of these studies the grass silage has been produced from swards which have received moderate or high rates of N fertiliser, and this does not simulate silage produced from mixed swards of grass and red clover, which normally receive either none or only small amounts of N fertilizer. For example, the grass in a grass-red clover sward will have a low N content due the relatively low amount of N transferred from red clover to the accompanying grass, particularly in the first full harvest year (Dahlin and Stenberg, 2010).

Mixed swards of grass and red clover are increasingly being sown on farms. In one of the few studies examining their use, Vanhatalo *et al*. (2006) found that cows offered a

grass-red clover silage had higher milk yields, but similar DMI, milk fat, and milk protein content, than cows offered a grass silage. Nevertheless, as in most red clover feeding studies, the silages offered were produced from a single harvest, rather than from successive harvests. In contrast, on commercial dairy farms, silages produced from all harvests within a season are normally offered to livestock, and thus it is important to quantify differences between harvests within a full growing season. This is important as changes in sward structure, maturity and fibre content of the component species in a grass-red clover sward over the course of the growing season will affect the nutritional quality and intake characteristics of the silage, and consequently milk production and milk composition (Vanhatalo *et al*., 2009). For example, a regrowth of a grass-red clover sward had a higher CP content and total complement of amino acids than its primary growth (Naadland *et al*. 2016).

The impact of offering red clover silage to dairy cows does not appear to have been examined previously on the island of Ireland. This is important as research carried out elsewhere may not be directly applicable to Ireland as the maritime climate can lead to unavoidable high humidity or rainfall at harvest, creating sub-optimal conditions for wilting (McEniry *et al*., 2013), which will impact on silage quality. Nevertheless, the majority of studies examining the impact of red clover inclusion in the diet have involved herbage ensiled under near optimum conditions. Consequently this study was conducted under Irish conditions, and compared the effects of offering dairy cows grass silages and grass-red clover silages produced from three consecutive harvests over a full production year (harvested early summer, late summer and autumn), on N and energy utilisation and subsequent animal performance.

Materials and Methods

This study was conducted at the Agri-Food and Biosciences Institute (AFBI), Hillsborough, Northern Ireland (54º27'N; 06º04'W). All experimental procedures were conducted under an experimental licence granted by the Department of Health, Social Services & Public Safety for Northern Ireland in accordance with the Animals (Scientific Procedures) Act 1986.

Experimental forages

A perennial ryegrass sward and a red clover/perennial ryegrass mixed sward (approximately 3 ha each) were established during autumn 2013. Crops were sown on 3 and 4 September 2013 following conventional seed bed preparation and sown using an air seed drill with a mounted spring harrow attachment (Stocks-Ag, UK; Twose, UK). Seed rates for red clover (*cv.* Merviot) and perennial ryegrass (*cv.* Navan) in the mixed sward were 9.9 kg/ha and 22.2 kg/ha, respectively, while the seed rate for the perennial ryegrass in the pure sward was 32.1 kg/ha. Neither sward received additional nitrogen fertiliser during establishment. The crops were harvested on three occasions during 2014, namely 12 June (H1), 7 August (H2), and 2 October (H3). Crops were mown at approximately 14.00 h at each harvest using a mower/conditioner unit (Claas, UK) and allowed to wilt in the swaths for periods of 48 hours, 72 hours and 72 hours, for Harvests 1, 2 and 3, respectively. Swards were placed in windrows using a trailed tractor-driven twin-rotor rower (Claas, UK) 2 - 3 hours prior to ensilage, and baled using a conventional round baler set at 90% density (Krone, UK). The crops were then wrapped using a conventional bale wrapper (McHale, Ireland) dispensing plastic film with 17 wraps per bale, weighed, and stacked. The yields for Harvest 1, 2 and 3 of the RCGS swards were 4.4, 4.0 and 1.6 t DM/ha, respectively, representing an annual yield of 10.0 t DM/ha. The yields for Harvest 1, 2 and 3 of the GS swards were 5.2, 3.4 and 1.8 t DM/ha respectively, representing an annual yield of 10.4 t DM/ha. Based on a visual assessment pre-mowing, the RCGS swards were estimated to contain approximately 20, 40 and 60% red clover at each of H1, H2 and H3, respectively.

During 2014 both the grass and the red clover/grass swards received 22 kg N/ha (Urea, 46% N) in March. The GS sward received a further 106 kg N/ha (CAN, 27% N) in April, followed by 122 kg N/ha after H1 in June, and a final application of 65 kg N/ha in August following H2. The RCGS sward received no additional N fertiliser during the growing season.

Animals and housing

Twenty-eight three-breed crossbred (Swedish-Red × Jersey/Holstein-Friesian) dairy cows (22 multiparous, six primiparous) with a mean lactation number of 3.1, a mean days-in-milk of 82 (s.d., 7.8) days, were used in this experiment. Cows were housed as a single group in a free-stall house with concrete flooring with access to individual cubicles that were fitted with rubber mats, and were bedded with sawdust twice daily. The cubicle-to-cow ratio was ≥1:1 at all times, thus meeting the recommendations of FAWC (1997). The floor area was scraped every 3 hours using an automated system.

Treatments

During a two-week pre-experimental period cows were offered grass silage plus 8.0 kg/day of concentrate via an in-parlour concentrate feeding system at a rate of 4.0 kg at each milking. Cows were allocated to one of two experimental treatments (grass silage, GS; or red clover/grass silage, RCGS) on the final day of the pre-experimental period, with cows within each treatment group balanced for parity (1, 2, 3 and ≥4), days in milk, and for milk yield, milk fat and protein content, live-weight (LW) and body condition score (BCS) during the week prior to the start of the study. Throughout the experiment all cows were offered 8.0 kg/day of a common concentrate through an in-parlour concentrate feeding system. This was divided into two equal meals, 4.0 kg at each milking. The ingredient composition of the concentrate (g/kg, fresh basis) was as follows: maize meal, 171; soya bean meal, 132; wheat, 128; rapeseed meal, 125; corn gluten, 124; soya hulls, 101; palm kernel, 99; wheat-feed, 45; molaferm (United Molasses, Belfast, UK), 40; lime flour, 9.5; palm oil, 9.5; salt, 8.2; calcined magnesite, 4.1; mineral-vitamin mix, 4; yeast (Actisaf, Lesaffre, Shannon, Ireland), 0.5.

Cows were offered the experimental forages during a 13-week experimental period. Silages produced from H1, H2 and H3 were offered successively, for periods of five weeks, five weeks and three weeks, respectively, with the duration of each period broadly reflecting the yields within each harvest of the grass-red clover sward. Fresh silage was offered daily at approximately 09.00 h (at 107% of the previous day's fresh weight intake), with uneaten silage removed the following day at approximately 08.00 h. Silage for cows on each treatment was mixed in a feeder wagon (Redrock Vari-Cut-12, Redrock, Armagh, Northern Ireland) for approximately six minutes prior to being

deposited in a series of feed boxes mounted on weigh scales. Cows accessed silage in these feed boxes via Calan gates (American Calan Inc., Northwood, NH, USA) linked to an electronic identification system, thus enabling individual cow intakes to be recorded daily. Cows had free access to fresh water at all times.

Cow measurements and sampling of feeds offered

All cows were milked twice daily (between 06.00 and 08.00 h and between 15.00 and 17.00 h) throughout the experiment using a 50-point rotary milking parlour, with milk yields automatically recorded at each milking, and a total daily milk yield for each cow for each 24-hour period calculated. Milk samples were taken during two consecutive milkings each week throughout the experiment, and analysed for fat, protein and lactose concentrations using an infrared milk analyser (Milkoscan Model 605; Foss Electric, Warrington, UK). A weighted concentration of each constituent was determined for the 24-hour sampling period.

Live-weight was recorded twice daily (immediately after each milking) using an automated weighbridge, and a mean weekly LW for each cow determined. The BCS of each cow was estimated fortnightly on a 1 – 5 scale, according to Edmonson *et al.* (1989) by a trained technician.

Samples of the experimental silages were taken daily throughout the experiment, dried at 85°C for 24 hours to determine oven DM (ODM) content, and milled through a mesh sieve with 1 mm apertures. Sub-samples of the dried milled silages were taken twice weekly and bulked for each week, with the bulked samples analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF), and ash concentrations. A fresh sample of each silage was taken weekly and analysed for concentrations of N, ammonia-N, gross energy (GE), fermentation acids (lactic, acetic, propionic, n-butyric, and iso-valeric acids), ethanol, propanol, metabolisable energy (ME), and for pH. The concentrate offered was sampled 3 times weekly, with one sub-sample dried at 85°C for 24 hours for ODM content, and the dried sample discarded. A second sub-sample was dried at 60°C for 48 hours prior to milling through a 1 mm sieve, bulked for each 14 day period, and analysed for N, NDF, ADF, ash, GE and starch concentrations.

Nutrient utilisation

During the final eight days of the feeding period within each harvest, four cows from each treatment (the same four cows at each harvest) were used in a nutrient utilisation study. The four cows selected from each treatment group were initially balanced for daily milk yield and LW. All eight cows were transferred into an experimental nutrient utilisation facility (at approximately 09.00 h) and tied by the neck in individual stalls, with their lying area comprising a rubber mat. Cows continued to be offered their experimental rations in a feed box located at the front of each stall. The experimental silages were offered *ad libitum* daily at 09:00 h, while uneaten silage was removed, with refused feed weighed and recorded the following day at 08:00 h. Concentrate (8.0 kg/day) was offered in two equal meals each day (4.0 kg/meal) at 06.30h and 16:30h, during milking. This concentrate was offered in plastic feed buckets which were placed within the feed boxes, and removed after all the concentrates had been consumed at each feeding time. Cows had access to fresh water at all times via a drinker located within each stall. Cows were weighed prior to, and on completion of each balance period, with the average live weight used in energy utilisation calculations.

Measurements of nutrient utilisation commenced 24 h after cows were placed in the nutrient utilisation facility, and comprised a 6-day feeding period (commencing two days before the first collection of faeces and urine), and a 6-day total faeces and urine collection period. Faeces was collected in a plastic collection tray (96 cm x 108 cm x 36 cm) placed behind each cow. Urine was collected into a 25 litre plastic container via a flexible plastic tube which was attached to a urine separation system. This was held in position over the vulva by attaching it using Velcro fasteners to a ‘patch’ glued (Bostik, France) either side of the cow’s tail head. Approximately 300 ml of 50% sulphuric acid was added to each urine collection container when empty to minimise nitrogen losses as ammonia. Containers were examined at approximately 20.00 h, and if more than 60% full, approximately, were replaced with an empty container. The total weight of faeces and urine produced during each 24 h collection period was recorded, and a sample of each (0.05 by weight) retained for subsequent analysis. Faeces and urine samples were stored in a fridge (4-6^{o}C) until the final day of the collection period, when the six daily faeces samples and six daily urine samples from each cow were bulked. The single bulked fresh urine sample for each cow was

analysed for GE and N concentrations, while the single bulked fresh faeces sample for each cows was analysed for N concentration. A single bulked dried faeces sample for each cow was dried at 85ºC for 100 h to determine ODM, with the dried sample subsequently milled and analysed for ADF, NDF, ash and GE concentrations. A milk sample was taken at each milking, bulked in proportion to yield, with samples bulked for the entire 6-day period and subsequently analysed for GE and N concentrations. Fresh silages offered were analysed daily for ODM, GE and N concentrations, while daily dried samples over the 6-day nutrient utilisation period were bulked and the single bulked sample for each period analysed for ADF, NDF and ash concentrations. The concentrate offered was sampled daily, bulked for the 6-day nutrient utilisation period, and its ODM concentration determined. The dry samples were subsequently analysed for GE, NDF, ADF, N and ash concentrations.

In vitro protein degradation kinetics using the Daisy^{II} incubator

Samples of the dry milled silages collected twice weekly were bulked for each harvest, and used to determine *in vitro* ruminal protein degradability using an ANKOM Daisy^{II} incubator (Ankom Technology®, Macedon, NY, USA). Samples (0.5 g) were weighed into F57 filter bags (in triplicate) (which had been pre-rinsed in acetone and allowed to dry), and then heat sealed. The four Daisy jars (2 litre capacity) were filled with a warmed (39°C) mixture of solution A (266 ml) and B (1330 ml) (v/v as 1:5; pH 6.8). The reagents used were: solution A (in 1 L of deionized water), KH_2PO_4 (10.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.1 g) and urea (0.5 g); solution B (in 100 ml of deionized water), Na_2CO_3 (15.0 g) and $Nsga_2S \cdot 9H_2O$ (1.0 g). Rumen fluid was then used to inoculate each Daisy jar. The rumen fluid used was collected three hours after morning feeding from two Holstein cows fitted with a ruminal cannula, thoroughly mixed together and poured into an insulated bottle prior to transport to the laboratory, where it was then filtered through four layers of cheese cloth. The rumen fluid was then poured into the Daisy jars (400 ml/jar), mixed with solution, and the inoculum was again warmed to 39°C. The jars were continuously purged with a stream of CO_2 gas to maintain anaerobic conditions.

The *in vitro* ruminal protein degradation kinetics were performed for each silage type and harvest according to the 'gradual addition/all out' schedule (Theodoridou and Yu, 2013), with samples incubated in the jars for 2, 4, 8, 12, 24 and 48 h. The bags

assigned to the 48 h incubation period were inserted first, followed 24 h later by those assigned to the 24 h period, with this repeated for the remaining bags at the appropriate time intervals. Each treatment were contained within separate jars and not mixed with other treatments. The whole process comprised three experimental runs. The number of bags for each treatment and in each experimental run were 2, 2, 2, 2, 6, 4 and 5 bags for incubation times 0, 2, 4, 8, 16, 24 and 48 h, respectively. The maximum number of bags in the each jar at any one time was 23.

The Daisy[II] rotated the jars continuously in the incubation chamber, and were mounted on slow turning rollers inside the fermentation cabinet which results in vessel rotation and filter. After the incubation, the bags were removed from the incubator and rinsed under a cold stream of tap water without detergent to remove excess rumen contents, and subsequently dried at 55° C for 48 h and reweighed. The soluble N fraction was determined by soaking bags containing ground samples in warm water (40° C) for 1.5 h, and was considered as the proportion of sample N that had disappeared from the bags at T0. The dried samples were kept in a refrigerated room (4° C). Within each treatment, samples for each harvest were subsequently bulked, and milled through a 1 mm sieve, and analysed for N using the Dumas method (Elementar, Vario Max CN).

Rumen degradation kinetics

In vitro rumen degradation kinetics of CP were determined using the first-order kinetics equation described by Ørskov and McDonald (1979), and modified by Robinson *et al*. (1986) and Dhanoa (1988) to include lag time: $R\,(t) = U + (100 - S - U) \times \exp[-Kd \times (t - T0)]$ where $R(t)$ is the residue present at t h incubation (%; S is the soluble fraction (%); U is the undegradable fraction (%); D is the potentially degradable fraction (%); $T0$ is the lag time (h); and Kd is the degradation rate (%/h).

The results were calculated using the NLIN (nonlinear) procedure of SAS (SAS Institute, Cary, NC, USA) with iterative least squares regression (Gauss–Newton method). Based on the nonlinear parameters estimated by the above equation (S, U and Kd), rumen-degraded feed CP (RDP), and rumen undegraded CP (RUP) were predicted according to the NRC 2001 model as:

$$(1)\ \text{RDP}\ (\%) = S + (D \times Kd) / (Kp + Kd),$$

$$(2)\ \text{RUP}\ (\%) = U + (D \times K\text{d}) / (K\text{p} + K\text{d}),$$

where D =100 − S − U (%) and Kp is the estimated rate of outflow of digesta from the rumen (%), which was assumed to be 0.06 per h, according to Tamminga *et al*. (1994).

Estimation of the intestinal digestibility of rumen undegraded crude protein

Intestinal digestibility of rumen undegraded feed protein was determined according to the protocol for ruminants (Calamiglia and Stern, 1995. Dried ground rumen residues, containing 15 mg of N, after 16 h of ruminal incubation, were exposed for 1 h to 10 ml of 0.05 mol L^{-1} HCl solution containing 1 g L^{-1} pepsin. The pH was then neutralized with 0.5 ml of 0.5 mol L^{-1} NaOH and 13.5 mL of pH 7.8 phosphate buffer containing 37.5 mg of pancreatin, which were added to the solution and incubated at 38°C for 24 h. After 24 h incubation, 3 ml of a 100% (wt/vol) trichloroacetic acid (TCA) solution was added to precipitate undigested proteins. The samples were centrifuged and the supernatant was analyzed for N by the kjeldahl method (Tecator Kjeltec Auto 2400/2460 Analyzer/Sampler System, Foss). Intestinal digestion of protein was calculated as TCA-soluble N divided by the amount of N in the 16 h residue sample.

Chemical analysis of feed, faeces and urine samples

Nitrogen concentrations of fresh samples were determined using the Kjeldahl method while N concentrations of dried samples were determined using the Dumas method (Elementar, Vario Max CN). Concentrations of NDF and ADF were determined using a Fibertec analyser (Fibertec FT122, Foss, Hillerød, Denmark) based on the method of Van Soest (1976), and ash concentrations were determined following combustion in a muffle furnace at 550°C for approximately 10 hours. Starch concentrations were determined using a Megazyme Kit (Megazyme International, Bray, Ireland). Gross energy concentrations of fresh silage, dry concentrate and faeces, and of freeze dried samples of milk and urine were determined using a bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument Co., Moline, Il., USA). Silage fermentation acids, ethanol, and propanol were determined using single-column, gas-liquid chromatography (Varian Star 3400 CX G.C.; equipped with a flame-ionisation detector), where samples were injected on-column. Ammonia nitrogen concentrations in silage, and silage pH, were determined as described by Steen (1989). The ME concentration of the fresh

silages was determined using near infrared reflectance spectroscopy (NIRS), using a calibration developed for grass silages, according to Park *et al.* (1998).

Statistical Analysis

Differences in the mean chemical composition of the GS and RCGS silage within each of Harvests 1 - 3 were tested for significance by Student's two-tailed t-test using Microsoft Excel (2013). *In vitro* protein degradation means were tested for significance by two-way analysis of variance, with 'runs' considered as replicates. Mean data for DMI, milk yield, milk composition, live weight (LW) and body condition score (BCS), as recorded during the period when each silage was offered, and over the entire experimental period, were analysed using ANOVA. When significant, appropriate pre-experimental variables were included as co-variates in the model when analysing corresponding dependant variables (pre-experimental milk yield for milk yield, pre-experimental milk composition values for milk fat, protein, fat-plus-protein, and mean daily milk yields; pre-experimental live weight for mean LW; pre-experimental BCS for mean and final BCS; pre-experimental LW for forage, concentrate, and total DMI). The effect of forage type on total diet digestibility coefficients, and N and GE parameters was examined using a two-way ANOVA. Means for protein degradation were analysed as a one way ANOVA. All data were analysed using GenStat (16^{th} edition, VSN International Ltd, Hemel Hempstead, UK).

Results

Chemical composition and in vitro protein degradability of silages

The concentrate offered had a DM, CP, ADF, NDF, ash, GE and starch concentration of 894 (s.d., 3.1) g/kg, 217 (s.d,. 2.54) g/kg DM, 159 (s.d., 6.7) g/kg DM, 325 (s.d., 4.0) g/kg DM, 76 (s.d., 1.5) g/kg DM, 18.0 (s.d., 0.04) MJ kg DM, and 229 (s.d., 8.7) g/kg DM, respectively. The volatile corrected ODM content of RCGS was higher than that of GS at H1 (P=0.014) while the reverse was true at H3 (P=0.005) (Table 1). At H1 forage CP tended to be higher in GS than RCGS (P=0.074), while at H3 it was lower in GS than RCGS (P=0.006). Both the ADF and NDF of GS at H1 was higher than in RCGS (P=0.001, P=0.002, respectively). At H2 the GS had a higher NDF content than RCGS (P=0.002) while the reverse tended to be the case for ADF content (P=0.059). Contents of acetic acid, propionic acid and n-butyric acid were higher in GS than RCGS at H1 (P=0.005, 0.017 and 0.001, respectively) and lower in GS than RCGS at H3 (P<0.001, 0.005 and 0.043, respectively). Acetic acid content was higher in RCGS than GS at H2 (P=0.034). The RCGS tended to have a lower lactic acid content than GS at H1 (P=0.087), while at H3 it was higher in RCGS than GS (P=0.044). Ammonia nitrogen was higher in GS than RCGS at H1 (P=0.002) and pH was lower in GS than RCGS at H2 (P=0.014). The ME content of the two silages were not significantly different at either H1 or H2, while tending (P=0.088) to be higher with the GS at H3.

Across the six forages offered there were no differences in D (potentially degradable fraction), or K_d (degradation rate) (Table 2). The potentially soluble fraction (S) was significantly higher in higher in GS than RCGS at H3 only. The undegradable fraction (U) was significantly higher in RCGS than GS at each of H2 and H3. The RCGS had a significantly higher rumen undegradable crude protein (RUCP) at H2 and H3, while the reverse was true for EDCP. While IVCPD was higher with RCGS at H1, it was unaffected by silage type at either of H2 or H3. IVCPDCP was higher with RCGS at H3, but did not differ at either of H1 or H2 (Table 2).

Cow performance

Silage DMI was lower in GS than RCGS treatments in H1 (P<0.001) and H2 (P=0.006), and across the entire experimental period (H1– H3: P<0.001; Table 3). Differences in total DMI were similar. Milk yield was unaffected by silage type at H1 (P>0.05), tended

to be lower for cows offered GS than RCGS at H2 (*P*=0.093), while being significantly lower for those offered GS at H3 (*P*=0.002). Milk fat concentration was unaffected by treatment at H1 and H2, but was higher in GS than RGCS at H3 (*P*<0.001). Similarly, milk protein concentration was unaffected by treatment at H1, tended to be significantly lower for cows offered RCGS than GS at H2 (*P*=0.080), and was lower for cows offered RCGS than GS at H3 (*P*<0.001). Milk fat + protein yield was unaffected by treatment at either of H1 or H2, but was lower with cows offered RCGS than GS at H3 (*P*=0.001). Cows offered RCGS were heavier (*P*=0.049) and had a higher condition score (*P*=0.042) than those offered GS at H1, while neither LW nor BCS was affected by treatment at either H2 or H3.

Nutrient utilisation

None of the digestibility parameters examined differed between GS and RCGS at H1 (P>0.05) (Table 4). DM digestibility, organic matter digestibility and digestible organic matter in DM were higher for GS than RCGS at H2 (*P*<0.001, P=0.003 and P<0.001, respectively) and H3 (*P*=0.006, P=0.007 and P=0.002, respectively). Digestibility of ADF and NDF was higher for GS than RCGS at H2 (*P*<0.001, P=0.017, respectively) and H3 (*P*=0.009 and P=0.037, respectively).

Faeces N/N intake was higher for RCGS than GS at H1 (*P*=0.019), but did not differ between silages at H2 and H3, while urine N/N intake was higher for GS than RCGS at H1 (*P*=0.008; Table 5). Manure N/N intake was higher for GS at H1 (*P*=0.005), while the reverse was observed at H2 (*P*=0.002). Milk N/N intake was higher for GS than RCGS at H2 and H3 (*P*=0.007 and 0.020, respectively). Retained N/N intake was higher for GS than RCGS at H2 (*P*=0.035), but unaffected at H1 and H3. At H1 faeces N/manure N was higher in RCGS than GS (*P*=0.010), while urine N/manure N was higher for GS than RCGS (*P*=0.010).

Gross energy digestibility and DE/GE were higher with GS than RCGS at H2 (P= 0.003, and P=0.002, respectively), but not at H1 and H3 (Table 6). Milk energy/ME intake tended to be higher with GS than RCGS at H1 (P = 0.094), while being higher at H3 (P<0.001). At H2 the proportion of digestible energy intake that was lost as faecal and urinary energy was higher in RCGS than GS (*P*=0.002 and 0.003, respectively)

while at H3 faecal E/DEI was unaffected by treatment while urine E/DEI was higher with GD (P=0.032).

Discussion

This study examined the performance and nutrient utilisation of dairy cows offered silages produced from three successive harvests of a red clover-perennial ryegrass sward, and a perennial ryegrass sward. While previous studies have examined the impact on cow performance of mixing red clover silages and grass silages post-harvest, few have examined silages produced from mixed swards across multiple harvests, as would be commonly practiced under commercial farm conditions.

Sward production

The annual DM production from the RCGS sward (10.0 t DM/ha) in the current study was similar to that recorded in a field scale study by Castle and Watson (1974) in western Scotland (10.9 t DM/ha). However, these yields were considerably lower than the 14.7 t DM/ha reported by Roberts *et al*. (1990) from a mixed red clover-grass sward in its first harvest year in southern Scotland.

While pure red clover crops have been reported to fix up to 250 kg N/ha (Smith *et al*., 1985), less is known about the N fixing potential of red clover within grass swards. In the current study the total annual DM produced from the mixed sward (which received only 22 kg N, in early spring) was only 0.4 t DM/ha less than that produced from the pure perennial ryegrass sward (10.4 t DM/ha) which received a total of 315 kg N/ha. This suggests that the amount of N the red clover component contributed to the sward was equivalent to a grass sward receiving approximately 263 kg N/ha, assuming the additional 0.4 t DM/ha was produced by 30 kg N/ha (Morrison *et al*., 1980) i.e. 315 – 30 – 22 kg N/ha.

Silage characteristics

Within each harvest, both the grass and grass-red clover silages were produced under the same climatic conditions, with both swards wilted for similar periods. However, silage DM contents indicate that wilting conditions were poor within the current study, as occurs frequently in Ireland. In general, red clover tends to have a higher moisture content than grass at any given time during the growth cycle (Hynes *et al*., 2018), and this likely accounts for the lower DM of RCGS than GS at H3, the former estimated to contain approximately 60% red clover. In contrast, the higher DM content of the RCGS

than GS at H1 is likely to reflect the lower total yield of crop resulting in more rapid drying than the heavier GS crop (Wright *et al*., 1997) and the low red clover proportion with this crop at first harvest (visually estimated to be approximately 20%).

Crude protein content of a pure red clover sward has been found to increase progressively with harvest. For example, in a trial in Ireland, CP increased from 155 g/kg at the first to 263 g/kg at the fourth harvest (Clavin *et al*., 2017). The increase in the CP content of the RCGS silages (98, 148 and 216 g/kg DM, for H1, H2 and H3, respectively) reflects the increasing CP content of the swards as red clover content increased with progress through the growing season, in addition to the seasonal increase in CP content as found by Clavin et al. (2017). This compares to a CP content of the GS at H3 of 158 g/kg DM, despite the much higher rate of N fertiliser applied to GS than RCGS.

The presence of vegetative red clover in the sward, together with the much lower application of inorganic fertiliser N, is likely to have contributed to the lower concentrations of ADF and NDF in RCGS than GS. The vegetative state of both sward types at H3 would have been responsible for the lower ADF and NDF contents at this harvest compared to H1 and H2. The higher pH for red clover silage than grass silage at H2 is similar to the 0.7 unit difference between the two silage types observed by Moorby *et al*. (2009). While this can be attributed to the high buffering capacity of red clover compared to perennial ryegrass (King *et al*., 2012), the reason that no such effect was observed at H3 is unclear. While red clover silages tend to have higher concentrations of VFA than grass silages (Moorby *et al*., 2009), no consistent trends in VFA content between RCGS and GS were observed in the current study due possibly to the lower DM content of RCGS than GS within harvests.

Although levels are variable, red clover PPO can oxidise phenols to produce quinones that bind with specific sites on some proteins reducing proteolysis in the rumen (Theodorou *et al.*, 2006). The presence of PPO may explain the higher proportion of CP that was undegraded in the rumen in RCGS compared to GS at H2 and H3 when assessed by the *in vitro* method. The digestibility of undegradable protein declined with successive harvests, thus suggesting less N to be readily available for intestinal absorption. Johansen *et al.* (2017) found total tract digestibility of CP in a perennial ryegrass-red clover silage to be slightly but significantly lower than that of the

corresponding grass silage, but rumen degradation rate of CP in red clover was faster than in perennial ryegrass. In our study although total degradation of protein in RGCS was lower than in GS, there was no difference in degradation rate between the two silages.

Intake and performance

Over the entire experimental period forage DM intakes with RCGS were 1.6 kg/day higher than for GS. However, this effect was not consistent across harvests, intakes with RCGS being higher at H1 and H2, but not at H3. In general, intakes increase with red clover inclusion in the diet. For example, Moorby *et al*. (2009), with a diet containing a mixture of red clover and grass silages (one third red clover on a DM basis), and Kuoppala *et al*. (2009), with a diet containing equal mixtures of red clover and grass silage on a DM basis, found intakes to be 1.1 and 2.0 kg DM/day higher respectively, than with pure grass silages. Halmemies-Beauchet-Filleau *et al*. (2012) also found an increase in forage DMI when red clover silage was mixed with grass silage, compared to a grass silage-only diet.

While *in vivo* digestibility and *in vitro* degradability characteristics of the two silage types did not differ for any parameter at H1, RCGS had a much higher DM content at H1 than at subsequent harvests, potentially contributing to the higher DM intake at this harvest. In addition, RCGS had a lower fibre content than GS, reflecting the visibly less mature state of the crop at harvest than at later harvests. However, the higher intakes with RCGS than GS at H2 occurred despite the two silages having similar degradability characteristics and DM content, and the RCGS having a lower digestibility coefficient for each of the parameters measured. Nevertheless, the NDF content of the RCGS was substantially lower than for the GS. In contrast at H3, although red clover content was at its highest, DM intake of RCGS was similar to that of GS at this harvest. As with H2, digestibility of RCGS was lower than that of GS, however the similar NDF contents between the two silages may have been a contributing factor (Weisbjerg and Soegaard, 2008), combined with the higher DM content of the GS. A similar effect was observed by Halmemies-Beauchet-Filleau *et al*. (2014).

Dry matter intake is partially inversely related to the concentration of NDF, and directly related to the rate of particle breakdown of feed and rumen digesta outflow (Kuoppala

et al., 2009). Rate of fermentation in the rumen and subsequent particle breakdown are considered to contribute to rate of rumen clearance (Moseley and Jones, 1984) which in turn influences DMI in legumes (Dewhurst, 2013). Legume leaves are more easily broken down into smaller particles than grass leaves (Mtengeti *et al*., 1996), and as a consequence, rumen fermentation is generally increased when red clover silage is offered (Vanhatalo *et al.,* 2009). However, this increase is not always associated with an increase in production of VFAs (Dewhurst *et al.,* 2003b).

In general, evidence within the literature suggests that red clover inclusion in the diet increases milk yields (Moorby *et al.,* 2009; Dewhurst, 2013), with this normally attributed to higher DM intakes. In agreement with these findings, over the entire experimental period milk yield tended to be higher with RCGS. However, this overall effect masks a number of underlying trends and effects within individual harvests. For example, there was a trend for the milk yield advantage to increase with increasing red clover content in the silages (P = 0.228, P = 0.093 and P = 0.002, for H1, H2 and H3, respectively), with a difference of 2.9 kg/d in favour of RCGS at H3. The absence of a milk yield benefit with RCGS at H1, despite the significantly higher intakes, may have been due to the low overall CP content in the diet (143 g/kg DM with RCGS compared to 169 g/kg DM with GS), a reflection of the low application of N fertiliser and the low red clover content. The additional energy deposited as body tissue with this treatment supports the RCGS diet having a low protein content. Despite similar intakes at H3, RCGS produced a significantly higher milk yield than GS. However, fat + protein yield was lower for RCGS than GS.

Offering RCGS had no effect on milk fat content at H1 and H2, while reducing milk fat content at H3. The poor milk fat response to red clover inclusion is in general agreement with the findings of a number of other studies (Dewhurst *et al.,* 2003a; Moorby *et al*., 2009; Vanhatalo *et al.* 2009; Johansen *et al*., 2017). Milk fat production is associated with the availability of acetic and butyric acid in the rumen, which requires degraded fibre to undergo bio-hydrogenation (Murphy *et al*., 2000). Low ADF and NDF digestibility leads to a reduction in beta-hydroxybutyrate available for de-novo fat synthesis within the mammary gland (Heinriches *et al*., 1997). Also, polyphenol oxidase (PPO) lowers rumen bio-hydrogenation of polyunsaturated fatty acids (PUFA), with PUFA known to inhibit milk fat synthesis in the mammary gland. Further, lipids may also be incorporated physically in protein complexes resulting from the

action of PPO (Halmemies-Beauchet-Filleau *et al.*, 2012). Digestibility of NDF and ADF was lower in RCGS than GS at H2 and H3. At H2 intake was higher in RCGS than GS and so despite the lower digestibility of NDF and ADF, intake of digestible fibre in the two treatments would have been similar. In contrast, there being no significant difference in intake between the two silages at H3, the lower digestibility of NDF and ADF with RCGS would explain the lower fat content of the milk in that treatment.

In a meta-analysis of 43 studies comparing grass and legume silages offered to dairy cows, in which 29 involved red clover-grass comparisons, milk protein from perennial ryegrass and red clover based diets was 31.9 g/kg and 31.5 g/kg, respectively (Johansen *et al.*, 2018), Similarly, in a review that included eight studies comparing red clover and perennial ryegrass silages, with the exception of one study which showed a slight increase in milk protein content due to red clover silage, milk protein was either unaffected, or was higher with grass silage than with red clover silage (Dewhurst *et al.,* 2003a). The results of the current study are in agreement with those from other studies, with milk protein unaffected at H1, tending to be higher with the grass silage at H2, and being significantly higher at H3. The effects in the latter harvests may have been due to PPO in red clover catalysing the synthesis of quinones which complex with, and inactivate enzymes such as proteases, hence reducing protein availability (Lee, 2014).

Nitrogen and energy utilisation

Effects of diet on N and energy utilisation parameters were inconsistent across harvests. For example, a higher proportion of N intake was excreted as faecal N and a lower proportion as urine N with the RCGS treatment at H1. However, at H2 there was a trend for urine N/N intake to be higher with RCGS, while manure N/N intake was higher. No differences were observed at H3. These effects largely reflect the relationships between milk protein yield and DM intake, and the increasing CP content of the RCGS diet moving from H1 to H3 (Cheng *et al*., 2011). For example, as the red clover content in the silages increased at H2 and H3, proportionately less N was partitioned to milk in RCGS than GS. Also, findings by Moorby *et al*. (2009) with treatments involving 3:1 and 3:2 ratios of grass silage: red clover silage vs grass silage

illustrated that whilst no differences were found in overall N balance, apparent partitioning of dietary N into milk was significantly lower in diets with 100% red clover.

There was no clear relationship between rumen degradation or intestinal digestion of protein with N utilisation efficiency. For example, a higher digestible intestinal protein content in RCGS than GS was associated with a higher proportion of N partitioned to faeces at H1. A higher ruminal undegraded protein was associated with a lower proportion partitioned to milk in the same treatment at H3. Faeces has less of an environmental impact than urine as it mineralises more slowly (Haynes and Williams, 1993). Urinary N is rapidly converted to NH_3 shortly after it is excreted (Varel *et al.*, 1999) and then to volatile nitrous oxides which are potent greenhouse gases (Kebreab *et al*., 2004). In contrast, faecal N is converted to NH_3 at a much slower rate and is retained in the soil contributing to accumulation of soil OM (Waghorn, 2008).

While Vanhatalo *et al.* (2009) found a positive effect on whole-body N balance by partially replacing grass silage with red clover silage, no such effect was observed in the current study at H1 and H3, in agreement with Bertilsson and Murphy (2003), while the opposite effect was observed at H2. However, as only four animals per treatment were used in the nutrient utilisation studies at each harvest, this was not ideal for identifying differences in N retention. Even with activated PPO (found in fresh samples vs ensiled), Moorby *et al*. (2009) found that when feeding fresh red clover with a CP content of 20% approximately, N utilisation by dairy cows was not improved relative to the inactivated PPO. They considered that a CP of 20%, similar to that at H3 in RCGS, was too high for PPO to complex with proteases to have an impact, resulting in less N available for milk protein synthesis, but increasing the likelihood of the creation of an environmental burden (Castillo *et al*., 2000).

Differences in energy utilisation between treatments again largely reflect differences in fat + protein yields, relative to nutrient intake. In addition, ammonia that results from the additional dietary protein degraded in the rumen is detoxified in the liver, requiring energy (Canfield *et al*., 1990), and this may have contributed to the lower milk energy/ME intake with the clover rich RCGS diets at H3.

System effects and practical considerations

Based on the quantities of herbage ensiled from each sward type, and silage DM intakes, one hectare of a red clover-grass mixture was able to provide sufficient silage for 811 'cow feeding days', while the respective value for grass silage was 989 'cow feeding days'. Although milk yield during the experimental period was higher in RCGS than GS treatments (2220 kg vs 2103 kg, respectively), milk fat + protein yield was higher in GS treatments (238 kg vs 231 kg, respectively). However, whilst milk yield was driven in part by the substantially higher intakes with RCGS than GS at H1, largely due to this being the first harvest following establishment, similar effects would not be expected to arise in subsequent years. Furthermore, limits to red clover persistence also need to be taken into account, with reseeding of red clover swards normally required ever 3 – 4 years. However, one of the main benefits of red clover inclusion in swards is the saving on inorganic N fertiliser which, in this study was estimated to be 263 kg N/ha over the course of the season. However, as red clover swards still have a requirement for phosphates and potash, the commonly practiced application of organic manures may lead to the crop may being oversupplied with N.

Furthermore, the study has clearly demonstrated the very different cow performance responses to red clover inclusion which can arise between individual harvests within a season, especially during the first full season following establishment, when the relative proportions of the two species changed considerably over the season. Indeed, this issue is rarely identified in published studies as most have involved offering silage from a single harvest. The variability in silage composition between harvests also creates very practical challenges, both commercially and experimentally. For example, the very different forage CP levels with RCGS (increasing from 98 to 216 g/kg DM, between H1 and H3, compared to 132 – 158 g/kg DM for GS) creates real practical difficulties in balancing the protein content of the diets offered. The use of a single concentrate type with both forages and across harvests, as was adopted in the current study, resulted in total ration protein levels being inadequate at H1, and excessive at H3. Similarly, while both swards were harvested on the same date at each of H1 - H3 in the current study, given the different growth patterns of the swards, different harvesting dates might have been adopted if a 'systems' type study had been undertaken. However, from an experimental point of view, this would have resulted in swards being harvested and ensiled under different sets of weather conditions, confounding the responses of the sward types. Similarly, while both forage types were

wilted for the same duration at each harvest, again differing periods of wilting might have been adopted if weather conditions had allowed. However, periods of rainfall during all three harvests in the current study dictated that longer periods of field wilting were not practical. This demonstrates the difficulty in achieving target DM concentrations with clover rich swards under typical Irish weather conditions, with this particularly evident at H3.

References

Bertilsson, J. and Murphy, M. 2003. Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. *Grass and Forage Science* 58: 309-322.

Calamiglia, S. and Stern, M. D. 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. *Journal of Animal Science* 73**:** 1459-1465.

Canfield, R., Sniffen, C. and Butler, W. 1990. Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. *Journal of Dairy Science* 73**:** 2342-2349.

Castillo, A. R., Kebreab, E., Beever, D. E. and France, J. 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *Journal of Animal and Feed Sciences* 9**:** 1-32.

Castle, M.E. and Watson, J. 1974. Red clover silage for milk production. *Journal of the British Grassland Society* 29: 101-108.

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

Clavin, D., Crossan, P., Grant, J. and O'Kiely, P. 2017. Red clover for silage: management impacts on herbage yield, nutritive value, ensilability and persistence, and relativity to perennial ryegrass. *Grass and Forage Science* 72: 414-431.

Dahlin, A.S.and Stenberg, M. 2010. Transfer of N from red clover to perennial ryegrass in mixed stands under different cutting strategies. *European Journal of Agronomy* 33: 149-156.

Dale, A.J., Laidlaw, A.S., Bailey, J.S. and Mayne, C.S. 2014. Effect of dairy slurry application rate and forage type on production, soil nutrient status and nitrogen-use efficiency. *Grass and Forage Science* 70: 44-58 .

Dewhurst, R. J., Fisher, W. J., Tweed, J. K. S. and Wilkins, R.J. 2003a. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. *Journal of Dairy Science* 86: 2598-2611.

Dewhurst, R. J., Evans, R. T., Scollan, N. D., Moorby, J. M., Merry, R. J.and Wilkins, R. J. 2003b. Comparison of grass and legume silages for milk production. 2. *In vivo* and *in sacco* evaluations of rumen function. *Journal of Dairy Science* 86 : 2612-2621.

Dewhurst, R. J., Delaby, L., Moloney, A., Boland, T. and Lewis, E. 2009. Nutritive value of forage legumes used for grazing and silage. *Irish Journal of Agricultural and Food Research* 48:167-187

Dewhurst, R. J. 2013. Milk production from silage: comparison of grass, legume and maize silages and their mixtures. *Agricultural and Food Science* 22**:** 57-69.

Dhanoa, M. 1988. On the analysis of dacron bag data for low degradability feeds. *Grass and Forage Science* 43**:** 441-444.

Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T. and Webster, G. 1989. A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* 72:68-28.

FAWC (1997) "Report on the welfare of dairy cattle". Farm Animal Welfare Committee. Ministry of Agriculture, Food and Fisheries.

Halmemies-Beauchet-Filleau, A., Vanhatalo, A., Toivonen, V., Heikkilä, T., Lee, M. and Shingfield, K. 2012. Effect of replacing grass silage with red clover silage on rumen lipid metabolism and milk fatty acid composition. *MTT Agrifood Research Finland, University of Helsinki, Finland, Helsinki, Finland***,** 40-41.

Halmemies-Beauchet-Filleau, A., Vanhatalo, A., Toivonen, V., Heikkilä, T., Lee, M. and Shingfield, K. 2014. Effect of replacing grass silage with red clover siilage on nutrient digestion, nitrogen metabolism, and milk fat composition in lactating cows fed diets containing 60:40 forage-to-concentrate ratio. *Journal of Dairy Science* 97: 3761-3776.

Haynes, R. and Williams, P. 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in Agronomy* 49, 119-199.

Heinriches, J., Jones, C. and Bailey, K. 1997. Milk components: Understanding the causes and importance of milk fat and protein variation in your dairy herd. *Dairy & Animal Science Fact Sheet* 5**:** 1e-8e.

Hynes, D., Laidlaw, S. and Ferris, C. 2018. Effect of management strategy on wilting of monocultures and mixtures of red clover and perennial ryegrass. *Proceedings of the 27th General Meeting of the European Grassland Federation, Cork,* pages 249-251.

Johansen, M., Søegaard, K., Lund, P. and Weisbjerg, M. R. 2017. Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows. *Journal of Dairy Science* 100**:** 8861-8880.

Johansen, M., Lund, P. and Weisbjerg, M. R. 2018. Feed intake and milk production in dairy cows fed different grass and legume species: a meta-analysis. *Animal* 12**:** 66-75.

Keady, T., Mayne, C. and Fitzpatrick, D. 2000. Prediction of silage feeding value from the analysis of the herbage at ensiling and effects of nitrogen fertilizer, date of harvest and additive treatment on grass silage composition. *The Journal of Agricultural Science* 134**:** 353-368.

Kebreab, E., Mills, J., Crompton, L., Bannink A., Dijkstra, J., Gerrits, W. and France, J. 2004. An integrated mathematical model to evaluate nutrient partition in dairy cattle between the animal and its environment. *Animal Feed Science and Technology* 112**:** 131-154.

King, C., McEniry, J. and O'Kiely, P., 2012. A note on the fermentation characteristics of red clover silage in response to advancing stage of maturity in the primary growth. *Irish Journal of Agricultural and Food Research*, pp.79-84.

Kuoppala, K., Ahvenjarvi, S., Rinne, M. and Vanhatalo, A. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 2. Dry matter intake and cell wall digestion kinetics. *Journal of Dairy Science* 92**:** 5634-5644.

Lee, M.R.F., Winters, A.L., Scollan, N.D., Dewhurst, R.J., Theodorou, M.K. and Minchin, F.R. 2004. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. *Journal of the Science of Food and Agriculture* 84: 1639-1645.

Lee, M.R.F., Theobold, V.J., Tweed, J.K.S., Winters, A.L. and Scollan, N.D. 2009. Effect of feeding fresh or conditioned red clover on milk fatty acids and niitrogen utilization in lactatign dairy cows. *Journal of Dairy Science*, 92: 1136-1147.
Lee, M. R. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. *Frontiers in Plant Science* 5**:** 694.

McEniry, J. King, C and O'Kiely, 2013. Silage fermantation characteristics of three common grassland species in response to advancing stage of maturity and additive application. *Grass and Forage Science* 69: 395-409.

Moorby, J. M., Lee, M. R. F., Davies, D. R., Kim, E. J., Nute, G. R., Ellis, N. M. and Scollan, N. D. 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. *Journal of Dairy Science* 92**:** 1148-1160.

Morrison, J., Jackson, M.V. and Sparrow, P. 1980. "The response of perennial ryegrass to fertilizer nitrogen in relation to climate and soil. Report of the joint ADAS/GRI grassland manuring trial-GM. 20". Technical Report, Grassland Reearch Institute, Hurley, 71 pages.

Moseley, G. and Jones, J.R. 1984. The physical digestion of perennial ryegrass (Lolium perenne) and white clover (Trifolium repens) in the foregut of sheep. British Journal of Nutrition 52: 381-390.

Mtengeti E.J., Wilman, D. and Mosley, G. 1996. Differences between twelve forrage species in physical breakdown when eaten. *Journal of Agricultural Science* 126: 287-293

Murphy, M., Åkerlind, M. and Holtenius, K. 2000. Rumen fermentation in lactating cows selected for milk fat content fed two forage to concentrate ratios with hay or silage. *Journal of Dairy Science* 83**:** 756-764.

Naadland, S.S., Steinshamn, H., Krizsan, S.J. and Randby, Å.T., 2016. Effect of replacing organic grass-clover silage from primary growth with regrowth on N digestion in dairy cows. *Animal Feed Science and Technology*, *220*, pp.57-66.

Ørskov, E. and McDonald, I. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *The Journal of Agricultural Science*, *92*(2), pp.499-503

Park, R. S., Agnew, R. E., Gordon, F. J. and Steen, R. W. J. 1998. The use of near infrared reflectance spectroscopy (NIRS) on undried samples of grass silage to predict chemical composition and digestibility parameters. Animal Feed Science and Technology 72: 155-167.

Peyraud, J., Le Gall, A. and Lüscher, A. 2009. Potential food production from forage legume-based-systems in Europe: an overview. *Irish Journal of Agricultural and Food Research* 48: 115-135.

Roberts, D.J., Lawson, A . and Fisher, G.E.J. 1990. A comparison of a red clover/grass sward with a grass sward plus fertilizer nitrogen under a three-cut silage regime. Proceedings of the British Grassland Society Second Research Conference British Grassland Society, Reading,Session III Paper 5.

Robinson, P., Fadel, J. and Tamminga, S. 1986. Evaluation of mathematical models to describe neutral detergent residue in terms of its susceptibility to degradation in the rumen. *Animal Feed Science and Technology* 15**:** 249-271.

Smith, R.R., Taylor, N.L and Bowley, S.R. 1985. Red clover. In: "Clover Science and Technology*"***,** (N.L. Taylor), ASA/CSSA/SSSA,Madison, Wisconsin, USA, pages 457-470.

Steen, R.W.J. and McIlmoyle, W.A. 1982. An evaluation of red clover silage for beef production. *Animal Production* 34: 95-101.

Steen R. W. J. 1989. A comparison of soyabean, sunflower, and fish meals as protein supplements for yearling cattle offered grass silage based diets. Anim. Prod., 48: 127-132.

Tamminga, S., van Straalen, M.W., Subnel, A.P.J., Meijer, R.G.N., Steg, A., Wever, C.j.G.and Blok, M.C. 1994. The Dutch protein evaluation system: The DVE/OEB system. *Livestock Production Science* 40: 139-155.

Theodoridou, K. and Yu, P. 2013. Effect of processing conditions on the nutritive value of canola meal and presscake. Comparison of the yellow and brown-seeded canola meal with the brown-seededcanola presscake. *Journal of the Science of Food and Agriculture* 9: 1986-1995

Theodorou, M. K., Kingston-Smith, A. H., Winters, A. L., Lee, M. R. F., Minchin, F. R., Morris, P. and MacRea, J. 2006. Polyphenols and their influence on gut function and health in ruminants: a review. *Environmental Chemistry Letters* 4**,** 121-126.

Vanhatalo, A., Gäddnäs, T. and Heikkilä, T., 2006. Microbial protein synthesis, digestion and lactation responses of cows to grass or grass-red clover silage diet supplemented with barley or oats. *Agricultural and Food Science*, *15*(3), pp.252-267.

Vanhatalo, A., Kuoppala, K., Ahvenjarvi, S. and Rinne, M. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. *Journal of Dairy Science* 92**:** 5620-5633.

Van Soest, P.J. 1976. Laboratory methods for evaluating the energy value of feedstuffs. In "Feed and Energy Sources for Livestock", (eds H. Swan and D Lewis), Butterworths, London, UK, pages 83-94.

Varel, V. H., Nienaber, J. A. and Freetly, H. C. 1999. Conservation of nitrogen in cattle feedlot waste with urease inhibitors. *Journal of Animal Science* 77**:** 1162-1168.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. *Animal Feed Science and Technology* 147**:** 116-139.

Weisbjerg, M. and Soegaard, K. 2008. Feeding value of legumes and grasses at different harvest times. *Biodiversity and animal feed: Future challenges for grassland production. Grassland Science in Europe* 13**:** 513-515.

Wright, D., Frost, J., Patterson, D. & Kilpatrick, D. 1997. The influence of weight of ryegrass per unit area and treatment at and after mowing on rate of drying. *Grass and Forage Science* 52**:** 86-98.

Table 1 Chemical composition of grass silage (GS) and red clover/grass silage (RCGS) offered during the 13 week experimental period at primary growth (H1) or first (H2) or second (H3) regrowth.

	H1				H2				H 3			
	GS	RCGS	s.e.	P-Value	GS	RCGS	s.e.	P-Value	GS	RCGS	s.e.	P-Value
VCODM (g/kg)	218	299	18.3	0.014	188	204	4.7		283	216	16.0	0.005
Crude protein (g/kg DM)	132	98	9.5		137	148	4.4		158	216	13.8	0.006
Ash (g/kg DM)	82	75	2.0		90	109	12.7	0.038	87	105	5.2	0.002
ADF (g/kg DM)	371	344	4.9	<0.001	362	383	3.8	0.059	262	269	6.1	
NDF (g/kg DM)	631	596	6.5	0.002	625	565	5.0	0.001	482	462	9.9	
pH	4.3	4.2	0.06		4.4	4.7	0.07	0.014	4.5	4.7	0.06	
Lactic acid (g/kg DM)	97.1	67.3	12.71		83.0	31.1	12.71		80.5	96.9	11.50	
Acetic acid (g/ kg DM)	22.6	12.5	2.09	0.005	24.2	38.7	2.90	0.034	25.9	36.3	3.36	<0.001
Propionic acid (g/kg DM)	2.4	0.7	0.38	0.017	1.9	3.8	0.47		0.5	3.7	0.78	0.005
n-Butyric acid (g/kg DM)	14.1	3.6	1.96	0.001	15.3	21.6	2.97		0.0	3.6	0.98	0.043
Propanol (g/kg DM)	0.4	0.0	0.15		0.0	0.0	0.00		0.0	0.1	0.07	
Ethanol (g/kg DM)	1.3	0.3	1.56		9.9	2.1	1.73	0.006	7.7	6.6	2.51	
Ammonia (g/kg total N)	17.4	9.2	1.60	0.002	18.7	17.0	0.16		10.3	14.3	1.04	0.027
Metabolisable energy (MJ/kg DM)	10.4	10.5	0.09		10.2	9.8	0.14		10.5	9.8	1.45	

VCODM-Volatile corrected oven dry matter, ADF-Acid detergent fibre, NDF-Neutral detergent fibre

Table 2. *In vitro* ruminal protein degradation kinetics of grass silage (GS) or red clover/grass silage (RCGS) produced from primary growth (H1), or first regrowth (H2), or second regrowth (H3)

	GS H1	RCGS H1	GS H2	RCGS H2	GS H3	RCGS H3	s.e.	P-Value
S (%CP)	53.4^{a}	55.9^{a}	34.3^{b}	27.3^{b}	63.6^{a}	32.0^{b}	4.76	0.017
D (%CP)	26.4	27.9	35.1	35.4	28.6	34.2	3.99	
U (%CP)	20.2^{c}	16.1^{c}	30.6^{b}	37.3^{a}	7.8^{d}	33.8^{ab}	1.64	<0.001
K_d (% h)	15.0	11.2	20.6	9.1	8.2	10.9	6.20	
RUCP (%CP)	29.3^{c}	25.8^{cd}	41.7^{b}	51.4^{a}	19.8^{d}	46.4^{ab}	2.30	0.001
EDCP (%CP)	70.7^{b}	74.2^{ab}	58.3^{c}	48.6^{d}	80.2^{a}	53.6^{cd}	2.30	0.001
IVCPD (%)	71.1^{b}	84.2^{a}	65.7^{c}	62.9^{cd}	55.6^{e}	58.9^{de}	1.35	<0.001
IVCPDCP (%)	20.9^{b}	21.7^{b}	27.5^{ab}	32.4^{a}	11.0^{c}	27.6^{ab}	2.14	0.009

Means with different letters $^{a, b, c}$ within the same row differ significantly. S.E.M.= Standard error of mean; S = potential soluble fraction in the *in vitro* ruminal incubation., D = potentially degradable fraction in the *in vitro* ruminal incubation., U=undegradable fraction., K_d = degradation rate., RUCP = Rumen Undegraded Crude Protein., EDCP = Effectively degraded Crude Protein., IVCPD= *In vitro* intestinal digestibility of CP, IVCPDCP= *In vitro* intestinal digestibility of the undegradable CP in the rumen

Table 3: Performance of dairy cows offered either grass silage (GS) or red clover/grass silage (RCGS) from primary growth (H1), or first regrowth (H2), or second regrowth (H3)

	Treatment			
	GS	RCGS	s.e.	P
H1				
Silage DMI (kg/day)	8.8	11.7	0.28	<0.001
Total DMI (kg/day)	15.9	18.8	0.28	<0.001
Milk yield (kg/day)	24.9	25.5	0.36	
Milk fat (g/kg)	47.3	47.3	1.10	
Milk protein (g/kg)	31.6	32.3	0.41	
Milk lactose (g/kg)	46.5	46.9	0.33	
Fat + protein yield (kg/day)	2.69	2.72	0.094	
Average live weight (kg)	549	558	2.9	0.049
Average body condition score	2.56	2.68	0.037	0.042
H2				
Silage DMI (kg/day)	9.2	10.4	0.28	0.006
Total DMI (kg/day)	16.4	17.5	0.28	0.006
Milk yield (kg/day)	22.1	23.1	0.44	
Milk fat (g/kg)	45.3	45.2	0.98	
Milk protein (g/kg)	32.0	30.8	0.47	
Milk lactose (g/kg)	45.4	45.4	0.47	
Fat + protein yield (kg/day)	2.47	2.43	0.090	
Average live weight (kg)	547	550	4.3	
Average body condition score	2.52	2.62	0.051	
H3				
Silage DMI (kg/day)	12.3	11.5	0.45	
Total DMI (kg/day)	19.5	18.7	0.45	
Milk yield (kg/day)	21.8	24.7	0.58	0.002
Milk fat (g/kg)	47.8	43.1	0.82	<0.001
Milk protein (g/kg)	35.1	31.4	0.45	<0.001
Milk lactose (g/kg)	44.5	45.2	0.55	
Fat + protein yield (kg/day)	2.74	2.41	0.085	
Average live weight (kg)	569	559	5.8	
Average body condition score	2.53	2.58	0.051	
H1-3				
Silage DMI (kg/day)	9.5	11.1	0.26	<0.001
Total DMI (kg/day)	16.7	18.3	0.26	<0.001
Milk yield (kg/day)	23.4	24.4	0.39	
Milk fat (g/kg)	46.8	45.8	0.82	
Milk protein (g/kg)	32.3	31.5	0.41	
Milk lactose (g/kg)	45.7	46.0	0.40	
Fat + protein yield (kg/day)	2.62	2.56	0.080	
Average live weight (kg)	552	555	3.71	
Average body condition score	2.54	2.64	4.21	
End of study live weight (kg)	569	559	5.8	
End of study body condition score	2.52	2.56	0.050	

Table 4: Digestibility of components of dry matter consumed by dairy cows offered either grass silage (GS) or red clover/grass (RCGS) silage produced from primary growth (H1), or first regrowth (H2) or second regrowth (H3)

	Treatment			
	GS	RCGS	s.e.	P
H1				
Digestibility (kg/kg)				
Dry matter (DM)	0.74	0.75	0.006	
Organic matter	0.75	0.76	0.006	
Digestible organic matter in DM	0.69	0.70	0.005	
ADF	0.71	0.71	0.006	
NDF	0.69	0.70	0.007	
H2				
Digestibility (kg/kg)				
Dry matter (DM)	0.74	0.68	0.006	<0.001
Organic matter	0.75	0.70	0.008	0.003
Digestible organic matter in DM	0.69	0.63	0.007	0.001
ADF	0.75	0.63	0.010	<0.001
NDF	0.69	0.55	0.031	0.017
H3				
Digestibility (kg/kg)				
Dry matter (DM)	0.76	0.71	0.009	0.006
Organic matter	0.77	0.73	0.007	0.007
Digestible organic matter in DM	0.70	0.65	0.007	0.002
ADF	0.78	0.72	0.010	0.009
NDF	0.71	0.66	0.012	0.037

ADF-Acid detergent fibre, NDF-Neutral detergent fibre

Table 5: Nitrogen utilisation of dairy cows offered either grass silage (GS) or red lover/grass silage (RCGS) produced from primary growth (H1), or first regrowth (H2), or second regrowth (H3)

	Treatment			
	GS	RCGS	s.e.	P
H1				
N utilisation (g/g)				
Faeces N/N intake	0.31	0.37	0.014	0.019
Urine N/N intake	0.33	0.22	0.019	0.008
Manure N/N intake	0.63	0.59	0.007	0.005
Milk N/N intake	0.28	0.29	0.016	
Retained N/N intake	0.09	0.12	0.017	
Faeces N/Manure N	0.48	0.62	0.027	0.010
Urine N/Manure N	0.52	0.38	0.027	0.010
H2				
N utilisation (g/g)				
Faeces N/N intake	0.29	0.32	0.010	
Urine N/N intake	0.26	0.33	0.019	
Manure N/N intake	0.56	0.65	0.012	0.002
Milk N/N intake	0.26	0.23	0.005	0.007
Retained N/N intake	0.19	0.13	0.015	0.035
Faeces N/Manure N	0.53	0.50	0.023	
Urine N/Manure N	0.47	0.50	0.023	
H3				
N utilisation (g/g)				
Faeces N/N intake	0.42	0.41	0.013	
Urine N/N intake	0.44	0.47	0.021	
Manure N/N intake	0.86	0.87	0.023	
Milk N/N intake	0.28	0.24	0.009	0.020
Retained N/N intake	-0.14	-0.11	0.018	
Faeces N/Manure N	0.50	0.46	0.021	
Urine N/Manure N	0.50	0.54	0.021	

Table 6: Gross energy digestibility and energy utilisation of dairy cows offered either grass silage (GS) or red clover/grass silage (RCGS) produced from primary growth (H1), first regrowth (H2), or second regrowth (H3)

	Treatment			
	GS	RCGS	s.e.	P
H1				
Gross energy digestibility	0.72	0.73	0.006	0.488
Energy utilisation (MJ/MJ)				
DE/GE	0.73	0.73	0.006	
Milk energy/ME	0.40	0.34	0.021	
Faeces E/DEI	0.38	0.37	0.011	
Urine E/DEI	0.03	0.03	0.003	
H2				
Gross energy digestibility	0.73	0.67	0.008	0.003
Energy utilisation (MJ/MJ)				
DE/GE	0.73	0.67	0.008	0.002
Milk energy/ME	0.39	0.40	0.009	
Faeces E/ DEI	0.37	0.49	0.015	0.002
Urine E/DEI	0.03	0.06	0.004	0.003
H3				
Gross energy digestibility	0.66	0.67	0.009	
Energy utilisation (MJ/MJ)				
DE/GE	0.66	0.67	0.009	
Milk energy/ME	0.51	0.42	0.011	0.001
Faeces E/ DEI	0.51	0.49	0.015	
Urine E/DEI	0.09	0.07	0.004	0.032

DEI- Digestible energy intake, GE- Gross Energy, ME- Metabolisable energy

EXPERIMENT 5

Cow performance, nutrient utilisation and the 'concentrate sparing effects' arising from the partial replacement of grass silage by red clover silage

Introduction

The increasing milk yield potential of dairy cows within the United Kingdom (UK) in recent years (AHDB Dairy, 2016) has led to a requirement for more nutrient dense diets. Increased diet nutrient density is often achieved through the adoption of higher concentrate feed levels, and this in turn has increased the demand for high quality protein ingredients such as soya bean meal and rapeseed meal, much of which is imported from outside of the UK. However, these imports suffer from variability in supply and price volatility (Jones et al., 2014), while the EU approval process for the import of genetically modified constituents for animal feeds impose further limits on their use.

Jones et al. (2014) considered that home grown protein crops such as beans, peas and lupins could substitute for up to 50% of imported protein constituents of concentrates. However, farms in the west of the UK are capable of producing high yields of forage, and so high protein leguminous forages such as red clover (RC), may also offer potential to reduce reliance on concentrate feeds (Wilkins and Jones, 2000). In addition to its potential to produce high annual yields of dry matter (DM) and crude protein (CP) (Dale et al. 2014; Clavin et al., 2017), red clover's ability to fix atmospheric nitrogen (N) reduces the need for N fertiliser (Frame et al. 1998). This can contribute to legume based systems having a lower carbon footprint than grass-based systems involving high levels or inorganic nitrogen (N) (DairyCo, 2012).

In generally, at a similar digestibility, intakes of red clover silage by dairy cows are higher than those of grass silage (2 – 2.5 kg higher: Dewhurst et al., 2003a; Johansen et al., 2017) due mainly to a faster rate of degradation and breakdown of particles than for grass silage. In addition, intakes of mixtures of red clover and grass silage are often higher than would be expected from the proportion of the two components (Dewhurst et al., 2003a; Vanhatalo et al., 2009; Halmemies-Beauchet-Filleau et al., 2014), but not always so (Moorby et al., 2009).

Milk production responses arising from replacing grass silage with red clover silage are highly variable. Dewhurst et al. (2003a) observed a response of 4 kg fat corrected milk (FCM), while milk yield responses of 2-2.5 kg (energy corrected milk; Bertilsson and Murphy , 2003; Johansen et al., 2017) are more normal. In common with the findings for intake, offering 50:50 mixtures of the two silages may result in FCM

production closer to that of the diet with red clover silage than grass silage (Experiment 1 in Dewhurst et al., 2003a; Johansen et al., 2017).

With regards milk composition, in general diets comprising primarily red clover have no effect or reduce milk fat and milk protein content relative to diets principally of grass silage (Dewhurst, 2013). However, replacing grass silage with red clover silage has been found to consistently increase polyunsaturated fatty acids, the concentration of 18:2 n-6 and 18:3 n-3 fatty acid increasing with increasing proportion of red clover silage in the diet (Moorby et al., 2009; Halmemies-Beauchet-Filleau et al., 2014). Although red clover silages usually have a higher CP content than grass silages, quinones resulting from the presence of polyphenol oxidase in red clover bind with proteins in the rumen, protecting them from degradation. As a consequence, the proportion of ingested nitrogen that is converted to non-protein nitrogen, and subsequently excreted in urine or as milk urea is reduced (Dewhurst, 2013)

Of the numerous studies which have compared the effect on dairy performance of offering dairy cows mixtures of grass-and red clover silage, only in the study of Moorby et al. (2009) did the grass silage have as high a crude protein content as the red clover silage. Therefore interpretation of any benefit derived from offering red clover silage on its own or in mixture with grass silage needs to take into account the higher CP content of the red clover component. Manipulating the CP content of the concentrate is a means of controlling the overall CP content of the diet.

The potential to increase milk production by replacing grass silage with red clover silage, either wholly or partly, presents the possibility of reducing the daily rate of concentrate offered to maintain a given level of output. However, in most studies comparing the performance of cows offered either grass silage or red clover silage, or mixtures of the two, comparisons have been made at equal concentrate levels, meaning that the potential concentrate sparing effects arising from red clover inclusion in the diet cannot be identified. Given that concentrates can contribute up to 70% of variable costs on dairy farms in the UK, the potential of a concentrate saving effect arising from replacing grass silage with red clover silage may offer a real opportunity to reduce feed costs. Consequently this study was designed to investigate the potential concentrate sparing effects arising when red clover silage partially replaced grass silage in the diet of dairy cows.

Materials and methods

This study was conducted at the Agri-Food and Biosciences Institute (AFBI), Hillsborough, Northern Ireland between 19 December 2017 and 15 May 2018. All experimental procedures described here were conducted under an experimental license granted by the Department of Health, Social Services & Public Safety for Northern Ireland in accordance with the Animals (Scientific Procedures) Act 1986.

Cows and pre-experimental management

Twenty eight multiparous Holstein dairy cows (mean parity, 3.0 (s.d., 1.20)) were housed as a single group in a free-stall cubicle house with concrete flooring. All cows had access to individual cubicles (cubicle:cow ratio >1:1 at all times) that were fitted with rubber filled mats and bedded with sawdust thrice weekly. During the three week period prior to commencement of the study, cows were offered grass silage, supplemented with 12.25 kg/day of a commercial concentrate via an out-of-parlour feeding system, and 1.0 kg/day of a commercial concentrate via an in-parlour feeding system (0.5 kg at each milking). Cows were a mean of 92 (s.d., 10.7) days calved at the commencement of the study.

Experimental design

The experiment comprised seven treatments arranged in a four period partially balanced change-over design. Treatments comprised grass silage supplemented with 8.5, 11.0, 13.5 or 16.0 kg concentrate per cow per day (GS8.5, GS11, GS13.5 and GS16, respectively), or grass silage and red clover silage mixed in ratios of 70:30, 50:50 or 30:70 (on a dry matter (DM) basis, all supplemented with 12.25 kg concentrate (RC30, RC50 or RC70, respectively). All concentrates were offered through two out-of-parlour feed stations. Cows were blocked according to milk fat plus protein yield during the week prior to the start of the experiment, and cows within each block were randomly allocated to one of the seven treatments during period 1. Each experimental period was 28 days, comprising a 21-day feed adaptation phase and a 7-day measurement phase. The four GS treatments (GS8.5 – GS16) were designed to establish a milk yield response curve to increasing levels of concentrate feeding, while the common concentrate levels with the three RC treatments was designed to allow the ‘concentrate sparing’ effects of red clover to be examined against the grass silage response curve.

Forages and concentrates offered, and diet preparation

The grass silage offered throughout the experiment (including the pre-experimental period) was produced from the first regrowth of a perennial ryegrass sward (harvested on 22 June). The red clover silage offered was produced from the first regrowth of a red clover sward (cv. Merviot) which has been sown the previous autumn at a seed rate of 13 kg/ha. The primary growth of the red clover sward had been harvested on 22nd May 2017 to remove perennial weeds and generate a pure sward, with the experimental silage subsequently harvested 8 weeks later on 18th July 2017. The sward received muriate of potash at a rate of 250 kg/ha following the harvest of the primary growth. Both the grass and red clover swards were mown using a Claas 3200 disc mower, raked into windrows and subsequently harvested using a John Deere 7450 self-propelled precision chop forage harvester. Both crops were allowed to wilt for approximately 24 hours and treated with Super MV50 bacterial inoculant (Biotal Ltd, Malvern Link, Worcestershire) at the time of harvest at 1 litre/t fresh herbage.

Rations were prepared and offered daily between 1000 and 1100 h, with uneaten feed removed the following day at 0800 h. Throughout the experiment the forage component of the diet was offered via a Redrock 11 FD Varicut diet feeder (Redrock, Collone, Co Armagh, Northern Ireland). Sufficient grass silage for all seven treatments was mixed for approximately five minutes, with the required quantity of silage for the four GS treatments then deposited in a series of feed boxes, each of which was resting on two load cells, and which cows were permitted to access via an electronic ear tag (BioControl AS, Rakkestad, Norway). This system allowed intakes of fresh food to be recorded during each individual meal, and total intakes over a 24 h period to be determined. The remaining grass silage from the diet feeder was deposited on a clean silo floor. Sufficient red clover silage for the three GS-RC treatments was similarly mixed for approximately five minutes using the diet feeder, and again deposited on a clean silo floor. The required quantities of grass silage and red clover silage for each of the GS-RC mixes were subsequently removed from the mixed piles, placed back in the diet feeder, and mixed for a further five minutes, before being placed in the feed boxes. All forages were offered at 1.075 of the intake for the previous day.

A key objective of this study was to offer iso-nitrogenous diets (target of 170 g/kg DM) with all seven treatments. This was achieved by offering two concentrate types (Table 2), with target crude protein contents of 165 and 236 g/kg DM, respectively. The relative proportions of each of the two concentrate types offered with each treatment (to achieve a total diet crude protein content of 170 g/kg DM) were calculated weekly throughout the experiment, based on the average forage DM intake and the crude protein contents of GS and RC silage for the previous seven days, and offered via the out-of-parlour feeders. In view of the large change in concentrate levels between some treatments at the start of each period, concentrate levels with all treatments were adjusted over a period of four days in week 1 of each period. Throughout the study cows had free access to water.

Cow measurements

Cows were milked twice daily at approximately 0630 and 1630 h using a 50-point rotary parlour (Boumatic, Madison, Wisconsin, USA), with milk yields recorded automatically at each milking, and a total daily milk yield for each cow for each 24-hour period calculated. Milk samples were collected during six consecutive milkings (day 5 – 7) during the final week of each period, a preservative tablet added to each sample (Lactab Mark III, Thompson and Cooper, Runcorn, UK), and individual samples analysed for fat, protein, and lactose contents using an infrared milk analyser (Milkoscan Model FT+; Foss Electric, Hillerød, Denmark). A weighted concentration of each constituent was determined for each 24-hour sampling period, and a mean concentration over the three day sampling period determined. Forage intakes of each individual cow were recorded daily using the feed intake recording system described above, while daily concentrate intakes were recorded by the out-of-parlour feeding system. The BCS of each cow was estimated by a trained technician during the final day of each period, according to Edmondson *et al.* (1989). Bodyweight was recorded twice daily immediately after milking using an automated weighbridge, and mean BW during the 7 day measurement period determined. Blood samples were collected on the final day of each measurement period prior to animals receiving their daily rations. Samples were collected from the tail of each cow and centrifuged at 1,810 *g* for 15 minutes to separate out the serum or plasma. Serum beta-hydroxybutyrate (BHB) concentrations were evaluated according to McMurray et al. (1984), while serum total protein and non-esterified fatty acid (NEFA) concentrations were analysed using

Boehringer Mannheim and WaKo (Wako Chemicals GmbH, Neuss, Germany) kits, respectively. Serum urea concentrations were determined using the Kinetic UV method (Roche Diagnostics Ltd.) and plasma glucose concentrations were assessed using the hexokinase method (Roche Diagnostics Ltd., Burgess Hill, UK). All analyses were undertaken using an Olympus AU640 analyser (Olympus, Center Valley, PA). Serum globulin was calculated as the difference between total protein and albumin.

Nutrient utilisation study

On completion of period four, total ration digestibility of four diets were examined using 16 cows (four per diet). These comprised the three GS-RC treatment diets, as described above (RC30, RC50 and RC70), and a fourth diet comprising grass silage as the sole forage, all supplemented with 12.25 kg concentrate/day. All cows were adapted to the diets offered for at least 21 days before measurements commenced. Cows were moved to a Digestibility Unit (eight cows during week 1 and eight cows during week 2 (2 per diet each week) and were tied by the neck in individual metabolism stalls, with their lying area comprising a solid rubber mat. Cows were milked while standing in these stalls, and accessed their experimental rations from feed boxes at the front of each stall. The forage part of the diets was offered ad *libitum* daily at 11:00 h, at proportionally 1.07 of the previous day's intake, while uneaten silage was removed the following day at 09:00 h. The experimental concentrates (12.25 kg/day), in proportions designed to achieve a total diet CP of 170 g/kg DM, were offered in four equal meals each day at 07:00, 11:00, 15:00 and 19:00 h, to simulate the out-of-parlour feeding regime used during the main experimental period. Concentrates were offered in plastic feed buckets which were placed within the feed boxes, with these removed after all the concentrates had been consumed at each feeding time. Cows had access to fresh water at all times via a drinker located within each stall.

Measurements of nutrient utilisation commenced 24 h after cows were moved to the Digestibility Unit, and comprised a 6-day feeding period, followed 48 hours later by a 6-day total faeces and urine collection period. Faeces were collected in a plastic collection tray (96 cm × 108 cm × 36 cm deep) placed behind each cow. Urine was collected into a 25 litre plastic container via a flexible plastic tube which was attached to a urine separation system. This was held in position over the vulva by attaching it using Velcro fasteners to a 'patch' glued (Bostik, France) either side of the cow's tail

head. Approximately 300 ml of 50% sulphuric acid was added to each urine collection container daily to achieve a pH of between 2.0 and 4.0 to minimise nitrogen (N) losses as ammonia. The total weight of faeces and urine produced during each 24 h collection period was recorded at approximately 08.30 h, and a sample of each (0.05 by weight) collected and stored in a fridge (approximately 4^{o}C), with samples for days 1 – 3, and 4 – 6 subsequently bulked, and analysed immediately. Milk samples were taken at each milking (2% volume sample), bulked in proportion to yield for days 1-3 and days 4-6, and subsequently analysed. Energy corrected milk yield (ECM) was determined as described by Chen and Yan (2015). During the 6-day nutrient utilisation study feed intakes and refusals of individual animals were measured and recorded daily using an electronic scale (Ohaus® Defender™ 3000 series, Ohaus Europe GmbH, Switzerland). Measurements of BCS and BW was recorded on the day prior to and the day following the 6-day digestibility measurement phase.

Chemical analysis

Samples of the silages offered were taken daily throughout the experiment and dried at 85°C for 24 hours to determine oven DM content. Sub-samples of the dried milled silages were taken three times weekly and bulked for every 14 days, with the bulked sample analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and ash concentrations. A fresh sample of the grass silage was taken weekly and analysed for concentrations of nitrogen (N), ammonia-N, lactic acid, acetic acid, ethanol, propanol and gross energy (GE), and for pH. A sample of each concentrate offered was taken weekly, bulked for each two week period, and subsequently dried at 85°C for 24 hours to determine oven DM content. Additional samples of each experimental concentrate were taken weekly, bulked for each two week period and dried at 60°C for 48 hours, and analysed for N, NDF, ADF, ash, GE and starch concentrations. Fresh faeces and urine were analysed for N content, while freeze dried urine samples were analysed for GE content. Faeces samples were dried at 85^{o}C for 96 hours to determine ODM content, with the dried samples subsequently milled and analysed for ADF, NDF, ash and GE concentrations. All dried forage, concentrate and faeces samples were milled through a 0.8 mm screen prior to analyses. Milk samples collected during the ration digestibility study were analysed for N and GE contents. All chemical analyses of forage, concentrate, faeces, urine and milk were performed as described by Hynes *et al*. (2016).

Statistical analysis

Energy-corrected milk yield was calculated using measured milk energy output (MJ/day) divided by milk energy content in one kg of standard milk (4.0% fat, 3.2% protein and 4.8% lactose) with the latter calculated as described by Hynes *et al*. (2016).

Averages of the daily individual animal data over the 7-day measurement phase for the feeding study and the 6-day digestibility measurement phase were used. All variables were analyzed employing linear mixed model with REML estimation using dietary treatment as the fixed factor and individual animal and period were used as random factors. Relevant covariates were included where appropriate. Residuals showed no deviation from normality. The differences between treatments were declared as non-significant at $P > 0.05$ and significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

To quantify the concentrate sparing of red clover silage compared with grass silage receiving concentrates, quadratic response curves to concentrate feeding were derived for the four grass silage treatments for a range of parameters, and the response for each parameters at a concentrate level of 12.25 kg/day determined from the curve. The difference between this calculated value for the grass silage only treatments, and the actual values observed with each of the RC treatments (all which had 12.25 kg concentrate) was determined. As the red clover value did not always exceed that of the grass silage with 12.25 kg concentrate sparing was either positive or negative.

Experimental data (feed intake, production parameters, energy balance and nutrient digestibility parameters) were analysed using GenStat (16th ed. Lawes Agricultural Trust, Rothamsted, UK).

Results

The two silages differed mainly in their CP, DM and energy content with the red clover silage having an ODM and CP 39 g/kg and 18 g/kg, respectively, higher than the grass silage but with a predicted ME 1.5 MJ/kg lower than the grass silage (Table 1). Fermentation of silages was satisfactory with both having a low pH and a low concentration of ammonia N in total N. Other than the CP of the high protein

concentrate being 39 g/kg higher than the low protein concentrate (LP), the two concentrates had similar chemical composition (Table 2).

Across the four GS treatments, the quantity of High CP concentrate offered declined while quantity of Low CP concentrate increased (Table 3). Similarly, across these treatments, grass silage DM intake declined while total DM intake increased. With the three RC clover treatments, replacing GS with RC silage increased total silage DM intake, and total intake. Across the three GS treatments, milk fat content and milk energy content decreased with increasing concentrate levels, while all of the other milk production parameters increased (Table 4). However with the exception of milk protein content, which increased with increasing RC clover inclusion ($P<0.05$), none of the other milk production parameters in Table 4 changed across the three red clover treatments. Across the four grass silage treatments, concentrations of total SFA (and of C18:0) decreased with increasing concentrate inclusion, while concentrations of MUFA and PUFA increased. Concentrations of total SFA and total MUFA were unaffected across the three RC treatments, while concentrations of PUFA decreased.

Table 6 presents the quantity of 'additional' concentrate that would be required with a grass silage diet (supplemented with 12.25 kg concentrate/day) in order to achieve the same level of performance as achieved with each of the RC treatments (supplemented with 12.25 kg concentrate/day). A few selected curves demonstrating the relationship between the concentrate rate response and output from the red clover treatments are presented in Figure 1. Negative values indicate that less concentrate than 12.25 kg was required to produce the equivalent of the red clover treatment. In only seven out of a total of 39 parameters examined was there a 'concentrate sparing' effect. Four of the seven were due to RC50 having a concentrate sparing effect i.e. for liveweight (1.15 kg), total DM intake (1.35 kg), milk fat content (>3.75 kg) and milk fat production (0.65 kg), fat + protein production (0.25kg). The remaining three were a concentrate sparing effect of 0.85 kg for DM intake and 0.45 kg for milk yield in the 30% red clover silage treatment and 2.15 kg for DM intake and 0.95 kg for milk fat content in the 70% red clover silage treatment.

Of the blood metabolites only β-hydroxybutyrate differed significantly between treatments, declining with increasing concentrate rate. The concentration in the red clover treatments did not differ from each other. Although non-esterified fatty acids

were analysed, there was insufficient variation among them to be statistically analysed, inferring that there were no statistical differences between treatments.

Intake of silage or total DM or milk yield did not differ between treatments in the nutrient balance study (Table 8). However, increasing red clover content resulted in a decrease in digestibility of DM, OM, GE and NDF. Nitrogen intake did not differ significantly between treatments and of the N outputs, only faeces N differed significantly between treatments with the output in the GS treatment lower than that of the three RC treatments (Table 9). Utilisation of N differed between GS and RC treatments with N output in faeces relative to N intake in GS significantly lower than in the RC treatments. There was a tendency for the proportion of N intake to be higher in GS than RC treatments, especially RC30 and RC50. There was also a tendency for manure N to comprise a lower proportion of faeces N in GS than the RC treatments and the reverse was found to apply to the proportion of manure that comprised urine N (Table 9).

There were no significant differences between treatments for GE intake or GE output although there was a tendency for GE output in faeces to increase with increase with red clover content (Table 10). Proportionately more intake of GE was lost as energy in faeces as red clover increased and there was a tendency for the proportion of GE intake lost in urine to be higher in GS than RC50.

Discussion

The objective of the study was to investigate and quantify the concentrate sparing effect when grass silage was partially replaced by red clover silage. All diets offered were designed to be iso-nitrogenous, with this achieved by offering two concentrates, each differing in CP content, in different ratios. Both silages offered had satisfactory fermentation characteristics, while the red clover silage had lower gross energy content and predicted ME content than the grass silage, although the latter was predicted using a calibration designed for grass silage. Nevertheless, in many studies involving comparisons of red clover silage with grass silage, the red clover used had a lower nutritive value e.g. Dewhurst et al. (2003b); Moorby et al. (2009); Johnston et al. (in review).

The approach used in the study involved developing a response curve to increasing levels of concentrate feeding with a grass silage based diet. As expected, total DM intake increased, and forage intake decreased with increasing levels of concentrate feeding, a reflection of substitution taking place. The decrease in grass silage DMI per kg increase in concentrate of 0.28 was lower than for some other estimates of substitution rates for grass silage e.g. 0.53 (Faverdin et al, 1991). As expected most of the milk production parameters responded to increasing levels of concentrate feeding, with a mean milk yield response of 0.88 kg milk/kg fresh concentrate, similar to that found in many studies. The exception to this was milk fat content, which decreased with increasing levels of concentrate feeding. This effect is likely a reflection of reduced rumen acetate production with the high concentrate diet, thus reducing de novo milk fat synthesis. This suggestion is supported by the reduction in C16:0 milk fat concentrations. In addition, it is likely that milk fat production was reduced by an inhibition of mammary lipid synthesis by specific fatty acid intermediates (as proposed by Bauman and Griinari (2001) and reviewed more recently by others, for example, Harvatine et al. (2008). The reduction in milk fat content was primarily driven by a fall in SFA concentrations, with concentrations of MUFA and PUFA actually increasing. The improved energy status, and higher intakes of cows associated with increasing concentrate levels was reflected in their greater live-weights.

Within the three red clover treatments, as has been observed in many other studies, total DM intake increased as the red clover silage content of the diet increased (e.g. Moorby et al., 2009). Vanhatalo et al. (2009) found that intake of a 50:50 mixture of grass silage and red clover silage was significantly higher than intake of either the pure grass or pure red clover constituents of the mixture, similar to other findings under Nordic conditions (Bertilsson and Murphy, 2003). In the current study, the increase in DM intake of 1.3 kg/day due to an increase in the proportion of red clover in the grass-red clover silage mixture from 0.3 to 0.7 was higher than the increase in intake (0.6 kg DM/day) found by Moorby et al. (2009) when the proportion of red clover was increased from 0.33 to 0.66. In contrast, Gidlund et al. (2017) found no increase in silage DM intake when red clover silage proportion was increased from 0.3 to 0.7. Replacing grass silage with red clover silage did not confer any marked benefit on milk production, milk composition or ECM, although the PUFA content of milk was increased by red clover inclusion. The absence of a milk fat response to red clover

inclusion is in agreement with the results of eight experiments reviewed by Dewhurst (2013), and a further three recently published i.e. Halmemies-Beauche-Filleau et al. (2014); Gidlund et al. (2017); Johansen et al. (2017). Intake of NDF was similar for all treatments and so *de novo* fat synthesis would be expected to be similar between treatments (Murphy et al., 2000) albeit that inclusion of red clover reduced OM digestibility. So the effect of red clover silage on milk fat content is inexplicable.

With regards the expected concentrate 'sparing' effects of red clover, the results were disappointing. With regards milk yield and ECM yield, there was no 'sparing'. Indeed by interpolation from the response curve developed from the response to supplementing a grass silage only diet, on average an additional 1.02 and 1.18 kg concentrate would have been required daily with the red clover treatments (mean of the three treatments) in order to achieve the same level of milk yield and energy corrected milk yield as was achieved when grass silage was supplemented with 12.25 kg concentrate/day. In contrast, Dewhurst et al. (2003a), in an experiment in which cows offered a grass or red clover silage were supplemented with 4 or 8 kg concentrates (although not designed specifically to study the concentrate sparing effect of red clover), milk production from red clover silage and 4 kg concentrate was equivalent to an estimated 6.1 kg of concentrate offered with grass silage. This suggested that pure red clover silage was sparing 2.1 kg concentrate (although daily intakes of the red clover silage was 2.6 kg DM higher than for the grass silage). Similarly there was no concentrate saving associated with the red clover treatments, compared to the grass silage treatments for milk protein or milk protein yield. Milk fat content on the other hand was associated with a positive concentrate saving with red clover inclusion, although

Within this study, in order to 'balance ration crude protein contents, a higher protein concentrate was offered with the grass silage treatments. However, Westreicheer-Kristen et al. (2018) have found that replacing soybean meal sequentially with red clover silage in TMR rations, while total tract CP digestibility of CP did not differ between treatments, CP degraded in the rumen increased and the amount of intestinally absorbable rumen undegraded protein was reduced by more than 50%. So in this study, the lower quality of CP in the red clover than that of the concentrate, may explain the lower than expected production from the treatment comprising 30% red

clover and 12.25 kg concentrate relative to the predicted production from a diet of grass silage and 12.25 kg concentrate.

The presence of PPO in red clover diets has been found to increase the proportion of C18 PUFA, total long chain PUFA and milk protein in red clover silage diet compared with grass diets offered to dairy cows (Lee et al., 2009a). Oxidation of plant diphenols to quinones is catalysed by PPO which in turn complex and inactivate lypolytic enzymes (Lee et al. 2007). The higher content of PUFA in diets containing red clover silage than grass silage in the current study suggests the possibility that PPO in these diets restricted biohydrogenation of unsaturated fatty acids.

Milk protein content and production did not respond strongly to red clover silage content. A similar mean milk protein content between milk from perennial ryegrass and from red clover silage based diets has been found in a meta-analysis of studies comparing milk production from these two types of diets (Johansen et al., 2018). Further, in this study proportionately more N was partitioned into faeces than urine in the diets containing red clover than grass silage suggesting inhibition of action of proteases in the rumen by quinones derived from the catalytic action of PPO on plant diphenols (Lee et al., 2009b). The resultant protection of plant proteins allows passage of a greater proportion of ingested N as bypass protein out of the rumen of cows offered red clover-based diets than grass-based diets. However, this was not reflected in a higher milk protein content by cows fed the red clover silage based diet, similar to the findings of Johnston et al. (in review) over three harvests within the one season.

Although body condition score did not differ significantly between any treatments, GS8.5 was calculated to be at negative energy balance due to mean ME intake by cows on this treatment estimated to be 54 MJ/d lower than those in 16GS but was not obvious from blood metabolite analysis.

Conclusions

A limitation to the study was the difficulty in creating iso-nitrogenous diets with protein of equal quality which would explain the relative low response to the RC30 diet which was similar to, or lower than, the GS11.5 diet for a range of parameters. Despite that, and the lower digestibility of DM and its components, total DM intake and milk fat responded positively to increases in the red clover silage content of the silage component of the diet. So in addition to the positive impact that replacing grass silage

partly with red clover silage had on DM intake (2.15 kg concentrate spared when replacing grass silage with 70% red cover silage) inclusion of red cover silage also spared concentrate for production of milk fat with an indication that the 50% red clover content diet performed higher for this and other effects than the content of red clover would suggest.

References

AHDB Dairy (2012) Grass Silage Analysis. Grass+ Factsheet 17. Agricultural and Horticultural Development Board, Kenilworth.

AHDB Dairy (2016) Dairy Statistics, an Insiders Guide. Agricultural and Horticultural Development Board, Kenilworth. 76 pp.

Bertilsson J. and Murphy M. (2003) effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. *Grass and Forage Science*, 58, 309-322.

Chen,X. J. and Yan, T. (2015) Development of enteric methane emission factors for Holstein-Friesian and Norwegian first lactation cows. *Acta Agricultura Scandinavica. Section A, Animal Science*, 65, 127-131.

Clavin D., Crosson P., Grant, J. and O'Kiely P. (2017) Red clover for silage: management impacts on herbage yield, nutritive value, ensilability and persistence, and relativity to perennial ryegrass. *Grass and Forage Science*, 72, 414-431.

DairyCo (2012) *Greenhouse Gas Emissions on British Dairy Farms*. Agricultural and Horticultural Development Board, Kenilworth. 55pp.

Dale A.J., Laidlaw A. S.; Bailey J. S.and Mayne C.S. (2015) Effect of dairy slurry application rate and forage type on production, soil nutrient status and nitrogen-use efficiency. Grass and Forage Science, 70, 44-58.

Dewhurst R. J., Fisher W.J., Tweed J.K.S. and Wilkins R.J. (2003).Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. *Journal of Dairy Science*, 86, 2598–2611.

Dewhurst R.J. (2013) Milk production from silage: comparison of grass, legume and maize silages and their mixtures. Agricultural and Food science, 22, 57-69

Edmondson A, Lean I, Weaver L.., Farver T. and Webster G. (1989) A body condition scoring chart for Holstein dairy cows Journal of Dairy Science, 72, 68-78.

Faverdin P., Dulphy, J.P., Coulson, J.B., Verite R., Garel J.P., Rouel J. and Marquis B. (1991) Substitution of roughage by concentrates for dairy cows. *Livestock Production Science*, 27, 137-156.

Frame, J., Charlton, J. and Laidlaw, A. S. 1997. *Temperate Forage Legumes*. CAB International, Wallingford. 327 pp.

Gidlund H., Hetta, M. and Huhtanen P. (2017) Milk production and methane emissions from dairy cows fed a low or high proportion of red clover silage and an incremental level of rapeseed expeller. *Livestock Science,* 197, 73–81.

Halmemies-Beauchet-Filleau A., Vanhatalo A., Toivonen V., Heikkilä T, Lee M.R.F. and Shingfield K.J. (2014) Effect of replacing grass silage with red clover silage on nutrient digestion, nitrogen metabolism, and milk fat composition in lactating cows fed diets containing a 60:40 forage-to-concentrate ratio. *Journal of Dairy Science,* 97, 3761-3776.

Hynes D. N., Stergiadis, S., Gordon A and Yan T. (2016) Effects of concentrate crude protein content on nutrient digestibility, energy utilization, and methane emissions in lactating dairy cows fed fresh-cut perennial grass. *Journal of Dairy Science*, 99, 8858-8866.

Johansen M., Soegaard K., Lund P. and Weisbjerg, M.R. (2017) Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows species. *Journal of Dairy Science*, 100, 8861-8880.

Johansen, M., Lund, P. and Weisbjerg, M. R. (2018). Feed intake and milk production in dairy cows fed different grass and legume species: a meta-analysis. *Animal,* 12**,** 66-75.

Johnston D.J., Laidlaw A.S., Theodoridou K, and Ferris C.P. (under review) Performance and nutrient utilisation of dairy cows offered silages produced from three successive harvests of either a red clover-perennial ryegrass sward or a perennial ryegrass sward. *Irish Journal of Agricultural and Food Research*.

Lee M.R.F. Parfitt L.J., Scollan N.D. and Minchin F.R. (2007) Lipolysis in red clover with different polyphenol oxidase activities in the presence and absence of rumen fluid. *Journal of the Science of Food and Agriculture*, 87, 1308-1314.

Lee M.R.F., Theobald V.J., Tweed J.K.S., Winters A.L. and Scollan N.D. (2009a) Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. *Journal of Dairy Science*, 92, 1136-1147.

Lee M. R. F., Tweed J.K.S., Minchin F.R. and Winters A.L. (2009b). Red clover polyphenol oxidase: Activation, activity and efficacy under grazing. *Aniimal Feed Science and Technology*, 149, 250-264

Jones P.J., Thomas D., Hazzledine M. and Rymer C. (2014) *Replacing soya in livestock feeds with UK-grown protein crops*. Centre of Agricultural Strategy, Report 19. University of Reading.

Wilkins R.J. and Jones R. (2000) Alternative home-grown protein sources for ruminants in the United Kingdom. *Animal Feed Science and Technology*, 85, 23-32.

McMurray C. H., Blanchflower W. J. and Rice D. A. (1984) Automated kinetic method for D-3-hydroxybutyrate in plasma or serum. Clinical Chemistry, 30, 421-425.

Moorby, J. M., Lee, M. R. F., Davies, D. R., Kim, E. J., Nute, G. R., Ellis, N. M. & Scollan, N. D. 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. *Journal of Dairy Science,* 92**,** 1148-1160.

Vanhatalo, A., Kuoppala, K., Ahvenjarvi, S. & Rinne, M. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. *Journal of Dairy Science,* 92**,** 5620-5633.

Westreicheer-KristenE., Blank R., Metges C. and Susenbbeth A. (2018) Protein values of diets for dairy cows with different proportions of crude protein originating from red clover versus soybean meal. *Animal Feed Science Technology*, 245, 126-135.

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Table 1. *Chemical composition of silages (g/kg volatile-corrected oven DM, unless stated otherwise) and concentrates (g/kg ODM, unless stated otherwise) (S.D in parenthesis)*

	Silage		Concentrate	
	Grass	Red clover	Low Protein	High Protein
Oven dry matter (g/kg)	230 (31.3)	269 (11.8)	886 (8.21)	885 (7.84)
VCODM (g/kg)	250 (40.9)	282 (11.8)		
Crude protein (g/kg DM)	142 (32.2)	160 (18.6)	175 (26.6)	214 (30.2)
Gross energy (MJ/kg DM)	19.3 (2.04)	17.8 (0.75)	18.2 (0.12)	18.2 (0.12)
NDF	507 (69.7)	445 (21.4)	258 (14.9)	262 (17.4)
ADF	303 (45.9)	305 (12.3)	142 (7.68)	142 (8.43)
Ash	91 (6.9)	110 (4.3)	78 (5.9)	86 (5.3)
Organic matter	902 (7.5)	879 (4.7)		
Metabolisable energy (MJ/kg DM)	11.2 (0.49)	9.69 (0.217)		
Starch (g/kg DM)			292 (59.7)	220 (46.2)
Silage fermentation variables:				
Ammonia N (g/kg of total N)	87 (27.0)	89 (20.8)		
Lactic acid	113 (41.0)	99.5 (24.3)		
Acetic acid	30.4 (13.8)	26.5 (8.70)		
Ethanol	7.7 (5.13)	3.7 (5.87)		
Propanol	2.7 (3.19)	0.1 (0.55)		
pH	3.88 (0.236)	4.17 (0.109)		
n-Butyric acid	2.5 (4.48)	0.94 (2.25)		
Propionic acid	1.8 (1.84)	1.06 (0.439)		

VCODM, volatile corrected oven DM; NDF, neutral detergent fibre; ADF, acid detergent fibre.

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Table 2. *Ingredient composition (g/kg, fresh basis) of the high and low crude protein concentrates offered as supplements during the experiment*

Ingredient composition	Concentrate type	
	Low protein	High protein
Maize	220	150
Barley	180	125
Wheat	130	50
Soya hulls	147	72
Sugar beet pulp	60	140
Rape meal	110	165
Soya bean meal	60	205
Molaferm	40	40
Megalac	20	20
Limestone flour	12.5	12.5
Salt	9.5	9.5
Calcined magnesite	6	6
Trace elements and vitamins	5	5

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Table 3. *Concentrate, silage and total dry matter intakes (kg/day) of dairy cows offered grass silage supplemented with 8.5, 11.0, 13.5 and 16.0 kg concentrate/day (GS8.5, GS11.0, GS13.5 and GS16.0, respectively), and grass and red clover silage mixtures containing 30% (RC30), 50% (RC50) or 70% (RC70) red clover (DM basis)*

	Treatment								
	GS8.5	GS11.0	RC30	RC50	RC70	GS13.5	GS16.0	s.e.d.	P Value
High protein concentrate (DM)	3.9^{de}	4.0^{e}	2.2^{c}	1.3^{b}	0.4^{a}	3.7^{d}	3.6^{d}	0.124	<0.001
Low protein concentrate (DM)	3.8^{a}	5.8^{b}	8.7^{d}	9.6^{e}	10.5^{f}	8.3^{c}	10.7^{f}	0.126	<0.001
Total concentrate	8.5^{a}	11.0^{b}	12.2^{c}	12.2^{cd}	12.2^{d}	13.5^{e}	16.0^{f}	0.0095	<0.001
Red clover silage	0	0	3.3^{b}	6.0^{c}	8.4^{d}	0	0	0.278	<0.001
Grass silage	12.0^{ef}	12.3^{f}	8.0^{c}	6.3^{b}	4.2^{a}	11.2^{e}	9.9^{d}	0.491	<0.001
Total silage	12.0^{bcd}	12.3^{cd}	11.3^{bc}	12.3^{cd}	12.6^{d}	11.1^{b}	9.9^{a}	0.577	<0.001
Total DMI	19.6^{a}	22.1^{b}	22.2^{bc}	23.2^{cd}	23.5^{d}	23.1^{bcd}	24.2^{d}	0.578	<0.001

In rows with superscripts, means without a common superscript are significantly different (P<0.05)

Table 4. *Milk production and body tissue reserves of dairy cows offered grass silage supplemented with 8.5, 11.0, 13.5 and 16.0 kg concentrate/day (GS8.5, GS11.0, GS13.5 and GS16.0, respectively), and grass and red clover silage mixtures containing 30% (RC30), 50% (RC50) or 70% (RC70) red clover (DM basis)*

	Treatment								
	GS8.5	GS11.0	RC30	RC50	RC70	GS13.5	GS16.0	s.e.d.	P-value
Milk yield (kg/day)	30.2^{a}	32.4^{b}	32.0^{b}	32.8^{b}	32.9^{b}	34.9^{c}	36.8^{d}	0.882	<0.001
Energy-corrected milk yield (kg/day)	30.0^{a}	32.2^{b}	31.5^{ab}	32.9^{bc}	32.4^{b}	34.4^{cd}	35.0^{d}	0.885	<0.001
Milk fat content (g/kg)	41.0^{b}	40.6^{b}	40.0^{b}	41.4^{b}	39.7^{b}	39.9^{b}	36.6^{a}	1.069	<0.001
Milk protein content (g/kg)	31.4^{a}	32.1^{abc}	32.0^{abc}	31.6^{ab}	32.1^{abc}	32.6^{cd}	32.8^{d}	0.329	<0.001
Milk lactose content (g/kg)	46.6	46.8	46.6	46.8	46.8	46.8	46.8	0.910	NS
Milk fat yield (kg/d)	1.23^{a}	1.31^{abc}	1.27^{ab}	1.35^{bc}	1.30^{abc}	1.38^{c}	1.34^{bc}	0.045	0.023
Milk protein yield (kg/d)	0.94^{a}	1.03^{b}	1.02^{c}	1.04^{b}	1.05^{b}	1.12^{c}	1.20^{d}	0.029	<0.001
Milk fat-plus-protein yield (kg/d)	2.17^{a}	2.34^{b}	2.29^{ab}	2.38^{bc}	2.34^{b}	2.50^{cd}	2.54^{d}	0.064	<0.001
Milk energy content (MJ/kg)	3.09^{b}	3.10^{b}	3.07^{b}	3.12^{b}	3.06^{b}	3.08^{b}	2.96^{a}	0.042	0.012
Milk energy yield (MJ/day)	92.8^{a}	99.6^{b}	97.5^{ab}	101.8^{bc}	100.3^{b}	106.7^{cd}	108.5^{d}	2.74	<0.001
Live-weight (kg)	645^{a}	649^{ab}	651^{ab}	660^{c}	654^{bc}	659^{c}	660^{c}	4.22	0.001
Body condition score	2.25	2.23	2.28	2.32	2.28	2.30	2.27	0.032	NS

Table 5. *Selected milk fatty acid concentrations (g/kg total fatty acids measured) of dairy cows offered grass silage supplemented with 8.5, 11.0, 13.5 and 16.0 kg concentrate/day (GS8.5, GS11.0, GS13.5 and GS16.0, respectively), and and grass and red clover silage mixtures containing 30% (RC30), 50% (RC50) or 70% (RC70) red clover (DM basis)*

	Treatment								
	GS8.5	GS11.0	RC30	RC50	RC70	GS13.5	GS16.0	s.e.d.	P-value
Total SFA[1]	72.6^{c}	72.6^{c}	70.6^{b}	71.0^{b}	70.4^{b}	71.4^{b}	69.3^{a}	0.555	<0.001
Total MUFA	22.4^{ab}	22.1^{a}	23.2^{bc}	22.8^{ab}	23.0^{ab}	22.9^{ab}	23.9^{c}	0.143	0.002
Total PUFA	2.27^{a}	2.35^{a}	2.74^{c}	2.83^{c}	3.03^{d}	2.47^{b}	2.75^{c}	0.063	<0.001
n3	0.642^{b}	0.595^{a}	0.722^{c}	0.765^{c}	0.864^{d}	0.583^{a}	0.565^{a}	0.0231	<0.001
n6	1.63^{a}	1.75^{b}	2.02^{d}	2.07^{de}	2.16^{ef}	1.89^{c}	2.18^{f}	0.0497	<0.001
C14	11.3^{abc}	11.6^{c}	11.1^{a}	11.2^{ab}	11.2^{ab}	11.5^{c}	11.4^{c}	0.138	0.008
C16	38.4^{cd}	38.6^{d}	37.1^{b}	37.5^{bc}	37.4^{b}	37.5^{bc}	35.8^{a}	0.468	<0.001
C18	9.84	9.19	9.71	9.64	9.02	9.40	8.80	0.357	0.070
CLA 9 11	0.554^{a}	0.581^{ab}	0.662^{cd}	0.615^{bc}	0.679^{de}	0.610^{abc}	0.730^{e}	0.0301	<0.001
C18t	2.22a	2.31a	2.78^{c}	2.70^{c}	2.90^{bc}	2.54^{d}	3.31^{b}	0.141	<0.001
C18:1 c 11	0.446a	0.468a	0.502^{c}	0.507^{c}	0.532^{c}	0.502^{c}	0.607^{b}	0.0268	<0.001
C18:2 c 9 12	1.44d	0.55a	1.80^{e}	1.84^{e}	1.94^{c}	0.68^{b}	1.94^{c}	0.047	<0.001
C18:3 c 9 12 15	0.520b	0.474a	0.595^{c}	0.640^{d}	0.729^{b}	0.462^{a}	0.446^{a}	0.0193	<0.001

In rows with superscripts, means without a common superscript are significantly different (P<0.05)
SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids, PUFA, poly unsaturated fatty acids

Table 6. The difference in concentrates that would be required (kg/day) with a grass silage diet to achieve the same the same level of performance as was achieved with a the red clover silage – grass silage mixtures, when the latter were supplemented with concentrates at a rate of 12.25 kg/cow/day (*Quadratic equations used to derive the response curves for grass silage diets are also presented)*

Parameter	Treatment			Equation
	RC30	RC50	RC70	
Total DM intake (kg/day)	-0.55	+1.35	+2.15	$TDMI = 8.49 + 1.744(Conc) – 0.048(Conc)^2$
Milk yield (kg/day)	-1.55	-0.75	-0.75	$MY = 1.486 + 0.516(PE) + 1.013(Conc) – 0.0056(Conc)^2$
Energy corrected milk yield (kg/day)	-2.05	-0.45	-1.05	$ECM = 1.533 + 0.111(PE) + 2.2(Conc) – 0.062(Conc)^2$
Milk fat content (g/kg)	+0.45	>3.75	+0.95	$MFC = 8.12 + 0.461(PE) + 2.588(Conc) – 0.129(Conc)^2$
Milk protein content (g/kg)	-1.05	-2.79	-0.64	$MPC = 2.241 + 0.844(PE) + 0.400(Conc) -0.009(Conc)^2$
Milk lactose content (g/kg)	-3.42	-1.25	-2.37	$MLC = 22.5 + 0.482(PE) + 0.203(Conc) – 0.007(Conc)^2$
Milk fat yield (kg/day)	-2.36	+0.65	-1.75	$MFY = -224.1 + 0.37(PE) + 137.3(Conc) – 4.93(Conc)^2$
Milk protein yield (kg/day)	-1.45	-1.05	-0.65	$MPY = 40.2 + 0.43(PE) + 46.9(Conc) – 0.53(Conc)^2$
Fat + protein yield (kg/day)	-0.95	-0.55	-1.15	$F + PY = -111.2 +389(PE) + 175.2(Conc) – 5.1(Conc)^2$
Milk energy yield (MJ/day)	-2.05	-0.45	-1.15	$MEY = 47.0 + 7.08(Conc) – 0.2(Conc)^2$
Liveweight (kg)	-2.15	+1.15	-1.15	$LW = -82.11 + 1.04(PE) + 6.09(Conc) – 0.14(Conc)^2$
Body condition score	-2.05	-0.45	-2.05	$BCS = 212.9 + 1.54 (Conc) – 0.04(Conc)^2$

Positive value indicates concentrate sparing of the red clover treatment.
PE, pre-experimental value; Conc, concentrate rate.

Table 7. *Blood metabolites of dairy cows offered grass silage supplemented with 8.5, 11.0, 13.5 and 16.0 kg concentrate/day (GS8.5, GS11.0, GS13.5 and GS16.0, respectively), and grass and red clover silage mixtures containing 30% (RC30), 50% (RC50) or 70% (RC70) red clover (DM basis)*

	Treatment								
Parameter	GS8.5	GS11.0	RC30	RC50	RC70	GS13.5	GS16.0	s.e.d.	P-value
Albumen (mmol/l)	30.1	29.8	30.7	30.8	30.2	30.5	30.5	0.815	NS
βHB (mmol/l)	0.48^{ab}	0.47^{bc}	0.47^{bc}	0.46^{abc}	0.51^{c}	0.47^{bc}	0.40^{a}	0.318	0.03
Glucose	3.77	3.75	3.79	3.78	3.79	3.81	3.87	0.469	NS
TP (g/)	71.2	72.1	73.4	72.8	72.2	72.3	71.8	1.254	NS
Urea (mmol/[)	3.97	3.52	3.55	3.39	3.71	3.62	3.44	0.186	0.059

βHB β-hydroxyburyrate; TP Total protein
In rows with superscripts, means without a common superscript are significantly different (P<0.05)

Table 8 . *Effect on DM intake, milk yield and total ration digestibility when the grass silage component of the diet (GS) was partially replaced with either 30%, 50% or 70% red clover (on a dry matter basis), with all diets supplemented with 12.25 kg concentrates/cow/day*

	Treatments					
Parameter	GS	RC30	RC50	RC70	s.e.d.	P
Silage DM intake	8.8	10.4	10.9	10.5	1.17	NS
Total DM intake	19.5	21.5	21.7	21.3	1.11	NS
Milk yield	28.9	30.5	28.5	29.5	2.75	NS
Digestibility coefficients:						
Dry matter	0.766^{a}	0.743^{ab}	0.735^{ab}	0.726^{b}	0.0098	0.017
Organic matter	0.752^{a}	0.726^{ab}	0.716^{b}	0.707^{b}	0.0108	0.013
Nitrogen	0.675	0.635	0.636	0.689	0.0243	NS
Gross energy	0.733	0.718	0.709	0.683	0.0010	0.007
ADF	0.680	0.600	0.631	0.611	0.0306	0.082
NDF	0.696^{a}	0.667^{ab}	0.647^{ab}	0.621^{b}	0.0179	0.021

In rows with superscripts, means without a common superscript are significantly different (P<0.05)

Table 9 *Effect on nitrogen utilisation when the grass silage component of the diet (GS) was partially replaced with either 30%, 50% or 70% red clover (on a dry matter basis), with all diets supplemented with 12.25 kg concentrates/cow/day*

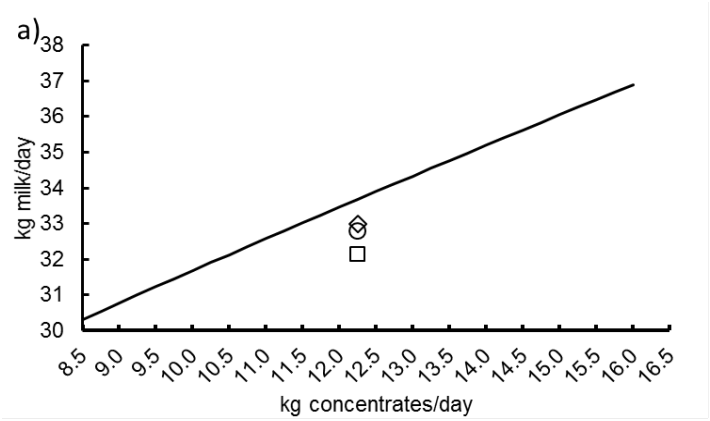
	Treatments					
Parameter	GS	RC30	RC50	RC70	s.e.d	P
N intake (g/d)						
Total N intake	498	536	547	519	22.6	NS
N output (g/d)						
Faeces N	164[a]	193[b]	204[b]	201[b]	10.8	0.027
Urine N	182	151	146	156	20.1	NS
Manure N	346	344	350	357	22.4	NS
Milk N	142	154	154	153	13.54	NS
N utilisation						
Faeces N/N intake	0.320[a]	0.357[ab]	0.372[b]	0.387[b]	0.0172	0.029
Urine N/N intake	0.355	0.279	0.264	0.302	0.0300	0.068
Milk N/N intake	0.276	0.286	0.280	0.295	0.0220	NS
Faeces N/Manure N	0.475	0.563	0.587	0.563	0.0351	0.06
Urine N/Manure N	0.525	0.437	0.413	0.437	0.0350	0.06

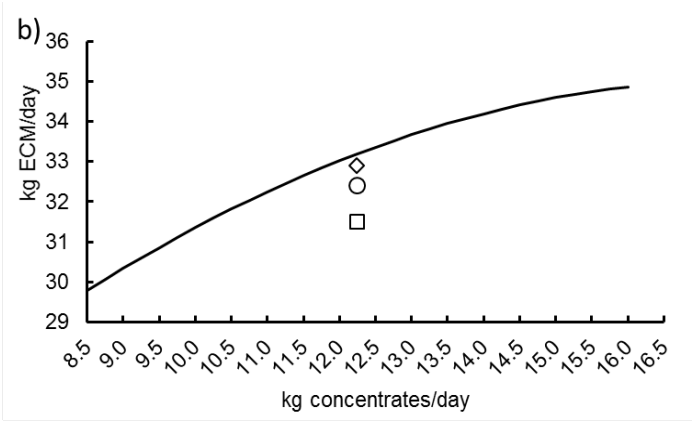
In rows with superscripts, means without a common superscript are significantly different (P<0.05)

Table 10. *Effect on energy utilisation when the grass silage component of the diet (GS) was partially replaced with either 30%, 50% or 70% red clover (on a dry matter basis), with all diets supplemented with 12.25 kg concentrates/cow/day*

	Treatments					
Parameter	GS	RC30	RC50	RC70	s.e.d.	P
GE intake (MJ/d)						
Total GE intake	364	387	391	369	19.8	NS
GE output (MJ/d)						
Faeces GE	82.8	97.2	102.2	103.2	6.50	0.054
Urine GE	14.5	12.3	12.0	13.2	1.60	NS
Milk GE	86.0	93.9	65.6	88.3	7.70	NS
GE utilisation						
Faeces GE/GE intake	0.228^{a}	0.251^{ab}	0.261b^{c}	0.281^{c}	0.0106	0.006
Urine GE/GE intake	0.040	0.032	0.031	0.036	0.0030	0.052
Milk GE/GE intake	0.236	0.242	0.245	0.240	0.0168	NS

In rows with superscripts, means without a common superscript are significantly different (P<0.05)


a)
kg milk/day
38
37
36
35
34
33
32
31
30
8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0 15.5 16.0 16.5
kg concentrates/day


b)
kg ECM/day
36
35
34
33
32
31
30
29
8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0 15.5 16.0 16.5
kg concentrates/day

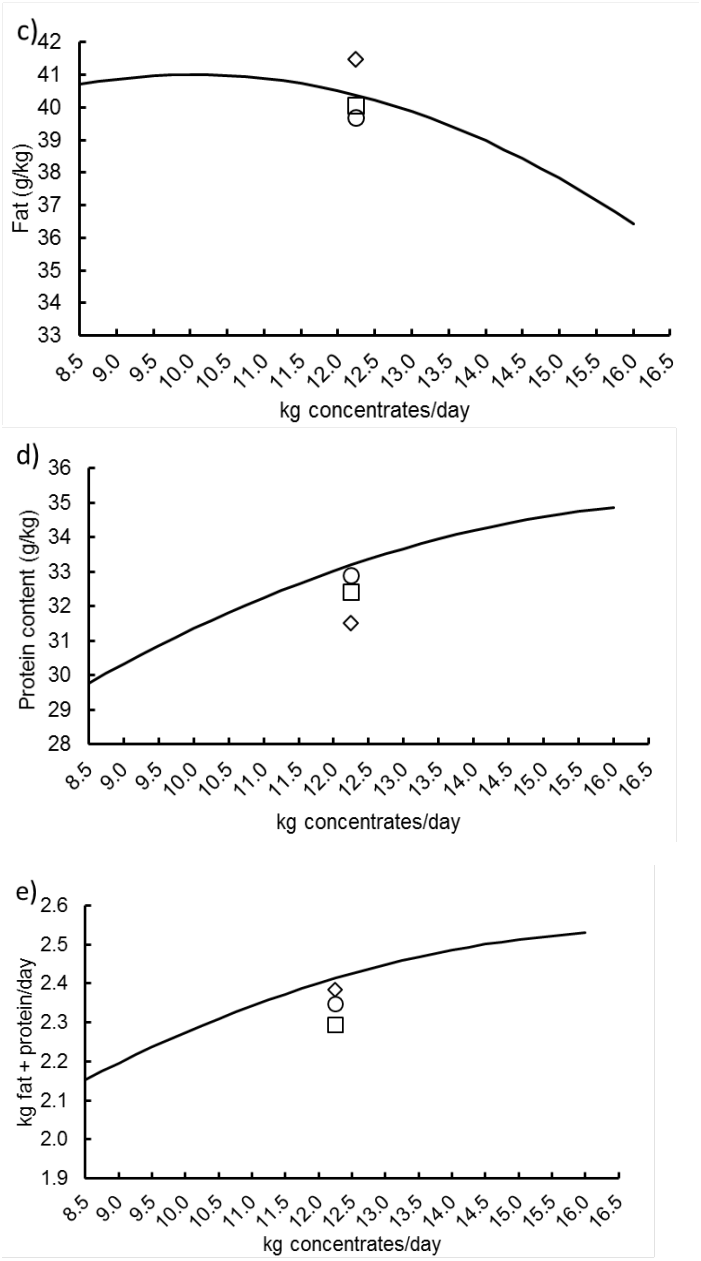


Figure 1. Response curves (a) milk yield, b) energy corrected milk yield, c) milk fat content, d) milk protein content and e) fat + protein yiled) for cows offered grass silage supplemented with either 8.5, 11.0, 13.5 or 16 kg concentrate/day, with the response of cows offered diets containing mixtures of grass silage and red clover silage (the latter at inclusion levels of 30% (□), 50% (◊) and 70% (○) DM), plus 12.5 kg concentrate/day, superimposed.

Experiment 6

Effect of management strategy on wilting of monocultures and mixture of red clover and perennial ryegrass

Introduction

Red clover is a forage that can achieve high yields of DM without the input of nitrogenous fertiliser (Frame et al., 1998). It also has attributes that lend it to replacing imported protein feeds, including a high protein content and high intake characteristics (Dewhurst et al., 2013). However, red clover can be difficult to ensile due to its high buffering capacity, low water soluble carbohydrate content and higher moisture content compared with grass treated under similar conditions. Further red clover can be slow to wilt relative to other forages. For example, it has been found to lose water more slowly than lucerne (Owens et al., 1999) which has been attributed to its lower leaf : stem ratio and wider stem diameter than Lucerne. However, less is known about the rate of moisture loss from red clover compared with perennial ryegrass under similar conditions. Fychan et al. (2016) found perennial ryegrass to dry faster than red clover during wilting prior to ensiling. Dry matter of perennial ryegrass increased from 246 g/kg at mowing to 399 g/kg at ensiling compared with 200 g DM/kg fresh weight at mowing and 323 g/kg at ensiling.

The weight of herbage per unit area has a profound effect on the rate of moisture loss in the swathe (Wright et al.,1997). Reducing weight of fresh grass from 6 to 3 kg/m^2 resulted in an increase in moisture loss of almost 50%. Treatment of the crop prior to ensiling may have an effect on the comparative rate of drying between the perennial ryegrass and red clover although Fychan et al. (2016) found that conditioning after mowing had similar beneficial effects on wilting of perennial ryegrass and red clover.

Physical characteristics of the swathe can affect the rate of loss of moisture (Jones and Harris 1979). Therefore, if perennial ryegrass is mixed with red clover the cut swathe may have a different physical structure to red clover grown alone. The following study was undertaken to investigate the effect of additional swathe management on wilting of red clover and perennial ryegrass in monoculture and mixture. A low rate of N fertilizer was applied to perennial ryegrass intended to produce a similar N content in the dry matter to the N content of DM in perennial ryegrass growing with red clover (Frame et al., 1998) so that the grass monoculture could be considered a component of the mixture.

Materials and methods

Swards. Red clover (cv. Merviot), perennial ryegrass (cv. Copeland) and a mixture of the two were sown on 7 September 2016 in plots of 0.1 ha each. Red clover (RC) and perennial ryegrass (PRG) were sown as monocultures at seed rates of 13 kg/ha and 30 kg/ha, respectively, and in a mixture (RC-PRG) at 8 kg/ha and 20 kg/ha for RC and PRG, respectively. After a 'clean-off' cut on 23 May 2017, 45 kg N/ha (as calcium ammonium nitrate) and 90 kg/ha K_2O (as muriate of potash) were applied to all plots, followed by 30 kg N/ha and 120 kg K_2O/ha after harvest 1 (H1) on 5 July 2017. A second harvest (H2) was taken on 25 September. The content of red clover in the mixture was 210 and 580 g/kg DM at H1 and H2, respectively.

Treatments and experimental design. The experiment (3 × 3 factorial design) consisted of the three sward types each subjected to three swathe management treatments, with each treatment replicated six times in a randomised block design. The three swathe management regimes were: Undisturbed herbage in the swathe representing 0.75 of the field area covered so there was full swathe weight per unit area; Herbage tedded by hand when the swathes in the trays were constituted, otherwise similar to Undisturbed ('Tedded, full wt/area'); Herbage tedded by hand as for Tedded, full wt/area, with fresh weight within the tray reduced by 25% (representing swathe distributed over the full field area) followed by tedding a second time during the morning of the second day ('Tedded, 0.75 wt/area'). Loss of fresh weight during wilting was measured by reconstituting swathes in wire mesh (13 mm square mesh) trays (0.5 m × 0.5 m base × 0.3 m high), weighing the trays and measuring loss at 2 h intervals from approximately 10.00 h and 12.00 h (H1 and H2, respectively) until 18.00 h (Day 1), and again at 08.00 h (Night 1) and from approximately 8.00 to 18.00 h (Day 2), and again at 08.00 h (Night 2). All times were accurately recorded so that rates of change with duration of wilting could be calculated.

Methods. At each harvest the weight of herbage to be used to reconstitute a swathe within a tray was determined before mowing by weighing herbage harvested with an Agria autoscythe (Agria, Moeckmuehl, Germany) in two 3 m long strips in each of the areas designated for harvesting (approximately 20 m × 10 m) for each sward type. Herbage harvested was weighed, sampled for ODM to be dried at 85°C for 24 h while the fresh weight yield was calculated, taking account of the area cut. Herbage for the

treatments in the trays was mown with a mower fitted with a steel flail conditioner, leaving a swathe covering 75% of the ground area. Average fresh herbage weight at the beginning of wilting for the Control and Tedded Full Wt/Area was 1450 g (5.80 kg/m^2) at H1 and 1117 g (4.35 kg/m^2) per tray at H2, while fresh herbage weight per tray for Tedded ¾Full Wt/Area was 1088 g (4.30 kg/m^2) at H1 and 836 g (3.26 kg/m^2) at H2. Immediately prior to setting up the trays, herbage was sampled for oven dry matter (ODM) and WSC concentration by taking samples within each plot for each of the six replicates, storing in a polystyrene box lined internally with ice-packs and processing for drying at 60^{o}C within 2 h of sampling. At the end of the wilting periods (46 and 44 h after measurements commenced at H1 at H2, respectively), herbage was sampled for determination of ODM and WSC from which the weight of DM and WSC in each tray at the beginning and end of wilting, and loss of DM and WSC were calculated.

Chemical analyses and meteorological data. Samples taken for ODM were dried at 85^{o}C for 24 h. Samples taken to determine WSC concentration of DM were dried at 65^{o}C for 48 h then milled through a 0.8 mm sieve. Concentration of WSC was determined by a Continuous Segmented Flow Analyzer (SEAL Analytical Ltd., Southampton, UK) following the method of McDonald and Henderson (1964).

Data on temperature and evapotranspiration were collected every 15 mins at an electronic weather station approximately 1 km from the experimental site. No rain fell during the days during wilting but dew was deposited on the herbage during the two nights of wilting at H2.

Statistical analyses: Differences between treatment means for consecutive periods during each day were analysed by repeated measurements analysis, and for nights by analysis of variance, with sward type, sward management and interaction as factors, and residual as error variance; GenStat for Windows, 16th Edition).

Results

Based on evapotranspiration (ET), conditions at Day 1 in H2 were more conducive to drying than Day 1 in H1, otherwise drying conditions were better in H1 than H2 (Table 1).

Interactions between swathe management regimes and forage type were not significant and so only main effects are presented.

Yield of perennial ryegrass was the lowest of the three swards at both harvests, and mean yield of all three at H1 was 4.64 t/ha compared with 2.98 t/ha at H2 (Table 2). At both harvests DM concentration of red clover herbage was significantly lower than DM concentration of PRG and DM concentration of the mixture at H2 did not differ significantly from red clover. Concentration of WSC in DM of PRG was significantly higher than in PRG-RC and RC at both harvests and WSC was significantly higher in PRG-RC than RC at H2.

The rate of fresh weight change of the forage types and swathe managements over consecutive days and nights during wilting are presented in Table 3. Fresh weight loss of PRG-RC during wilting was generally similar to PRG during H1 and RC during H2. In three of the four days (to 18.00 h) over the two harvests RC lost FWt significantly faster than PRG and rate of loss was higher in Day 1 of H2 than Day 1 of H1, despite drying conditions suggesting the contrary. During nights at H2 herbage gained weight. Rate of loss of FWt in Day 2 at each harvest was approximately 30-50% of the loss in Day 1. The fastest rate of loss of fresh weight was generally between 12.00 and 14.00 for all sward types, although the greatest differences between sward types was not confined to that period (generally within the 2 hours before or after) (Table 4).

Tedding alone had a small but significant effect on fresh weight loss in Day 1 at each harvest (Table 3). However, tedding twice and reducing the herbage weight per unit area had a more marked effect on fresh weight loss in Day 1 than tedding alone (25 and 22 % increase over the Control at H1 and H2, respectively) and increased loss by 13% in Day 2 of both harvests. The daily profile rate of fresh weight loss (Table 5) indicates that the differences between the extreme treatments (usually between the control and the treatment involving twice tedded and reduced herbage weight per unit area) were, as for largest differences between sward types, in the 12.00 – 14.00 hour period.

At the end of the wilting period at both harvests PRG continued to have a higher mean OMD than RC (Table 6), with ODM for the mixture midway between the two monocultures at H1 and no significant difference between RC and PRG-RC at H2.

Concentration of WSC at the end of wilting was significantly higher for PRG than both PRG-RC and RC at both harvests. The percentage of the original DM and WSC lost during wilting did not differ significantly between the three sward types. The overall mean loss of DM was 7.0 and 12.7 % of the original weight at H1 and H2, respectively. Swathe management had no significant effect on the percentage of DM or WSC weight lost during wilting.

Discussion

The use of a mesh tray to confine a segment of swathe enabled measurements to be made on the change in its weight at intervals, and for rate of change to be calculated. However an edge effect is created around the tray as it is isolated from the remainder of the swathe. Frost et al. (1995) estimated that this loss could account for an additional 8-23% fresh matter loss and so the trays are a source of bias. However, provided all treatments were affected similarly the method would not have affected precision i.e. relative effect between treatments due to either sward type or swathe management.

Change in fresh weight was taken to be a measure of change in moisture content. The mean loss of fresh weight at the end of wilting at each harvest calculated from the data in Table 3 was 506 g/kg and 454 g/kg at H1 and H2, respectively. However, DM was also lost during wilting i.e. 7.1% and 12.7% of original DM, meaned across all treatments in H1 and H2, respectively (Table 6). So across all treatments, the mean loss of DM at H1 and H2 was 13.3 and 32.6 g/kg of original fresh weight. This represents an overestimate of moisture loss during wilting of i.e. 1% and 5%, respectively, for H1 and H2 if change in fresh weight is taken as loss of moisture during wilting.

The ability of red clover to produce high DM yields compared with perennial ryegrass when both received modest dressings of inorganic N was demonstrated in this experiment, red clover producing significantly more DM than perennial ryegrass at both harvests. The yield of the mixture at the second harvest produced the highest yield of the three swards. The high yield from red clover-grass mixtures has been underscored by data from a trial in south west Scotland where, under farming

conditions, a red clover- grass mixture produced the equivalent of 87% the yield of a grass sward receiving 310 kg N/ha (Roberts et al., 1990).

The lower content of DM in red clover than perennial ryegrass at both harvests agrees with the perception of red clover's moisture content and is supported by studies such as McEniry et al. (2014). Although the rate of loss of moisture from red clover was slightly faster than the rate of loss from grass at the time of day when the rate was highest, the outcome did not markedly change the moisture content relative to that of grass. The sensitivity of red clover leaves to drought does not seem to have a major impact on the loss of moisture from the plant. The crops were conditioned with a steel tined conditioner at mowing to aid wilting. Fychan et al. (2016) have found that a high proportion of leaf material from legumes such as red clover is lost especially when they are subjected to this type of conditioning. The lack of an interaction between sward type and swathe management suggests that opportunities to manage the swathe to hasten wilting relative to perennial ryegrass are limited.

Although the mixture between red clover and perennial ryegrass usually responded to treatments as its content of red clover would suggest, its loss of moisture in Day 1 in H2 was inexplicably significantly faster than moisture loss from either of the monocultures. The mixture may have resulted in the swathe having a sufficiently different structure to the other two sward types as it comprised approximately 50% of each herbage type (Rotz, 1995). This may have produced a microclimate conducive to moisture loss. Alternative it may have been a chance effect.

Method of swathe management had more impact on the rate of moisture loss than sward type. As swathes in both tedding treatments were subjected to the same disturbance in Day 1, any difference between the two would have been due to differences in herbage weight per unit area. Consideration of moisture content rather than dry matter content can be more helpful in evaluating the impact of wilting. At H1, mean moisture content of 895 g/kg at the beginning of wilting was reduced to 598 g/kg and 542 g/kg for the tedded treatment with 5.8 kg herbage/m^2 and tedded with 4.35 kg/m^2, respectively – a loss of 297 g/kg and 353 g/kg. At H2, the corresponding losses were 243 and 297 g/kg from initial moisture content of 809 g/kg. Wright et al. (1997) found that reducing fresh weight per unit area from 6 to 3 kg/m^2 increased the rate of moisture loss in herbage with an initial ODM of about 180 g/kg from 240 g/kg to 280

g/kg in the first day of wilting. This represented a loss of approximately 60 and 100 g/kg for the 6 and 3 kg fresh matter/m^2 treatments, respectively – the lighter fresh herbage weight having a loss rate about 67% higher than the heavier weight treatment. The 22% faster rate in this study of the lighter than the heavier fresh material treatment at both harvests falls short of the expected 30% increase, based *pro rata* on the data of Wright et al. (1997). Conditioning at mowing may have resulted in the capability of the crops to lose moisture quickly (Fychan et al., 2016) without further treatment resulting in a reduction in differences between swathe management treatments.

Fresh weight reduction was greater in the first than second day of wilting at both harvests by a factor of two to three. It is not possible to separate the effect of tedding and fresh weight per unit area on the rate of drying in Day 2 although Wright et al. (1997) report that the latter has a continuing effect over the 2 day wilting period.

Conclusions

Red clover lost moisture only slightly faster than perennial ryegrass during wilting and that was confined to the period of generally greatest wilting rate for all types, i.e. around the middle of the day. Most mowers are fitted with conditioners, mainly the steel tined type. As conditioning overcomes some of the limitations to moisture loss from herbage, external factors become more important in determining the rate of loss of moisture. While tedding has some impact, the herbage weight per unit area reducing the amount of herbage per unit area for all sward types in this study resulted in faster wilting than disturbing the swathe by tedding alone. So wilting rate will be increased by scattering the herbage over the whole area during tedding, rather than confining herbage to the area that the swathe lies after cutting which is usually about 75% of the potential area.

References

Dewhurst, R.J. (2013) Milk production from silage: comparison of grass, legume and maize silages and their mixtures. *Agricultural and Food Science*, 22, 57-69

Frame J., Charlton J.F.L. and Laidlaw A.S. (1997) *Temperate Forage Legumes*, CAB International, Wallingford, UK, 336 pp.

Fychan R., Leemans D.K., Scott M.B., Theobald V.J., Sanderson R. and Marley C.L. (2016) Effect of mower/conditioner type during legume or grass harvesting on silage quality. *Proceedings of the 26th General Meeting of the European Grassland Federation*, Vol. 21, 176-178.

Frost, J.P., Poots, R., Knight, A., Gordon, F.J. and Long, F.N.J. (1995) Effect of forage matting on rate of grass drying, rate of silage fermentation, silage intake and digestibility of silage by sheep. *Grass and Forage Science*, 50, 21-30.

Hunt, I.V., Frame, J. and Harkess, R.D. (1975) Potential productivity of red clover varieties in south west Scotland. *Journal of the British Grassland Society*, 30, 209-216.

Jones, L. and Harris, C.E. (1979) Plant and Swath limits to drying. In: *Forage Conservation in the 80s.* Occasional Symposium No. 11. (Thomas, C. (ed.). British Grassland Society, Hurley. Pp. 53-60.

McDonald, P. and Henderson, A.R. (1964) Determination of water soluble carbohydrates in grass. *Journal of the Science of Food and Agriculture*, 15, 395-398.

McEniry, J., King, C. and O'Kiely, P. (2013) Silage fermentation characteristics of three common grassland spsecies in response to advancing stage of maturity and additive application. *Grass and Forage Science*, 69, 393-404.

Owens, V.N., Albrecht, K.A. and Muck, R.E. (1999) Protein degradation and ensiling characteristics of red clover and alfalfa wilted under varying levels of shade. *Canadian Journal of Plant Sciences*, 79, 209-222.

Roberts, D.J., Lawson, A. and Fisher, G.E.J. (1990) A comparison of a red clover-grass sward with a grass sward plus fertilizer nitrogen under a three-cut silage regime. In: *British Grassland Society Second Research Conference*, Session III, Paper 5. British Grassland Society, Reading.

Wright D.A., Frost J.P., Patterson D.C. and Kilpatrick D.J. (1997) The influence of weight of ryegrass per unit area and treatment at and after mowing on rate of drying. *Grass and Forage Science*, 52, 86-98.

Table 1. Mean air temperature (Temp, ^{o}C) and evapotranspiration (ET, mm h^{-1}) for Day and Night periods during Harvests 1 (H1) and 2 (H2)

	Day 1		Night 1		Day 2		Night 2	
	Temp	ET	Temp	ET	Temp	ET	Temp	ET
H1	16.2	0.188	14.2	0.021	18.2	0.160	15.8	0.031
H2	16.1	0.228	11.9	0.004	13.7	0.078	14.1	0.023

Table 2. a) Forage DM yield, b) DM concentration in fresh weight (FWt) and c) WSC concentration in DM at start of wilting at Harvests 1 and 2.

	Sward Type				
Harvest	PRG	PRG/RC	RC	s.e.d.	*P*
a) DM yield (t DM/ha)					
1	3.58^{c}	4.73^{d}	5.62^{a}	0.062	0.004
2	2.00^{c}	4.09^{a}	2.84^{b}	0.120	0.035
b) DM concentration (g ODM/kg FWt)					
1	117^{a}	102^{b}	95^{b}	4.98	0.004
2	266^{a}	145^{b}	161^{b}	11.98	<0.001
c) WSC concentration (g WSC/kg DM)					
1	96.0^{a}	59.3^{b}	59.6^{b}	6.80	0.005
2	206.9^{a}	111.6b	96.0^{c}	3.40	<0.001

Means that do not have a common superscript within a row that has significant differences are significantly different at P<0.05.

Table 3. Rate of change in fresh weight (g/kg original fresh weight/h) during the day and night for 46 and 44 hours, respectively, for Harvests 1 and 2 for, a) sward types and b) swathe management

a)			Sward type				
Harvest	Day	Period	PRG	PRG-RC	RC	s.e.d.	*P*
1	Day1	10.00-18.00	38.5^{b}	38.3^{b}	39.6^{a}	0.610	0.042
	Night 1	18.00-8.00	1.47	1.47	1.51	0.062	NS
	Day 2	8.00-18.00	15.1^{c}	16.0^{b}	16.9^{a}	0.272	<0.001
	Night 2	18.00-8.00	0.9	1.1	1.2	0.062	0.098
2	Day1	12.00-18.00	55.3^{a}	59.3^{b}	56.1^{a}	1.32	0.007
	‡Night 1	18.00-8.00	-5.8^{a}	-5.1^{b}	-5.3^{b}	0.132	<0.001
	Day 2	8.00-18.00	18.3^{b}	20.7^{a}	21.0^{a}	0.658	<0.001
	‡Night 2	18.00-8.00	-0.94	-0.99	-0.55	0.217	NS

b)			Swathe management				
Harvest	Day	Period (h)	Control Full wt/ Area	Tedded Full wt/ Area	Tedded x 2 ¾Full wt/ Area	s.e.d.	Interaction: *P*
1	Day1	10.00-18.00	35.2^{c}	37.1^{b}	44.1^{a}	0.610	<0.001
	Night 1	18.00-8.00	1.5	1.5	1.4	0.109	NS
	Day 2	8.00-18.00	15.3^{b}	15.3^{b}	17.3^{a}	0.272	<0.001
	Night 2	18.00-8.00	0.9	1.1	1.1	0.137	NS
2	Day 1	12.00-18.00	52.0^{c}	54.9^{b}	63.8^{a}	1.32	<0.001
	‡Night 1	18.00-8.00	-4.9^{b}	-4.8^{b}	-6.5^{a}	0.132	<0.001
	Day 2	8.00-18.00	19.1^{b}	19.1^{b}	21.6^{a}	0.658	<0.001
	‡Night 2	18.00-8.00	-0.3^{b}	-0.6^{b}	-1.6^{a}	0.217	<0.001

Means that do not have a common superscript within a row that has superscripts are significantly different at P<0.05.
Interactions between sward types and time are presented in Table 4 and between swathe management and time are presented in Table 5.
‡Negative represents a rate of fresh weight gain.

Table 4. Interaction data sets of means of rate of change in fresh weight (g/kg original fresh weight/h) for each sward type during the day over two days of wilting at Harvests 1 and 2.

			Sward type			Interaction:	
Harvest	Day	Period (h)	PRG	PRG-RC	RC	s.e.d.	*P*
1	Day1	10.00-12.00	34.8	33.7	36.2		
		12.00-14.00	55.9	56.1	58.2	1.67	NS
		14.00-16.00	35.7	36.4	35.5		
		16.00-18.00	27.5	27.0	28.6		
	Day 2	8.00-10.00	9.6^{e}	9.8^{e}	10.0^{e}		
		10.00-12.00	16.8^{d}	17.1^{cd}	17.6^{cd}		
		12.00-14.00	21.3^{abc}	22.3^{ab}	23.8^{a}	1.20	0.015
		14.00-16.00	19.1^{cd}	19.9^{bc}	22.3^{ab}		
		16.00-18.00	8.8^{e}	10.8^{e}	10.8^{e}		
2	Day1	12.00-14.00	90.8	96.9	92.9		
		14.00-16.00	56.3	59.3	55.7	3.39	NS
		16.00-18.00	18.7	21.9	19.8		
	Day 2	8.00-10.00	18.7^{d}	17.1^{d}	17.6^{d}		
		10.00-12.00	27.0^{ab}	28.2^{a}	30.5^{a}		
		12.00-14.00	21.8^{cd}	26.0^{ab}	24.9^{bc}	1.83	0.018
		14.00-16.00	17.6^{d}	22.6^{bc}	23.3^{bc}		
		16.00-18.00	6.2^{e}	9.4^{e}	8.5^{e}		

Means within a significant interaction data set that do not have a common superscript are significantly different at P<0.05.

Table 5. Interaction data sets of means of rate of change in fresh weight (g/kg original fresh weight/h) for each swathe management during the day over two days of wilting at Harvests 1 and 2

Harvest		Period (h)	Swathe management			Interaction:	
			Control Full wt/ Area	Tedded Full wt/ Area	Tedded Full wt/ Area	s.e.d.	*P*
1	Day1	10.00-12.00	30.9^{de}	34.2^{d}	39.7^{c}		
		12.00-14.00	51.9^{b}	53.0^{b}	65.4^{a}	1.67	<001
		14.00-16.00	32.7^{d}	34.4^{d}	40.5^{c}		
		16.00-18.00	25.4^{f}	26.9^{ef}	30.8^{de}		
	Day 2	8.00-10.00	9.2^{d}	9.0^{d}	11.3^{d}		
		10.00-12.00	16.3^{c}	16.3^{c}	18.9^{bc}		
		12.00-14.00	21.4^{b}	20.9^{b}	25.1^{a}	1.21	<0.001
		14.00-16.00	20.0^{b}	19.7^{b}	21.6^{b}		
		16.00-18.00	9.7^{d}	10.8^{d}	9.8^{d}		
2	Day1	12.00-14.00	82.5^{c}	91.3^{b}	106.9^{a}		
		14.00-16.00	53.6^{e}	54.1^{e}	63.5^{d}	3.39	<0.001
		16.00-18.00	20.0^{f}	19.3^{f}	21.1^{f}		
	Day 2	8.00-10.00	16.7	16.7	19.9		
		10.00-12.00	25.7	28.7	31.3		
		12.00-14.00	23.3	22.8	26.6	1.83	0.078
		14.00-16.00	21.7	19.4	22.5		
		16.00-18.00	8.2	7.9	7.9		

Means within a significant interaction data set that do not have a common superscript are significantly different at P<0.05.

Table 6. Mean concentration of DM in fresh weight and WSC in DM after wilting and loss of weight during wilting of DM and WSC (as % of pre-wilting weight, T_0) for different a) sward types and b) swathe managements.

a)		Sward type				
	Harvest	PRG	PRG/RC	RC	s.e.d.	P
ODM	H1	205.4[a]	190.6[b]	173.5[c]	4.53	<0.001
(g/kg FWt)	H2	321.8[a]	213.2[b]	235.5[b]	5.50	<0.001
WSC	H1	90.2[a]	59.3[b]	57.1[b]	5.83	<0.001
(g/kg DM)	H2	175.4[a]	94.1[b]	78.2[b]	8.76	<0.001
DM Wt loss	H1	6.9	5.2	9.0	2.52	NS
(% T_0 Wt)	H2	17.9[a]	11.1[b]	9.0[b]	3.65	0.047
WSC Wt loss	H1	2.0	-4.7	2.1	9.73	NS
(% T_0 Wt)	H2	23.7	21.4	30.7	9.70	NS

b)		Swathe management				
	Harvest	Control Full wt/ Area	Tedded Full wt/ Area	Tedded x 2 ¾Full wt/ Area	s.e.d.	*P*
ODM	H1	177.0[b]	177.4[b]	215.0[a]	4.53	<0.001
(g/kg FWt)	H2	242.8[b]	259.3[ab]	268.4[a]	5.50	0.007
WSC	H1	63.2	69.7	73.8	5.83	NS
(g/kg DM)	H2	112.2	117.2	78.2	6.20	NS
DM Wt loss	H1	7.9	8.0	5.3	2.52	NS
(% T_0 Wt)	H2	15.2	10.7	12.1	3.65	NS
WSC Wt loss	H1	-8.9	8.7	-0.4	9.73	NS
(% T_0 Wt)	H2	32.8	21.7	21.4	9.70	NS

Means that do not have a common superscript within a row that has significant differences are significantly different at P<0.05
Negative indicates a gain.

Experiment 7

The effect of increasing the proportion of red clover in a grass clover mixture on ensiling characteristics and in-silo losses

Introduction

Red clover, similar to all other forage legumes, generally has a higher crude protein content than grasses. That attribute combined with high voluntary intake characteristics when offered to ruminants (Dewhurst et al., 2003) qualifies it as a potential replacement for imported protein feeds for ruminants. However, as it usually has a lower water soluble carbohydrate (WSC) content, a lower dry matter content (DM), and a higher buffering capacity than forage grasses, it is often grown with grass to improve its ensilability (Clavin et al., 2016). Despite these shortcomings as a crop perceived to be difficult to ensile, King et al. (2012) found that even when red clover with a low DM content (in the range 140 to 170 g/kg) was ensiled, an additive was not required to produce an adequate fermentation. However, McEniry et al. (2013) found that when first harvest red clover was ensiled, treatment with formic acid improved fermentation characteristics.

When offering mixtures of red clover and grass silage to dairy cows, synergism between the two components has been suggested. For example, in some experiments higher milk yields have been recorded when cows were offered mixtures of grass and red clover than when the two silage types have been offered on their own (Bertilsson and Murphy, 2003; Vanhatalo et al., 2009). In addition to the two silages interacting after ingestion, there is also potential for the two components to interact when ensiled together. Perennial ryegrass, when ensiled with red clover, may contribute WSC to the mixture and hence increase the rate of fermentation and cause pH to decline. Red clover has the potential to contribute polyphenol oxidase (PPO) to the mixed forage during ensilage (Marley et al., 2003) which may reduce proteolysis in the ensiled mixture, and thus reduce the production of ammonia-N. However, when this was tested experimentally in 1.5 L anaerobic jars the effect of PPO was not obvious. For example, mixing of the two forages did not result in inhibition of production of ammonia-N compared to production of ammonia-N in the monocultures during ensiling (Krawutschke et al., 2012). In order to investigate the possibility of the two forage types interacting during ensilage in silos that would be more akin than jars to commercial silos, the following study was undertaken using tube silos with the capacity to hold approximately 6 kg of fresh herbage.

Materials and Methods

Establishment and harvesting of swards. Red clover (cv. Merviot) and perennial ryegrass (cv. Copeland) were each sown as monocultures into a 0.1 ha plot on 7 September 2016 at 15 and 30 kg/ha, respectively. Seed was sown into a conventionally prepared seed bed with a StocksAG Turbo Jet Wizard (Stocks AG Ltd., Wisbech, UK) and a 6 m spring tine harrow (Garnett Farms Engineering Ltd., Cheshire, UK). Plots were rolled with a 3 tonne flat roller.

Both plots were harvested on 22 May 2017 (herbage removed was not used in this experiment) after which 45 kg N/ha as calcium ammonium nitrate (CAN) and 90 kg K_2O as muriate of potash were applied with a Vicon SuperFlow spreader (Kverneland Group UK Ltd., Merseyside, UK). A further 30 kg N/ha were applied as CAN to the perennial ryegrass plot and 120 kg K_2O as muriate of potash were applied to both plots on 19 July, after the plots had been mown and the crop lifted.

Prior to mowing, 5 strips each approximately 3 m long, were cut to 4 cm above ground level with an Agria autoscythe to assess yields. Swards were mown on 17 July and 6 September 2017 with a Claas 3200 disc mower and left to wilt for 24 h. After windrowing the crop was lifted and chopped with a John Deere 7450 self-propelled precision chop forage harvester and transported to the yard. The chopped forages were deposited in piles on clean polythene sheets, with these piles kept covered with sheeting to prevent evaporation.

Experimental design and filling the mini-silos. At each harvest the experiment involved five ‘forage treatments’, comprising red clover and grass silage mixed in different fresh weight ratios, namely 0:100, 25:75, 50:50, 75:25 and 100:0. A total of 90 mini-silos were filled at each harvest, 18 for each of the five ratios. For each ‘forage treatment’ this allowed three mini silos to be opened at each of six destructive sampling times, namely at days 3, 6, 12, 24, 48 and 96 days after filling.

Mini silos were filled in ‘three replicated blocks’, each of 30 silos. Sufficient grass and red clover to fill all silos within the first block were removed from the two covered piles, and placed in two ‘sub-piles’. These sub-piles were turned three times to ensure their contents were homogenous, before silo filling commenced. Within each block, silos

were randomised for sampling time (silos 1 – 5, sampling time 1: silos 6 – 10, sampling time 2, etc) and the five silos within each sampling time randomised for forage treatment.

Sufficient grass and red clover (15.5 kg of each: sufficient to fill one silo of each of the grass : red clover treatments) was then removed from the 'sub-piles' in separate bins. The required quantity of each herbage was removed from these two bins to achieve the required ratio (based on a total of 6.2 kg fresh forage), and mixed on a tray. The sample was then spread over the tray and Super MV50 bacterial inoculant (Biotal Ltd, Malvern Link, Worcestershire) was applied to the forage with a garden sprayer at the equivalent of 3 litres per ton fresh forage, and the sample remixed. Six kg of forage was then weighed out from the 6.2 kg of treated forage and packed into a plastic pipe mini-silo 0.91 m long and 0.15 m diameter, as described by Keady et al. (1996) with a perforated plastic disc and valve at the base to allow effluent to be drained when the valve was opened. Pressure was applied on the silage by applying a weight of 10.5 kg on a perforated disc resting on the packed forage. An air lock on the sealed screw cap allowed escape of gases. Mini-silos were suspended on racks arranged in rows, in their randomised order. The process was then repeated for the 30 silos in each of blocks 2 and 3. During filling of each of the three blocks a composite sample of each forage was taken, and dried at 65^{o}C, to determine ODM and for subsequent analysis.

Measurements. Herbage yield was determined from the weight of herbage removed from the strips mown prior to cutting, and its ODM content. Effluent was drained and collected from each silo prior to opening, the weight recorded. At harvest 2 the filled silo was weighed before it was suspended on the rack and again before it was emptied after the effluent had been drained. From the samples taken per block at time of filling ODM, ADF, NDF, N and WSC were determined. Concentrations of NDF and ADF were determined using a Fibertec analyzer (Fibertec FT122, Foss, Hillerød, Denmark) based on the method of Van Soest (1976), and ash concentrations were determined following combustion in a muffle furnace at 550°C for approximately 10 h. Concentration of N was determined using the Kjeldahl method (Tecator Kjeltec Auto 2400/2460 Analyzer/Sampler System, Foss). Concentration of WSC was determined by a Continuous Segmented Flow Analyzer (SEAL Analytical Ltd., Southampton, UK) following the method of McDonald and Henderson (1964).

Silos were emptied at the designated time intervals, and 1.5 kg of the contents were retained, of which 600 kg were dried at 65^{o}C for determination of ODM, N and WSC content. The remainder was frozen and retained for determination of NH_3–N, pH, lactic, acetic, and propionic acids, ethanol, propanol and VCODM concentrations, N, ammonia-N, fermentation acids (lactic, acetic, propionic, n-butyric, and isovaleric acids), ethanol, and propanol, and for pH. Silage fermentation acids, ethanol, and propanol were determined using single-column GLC (Varian Star 3400 CX GC, equipped with a flame-ionization detector, Varian Inc., Palo Alto, CA), where samples were injected on-column. Dry weights were corrected for volatile losses following the methods of Porter and Murray (2001).

Aerobic stability of silages. The aerobic stability of silage in each mini-silo sampled at day 96 for each harvest was determined. When the silo was opened, after the samples were taken for analysis as already described, a further sample of 3 kg from each silo was placed in a 'conical' pile inside a clean polystyrene box measuring 55 x 35 x 18 cm. These boxes were then randomly placed on the floor, within their blocks, under controlled environmental conditions within a metabolism chamber. Temperature within the pile was determined every 15 minutes by inserting a probe linked to a temperature data logger (ThermaData logger – model TDF; Electronic Temperature Instruments Ltd., Worthing, Sussex), into the geometrical centre of each pile. A polystyrene lid was placed loosely on each box (with a 1.0 cm gap left uncovered along the 'long' side of each box) to prevent the silage from drying, but to allow air to enter the box. In addition, ambient temperature within the chamber at the level of the boxes was recorded by positioning outside the boxes two probes with aluminium 'shades' shielding the probes from radiant energy from lights,

Temperatures were recorded at 15-minute intervals over a 20-day period. Criteria to determine aerobic stability were: hours until a 3^{o}C temperature rise above ambient was observed, hours until the maximum temperature was observed, maximum temperature observed (^{o}C), and average temperature during the first nine days of the recording period.

Statistical analysis. The data per harvest were analysed as a split plot experimental design with red clover:grass ratio as main plot and sampling time as the split plot using GenStat (16th ed. Lawes Agricultural Trust, Rothamsted, UK). An exception was the

analysis of the indices for aerobic stability where the data for the two harvests were included in the one analysis and analysed as a split-split plot design, with harvest as the second split. Data for some silage characteristics were transformed where zeros were prevalent by adding '+1' to each datum in the data set, and by taking square roots for analysis where the distribution of data was skewed and so were required to be normalised.

Results

At both harvests the oven dry matter (ODM) content was 50-60 g/kg higher for chopped grass at the point of ensiling, than for chopped red clover, with the DM content of the Harvest 2 herbages being lower that for the Harvest 1 herbages (Table 1). Fibre contents (both ADF and NDF) were lower at Harvest 2 than Harvest 1, with NDF of grass higher than that of red clover at both harvests. While the N content of red clover was consistently above that of grass, WSC of red clover was particularly low, especially at the second harvest.

During ensilage, the amount of effluent collected before the silos were destructively sampled increased with red clover inclusion level, so that by day 96 effluent accounted for 32 % of weight loss in red clover silage compared with 1.3 % in the grass silage. Fresh weight losses in effluent accounted for all but 0.4 to 1.5 % of total losses (Table 2; Figure 1). Proportionately, total effluent losses were greater during the early phase of ensiling with mixtures containing a greater proportion of red clover.

The chemical composition of the silages removed from the mini-silos at harvests 1 and 2 are presented in Tables 3 and Table 4, respectively. The differences in VCODM of the silages of the two monocultures, meaned over all the sampling dates, were smaller than that of the herbages at ensiling. At Harvest 1, which involved ensiling herbage with a high DM content, the pH of all forages after 3 days of fermentation was similar, irrespective of red clover content. However, after 96 days, the pH of the pure red clover silage was almost 0.5 pH units higher than the pure grass silage. In contrast, the pH of the low DM silages at the second harvest after 3 days fermentation differed according to red clover content, the pure red clover silage having a pH more than 0.5 pH units higher than that of the grass silage, with a gradation according to red clover

content. Thereafter, the rate of decline in pH was faster in the grass and low red clover silages than the high content-red clover silages.

The concentration of lactic acid was on average 44% higher at Harvest 1 than Harvest 2. While there was a tendency for the concentration in RC50 and RC75 silages to be higher than the other silages in Harvest 1 ($p=0.077$), at Harvest 2 these two silages, again, had the highest concentrations which with were very highly significantly higher than lactic acid concentrations of the other treatments and RC100 had a significantly lower concentration than all other treatments. At Harvest 2 the significant interaction was due to lactic acid declining in the pure red clover silage after 12 days of fermentation in contrast to all other silages which continued to increase in content to day 48.

Harvest 1 silages had an average of 36% more acetic acid than Harvest 2 silages. Acetic acid increased significantly with increase in red clover content in Harvest 1 silage, interacting with sampling date (Table 3b) due to concentration increasing faster in the silages with high red clover contents, especially RC100, than those with lower contents. At harvest 2, acetic content of RC100 was significantly higher than the content of other silages.

Propionic acid content was low (average 0.22 g/kg fresh wt) and not significantly different between Harvest 1 silages. Harvest 2 silages RC75 and RC100 had low concentrations of propionic acid but significantly more than the other silages, with RC100 significantly higher than RC75 and was detected only from day 24.

In silages from both harvests ethanol concentration declined with increasing red clover content. The significant interactions for silages at both harvests were due to the difference being widest at day 96 in Harvest 1 silages, and from day 48 in Harvest 2 silages. Propanol was detected after 96 days in harvest 1 and 12 days in harvest 2, with the red clover dominant silages having the higher concentration.

Nitrogen content of the silages increased progressively with red clover content but was unaffected by length of time of ensilage for herbage from both harvests. Silages from the first harvest had no significant differences in NH_3-N between the treatments although there was a small but significant increase in NH_3-N with time, after a decline

from the third to sixth day of the fermentation. Ammonia-N in silage from the later harvest had a tendency for the grass silage to have a lower content in RC100 silage. The mean proportion of NH_3-N in silages from both harvests was less than 70 g/kg total N.

Water soluble content early in the fermentation of silages from both harvests was 4 – 5 times more in the grass silage than red clover silage. There was a significant interaction due to the rate of fall in content of WSC being faster in the grass and RC25 than in RC75 and RC100 silages.

Exposing the silages to air for 20 days, RC0 silage reached 3°C above ambient significantly sooner than the RC100 silage (Figure 2; Table 5). The maximum was reached significantly sooner in silages RC0 and RC25 than the other silages with a tendency for the maximum in RC0 to be higher than all other treatments. The mean temperature above ambient for 20 days exposure to air was significantly higher for RC25 and RC100 than all other treatments but the mean temperature for 9 days exposure did not differ significantly between treatments. There was a tendency for the first maximum to be reached over 9 days sooner for RC0 and RC25 than all other treatments. Time to the first maximum during 9 days exposure increased significantly with red clover content and there was a tendency for the magnitude of the maximum to decrease with increase in red clover content.

Discussion

The objective of this study was to investigate if ensiling mixtures of red clover and grass would result in silage of higher quality than would be calculated from the proportion of each herbage type in the mixture. The two harvests produced a contrast between silages produced from herbages that had been well wilted (Harvest 1) and herbages that had low dry matter and low WSC content (Harvest 2). Further, the mini silos were stored in an open fronted unheated shed with mean air temperatures during the 96 days for ensilage of herbage of Harvest 1 and Harvest 2 of 12.8 and 8.8°C, respectively. Despite these contrasts, all silages of both species were of acceptable quality (AHDB Fact Sheet No. 17) based on pH (maximum means for Harvest 1 and 2 at day 96 were 4.53 and 4.67, respectively) and NH_3-N (overall mean at day 96 of 72 and 66 g/kg total N, respectively), albeit that a bacterial inoculant was applied

during ensilage. Further, butyric acid was not detected in any of the silage samples in the experiment. The greater amount of effluent produced by the red clover silage in mixtures is in line with the amount of effluent produced by silages of maturing red clover (21.7% of original weight) compared with perennial ryegrass (6.6% original weight) for herbage of DM contents of 185 g DM/kg and 167 g DM/kg, respectively (McEniry et al., 2014).

Generally, perennial ryegrass silage has a higher pH than red clover silage when conditions are similar (King et al., 2012b; McEniry et al., 2014) presumably due to the higher buffering capacity of red clover silage. However, exceptionally, Krawutschke et al. (2012) did not find differences in pH between the two silage types either in monoculture or and mixtures.

However, converting means to g/kg DM allows comparison with data in other studies. Hence perennial ryegrass and red clover at the first harvest had similar lactic and acetic acid concentrations in the fresh weight but when converted to dry weight concentrations red clover had 10 and 7 g/kg DM more than perennial ryegrass, respectively, which in turn agrees with the findings of Krawutschke et al. (2012). Ethanol concentrations were low in all silages (<10 g/kg DM) suggesting that silages were relatively free of yeast fermentation in contrast to the perennial ryegrass and red clover silages in the study of McEniry et al. (2014) in which ethanol levels could reach 54.8 g/kg DM.

In the progression of changing red clover with perennial ryegrass, in this study the largest progressive change in pH in both harvests was found when 25% of red clover was replaced by perennial ryegrass, a change that had a disproportionate influence on improving pH, an important ensilability index (Driehuis et al., 1997). Also, particularly in Harvest 2, herbage lactic acid concentration increased from 7.9 to 11.7 g/kg fresh silage (50.3 to 73.1 g/kg DM) when 25% of the red clover fresh weight was replaced by fresh perennial ryegrass. Therefore benefit to red clover silage ensilability could be accrued by addition of grass to red clover prior to ensiling.

It was hypothesised when this study was initiated that by including red clover with perennial ryegrass the PPO in red clover would contribute to inhibition of proteolysis of protein in grass, in addition to protein in clover, and reduce the amount of NH_3-N

produced in the mixture (Marley et al.,2003). However, the proportion of N as NH_3-N was not high in the pure grass silage and so there was not much opportunity for the activity of PPO to be obvious. The pH declined quickly in the silages i.e. the mean pH over all treatments was 4.8 for Harvest 1 silage (Table 4) and 4.77 for Harvest 2 silage (Table 5) by 3 days after the silos were filled and so the environment would rapidly have become too acidic for protease activity.

High concentration of acetic acid is considered to be responsible for aerobic stability in silages (Danner et al., 2003) and generally legume silages are more stable than grass silages. Therefore the generally higher stability of the pure red clover silages may be explained by their higher acetic acid levels.

Conclusion

Although most of the characteristics of silage from mixtures of the two herbages could have been predicted from the characteristics of the two components, some of the mixtures had lower pH, higher lactic acid concentration and lactic acid as a proportion of total fermentation products and lower acetic acid concentration than would have been predicted from the monocultures. This applied especially to the 75% red clover treatment and as the first three of these characteristics are highly beneficial, this presents a case for mixing red clover herbage in a ratio of 3:1 with grass on a fresh weight basis to maintain the benefits of red clover and of apparent synergy with grass.

References

Bertilsson, J. and Murphy, M. (2003) Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. *Grass and Forage Science*, 58, 309-322.

Clavin, D., Crosson, P., Grant,J and O'Kiely, P. (2017) Red clover for silage: management impacts on herbage yield, nutritive value , ensilability and persistence, and relativity to perennial ryegrass*. Grass and Forage Science*, 72, 414-431.

Danner, H., Holzer, M., Mayrhuber, E. and Braun, R. (2003) Acetic acid increases stability of silage under aerobic conditions. *Applied and Environmental Microbiology*, 69, 562-567.

Dewhurst, R.J., Fisher, W.J., Tweed, J.K.S. and Wilkins, R.J. (2003) Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. *Journal of Dairy Science*, 86, 2598-2611.

Driehuis, F., Van Winkselaar, P.G., Van Vuuren, A.M. and Spoelstra, S.F. (1997) The effect of a bacterial inoculant on rate of fermentation and chemical composition of high dry matter silages. *Journal of Agricultural Science*, 128, 323-329.

King, C., McEniry, J., and O'Kiely, P. (2012) A note on the fermentation characteristics of red clover silage in response to advancing stage of maturity in the primary growth. Irish *Journal of Agricultural and Food Research*, 51, 79-84.

King, C., McEniry, J., Richardson, M and O'Kiely, P. (2012a) Ensilage characteristics of perennial ryegrass grown under two nitrogen fertiliser inputs and red clover, each harvested at five dates in the primary growth. *16th International Silage Conference*, Hämeenlinna, Finland. 195-196.

Krawutschke, M., Thaysen, J., Weiher, N., Taube, F. and Gierus, M. Effectts of inoculants and wilting on silage fermentation and nutritive characteristics of red clover-grass mixtures. *Grass and Forage Science*, 68, 326-338.

Marley, C.L., Fychan, R., Fraser, M.D., Winters, A. and Jones, R. (2003) Effect of sowing ratio and stage of maturity on harvest yield, persistency and chemical composition of fresh and ensiled red clover/lucerne bi-crops. *Grass and Forage Science,* 58, 397-406.

McEniry, J., King, C. and O'Kiely, P. (2014) Silage fermentation characteristics of three common grassland species in response to advancing stage of maturity and additive application. *Grass and Forage Science*, 69, 393-404.

Keady, T.W.J., Mayne, C.S. and Fitzpatrick, D.A. (2000) Prediction of silage feeding value from the analysis of the herbage at ensiling and effects of nitrogen fertilizer, date of harvest and additive treatment on grass silage composition. *Journal of Agricultural Science*, 134, 353-368.

Porter, M.G. and Murray, R.S. (2001) The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass and Forage Science*, 56, 405-411.

Vanhatalo, A., Kuoppala, K., Ahvenjärvi, S. and Rinne, M. (2009) Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. *Journal of Dairy Science*, 92, 5620-5633.

Van Soest, P.J. (1976) Laboratory methods for evaluating the energy value of feedstuffs. In*: Feed and Energy Sources for Livestock*. Eds. Swan, H. and Lewis, D, pages 83-94. Butterworth: London

Table 1. Chemical composition of chopped forage ensilage at Harvest 1 (18 July) and Harvest 2 (7 September).

	Harvest 1				Harvest 2			
	PRG		RC		PRG		RC	
g/kg DM	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Oven dry matter	323	41.7	267	11.3	180	4.5	121	2.8
Acid detergent fibre	338	24.1	294	10.1	273	10.4	272	12.8
Neutral detergent fibre	616	40.4	469	6.0	527	11.0	431	19.4
Nitrogen	17.5	1.1	27.1	1.0	19.0	0.4	33.0	0.2
Water soluble carbohydrate	146	22.6	46.4	4.1	157	6.3	24.1	2.61

PRG, Perennial ryegrass; RC, Red clover.

Table 2. Fresh weight loss in Harvest 2 during 96 days of ensilage, expressed as a percentage of the original weight of herbage in the mini-silo, and effluent collected as a percentage of original weight (a), and interaction table of individual losses for each forage treatment- opening time period.

a)	Red clover %							Days from filling silo							
	0	25	50	75	100	s.e.m.	P	3	6	12	24	48	96	s.e.m.	P
% Wt loss	0.8	4.5	11.1	18.0	27.8	0.40	<0.001	7.7	11.0	11.7	13.3	14.7	16.2	0.46	<0.001
% Wt loss as effluent	0.2	38	11.0	16.5	26.9	0.67	<0.001	6.7	10.1	10.9	12.4	14.3	15.7	0.53	<0.001

b)		Red clover%										
	Days	0	25	50	75	100		0	25	50	75	100
% Wt loss							% Wt. lost as effluent					
	3	0.8	0.7	5.3	8.4	23.5		0	0	4.2	6.9	22.3
	6	0.5	2.5	8.5	17.1	26.2		0	1.4	10.6	12.5	25.8
	12	0.5	3.7	9.9	17.0	27.6		0	3.1	9.2	15.8	26.4
	24	0.4	5.2	13.1	19.5	28.2		0	4.6	12.5	18.1	27.0
	28	1.3	7.5	13.4	22.3	29.2		0.5	5.9	13.4	21.9	29.7
	96	1.3	7.7	16.1	23.8	32.0		0.9	7.6	15.9	23.7	30.2
		s.e.m. = 1.02; P = <0.001						s.e.m. = 1.27; P = <0.001				

Table 3. Effect of red clover percentage at ensiling and time of sampling (Harvest 1) on silage composition expressed as a) main effects and b) interactions, when significant. Units g/kg VCODM except for pH (no units) and NH_3-N (g/kg total N)

a)

	Red clover (%)							Days from filling silo							
	0	25	50	75	100	s.e.m.	P	3	6	12	24	48	96	s.e.m.	P
VCODM	315	302	293	286	273	5.90	0.009	300	298	296	291	291	287	2.80	0.014
‡pH	4.31	4.31	4.33	4.36	4.50	0.0165	<0.001	4.77	4.57	4.31	4.20	4.15	4.18	0.021	<0.001
Lactic	55.9	58.8	66.2	67.7	65.4	2.06	0.014	31.8	41.5	65.0	81.1	81.7	75.8	2.19	<0.001
‡Acetic	17.0	19.2	20.1	21.4	24.2	0.36	<0.001	16.9	18.6	20.6	21.3	22.0	22.9	0.34	<0.001
Ethanol	4.23	3.13	2.93	2.76	2.97	0.168	0.002	2.71	2.87	2.60	3.59	2.93	4.52	0.212	<0.001
LA/TFP	0.70	0.70	0.72	0.71	0.69	0.006	0.037	0.61	0.65	0.73	0.76	0.76	0.72	0.008	<0.001
Total N	17.7	20.0	20.5	23.2	26.6	0.48	<0.001	20.5	21.4	22.0	21.8	21.5	22.4	0.38	0.026
NH3-N	68	66	68	69	68	1.9	NS	68	58	63	71	74	72	2.3	<0.001
WSC	42.2	29.70	24.4	17.3	9.7	-	-	54.1	36.7	22.4	12.5	13.7	8.60	-	-
‡sqrt[WSC]	6.14	5.05	4.64	3.86	2.99	0.209	<0.001	7.10	5.78	4.50	3.43	3.53	2.87	0.190	<0.001

‡Significant interactions; LA/FP, lactic acid as a proportion of all products of fermentation

b) Table of significant interactions

	Red clover%				
Days	0	25	50	75	100
pH					
3	4.74	4.72	4.75	4.81	4.83
6	4.51	4.53	4.56	4.58	4.66
12	4.32	4.29	4.30	4.31	4.34
24	4.17	4.16	4.16	4.19	4.32
48	4.06	4.09	4.09	4.14	4.37
96	4.08	4.06	4.1	4.13	4.53
	Interaction s.e.m. = 0.046; P = 0.025				
Ethanol (transformed)					
3	0.944	0.969	0.910	0.896	0.925
6	0.949	0.939	0.884	0.890	0.944
12	0.859	0.776	0.916	0.894	0.892
24	1.264	1.076	0.918	0.929	0.885
48	1.106	0.884	0.881	0.818	0.898
96	1.562	1.163	1.028	0.929	0.888
	Interaction s.e.m. = 0.064; P = 0.001				
Acetic					
3	4.39	5.05	4.87	5.57	5.43
6	5.03	5.61	5.65	5.66	5.65
12	5.70	6.02	6.19	6.04	6.45
24	5.60	6.04	6.22	6.33	6.60
48	5.76	6.14	6.18	6.43	7.27
96	5.55	6.01	6.07	6.79	8.10
	Interaction s.e.m. = 0.215; P = 0.007				
Propionic (transformed)					
3	1.21	1.22	1.16	1.17	1.19
6	1.16	1.16	1.14	1.15	1.17
12	1.24	1.19	1.21	1.19	1.20
24	1.38	1.29	1.26	1.25	1.23
48	1.22	1.14	1.17	1.17	1.21
96	1.26	1.25	1.26	1.29	1.36
	Interaction s.e.m. = 0.042; P = 0.014				
WSC (transformed)					
3	9.53	7.66	7.25	6.63	4.44
6	7.67	7.24	6.01	4.69	3.29
12	6.46	5.49	4.25	3.72	2.59
24	4.56	3.98	3.49	2.85	2.29
48	4.89	3.26	3.78	2.69	3.03
96	3.76	2.66	3.07	2.55	2.29
	Interaction s.e.m. = 0.435; P = 0.005				

Table 4. Effect of red clover percentage at ensiling and time of sampling (Harvest 2) on silage composition expressed as a) main effects and b) interactions, when significant. Units g/kg DM except for pH (no units) and NH_3-N (g/kg total N)

a)

	Red clover (%)							Days from filling silo							
	0	25	50	75	100	s.e.m.	P	3	6	12	24	48	96	s.e.m.	P
‡VCODM	177	167	163	160	157	0.800	<0.001	164	166	169	165	164	163	1.30	0.084
‡pH	4.15	4.18	4.23	4.30	4.62	0.010	<0.001	4.63	4.38	4.34	4.17	4.14	4.12	0.020	<0.001
Lactic	55.8	61.2	68.6	72.2	50.1	1.89	<0.001	30.6	44.0	69.2	59.5	82.1	84.3	4.17	<0.001
Acetic	21.7	22.7	22.6	22.6	25.8	0.57	0.007	14.9	18.2	27.1	19.6	27.9	31.0	1.14	<0.001
‡Ethanol	9.67	7.68	5.00	4.34	3.40	0.228	<0.001	4.32	4.85	5.05	6.10	8.14	7.63	0.238	<0.001
LA/FP	0.63	0.65	0.70	0.71	0.62	0.010	<0.001	0.61	0.65	0.67	0.69	0.68	0.66	0.007	<0.001
Total N	19.3	20.9	23.7	26.9	31.7	0.418	<0.001	23.4	23.3	28.4	23.5	23.9	24.5	0.93	0.002
NH3-N	49	52	52	50	53	1.1	0.076	36	39	52	54	60	66	3.2	<0.001
WSC	42.4	31.9	28.4	22.1	9.98		63.5	41.8	26.7	15.2	8.6	6.0			
‡sqrt[WSC]	5.98	5.13	4.94	4.30	2.88	0.193	<0.001	7.72	6.19	4.94	3.73	2.90	2.40	0.206	<0.001

‡Significant interaction

b) Table of significant interactions

	Red clover%				
Days	0	25	50	75	100
VCODM					
3	182	168	160	156	153
6	182	167	164	159	157
12	182	172	167	162	161
24	176	164	162	163	161
48	173	167	162	161	158
96	169	166	164	161	154
	Interaction s.e.m. = 1.30; P = <0.001				
pH					
3	4.41	4.51	4.60	4.66	4.97
6	4.23	4.29	4.37	4.43	4.57
12	4.31	4.31	4.29	4.34	4.44
24	4.07	4.06	4.08	4.15	4.48
48	3.99	3.98	4.02	4.14	4.57
96	3.90	3.94	4.00	4.10	4.67
	Interaction s.e.m. = 0.009; P = <0.001				
Propionic (Transformed)					
3	1.000	1.000	1.000	1.000	1.000
6	1.000	1.000	1.000	1.000	1.000
12	1.000	1.000	1.000	1.000	1.000
24	1.000	1.000	1.000	1.000	1.000
48	1.000	1.010	1.023	1.000	1.066
96	1.070	1.095	1.091	1.053	1.216
	Interaction s.e.m. = 0.0048.; P = <0.001				
Ethanol (Transformed)					
3	0.977	0.912	0.816	0.790	0.838
6	1.010	0.975	0.949	0.794	0.730
12	1.040	1.030	0.896	0.874	0.751
24	1.400	1.130	0.822	0.815	0.717
48	1.670	1.310	0.980	0.904	0.701
96	1.530	1.350	0.932	0.823	0.780
	Interaction s.e.m. = 0.0426; P = <0.001				
WSC (transformed)					
3	9.79	8.85	8.19	7.57	4.21
6	8.31	6.06	4.49	5.66	4.44
12	6.59	5.91	5.51	4.43	2.28
24	5.06	4.58	3.87	3.09	2.05
48	3.34	3.21	2.84	2.77	2.36
96	2.80	2.15	2.75	2.31	1.97
	Interaction s.e.m. = 0.462; P = 0.001				

Table 5. Aerobic stability indices of silages made from red clover and perennial ryegrass monocultures and mixtures

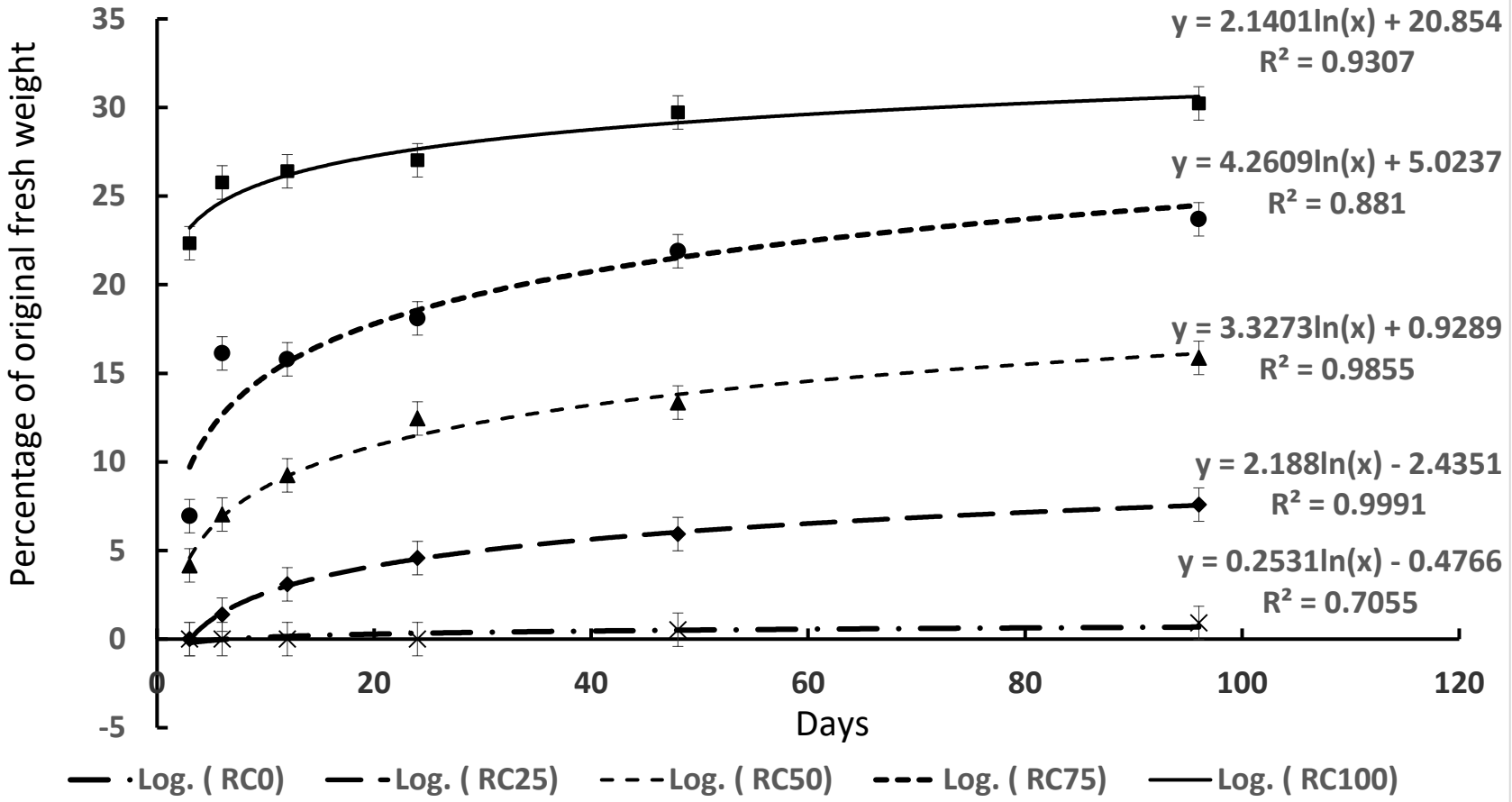
	Red clover %							Harvest			
Index	0	25	50	75	100	s.e.m.	P	H1	H2	s.e.m.	P
Time to 3°C a.a. (h)	69.0	73.5	88.9	99.3	112.0	7.68	0.021	97.2	79.9	4.74	0.03
Time to max. temp. during 20 days (h)	160	137	283	313	245	4.5	0.027	267	188	19.2	0.017
Max. temp. during 20 days (h)	15.9	12.8	12.4	12.0	13.5	0.83	0.062	15.0	11.6	0.78	0.013
†Mean temp. a.a. during 20 days (°C)	5.40	1.08	5.16	5.00	3.81	0.32	0.029	5.90	3.48	0.21	<0.001
Mean temp. a.a. during 9 days (°C)	4.6	3.3	3.2	2.5	1.4	0.89	NS	3.7	2.3	0.50	0.079
1st max temp. a.a. during 9 days (°C)	13.2	11.7	8.3	7.9	7.5	2.04	0.082	10.1	9.3	1.27	NS
Time to 1st max. temp. a.a. (h)	88.9	91.3	111.1	116.4	132.5	7.47	0.016	121.0	95.1	5.15	0.006
Max temp. a.a. during 9 days (°C)	13.1	12.6	8.6	8.1	7.5	1.46	0.071	10.7	9.3	1.04	NS
Time to max temp. a.a. during 9 days (h)	88.9	110.5	125.7	136.6	132.5	16.25	NS	142.5	95.1	5.99	<0.001

a.a. above ambient

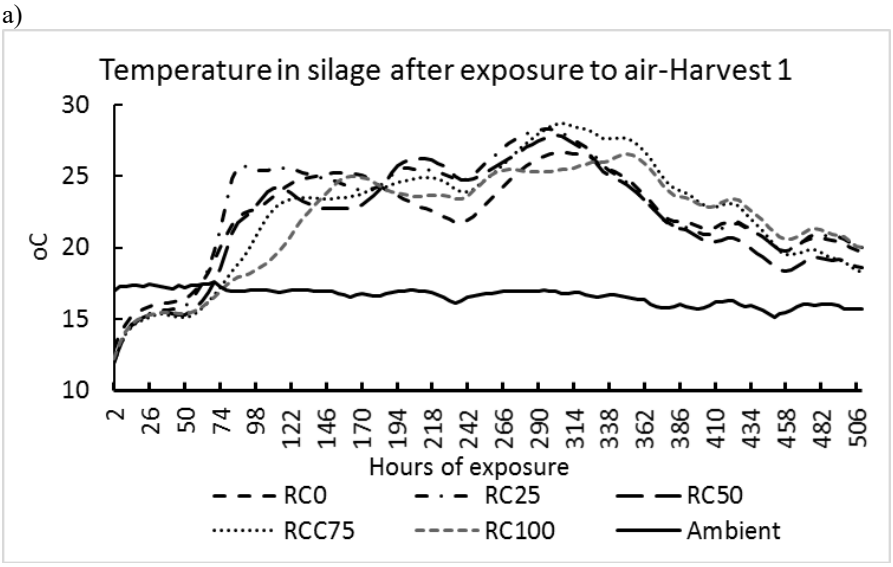
†Interaction table for mean temperature during 20 days (°C)

	Red clover %				
	0	25	50	75	100
H1	7.7	5.0	6.0	6.0	4.9
H2	3.2	3.2	4.3	4.0	2.7

Figure 1. Accumulated effluent drained from minisilos at each sampling (Days = days after filling). Bars = +/_ s.e.m.



a)



b)

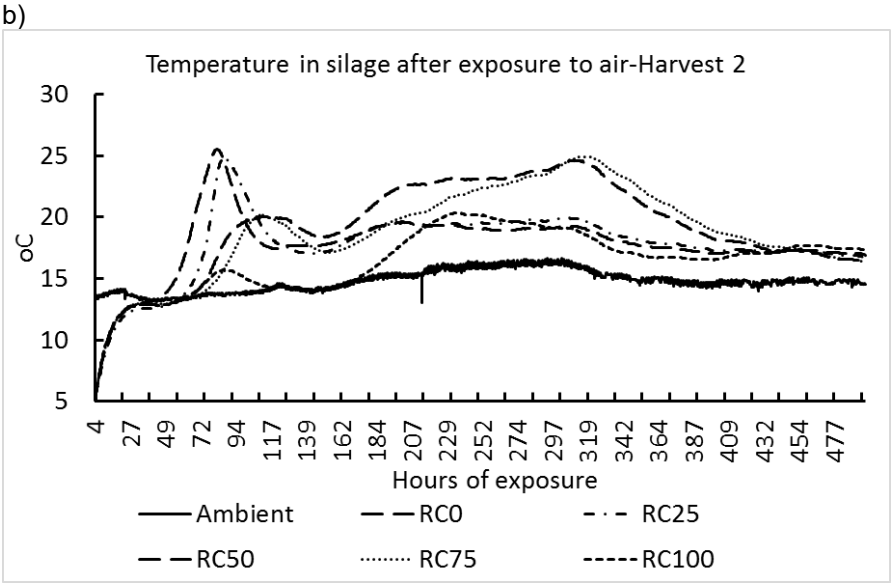


Figure 2. Mean temperature profile within silage from grass-red clover herbage mixtures exposed to air in a constant environment for silage from a) Harvest 1 and b) Harvest 2 (RC0 0%, RC25 25%, RC50 50%, RC75 75% and RC100 100% red clover)