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Performance, metabolism and nitrogen use efficiency in dairy cows fed low protein, legume silage-based diets: a systematic review and meta-analysis

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ABSTRACT

The primary focus of low CP diets and the inclusion of legume silages for dairy cows is to decrease feed costs and mitigate the environmental impact of milk production. This systematic review and meta-analysis were conducted to evaluate the effects of dietary CP level on the performance, metabolism, and N use efficiency (**NUE;** g milk N output/kg N intake) of dairy cows fed legume-based rations. A total of 36 production trials with 102 treatment means were included, and the effect of dietary CP level was estimated using the raw-mean difference between control (high CP) and low CP diets. Publication bias was examined using Begg's and Egger's tests. Meta-regression and subgroup analyses were performed to explore the heterogeneity of the response variables. Reducing dietary CP from 171 g/kg DM to 145 g/kg DM in forage legume-based diets resulted in decreased DM intake (−0.62 kg/d), milk yield (−1.41 kg/d), milk protein (−0.22 g/kg), milk urea N (**MUN**; −3.47 mg/dL), plasma urea N (−1.85 mmol/L) and condition score (−0.03) in dairy cows. Similarly, nutrient intake, diet digestibility, total urine output, N excretion through milk, urine and faeces, urine N/total N intake, rumen ammonia-N and molar proportion of butyrate were decreased ($P < 0.05$) in cows receiving low CP diets compared with those fed the control. In contrast, low CP diets increased ($P < 0.05$) the faecal N/total N intake, NUE, and plasma content of non-esterified fatty acids. Subgroup analyses revealed that the effect size of DM intake, milk yield, MUN, urinary N excretion and rumen ammonia-N content had less of a negative impact ($P < 0.05$) when cows received dietary CP levels of 140–155 g/kg DM than < 140 g/kg DM. The inclusion of rumen-protected methionine in low CP diets increased ($P = 0.04$) DM intake and tended to improve ($P = 0.08$) the milk protein content of dairy cows. Feeding lucerne silage-based low CP diets showed an improvement ($P < 0.05$) in apparent diet digestibility but reduced milk yield (−1.46 kg/d) relative to red clover silage-based rations. The inclusion rate of legume silages in low-CP diets beyond 40% of the forage DM reduced ($P < 0.01$) DM intake and milk protein content. We conclude that legume silage-based low CP diets enhance NUE but have adverse effects on dairy cow performance that can partially be mitigated by including rumen-protected methionine and limiting their proportion in the forage component of the diet.

Implications

Feeding low-protein, legume silage-based diets offers practical strategies for reducing the environmental impact of dairy farming by enhancing nitrogen use efficiency. However, these diets may compromise feed intake and milk production, potentially affecting overall farm performance. The inclusion of rumen-protected methionine and careful management of legume silage proportions in low protein diets can help to mitigate the performance losses. Future research on low–protein diets should focus on optimising legume silage inclusion rates and amino acid supplementation to develop cost-effective, environmentally sustainable feeding strategies that maintain production efficiency across diverse farming systems.

Introduction

The principle objective in reducing the CP content of dairy cow diets is to decrease feed costs and minimise the excretion of N

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through urine and manure (Sinclair et al., 2014; Yang et al., 2022). The most effective approach to improve N utilisation and decrease N loss is to avoid overfeeding protein (Broderick et al., 2015; Chowdhury et al., 2023, 2024). Extensive research has been conducted to determine the optimal dietary protein concentration that maximises the conversion of protein into milk N and improves N use efficiency (**NUE**; g milk N output/kg dietary N intake; Letelier et al., 2022; Chowdhury et al., 2023; Seleem et al., 2023).

Feeding low CP diets to early or mid-lactation dairy cows has been widely studied, although the response has not always been consistent, possibly due to a wide variety of dietary ingredients, supplementation strategies and treatments being based on CP content rather than the metabolisable protein (**MP**) supply (Lee et al., 2015; Oh et al., 2019; Van den Bossche et al., 2023). Most nutritional systems (e.g. INRA, 2018; NASEM, 2021; NRC, 2001; Thomas, 2004) consider dietary CP as containing two major fractions: that which is degraded in the rumen and available for microbial CP synthesis (rumen degradable protein, **RDP**), and that which by-passes the rumen and is subsequently available for digestion and absorption in the small intestine (rumen undegradable protein, **RUP**). The combination of digestible microbial CP, along with digestible RUP, provides the MP supply to the dairy cow for maintenance, milk performance, tissue deposition, and foetal growth (Sinclair et al., 2014). A meta-analysis of 207 production trials (Huhtanen et al., 2008) identified dietary CP content and rumen protein balance as key predictors of apparent milk NUE. Similarly, Chowdhury et al. (2023, 2024) reported that dietary CP concentration could be reduced to around 150 g/kg DM without affecting performance if the diets met the cow's MP requirements. However, other dietary and animal variables such as forage source, parity and days of lactation can also have a strong influence on nutrient utilisation and milk performance in dairy cows (Broderick, 2018; Letelier et al., 2022).

Home-grown forage legumes are attractive silages to include in the diet of dairy cows as they reduce the requirement for purchased protein sources because of their high CP content compared with grass or maize silages (Dewhurst, 2013; Sinclair et al., 2015). However, forage protein is more degradable in the rumen than vegetable protein sources such as soybean or rapeseed meals, making it more challenging to meet the MP requirements of high-producing dairy cows (Chowdhury et al., 2023). Indeed, a meta-analysis showed that cows' feed intake and milk yield response to different grass and legume species were variable when various combinations of grass or legume silage were fed (Johansen et al., 2017). The effect of reducing the dietary CP concentration on the performance of dairy cows fed legume silage-based diets, particularly those based on red clover, peas or beans forages, has however, not been widely studied. Moreover, there may be other factors (animal and dietary) such as CP level, legume silage inclusion rate, supplementation of rumen-protected amino acid (**RP-AA**), parity and days in milk that can influence the performance of dairy cows when a low CP diet based on legume silages is fed.

In addition to providing an adequate supply of MP, the amino acid (**AA**) content of the protein reaching the small intestine is crucial for high-yielding dairy cows (Lean et al., 2018). Supplementing low-CP diets with rumen-protected lysine, rumen-protected methionine (**RPM**), or both, has been evaluated in several studies (Giallongo et al., 2016; Lee et al., 2015; Van den Bossche et al., 2023) to enhance performance and NUE in dairy cows. However, responses have not always been consistent, as reported in the meta-analyses of Patton (2010), Zanton et al. (2014), and Wei et al. (2022). Despite this, Lee et al. (2012) and Giallongo et al. (2016) reported that feeding MP-deficient diets decreased feed intake, milk yield and milk composition in lactating cows, and suggested that a combination of RP-AA has the potential to improve milk performance under such dietary conditions.

The hypothesis of the current study was that reducing the dietary protein concentration in legume silage-based diets for lactating dairy cows would reduce milk production but improve NUE, and that supplementing with RP-AA would restore performance. The objective of the study was to undertake a systematic review and meta-analysis of research studies that have investigated the effect of low–protein diets based on legume silages on the performance and metabolism of early and mid-lactation cows.

Material and methods

Literature search strategy

The systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (Moher et al., 2009). A comprehensive literature search was carried out using the following electronic databases: Science Direct (https://www.sciencedirect.com/), Web of Science (https://apps.webofknowledge.com/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/). The studies were retrieved from 1980 to 2021, and the search terms included were "dairy cow", "protein", "milk", "performance", "legume silage", "nitrogen", and "efficiency".

Study selection and inclusion criteria

A total of 580 publications were identified through the database search and were initially checked for duplicates. Around 205 duplicate studies were removed, and the titles and abstracts of the remaining records were screened. The following inclusion criteria were incorporated for screening the full-text articles: (1) the study or experiments within the study were conducted in early or mid-lactation dairy cows (after calving to 220 days postpartum), and articles were reported in English; (2) the dairy cows in the control and treatment groups were housed in the same environment; (3) the diets were fed *ad libitum* as a total or partial mixed ration, and the forage component included legume silage or was partially replaced with grass or maize silage; (4) the CP content of the control (high protein) and treatment (low protein) diets varied from 156 to 220 and 110 to 155 g/kg DM, respectively; (5) the low protein or MP-deficient diets were supplemented with or without bypass protein or essential AA. The eligible studies consisted of 36 peer-reviewed journal articles, including 2 published articles from the author's own studies (Chowdhury et al., 2023, 2024). However, there was a lack of low protein studies specifically examining peas and beans silages, and only a few studies reported or predicted RDP, RUP, or MP. A flow diagram of all of the records screened and included in the meta-analysis is shown in Fig. 1. Additionally, a summary of the studies included in the systematic review and the meta-analysis is presented in Supplementary Table S1.

Data extraction and calculation

A systematic map was constructed using a Microsoft Excel spreadsheet to extract the data from the selected studies. The following variable data from the control and low protein treatments were extracted for effect size estimation: feed intake, milk performance, feed efficiency, BW, body condition score, nutrient intake and apparent digestibility, urine and plasma metabolites, N output and efficiency and rumen fermentation kinetics. Most of the studies reported a pooled SD or SE for the variables in the control and low protein treatments. In the meta-analysis, only the SD was used as the measure of variance, and if SE was reported, then, the SD was calculated by multiplying the SE by the square root of the

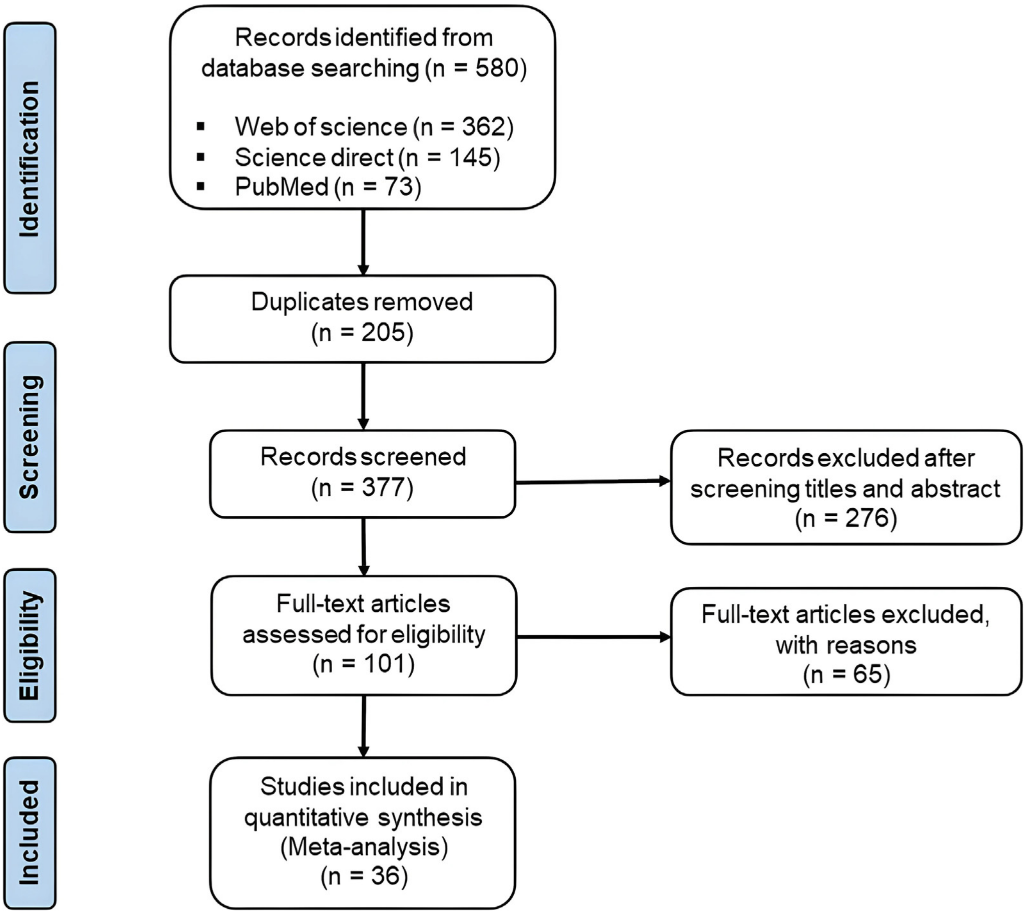


Fig. 1. Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram of all of the records screened and included in the meta-analysis that investigated the response of dairy cows to dietary protein levels in diets based on forage legumes.

sample size (Salami et al., 2020). The main influencing factors (covariates) that may have affected the performance response and were included in the analysis were parity (multiparous or mixed), days in milk, experimental design (continuous vs Latin square) and duration, forage to concentrate ratio, silage type (lucerne or red clover silage) and legume silage inclusion rate, AA supplementation in low protein diets (RPM, rumen-protected lysine or both, and no AA), and level of CP in the treatment group.

Statistical analysis

A comprehensive meta-analysis software (version 3, Biostat Inc., Englewood, USA) was used to perform the meta-analysis and generate forest plots. The effects of low–protein diets on performance variables were examined using random-effect models, assuming heterogeneity existed among the study results (Borenstein et al., 2009). The effect size of low-protein diets for each or overall study was expressed as the raw mean difference (**RMD**) at a 95% confidence interval level. The RMD was calculated as the mean differences between each study's treatment and control groups (Torres et al., 2020). The treatment means of the random-effect model were weighted by the individual variances as per the method described by DerSimonian and Laird (1986). The significance of RMD was declared when $P < 0.05$.

Variations of the treatment effect across the studies were estimated using the χ^2 and I^2 tests to define the percentage of variation due to heterogeneity (Lean et al., 2018). Types of heterogeneity were defined as low, $I^2 < 25\%$; moderate, $I^2 = 25–50\%$; and high, $I^2 > 50\%$; negative I^2 value was denoted as zero (Higgins et al., 2003). Publication bias was checked statistically with the funnel plot asymmetry by using Begg's (Begg and Mazumdar, 1994) and Egger's (Egger et al., 1997) regression tests. The significance of publication bias was declared at $P < 0.05$. A meta-regression analysis was performed with predefined covariates (both continuous and categorical) to explore the heterogeneity among the response variables. Those response variables with a lack of publication bias and high heterogeneity ($I^2 > 50\%$) or heterogeneity test at $P < 0.05$ were included in the meta-regression analysis. The adjusted R^2 value was calculated for all covariates, representing the proportion of study variance.

Based on significant ($P < 0.05$) results in the meta-regression, along with categorical covariates (parity, legume silage type, RP-AA supplementation in the low protein diets), the other covariates were divided into different groups or subgroups (days in milk <100 or ≥ 100), experimental duration (≤ 50 or > 50 days), legume silage inclusion rate of the forage DM (10–20, 21–40 or 41–60%), and the CP content (<140 or ≥ 140 g CP/kg DM) in the low CP (treatment) group) for each response variable. The subgroup meta-analyses were conducted using a similar random-effect model at a 95% confidence interval level. A mixed model was also applied within the subgroup analysis to examine differences between groups for the effect size of each categorical covariate of respective response variables. A Bonferroni multivariate posthoc comparison test was performed for covariate "AA supplementation" to determine the effect size that differed significantly from each other. Descriptive statistics for the chemical composition of the low and high (control) protein diets among the studies were conducted using GenStat (VSNI, 19th Edition, UK). The differences in chemical composition between the diets were also evaluated using an unpaired parametric *t*-test in GenStat.

Results

Study characteristics and diet composition

A total of 36 studies with 102 treatment means were included in the meta-analysis. The studies were conducted from 1980 in eight different countries (27 from the United States, four from the United Kingdom, two from Iran, and one each from Canada, Italy, Israel, Pakistan and China). Holstein-Friesian dairy cows, either multiparous or mixed, were used in the studies, which were either crossover or continuous in design, with all the studies feeding the diets as a total mixed ration (Supplementary Table S1). The mean forage-to-concentrate ratio across all studies was 53:47 on a DM basis. The diets were based on either lucerne hay/silage (92%) or red clover silage (8%), with an average inclusion rate of legume silage of 40% of forage DM. The mean CP content of the control diets was 26 g/kg DM higher ($P < 0.05$) than the low CP diets (Table 1). The mean RDP, RUP and MP content of the control diets was also 12.2, 11.9 and 14.5 g/kg DM higher than the low protein diets, respectively. There were no other differences in chemical composition between the diets except for starch, which was 34 g/kg DM higher in the low CP compared with the control diets.

Feed intake and performance

The mean DM intake of cows fed the control diets was 24.1 kg/d, which was 0.62 kg/d higher ($P < 0.01$) than in animals fed the low CP diets (Table 2, Supplementary Fig. S1). Similarly, feeding low CP diets decreased ($P < 0.05$) the daily milk yield, energy-corrected milk and fat-corrected milk yield (adjusted to 40 or 35 g fat/kg) by 1.41, 1.29, 0.73, or 1.31 kg/d, respectively. Neither milk fat nor lactose content was affected by dietary CP content. In contrast, milk protein, milk urea and milk urea N (**MUN**) concentrations were 0.22 g/kg, 6.39 mg/dL and 3.47 mg/dL lower ($P < 0.01$) in cows fed the low CP compared with the control diets. Feed efficiency tended ($P = 0.06$) to be lower when expressed as milk yield per kg DM intake, and the efficiency, when calculated using the 3.5% fat-corrected milk yield, was lower (RMD = –0.05; $P < 0.01$) in cows fed the low protein compared with the control diets. The lowest mean body condition score (RMD = –0.03; $P = 0.01$) was recorded in cows fed the low CP compared with the control diets. However, there was no effect ($P > 0.05$) of feeding low CP diets on BW or condition score change across the studies.

Nutrient intake and apparent total tract digestibility

The mean intake of DM, organic matter, CP, NDF and ADF were 0.65, 0.59, 0.74, 0.27 and 0.19 kg/d lower, respectively ($P < 0.05$), in cows fed low CP compared with the control diets (Table 3, Supplementary Fig. S2). Similarly, apparent total tract digestibility of DM, organic matter, CP, NDF and ADF were 12.5, 13.1, 43.9, 19.1 and 27.0 g/kg lower, respectively ($P < 0.01$), in cows receiving low CP diets than those fed the control, which had means of 684, 704, 661, 492 and 449 g/kg, respectively.

Urine and plasma metabolites

No difference in urine metabolite concentration (allantoin, uric acid and total purine derivatives) was observed between cows receiving the control or low CP diets (Table 4, Supplementary Fig. S3). Compared with low CP diets, daily total urine output was 3.04 L higher ($P < 0.01$) in cows fed the control. Plasma metabolites, including glucose, β-hydroxybutyrate, total glycerides and creatinine levels, did not differ ($P > 0.05$) between cows fed the control or low CP diets. In contrast, reducing the dietary CP content increased ($P = 0.01$) the plasma concentration of non-esterified fatty acids, which was 0.03 mmol/L higher than those receiving the control diet. The mean concentration of plasma urea-N (**PUN**) was 1.85 mmol/L lower ($P < 0.01$) in cows fed the low CP compared with the control diets.

Nitrogen intake, use efficiency and fermentation kinetics

Dietary N intake was reduced ($P < 0.01$) by 107 g/d when cows received legume-based low CP diets than those fed the control (Table 5, Supplementary Fig. S4). Likewise, daily N excretion in milk, urine or faeces was 4.26, 13.6 and 69.3 g, respectively, lower ($P < 0.01$) in cows fed low CP than the control diets. In contrast, the apparent NUE was approximately 36.9 g/kg higher ($P < 0.01$) in cows fed low CP diets than in the control. Similarly, feeding a low CP diet increased the partitioning of dietary N into faecal N (RMD = 46.7 g/kg; $P < 0.01$) but reduced ($P < 0.01$) urine N by 70 g/kg of total N intake compared with those receiving the control diet. There was no difference ($P > 0.05$) between cows fed the control or low CP diets in rumen pH or molar proportion of rumen

Table 1
Descriptive statistics of the chemical composition of high (control) and low protein diets for studies included in the meta-analysis that investigated the response of dairy cows to dietary protein levels in diets based on forage legumes.

Item	Mean		Median		Maximum		Minimum		SE		N
	Control	Low CP	Control	Low CP	Control	Low CP	Control	Low CP	Control	Low CP	
DM, g/kg	542	540	532	529	679	685	379	379	9.116	9.381	61
OM, g/kg DM	930	933	928	931	951	958	906	906	1.472	1.674	67
*CP, g/kg DM	171	145	170	149	220	155	156	110	1.160	0.937	102
NDF, g/kg DM	316	316	315	316	393	415	224	219	4.178	4.440	100
ADF, g/kg DM	199	197	197	195	281	281	115	109	4.014	4.123	93
EE, g/kg DM	38.8	42.2	35.3	37.7	74.0	76.7	18.6	16.6	1.742	2.025	69
*Starch, g/kg DM	226	260	230	273	315	362	104	154	7.142	7.610	55
Ca, g/kg DM	8.92	9.01	9.00	9.55	11.7	13.3	5.87	5.13	0.224	0.244	44
P, g/kg DM	4.24	4.11	4.00	3.98	5.20	5.30	3.70	3.40	0.076	0.080	44
NE_L, g/kg DM	6.64	6.60	6.61	6.61	7.82	7.82	4.46	4.58	0.053	0.054	80
*RDP, g/kg DM	105	92.8	102	95.1	127	109	86.6	70.9	1.440	1.556	42
*RUP, g/kg DM	62.6	50.7	62.3	49.0	77.0	75.6	50.6	35.0	0.986	1.110	44
*MP, g/kg DM	110	95.5	114	93.5	120	109	94.8	86.8	2.261	1.651	16

Abbreviations: OM = organic matter; EE = ether extract; NE_L = Net energy for lactation; RDP = rumen degradable protein; RUP = rumen undegradable protein; MP = metabolisable protein; N = the number of comparisons between high (control) and low CP (treatment) diets.

[*] Means between control (high CP) and low CP diet statistically differ at $P < 0.05$.

Table 2
Summary effect size estimates for intake, milk performance, BW, and condition of dairy cows fed high (control) or low CP diets based on forage legumes in a random-effect meta-analysis.

Item	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P*–value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P*–value	Q value	*P*–value	I^2 (%)	Begg's test	Egger's test	
DM intake, kg/d	24.1	0.23	−0.62	0.09	−0.80	−0.45	<0.01	176	<0.01	46.5	0.82	0.91	95
Milk yield, kg/d													
Milk	37.3	0.57	−1.41	0.15	−1.71	−1.11	<0.01	207	<0.01	51.7	0.76	0.89	101
ECM	37.4	0.52	−1.29	0.24	−1.75	−0.82	<0.01	57.9	0.16	17.1	0.84	0.48	49
4% FCM	36.3	1.24	−0.73	0.27	−1.26	−0.19	0.01	17.3	0.57	0.00	0.80	0.80	20
3.5% FCM	38.0	0.74	−1.31	0.19	−1.68	−0.93	<0.01	42.8	0.27	11.2	0.78	0.51	39
Composition, g/kg													
Fat	36.3	0.39	−0.12	0.14	−0.39	0.16	0.40	93.5	0.41	2.63	0.74	0.13	92
Protein	31.4	0.21	−0.22	0.05	−0.32	−0.12	<0.01	112	<0.01	78.1	0.75	0.91	98
Lactose	48.1	0.18	−0.02	0.03	−0.09	0.05	0.56	79.6	0.62	0.00	0.45	0.39	85
Urea, mg/dL	23.9	1.41	−6.39	0.58	−7.51	−5.26	<0.01	17.4	0.07	42.6	0.44	0.72	11
MUN, mg/dL	12.7	0.27	−3.47	0.18	−3.83	−3.11	<0.01	756	<0.01	89.6	0.38	0.11	80
Feed efficiency, %													
Milk yield/DMI	1.58	0.02	−0.01	0.01	−0.03	0.00	0.06	57.6	0.31	8.05	0.88	0.55	54
ECM/DMI	1.55	0.02	−0.02	0.01	−0.04	0.00	0.09	61.6	0.01	41.6	0.09	0.15	37
4% FCM/DMI	1.62	0.09	−0.03	0.03	−0.08	0.03	0.34	12.2	0.20	26.0	0.72	0.16	10
3.5% FCM/DMI	1.59	0.03	−0.05	0.01	−0.07	−0.02	<0.01	14.7	0.80	0.00	0.51	0.79	21
Body performance													
BW, kg	652	6.70	−1.55	1.31	−4.11	1.01	0.24	44.2	0.70	0.00	0.13	0.11	51
BWC, kg/d	0.09	0.08	−0.02	0.02	−0.06	0.01	0.22	83.2	0.09	19.5	0.75	0.40	68
BCS	2.87	0.04	−0.03	0.01	−0.06	−0.01	0.01	23.5	0.91	0.00	0.30	0.20	35
BCS change	0.03	0.01	0.00	0.00	−0.01	0.00	0.28	32.8	0.14	23.7	0.95	0.17	26

Abbreviations: ECM = energy-corrected milk yield; FCM = fat-corrected milk yield; MUN = milk urea N; DMI = DM intake; BWC = BW change; BCS = body condition score (1–5 scale).
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.
Publication bias was examined using Begg's and Egger's regression (Funnel) test.

Table 3
Summary effect size estimates for intake performance (intake data was included just from the digestibility studies) and apparent total tract nutrient digestibility of dairy cows fed high (control) or low CP diets based on forage legumes in a random-effect meta-analysis.

Item	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (*P*–value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	*P*–value	Q value	*P*–value	I^2 (%)	Begg's test	Egger's test	
Intake, kg/d													
DM	24.2	0.25	−0.65	0.09	−0.83	−0.48	<0.01	139	<0.01	35.9	0.82	0.07	39
OM	22.4	0.36	−0.59	0.18	−0.95	−0.23	0.01	38.0	0.12	23.8	0.16	0.73	30
CP	3.99	0.06	−0.74	0.04	−0.82	−0.67	<0.01	240	<0.01	84.2	0.36	0.31	39
NDF	7.36	0.28	−0.27	0.07	−0.41	−0.14	<0.01	56.8	0.01	43.7	0.37	0.19	33
ADF	4.82	0.20	−0.19	0.03	−0.26	−0.13	<0.01	42.4	0.18	17.5	1.00	0.06	36
Digestibility, g/kg													
DM	684	7.0	−12.5	2.86	−18.1	−6.91	<0.01	215	<0.01	78.1	0.76	0.70	48
OM	704	8.1	−13.1	2.51	−18.0	−8.19	<0.01	89.2	<0.01	58.5	0.96	0.77	38
CP	661	7.4	−43.9	3.56	−50.9	−37.0	<0.01	105	<0.01	58.1	0.95	0.90	45
NDF	492	15.5	−19.1	4.46	−27.8	−10.3	<0.01	138	<0.01	68.7	0.75	0.60	44
ADF	449	18.1	−27.0	5.35	−37.5	−16.5	<0.01	149	<0.01	75.1	0.68	0.51	38

Abbreviations: OM = organic matter.
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.
Publication bias was examined using Begg's and Egger's regression (Funnel) test.

acetate or propionate; however, butyrate was reduced ($P < 0.01$) by 0.45 mol per 100 mol of volatile fatty acids when a low CP diet was fed (Table 5, Supplementary Fig. S5).

Heterogeneity, publication bias and meta-regression

A high heterogeneity was observed ($I^2 > 50\%$; $P < 0.05$) for DM intake, milk yield, milk protein, MUN, PUN, CP and NDF intake, nutrients digestibility, urinary N excretion, NUE, faecal and urine N/ total N intake and rumen NH_3-N level. However, there was no substantial evidence in Begg's and Egger's tests to indicate publication bias across the studies for each response variable (Tables 2–5). The response variables which showed significant heterogeneity were subjected to meta-regression analysis using preselected covariates to identify the key sources of variation (Table 6). Among the covariates, the level of CP in the diet, the type of legume silage and its inclusion rate, days in milk and AA supplementation were the major factors that influenced the response variables. Other covariates such as parity and experimental duration also showed a significant correlation ($P < 0.05$) with DM intake, milk yield, milk protein, PUN and urinary N excretion.

Subgroup analysis

Subgroup analysis indicated that DM and CP intake, DM digestibility, milk yield, MUN, urinary N excretion, and rumen

Table 4
Summary effect size estimates for urine and blood metabolites of dairy cows fed high (control) or low CP diets based on forage legumes in a random-effect meta-analysis.

Item	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (P–value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	P–value	Q value	P–value	I^2 (%)	Begg's test	Egger's test	
Urine metabolites													
Allantoin, mmol/L	24.1	1.67	1.37	2.08	−2.70	5.44	0.51	0.73	1.00	0.00	1.00	0.27	18
Uric acid, mmol/L	2.37	0.23	0.21	0.26	−0.29	0.72	0.41	0.64	1.00	0.00	0.47	0.64	18
Total PD, mmol/L	26.6	1.82	1.72	2.26	−2.72	6.15	0.45	0.59	1.00	0.00	0.91	0.20	18
Urine output, L/d	25.0	1.23	−3.04	0.28	−3.59	−2.49	<0.01	41.1	0.22	14.8	0.20	0.06	36
Plasma metabolites													
Glucose, mmol/L	3.56	0.06	−0.01	0.02	−0.06	0.03	0.51	38.3	0.14	21.7	0.99	0.82	31
BHB, mmol/L	0.55	0.07	0.02	0.01	−0.01	0.05	0.13	6.39	0.85	0.00	1.00	0.65	12
NEFA, mmol/L	0.25	0.03	0.03	0.01	0.01	0.06	0.01	2.67	0.85	0.00	1.00	0.94	7
TG, mmol/L	0.15	0.02	0.01	0.01	0.00	0.02	0.14	5.02	0.89	0.00	0.31	0.16	11
Creatinine, mg/dL	1.40	0.15	−0.03	0.02	−0.08	0.01	0.15	3.89	0.57	0.00	1.00	0.88	6
PUN, mmol/L	5.64	0.23	−1.85	0.13	−2.11	−1.59	<0.01	493	<0.01	89.2	1.01	0.05	54

Abbreviations: PD = purine derivatives; BHB = β-hydroxybutyric acid; NEFAs = non-esterified fatty acids; TG = total glycerides; PUN = plasma urea N.
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.
Publication bias was examined using Begg's and Egger's regression (Funnel) test.

Table 5
Summary effect size estimates for nitrogen intake, output, efficiency, and rumen fermentation kinetics of dairy cows fed high (control) or low CP diets based on forage legumes in a random-effect meta-analysis.

Item	Control		Effect (Random effect) size and 95% CI					Heterogeneity test			Funnel test (P–value)		N
	Mean	SE	RMD	SE	Lower limit	Upper limit	P–value	Q value	P–value	I^2 (%)	Begg's test	Egger's test	
N intake, g/d	668	6.02	−107	7.43	−121	−92.3	<0.01	291	<0.01	82.5	0.48	0.65	52
N output, g/d													
Milk	187	2.47	−4.26	1.29	−6.78	−1.74	<0.01	52.3	0.24	11.99	0.14	0.84	47
Faecal	226	5.72	−13.6	2.48	−18.5	−8.75	<0.01	67.1	0.05	25.4	0.99	0.97	51
Urine	218	6.33	−69.3	4.24	−77.6	−61.0	<0.01	435	<0.01	86.9	0.23	0.23	58
N efficiency, g/kg													
Faecal	355	7.9	46.7	4.71	37.5	56.0	<0.01	169	<0.01	75.7	0.61	0.58	42
Urine	316	9.7	−70.0	4.65	−79.1	−60.8	<0.01	96.9	<0.01	64.9	0.17	0.14	35
NUE	284	3.0	36.9	1.87	33.3	40.6	<0.01	147	<0.01	50.5	0.14	0.10	74
Rumen fermentation													
Rumen pH	6.25	0.04	0.02	0.02	−0.05	0.05	0.10	28.4	0.65	0.00	0.62	0.79	33
NH_3-N, mg/dL	10.5	0.74	−3.38	0.32	−4.01	−2.75	<0.01	69.5	<0.01	61.2	0.51	0.24	28
Acetate, mol/100 mol	58.8	0.75	−0.15	0.41	−0.95	0.64	0.71	33.5	0.22	16.3	0.32	0.22	29
Propionate, mol/100 mol	23.1	0.67	−0.32	0.37	−1.04	0.39	0.38	32.9	0.17	21.0	0.82	0.96	27
Butyrate, mol/100 mol	12.0	0.28	−0.45	0.12	−0.69	−0.21	<0.01	23.3	0.72	0.00	0.56	0.96	29

Abbreviations: Faecal N efficiency = faecal N/total N intake; Urine N efficiency = urine N/total N intake; NUE = apparent N use efficiency of milk production (milk N/N intake); NH_3-N = rumen ammonia-N.
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.
Publication bias was examined using Begg's and Egger's regression (Funnel) test.

NH_3-N concentration were reduced (P < 0.05) in cows receiving < 140 g CP/kg DM compared with those fed ≥ 140 g CP/kg DM (Table 7). In contrast, there was a tendency for the dietary partitioning of N into faeces to be increased (RMD = 57.7 vs 40.6; P = 0.06) when cows received CP < 140 g/kg DM compared with those receiving ≥ 140 g CP/kg DM. A tendency for a decrease in PUN (RMD = −2.20 vs −1.71; P = 0.08) was also observed when cows received CP < 140 g/kg DM.

Compared with the control, the DM intake was reduced (P < 0.05) in cows when fed either lucerne or red clover silage-based low CP diets (Table 8). The NDF intake, DM, organic matter, NDF and ADF digestibility, daily urinary N excretion, and the urinary N/total N intake were reduced (P < 0.05) in cows fed low CP diets based on either red clover or lucerne silage compared with control diets; however, the RMD was substantially lower when cows received low protein diets based on red clover silage than lucerne. In contrast, milk yield was reduced (RMD = −1.54 kg/d; P < 0.01) in cows fed lucerne silage-based low CP diets compared with control, with daily milk yield being 1.46 kg lower (P = 0.01) than those receiving red clover silage-based rations. Likewise, the faecal N/total N intake was increased (P < 0.01) in cows fed low CP diets based on either lucerne or red clover silage compared with the control, with the RMD being lower (RMD = 42.6 vs 78.3; P = 0.01) in cows fed lucerne than red-clover–based low CP diets.

Dry matter intake was reduced (P < 0.01) in cows when legume silage inclusion in the low CP diets increased from 21 to 60% of the forage DM (Fig. 2a). Similarly, the milk protein concentration was reduced (P < 0.01) when legume silage inclusion rates were increased to 41–60% of the forage DM (Fig. 2b). In contrast, the urinary N excretion was decreased (P < 0.01) in cows fed up to 60% inclusion of legume silage–based low CP diets (Fig. 2c). Additionally, the DM intake was 0.71 kg/d lower (P = 0.04) in cows receiving legume silage-based low CP diets without supplementation of AA than those with added RPM (Fig. 3a). Similarly, there was a tendency (P = 0.08) for milk protein content to be increased when a legume-based low CP diet was offered with RPM (Fig. 3b). The con-

Table 6

Covariates effect on intake (kg/d), nutrient digestibility (g/kg), milk yield (kg/d), milk protein (g/kg), milk urea N (mg/dL), plasma urea N (mmol/L), urine N (g/d), apparent milk N use efficiency (g/kg), urine and faecal N/total N intake (g/kg) and rumen ammonia-N (mg/dL), of dairy cows fed high (control) or low CP diets based on forage legumes in a random-effect meta-regression analysis.

Variables			[1]Covariates, meta-regression coefficient and associated *P*–value														Adj. R^2	N
	Intercept		Forage type		Legume inclusion		AA (%)		Parity		DIM		EXPD		CP level			
	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value	Coef.	*P* –value		
DM intake	−8.98	<0.01	−0.59	0.05	−0.01	0.08	0.31	0.02	1.24	0.02	0.00	<0.01	0.00	0.16	0.06	<0.01	0.97	95
CP intake	−4.23	<0.01	−0.04	0.83	0.00	0.80	0.21	0.06	0.10	0.54	0.00	0.56	0.00	0.43	0.02	<0.01	0.00	39
NDFI	0.84	0.55	−0.72	0.01	0.00	0.60	0.07	0.72	−0.27	0.25	−0.01	0.07	0.00	0.32	0.00	0.89	0.48	33
DMD	−78.2	0.12	−51.7	0.01	−0.01	0.97	10.2	0.08	−2.44	0.78	−0.25	0.19	−0.01	0.95	0.64	0.05	0.39	48
OMD	−30.6	0.47	−61.7	<0.01	0.22	0.38	77.7	0.11	5.78	0.44	−0.36	0.02	0.09	0.58	0.29	0.25	0.63	38
CPD	−204	0.01	−45.9	0.08	−0.21	0.57	2.80	0.72	6.70	0.57	0.16	0.52	−0.08	0.74	1.05	0.04	0.31	45
NDFD	−78.6	0.30	−91.0	0.00	0.46	0.31	8.13	0.37	23.4	0.09	−0.35	0.20	0.31	0.29	0.40	0.41	0.38	44
ADFD	−45.1	0.69	−88.3	0.01	0.74	0.20	−1.00	0.93	23.3	0.24	−0.31	0.36	0.23	0.57	0.07	0.93	0.10	38
Milk yield	−15.4	0<.00	1.57	0.00	0.02	0.13	−0.22	0.45	3.73	<0.01	0.01	<0.01	−0.01	0.05	0.09	<0.01	0.65	101
MP	−2.24	0.01	0.28	0.20	−0.01	<0.01	−0.42	<0.01	−1.24	0.14	0.00	0.59	−0.00	0.26	0.02	<0.01	1.00	98
MUN	−15.5	<0.01	1.07	0.08	−0.02	0.15	−1.41	<0.01	0.73	0.06	0.01	0.04	0.00	0.87	0.09	<0.01	0.45	80
PUN	−9.91	<0.01	0.47	0.25	0.00	0.82	−0.90	<0.01	0.94	<0.01	0.01	0.03	0.01	0.01	0.05	<0.01	0.49	54
Urine N	−353	<0.01	−62.0	0.00	−0.63	0.03	−20.6	0.00	14.0	0.11	0.31	0.01	0.27	0.01	1.91	<0.01	0.63	58
FNE	273	<0.01	64.3	0.00	−0.03	0.39	0.97	0.37	−2.94	0.06	0.00	1.00	−0.03	0.13	−0.14	0.00	0.50	42
UNE	−101	0.25	−87.7	0.00	−0.36	0.32	−13.6	0.23	−11.1	0.50	−0.12	0.62	0.29	0.12	0.47	0.38	0.64	35
NUE	76.5	0.08	12.4	0.07	0.15	0.32	6.67	0.11	4.21	0.38	−0.08	0.15	−0.01	0.83	−0.30	0.28	0.44	74
NH_3-N	−8.54	0.03	–	–	−0.01	0.67	−1.30	0.13	0.28	0.14	−0.02	0.06	0.01	0.73	0.06	0.03	0.33	28

Abbreviations: NDFI = NDF intake; DMD = DM digestibility; OMD = organic matter digestibility; CPD = CP digestibility; NDFD = NDF digestibility; ADFD = ADF digestibility; MP = milk protein; MUN = milk urea N; PUN = plasma urea N; NUE = apparent milk N use efficiency (milk N/N intake); FNE = faecal N/total N intake; UNE = urine N/total N intake; NH_3-N = Rumen ammonia N.

[1] Coef. = coefficient; AA = amino acid; DIM = days in milk of cows; EXPD = duration of the experiment (days); Adj. = adjusted; N = number of comparisons between high (control) and low CP (treatment) diets; Adjusted R^2 value presented for all covariates.

Table 7

Covariate (CP level: ≥140 or < 140 g CP/kg DM) effect size estimates for DM and CP intake and digestibility, milk yield, milk protein, milk and plasma urea N, urinary N excretion, faecal N/total N intake, and rumen ammonia-N of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis.

Variables	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P*–value[1]	N
		RMD	SE	Lower limit	Upper limit	*P*–Value	Q value	*P*–value	I^2 (%)		
DM intake, kg/d DM	<140	−1.01	0.20	−1.39	−0.62	<0.01	31.8	0.02	46.5	0.03	18
	≥140	−0.53	0.10	−0.73	−0.34	<0.01	133	<0.01	42.9		77
CP intake, kg/d DM	<140	−0.92	0.06	−1.04	−0.79	<0.01	88.5	<0.01	86.4	<0.01	13
	≥140	−0.67	0.04	−0.75	−0.59	<0.01	109	<0.01	77.1		26
DM digestibility, g/kg	<140	−21.5	5.16	−31.6	−11.4	<0.01	51.6	<0.01	72.9	0.04	15
	≥140	−8.41	3.48	−15.2	−1.59	0.02	159	<0.01	79.9		33
CP digestibility, g/kg	<140	−46.5	6.68	−59.6	−33.4	<0.01	12.1	0.35	9.42	0.65	12
	≥140	−43.0	4.03	−50.9	−35.1	<0.01	83.2	<0.01	61.5		33
Milk yield, kg/d	<140	−2.45	0.38	−3.19	−1.71	<0.01	30.2	0.05	37.1	<0.01	20
	≥140	−1.22	0.16	−1.54	−0.91	<0.01	159	<0.01	49.8		81
Milk protein, g/kg	<140	−0.39	0.13	−0.64	−0.13	<0.01	37.9	0.01	49.8	0.17	20
	≥140	−0.19	0.05	−0.30	−0.09	<0.01	72.5	0.63	0.00		78
MUN, mg/dL	<140	−4.27	0.37	−5.00	−3.53	<0.01	242	<0.01	93.0	0.02	18
	≥140	−3.23	0.20	−3.63	−2.83	<0.01	470	<0.01	87.0		62
PUN, mmol/L	<140	−2.20	0.23	−2.66	−1.74	<0.01	156	<0.01	91.0	0.08	15
	≥140	−1.71	0.15	−2.00	−1.41	<0.01	281	<0.01	86.5		39
Urine N, g/d	<140	−83.1	7.97	−98.7	−67.5	<0.01	108	<0.01	87.0	0.04	15
	≥140	−64.1	4.83	−73.6	−54.7	<0.01	293	<0.01	85.6		43
FNE, g/kg	<140	57.7	7.24	43.5	71.9	<0.01	42.9	<0.01	67.3	0.06	15
	≥140	40.6	5.41	30.0	51.2	<0.01	94.9	<0.01	72.6		27
NH_3-N, mg/dL	<140	−4.53	0.57	−5.64	−3.42	<0.01	15.8	0.01	68.3	0.02	6
	≥140	−2.95	0.34	−3.61	−2.28	<0.01	38.3	0.01	45.1		22

Abbreviations: MUN = Milk urea N; PUN = plasma urea N; FNE = faecal N/total N intake; NH_3-N = Rumen ammonia N.
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.

[1] *P*–value of a mixed model between sub-groups (<140 vs ≥140).

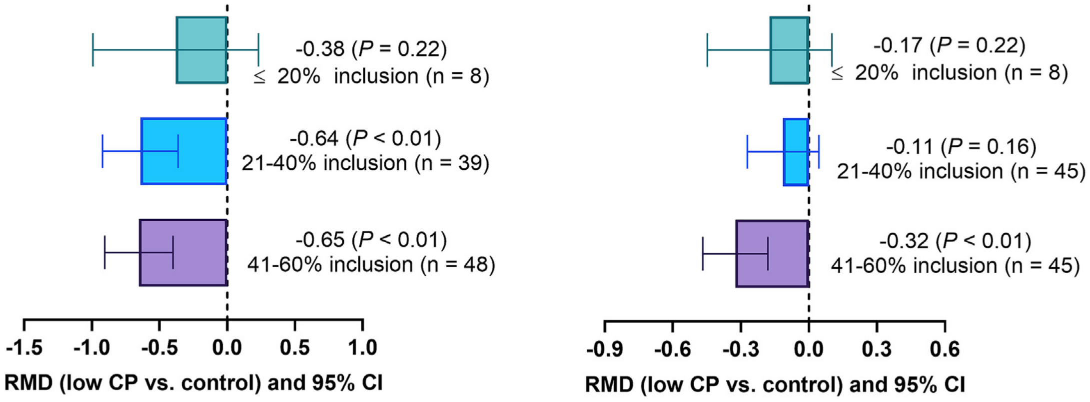
centration of MUN was decreased ($P < 0.05$) in cows fed low CP diets either with or without added AA (Fig. 3c). Similarly, PUN content was reduced ($P < 0.01$) in cows fed legume-based low CP diets with added RPM or without any additional AA (Fig. 3d). In addition, the MUN content and urinary N excretion were 2.36 mg/dL and 33.5 g/d higher ($P < 0.05$) in cows fed legume silage-based low CP diets with added rumen-protected lysine and RPM, respectively, compared with those receiving no added AA (Fig. 3c and 3e).

Table 8
Covariate (predominant silage type: lucerne (LS) or red clover (RCS)) effect size estimates for DM and NDF intake, nutrients digestibility, milk yield, urinary N excretion, urine and faecal N/total N intake of dairy cows fed high (control) or low CP diets based on lucerne or red clover silages in a subgroup random-effect meta-analysis.

Variables	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P*–value[1]	N
		RMD	SE	Lower limit	Upper limit	*P* value	Q value	*P* value	I^2 (%)		
DM intake, kg/d DM	LS	−0.59	0.09	−0.77	−0.41	<0.01	166	<0.01	48.3	0.13	87
	RCS	−1.12	0.34	−1.79	−0.45	0.01	3.51	0.83	0.00		8
NDF intake, kg/d DM	LS	−0.21	0.07	−0.35	−0.08	0.01	44.2	0.02	39.0	0.02	28
	RCS	−0.69	0.19	−1.07	−0.32	<0.01	2.30	0.68	0.00		5
DM digestibility, g/kg	LS	−10.5	2.97	−16.3	−4.70	<0.01	196	<0.01	78.6	0.04	43
	RCS	−30.1	8.77	−47.3	−12.9	<0.01	10.5	0.03	62.0		5
OM digestibility, g/kg	LS	−10.5	2.57	−15.6	−5.49	<0.01	71.6	<0.01	55.3	0.01	33
	RCS	−30.6	6.66	−43.6	−17.5	<0.01	9.20	0.06	56.5		5
NDF digestibility, g/kg	LS	−14.6	4.58	−23.6	−5.60	<0.01	121	<0.01	68.5	0.01	39
	RCS	−54.4	12.9	−79.6	−29.2	<0.01	7.03	0.13	43.1		5
ADF digestibility, g/kg	LS	−21.6	5.59	−32.6	−10.7	<0.01	132	<0.01	75.8	0.01	33
	RCS	−63.2	14.5	−91.5	−34.8	<0.01	8.36	0.08	52.1		5
Milk yield, kg/d	LS	−1.54	0.16	−1.85	−1.23	<0.01	189	<0.01	51.4	0.01	93
	RCS	−0.08	0.50	−1.06	0.90	0.87	6.60	0.47	0.00		8
Urine N, g/d	LS	−67.3	4.20	−75.5	−59.0	<0.01	407	<0.01	86.5	0.01	56
	RCS	−137	24.6	−185	−88.7	<0.01	4.00	0.05	75.0		5
FNE, g/kg	LS	42.6	4.94	32.9	52.2	<0.01	157	<0.01	77.1	0.01	37
	RCS	78.3	13.6	51.7	105	<0.01	5.16	0.27	22.5		5
UNE, g/kg	LS	−64.4	4.27	−72.8	−56.0	<0.01	74.9	<0.01	57.3	<0.01	33
	RCS	−162	17.4	−196	−128	<0.01	1.63	0.20	38.8		2

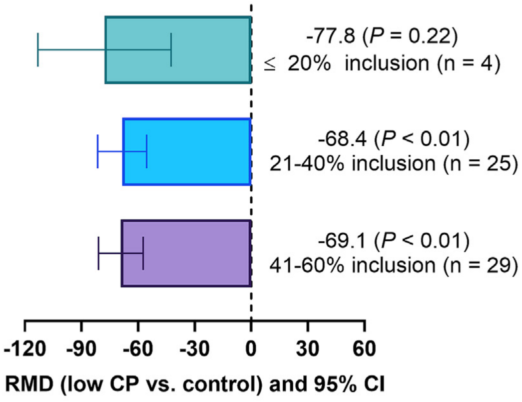
Abbreviations: OM = organic matter; FNE = faecal N/total N intake; UNE = urine N/total N intake;
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.

[1] *P*–value of a mixed model between sub-groups.



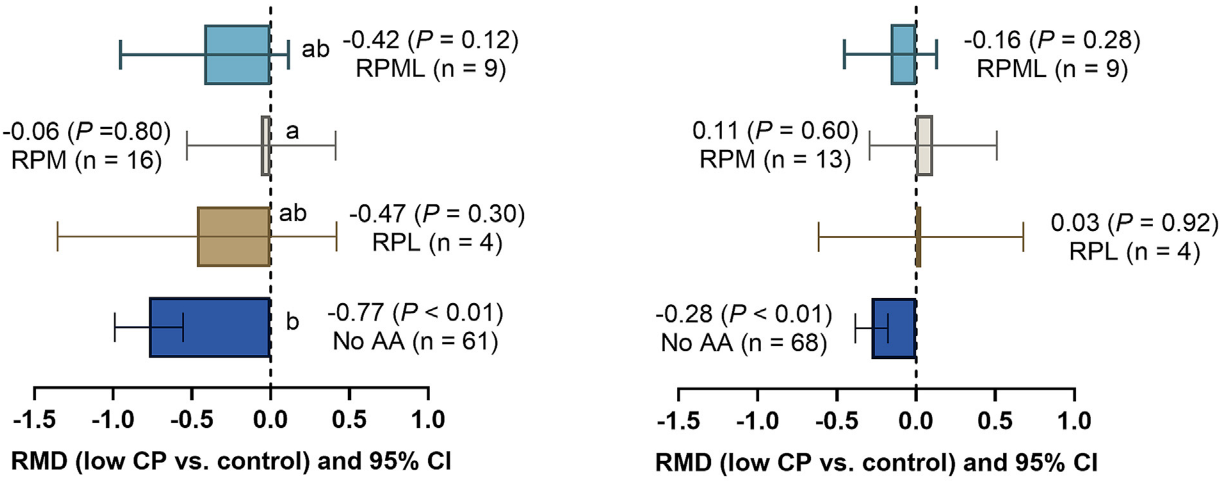
a) Dry matter intake (kg/d)

b) Milk protein (g/kg)



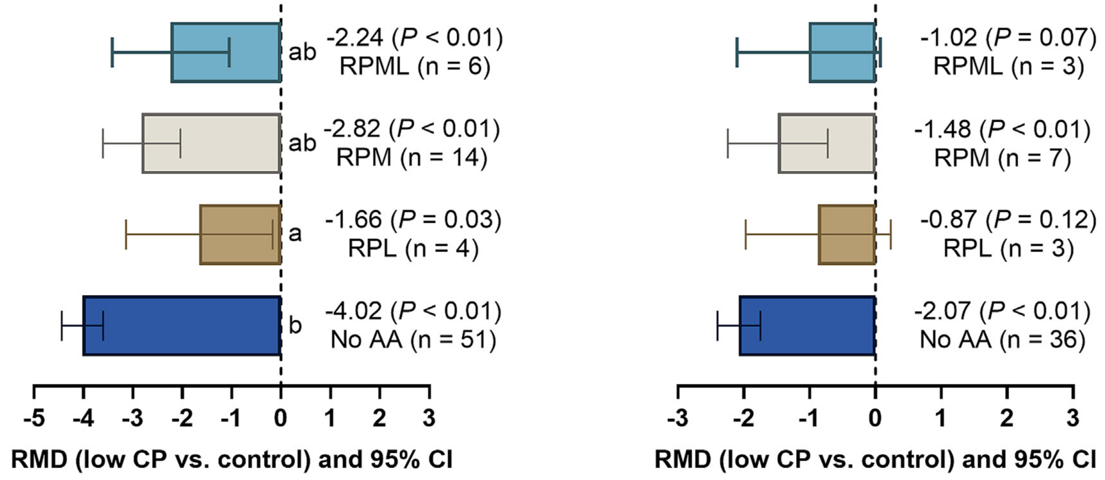
c) Urinary N (g/d)

Fig. 2. Covariate (legume silage inclusion rate on forage DM: ≤20%, 21–40% or 41–60%) effect size estimates for (a) DM intake (kg/d), (b) milk protein (g/kg) and (c) urinary N excretion (g/d) of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis. *P*-values within parentheses are used to compare with control values. RMD = raw mean differences between high (control) and low CP diets. *P*–value between groups (10–20, 21–40 or ≥60%) for DM intake, *P* = 0.71; milk protein, *P* = 0.15; and urinary N, *P* = 0.89. The error bar indicates a 95% level confidence interval. Abbreviations: CI = confidence interval.



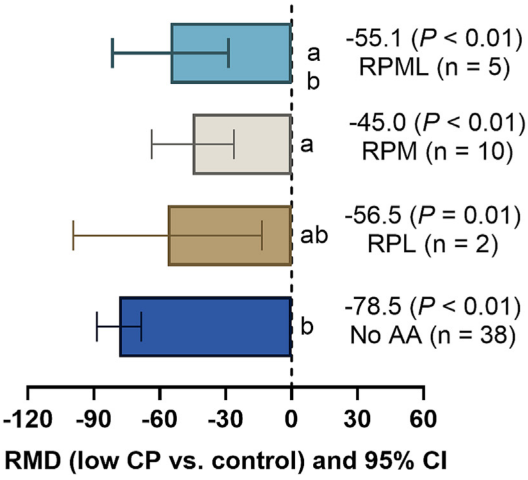
a) Dry matter intake (kg/d)

b) Milk protein (g/kg)



c) Milk urea N (mg/dL)

d) Plasma urea N (mmol/L)



e) Urinary N (g/d)

Fig. 3. Covariate (low CP diet without (No AA) or with added amino acids: Rumen-protected lysine (RPL), Rumen-protected methionine (RPM) or Rumen-protected methionine-lysine (RPML)) effect size estimates for a) DM intake (kg/d), b) milk protein (g/kg), c) milk urea N (mg/dL), d) plasma urea N (mmol/L) and e) urinary N excretion (g/d) of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis. *P*-values within parentheses are used to compare with control values. RMD = raw mean differences between high (control) and low CP diets. *P*–value between groups (No AA, RPL, RPM, RPML) for DM intake, $P = 0.04$; milk protein, $P = 0.08$; milk urea N, $P < 0.01$; plasma urea N, $P = 0.26$; and urinary N, $P = 0.04$. The error bar indicates a 95% level confidence interval. RMD with different letters differ significantly ($P < 0.05$). Abbreviations: CI = confidence interval.

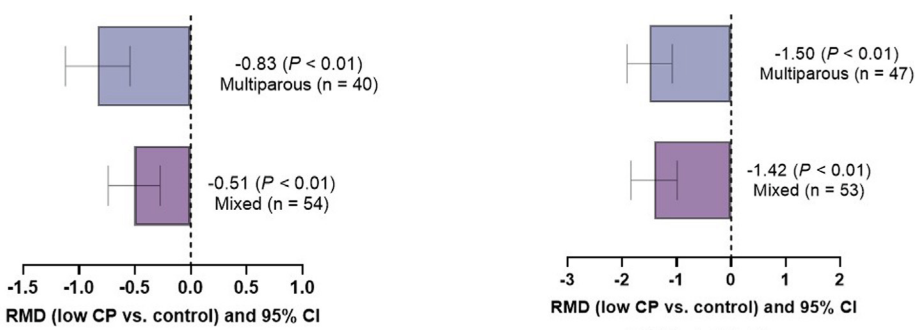
Table 9
Covariate (days in milk (DIM): ≥100 or < 100 DIM)) effect size estimates for DM intake, OM digestibility, milk yield, milk and plasma urea N, and urinary N excretion of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis.

Variables	Sub-group	Effect (Random effect) size and 95% CI					Heterogeneity test			*P*–value[1]	N
		RMD	SE	Lower limit	Upper limit	*P*–value	Q value	*P*–value	I^2 (%)		
DM intake, kg/d DM	≥100 DIM	−0.61	0.12	−0.84	−0.38	<0.01	111	<0.01	52.2	0.85	54
	<100 DIM	−0.65	0.15	−0.93	−0.36	<0.01	61.5	0.02	34.9		41
OM digestibility, g/kg	≥100 DIM	−18.5	3.50	−25.3	−11.6	<0.01	41.7	<0.01	56.9	0.03	19
	<100 DIM	−7.70	3.52	−14.6	−0.80	0.03	41.6	<0.01	56.7		19
Milk yield, kg/d	≥100 DIM	−1.52	0.20	−1.92	−1.12	<0.01	127	<0.01	56.8	0.42	56
	<100 DIM	−1.26	0.24	−1.73	−0.79	<0.01	79.7	<0.01	44.8		45
MUN, mg/dL	≥100 DIM	−3.27	0.25	−3.77	−2.78	<0.01	328	<0.01	87.8	0.26	41
	<100 DIM	−3.68	0.26	−4.20	−3.17	<0.01	412	<0.01	90.8		39
PUN, mmol/L	≥100 DIM	−1.90	0.16	−2.21	−1.58	<0.01	322	<0.01	89.1	0.63	36
	<100 DIM	−1.76	0.23	−2.22	−1.31	<0.01	153	<0.01	88.9		18
Urine N, g/d	≥100 DIM	−64.9	5.85	−76.4	−53.5	<0.01	320	<0.01	90.9	0.27	30
	<100 DIM	−74.3	6.30	−86.7	−62.0	<0.01	111	<0.01	75.7		28

Abbreviations: OM = organic matter; MUN = milk urea N; PUN = plasma urea N.
CI = confidence interval; RMD = the raw mean differences between high (control) and low CP diets at 95% confidence interval; Q = χ^2 statistic of heterogeneity; I^2 = percentage of the total variation of effect size estimates; N = the number of comparisons between high (control) and low CP (treatment) diets.

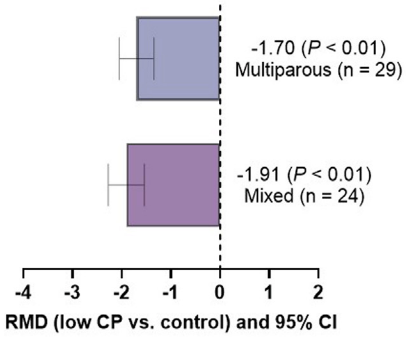
[1] *P*–value of a mixed model between sub-groups.

The DM intake, organic matter digestibility, milk yield, MUN, PUN, and daily urinary N excretion were reduced ($P < 0.01$) in cows that were either < 100 or ≥ 100 days in milk when fed legume silage-based low CP diets (Table 9). However, there was no difference between the two groups in the RMD of the response variables except for organic matter digestibility, which was decreased (RMD = −18.5 vs −7.70; $P = 0.03$) when cows were ≥ 100 days in milk and fed legume-based low CP diets. The DM intake, milk yield and PUN content were reduced ($P < 0.01$) in both multiparous and mixed cows when legume silage-based low CP diets were fed compared with the control (Fig. 4a-c). In addition, the PUN level was 0.21 mg/dL lower ($P < 0.01$) in mixed parity than in multiparous cows (Fig. 4c). Feeding low CP diets for a short (≤ 50 days) or long period (>50 days) both resulted in a reduction ($P < 0.01$) in milk yield, PUN content and urinary N excretion of dairy cows (Fig. 5a-c). The daily urinary N excretion was 32 g higher ($P < 0.01$) in cows when legume-based low CP diets were fed over 50 days than a short period (≤ 50 days; Fig. 5c).



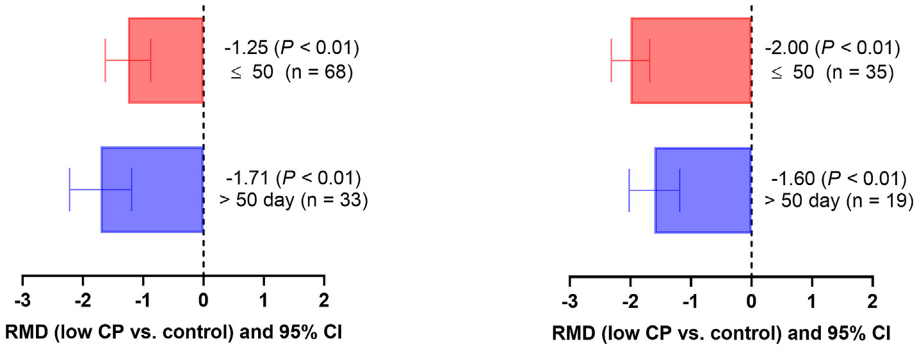
a) Dry matter intake (kg/d)

b) Milk yield (kg/d)



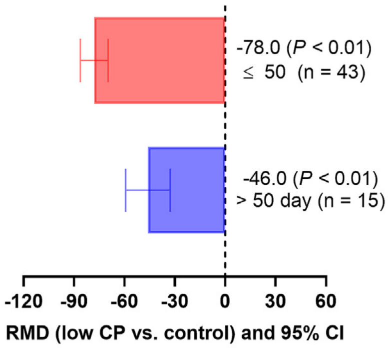
c) Plasma urea N (mmol/L)

Fig. 4. Covariate (parity: multiparous cow or mixed cow (used primiparous and multiparous)) effect size estimates for a) DM intake (kg/d), b) milk yield (kg/d), and c) plasma urea N (mmol/L) of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis. *P*-values within parentheses are used to compare with control values. RMD = raw mean differences between high (control) and low CP diets. *P*–value between groups (multiparous vs mixed) for DM intake, $P = 0.17$; milk yield, $P = 0.13$; and plasma urea N, $P < 0.01$. The error bar indicates a 95% level confidence interval. Abbreviations: CI = confidence interval.



a) Milk yield (kg/d)

b) Plasma urea N (mmol/L)



c) Urinary N (g/d)

Fig. 5. Covariate (experimental duration (days): ≤ 50 (short) or >50 (long) days) effect size estimates for a) milk yield (kg/d), b) plasma urea N (mmol/L), and c) urinary N excretion (g/d) of dairy cows fed high (control) or low CP diets based on forage legumes in a subgroup random-effect meta-analysis. *P*-values within parentheses are used to compare with control values. RMD = raw mean differences between high (control) and low CP diets. *P*–value between groups (≤50 days vs > 50 days) for milk yield, *P* = 0.16; plasma urea N, *P* = 0.14; and urinary N, *P* < 0.01. The error bar indicates a 95% level confidence interval. Abbreviations: CI = confidence interval.

Discussion

The dietary CP content of the legume silage-based rations was reduced in the treatment diet of all included studies by decreasing the concentration of vegetable proteins, including soybean meal, heat-treated/expeller soybean or rapeseed meal, which resulted in differences in the RDP, RUP and MP content between control and low protein diets. However, only 22.2% of studies (n = 8) reported the predicted MP supply, with 11.1% of studies (n = 4) that lowered the dietary CP content and simultaneously maintained the MP supply at or around that of the control diets (e.g. Chowdhury et al., 2023, 2024). Thus, our current meta-analysis is solely based on CP level rather than RDP, RUP or MP due to the limited number of studies that reported or predicted RDP, RUP and MP values. Some studies (42%) supplemented RP-AA in MP-deficient diets to offset the negative impact on the performance of dairy cows by enhancing the postruminal supply of limiting essential AA. Differences between diets in starch content were due to the inclusion of processed or ground maize, wheat or barley in low CP diets when metabolisable energy was not limited (Ipharraguerre and Clark, 2005; Liu and VandeHaar, 2020; Recktenwald et al., 2014).

Feed intake

Dry matter intake was reduced in cows fed low CP, legume silage-based diets, which could be attributed to an impaired rumen function due to an insufficient supply of RDP (105 vs 92.8 g/kg DM, Table 1), which may depress fibre digestion and rumen passage rate, resulting in a lower feed intake (Allen, 2000). However, a significant heterogeneity for DM intake was observed, and the variation was due to the influence of covariates. For example, DM intake was lowest when cows received less than 140 g CP/kg DM, indicating a positive relationship between the dietary concentration of CP and DM intake, which supports the findings of Barros et al. (2017), who reduced the concentration of dietary CP from 162 to 118 g/kg DM and reported that DM intake was reduced linearly with decreasing dietary CP concentration. A meta-analysis by Huhtanen and Hetta (2012) also reported a similar trend, but this response is not always evident because of the inconsistent effect of dietary CP levels on DM intake (Broderick et al., 2015; Liu and VandeHaar, 2020; Olmos Colmenero and Broderick, 2006). According to Hristov and Giallongo (2014), the negative effect of low CP diets on DM intake is due to a lower supply of MP in high-yielding dairy cows, whilst Sinclair et al. (2014) reported that the dietary CP level could be reduced to around 140 g/kg DM without affecting DM intake if the diet meets the cows' MP requirements.

Another factor that can decrease the DM intake of cows fed diets deficient in MP is a reduction in the postruminal supply of essential AA, as reported by Lee et al. (2012) and Giallongo et al. (2016). In a subgroup analysis, the DM intake of cows was not negatively affected by low CP diets supplemented with RP-AA and, in some cases, was increased when supplemented with RPM, possibly due to the balance of available AA within the microbial CP synthesised in the rumen (Li et al., 2022). Our recent study (Chowdhury et al., 2024) concluded that dietary strategies should aim to optimise microbial CP synthesis to correct MP or essential AA supply and to mitigate the anticipated reduction in DM intake by feeding low CP (≤ 150 g/kg DM) diets. However, two other meta-analyses by Patton (2010) and Zanton et al. (2014) reported an inconsistent effect of RPM on the DM intake of milking cows, which may have occurred due to the deficiency of other rate-limiting essential AA (Patton, 2010), excessive inclusion of RPM (Robinson et al., 2000), the use of different synthetic sources of RPM with different bioavailabilities (Zanton et al., 2014) or MP not being deficient as predicted.

Broderick et al. (2001) reported that feed intake in dairy cows was affected by forage type and that the daily DM intake of a lucerne-based diet was 1.20 kg higher than a red clover silage-based ration (Broderick, 2018). However, a reduced DM intake in cows fed lucerne or red clover silage-based low CP diets was observed in the current study, but the effect size between forages did not alter. This finding is in agreement with the meta-analyses by Johansen et al. (2017) and Steinshamn (2010), who reported a similar DM intake when lucerne or red-clover silage-based diets were fed to lactating dairy cows. Furthermore, the increase in DM intake associated with the inclusion of legume silage is influenced by the type of silage being replaced (Moorby et al., 2009; Schulz et al., 2018; Sinclair et al., 2015). The DM intake did not alter when legume silage substituted approximately 20% of non-legume forages; however, increasing the proportion up to 60% reduced intake, an effect in accordance with previous observations by Sinclair et al. (2015) and Schulz et al. (2018), who investigated the effects of different inclusion levels of legume silages in the diet of dairy cows.

Milk performance

Reduced milk yield observed in dairy cows fed legume silage-based low CP diets may be attributed to a decrease in DM intake. The greatest reduction in milk yield was found when cows received <140 g CP/kg DM diet, and according to Lee et al. (2012) and Alstrup et al. (2014), reducing dietary CP concentration below 140 g/kg DM can negatively affect milk production. A very low CP concentration in dairy cows' diet can decrease the postruminal supply of MP, leading to reduced milk and milk protein yield (Giallongo et al., 2016; Hristov and Giallongo, 2014), suggesting a strong correlation between intestinal MP supply and milk yield (Daniel et al., 2016). However, there was a lack of studies that reported predicted MP supply, and it is recommended that all future studies report this rather than just CP.

Feeding legume-based diets can improve milk yield in dairy cows compared with those receiving grass-silage–based rations (Dewhurst et al., 2003; Steinshamn, 2010). A meta-analysis by Johansen et al. (2017) reported that feeding legume-based diets increased milk yield by 1.60 kg/d than grass silage-based rations. Cows had a comparable milk production when lucerne or red clover silage-based rations were fed rather than white clover-based diets (Johansen et al., 2017). In the current study, a reduced milk yield in response to low dietary protein was observed in cows fed lucerne-based rations, but no significant difference was observed when cows received low CP diets based on red clover silage. The possible reason for the milk yield difference between cows fed lucerne or red clover silage-based diets could be the decrease in MP when reducing dietary protein concentration, which is greater for lucerne due to a higher RUP associated with the action of polyphenol oxidase in red clover silage.

The concentration of milk protein was reduced in cows fed low CP diets based on legume silages but was similar to the control diet when either RPM, rumen-protected lysine or both was added to the low CP diet, highlighting that milk protein synthesis depends on the availability of essential AA to the mammary gland (Doepel and Lapierre, 2010; Hristov et al., 2005; Huhtanen and Hristov, 2009). Several authors (Giallongo et al., 2016; Giallongo et al., 2015; Lee et al., 2015) have demonstrated that methionine and lysine are the key limiting AA for milk protein production in cows fed maize silage and lucerne-based rations. However, a decrease in milk protein content was observed with an increasing proportion of legume silages in our subgroup analysis, which might have been due to a limited metabolisable energy content in legumes (Steinshamn, 2010), which leads to a lower supply of rumen available energy and subsequent microbial CP flow to the duodenum. A meta-analysis by Daniel et al. (2016) indicated that increasing metabolisable energy supply increases the proportion partitioned to body reserves, whilst increasing MP supply increases the proportion of energy partitioned towards milk production. Therefore, optimising the metabolisable energy-to-MP ratio could enhance nutrient partitioning and milk performance, with potential variations depending on the stage of lactation.

Nutrient intake and digestibility

The negative effect of low CP diets on nutrient digestibility, including fibre, could be attributed to a deficiency of rumen degradable N, which is required by cellulolytic bacteria to degrade ingested carbohydrates (Atasoglu et al., 2001). The lowest concentration of rumen NH_3-N was also observed in the current meta-analysis when cows received less than 140 g CP/kg DM diets. Feeding legume-based diets that are very low in CP can limit the supply of rumen available N, which leads to a decrease in microbial CP synthesis and rumen fermentation (Broderick, 2018; Lee et al., 2012). A meta-analysis by Huhtanen et al. (2009) noted that the apparent total-tract organic matter digestibility in lactating cows was negatively correlated to DM intake. In addition, a significant reduction in NDF intake and apparent organic matter and fibre digestibility was observed in the current study when cows received low CP diets based on red clover silage, which could be attributed to a lower silage non-protein N content and greater concentration of acid detergent insoluble-N, or the enzyme polyphenol oxidase in red clover silage (Lee, 2014). Polyphenol oxidase may interact with plant proteins, including proteases, and depress fibre degradation, resulting in a reduced microbial CP synthesis in the rumen due to a lower supply of RDP (Broderick, 2018).

Plasma metabolites and urea nitrogen

The plasma concentration of β-hydroxybutyrate was numerically increased, and non-esterified fatty acid concentration was substantially increased in cows fed legume silage-based low CP diets, confirming the mobilisation of body fat. Law et al. (2009) also noted that the plasma concentration of β-hydroxybutyrate was increased by 0.08 mmol/L in cows fed 114 g CP/kg DM than the control CP concentration of 173 g/kg DM. Similarly, Halmemies-Beauchet-Filleau et al. (2017) observed that reducing dietary CP content from 171 to 156 g/kg DM in red clover and grass silage-based rations increased plasma concentrations of non-esterified fatty acids by 0.08 mmol/L in early lactation Holstein cows. Therefore, lowering dietary CP concentration during the early stages of lactation is challenging for high-yielding dairy cows.

The decrease in total N intake and CP digestibility in dairy cows fed legume silage–based low CP diets reduced the concentration of PUN, which was associated with a significant reduction in MUN content and N excretion in the current study. However, high heterogeneity was observed for both PUN and MUN contents due to the level of CP or supplementation of RP-AA in the low CP diets. The subgroup analyses showed that the lowest milk or plasma urea N content reduction occurred when cows received ≥ 140 g CP/kg DM or when diets were supplemented with RP-AA. In general, the concentration of PUN in dairy cows is closely related to dietary CP level (Recktenwald et al., 2014). Another factor that may influence the variation in PUN level in cows is parity, and previous studies (Barton et al., 1996) have established that multiparous cows have a higher PUN content than first lactation animals, which agrees with the current findings.

Nitrogen output, use efficiency and rumen fermentation

Nitrogen excretion mainly depends on the concentration of dietary CP, total N or RDP intake, and a linear relationship exists between dietary N intake and urinary or faecal N output (Castillo et al., 2000). Several studies (Lee et al., 2012; Niu et al., 2016; Oh et al., 2019) have reported that low CP diets significantly decreased urinary N emission rather than faecal N, which is consistent with the current findings. Similar to MUN, a lower excretion of urinary N was observed when animals received < 140 g CP/kg DM or a ration without added AA, indicating a positive correlation between MUN and urinary N output, which supports previous studies (Kauffman and St-Pierre, 2001; Spek et al., 2013, Chowdhury et al., 2024). In contrast, feeding legume-based low CP diets with added RPM slightly elevated urinary N excretion in cows compared with those fed non-AA supplemented diets, possibly due to a lack of change in milk protein synthesis with RPM, assuming that the excess N is excreted in the urine. Broderick (2018) also observed a similar effect when the diet was supplemented with rumen-protected lysine.

Compared with the lucerne-based diet, there was a substantial decrease in urinary N excretion and increased excretion of faecal N as a proportion of total N intake in dairy cows fed red clover silage-based rations. This effect supports the findings of Broderick (2018), who investigated N utilisation in lactating dairy cows and growing lambs fed lucerne or red clover-based diets. The efficient utilisation of N is associated with feeding legume silage-based low protein diets (Chowdhury et al., 2023), and low CP diets increased the apparent milk NUE in the current study, which was related to a reduced urinary N excretion (Chowdhury et al., 2024). The excretion of urinary N was slightly increased when cows were fed legume silage-based low CP diets for more than 7 weeks in the current analysis, which might possibly be associated with urea recycling adaptation to low CP diets.

Feeding legume silage-based low CP diets reduced the molar proportion of rumen butyrate, which is in agreement with the findings of Cui et al. (2019), who reported a tendency (P = 0.05) towards a lower concentration of rumen butyrate in lambs fed either CP or energy-deficient diets. In contrast, some studies (Aguerre et al., 2016; Nursoy et al., 2018) have reported no significant effect of dietary CP concentration on rumen volatile fatty acid concentration, except for branched-chain volatile fatty acids, including the molar proportion of valerate or *iso*-valerate, which might be a potential marker of rumen N deficiency (Cabrita et al., 2003; Leduc et al., 2017).

Limitations and strengths

The current meta-analysis was limited to early and mid-lactating high-yielding dairy cows. Therefore, the outcomes may not be appropriate for late or low-producing cows, as the lowest yield in the data analysed was 22 kg/cow/d. Most studies in the literature that have fed legume silages were based on lucerne, and there are few studies that have fed low–protein diets containing red clover silages. Therefore, further studies on low CP diets based on red clover and other legume silages such as peas or beans are required. Some performance outcomes contained variations across the studies due to the level of CP in the diet, rate of legume silage inclusion, and supplementation of RP-AA in low CP diets. However, the meta-analysis did not include other dietary factors such as starch level, RDP and RUP content, and the concentration of MP due to a very limited number of studies on legume silages that reported these values. Therefore, future studies should report the measured or predicted RDP, RUP and MP in addition to dietary CP concentration. In addition, a subsequent meta-analysis could compare responses to low dietary CP in legume-based diets to responses to low dietary CP in grass silage-based rations. Regardless of these limitations, the main strength of the current study was that there was no publication bias for the response variables, and there was a systematic characterisation of a pooled dataset from the literature to provide an overall summary of dairy cow performance, metabolism and N use efficiency.

Conclusion

Feeding low protein diets based on legume silages negatively impacted the performance of dairy cows by reducing intake, milk yield, milk protein content, condition score, diet digestibility, and rumen NH_3-N and molar proportion of butyrate, but improved apparent NUE, which was associated with a reduced N excretion in urine and decreased plasma and milk urea N content. The dietary concentration of CP, legume type and its inclusion rate, and RP-AA supplementation were strongly related to some but not all performance outcomes and, consequently, raised heterogeneity. Feeding very low CP content diets (<140 g/kg DM) negatively impacted DM intake and milk performance. Supplementation of RP-AA in low CP diets did not alter DM intake or milk protein content compared with high protein diets. However, providing RPM increased DM intake and had a tendency for milk protein content to be increased when compared with no AA supplement, and MUN concentration was higher in cows receiving rumen-protected lysine than no additional AA. Compared with red clover silage-based rations, lucerne-based low CP diets improved apparent nutrient digestibility, but reduced milk yield. Future studies investigating low-protein diets based on legume silages should focus on red clover-based rations and other legumes, and the dietary effects of RDP, RUP, and MP should be reported rather than just CP.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101433) can be found at the foot of the online page, in the Appendix section.

Ethics approval

Not applicable.

Data and model availability statement

The data were not deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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M.R. Chowdhury: Writing – original draft, Visualisation, Validation, Software, Project administration, Methodology, Formal analysis, Data curation. **R.G. Wilkinson:** Writing – review & editing, Supervision. **L.A. Sinclair:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualisation.

Declaration of interest

None.

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References

Aguerre, M.J., Capozzolo, M.C., Lencioni, P., Cabral, C., Wattiaux, M.A., 2016. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. Journal of Dairy Science 99, 4476–4486. https://doi.org/10.3168/jds.2015-10745.

Allen, M.S., 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. Journal of Dairy Science 83, 1598–1624. https://doi.org/10.3168/JDS.S0022-0302(00)75030-2.

Alstrup, L., Weisbjerg, M.R., Hymøller, L., Larsen, M.K., Lund, P., Nielsen, M.O., 2014. Milk production response to varying protein supply is independent of forage digestibility in dairy cows. Journal of Dairy Science 97, 4412–4422. https://doi.org/10.3168/jds.2013-7585.

Atasoglu, C., Newbold, C.J., Wallace, R.J., 2001. Incorporation of [15N]Ammonia by the Cellulolytic Ruminal Bacteria Fibrobacter succinogenesBL2, Ruminococcus albus SY3, and Ruminococcus flavefaciens 17. Applied and Environmental Microbiology 67, 2819–2822. https://doi.org/10.1128/AEM.67.6.2819-2822.2001.

Barros, T., Quaassdorff, M.A., Aguerre, M.J., Colmenero, J.J.O., Bertics, S.J., Crump, P. M., Wattiaux, M.A., 2017. Effects of dietary crude protein concentration on late-lactation dairy cow performance and indicators of nitrogen utilization. Journal of Dairy Science 100, 5434–5448. https://doi.org/10.3168/jds.2016-11917.

Barton, B.A., Rosario, H.A., Anderson, G.W., Grindle, B.P., Carroll, D.J., 1996. Effects of dietary crude protein, breed, parity, and health status on the fertility of dairy cows. Journal of Dairy Science 79, 2225–2236. https://doi.org/10.3168/jds.S0022-0302(96)76599-2.

Begg, C.B., Mazumdar, M., 1994. Operating characteristics of a rank correlation test for publication bias. Biometrics 50, 1088. https://doi.org/10.2307/2533446.

Borenstein, M., Hedges, L.V., Higgins, J.P.T., Rothstein, H.R., 2009. Fixed-effect versus random-effects models. In: Borenstein, M., Hedges, L.V., Higgins, J.P.T., Rothstein, H.R. (Eds.), Introduction to Meta-Analysis. Wiley, Chichester, UK, pp. 77–86.

Broderick, G.A., 2018. Utilization of protein in red clover and alfalfa silages by lactating dairy cows and growing lambs. Journal of Dairy Science 101, 1190–1205. https://doi.org/10.3168/jds.2017-13690.

Broderick, G.A., Walgenbach, R.P., Maignan, S., 2001. Production of lactating dairy cows fed alfalfa or red clover silage at equal dry matter or crude protein contents in the diet. Journal of Dairy Science 84, 1728–1737. https://doi.org/10.3168/jds.S0022-0302(01)74608-5.

Broderick, G.A., Faciola, A.P., Armentano, L.E., 2015. Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows. Journal of Dairy Science 98, 5672–5687. https://doi.org/10.3168/JDS.2015-9563.

Cabrita, A.R.J., Fonseca, A.J.M., Dewhurst, R.J., Gomes, E., 2003. Nitrogen supplementation of corn silages. 2. assessing Rumen function using fatty acid profiles of bovine milk. Journal of Dairy Science 86, 4020–4032. https://doi.org/10.3168/JDS.S0022-0302(03)74013-2.

Castillo, A., Kebreab, E., Beever, D., France, J., 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. Journal of Animal and Feed Sciences 9, 1–32. https://doi.org/10.22358/jafs/68025/2000.

Chowdhury, M.R., 2022. Low protein diets based on high protein forages for dairy cows: effects on performance, metabolism and nitrogen use efficiency. PhD Thesis,Harper Adams University, Edgmond, Newport, UK.

Chowdhury, M.R., Wilkinson, R.G., Sinclair, L.A., 2023. 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. Journal of Dairy Science 106, 1773–1789. https://doi.org/10.3168/jds.2022-22607.

Chowdhury, M.R., Wilkinson, R.G., Sinclair, L.A., 2024. 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. Journal of Dairy Science 107, 3543–3557. https://doi.org/10.3168/JDS.2023-23987.

Cui, K., Qi, M., Wang, S., Diao, Q., Zhang, N., 2019. Dietary energy and protein levels influenced the growth performance, ruminal morphology and fermentation and microbial diversity of lambs. Scientific Reports 9, 16612. https://doi.org/10.1038/s41598-019-53279-y.

Daniel, J.B., Friggens, N.C., Chapoutot, P., Van Laar, H., Sauvant, D., 2016. Milk yield and milk composition responses to change in predicted net energy and metabolizable protein: a meta-analysis. Animal 10, 1975–1985. https://doi.org/10.1017/S1751731116001245.

DerSimonian, R., Laird, N., 1986. Meta-analysis in clinical trials. Controlled Clinical Trials 7, 177–188. https://doi.org/10.1016/0197-2456(86)90046-2.

Dewhurst, R.J., 2013. Milk production from silage: comparison of grass, legume and maize silages and their mixtures. Agricultural and Food Science 22, 57–69. https://doi.org/10.23986/afsci.6673.

Dewhurst, R.J., Fisher, W.J., Tweed, J.K.S., Wilkins, R.J., 2003. Comparison of grass and legume silages for milk production. 1. production responses with different levels of concentrate. Journal of Dairy Science 86, 2598–2611. https://doi.org/10.3168/jds.S0022-0302(03)73855-7.

Doepel, L., Lapierre, H., 2010. Changes in production and mammary metabolism of dairy cows in response to essential and nonessential amino acid infusions. Journal of Dairy Science 93, 3264–3274. https://doi.org/10.3168/JDS.2009-3033.

Egger, M., Smith, G.D., Schneider, M., Minder, C., 1997. Bias in meta-analysis detected by a simple, graphical test. British Medical Journal 315, 629–634. https://doi.org/10.1136/bmj.315.7109.629.

Giallongo, F., Hristov, A.N., Oh, J., Frederick, T., Weeks, H., Werner, J., Lapierre, H., Patton, R.A., Gehman, A., Parys, C., 2015. Effects of slow-release urea and rumen-protected methionine and histidine on performance of dairy cows. Journal of Dairy Science 98, 3292–3308. https://doi.org/10.3168/JDS.2014-8791.

Giallongo, F., Harper, M.T., Oh, J., Lopes, J.C., Lapierre, H., Patton, R.A., Parys, C., Shinzato, I., Hristov, A.N., 2016. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. Journal of Dairy Science 99, 4437–4452. https://doi.org/10.3168/JDS.2015-10822.

Halmemies-Beauchet-Filleau, A., Shingfield, K.J., Simpura, I., Kokkonen, T., Jaakkola, S., Toivonen, V., Vanhatalo, A., 2017. Effect of incremental amounts of camelina oil on milk fatty acid composition in lactating cows fed diets based on a mixture of grass and red clover silage and concentrates containing camelina expeller. Journal of Dairy Science 100, 305–324. https://doi.org/10.3168/jds.2016-11438.

Higgins, J.P.T., Thompson, S.G., Deeks, J.J., Altman, D.G., 2003. Measuring inconsistency in meta-analyses. British Medical Journal 327, 557–560. https://doi.org/10.1136/bmj.327.7414.557.

Hristov, A.N., Giallongo, F., 2014. Feeding protein to dairy cows - what should be our target?, Proceedings of the 23rd Tri-State Dairy Nutrition Conference, 14-16 April 2014, Fort Wayne, Indiana, USA, pp. 75–84.

Hristov, A.N., Price, W.J., Shafii, B., 2005. A meta-analysis on the relationship between intake of nutrients and body weight with milk volume and milk protein yield in dairy cows. Journal of Dairy Science 88, 2860–2869. https://doi.org/10.3168/jds.S0022-0302(05)72967-2.

Huhtanen, P., Hetta, M., 2012. Comparison of feed intake and milk production responses in continuous and change-over design dairy cow experiments. Livestock Science 143, 184–194. https://doi.org/10.1016/j.livsci.2011.09.012.

Huhtanen, P., Hristov, A.N., 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. Journal of Dairy Science 92, 3222–3232. https://doi.org/10.3168/jds.2008-1352.

Huhtanen, P., Nousiainen, J.I., Rinne, M., Kytölä, K., Khalili, H., 2008. Utilization and partition of dietary nitrogen in dairy cows fed grass silage-based diets. Journal of Dairy Science 91, 3589–3599. https://doi.org/10.3168/jds.2008-1181.

Huhtanen, P., Rinne, M., Nousiainen, J., 2009. A meta-analysis of feed digestion in dairy cows. 2. the effects of feeding level and diet composition on digestibility. Journal of Dairy Science 92, 5031–5042. https://doi.org/10.3168/jds.2008-1834.

National Institute for Agricultural Research (INRA), 2018. INRA feeding system for ruminants, Feeding system for ruminant, 2nd edition. Wageningen Academic

Publishers, Leiden, The Netherlands. https://doi.org/10.3920/978-90-8686-292-4.

Ipharraguerre, I.R., Clark, J.H., 2005. Varying protein and starch in the diet of dairy cows. II. effects on performance and nitrogen utilization for milk production. Journal of Dairy Science 88, 2556–2570. https://doi.org/10.3168/jds.s0022-0302(05)72932-5.

Johansen, M., Lund, P., Weisbjerg, M.R., 2017. Feed intake and milk production in dairy cows fed different grass and legume species: a meta-analysis. Animal 12, 66–75. https://doi.org/10.1017/S1751731117001215.

Kauffman, A.J., St-Pierre, N.R., 2001. The relationship of milk urea nitrogen to urine nitrogen excretion in holstein and jersey cows. Journal of Dairy Science 84, 2284–2294. https://doi.org/10.3168/jds.S0022-0302(01)74675-9.

Law, R.A., Young, F.J., Patterson, D.C., Kilpatrick, D.J., Wylie, A.R.G., Mayne, C.S., 2009. Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation. Journal of Dairy Science 92, 1001–1012. https://doi.org/10.3168/jds.2008-1155.

Lean, I.J., de Ondarza, M.B., Sniffen, C.J., Santos, J.E.P., Griswold, K.E., 2018. Meta-analysis to predict the effects of metabolizable amino acids on dairy cattle performance. Journal of Dairy Science 101, 340–364. https://doi.org/10.3168/jds.2016-12493.

Leduc, M., Gervais, R., Tremblay, G.F., Chiquette, J., Chouinard, P.Y., 2017. Milk fatty acid profile in cows fed red clover- or alfalfa-silage based diets differing in rumen-degradable protein supply. Animal Feed Science and Technology 223, 59–72. https://doi.org/10.1016/j.anifeedsci.2016.11.001.

Lee, M.R.F., 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Frontiers in Plant Science 5, 694. https://doi.org/10.3389/fpls.2014.00694.

Lee, C., Hristov, A.N., Cassidy, T.W., Heyler, K.S., Lapierre, H., Varga, G.A., de Veth, M. J., Patton, R.A., Parys, C., 2012. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. Journal of Dairy Science 95, 6042–6056. https://doi.org/10.3168/jds.2012-5581.

Lee, C., Giallongo, F., Hristov, A.N., Lapierre, H., Cassidy, T.W., Heyler, K.S., Varga, G. A., Parys, C., 2015. Effect of dietary protein level and rumen-protected amino acid supplementation on amino acid utilization for milk protein in lactating dairy cows. Journal of Dairy Science 98, 1885–1902. https://doi.org/10.3168/JDS.2014-8496.

Letelier, P., Zanton, G.I., Wattiaux, M.A., 2022. Production performance of Holstein cows at 4 stages of lactation fed 4 dietary crude protein concentrations. Journal of Dairy Science 105, 9581–9596. https://doi.org/10.3168/jds.2022-22146.

Li, Y., Wei, J., Dou, M., Liu, S., Yan, B., Li, C., Khan, M.Z., Zhang, Y., Xiao, J., 2022. Effects of rumen-protected methionine supplementation on production performance, apparent digestibility, blood parameters, and ruminal fermentation of lactating Holstein dairy cows. Frontiers in Veterinary Science 9, 981757. https://doi.org/10.3389/fvets.2022.981757.

Liu, E., VandeHaar, M.J., 2020. Relationship of residual feed intake and protein efficiency in lactating cows fed high- or low-protein diets. Journal of Dairy Science 103, 3177–3190. https://doi.org/10.3168/jds.2019-17567.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., Altman, D., Antes, G., Atkins, D., Barbour, V., Barrowman, N., Berlin, J.A., Clark, J., Clarke, M., Cook, D., D'Amico, R., Deeks, J.J., Devereaux, P.J., Dickersin, K., Egger, M., Ernst, E., Gøtzsche, P.C., Grimshaw, J., Guyatt, G., Higgins, J., Ioannidis, J.P.A., Kleijnen, J., Lang, T., Magrini, N., McNamee, D., Moja, L., Mulrow, C., Napoli, M., Oxman, A., Pham, B., Rennie, D., Sampson, M., Schulz, K.F., Shekelle, P.G., Tovey, D., Tugwell, P., 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Annals of Internal Medicine 151, 264–269. https://doi.org/10.1371/journal.pmed.1000097.

Moorby, J.M., Lee, M.R.F., Davies, D.R., Kim, E.J., Nute, G.R., Ellis, N.M., Scollan, N.D., 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. Journal of Dairy Science 92, 1148–1160. https://doi.org/10.3168/jds.2008-1771.

National Academies of Sciences, Engineering, and Medicine (NASEM), 2021. Nutrient Requirements of Dairy Cattle, 8th revised edition. National Academies Press, Washington, DC, USA. https://doi.org/10.17226/25806.

NRC (National Research Council), 2001. Nutrient requirements of dairy cattle, 7th revised edition. National Academy Press, Washington, DC, USA.

Niu, M., Appuhamy, J.A.D.R.N., Leytem, A.B., Dungan, R.S., Kebreab, E., 2016. Effect of dietary crude protein and forage contents on enteric methane emissions and nitrogen excretion from dairy cows simultaneously. Animal Production Science 56, 312–321. https://doi.org/10.1071/AN15498.

Nursoy, H., Ronquillo, M.G., Faciola, A.P., Broderick, G.A., 2018. Lactation response to soybean meal and rumen-protected methionine supplementation of corn silage-based diets. Journal of Dairy Science 101, 2084–2095. https://doi.org/10.3168/jds.2017-13227.

Oh, J., Harper, M., Hristov, A.N., 2019. Effects of lowering crude protein supply alone or in a combination with essential oils on productivity, rumen function and nutrient utilization in dairy cows. Animal 13, 2510–2518. https://doi.org/10.1017/s1751731119001083.

Olmos Colmenero, J.J., Broderick, G.A., 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. Journal of Dairy Science 89, 1704–1712. https://doi.org/10.3168/jds.S0022-0302(06)72238-X.

Patton, R.A., 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: a meta-analysis. Journal of Dairy Science 93, 2105–2118. https://doi.org/10.3168/jds.2009-2693.

Recktenwald, E.B., Ross, D.A., Fessenden, S.W., Wall, C.J., Van Amburgh, M.E., 2014. Urea-N recycling in lactating dairy cows fed diets with 2 different levels of dietary crude protein and starch with or without monensin. Journal of Dairy Science 97, 1611–1622. https://doi.org/10.3168/jds.2013-7162.

Robinson, P.H., Chalupa, W., Sniffen, C.J., Julien, W.E., Sato, H., Fujieda, T., Ueda, T., Suzuki, H., 2000. Influence of abomasal infusion of high levels of lysine or methionine, or both, on ruminal fermentation, eating behavior, and performance of lactating dairy cows. Journal of Animal Science 78, 1067–1077. https://doi.org/10.2527/2000.7841067x.

Salami, S.A., Moran, C.A., Warren, H.E., Taylor-Pickard, J., 2020. A meta-analysis of the effects of slow-release urea supplementation on the performance of beef cattle. Animals 10, 657. https://doi.org/10.3390/ani10040657.

Schulz, F., Westreicher-Kristen, E., Knappstein, K., Molkentin, J., Susenbeth, A., 2018. Replacing maize silage plus soybean meal with red clover silage plus wheat in diets for lactating dairy cows. Journal of Dairy Science 101, 1216–1226. https://doi.org/10.3168/jds.2017-13605.

Seleem, M.S., Wu, Z.H., Xing, C.Q., Zhang, Y., Hanigan, M.D., Bu, D.P., 2023. Impacts of rumen-encapsulated methionine and lysine supplementation and low dietary protein on nitrogen efficiency and lactation performance of dairy cows. Journal of Dairy Science 107, 2087–2098. https://doi.org/10.3168/jds.2023-23404.

Sinclair, L.A., Edwards, R., Errington, K.A., Holdcroft, A.M., Wright, M., 2015. Replacement of grass and maize silages with lucerne silage: effects on performance, milk fatty acid profile and digestibility in Holstein-Friesian dairy cows. Animal 9, 1970–1978. https://doi.org/10.1017/S1751731115001470.

Sinclair, K.D., Garnsworthy, P.C., Mann, G.E., Sinclair, L.A., 2014. Reducing dietary protein in dairy cow diets: implications for nitrogen utilization, milk production, welfare and fertility. Animal 8, 262–274. https://doi.org/10.1017/S1751731113002139.

Spek, J.W., Dijkstra, J., van Duinkerken, G., Hendriks, W.H., Bannink, A., 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: a meta-analysis. Journal of Dairy Science 96, 4310–4322. https://doi.org/10.3168/jds.2012-6265.

Steinshamn, H., 2010. Effect of forage legumes on feed intake, milk production and milk quality-a review. Animal Science Papers and Reports 28, 195–206.

Thomas, C., 2004. Feed into milk: a new applied feeding system for dairy cows. Nottingham University Press, Nottingham, UK.

Torres, R.N.S., Moura, D.C., Ghedini, C.P., Ezequiel, J.M.B., Almeida, M.T.C., 2020. Meta-analysis of the effects of essential oils on ruminal fermentation and performance of sheep. Small Ruminant Research 189, 106148. https://doi.org/10.1016/j.smallrumres.2020.106148.

Van den Bossche, T., Goossens, K., Ampe, B., Haesaert, G., De Sutter, J., De Boever, J.L., Vandaele, L., 2023. Effect of supplementing rumen-protected methionine, lysine, and histidine to low-protein diets on the performance and nitrogen balance of dairy cows. Journal of Dairy Science 106, 1790–1802. https://doi.org/10.3168/jds.2022-22041.

Wei, C., He, T., Wan, X., Liu, S., Dong, Y., Qu, Y., 2022. Meta-analysis of rumen-protected methionine in milk production and composition of dairy cows. Animals 12, 1505. https://doi.org/10.3390/ani12121505.

Yang, C.T., Ferris, C.P., Yan, T., 2022. Effects of dietary crude protein concentration on animal performance and nitrogen utilisation efficiency at different stages of lactation in Holstein-Friesian dairy cows. Animal 16, 100562. https://doi.org/10.1016/j.animal.2022.100562.

Zanton, G.I., Bowman, G.R., Vázquez-Añón, M., Rode, L.M., 2014. Meta-analysis of lactation performance in dairy cows receiving supplemental dietary methionine sources or postruminal infusion of methionine. Journal of Dairy Science 97, 7085–7101. https://doi.org/10.3168/jds.2014-8220.