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Reducing dietary protein and supplementation with starch or rumen-protected methionine and its effect on performance and nitrogen efficiency in dairy cows fed a red clover and grass silage-based diet

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ABSTRACT

The increasing cost of milk production, in association with tighter manure N application regulations and challenges associated with ammonia emissions in many countries has increased interest in feeding lower crude protein (CP) diets based on legume silages. Most studies have focused on alfalfa silage, and there is a lack of information on low CP diets based on red clover silage. Our objectives were to examine the effects of dietary CP content and supplementing a low CP diet with dietary starch or rumen-protected Met (RPMet) on the performance, metabolism, and nitrogen-use-efficiency (NUE; milk N output/N intake) in dairy cows fed a red clover and grass silage-based diet. Fifty-six Holstein-Friesian dairy cows were blocked and randomly allocated to 1 of 4 diets over a 14-week feeding period. Diets were based on red clover and grass silages at a ratio of 50:50 (dry matter (DM) basis) and were fed as a total mixed ration, with a forage-to-concentrate ratio of 53:47 (DM basis). The diets were formulated to supply a similar metabolizable-protein (MP) content, and have a CP concentration of 175 g/kg DM (CON), or 150 g/kg DM (LP = low protein), or LP supplemented with additional barley as a source of starch (+ 64 g/kg DM; LPS) or RPMet (+ 0.3 g/100 g MP; LPM). At the end of the 14-week feeding period, 20 cows (5 per treatment) continued to be fed the same diets for a further 6 d, and total urine output and fecal samples collected. We observed that dietary treatment did not affect DM intake, with a mean of 21.5 kg/d, however, there was an interaction between diet and week with intake being highest in cows fed LPS in wk 4 and CON in wk 9 and 14. Mean milk yield, 4% fat corrected milk and energy corrected milk were not altered by treatment. Similarly, we found no effect of dietary treatment on milk fat, protein or lactose content. In contrast, milk and plasma urea concentrations were highest in cows fed CON. The concentration of blood plasma β-hydroxybutyrate was highest in cows receiving LPM and lowest in LPS. Apparent NUE was 28.6% in cows fed CON, and was higher in cows fed any of the low protein diets (LP, LPS or LPM) with a mean value of 34.2%. The sum of milk fatty acids with a chain length below C16:0 was also highest in cows fed CON. We observed that dietary treatment did not affect the apparent whole-tract nutrient digestibility of organic matter, N, neutral detergent fiber and acid detergent fiber, with mean values of 0.785, 0.659, 0.660 and 0.651 kg/kg respectively, but urinary N excretion was approximately 60 g/d lower in cows fed the low CP diets compared with CON. We conclude that reducing the CP content of red clover and grass silage-based diets from 175 to 150 g/kg DM while maintaining MP supply did not affect performance, but reduced the urinary N excretion and improved NUE, and that supplementing additional starch or RPMet had little further effect.
Keywords: nitrogen use efficiency, low protein, red clover, starch

INTRODUCTION

There is considerable interest in reducing dietary CP concentrations and making greater use of home grown forage legumes in dairy cow diets due to the volatile cost of protein feeds, and to reduce N and ammonia emissions from dairy herds (Chowdhury et al., 2023; Defra, 2019; Lavery and Ferris, 2021). Diets high in CP typically result in a low N use efficiency (**NUE**; milk N output/N intake), with excess N excreted to the environment, which may potentially contribute to the deterioration of terrestrial and aquatic ecosystems (Groenestein et al., 2019). It has been shown that dietary protein levels can be reduced in dairy cow diets to around 140–150 g/kg dietary DM without any major impact on performance, health or fertility if the diet meets the cows MP requirements (Sinclair et al., 2014). However, other studies have reported that lowering the

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dietary CP concentration from 173 to 144 g/kg DM reduced DM intake and resulted in a decreased milk yield by 3.6 kg/d, and was also associated with a lower milk fat and protein content (Law et al., 2009; Barros et al., 2017a). Hristov and Giallongo (2014) observed that feeding diets with a CP concentration lower than 150 g/kg DM would also decrease milk production in high yielding dairy cows, mainly by reducing DM intake.

Most studies that have investigated feeding low CP, legume-based diets have focused on alfalfa silage (Olmos Colmenero and Broderick, 2006; Lee et al., 2015; Barros et al., 2017), despite the importance of red clover silage in many countries (Moorby et al., 2016). Red clover silage is high in CP (178 to 194 g CP/kg DM; Broderick, 2018; Schulz et al., 2018), and it's inclusion at up to 66% of the forage component of the diet improved intake, milk yield, and the proportion of PUFA in milk compared with grass silage (Dewhurst, 2013; Moorby et al., 2016). In addition to its high CP content, red clover silage contains polyphenol oxidase (**PPO**), which can protect CP from microbial degradation in the rumen, increasing its RUP content (Lee, 2014). Moreover, red clover silage can increase microbial protein (**MCP**) synthesis in the rumen (Merry et al., 2006), and improve blood plasma concentrations of AA, except for Met (Vanhatalo et al., 2009). Reducing the CP content of the diet in our previous study on red clover silage (Chowdhury et al., 2023) decreased DM intake but did not significantly affect milk yield, although the short-term, change over study design did not permit an accurate assessment of body tissue change to be made.

Ways to mitigate a potential reduction in performance due to lower dietary CP concentrations is to increase MCP synthesis in the rumen by increasing rumen fermentable energy, or to improve the utilization of MP by supplementing with rumen-protected EAA (Lee et al., 2012a; Sinclair et al., 2014; Giallongo et al., 2016). Feeding additional rumen degradable starch has been shown to increase MCP synthesis in the rumen, increasing the capture of recycled urea and improving ruminal N utilization (Davies et al., 2013). The effect of feeding rumen-protected EAA on dairy cow performance is variable (Robinson, 2010), although supplementation with rumen-protected AA such as Met (**RPMet**), which is the first limiting in red clover silage based diets as suggested by Broderick (2018), may improve performance and NUE in low protein (150 g CP/kg DM) diets (Sinclair et al., 2014). To the best of our knowledge no study has examined the effect of dietary starch concentration or RPMet in low CP diets based on red clover and grass silages.

We hypothesized that reducing the dietary CP content in a red clover and grass silage-based diet would improve NUE, but may decrease intake or performance, and that supplying additional rumen fermentable energy in the form of dietary starch, or supplementation with RPMet, may enhance intake, milk or milk component yield, and NUE. The objectives of our study were to determine the effects of reducing the dietary CP content and supplementation with additional rumen fermentable energy in the form of barley starch, or RPMet on the performance, metabolism, urinary N excretion and NUE in dairy cows fed a diet based on red clover and grass silages for 14 weeks.

MATERIALS AND METHODS

Animals and Housing

All procedures, including animal care and experimentation, were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986 (amended 2012), and received local ethical approval by Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

Fifty-six Holstein-Friesian dairy cows (8 primiparous and 48 multiparous) that were 39 ± 13 DIM (mean ± SD), producing 42.3 ± 6.83 kg milk per d with a mean BCS of 2.8 ± 0.30 (where 1 = emaciated and 5 = obese; scored at 0.25 units; Ferguson et al., 1994) and BW of 671 ± 84 kg measured during the week before allocation were used. Cows were loose housed in a barn containing free stalls fitted with mattresses. Stalls were bedded twice weekly with lime and sawdust, and passageways scraped every 6 h by an automatic scrapper. To determine N-balance and diet digestibility, at the end of the 14-week feeding period 20 representative multiparous cows (5 blocks) were transferred to individual tied stalls fitted with rubber mattresses for 6 d, which included 2 d adaptation and 4 d collection of urine and feces.

Forages and Diet

A first and second cut of grass silage were made from grass (*Lolium perenne*) that was mown at the leafy stage on 25 May and 5 July 2019, wilted for 36 h and harvested with a self-propelled precision-chop forage harvester (John Deere 7840i) with an additive (cfu/g fresh material applied; 100,000 *Lactobacillus plantarum*, 100,000 *Pediococcus pentacaseus,* 100,000 *Lactobacillus buchneri* and 20,000 *Propionibacterium propionici*). The red clover (*Trifolium pretense*, var. AberClaret) was mown at early bloom, wilted for 24 h and harvested using the same forage harvester on 5 July 2019, with the same additive applied at the same rate. The grass and red clover silages were ensiled in separate roofed, concrete walled silage clamps.

Based on measurements during the 7 d before allocation, the cows were blocked by parity, DIM, and milk yield and randomly allocated 1 of 4 experimental diets for 14 weeks. During the pre-study period, all cows were fed the same diet that was composed (g/kg DM) of: corn silage 382, alfalfa silage 168, rolled barley 136, canola meal 80, wheat distillers dark grains 80, molasses 51, Hi-Pro Soybean meal 33, Palm kernel 22, rumen-protected fat (Megalac®, Volac International Ltd., Hertfordshire, UK), minerals and vitamins 14, and formulated to contain 176 g CP/kg DM. The treatment diets were: high CP diet containing 175 g CP/kg DM (**CON**); low CP diet containing 150 g CP/kg DM (**LP**); LP diet with additional dietary starch achieved by replacing sugar beet pulp and soyhulls with ground barley (+ 64 g starch/kg DM; **LPS**) or LP diet with added synthetic form of RPMet (MetaSmart ®; Adisseo Feeds; +0.3 g/100 g MP; **LPM**). The diets were fed as a TMR with a 50:50 ratio (DM basis) of red clover to grass silage, and a forage-to concentrate ratio of 53:47 (DM basis; Table 1). The diets were formulated to produce an average of 37 kg of milk per day over the study period according to Thomas (2004), and contain a similar MP content but differ in their CP, starch or Met concentration. To maintain the MP supply of the low CP diets, additional rumen-protected protein (heat-treated rapeseed meal and xylose-treated soybean meal) were used. During the N-balance and digestibility period the cows remained on the same dietary treatments, and were fed at the same time and rate, and had continuous access to fresh drinking water at all times.

A forage mixer wagon (MixMax 10, Hi-Spec Engineering Limited; Carlow, Ireland) calibrated to ± 1 kg was used daily to mix the dietary ingredients for 10 min, and the TMR was fed through electronic feed bins (Insentec B.V.), fitted with automatic animal identification and weighing system calibrated to ± 0.1 kg. A total of 32 feed bins were used with an average ratio of 1.1 cows per bin. At approximately 0800 h, fresh feed was delivered once daily at a rate of 1.05 of the previous days intake. Refusals were removed 3 times per week before feeding. All animals had continuous access to fresh drinking water.

Sampling Procedure

Forage samples were collected twice per week, the sample split and the DM determined using a forced-air oven at 105°C, and the ratio of red clover to grass silage adjusted weekly to maintain the ratio of 50:50, and the forage to concentrate ratio of 53:47 (DM basis), with the second sample stored at −20°C for subsequent analysis. The TMR were sampled weekly, and stored at −20°C for subsequent analysis. The particle size of the forages and TMR were determined once per month by manual shaking (Kononoff et al., 2003) using a modified Penn State Particle Separator as described by Tayyab et al. (2018).

The in situ degradability of the red clover and grass silages was determined using 3 mature, nonlactating Holstein-Friesian that had previously been fitted with a rumen-cannulas (10 cm in diameter, Bar Diamond) with incubation times of 0, 4, 8, 16, 24, 48 and 96 h as described by Huntington and Givens (1997). Further details on cows, diet, and the in situ degradability of the forages are provided by Chowdhury et al. (2023).

The cows were milked through a 40-point internal rotary parlor (GEA AutoRotor Magnum 40) twice daily at approximately 0600 h and 1600 h, with milk yield recorded at each milking and samples collected fortnightly during the morning and evening milking with a preservative added (2-Bromo-2-nitropropane-1,3-diol) before subsequent analyses. Additional milk samples were collected without a preservative at wk 0 and 6 of the study, and stored at −20°C for milk fatty acid (**FA**) determination. The BW and BCS of the cows were recorded after the evening milking at wk 0, 2, 4, 6, 8, 10, 12 and 14.

Blood samples were collected by jugular venipuncture at 1100 h from 10 representative cows per treatment during wk 0, 4, 8 and 14 of the study. The samples were collected into vacutainers containing sodium heparin for BHB and urea determination, or potassium oxalate for glucose determination. Immediately after collection, the samples were centrifuged at 1,600 × g for 15 min at 4°C, and the plasma separated and stored at −20°C for subsequent analysis.

During N-balance and diet digestibility determination the cows (n = 20) were milked twice daily at approximately 0600 and 1600 h using a portable milking machine (Milkline, London, UK). Milk yield was recorded at each milking, and 4 consecutive milk samples (2 morning and 2 evening milking) were collected for subsequent analyses of milk composition. Samples of the TMR were collected daily within 5 min of feeding. The total volume of urine was collected daily during the sampling period using a modified catheter bag secured over the vulva of the cow with Velcro® straps and connected to a 25 L barrel (Johnson et al., 2023). To avoid volatilization of N compounds, 1.8 L of 20% H_2SO_4 (vol/vol) was added to the barrel to reduce the pH of the urine below 3.0 (Schulz et al., 2018). The urine and acid were agitated throughout the day to ensure that they were well mixed. A sub-sample of approximately 1% of the urine was obtained daily, pH immediately recorded and stored at −20°C. Spot fecal samples (approximately 350 g/d per cow) were collected from all cows at 0600, 1100, 1600 and 2100 h for 4 consecutive days (Morris et al., 2018).

Chemical Analysis

Forage and TMR samples were composited by month (performance study) or week (N balance study), and sub-samples analyzed according to AOAC (2012) for DM (934.01, intra-assay coefficient of variation (CV) of 0.12%) by drying samples in a forced-air oven at 60°C. Dried forage and TMR samples were milled using a Wiley mill (Thomas Scientific) fitted with a 1 mm screen before analyses of total N (988.05; CP = 6.25 × total N; intra-assay CV of 0.15%), ether extract (920.39; intra-assay CV of 1.19%), ash (942.05; intra-assay CV of 0.50%) and acid insoluble ash (intra-assay CV of 3.28%) content (Van-Keulen and Young, 1977). The NDF and ADF contents were determined using heat-stable α-amylase (Sigma; intra-assay CV of 0.73 and 1.0% for NDF and ADF, respectively) as per the method of Van Soest et al. (1991). The TMR samples were also analyzed for starch (intra-assay CV of 4.1%) according to ISO 6493 (ISO, 2000) at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK). Forage pH and ammonia-N were determined using the methods of MAFF (1986). Volatile fatty acids, lactic acid and the alcohol content of the red clover and grass silages were analyzed at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) using a water extraction method followed by HPLC and GLC.

Milk samples were analyzed for fat, total protein, lactose, urea and SCC at National Milk Laboratories (Wolverhampton, UK) using a near-midinfrared method and calibrated by the method of AOAC (2012). Feed and milk FAME were prepared according to the protocol of Jenkins (2010) and Feng et al. (2004), respectively. The individual FAME was determined by

Table 1. Dietary ingredients and predicted chemical composition of a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages

Item	Diet[1]			
	CON	LP	LPS	LPM
Dietary ingredients (g/kg DM)				
Red clover silage	262	262	263	262
Grass silage	262	262	263	262
Barley (ground)	146	146	267	145
Soybean meal	50.0	0.00	0.00	0.00
SoyPass[2]	8.33	33.7	33.8	33.7
Soy hulls	154	154	117	154
Molassed sugar beet pulp	39.6	85.3	0.00	85.2
Rapeseed meal	41.6	0.00	0.00	0.00
NovaPro[3]	16.7	37.1	37.1	37.0
MetaSmart[4]	0.00	0.00	0.00	1.66
Rumen-protected fat (Megalac)[5]	14.6	14.6	14.6	14.5
Minerals and vitamins[6] (g/kg DM)	5.00	5.00	5.00	5.00
Predicted composition[7]				
Forage: Concentrate (DM basis)	0.53	0.53	0.53	0.53
CP	176	152	153	152
Starch	94	95	161	95
MPE, g/kg DM	103	100	102	100
MPN, g/kg DM	121	110	110	110
MP (% of requirements)	100	97.0	99.0	97.0
Methionine (g/100 g MP)	2.00	2.00	2.00	2.30

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+ 64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP).

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

[3]Hot pressed, xylose-treated rapeseed meal (KW Alternative Feeds).

[4]Rumen-protected methionine (isopropyl ester of the hydroxy analog of methionine; Adisseo Feeds).

[5]A calcium soap of long chain fatty acids (% of DM: C16:0 = 51, C18:0 = 4, C18:1 *cis*-9 = 36, C18:2 *cis*-9, *cis*-12 = 7, C18:3 *cis*-9, *cis*-12, *cis*-15 = 0.2. Volac International Ltd., Hertfordshire, UK).

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

[7]Calculated using Feed into Milk (Thomas, 2004). MPN = metabolizable protein-rumen nitrogen limited; MPE = metabolizable protein-rumen energy limited.

gas-liquid chromatography (Hewlett Packard 6890), using a CP-Sil 88 column (100 m × 0.25 mm i.d. × 0.2 um film, Agilent Technologies) as described by Lock et al. (2006). Individual FA was calculated using a mixed reference standard (Sigma-Aldrich).

Fecal samples were pooled by cow and dried in a forced-air oven at 60°C. A Cuisinart electric grinder (SG20U) was used to mill dried fecal samples (1 mm) before analysis of total N, ash, NDF, ADF and acid insoluble ash (Van Keulen and Young, 1977). Subsamples of urine were composited within cow, filtered with N free filter paper, and analyzed for total N (AOAC, 2012; 976.06). Plasma samples were analyzed for glucose, urea and BHB (Kit-Catalogue no. GL 1611, UR 221 and RB 1008 with an intra-assay CV of 2.11, 5.18 and 4.69%, respectively) using a Cobas Miras Plus Autoanalyzer (ABX Diagnostics).

Calculations

Based on the daily intake of fresh feed and the analyzed DM content of the diets, the DMI of each cow was calculated. We determined the fecal DM output, digested nutrients, and the apparent whole-tract digestibility coefficients of DM, N, OM, NDF and ADF using acid insoluble ash as an internal marker as described by Van Keulen & Young (1977). Energy-corrected milk (3.14 MJ/kg) yield was calculated using milk yield and composition records according to Sjaunja et al. (1991) as ECM = milk yield × [383 × fat (g/kg) × 100 + 242 × protein (g/kg) × 100 + 165.4 × lactose (g/kg) × 100 + 207)/3140. The 4% FCM yield was calculated by correcting the milk yield to 40 g/kg of milk fat (4% FCM yield = milk yield × fat% /4). The estimated apparent NUE % = (kg milk N/kg dietary N intake) x 100, and milk n = milk protein/6.38. Nitrogen balance was determined by subtracting N output (urinary N + fecal N + milk N) from the total N intake. The geometric mean ($\mathbf{X_m}$) and its SD of TMR and forage particle size were determined using the equations provided by ASABE (2007). The physically-effective fiber of silage and TMR was calculated using the NDF content and physical effectiveness factor (Tayyab et al., 2018).

The in situ DM and CP disappearance data were fitted to the exponential equation $P = a + b\ (1 - \exp^{-ct})$ using Sigmaplot Version 12.0 (Jandel Engineering) as described by Ørskov and McDonald (1979), where; P = disappearance (%) at t time, a = soluble fraction, b = potentially degradable fraction, c = the rate of degradation (per h) of b, t = incubation time (h). The ruminal effective degradability (**ED**) was calculated assuming a rumen passage rate (k) of 0.08%/h as: ED = a + b [c/(c + k)].

Statistical Analysis

Statistical analysis was conducted using GenStat 19th Edition (VSN International Ltd.), UK), with variables such as feed intake, milk performance, BW, BCS, and blood parameters analyzed as repeated measures ANOVA including covariates of the pre-experimental period according to the following model: $Y_{ijk} = \mu + B_i + P_j + T_k + P_j.T_k + COV + \varepsilon_{ijk}$ where Y_{ijk} = dependent variable; μ = overall mean; B_i = random effect of blocks; P_j = main effect of protein treatment; T_j = effect of time (time of day or week of study depending on the variable) and $P_j.T_k$ = interaction between dietary protein treatment and time; COV is the effect of the covariate; and ε_{ijk} = residual error. Other parameters such as whole tract digestibility and N partitioning were analyzed by ANOVA according to the model: $Y_{ijk} = \mu + B_i + P_j + \varepsilon_{ij}$ where Y_{ij} = dependent variable; μ = overall mean; B_i = random effect of blocks; P_j = main effect of protein treatment; and ε_{ijk} = residual error. In situ degradability data were also analyzed by ANOVA using the model $Y_{ij} = \mu + F_i + A_j + \varepsilon_{ij}$ where Y_{ij} = dependent variable; μ = overall mean; F_i = effect of forage; A_j = random effect of animal; and ε_{ij} = residual error.

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

RESULTS

Forage and Diet Analysis

Our red clover silage was 57 g/kg DM higher in CP, but had a lower content of NDF, ammonia-N and pH compared with the grass silage (Table 2). The DM, OM, NDF, ADF and EE concentrations of the TMR were similar, whereas the CP concentration was 21 g/kg DM higher in CON than other 3 diets (LP, LPS, or LPM). The total FA content of the diets was also comparable, although LPM was 1.4 g/kg DM lower in FA than the other 3 diets, which had a mean of 19.9 g FA/kg DM. The calculated ED of DM and CP at a rumen outflow rate of 8% per hour was 7.5 and 7.6% higher in the grass compared with the red clover silage while the particle size distribution of each of the TMR was similar (Supplementary Tables 1 and 2),

Table 2. Nutrient composition (g/kg DM), fermentation profile, fatty acid content, and particle size distribution of grass silage, red clover silage, and a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages

	Forages		Diet[1]			
Item	Grass silage	Red clover silage	CON	LP	LPS	LPM
DM (g/kg)	378	355	484	472	470	475
CP	126	183	173	151	153	152
OM	906	881	914	915	920	913
NDF	475	372	384	399	362	397
ADF	282	307	267	275	252	269
Starch	—	—	95	94	158	94
Ether extract	21.7	16.5	24.6	26.9	27.7	27.0
Ash	94.3	119	85.9	84.8	79.9	86.7
Fermentation profile (g/kg DM)						
pH	4.11	3.96	—	—	—	—
Ammonia-N (g/kg total N)	73.1	51.3	—	—	—	—
Lactate	67.1	70.5	—	—	—	—
Ethanol	2.87	0.76	—	—	—	—
Acetate	27.5	18.9	—	—	—	—
Propionate	0.51	0.37	—	—	—	—
Iso-butyrate	—	0.55	—	—	—	—
Butyrate	0.77	0.20	—	—	—	—
Acetate: propionate	54.1	50.4	—	—	—	—
Amino acids (% of CP)						
Histidine	1.1	1.7	1.8	1.8	2.1	2.1
Leucine	5.5	3.9	6.1	6.3	6.5	6.3
Lysine	3.2	4.8	4.4	4.5	4.1	4.6
Methionine	1.5	0.9	1.3	1.2	1.3	1.8
Threonine	3.2	3.9	3.6	3.7	3.9	3.8
Fatty acid (g/kg DM)						
C16:0	2.42	2.26	5.33	5.18	5.16	4.43
C18:0	0.23	0.29	0.65	0.62	0.60	0.56
C18:1*cis*-9	0.57	0.26	4.29	4.31	4.31	3.81
C18:2 *cis*-9, *cis*-12	2.14	2.41	3.75	3.74	4.00	3.61
C18:3 *cis*-9, *cis*-12, *cis*-15	4.44	4.60	3.20	3.17	3.38	3.34
Total fatty acids	19.8	16.3	19.9	19.7	20.1	18.5

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP).

Performance and Metabolism

Feed Intake and Animal Performance. Dry matter intake did not differ between cows fed any of the treatment diets, with a mean value of 21.5 kg/d (Table 3), although we found an interaction ($P = 0.007$) between diet and week (Supplementary Figure 1), with intake being highest in cows fed LPS in wk 4 and CON in wk 9 and 14. Milk yield, FCM yield, or ECM yield did not differ between cows fed any of the diets, with mean values of 37.3, 37.8 and 36.4 kg/d, respectively. Similarly, diet had no effect on milk fat, protein, or lactose contents, or SCC. In contrast, MUN concentration was approximately 32% higher ($P < 0.001$) in cows fed CON at 9.4 mg/dL than those fed LP, LPS, or LPM that had a mean value of 6.5 mg/dL. Feed conversion efficiency was not affected by diet, with a mean value of 1.75 kg milk/kg DMI; however, we found a tendency ($P = 0.082$) for ECM/kg DM intake to be lower in cows fed CON compared with LPM. Diet had no effect on BW or BCS, with mean values of 671 kg and 2.65 respectively. Apparent NUE was lowest in cows fed CON at 28.6%, which was 5.6 percentage units lower ($P < 0.001$) than those fed any of the low CP diets (LP, LPS, or LPM) that had a mean of 34.2%.

Plasma Metabolites The mean concentration of plasma glucose was 3.98 mmol/L and was not affected by diet (Figure 1a). In contrast, the mean concentration of plasma BHB was highest (0.92 mmol/L, $P = 0.003$) in cows receiving LPM, which increased over the study period (Figure 1b), and lowest in LPS (0.70 mmol/L), but was similar in animals fed CON or LP with a mean value of 0.79 mmol/L. We found an effect of diet on the mean concentration of plasma urea (Figure 1c), which was 1.44 mmol/L higher (3.62 mmol/L, $P < 0.001$) in cows fed CON compared with those fed any of the low protein diets, with a mean of 2.18 mmol/L. We also observed an effect of sampling week ($P = 0.008$) and interaction between diet and week ($P = 0.002$) for plasma urea, which was similar between treatments at wk 0 (5.03 mmol/L), but was higher in cows fed CON, at wk 4, 8 and 14 with a mean value of 3.60, 3.62, and 3.64 mmol/L respectively, compared with those fed any of the low protein diets with a mean of 2.07, 1.98 and 2.48 mmol/L respectively, which did not differ.

Milk Fatty Acid Profile The milk fat proportions of C10:0 and C12:0 were 0.22 and 0.32 g/100 g higher respectively ($P < 0.05$) in cows fed CON or LPS compared with LP or LPM (Table 4). Similarly, milk fat proportions of C14:0 and C15:1 *cis*-10 were 0.70 and 0.03 g/100 g higher respectively ($P < 0.05$) in cows fed CON compared with those fed any of the low protein diets, with LPS having an intermediate value for C14:0. The lowest ($P < 0.05$) milk fat proportion of C17:0 and C17:1 *cis*-10 was observed in cows fed LPS, with CON having an intermediate value for milk C17:1 *cis*-10. In contrast, the proportion of C18:1 *trans*-12 was 0.033 g/100 g higher ($P < 0.001$) in milk from cows fed LPS compared with the other diets. We found a tendency ($P < 0.10$) for C18:0 to be higher in milk from cows fed LP than CON, and for C18:1 *cis*-9 to be higher in milk from LP than LPS. We also observed that dietary treatment did not affect the total milk fat proportion of SFA, MUFA, PUFA, linear odd-chain, or odd-and branched-chain FA (**OBCFA**), but there was 1.35 g/100 g higher proportion of FA with a chain length < C16:0 ($P = 0.032$) in milk from cows fed CON compared with those fed LP or LPM that had a mean of 24.5 g/100 g, with cows fed LPS having an intermediate value. In contrast, the total milk fat proportion of chain length > C16:0 was 1.95 g/100 g higher ($P = 0.048$) in cows fed LP than those fed CON or LPS that had a mean of 34.9 g/100 g, with cows fed LPM having an intermediate value.

N -balance and diet digestibility We found no effect of diet on DMI of the cows during the N-balance and digestibility period, with a mean value of 21.5 kg/d, and there was no effect of dietary treatment on the total milk yield, FCM yield, or ECM yield, with mean values of 36.6, 41.2 and 38.0 kg/d, respectively (Supplementary Table 3). The intake of N was highest ($P = 0.020$) in cows fed CON at 618 g/d, lowest in LP or LPM with a mean of 516 g/d, and intermediate in LPS (Table 5). Diet had no effect on fecal N or milk N excretion, but daily urinary N output was 65.7 g higher ($P = 0.011$) in cows fed CON compared with those fed LP or LPM with a mean of 92.1 g/d, while cows receiving LPS had an intermediate value. We also observed a difference ($P = 0.017$) between di-

Table 3. Feed intake, milk performance, BW and body condition of dairy cows fed a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages (n = 14 cows per treatment)

	Diet[1]					*P*-value		
Item	CON	LP	LPS	LPM	SEM	D	T	Int.
Intake (kg DM/d)	22.0	21.1	21.7	21.2	0.59	0.371	<0.001	0.007
Production (kg/d)								
Milk yield	37.9	36.3	37.8	37.1	0.73	0.170	<0.001	0.957
FCM[2] yield	37.2	37.2	38.6	38.1	1.61	0.776	0.003	0.919
ECM yield	36.5	35.6	37.1	36.4	1.05	0.629	0.001	0.930
Composition (g/kg)								
Fat	39.4	40.9	41.6	41.3	1.67	0.581	0.021	0.755
Protein	29.9	30.1	31.3	30.7	0.68	0.400	<0.001	0.820
Lactose	45.7	45.8	46.1	45.5	0.44	0.672	0.273	0.872
SCC ($\log_{10}$)	3.24	3.17	3.29	3.59	0.290	0.641	0.236	0.678
MUN (mg/dL)	9.44[a]	6.91[b]	6.70[b]	5.77[b]	0.534	<0.001	0.014	0.420
Yield (kg/d)								
Fat	1.49	1.49	1.55	1.50	0.068	0.801	0.014	0.899
Protein	1.14	1.09	1.16	1.12	0.033	0.263	0.054	0.534
Lactose	1.75	1.67	1.72	1.66	0.049	0.318	0.067	0.498
Feed efficiency								
FCM/DMI	1.66	1.75	1.77	1.82	0.081	0.130	0.026	0.042
ECM/DMI	1.62	1.68	1.70	1.74	0.061	0.082	0.006	0.010
Body performance								
BW (kg)	667	665	678	673	8.0	0.605	<0.001	0.854
BW change (kg/d)[3]	0.05	0.02	0.13	0.18	0.126	0.600	—	—
BCS	2.67	2.63	2.70	2.59	0.051	0.199	0.138	0.075
BCS change[3]	−0.03	−0.10	−0.02	−0.16	0.083	0.340	—	—
NUE[4] (%)	28.6[b]	33.9[a]	34.1[a]	34.7[a]	1.31	<0.001	0.011	0.003

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP). D = main effect of diet; T = main effect of week; Int. = interaction between diet and week.

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

[3]Change over the 14 weeks feeding period.

[4]NUE = apparent nitrogen use efficiency (kg milk N output/ kg N intake) x 100.

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

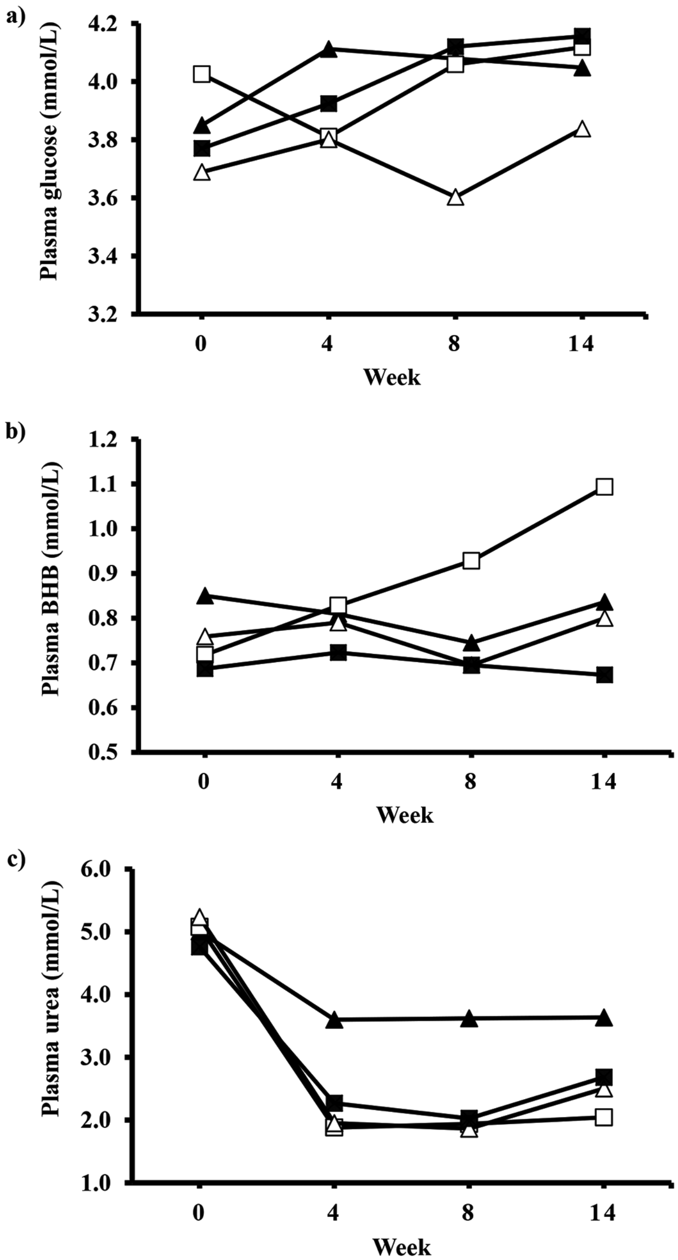

Figure 1. Plasma glucose (a), BHB (b) and urea (c) concentrations in dairy cows fed a control (CON, ▲), low protein (LP, Δ), LP with added dietary starch (LPS, ■) or RPMet (LPM, □) diets based on red clover and grass silages. For plasma glucose; pooled SEM = 0.196; diet, $P = 0.178$, week, $P = 0.556$ and diet × week, $P = 0.802$. For plasma BHB; pooled SEM = 0.067; diet, $P = 0.003$, week, $P = 0.182$ and diet × week, $P = 0.321$. For plasma urea; pooled SEM = 0.189; diet, $P < 0.001$, week, $P = 0.008$ and diet × week, $P = 0.002$ (n = 10 cows per treatment).

ets on apparent NUE for milk production, which was increased in cows fed LP or LPM with a mean of 34.7, approximately 6.3 percentage units higher than those fed CON, with LPS having an intermediate value. The mean urea-N concentration in milk and urine was 4.05 and 55.9 mg/dL higher ($P < 0.05$) in cows fed CON compared with those fed any of the low protein diets with mean values of 7.85 and 79.1 mg/dL, respectively. We found a positive relationship ($R^2 = 0.52$; $P < 0.001$) between milk urea concentration and urinary N output (Supplementary Figure 2).

We found no effect of diet on the intake or fecal output of DM, OM, NDF and ADF (Table 6). Digestible N (g/d) was highest ($P = 0.037$) in cows fed CON, lowest in LP, with LPS or LPM having an intermediate value of 0.35 kg/d. We observed no difference between diets on the apparent whole-tract digestibility of DM, OM, N, NDF and ADF, with mean values of 0.766, 0.785, 0.660, 0.660 and 0.652 kg/kg, respectively.

DISCUSSION

Forage and Diet Characterization

The CP concentration of our red clover silage was 45% higher than the grass silage, which is comparable to our previous work (Chowdhury et al., 2023), and with that reported by Broderick (2018) and Dewhurst et al. (2003b). In general, legumes contain less fiber and more protein than grass silage (Dewhurst, 2013), and we observed that the NDF content was 103 g/kg DM higher in the grass silage compared with the red clover silage, which was also reflected in a greater content of physically-effective fiber in the grass silage (Supplementary Table 1). The grass silage had a higher pH, which may be explained by a higher DM content and a more restricted fermentation, although the lactic, acetic and propionic acid content was similar in both forages when expressed on a DM basis.

The soluble fraction of DM and CP in the grass silage was higher than the red clover silage, a finding in accordance with previous work by Dewhurst et al. (2003a). Previous studies have shown that the potential degradable CP fraction of red clover silage is higher than grass silage (Dewhurst et al., 2003a; Purwin et al., 2014), which is consistent with our findings. In our study, the calculated ED of DM and CP was lower in red clover compared with the grass silage, which might be related to the presence of the enzyme PPO in red clover (Lee, 2014). This enzyme reacts with phenols in the presence of oxygen to produce quinones, which inhibit the role of proteases that degrade forage proteins (Jones et al., 1995; Broderick et al., 2001). The higher concentration of ammonia in the grass silage could also partially explain the high calculated ED compared with the red clover silage (Purwin et al., 2014).

Table 4. Milk fatty acid composition of dairy cows fed a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages (n = 14 cows per treatment)

	Diet[1]					
Fatty acid (g/100 g)	CON	LP	LPS	LPM	SEM	*P*-value
C4:0	1.67	1.72	1.65	1.68	0.035	0.522
C6:0	1.53	1.53	1.55	1.50	0.022	0.474
C8:0	1.10	1.07	1.13	1.05	0.021	0.062
C10:0	2.86[a]	2.65[b]	2.87[a]	2.64[b]	0.071	0.041
C11:0	0.06	0.05	0.07	0.06	0.006	0.122
C12:0	3.62[a]	3.28[b]	3.63[a]	3.33[b]	0.100	0.029
C13:0	0.10	0.08	0.11	0.10	0.006	0.076
C14:0	12.4[a]	11.7[b]	12.0[ab]	11.7[b]	0.18	0.036
C14:1 *cis*-9	1.08	1.02	1.08	1.05	0.034	0.494
C15:0	1.21	1.09	1.15	1.19	0.039	0.158
C15:1 *cis*-10	0.21[a]	0.19[b]	0.18[b]	0.18[b]	0.007	<0.001
C16:0	37.9	37.4	38.2	37.7	0.44	0.620
C16:1 *cis*-9	1.35	1.40	1.47	1.46	0.073	0.661
C17:0	0.54[a]	0.55[a]	0.49[b]	0.55[a]	0.010	<0.001
C17:1 *cis*-10	0.28[ab]	0.30[a]	0.25[b]	0.29[a]	0.007	<0.001
C18:0	8.66	9.41	8.93	9.22	0.213	0.078
C18:1 *trans*-8	0.22	0.23	0.19	0.24	0.022	0.457
C18:1 *trans*-9	0.26	0.10	0.17	0.15	0.057	0.274
C18:1 *trans*-10	0.91	0.95	0.79	0.92	0.067	0.365
C18:1 *trans*-11	0.84	0.83	0.81	0.85	0.024	0.687
C18:1 *trans*-12	0.15[c]	0.17[bc]	0.20[a]	0.18[b]	0.005	<0.001
C18:1 *cis*-9	19.0	20.2	18.8	19.7	0.42	0.081
C18:2 *cis*-9, *cis*-12	2.01	2.18	2.26	2.18	0.069	0.106
C18:2 *cis*-9, *cis*-12	0.35	0.36	0.39	0.35	0.010	0.087
CLA *cis*-9, *trans*-11	0.82	0.88	0.86	0.86	0.022	0.327
CLA *trans*-10, *cis*-12	0.03	0.03	0.03	0.03	0.002	0.386
C18:3 *cis*-9, *cis*-12, *cis*-15	0.33	0.34	0.32	0.33	0.009	0.302
C18:3 *cis*-6, *cis*-9, *cis*-12	0.06	0.07	0.07	0.07	0.002	0.241
C20:0	0.01	0.02	0.01	0.01	0.001	0.285
C20:3 *cis*-11, *cis*-14, *cis*-17	0.12	0.12	0.12	0.12	0.004	0.665
C21:0	0.07	0.07	0.07	0.06	0.003	0.246
C22:0	0.04	0.04	0.04	0.04	0.003	0.815
EPA[2]	0.11	0.10	0.09	0.10	0.004	0.170
DHA[3]	0.07	0.07	0.07	0.07	0.002	0.990
Summation						
<C16:0	25.8[a]	24.4[b]	25.5[ab]	24.5[b]	0.38	0.032
C16:0 + C16:1	39.3	38.9	39.6	39.1	0.48	0.744
>C16	35.0[b]	36.9[a]	34.9[b]	36.2[ab]	0.56	0.048
SFA[4]	71.8	70.6	71.9	70.9	0.50	0.185
MUFA[5]	24.3	25.3	24.0	25.1	0.45	0.144
PUFA[6]	3.88	4.15	4.22	4.11	0.105	0.137
LOCFA[7]	1.98	1.84	1.88	1.97	0.054	0.211
OBCFA[8]	2.47	2.33	2.31	2.44	0.051	0.090

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP).

[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]SFA = saturated fatty acids are defined as fatty acids with no double bonds.

[5]MUFA = monounsaturated fatty acids are defined as fatty acids with one double bond.

[6]PUFA = 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).

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

Animal Performance, Metabolism and Milk fatty Acid Profile

Reducing dietary CP concentration did not affect the DMI of cows fed our red clover and grass silage-based diets, a finding in agreement with that observed by Broderick et al. (2015) who reported a similar DMI when dietary CP concentration was reduced from 170 to 150 g/kg DM in an alfalfa and corn silage-based diet.

Table 5. Nitrogen partitioning in dairy cows fed a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages (n = 5 cows per treatment)

Item	Diet[1]				SEM	*P*-value
	CON	LP	LPS	LPM		
N intake and excretion (g/d)						
Intake	618[a]	521[b]	530[ab]	510[b]	22.7	0.020
Fecal	182	214	169	169	19.1	0.343
Milk	174	179	173	177	6.6	0.885
Urine	156[a]	90.3[b]	106[ab]	93.9[b]	12.72	0.011
N balance[2]	106	37.3	82.0	70.6	22.00	0.226
N partitioning (%)						
Fecal	29.8	41.7	31.8	32.9	3.84	0.191
Urine	25.1[a]	17.4[b]	19.9[ab]	18.4[ab]	1.59	0.023
NUE[3]	28.4[b]	34.5[a]	32.6[ab]	34.9[a]	1.31	0.017
Urea-N concentration (mg/dL)						
Milk	11.9[a]	8.15[b]	8.36[b]	7.05[b]	0.753	0.004
Urine	135[a]	63.1[b]	95.9[b]	78.3[b]	8.58	<0.001

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP). Measured using 20 cows (5 cows per treatment diet, except plasma urea-N, which was calculated using the week-14 data from study 1a.

[2]N balance (g/d) = Intake N – (Milk N + Fecal N + Urine N).

[3]NUE = apparent nitrogen use efficiency (kg milk N output/ kg N intake) x 100.

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

Table 6. Intake, fecal output, digested values (kg/d) and apparent whole-tract digestibility (kg/kg) of nutrients in dairy cows fed a control (CON), low protein (LP), LP with added dietary starch (LPS) or RPMet (LPM) diets[1] based on red clover and grass silages (n = 5 cows per treatment)

Item	Diet[1]				SEM	*P*-value
	CON	LP	LPS	LPM		
DM						
Intake	22.1	21.4	21.6	21.0	0.91	0.848
Fecal output	4.95	5.82	4.66	4.67	0.482	0.324
Digested	17.2	15.6	16.9	16.3	0.90	0.626
Digestibility (kg/kg)	0.773	0.726	0.785	0.779	0.0226	0.278
N						
Intake	0.62[a]	0.52[b]	0.53[ab]	0.51[b]	0.023	0.020
Fecal output	0.18	0.21	0.17	0.17	0.019	0.343
Digested	0.44[a]	0.31[b]	0.36[ab]	0.34[ab]	0.028	0.037
Digestibility (kg/kg)	0.702	0.583	0.682	0.671	0.0384	0.191
OM						
Intake	20.3	19.7	20.0	19.2	0.84	0.818
Fecal output	4.16	4.89	4.00	3.87	0.418	0.354
Digested	16.2	14.8	16.0	15.4	0.84	0.637
Digestibility (kg/kg)	0.792	0.748	0.801	0.800	0.0216	0.308
NDF						
Intake	8.07	7.99	7.28	7.67	0.369	0.442
Fecal output	2.58	3.02	2.50	2.41	0.251	0.373
Digested	5.50	4.97	4.78	5.25	0.374	0.569
Digestibility (kg/kg)	0.675	0.621	0.659	0.686	0.0313	0.502
ADF						
Intake	5.71	5.42	5.26	5.15	0.229	0.372
Fecal output	1.88	2.19	1.72	1.69	0.190	0.269
Digested	3.83	3.22	3.54	3.46	0.274	0.495
Digestibility (kg/kg)	0.670	0.590	0.675	0.670	0.0371	0.348

[1]CON = Control (175 g CP/kg DM); LP = low protein (150 g CP/kg DM); LPS = LP with added starch (+64 g/kg DM); LPM = LP with added RPMet (+0.3 g/100 g MP). Measured using 20 cows (5 cows per treatment diet).

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

A similar response has also been reported by Kidane et al. (2018) and Olmos Colmenero and Broderick (2006), who fed lower CP concentrations (175 to 130 g CP/kg DM, and 194 to 135 g CP/kg DM, respectively). In contrast, our previous study (Chowdhury et al., 2023) reported a decrease in DMI of 1.6 kg/d when the dietary CP concentration was reduced from 175 to 150 g/kg DM in a red clover and grass silage-based diet. This may have been due to insufficient rumen degradable N that impaired fibrolytic bacteria activity (Allen, 2000), as whole tract apparent fiber digestibility was also decreased.

The inclusion of additional starch or RPMet to our low CP diet did not affect DMI, a finding in agreement with that observed by others who have examined the effect of MP deficient diets based on alfalfa-corn silage supplemented with rumen-protected EAA (Lee et al., 2012a; Recktenwald et al., 2014; Giallongo et al., 2016). Meta-analysis that have investigated the effect of RPMet on DMI in dairy cows have also reported a lack of a response (Robinson, 2010), although few studies have investigated the effect in red clover and grass silage based diets. In contrast, others have reported both an increase or decrease in DMI to RPMet supplementation (Patton, 2010; Zanton et al., 2014), which may be attributed to factors such as the source of RPMet (Zanton et al., 2014), the concentration of Met supplementation, or the presence of other limiting EAA (Patton, 2010). Supplementation with additional starch also has variable effects on DMI, with an increase or no effect having been reported when moderate levels of dietary starch (rolled wheat, 220 g starch/kg DM) have been fed (McCaughern et al., 2020), or a decrease when high levels of starch that result in sub-acute ruminal acidosis have been fed (Zebeli et al., 2012). The starch content was approximately 64 g/kg DM higher in LPS than LP, although the highest starch concentration of 158 g/kg DM in our study was still comparatively low, and unlikely to have had a major negative effect on rumen pH.

In line with previous studies (Olmos Colmenero and Broderick, 2006; Bahrami-Yekdangi et al., 2014; Hynes et al., 2016) that have fed alfalfa silage, hay, or grass silage diets, we did not find an effect of dietary CP on milk yield or milk composition in early lactation cows fed red clover silage. A similar result was reported by Barros et al. (2017) when the concentration of CP was reduced from 162 to 144 g/kg DM in an alfalfa and corn silage based diet. The similar milk performance in our cows across all of the treatments suggests that the dietary CP concentration and MP supply in our low CP red clover and grass silage-based diets was sufficient to maintain production performance. However, further reducing dietary CP concentration below 150 g/kg DM may negatively affect milk production (Lee et al., 2012a; Alstrup et al., 2014). We also observed that milk performance did not improve with supplementation with either RPMet or dietary starch. The lack of a response to RPMet is also surprising as the benefits to EAA supplementation are likely to be greatest when feeding low CP diets (Sinclair et al., 2014). The similarity in DMI between treatments may possibly explain the lack of an effect on milk yield, or that other EAA limited milk yield and milk protein synthesis. Several other studies have also reported a lack of an effect of additional starch (McCaughern et al., 2020) or rumen-protected EAA (Giallongo et al., 2015, 2016; Lee et al., 2015) on the lactation performance of dairy cows.

In our previous study (Chowdhury et al., 2023), feeding a low CP (150 g/kg DM) red clover and grass silage-based diet had no significant effect on BW or BCS, although the short-term, change-over study design did not permit an accurate assessment. In line with results from previous studies (Giallongo et al., 2016; Hynes et al., 2016; Barros et al., 2017a), reducing dietary CP in our current study did not alter BW or BCS, most probably because of a lack of an effect on DMI.

Feeding low CP diets, either without or with supplementation with starch or RPMet, decreased plasma and milk urea concentrations, an effect in accordance with most studies that have investigated the effects of dietary protein or MP deficient diets, either without or with the addition of rumen-protected EAA (Chowdhury et al., 2023; Lee et al., 2012a; Van den Bossche et al., 2023). It is generally accepted that feeding higher dietary CP levels increases ammonia absorption from the rumen which is reflected in a higher concentration of urea in the blood, and a greater output of milk urea (Bach et al., 2000). A study by Olmos Colmenero and Broderick (2006) confirmed that both BUN and MUN were highly correlated ($r = 0.83$), and in our study both MUN and plasma urea-N were lowest in cows fed any of the low CP diets.

The higher concentration of plasma BHB in cows fed LPM could be attributed to a mobilization of body reserves which might be associated with a lower BCS, although we found no effect on BW or BCS. The relationship between RPMet supplementation and BHB levels is complex, and may depend on factors such as dietary energy intake level and source, hepatic health, and genetic variation. Moreover, supplementation of Met leads to increased protein synthesis that might indirectly affect the balance of energy metabolism (Sinclair et al., 2014). Law et al. (2009) noted that the plasma concentration of BHB was increased by 0.08 mmol/L in cows fed a very low CP diet (114 g CP/kg DM), but there was no effect of diet on BCS change. In contrast, several other studies (Alstrup et al., 2014;

Kaufman et al., 2020) have reported no effect of dietary CP on plasma BHB concentration. In line with previous reports (Bach et al., 2000; Bahrami-Yekdangi et al., 2014; Giallongo et al., 2016) we did not observe an effect of dietary CP level on plasma glucose concentration. However, plasma metabolites such as glucose are often not affected by the inclusion of RPMet in low CP or MP deficient diets (Giallongo et al., 2015, 2016).

Dietary CP concentration had little influence on the individual FA profile of the milk in our study. The highest proportion of FA of chain length < C16:0 and OBCFA were observed in milk from cows fed CON or LPM, which could be attributed to higher levels of RDP which may have led to an increased rumen-microbial growth, as OBCFA are principally derived from rumen microbial lipids, and have been suggested as markers to predict MCP synthesis (Vlaeminck et al., 2006). A study by Leduc et al. (2017) reported a positive relationship between the supply of RDP and yield of OBCFA, while other work (Giallongo et al., 2015) also reported an increased yield of OBCFA in milk when the diet was supplemented with RPMet or Met analog. In contrast, Giallongo et al. (2016) reported that rumen-protected EAA, including Met, had little or no effect on milk FA composition. The milk FA proportions of chain length > C16:0, along with C18:2 *cis*-9, *trans*-12, were increased when cows were fed LP compared with CON. This is difficult to explain as the FA profile of all diets was similar. We also did not expect the higher proportion of intermediary biohydrogenation products such as C18:1 *trans*-12 in the milk fat of cows fed the low CP diets, which may possibly due to a higher supply of rumen protected C18:1-enriched expeller rapeseed meal (Hristov et al., 2011). Apart from feed FA, the other source of C18:0 in milk is body adipose tissue mobilization (Alstrup et al., 2014), but we did not observe any differences in BW or BCS change

Digestibility and N-balance

It has been suggested that whole tract nutrient digestibility in dairy cows may be reduced if the CP content is below 165 g/kg DM (Olmos Colmenero and Broderick, 2006). Similarly, other studies (Chowdhury et al., 2023; Lee et al., 2012b; Giallongo et al., 2015) have reported a lower nutrient digestibility when low CP (135 to 150 g/kg DM) or MP deficient (−5 to −15% below requirement) diets were fed to dairy cows. The effect of low protein diets on nutrient digestibility could be due to a lower supply of RDP and an insufficient concentration of rumen ammonia, which may decrease the growth of rumen microorganisms and result in a depressed fiber digestibility and intake (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a). However, we did not observe any difference between diets in apparent whole-tract nutrient digestibility or intake, which indicates that rumen function was not impaired when low CP (150 g/kg DM) or marginally MP-deficient (−2% less than requirements) diets were fed, as long as RDP supply is sufficient.

We observed that addition of starch or RPMet had no effect on nutrient digestibility. The precise role of rumen-protected EAA including Met, Lys or His on feed intake and digestibility is not clear and difficult to predict (Lee et al., 2012a; Sinclair et al., 2014; Giallongo et al., 2015). A study by Davies et al. (2013) also reported that diets with additional rumen degradable starch had no effect on total-tract nutrient digestibility in beef heifers. To our knowledge, limited research exists that has examined the effect of supplementary starch on nutrient digestibility and metabolism on low protein, red clover-based diets fed to dairy cows.

A meta-analysis undertaken by Huhtanen and Hristov (2009) reported that increasing the capture of N for milk protein synthesis improves NUE, and that a higher concentration of N in urine leads to an inefficient partitioning of N. In agreement with previous studies (Olmos Colmenero and Broderick, 2006; Lee et al., 2012a; Kidane et al., 2018), we observed an increase in apparent NUE of approximately 20% when the content of dietary CP was reduced. Reducing the dietary CP concentration from 175 to 150 g/kg DM in our study had little effect on milk N output, but we found that it decreased urinary N output by 50 to 66 g/d. Over a 305-d lactation feeding these low CP diets would reduce urinary N excretion by 15 to 20 kg N/cow. Additionally, as urinary urea is the principal source of atmospheric release of ammonia from dairy systems (Defra 2019), feeding low CP diets can reduce ammonia emissions and the formation of fine particulate material in the atmosphere. Similarly to our findings, Giallongo et al. (2015) also reported a substantial decrease in urinary N excretion of 55 g/d when the dietary CP content was reduced from 167 to 148 g/kg DM in dairy cow rations. Lavery and Ferris (2021) also reported a negative and linear relationship between the intake of dietary N and urinary N excretion in dairy cows. Previous studies (Spek et al., 2013; Hynes et al., 2016) have reported a positive relationship between urinary N output and MUN in dairy cows, which is consistent with our current findings when dairy cows were fed red clover and grass silage-based diets. This relationship has the potential to allow dairy farmers to more accurately assess the effects of dietary protein on urinary N excretion when feeding diets based on red clover and grass silages.

CONCLUSIONS

Reducing the dietary CP concentration from 175 to 150 g/kg DM in a red clover and grass silage-based diet did not affect intake or animal performance. Feeding low CP diets that were marginally deficient in MP with additional barley starch or RPMet had no beneficial effect on DM intake, milk yield or composition, BW or BCS. The concentration of MUN was decreased by 31.6%, and the apparent NUE increased by approximately 20% in dairy cows when fed a low CP diet either without or with added starch or RPMet. We conclude that reducing the concentration of CP from 175 to 150 g/kg DM in red clover and grass silage-based diets reduced the environmental impact of milk production without negatively affecting dairy cow performance if the diets are formulated to meet RDP and MP requirements, although the addition of starch or RPMet to the diet may have no benefit.

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