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Feeding lower-protein diets based on red clover and grass or alfalfa and corn silage does not affect milk production but improves nitrogen use efficiency in dairy cows

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ABSTRACT

Reducing the dietary crude protein (CP) concentration can decrease the financial cost and lower the environmental impact of milk production. Two studies were conducted to examine the effects of reducing the dietary CP concentration on animal performance, nutrient digestibility, milk fatty acid (FA) profile, and nitrogen use efficiency (NUE; milk N/N intake) in dairy cows fed legume silage-based diets. Thirty-six multiparous Holstein-Friesian dairy cows that were 76 ± 14 (mean ± SD) days in milk and 698 ± 54 kg body weight were used in a 3 × 3 Latin square design in each of 2 studies, with 3 periods of 28 d. In study 1, cows were fed diets based on a 50:50 ratio of red clover to grass silage [dry matter (DM) basis] containing 1 of 3 dietary CP concentrations: high (H) = 175 g of CP/kg of DM; medium (M) = 165 g of CP/kg of DM; or low (L) = 150 g of CP/kg of DM. In study 2, cows were fed 175 g of CP/kg of DM with a 50:50 ratio of alfalfa to corn silage (H50) or 1 of 2 diets containing 150 g of CP/kg of DM with either a 50:50 (L50) or a 60:40 (L60) ratio of alfalfa to corn silage. Cows in both studies were fed a total mixed ration with a forage-to-concentrate ratio of 52:48 (DM basis). All diets were formulated to meet the MP requirements, except L (95% of MP requirements). In study 1, cows fed L ate 1.6 kg of DM/d less than those fed H or M, but milk yield was similar across treatments. Mean milk protein, fat, and lactose concentrations were not affected by diet. However, the apparent total-tract nutrient digestibility was decreased in cows fed L. The NUE was 5.7 percentage units higher in cows fed L than H. Feeding L also decreased milk and plasma urea concentrations by 4.4 mg/dL and 0.78 mmol/L, respectively. We found no effect of dietary treatment on the milk saturated or monounsaturated FA proportion, but the proportion of polyunsaturated FA was increased, and milk odd- and branched-chain FA decreased in cows fed L compared with H. In study 2, DM intake was 2 kg/d lower in cows receiving L50 than H50. Increasing the alfalfa content and feeding a low-CP diet (L60) did not alter DMI but decreased milk yield and milk protein concentration by 2 kg/d and 0.6 g/kg, respectively, compared with H50. Likewise, milk protein and lactose yield were decreased by 0.08 kg/d in cows receiving L60 versus H50. Diet had no effect on apparent nutrient digestibility. Feeding the low-CP diets compared with H50 increased the apparent NUE by approximately 5 percentage units and decreased milk and plasma urea concentrations by 7.2 mg/dL and 1.43 mmol/L, respectively. Dietary treatment did not alter milk FA profile except *cis*-9,*trans*-11 conjugated linoleic acid, which was higher in milk from cows receiving L60 compared with H50. We concluded that reducing CP concentration to around 150 g/kg of DM in red clover and grass or alfalfa and corn silage-based diets increases the apparent NUE and has little effect on nutrient digestibility or milk performance in dairy cows.

Key words: crude protein, red clover, alfalfa, dairy cow, nitrogen efficiency

INTRODUCTION

The increasing global price of soybean meal in association with tighter regulations on the disposal of slurry and manure, and greater public scrutiny of the sustainability of dairy farming, has led to renewed interest in alternative strategies for feeding protein to dairy cows (Lavery and Ferris, 2021). Two obvious approaches are to increase the use of high-protein homegrown forage legumes and to reduce protein concentrations in the diet of dairy cows (Sinclair et al., 2014).

Alfalfa and red clover are the most popular forage legumes grown globally and are common in dairy cat-

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tle diets in North America and northern and western Europe (Moorby et al., 2016). Both alfalfa and red clover silages contain CP levels of approximately 180 to 200 g/kg of DM under typical management conditions. In comparison, grass silage ranges from 100 to 160 and corn silage from 70 to 90 g of CP/kg of DM (Dewhurst et al., 2010; Tayyab et al., 2019; Johnston et al., 2020). Alfalfa and red clover can be grown in a range of soil types and weather conditions, and produce high DM yields without artificial N fertilizer input (Dewhurst et al., 2003). Several studies have reported that the inclusion of up to 66% red clover or alfalfa silage in the forage component of dairy cow diets improved DMI, milk yield, and the content of PUFA in milk (Moorby et al., 2009; Dewhurst, 2013; Sinclair et al., 2015). The use of red clover compared with grass silage has also been reported to increase the efficiency of microbial protein (**MCP**) synthesis in the rumen and improve the omasal flow of AA (Moorby et al., 2009, 2016), except for methionine, which was lower in cows fed red clover compared with grass silage (Lee et al., 2009). However, higher inclusion rates of alfalfa silage of up to 75% have been shown to have a negative effect on milk production (Sinclair et al., 2015; Thomson et al., 2017).

Feeding diets that are high in CP (>175 g CP/kg DM) can improve the supply of MP to the intestine, but high-CP diets typically result in low nitrogen use efficiency (**NUE**; milk N/N intake), with increased RDP leading to the production of excess ammonia in the rumen (Hristov et al., 2015). This excess ammonia is absorbed into the blood, where it is converted to urea in the liver and subsequently excreted in the urine (Schwab and Broderick, 2017). Excreted N can subsequently be lost to the environment, contributing to the deterioration of terrestrial and aquatic ecosystems (Schwab and Broderick, 2017). In contrast, feeding lower-CP diets (<165 g CP/kg DM) has been shown to reduce urinary N excretion by increasing N utilization (Olmos Colmenero and Broderick, 2006). However, concentrations less than 150 g CP/kg DM may decrease milk production by reducing DMI in high-yielding dairy cows (Hristov and Giallongo, 2014; Sinclair et al., 2014).

Despite the potential negative effects of low-CP diets on intake and performance, it has been reported that the CP concentration in dairy cow diets can be reduced to around 140 to 150 g/kg DM with no negative effects on dairy cow performance, health, or fertility if the diets are formulated appropriately to maximize MCP synthesis and supply sufficient MP (Sinclair et al., 2014). However, the effects of low-CP diets that are based on high inclusion rates of red clover or alfalfa silage are unclear. Most studies that have evaluated the inclusion of legume silages have fed CP concentrations of approximately 170 g/kg DM, and limited knowledge exists on the effects of reducing the dietary protein concentration in legume silage-based diets on milk performance and N utilization, particularly for red clover (Moorby et al., 2016; Broderick, 2018; Johansen et al., 2018). Forage legumes contain an excess of RDP, as most of the N is released rapidly in the rumen (Sinclair et al., 2009), even in red clover silages, where the action of the enzyme polyphenol oxidase (**PPO**) can reduce the degradability of CP (Lee, 2014). As a consequence, diets based on forage legume silages can be deficient in digestible undegradable protein or may be imbalanced in AA, which can lead to a reduced milk yield (Westreicher-Kristen et al., 2018).

We hypothesized that feeding low-protein diets based on legume (red clover or alfalfa) silages or altering the inclusion rate of alfalfa in a low-CP diet of lactating cows would improve NUE without affecting milk performance if the MP supply was maintained. The objective of our 2 studies was to determine the effect of reducing dietary CP concentration in diets based on high-protein forage legumes. The first study (study 1) examined the effects of reducing dietary CP concentration in a red clover and grass silage-based diet on intake, milk performance, diet digestibility, and NUE in dairy cows. The second study (study 2) examined the effects of dietary CP concentration in diets with different proportions of alfalfa and corn silage.

MATERIALS AND METHODS

Animals and Housing

All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (amended 2012) and received local ethical approval (Harper Adams University, Newport, Shropshire, United Kingdom). Study 1 was conducted from October 2018 to January 2019, and study 2 from January to April 2019.

Study 1 used 18 multiparous lactating dairy cows producing (mean ± SD) 45.3 ± 5.72 kg of milk per day. Cows were 71 ± 14 DIM, with BW 690 ± 48 kg, and BCS 2.6 ± 0.31 (where 1 = emaciated and 5 = obese; scored to 0.25 units; Ferguson et al., 1994). Study 2 used a separate group of 18 Holstein-Friesian multiparous dairy cows, yielding 46.5 ± 4.78 kg of milk per day. These cows were 81 ± 13 DIM, with a mean BW of 705 ± 59 kg, and BCS of 2.6 ± 0.32. All cows were housed in the same area of an open span building fitted with freestalls and mattresses. Stalls were bedded twice

Table 1. Dietary ingredients and predicted chemical composition of diets based on red clover and grass silage or alfalfa and corn silage, fed to dairy cows in studies 1 and 2[1]

	Study 1			Study 2		
Item	H	M	L	H50	L50	L60
Dietary ingredient (g/kg DM)						
Red clover silage	262	262	262	0.00	0.00	0.00
Alfalfa silage	0.00	0.00	0.00	262	262	315
Grass silage	262	262	262	0.00	0.00	0.00
Corn silage	0.00	0.00	0.00	262	262	210
Rolled wheat	144	156	173	129	158	163
Soy hulls	144	156	173	129	158	163
Molassed sugar beet	77.0	77.0	77.0	77.0	77.0	77.1
Soybean meal	74.9	8.3	0.00	95.8	29.1	7.3
SoyPass[2]	0.00	41.6	20.8	0.00	20.8	37.5
Rapeseed meal	12.5	4.16	0.00	20.8	0.00	0.00
RapeTec[3]	0.00	8.3	8.3	0.00	8.3	4.2
Rumen-protected fat	18.7	18.7	18.7	18.7	18.7	18.8
Minerals and vitamins[4]	5.00	5.00	5.00	5.00	5.00	5.00
Predicted composition[5] (g/kg DM)						
Forage:concentrate (DM basis)	0.52	0.52	0.52	0.52	0.52	0.52
ME (MJ/kg DM)	11.9	11.9	11.9	12.0	11.9	11.8
CP	175	165	150	175	150	152
MPE	104	104	98	109	104	104
MPE (% of requirements)	100	100	95	105	100	100
MPN	118	115	106	121	104	104
MPN (% of requirements)	118	111	102	116	100	100

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage.

[2]Xylose-treated soybean meal (KW Alternative Feeds).

[3]Heat-treated rapeseed meal (SC Feeds).

[4]Mineral and vitamin premix (KW Alternative Feeds), providing (g/kg) 220 calcium, 30 phosphorus, 80 magnesium, 80 sodium, (mg/kg) 760 copper, 30 selenium, 1,000,000 IU of vitamin A, 300,000 IU of vitamin D_3, 3,000 IU of vitamin E, 2.5 mg/kg vitamin B_{12}, 135 mg/kg biotin.

[5]Predicted composition of diets were calculated using Feed Into Milk (Thomas, 2004). MPE = metabolizable protein-rumen energy limited; MPN = metabolizable protein-rumen nitrogen limited.

weekly with sawdust and lime, with automatic scrapers that scraped the passageways at 6-h intervals. All cows had constant ad libitum access to fresh drinking water.

Experimental Design

Each study was a 3 × 3 Latin square design with 3 periods and 3 dietary treatments. Experimental periods were 28 d in duration, which included a 21-d adaptation to the diets and a 7-d sampling period. Cows were blocked by milk yield recorded in the week before the start of the study and DIM, and randomly assigned to 1 of 3 dietary treatments in each study. In study 1, the red clover and grass silage-based diets were formulated to supply 3 different concentrations of dietary CP: 175 (high, **H**), 165 (medium, **M**), or 150 (low, **L**) g CP/kg DM. In study 2, the treatment diets were formulated to contain 175 g CP/kg DM with a 50:50 ratio of alfalfa to corn silage (**H50**), 150 g CP/kg DM with 50:50 alfalfa to corn silage (**L50**), or 150 g CP/kg DM with 60:40 alfalfa to corn silage (**L60**).

Diets and Feeding

The animals were fed the diets as a TMR that was formulated to produce an average of 37 kg of milk per day according to Thomas (2004). All diets were formulated to be isoenergetic (ME basis) and to contain a similar MP content, except in study 1, where diet L was predicted to supply 95% of MP requirements. To maintain the MP supply, rumen-protected protein sources (xylose-treated soybean meal and heat-treated rapeseed meal) were used in the formulation of the low-CP diets (Table 1). The forage-to-concentrate ratios in both studies were maintained at 52:48 (DM basis).

In study 1, the red clover was fed 50:50 (DM basis) with grass silage. First cuts of both red clover (*Trifolium pratense*) and grass silages (*Lolium perenne*) were mown at a leafy stage on May 23 and June 10, 2018, respectively, wilted for 24 h, harvested with a self-propelled, precision chop forage harvester (John Deere 7840i), and ensiled in separate concrete clamps with an additive (Axphast Gold; Biotal) applied at the rate of 2.0 L/t. In study 2, a primary growth of alfalfa (*Medicago sativa*) was mown at early bloom, wilted for 36 h, harvested using a self-propelled forage harvester on June 2, 2019, and ensiled in a concrete clamp with an additive added at the rate of 2.0 L/t (Axcool Gold, Biotal). The corn silage was harvested on October 26, 2019, using a self-propelled forage harvester and ensiled in a concrete clamp with an additive (Corncool Gold, Biotal) applied at the rate of 2 L/t. The alfalfa silage was fed 50:50 (DM basis) with corn silage in H50 and L50, and increased to 60:40 in L60, the maximum inclusion rate of alfalfa that still provided the same predicted MP supply.

In both studies, dietary ingredients were mixed daily for 10 min using a forage mixer (MixMax 10, Hi-Spec Engineering Limited) calibrated to ±1 kg and fed through roughage intake control feed bins (Insentec B.V.), fitted with automatic animal identification and weighing system calibrated to ±0.1 kg, with a cow per feed bin ratio of 1.2. Fresh feed was delivered once daily at approximately 0800 h at the rate of 1.05 of the previously recorded intakes, with intake calculated daily and refusals collected 3 times weekly before feeding.

Experimental Routine

Forage samples were collected twice weekly and dried in a forced-air oven at 105°C, and the ratio of the forages was adjusted to the desired value. Fresh forages and TMR were sampled daily during the final week of each period, stored at −20°C, and pooled within period before subsequent analyses. Additional TMR samples were collected at 0, 4, 8, and 24 h after feeding and fresh forage at approximately 1000 h on d 22 to 24 of each period for the determination of particle size (**PS**) distribution. All TMR and forage samples were separated into 6 fractions using a modified Penn State Particle Separator, as described by Tayyab et al., (2018), by manual shaking (Kononoff et al., 2003).

Cows were milked twice daily at approximately 0600 and 1600 h in a 40-point internal rotary parlor (GEA AutoRotor Magnum 40). During the final week of each period, milk yield was recorded at each milking, and 4 samples were collected at 2 consecutive morning and evening milkings for subsequent analyses of milk composition. Body weight and BCS were recorded following the afternoon milking at the start of the first experimental period, and then at the end of periods 1, 2, and 3.

In studies 1 and 2, fecal grab samples (approximately 350 g/d per cow) were collected during the final week of each study period from 4 representative cows per treatment at 1000 and 1600 h for 5 consecutive days, and stored at −20°C for subsequent analyses. Blood samples were collected by jugular venipuncture during the final week of each study period from 4 representative cows per treatment into heparinized and fluoride oxalate tubes (Becton Dickinson and Company) over 2 consecutive days at 0800, 0900, 1100, and 1300 h. The samples were centrifuged at 1,600 × g for 15 min at 4°C to separate the plasma. Plasma derived from heparinized tubes was immediately analyzed for ammonia, with further subsamples stored at −20°C for subsequent analysis of urea and BHB, whereas glucose was analyzed using plasma obtained from fluoride oxalate tubes.

In Situ Degradability of the Forages

Three nonlactating Holstein-Friesian cows with a mean BW of 650 ± 28 kg that had previously been fitted with a rumen cannula 10 cm in diameter (Bar Diamond) were housed in a straw-bedded yard and fed a basal ration at maintenance level (Thomas, 2004). The ration contained (g/kg, DM basis) 264 alfalfa silage, 176 corn silage, 354 chopped wheat straw, 86 Spey syrup (Trident, AB Agri Ltd.), 32 rapeseed meal, 32 wheat distillers dark grains, 14 soybean meal, 9 palm kernel meal, 3 molasses, 12 magnesium chloride, 12 dry cow minerals, 4 g/kg Provimi LiFT (a package of vitamins and cofactors), and 2 g/kg DM of Vistacell Ultra (a live yeast; AB Vista). Dietary ingredients were mixed with the same forage mixer as the cows in the performance study, and diet was offered daily at 0800 h through individual feed bins (American Calan). All cows had continuous access to fresh drinking water.

The in situ degradability of the silages used in both studies was determined following the method reported by Huntington and Givens (1997). In situ nylon bags (Sericol) with a pore size of 42 μm were filled with 8 ± 0.1 g of fresh forage and placed in the rumen of each cow in duplicate 30 min after feeding, and recovered after 4, 8, 16, 24, 48, and 96 h. Following ruminal incubation, the bags were immediately placed in cold water to inhibit further microbial degradation and rinsed for 10 min in the cold rinse cycle of an automatic washing machine (model WAK24210GC, Bosch). For each forage, 0-h time points were also determined using samples without ruminal incubation. All bags were then dried for 48 h in a forced-air oven at 60°C, and the duplicates of the same sample dried residues composited within time point and animal and analyzed for total N.

Chemical Analyses

Subsamples of forage and TMR were bulked by period and dried for 48 h in a forced-air oven at 60°C and analyzed for DM according to AOAC International (2012; 934.01, intra-assay CV of 1.18%). Dried feed samples were then ground in a Wiley mill (Thomas Scientific) through a 1.0-mm sieve before analyses for ash (942.05), CP (988.05), and ether extract (920.39), with intra-assay CV of 0.53, 0.80, and 7.52%, respectively (AOAC International, 2012). The acid-insoluble ash content (intra-assay CV of 5.41%) was determined as per the method of Van Keulen and Young (1977). Acid detergent fiber and NDF were determined as per the method of Van Soest et al. (1991) using heat stable α-amylase for NDF analysis (Sigma; intra-assay CV of 1.87 and 1.05% for NDF and ADF, respectively). Forage ammonia-N and pH were determined using fresh samples following the method of MAFF (1986). Fresh forage samples were also analyzed for their content of lactate, ethanol, acetate, propionate, isobutyrate, and butyrate by Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) using a water extraction technique followed by high-performance liquid and gas-liquid chromatography.

Milk samples were analyzed for fat, total protein, lactose, and urea by the near-mid-infrared method at National Milk Laboratories (Wolverhampton, UK). Fatty acids in milk were analyzed by extracting milk fat by centrifugation and methylation using sodium methoxide, according to the method of Feng et al. (2004). The FAME of the feed fatty acids (**FA**) was prepared according to the protocol of Jenkins (2010). The individual milk and feed FAME was determined by gas-liquid chromatography (Hewlett Packard 6890), fitted with a CP-Sil 88 column (100 m × 0.25-mm i.d. × 0.20-um film, Agilent Technologies) as described by Lock et al. (2006). A mixed reference standard (Sigma-Aldrich) was routinely run, and individual FA calculated.

Fecal samples were composited by cow and period, and dried in a forced-air oven at 60°C until constant weight. The dried fecal samples were then milled (1 mm) using a Cuisinart electric grinder (SG20U) and analyzed for acid-insoluble ash, total N, NDF, ADF, and ash. Blood plasma samples were analyzed for ammonia BHB, glucose, and urea (Randox Laboratories; kit catalog no. AM 1015, RB 1008, GL 1611, and UR 221, respectively, with intra-assay CV of 9.61, 5.94, 1.08, and 5.12%, respectively) using a Cobas Miras Plus Autoanalyzer (ABX Diagnostics).

Calculations

Dry matter intake was calculated from the recorded daily fresh feed intake for each cow and the analyzed DM content of the TMR. Fecal DM output, digested nutrients, and apparent total-tract digestibility coefficients of DM, OM, N, NDF, and ADF were determined using acid-insoluble ash as an internal marker, as per the method of Van Keulen and Young (1977). The 4% FCM yield was determined by adjusting the milk yield to 40 g of fat per kilogram of milk (4% FCM yield = milk yield × fat %/4), and ECM was computed as (3.14 MJ/kg) = milk yield × [383 × fat (g/kg) × 100 + 242 × protein (g/kg) × 100 + 165.4 × lactose (g/kg) × 100 + 207]/3,140 (Sjaunja et al., 1991). Apparent NUE was calculated as milk N/dietary total N intake, with the N secreted in milk determined as total milk protein/6.38. The forage and TMR PS geometric mean ($\mathbf{X_m}$) and standard deviation of X_m were determined using the equations by ASABE (2007). The physically effective fiber (**peNDF**) was calculated by multiplying the physical effectiveness factor with the dietary NDF content (Maulfair et al., 2010).

The in situ DM and CP degradability data were fitted in Sigmaplot (version 12.0, Jandel Engineering) as described by Ørskov and McDonald (1979):

$$p = a + b\,(1 - \exp^{-ct}),$$

where p is the disappearance percentage at t time, a is the immediately soluble fraction, b is the potentially degradable fraction, c is the degradation rate (per hour) of b, and t is the incubation period (h). The effective ruminal degradability (**ED**) was computed using an 8% per hour rumen passage rate (k) as follows:

$$ED = a + b\,[c/(c + k)].$$

Statistical Analysis

Data from each study were analyzed by ANOVA as a row and column design (Mead et al., 1993), using GenStat 18th edition (VSN International Ltd.), with diet and period as fixed effects and cow as random effect. All data were checked for normality using descriptive statistics before running the ANOVA model in GenStat. The model used was

$$Y_{ijk} = \mu + D_i + P_j + A_k + E_{ijk},$$

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

RESULTS

Forage and Diet Characteristics

Study 1. We observed that the red clover silage contained a higher DM content than the grass silage but was lower in NDF and total fat (Table 2). Both forages had CP contents above 160 g/kg DM, with the concentration in the red clover being 11 g/kg DM higher than the grass silage. The lactate and acetate contents of the red clover silage were 26.0 and 16.3 g/kg DM lower, respectively, than the grass silage, and the concentrations of ammonia-N were similar in both forages. Both silages also had a similar long-chain PUFA content, with the content of C18:3 *cis*-9,*cis*-12,*cis*-15 in the grass silage being 3.88 g of FA/kg of DM higher than the red clover silage. The X_m of the PS of the grass silage was higher than the red clover silage. Similarly, the fractions of $peNDF_{>4mm}$ and $peNDF_{>8mm}$ were higher in grass silage than in the red clover silage. The DM, OM, NDF, ADF, and ether extract concentrations of the 3 TMR were similar, whereas the CP concentration was higher in H than M or L (Table 3). The total FA content in H was 1.9 g of FA/kg of DM higher than the other diets. The mean X_m, physical effectiveness factor $(\mathbf{pef})_{>4mm}$, and $peNDF_{>4mm}$ were similar for all 3 diets, but we found a tendency for an interaction ($P = 0.06$) between diet and sampling time for $pef_{>8mm}$ (Supplemental Table S1, https://doi.org/10.6084/m9.figshare.21878619, Sinclair, 2023), which was lower at 4 h after the morning feed for diet H, but at 24 h postfeeding was higher in H compared with the other 2 diets.

Study 2. Our alfalfa silage was lower in DM and OM and higher in NDF and ADF than the corn silage (Table 2). We observed that the CP concentration of the alfalfa silage was nearly twice that of the corn silage. In contrast, the ammonia-N content of our corn silage was 10.4 g/kg of total N higher than the alfalfa silage, and the pH of the alfalfa silage was 0.55 pH units lower. The lactate content of the alfalfa silage was 43.4 g/kg of DM higher than the corn silage, and the content of acetate was 9.4 g/kg of DM lower. We further found that the long-chain PUFA content was higher in the corn silage, with the concentration of C18:2 *cis*-9,*cis*-12 being 8.22 g of FA/kg of DM higher than the alfalfa silage. In contrast, the C18:3 *cis*-9,*cis*-12,*cis*-15 content of the corn silage was 4.32 g of FA/kg of DM lower than the alfalfa silage. We observed that the X_m of the PS of our alfalfa silage was higher than that of our corn silage. The DM, OM, NDF, ADF, and ether extract concentrations of the 3 TMR were similar, whereas the CP concentration varied across the treatments (Table 3). The total FA content in H50 was 1.8 g of FA/kg of DM higher than the other 2 diets. We found a tendency ($P < 0.10$) for the mean X_m, pef, and peNDF to be numerically higher in L50 or L60 compared with H50 (Supplemental Table S2, https://doi.org/10.6084/m9.figshare.21878619, Sinclair, 2023). In contrast, we found a tendency ($P = 0.061$) for a higher mean small PS fraction (<4 mm) in H50 compared with L50 or L60, whereas sampling time post-feeding did not affect the mean PS fractions of the diet except for the medium-length (8–19 mm) fraction, which increased ($P = 0.050$) until 8 h after feeding.

In Situ Forage Degradability

Study 1. We found a tendency for a higher ($P = 0.094$) soluble fraction (a) of DM in the red clover compared with the grass silage (Table 4). In contrast, the red clover silage had a lower ($P = 0.014$) potentially degradable DM fraction (b) compared with the grass silage. However, we observed no effect ($P > 0.05$) of forage on the extent of degradation (a + b) or the rate (c) of the potentially degradable fraction of DM. The ED of DM was 7.12% higher ($P = 0.030$) in the red clover compared with the grass silage. The soluble fraction of the CP was 101 g/kg of DM higher ($P = 0.011$) in the grass silage compared with the red clover. In contrast, the potentially degradable fraction of CP was 109 g/kg of DM higher ($P = 0.004$) than the grass silage. However, we found no effect ($P > 0.05$) of forage on the extent of degradation or the rate of the potentially degradable fraction of CP, whereas the ED of the CP was 4.5% lower ($P = 0.008$) in the red clover than the grass silage.

Study 2. Our corn silage had a higher ($P < 0.05$) soluble fraction of DM compared with the alfalfa silage (Table 4). In contrast, the potentially degradable fraction of the DM was 123 g/kg of DM higher ($P < 0.05$) in the alfalfa compared with the corn silage. Likewise, the rate of degradation of the potentially degradable fraction of DM was higher ($P < 0.05$) in the alfalfa compared with the corn silage. We observed a difference in the ED of the DM, which was 22.1% higher ($P < 0.001$) in the corn than the alfalfa silage. The corn silage also had a higher ($P < 0.05$) soluble fraction of CP compared with the alfalfa silage. In contrast, the potentially degradable fraction of CP was 150 g of CP/kg of DM higher ($P < 0.05$) in the alfalfa compared with the corn silage. Similarly, the rate of degradation of the potentially degradable fraction of CP was higher ($P < 0.05$) in the alfalfa compared with the corn silage.

Table 2. Nutrient composition (g/kg DM), fermentation profile, fatty acid content, and particle size distribution of red clover, grass silage, alfalfa silage, and corn silage fed to dairy cows in studies 1 and 2

	Study 1		Study 2	
Item[1]	Red clover silage	Grass silage	Alfalfa silage	Corn silage
DM (g/kg)	421	320	307	340
CP	178	167	188	98.2
OM	877	892	870	964
NDF	371	510	399	380
ADF	311	309	313	198
EE	14.9	35.9	23.7	32.5
Ash	123	108	130	35.8
Fermentation profile (g/kg DM)				
pH	4.26	3.98	4.22	3.67
Ammonia-N (g/kg total N)	54.3	54.7	66.0	76.4
Lactate	64.0	90.0	104	60.6
Ethanol	0.99	3.74	1.21	4.33
Acetate	12.2	28.5	23.8	33.2
Propionate	0.25	0.75	0.98	3.07
Iso-butyrate	0.29	—	0.45	—
Butyrate	0.13	0.27	0.16	0.15
Acetate:propionate	0.12	0.12	0.08	0.03
Fatty acid (g/kg DM)				
C16:0	1.86	2.43	2.85	3.26
C18:0	0.32	0.26	0.36	0.57
C18:1 *cis*-9	0.28	0.42	0.40	5.76
C18:2 *cis*-9,*cis*-12	2.01	2.21	3.13	11.35
C18:3 *cis*-9,*cis*-12,*cis*-15	4.08	7.96	5.38	1.06
Total fatty acids	12.0	17.5	19.9	27.3
Fraction[2] (% DM)				
>44 (mm)	0.00	0.00	0.00	0.00
33 to 44 (mm)	4.50	4.80	2.53	0.39
19 to 32.9 (mm)	19.1	24.0	40.2	11.7
8 to 19 (mm)	50.5	56.1	46.2	62.0
4 to 8 (mm)	9.71	8.60	4.90	14.6
<4 (mm)	16.2	6.54	6.21	11.3
X_m (mm)	15.3	18.4	20.6	14.5
SD_{gm}	2.04	1.78	1.77	1.81
$pef_{>4}$ (%)	83.8	93.5	93.8	88.7
$pef_{>8}$ (%)	74.1	84.9	88.9	74.2
$peNDF_{>4}$ (%)	31.1	47.7	37.4	33.8
$peNDF_{>8}$ (%)	27.5	43.3	35.5	28.2

[1]EE = ether extract.

[2]Fraction of forages at 0 h postfeeding; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fiber.

However, the ED of the CP was comparable for both forages.

Feed Intake and Animal Performance

Study 1. The DMI was 1.6 kg/d lower ($P = 0.001$) in cows receiving L than H or M (Table 5). However, diet had no effect on milk yield, ECM yield, or FCM yield, with means of 34.8, 34.9, and 36.7 kg/d, respectively. Similarly, diet did not ($P > 0.05$) affect milk protein, fat, or lactose contents. In contrast, the milk urea concentration was 4.4 mg/dL lower ($P = 0.018$) in cows receiving L than H, with those fed M having an intermediate value. Feed efficiency was 0.1 kg/kg of DM higher ($P < 0.05$) in cows fed L compared with M, with those receiving H having an intermediate value. Dietary treatment did not affect ($P > 0.05$) mean BW or BCS, although BCS change was numerically lower in cows fed L compared with M or H.

Study 2. The DMI of cows receiving L50 was 2 kg/d lower ($P = 0.019$) than when receiving H50 (Table 5). Milk yield was 2 kg/d lower ($P = 0.010$) in cows receiving L60 compared with H50, but we found no difference ($P > 0.05$) in ECM or FCM yield between treatments. Diet also had no effect ($P > 0.05$) on milk fat or lactose contents. In contrast, milk protein concentration was 0.6 g/kg lower ($P = 0.024$) in cows receiving L60 than H50. We also observed that cows fed L60 had lower ($P < 0.05$) milk protein and lactose yields compared with those fed H50. The concentration of milk urea was 7.2 mg/dL higher ($P < 0.001$) in cows fed H50 compared with those fed L50 or L60. Feed efficiency was

Table 3. Nutrient composition (g/kg DM), fatty acid content, and particle size distribution of diets based on red clover and grass silage or alfalfa and corn silage, fed to dairy cows in studies 1 and 2[1]

	Study 1			Study 2		
Item[2]	H	M	L	H50	L50	L60
DM (g/kg)	489	481	481	453	442	439
CP	174	165	153	172	150	152
OM	906	906	906	919	920	913
NDF	383	384	376	348	355	356
ADF	275	281	281	233	243	248
EE	25.6	26.0	26.8	29.5	29.5	30.3
Ash	94.3	94.0	93.6	81.1	79.9	87.2
Fatty acid (g/kg DM)						
C16:0	6.11	5.46	5.89	6.99	5.83	6.46
C18:0	0.77	0.62	0.64	0.80	0.70	0.74
C18:1*C*9	4.13	3.67	3.80	5.37	4.48	4.40
C18:2n-6	3.85	3.53	3.71	6.31	5.88	5.71
C18:3n-3	3.77	3.70	3.88	2.17	2.31	2.83
Total fatty acids	21.9	19.4	20.6	23.9	21.5	22.7
Fraction[3] (% DM)						
>44 (mm)	0.00	0.00	0.00	0.00	0.00	0.00
33 to 44 (mm)	2.84	2.49	2.58	1.06	0.78	0.98
19 to 32.9 (mm)	16.0	15.3	14.4	15.8	15.6	17.0
8 to 19 (mm)	45.3	44.8	45.5	45.3	47.8	45.4
4 to 8 (mm)	10.2	9.31	9.94	12.7	13.0	12.9
<4 (mm)	25.7	28.1	27.5	25.2	22.8	23.7
X_m (mm)	12.7	12.3	12.3	12.4	12.7	12.7
SD_{gm}	2.19	2.21	2.19	2.14	2.09	2.13
$pef_{>4}$ (%)	74.3	71.9	72.5	74.8	77.2	76.3
$pef_{>8}$ (%)	64.1	62.6	62.5	62.1	64.2	63.4
$peNDF_{>4}$ (%)	28.4	27.6	27.2	26.0	27.4	27.2
$peNDF_{>8}$ (%)	24.5	24.1	23.5	21.6	22.8	22.6

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage.

[2]EE = ether extract.

[3]Fraction of the diets at 0 h postfeeding; X_m = geometric mean particle size; SD_{gm} = SD of X_m; pef = physical effectiveness factor; peNDF = physically effective fiber.

Table 4. In situ DM and CP degradability coefficients of red clover, grass silage, alfalfa silage, and corn silage fed to dairy cows in studies 1 and 2

	Study 1				Study 2			
Item[1]	Red clover silage	Grass silage	SEM	*P*-value	Alfalfa silage	Corn silage	SEM	*P*-value
DM degradation coefficient (g/kg DM)								
a	278	246	1.1	0.094	200	406	0.1	<0.001
b	565	611	0.8	0.014	580	457	1.2	0.002
a+b	844	857	0.5	0.124	779	863	0.6	<0.001
c	0.08	0.07	0.007	0.243	0.09	0.06	0.005	0.028
ED	557	520	0.8	0.030	498	608	0.6	<0.001
CP degradation coefficient (g/kg total N)								
a	323	424	1.6	0.011	428	538	1.1	0.002
b	549	440	1.3	0.004	422	272	1.1	<0.001
a+b	872	864	0.5	0.318	850	810	0.5	0.004
c	0.10	0.09	0.009	0.416	0.11	0.08	0.008	0.045
ED	627	655	0.4	0.008	672	673	0.3	0.843

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

Table 5. Intake, milk performance, BW, and body condition of dairy cows fed diets based on red clover and grass silage or alfalfa and corn silage in studies 1 and 2[1]

	Study 1					Study 2				
Item	H	M	L	SEM	P-value	H50	L50	L60	SEM	P-value
Intake (kg DM/d)	25.0[a]	25.2[a]	23.5[b]	0.33	0.001	26.2[a]	24.2[b]	25.0[ab]	0.45	0.019
Production (kg/d)										
Milk yield	35.0	34.7	34.6	0.51	0.810	40.9[a]	39.8[ab]	38.9[b]	0.43	0.010
ECM yield	34.8	35.0	34.8	0.54	0.932	38.7	37.6	37.2	0.58	0.175
FCM[2] yield	36.1	37.0	36.8	0.75	0.692	39.1	38.1	38.3	0.92	0.703
Composition (g/kg)										
Fat	41.4	42.9	42.6	0.68	0.252	38.3	38.3	39.4	0.86	0.553
Protein	32.0	31.6	31.6	0.23	0.422	30.7[a]	30.2[ab]	30.1[b]	0.14	0.024
Lactose	45.2	45.6	45.4	0.15	0.164	45.2	45.0	45.4	0.17	0.313
Milk urea (mg/dL)	21.8[a]	19.7[ab]	17.4[b]	1.02	0.018	24.0[a]	16.6[b]	17.0[b]	0.53	<0.001
Yield (kg/d)										
Fat	1.45	1.48	1.47	0.030	0.692	1.56	1.52	1.53	0.037	0.703
Protein	1.11	1.09	1.09	0.015	0.391	1.25[a]	1.20[ab]	1.17[b]	0.015	0.002
Lactose	1.58	1.58	1.57	0.025	0.938	1.85[a]	1.80[ab]	1.77[b]	0.023	0.047
Feed efficiency										
Milk/DMI	1.40[ab]	1.38[b]	1.48[a]	0.022	0.009	1.57	1.64	1.56	0.031	0.158
ECM/DMI	1.39[ab]	1.38[b]	1.48[a]	0.027	0.030	1.48	1.56	1.50	0.032	0.193
Body performance										
BW (kg)	685	680	684	4.5	0.713	715	708	702	4.2	0.113
BW change[3] (kg/d)	0.14	−0.10	−0.01	0.246	0.792	0.66	−0.04	0.12	0.217	0.070
BCS	2.54	2.61	2.57	0.039	0.477	2.68	2.65	2.64	0.045	0.804
BCS change[3]	−0.01	0.04	−0.04	0.061	0.600	0.13	0.00	0.04	0.056	0.293

[a,b]Means within a row within a study with different superscripts differ ($P < 0.05$).

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage.

[2]FCM = 4% fat-corrected milk yield.

[3]Change over the 28-d period.

comparable across all 3 treatments. We found no effect ($P > 0.05$) of diet on mean BW or BCS, but we found a tendency ($P = 0.070$) for BW gain to be higher in cows receiving H50 than L50.

Nutrient Intake and Apparent Total-Tract Digestibility

Study 1. We observed that DM, OM, N, and NDF intakes were lower ($P < 0.05$) in cows receiving L compared with H or M (Table 6). In contrast, the fecal outputs of DM, OM, and ADF were higher ($P < 0.05$) in cows fed L compared with those receiving H, whereas cows receiving L had a lower ($P < 0.05$) amount digested and a lower digestibility of DM, OM, N, NDF, and ADF compared with those fed either of the other 2 diets. In general, we found that the total-tract digestibility in cows receiving H or M was comparable, although total N digested was lower ($P < 0.001$) in animals when fed diet M compared with H. As a proportion of total N intake, N output in feces was 11 percentage units higher ($P = 0.001$) in cows fed L at 45.1% than those fed H or M. The NUE was approximately 5 percentage units higher in cows fed L than H or M.

Study 2. The intakes of DM, OM, N, and NDF were lower ($P < 0.05$) in cows fed L50 compared with H50, with those fed L60 being intermediate, except for total N intake, which was similar in cows fed L50 or L60 at 0.59 kg/d (Table 6). We observed that the amounts of DM and OM digested were higher ($P < 0.05$) in cows fed H50 compared with L50, with those receiving L60 having intermediate values. Similarly, the amount of N digested was 0.14 kg/d higher ($P < 0.001$) in cows receiving H50 than L50 or L60. However, we found that dietary treatment had no effect ($P > 0.05$) on apparent whole-tract digestibility of DM, OM, N, NDF, or ADF.

Milk N output was higher ($P = 0.011$) in cows receiving H50 compared with L60, but, as a proportion of total N intake, we found no effect ($P > 0.05$) of diet on fecal N output. In contrast, the apparent NUE was approximately 5 percentage units higher ($P < 0.001$) in cows receiving L50 or L60 compared with H50.

Blood Plasma Metabolites

Study 1. Dietary treatment had no effect ($P > 0.05$) on the concentrations of plasma ammonia, glucose, or BHB (Table 7). In contrast, mean plasma urea concentration was lower ($P = 0.011$) in cows receiving L, but similar in those receiving H or M, and increased with time after feeding (Figure 1a).

Table 6. Intake, output, and apparent total-tract digestibility (kg/d) of nutrients and nitrogen partitioning (%) in dairy cows fed diets based on red clover and grass silage or alfalfa and corn silage in studies 1 and 2[1]

	Study 1					Study 2				
Item	H	M	L	SEM	*P*-value	H50	L50	L60	SEM	*P*-value
DM										
Intake	25.4[a]	25.2[a]	23.6[b]	0.448	0.018	26.3[a]	23.8[b]	25.0[ab]	0.521	0.010
Fecal output	6.13[b]	6.77[ab]	7.65[a]	0.408	0.050	8.04	7.32	7.78	0.388	0.433
Digested	19.3[a]	18.5[a]	16.0[b]	0.402	<0.001	18.3[a]	16.4[b]	17.2[ab]	0.441	0.028
Digestibility (kg/kg)	0.759[a]	0.734[a]	0.677[b]	0.0144	0.002	0.695	0.690	0.691	0.0118	0.940
N										
Intake	0.71[a]	0.67[a]	0.58[b]	0.012	<0.001	0.73[a]	0.57[b]	0.61[b]	0.013	<0.001
Fecal output	0.22	0.24	0.26	0.016	0.301	0.24	0.21	0.22	0.011	0.170
Milk output	0.18	0.17	0.17	0.003	0.597	0.19[a]	0.18[ab]	0.18[b]	0.002	0.011
Digested	0.48[a]	0.42[b]	0.32[c]	0.015	<0.001	0.49[a]	0.36[b]	0.31[b]	0.028	<0.001
Digestibility (kg/kg)	0.683[a]	0.635[a]	0.549[b]	0.0218	0.001	0.669	0.628	0.634	0.0120	0.055
OM										
Intake	23.0[a]	22.9[a]	21.4[b]	0.40	0.018	24.2[a]	21.9[b]	22.9[ab]	0.48	0.011
Fecal output	5.17[b]	5.72[ab]	6.49[a]	0.351	0.047	6.96	6.33	6.70	0.346	0.458
Digested	17.9[a]	17.1[a]	14.9[b]	0.36	<0.001	17.2[a]	15.5[b]	16.2[ab]	0.42	0.031
Digestibility (kg/kg)	0.775[a]	0.752[a]	0.698[b]	0.0137	0.002	0.713	0.708	0.709	0.0116	0.956
NDF										
Intake	9.72[a]	9.68[a]	8.86[b]	0.190	0.006	9.17[a]	8.43[b]	8.90[ab]	0.180	0.031
Fecal output	3.07	3.22	3.77	0.220	0.085	3.92	3.60	3.76	0.205	0.556
Digested	6.65[a]	6.46[a]	5.09[b]	0.211	<0.001	5.25	4.83	5.14	0.157	0.176
Digestibility (kg/kg)	0.684[a]	0.669[a]	0.576[b]	0.0205	0.003	0.575	0.572	0.577	0.0176	0.982
ADF										
Intake	6.98	7.08	6.62	0.130	0.050	6.15	5.76	6.18	0.120	0.050
Fecal output	2.68[b]	2.84[ab]	3.43[a]	0.193	0.031	3.19	3.03	3.19	0.160	0.718
Digested	4.31[a]	4.25[a]	3.20[b]	0.166	<0.001	2.96	2.73	2.99	0.118	0.278
Digestibility (kg/kg)	0.616[a]	0.602[a]	0.487[b]	0.0242	0.002	0.482	0.476	0.485	0.0207	0.957
N partitioning (%)										
Fecal	31.7[b]	36.5[b]	45.1[a]	2.18	0.001	33.1	37.2	36.6	1.20	0.055
NUE[2]	24.7[b]	25.6[b]	30.4[a]	0.40	<0.001	26.5[b]	32.6[a]	29.9[a]	0.77	<0.001

[a,b]Means within a row within a study with different superscripts differ ($P < 0.05$).

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage. Measured using 12 cows for each study (4 cows for each treatment group).

[2]NUE = apparent nitrogen use efficiency (milk N/N intake).

Study 2. Dietary treatment did not affect ($P > 0.05$) plasma ammonia, glucose, or BHB concentrations (Table 7). In contrast, plasma urea concentration was higher ($P < 0.001$) in cows fed H50 compared with L50 or L60, and increased immediately after feeding (Figure 1b).

Milk Fatty Acid Profile

Study 1. The highest milk fat proportions of C8:0, C11:0, C12:0, C15:0, C15:1 *cis*-10, and C16:1 were observed in cows fed H, and lowest among those fed M ($P < 0.05$), except for C15:0 and C15:1 *cis*-10, which were lowest in milk from cows fed L (Table 8). In contrast, the milk fat proportion of C18:2 *cis*-9,*cis*-12 was higher ($P = 0.002$) in cows receiving L or M than H. Milk fat proportion of C18:1 *trans*-9 was higher ($P < 0.05$) in cows when fed M than L, whereas C18:1 *trans*-12 was higher in those fed L than H. We found that diet had no effect ($P > 0.05$) of diet on milk FA of chain length >C16:0 or C16:0 + 16:1, but those <C16:0 were higher ($P = 0.040$) in cows fed H compared with M. Also, dietary treatment did not affect the total milk fat proportions of SFA or MUFA, but the PUFA proportion was higher ($P = 0.017$) in cows receiving L than H. The total proportions of milk fat linear odd-chain FA or odd- and branched-chain FA (**OBCFA**) were higher ($P < 0.05$) in cows fed H compared with L.

Study 2. Diet had no effect on the milk FA profile except for CLA *cis*-9,*trans*-11, which was higher ($P = 0.019$) in cows when receiving L60 compared with H50, and intermediate in those fed L50 (Table 8). Similarly, we found that CLA *trans*-10,*cis*-12 tended ($P = 0.071$) to be higher in cows receiving L60 compared with those fed H50. Diet had no effect on the milk FA proportion of chain length higher or lower than C16:0, and C16:0 + 16:1. Similarly, we found no effect of dietary treatment on the total milk fat proportions of SFA, MUFA, PUFA, linear odd-chain FA, or OBCFA.

Table 7. Plasma ammonia, BHB, and glucose concentrations in dairy cows fed diets based on red clover and grass silage or alfalfa and corn silage in studies 1 and 2[1]

Item	Study 1							Study 2						
					P-value							*P*-value		
	H	M	L	SEM	D	T	Int	H50	L50	L60	SEM	D	T	Int
Ammonia (μmol/L)	35.2	35.6	34.4	2.36	0.690	<0.001	0.768	40.2	41.4	41.7	4.75	0.720	0.004	0.972
BHB (mmol/L)	0.76	0.73	0.79	0.065	0.630	<0.001	0.856	0.49	0.49	0.53	0.046	0.640	<0.001	0.087
Glucose (mmol/L)	3.72	3.67	3.68	0.108	0.832	<0.001	0.492	3.63	3.61	3.61	0.068	0.952	<0.001	0.505

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage. D = main effect of diet; T = main effect of time; Int. = interaction between diet and time.

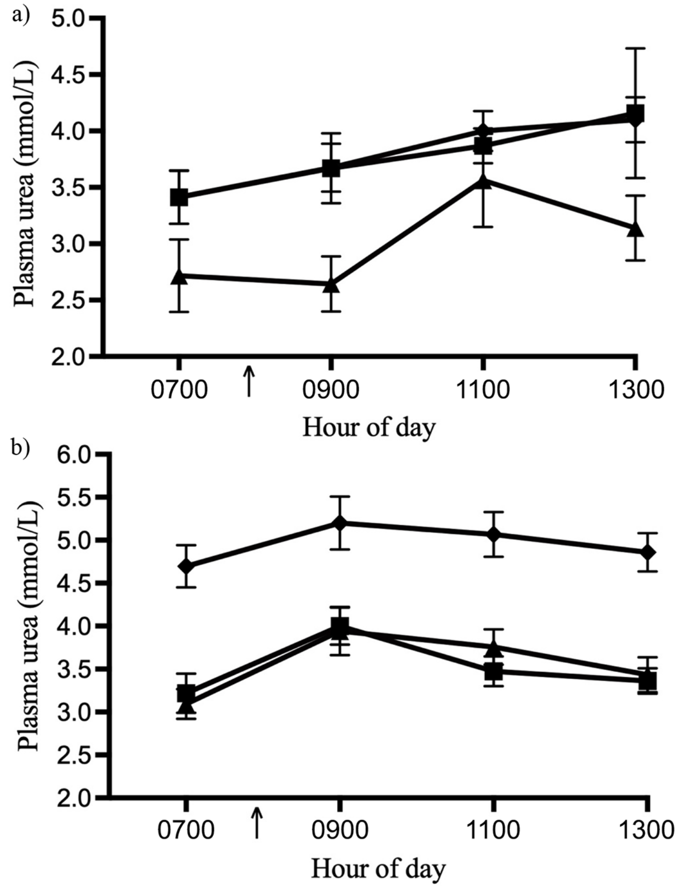

Figure 1. Plasma urea concentration in dairy cows fed a high- (H, ◆), medium- (M, ■), or low-CP (L, ▲) diet based on red clover and grass silage (study 1, a), a high-CP diet with 50:50 ratio of alfalfa to corn silage (H50, ◆), or a low-CP diet with either a 50:50 (L50, ■) or 60:40 (L60, ▲) ratio of alfalfa to corn silage (study 2, b). For study 1: pooled SEM = 0.276; diet, $P = 0.011$; time, $P = 0.006$; and diet × time, $P = 0.598$. For study 2; pooled SEM = 0.326; diet, $P < 0.001$; time, $P < 0.001$; and diet × time, $P = 0.781$. The arrow indicates the time of feeding. Error bars indicate SEM.

DISCUSSION

Feed Characteristics and Particle Size Distribution

The chemical compositions of the forages used in both of our studies were comparable to previous work by Broderick (2018) and Sinclair et al. (2015). In line with Broderick (2018), the grass silage had the highest concentration of NDF, although the content of CP was marginally lower than that of the red clover or alfalfa silage. Dewhurst (2013) also reported that grass silage usually contains less CP but more fiber than legume forages, although factors such as variety, fertilizer application rate, and stage of maturity at harvest can all influence the content. According to Broderick (2018), red clover silage contains 20 g/kg of DM less CP than alfalfa silage when harvested at a similar

Table 8. Milk fatty acid composition of dairy cows fed diets based on red clover and grass silage or alfalfa and corn silage in studies 1 and 2[1]

	Study 1: Red clover and grass silage-based diet					Study 2: Alfalfa and corn silage-based diet				
Fatty acid (g/100 g)	H	M	L	SEM	*P*-value	H50	L50	L60	SEM	*P*-value
C4:0	1.47	1.46	1.45	0.020	0.870	1.57	1.59	1.60	0.022	0.517
C6:0	1.39	1.37	1.39	0.011	0.348	1.39	1.40	1.40	0.014	0.855
C8:0	1.01[a]	0.98[b]	0.99[ab]	0.009	0.034	0.99	0.98	0.98	0.009	0.766
C10:0	2.54	2.47	2.54	0.048	0.448	2.50	2.37	2.41	0.040	0.091
C11:0	0.31[a]	0.29[b]	0.30[ab]	0.005	0.009	0.08	0.05	0.06	0.007	0.083
C12:0	3.39[a]	3.18[b]	3.29[ab]	0.049	0.015	3.28	3.23	3.14	0.044	0.085
C13:0	0.09	0.09	0.08	0.003	0.109	0.11	0.10	0.10	0.003	0.057
C14:0	12.08	11.78	11.94	0.088	0.073	11.7	11.7	11.5	0.09	0.202
C14:1 *cis*-9	1.25	1.20	1.23	0.030	0.530	1.21	1.32	1.22	0.040	0.122
C15:0	1.26[a]	1.22[ab]	1.19[b]	0.016	0.014	1.25	1.21	1.22	0.026	0.480
C15:1 *cis*-10	0.23[a]	0.21[ab]	0.21[b]	0.001	0.023	0.18	0.21	0.21	0.020	0.256
C16:0	39.41	39.52	39.55	0.231	0.904	38.8	38.9	39.6	0.27	0.126
C16:1 *cis*-9	1.82[a]	1.72[b]	1.73[ab]	0.030	0.031	1.70	1.71	1.70	0.050	0.970
C17:0	0.52	0.52	0.52	0.005	0.877	0.48	0.47	0.50	0.010	0.231
C17:1 *cis*-10	0.28	0.27	0.27	0.001	0.053	0.26	0.27	0.26	0.000	0.494
C18:0	7.68	7.79	7.90	0.130	0.479	8.27	8.21	8.22	0.125	0.926
C18:1 *trans*-8	0.25	0.26	0.26	0.010	0.676	0.15	0.14	0.15	0.009	0.816
C18:1 *trans*-9	0.11[ab]	0.14[a]	0.09[b]	0.014	0.034	0.36	0.36	0.33	0.022	0.670
C18:1 *trans*-10	0.99	1.01	1.04	0.039	0.702	0.59	0.57	0.61	0.022	0.482
C18:1 *trans*-11	0.72	0.75	0.74	0.012	0.345	0.99	0.96	0.92	0.027	0.234
C18:1 *trans*-12	0.15[b]	0.16[ab]	0.17[a]	0.003	0.022	0.32	0.35	0.34	0.011	0.115
C18:1 *cis*-9	19.15	19.51	18.97	0.220	0.228	19.5	19.4	19.2	0.21	0.506
C18:2 *cis*-9,*cis*-12	1.96[b]	2.17[a]	2.17[a]	0.042	0.002	2.08	2.20	2.18	0.097	0.662
C18:3 *cis*-9,*cis*-12,*cis*-15	0.27	0.27	0.27	0.003	0.097	0.54	0.52	0.50	0.020	0.456
CLA *cis*-9,*trans*-11	0.80	0.81	0.81	0.010	0.669	0.22[b]	0.23[ab]	0.25[a]	0.010	0.019
CLA *trans*-10,*cis*-12	0.03	0.03	0.04	0.002	0.237	0.027	0.031	0.032	0.0010	0.071
C20:0	0.01	0.01	0.01	0.001	0.965	0.04	0.04	0.04	0.001	0.534
C20:3 *cis*-11,*cis*-14,*cis*-17	0.13	0.13	0.13	0.003	0.907	0.16	0.16	0.15	0.004	0.240
C21:0	0.06	0.05	0.06	0.003	0.789	0.07	0.06	0.06	0.004	0.396
C22:0	0.04	0.04	0.04	0.001	0.516	0.12	0.11	0.11	0.003	0.090
EPA[2]	0.12	0.11	0.12	0.005	0.615	0.13	0.14	0.11	0.022	0.471
DHA[3]	0.06	0.02	0.03	0.022	0.366	0.06	0.06	0.06	0.002	0.257
Summation										
<C16	25.0[a]	24.2[b]	24.6[ab]	0.20	0.040	24.3	24.2	23.8	0.15	0.116
C16 + 16:1	41.2	41.3	41.3	0.24	0.987	40.5	40.6	41.3	0.29	0.154
>C16	33.8	34.5	34.1	0.35	0.334	35.2	35.2	34.9	0.32	0.737
SFA[4]	71.3	70.8	71.3	0.25	0.301	70.7	66.7	71.0	2.24	0.326
MUFA[5]	25.0	25.2	24.7	0.24	0.309	25.5	24.0	25.2	0.91	0.502
PUFA[6]	3.81[b]	3.99[ab]	4.03[a]	0.056	0.017	3.80	3.73	3.86	0.157	0.837
LOCFA[7]	2.24[a]	2.17[ab]	2.14[b]	0.021	0.010	2.00	1.82	1.96	0.068	0.148
OBCFA[8]	2.75[a]	2.65[b]	2.62[b]	0.023	0.001	2.44	2.27	2.43	0.081	0.259

[a,b]Means within a row within a study with different superscripts differ ($P < 0.05$).

[1]Study 1 based on red clover and grass silage; H = high- (175 g CP/kg DM), M = medium- (165 g CP/kg DM), and L = low-CP diets (150 g CP/kg DM). Study 2 based on alfalfa and corn silage; H50 = 175 g CP/kg DM with 50:50 alfalfa to corn silage, L50 = 150 g CP/kg DM with 50:50 alfalfa to corn silage, and L60 = 150 g CP/kg DM with 60:40 alfalfa to corn silage.

[2]EPA = eicosapentaenoic acid; C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17.

[3]DHA = docosahexaenoic acid; C22:6 *cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19.

[4]Saturated fatty acids are defined as fatty acids with no double bonds.

[5]Monounsaturated fatty acids are defined as fatty acids with one double bond.

[6]Polyunsaturated fatty acids are defined as fatty acids with more than one double bond.

[7]LOCFA = linear odd-chain fatty acids: ΣLOCFA = (C11:0 + C13:0 + C15:0 + C17:0 + C21:0).

[8]OBCFA = linear odd- and branched-chain fatty acids: ΣOBCFA = (C11:0 + C13:0 + C15:0 + C15:1 + C17:0 + C17:1 + C21:0).

growth stage or NDF content. However, in our studies the CP content of red clover silage was only 10 g/kg of DM lower than the alfalfa silage. The higher pH of red clover and alfalfa silages compared with grass or corn silage is a common feature for legume forages due to their higher CP and ash content, and reflects a high buffering capacity with a low concentration of soluble CP (Dewhurst et al., 2010). The alfalfa silage had the highest concentration of lactic acid, which is consistent with Sinclair et al. (2015), who reported a 7-g/kg DM higher content of lactate in alfalfa compared with corn or grass silage. Likewise, Dewhurst et al. (2010) also noted a higher concentration of lactate and acetate in red clover than grass or corn silage. The PS distributions of our grass and corn silages were similar to that reported by Tayyab et al. (2018) except for the long PS (mainly >44 mm), which was not found in our current study. The mean X_m and peNDF were highest in our alfalfa and grass silages, respectively, primarily due to the greater content of the long-PS (19 to 32.9 mm) fraction of our alfalfa silage and the higher NDF content of our grass silage.

The nutrient composition of the TMR was similar in both of our studies except for CP, as we predicted, although we found a tendency for an interaction for $pef_{>8mm}$ in our study 1, which indicated that eating behavior differed between the dietary treatments (Kononoff et al., 2003; Tayyab et al., 2019). The observation that the mean $pef_{>8mm}$ was lowest at 4 h and highest at 24 h after the morning feed for diet H in study 1 could be attributable to an increase in the consumption of large PS (>8 mm) during the first 4 h and short PS (<8 mm) between 4 and 24 h after feeding. In study 2, a numerically higher proportion of small-PS (<4 mm) and lower content of medium-PS (8–19 mm) fractions was the major factor causing the higher X_m, pef, and peNDF in H50 than the other 2 diets. Moreover, the consumption of medium (8–19 mm) fractions by our cows was lower during the first 8 h following the morning feeding in study 2, mainly due to the preference for a short PS (Kononoff et al., 2003).

In Situ Degradability

The soluble CP fraction in our grass silage was higher than the red clover silage, whereas the soluble DM was lower, a finding in agreement with Purwin et al. (2014), who examined the soluble fractions of different legumes and grass silages. Red clover silage has a higher concentration of potentially degradable CP compared with grass or other non-legume silages, as reported in a series of other studies (Dewhurst et al., 2003; Purwin et al., 2014; Damborg et al., 2018). Similarly, several studies (Dewhurst et al., 2003; Damborg et al., 2018) have shown that red clover silage has a greater content of ED of CP than grass silage, with the higher content of NPN relative to neutral detergent insoluble CP being responsible for a rapid ruminal degradation of legume forage proteins. In contrast, red clover had a slightly higher ED of the DM compared with grass silage and therefore would be predicted to supply more rumen available energy for microbial growth (Thomas, 2004).

The contents of the immediately soluble fractions of DM and CP were lower in our alfalfa silage, but the potentially degradable fractions and the rates of degradation for DM and CP were higher than corn silage, a finding in accordance with Damborg et al. (2018). In comparison to alfalfa silage, red clover silage had a lower ruminal degradability of CP in our studies. This may be attributable to the presence of *o*-quinones that are synthesized by the enzyme PPO present in red clover, reducing protein degradation by creating a cross-linked complex with soluble and other dietary proteins inhibiting the function of proteases (Broderick et al., 2004).

Intake and Animal Performance

Our cows had a reduced DMI in both studies when the dietary CP concentration was decreased from 175 to 150 g/kg of DM, a finding consistent with other studies (Alstrup et al., 2014; Giallongo et al., 2016; Barros et al., 2017). In a review of the literature, Sinclair et al. (2014) reported that the DMI responds negatively when the concentration of dietary CP decreased between 220 to 140 g/kg of DM. The reduced DMI in cows fed diet L (study 1) or L50 (study 2) could be related to a lower supply of available N in the rumen, which depressed the activity of fiber-degrading bacteria, resulting in a lower intake. However, plasma urea concentrations in both of our studies were within the range generally considered to indicate that ruminal N supply was not limiting (Reynolds and Kristensen, 2008). In contrast, Broderick et al. (2015) reported no difference in DMI in lactating dairy cows fed alfalfa and corn silage-based diets containing 170 to 150 g of CP/kg of DM. Likewise, Olmos Colmenero and Broderick (2006) noted that dietary CP concentration ranging from 135 to 194 g of CP/kg of DM had no effect on DMI in Holstein dairy cows fed alfalfa and corn silage-based rations. Increasing the inclusion rate of alfalfa silage from 50 to 60% in our study 2 did not affect the DMI of the cows (L60), supporting the findings of Arndt et al. (2015), who reported no difference in DMI when between 20 and 80% of corn silage was replaced with alfalfa silage. In contrast, Sinclair et al. (2015) observed a decrease in DMI when alfalfa replaced 60% of the corn silage.

Reducing dietary CP concentration has often been reported to reduce lactation performance (Hristov and Giallongo, 2014). For example, Giallongo et al. (2016) reported that decreasing the concentration of dietary CP from 165 to 145 g/kg of DM (resulting in a 5 to 10% deficiency in MP supply) reduced milk yield by approximately 4.3 kg/d when cows were fed a 1:2 (DM basis) alfalfa haylage and corn silage-based ration. In contrast, feeding a low-CP diet had no effect on milk yield or composition in our studies, supporting the findings of Barros et al. (2017), who also reported that feeding a low-CP (144 g of CP/kg of DM) alfalfa and corn silage-based diet (48:52 ratio, DM basis) had no effect on milk performance. We predicted that the supply of MP was similar across all the diets, which would be anticipated to result in a similar level of performance, irrespective of dietary CP content (Thomas, 2004). The exception was diet L in study 1, which was predicted to supply 95% of MP requirements, principally due to the high CP content of the grass silage and the subsequent difficulty in formulating a low-CP diet while providing sufficient supplementary RUP to meet the MP requirements. Despite this, performance was similar to animals fed H or M, which may possibly be attributable to inaccuracies in the prediction of MP requirements or to a greater mobilization of body tissues to meet nutrient demand. Body weight change did not, however, differ between treatments in our study 1, although the short-term changeover study design that we used may not have permitted an accurate assessment, and longer-term studies are required.

Milk yield and milk protein concentration have been found to be reduced when dairy cows were fed a high compared with a low proportion of alfalfa-based diet (75:25 vs. 25:75 alfalfa to corn silage ratio; Thomson et al., 2017), which supports our findings in study 2. The decrease in milk yield and milk protein content with increasing alfalfa proportion might have been due to the ME density of the alfalfa silage, which may have led to a lower supply of rumen available energy and subsequent MCP flow to the duodenum. However, several studies have reported that the replacement of corn with alfalfa silage has little consistent effect on milk yield or composition (Hassanat et al., 2013; Arndt et al., 2015; Sinclair et al., 2015). It has also been suggested that milk and milk protein yield could be improved by improving the AA profile of the MP, particularly the limiting essential AA such as lysine, methionine, or histidine (Lee et al., 2012b; Giallongo et al., 2016).

Whole-Tract Digestibility and N Efficiency

The apparent whole-tract digestibilities of DM, OM, N, NDF, and ADF in both of our studies were similar to previous reports that have examined the effects of dietary CP concentration in red clover or alfalfa silage-based diets (Broderick et al., 2008; Lee et al., 2011). Similar to other studies (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a), the apparent digestibilities of DM, OM, N, NDF, and ADF were decreased when a low-CP diet (L) was fed to our dairy cows in study 1, but this was not observed for the low-protein diets (L50 and L60) in study 2, although we acknowledge that the low frequency of fecal sampling in our study could have influenced the results (Morris et al., 2018). In contrast, Niu et al. (2016) reported no change in the apparent digestibility of alfalfa and corn silage-based rations, except for OM and CP, which were decreased when the dietary concentration of CP was reduced from 185 to 152 and from 155 to 137 g/kg of DM, respectively. Olmos Colmenero and Broderick (2006) suggested that a CP concentration below 165 g/kg of DM could contribute to a lower nutrient digestibility, supporting our finding from study 1.

No difference was found in nutrient digestibility when alfalfa replaced corn silage at a higher rate (L50 and L60) in our study 2. Likewise, Sinclair et al. (2015) and Arndt et al. (2015) reported that the replacement of corn with alfalfa silage did not affect apparent nutrient digestibility, except for fiber digestibility, which was increased by the inclusion rate of alfalfa silage (Arndt et al., 2015). Alternatively, feeding alfalfa-based diets may enhance the duodenal flow of indigestible fiber, resulting in an increased sloughing of endogenous cells from the intestinal wall, with a resultant reduction in the apparent digestibility of CP (Dewhurst, 2013).

Several studies have reported that reducing dietary N intake can increase apparent NUE in lactating dairy cows when fed either alfalfa or alfalfa and corn silage-based rations (Broderick et al., 2015; Hristov et al., 2015; Niu et al., 2016), a finding in agreement with our results, where reducing dietary CP from 175 to 150 g/kg of DM while maintaining the MP supply increased NUE by approximately 25%. We found no difference in N output in milk or NUE when alfalfa replaced corn silage (L50 vs. L60) in our study 2, although numerically NUE was lower in cows fed L60 than L50. Increasing the rate of inclusion of alfalfa silage in corn silage-based diets up to 75 or 80%, has been shown to decrease NUE, which may be associated with a lower milk yield, oversupply of RDP, or limitation of rumen available energy in high alfalfa silage-based rations (Arndt et al., 2015; Thomson et al., 2017).

Plasma Metabolites and Milk Fatty Acid Profile

We detected a reduction in plasma urea in cows receiving the low-CP diets (L, L50, or L60) compared with the high-CP diets (H or H50), which could be attributable to a lower content of degradable N in the rumen with these diets (Sinclair et al., 2012; Alstrup et al., 2014). Moreover, the lower concentration of milk urea in cows receiving the low-CP diets in our studies was associated with a reduction in plasma urea content, which is supported by Olmos Colmenero and Broderick (2006), who reported that milk and plasma urea N were highly correlated ($r = 0.83$). Alstrup et al. (2014) reported no difference in plasma glucose or BHB concentrations in cows when fed a corn and grass silage-based low-CP diet (139 g/kg of DM) compared with a higher-CP diet (157 g CP/kg of DM), whereas plasma urea was reduced by 1.17 mmol/L. When we increased the proportion of alfalfa silage in the diet in study 2 (L50 vs. L60), this did not affect the concentration of plasma metabolites, although plasma BHB was numerically higher in cows when fed the high-alfalfa diet (L60). The highest concentration of plasma BHB was also associated with a high inclusion rate of alfalfa in the study of Sinclair et al. (2015), an effect that may partially be associated with a higher ruminal molar concentration of butyrate in cows fed this forage (Hassanat et al., 2013).

Milk FA profile principally depends on the FA composition of the diet consumed by the cow and the degree of biohydrogenation in the rumen (Lashkari et al., 2019). In our current studies the milk FA proportion of CLA *cis*-9,*trans*-11 was not affected by dietary CP concentration, but CLA *cis*-9,*trans*-11 increased with alfalfa inclusion rate (L60 vs. H50) in study 2. Increased milk FA proportion of CLA *cis*-9,*trans*-11 can be associated with a greater dietary supply of C18:2 *cis*-9,*cis*-12 or C18:3 *cis*-9,*cis*-12,*cis*-15, although the dietary concentrations of these fatty acids were similar in L50 and L60. The lower proportion of CLA in the milk fat of cows fed H50 may also partly be due to a higher DM and fiber intake, which increased the passage rate and reduced the time available for ruminal biohydrogenation.

The highest proportion of total milk PUFA (mainly C18:2 *cis*-9,*cis*-12) and C18:1 *trans*-12 in the milk from cows fed the low-CP diet (L) in our study 1 could be related to a lower dietary supply of RDP that might have reduced rumen microbial metabolism and subsequent biohydrogenation of long-chain FA in the rumen (Leduc et al., 2017). Moreover, forage type also had an influence on milk FA content (Lashkari et al., 2019). For example, milk from cows fed red clover and grass silage-based diets in our study 1 had higher total PUFA than milk from cows fed the alfalfa and corn silage-based rations in study 2. The higher proportion of milk PUFA in lactating cows fed red clover-based diets could be due to the action of PPO reducing the ruminal biohydrogenation of long-chain FA (Van Ranst et al., 2011). The exact mechanism by which PPO reduces FA biohydrogenation in the rumen is unclear (Lee, 2014), but it has been suggested that encapsulation of plant lipids in a phenol-protein complex reduces the accessibility of lipids to microbial lipolysis, lowering biohydrogenation (Van Ranst et al., 2011). In contrast, Lee et al. (2011) and Giallongo et al. (2016) reported a lower concentration of PUFA in milk fat when an alfalfa and corn silage-based low-CP diet (145 to 148 g/kg of DM) was fed to dairy cows, an effect that was attributed to the inclusion of dietary heat-treated or expeller soybean meal, which contained more saturated fat. The low-CP diets in our current studies also contained more RUP sources, which may have altered the milk FA profile.

Milk OBCFA and <C16 FA in ruminants have been suggested to be markers that could be used to predict MCP synthesis (Vlaeminck et al., 2006). In our study 1, the yields of milk <C16 (mainly C8:0, C11:0, and C12:0) and OBCFA (mainly C15:0 and C15:1) were lower when fed L or M compared with H, a finding in agreement with previous work (Vlaeminck et al., 2006; Giallongo et al., 2016; Leduc et al., 2017), where the microbial synthesis of OBCFA was decreased when diets low in RDP were fed. Indeed, a positive relationship has been found between dietary N supply and MCP synthesis in ruminants (Sinclair et al., 1995).

CONCLUSIONS

Reducing the dietary CP concentration from 175 to 150 g/kg of DM in a red clover and grass silage or alfalfa and corn silage-based diet that were predicted to meet or be marginally deficient in MP supply decreased DMI but had no effect on milk yield or composition, whereas increasing the proportion of alfalfa in a low-CP diet from 50 to 60% of the forage DM reduced milk yield and milk protein content but not DMI or ECM yield. The apparent whole-tract nutrient digestibility was decreased by reducing dietary CP in a red clover and grass silage-based diet but not in an alfalfa and corn silage-based diet. Importantly, feeding a low-CP diet based on either red clover and grass silage or alfalfa and corn silage improved the apparent nitrogen use efficiency in dairy cows by approximately 25%.

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