Animal 15 (2021) 100300

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effects of faba bean, blue lupin and rapeseed meal supplementation on nitrogen digestion and utilization of dairy cows fed grass silage-based diets

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ARTICLE INFO

Article history:
Received 28 November 2020
Revised 18 May 2021
Accepted 25 May 2021
Available online 24 June 2021

Keywords:
Digestibility
Grain legume
Milk production
Nitrogen use efficiency
Rumen fermentation

ABSTRACT

There is increasing interest in using locally produced protein supplements in dairy cow feeding. The objective of this experiment was to compare rapeseed meal (**RSM**), faba beans (**FBs**) and blue lupin seeds (**BL**) at isonitrogenous amounts as supplements of grass silage and cereal based diets. A control diet (**CON**) without protein supplement was included in the experiment. Four lactating Nordic Red cows were used in a 4 × 4 Latin Square design with four 21 d periods. The milk production increased with protein supplementation but when expressed as energy corrected milk, the response disappeared due to substantially higher milk fat concentration with CON compared to protein supplemented diets. Milk protein output increased by 8.5, 4.4 and 2.7% when RSM, FB and BL were compared to CON. The main changes in rumen fermentation were the higher propionate and lower butyrate proportion of total rumen volatile fatty acids when the protein supplemented diets were compared to CON. Protein supplementation also clearly increased the ruminal ammonia N concentration. Protein supplementation improved diet organic matter and NDF digestibility but efficiency of microbial protein synthesis per kg organic matter truly digested was not affected. Flow of microbial N was greater when FB compared to BL was fed. All protein supplements decreased the efficiency of nitrogen use in milk production. The marginal efficiency (amount of additional feed protein captured in milk protein) was 0.110, 0.062 and 0.045 for RSM, FB and BL, respectively. The current study supports the evidence that RSM is a good protein supplement for dairy cows, and this effect was at least partly mediated by the lower rumen degradability of RSM protein compared to FB and BL. The relatively small production responses to protein supplementation with simultaneous decrease in nitrogen use efficiency in milk production suggest that economic and environmental consequences of protein feeding need to be carefully considered.

Implications

Locally produced protein sources are in great demand to improve the self-sufficiency and sustainability of milk production. Dairy cows were offered diets supplemented with rapeseed meal, faba beans or blue lupin seeds at isonitrogenous amounts, and compared with a control diet without protein supplementation. Rapeseed meal increased milk protein production compared to faba bean and blue lupin, which may be explained by the lower rumen degradability of rapeseed meal protein. All protein supplements increased milk protein production compared to control, but on average only by 5% and led to decreased nitrogen use efficiency potentially linked with negative environmental impacts.

Introduction

Protein supplementation of the diet is an effective method to increase the supply of energy and nutrients limiting milk synthesis of dairy cows. Provision of protein supplements increases the flow of undegradable feed protein into the small intestine and modifies the composition of undegraded feed amino acids available for absorption (Korhonen et al., 2002; Rinne et al., 2015). Other factors contributing to increased milk production include an improved ruminal microbial protein synthesis (Hoover, 1986), higher feed intake (Huhtanen et al., 2008a) and improved diet digestibility

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https://doi.org/10.1016/j.animal.2021.100300

(Nousiainen et al., 2009). All these factors have led to a practice of routine protein supplementation of dairy cow diets in intensive production in Europe, and subsequently into high dependency of soya bean protein imported to European Union (European Commission, 2018), which can be seen as an environmental, economic and political risk.

Locally produced protein supplements have thus gained a lot of interest particularly as e.g. rapeseed meal (**RSM**) has proven to be as good or even superior protein supplement for dairy cows compared to soya bean meal (Huhtanen et al., 2011; Rinne et al., 2015). Grain legumes such as faba bean (**FB**), lupins and peas are also potential local alternatives to soya bean protein. The economic competitiveness of the different crops depends largely on their agronomic performance, but assessment of that is beyond the scope of this article.

An intriguing question is whether protein supplementation can be considered necessary for dairy cows. Protein supplements are typically the most expensive components of the ration. Their use decreases the nitrogen use efficiency (**NUE**) of milk production (Huhtanen et al., 2008b) and increases the environmental load of milk production. The marginal efficiency of increased dietary protein is generally low as e.g. approximately only 10% of the additional CP given as soybean supplement was recovered in milk protein based on the meta-analysis of Huhtanen et al. (2011). Indeed, cows can produce milk even on diets solely based on non-protein-nitrogen by relying on the microbial protein synthesis in the rumen as proven already by Virtanen (1966). Even for the current dairy cow fed a high quality diet, the majority of the metabolized amino acids originate from microbial protein. As an example, 76% of the non-ammonia N flowing to duodenum was of microbial origin on a diet without protein supplementation in Rinne et al. (2015). Further, the term "protein requirement" can be misleading for lactating dairy cows because the milk output can be considered a response to the supply of nutrients rather than a direct requirement (Huhtanen & Nousiainen, 2012).

Several studies have revealed that FB and blue lupin (**BL**) can replace soybean based protein in dairy cow diets without reduction in milk production (e.g. Froidmont & Bartiaux-Thill, 2004; Cherif et al., 2018; Johnston et al., 2019; Mendowski et al., 2019). However, when compared with RSM, both FB (Puhakka et al., 2016; Ramin et al., 2017; Lamminen et al., 2019) and BL (Puhakka et al., 2017) resulted in lower milk production when supplementing a grass silage-based diet.

The objective of this study was to evaluate the feed N use efficiency and digestive responses of dairy cows to different plant-based protein supplements on a high quality grass silage and cereal based diet. The supplements chosen for this study were RSM, FB and BL, which all have the potential to decrease the dependency of imported soya bean protein. Further, we assessed the responses to a diet without protein supplementation to evaluate the overall need for protein supplementation. Our hypotheses were that protein supply is increased in response to protein supplementation but simultaneously NUE decreases, and that when given in isonitrogenous amounts, RSM is superior in metabolizable protein supply compared to the grain legumes FB and BL, which do not differ from each other.

Material and methods

Animals and diets

The effect of different protein supplements RSM (*Brassica napus* subsp. *oleifera*), FB (*Vicia faba* var. Kontu) and BL (*Lupinus angustifolius* var. Haags Blaue) were compared as protein supplements for lactating cows with no protein supplement as a negative control (**CON**). The FB was grown in Jokioinen, Finland (60.5°N 23.3°E) and BL in Helsinki, Finland (60.2°N, 24.9°E), while RSM was a commercial product (A-Rehu, Seinäjoki, Finland). Four multiparous Nordic Red cows fitted with rumen cannulas (Bar Diamond Inc., Parma, ID, USA) were used as experimental animals. The average BW of the cows was 601 (SD 3.7) kg and they were on average 53 (SD 9.7) days into lactation at the beginning of the experiment. Three of the cows were in their second lactation and one in her third lactation. The experimental design was a 4 × 4 Latin square with 21-day periods. The cows were kept in tie stalls.

The basal diet consisted of grass silage supplemented with 12 kg/day experimental concentrates containing barley, oats, molassed sugar beet pulp, minerals, vitamins and the experimental protein supplements. Control treatment contained no protein supplement, while in the other treatments, part of cereals was replaced by RSM, FB or BL (Table 1). All concentrate components were mixed and pelleted at the feed mill of Natural Resources Institute Finland (Jokioinen, Finland). Daily amount of protein supplements RSM, FB and BL given were 2.62, 3.19 and 2.82 kg DM/d, respectively, to provide the cows with isonitrogenous levels of supplementation. All concentrate mixtures contained 0.3 kg commercial mineral and vitamin supplement (MahtiMira, Hiven Oy, Paimio, Finland) including 217 g Ca, 110 g Na and 65 mg g/kg with no P. The concentrations of Cu, Zn, Mn, Co, Se, I and vitamin E were 296, 1 600, 330, 27, 29, 43 and 1 140 mg/kg, respectively, while those of vitamin D3 and vitamin A were 52 000 and 158 000 IU/kg. The silage was made from a first cut of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) sward at Jokioinen, Finland. The grass was wilted slightly, precision-chopped and ensiled in a horizontal silo using a formic acid based additive (AIV2 Plus, Eastman, Oulu, Finland) dosed at 5 l/ton fresh matter.

Experimental procedures and analyses

Concentrate feeds and silage were fed from separate troughs. Concentrates were fed in four batches (3 kg each) at 0600, 0900, 1600 and 1900 h. During the first 15 days of each period, silage was fed *ad libitum* (refusals 0.05–0.10). The feed intake was restricted to 0.95 of the *ad libitum* intake from days 16 to 21 to minimize fluctuations in daily feed intake during the sampling period. The data of feed intake and milk yield from days 16 to 21 of each period were used for calculating the results. Milking times were at 0700 and 1700 h. The milk was analysed for fat, protein, lactose and urea (Valio Ltd. Seinäjoki, Finland; infrared analyzer (MilkoScan FT6000, Foss Electric, Hillerød, Denmark) from samples collected on four consecutive milkings during days 18–20.

Representative feed samples were collected daily from the last week of each period and bulked for subsequent analyses of DM, ash, CP, crude fat, starch, NDF and indigestible NDF. Silage samples were analysed for every period but concentrate samples were combined over periods to yield one sample per feed for the whole experiment since they originated from a single batch. Silage samples were also analysed for pH, ammonia N, lactic acid, volatile fatty acids, ethanol and water soluble carbohydrates. Feed samples were analysed using standard procedures described by Ahvenjärvi et al. (2018) except for the protein fractions in the protein supplements, which were analysed according to Licitra et al. (1996). The N analyses of silages, concentrate feeds, faecal samples, omasal small and large particles and liquid, and microbes were determined with AOAC-968.06 method using Leco FP 428 nitrogen analyser (Leco Corp., St Joseph, MI, USA), while the Kjeldahl method (AOAC-984.13 using Cu as a digestion catalyst and Foss Kjeltec 2400 Analyzer Unit (Foss Tecator AB, Höganäs, Sweden)) was used for combined omasal samples and urine. The effective ruminal protein degradability (**EPD**) based on the protein fractions (Fox et al.,

Table 1
Composition of the experimental silage (n = 4) and concentrate mixtures (n = 1) without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL) fed to dairy cows.

		Pelleted concentrate mixture			
Item	Silage	CON	RSM	FB	BL
Proportional composition (g/kg DM)					
Barley	–	428	302	274	288
Oats	–	418	295	268	281
Molassed sugar beet pulp	–	126	126	126	129
Mineral mixture	–	28	28	28	29
RSM	–	–	249	–	–
FB	–	–	–	303	–
BL	–	–	–	–	273
DM (g/kg)	256	880	881	881	873
In DM (g/kg)					
Ash	82.9	60.7	70.6	63.6	63.3
CP	166	110	169	164	170
Crude fat	na[1]	19.2	34.3	21.6	33.0
NDF	532	208	227	180	226
Indigestible NDF	105	60.3	88.9	45.4	50.0
Starch	na	461	325	430	327
Feed values					
ME[2] (MJ/kg DM)	10.9	11.8	11.5	11.8	11.8
MP[3] (g/kg DM)	88	92	104	101	99
PBV[4] (g/kg DM)	35	−26	20	17	25

[1] na = not analysed.
[2] Metabolizable energy calculated according to Luke (2021).
[3] Metabolizable protein (MP) calculated as the sum of microbial protein synthesized in the rumen and rumen undegradable feed protein according to Luke (2021).
[4] Protein balance in the rumen (PBV) calculated as the difference between rumen degradable protein and protein used for microbial protein synthesis according to Luke (2021).

2003) was calculated for the protein supplements using a ruminal outflow rate of 0.08 per hour and the following formula:

$$\text{EPD (g/g)} = (A + B1 \times [2/(2 + 0.08)] + B2 \times [0.1/0.1 + 0.08)] + B3 \times [0.002/(0.002 + 0.08)])/1\ 000,$$

where the protein fractions A, B1, B2 and B3 were given in g/kg total N and fraction A was considered instantly degraded while the degradation rates of B1, B2 and B3 were 2, 0.1 and 0.02 per hour, respectively.

Rumen fermentation, diet digestibility and omasal digesta flow were determined as described in Rinne et al. (2015). In brief, rumen fluid samples were taken through ruminal cannula on a single day of each period eight times at 1.5 h intervals. Total collection of faeces and urine was conducted over the four last days of each period. The digesta flow from rumen to the lower tract was based on omasal sampling over four days with three samplings per day. The results were calculated using a triple marker method with CrEDTA, Yb-acetate and indigestible NDF as markers for liquid, small particle and large particle flows, respectively, and ^{15}N as the microbial marker.

Calculations and statistical analysis

The metabolizable energy (**ME**) concentration of the silage was based on the digestible organic matter concentration in DM (DOM, g/kg DM) determined *in vitro* using a pepsin-cellulase based method (Huhtanen et al., 2006). The equation used was as follows: ME (MJ/kg DM) = DOM (g/kg DM) × 16 (MJ/kg DOM)/1 000 (Luke, 2021). For concentrate feeds, the ME concentration was based on digestible nutrients using crude fibre concentrations, digestibility coefficients and ME-values of digestible nutrients presented by Luke (2021). The intake of ME was calculated from feed ME-values and DM intake applying the correction equation taking into account the associative effects on diet digestion (level of feed intake and diet composition) according to Luke (2021). The efficiency of energy use for milk energy yield was calculated as energy excreted in milk/(ME intake − ME for maintenance), where ME for maintenance was based on Luke (2021). The concentrations of metabolizable protein (**MP**) and protein balance in the rumen (**PBV**) were calculated as described in Luke (2021). Nitrogen use efficiency of milk production was calculated as N excreted in milk (g)/feed N intake (kg). The efficiency of MP intake for milk protein yield was calculated as milk protein yield (g)/MP intake (kg) and N balance as N intake minus N excreted in milk, faeces and urine.

The data were analysed statistically using the GLM procedure of the SAS software for Windows version 9.1.3 (SAS Institute Inc., Cary, NC, USA) with the following model:

$$Y_{ijk} = \mu + A_i + P_j + D_k + e_{ijk},$$

where A, P and D are the animal, period and diet effects, and animal was considered as a fixed effect. The effect of experimental diets on variation in rumen pH and ammonia N over time was assessed using the MIXED procedure with a model for repeated measurements. The experimental hypotheses were tested using predetermined contrasts to evaluate the effect of protein supplementation (CON vs RSM + FB + BL), the comparison of RSM against the grain legumes (RSM vs FB + BL) and finally the comparison of the two grain legumes (FB vs BL). The data presented in the Tables are based on Least Square Means. Probability values less than 0.05 were considered statistically significant and less than 0.10 to indicate a tendency for significance.

Results

The *in vitro* organic matter digestibility of the grass silage was high (0.773) and CP concentration also relatively high at 166 g/kg DM (Table 1). The preservation quality of the silage was good as evidenced by low pH (4.05) and proportion of ammonia N in total N (42 g/kg). The lactic, acetic, propionic and butyric acids, ethanol and water soluble carbohydrate concentrations were 65.5, 17.4, 0.71, 0.66, 19.6 and 33.2 g/kg DM, respectively.

The CP concentration of CON was lower than in the other concentrates according to the experimental design, while the CP concentration of RSM, FB and BL was similar. The formulation of the

Table 2
CP concentration and Cornell N fractions of the protein supplements used in the experimental concentrate mixtures (n = 1 per feed) fed to dairy cows.

Item	Rapeseed meal	Faba beans	Blue lupin seeds
CP (g/kg DM)	373	287	322
Cornell N fractions[1] (g/kg total N)			
A	144	128	218
B_1	101	621	478
B_2	638	155	276
B_3	61	79	18
C	56	17	10
Effective protein degradability[2]	0.597	0.813	0.831

[1] Determined according to Licitra et al. (1996), where A = non-protein-nitrogen, B_1 = true protein soluble in mineral buffer, B_2 = true protein insoluble in mineral buffer but soluble in neutral detergent, B_3 = true protein bound to NDF, C = protein bound to ADF.
[2] Calculated based on the Cornell N fractions.

feeds also resulted in higher starch in CON than in the other feeds, and the starch concentration was higher and crude fat concentration was lower in FB than in RSM and BL reflecting the intrinsic differences in the protein supplements. The calculated ME concentration of RSM was lower than in the other concentrates due to its lower digestibility, but it had the highest calculated MP concentration because of the lower ruminal CP degradation of rapeseed protein (feed value calculations based on Luke, 2021). The protein fractions were analysed separately for each of the protein ingredients (Table 2). RSM had clearly lower B_1 and higher B_2 proportions than FB and BL which resulted in lower calculated ruminal degradability of RSM compared to the grain legumes.

Silage and total DM intakes increased in response to protein supplementation ($P < 0.05$) but this was due to RSM and FB, whereas BL did not numerically differ from CON, and was significantly lower than FB ($P < 0.05$; Table 3). Silage DMI increased with 1.28 and 1.29 kg per kg more CP intake with RSM and FB, respectively as compared with CON. Protein supplementation increased CP intake compared with CON ($P < 0.001$), but for ME and MP intake, results reflected DMI in a way that BL had lower intakes. All diets were clearly positive in terms of PBV, but CON was clearly lower than the protein supplemented diets ($P < 0.001$).

The protein supplemented diets had higher milk, milk protein and lactose production compared with CON ($P < 0.05$) but there were no significant effects on ECM or fat yield (Table 4). Protein yield was higher for RSM than FB and BL ($P < 0.05$). The concentration of milk fat was higher for CON than for the other diets ($P < 0.05$) and milk protein concentration tended to be higher ($P < 0.1$) for RSM than for FB and BL, and for FB than BL. The concentration of milk urea increased ($P < 0.001$) and NUE decreased ($P < 0.001$) with protein supplementation. The efficiency of MP use tended to be higher ($P < 0.1$) and ME was used more efficiently ($P < 0.05$) with CON compared to the protein supplemented diets.

Protein supplementation clearly increased the average ammonia N concentration in the rumen fluid ($P < 0.001$; Table 5), and diet × time interaction for CON vs RSM + FB + BL was significant ($P < 0.001$; Fig. 1). Average rumen pH tended ($P < 0.1$) to be higher and ammonia N concentration lower in RSM compared with the other protein supplements, and similar differences were found in BL vs FB. For the proportions of single volatile fatty acids, protein supplementation increased the proportions of propionic acid and isobutyric acid but decreased that of butyric acid ($P < 0.05$).

In the omasal canal (Table 6), higher microbial N flow was found for FB than for BL ($P < 0.01$) and higher non-microbial N flow for RSM compared to FB and BL ($P < 0.05$). True ruminal digestibility of N was 5.6 percentage units lower in RSM when compared to FB and BL. N excretion into urine was higher for RSM, FB and BL compared with CON ($P < 0.001$). Also N balance was more positive in response to protein supplementation ($P < 0.01$). There was a slight but significant increase in apparent total OM digestibility of the diets in response to protein supplementation ($P < 0.05$) and a similar trend was even more clear ($P < 0.01$) for total NDF digestibility.

Discussion

Feeds and diets

The protein feeds used in the current study had typical CP concentrations (see e.g. Feedipedia, 2021; Luke, 2021), that differed from each other with RSM having the numerically highest and FB the lowest CP concentration. The EPD values for RSM, FB and BL in the Finnish Feed Tables (Luke, 2021) are 0.63, 0.80 and 0.85, which are in close relationship with the EPD values based on the

Table 3
Feed and nutrient intake of dairy cows fed grass silage-based diets supplemented with concentrate mixtures without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL).

	Protein supplementation				SEM	*P-value*		
Item	CON	RSM	FB	BL		CON vs RSM + FB + BL	RSM vs FB + BL	FB vs BL
Feed intake (kg DM/day)								
Silage	12.0	13.1	13.0	12.1	0.20	0.025	0.074	0.018
Concentrate	10.5	10.6	10.6	10.4	-			
Total	22.5	23.6	23.5	22.4	0.22	0.031	0.058	0.013
Nutrient intake								
OM[1] (kg/day)	20.9	21.8	21.8	20.8	0.20	0.043	0.088	0.013
Total CP (kg/day)	3.14	3.95	3.88	3.76	0.306	<0.001	0.016	0.036
CP from concentrates (kg/day)	1.15	1.79	1.74	1.77	0.007	<0.001	0.009	0.017
CP from silage (kg/day)	1.99	2.16	2.14	1.99	0.030	0.019	0.046	0.012
Starch (kg/day)	4.83	3.43	4.53	3.40	0.019	<0.001	<0.001	<0.001
NDF (kg/day)	8.55	9.33	8.80	8.78	0.133	0.034	0.017	0.937
iNDF[2] (kg/day)	1.89	2.30	1.84	1.78	0.026	0.027	<0.001	0.155
ME[3] (MJ/day)	236	247	248	238	2.1	0.012	0.189	0.015
MP[4] (kg/day)	1.97	2.19	2.15	2.04	0.018	<0.001	0.005	0.005
PBV[5] (g/day)	222	745	711	756	6.2	<0.001	0.187	0.002

[1] OM = organic matter.
[2] iNDF = indigestible NDF.
[3] ME = Metabolizable energy.
[4] MP = Metabolizable protein according to Luke (2021).
[5] PBV = Protein balance in the rumen according to Luke (2021).

Table 4
Milk production and composition of dairy cows fed grass silage-based diets supplemented with concentrate mixtures without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL).

	Protein supplementation				SEM	*P-value*		
Item	CON	RSM	FB	BL		CON vs RSM + FB + BL	RSM vs FB + BL	FB vs BL
Production per day								
Milk (kg)	33.0	35.4	34.6	35.4	0.49	0.010	0.560	0.278
ECM[1] (kg)	34.5	34.6	34.2	34.6	0.57	0.950	0.819	0.671
Fat (g)	1 505	1 412	1 431	1 450	33.9	0.105	0.515	0.712
Protein (g)	1 047	1 136	1 093	1 075	15.0	0.021	0.029	0.443
Lactose (g)	1 492	1 590	1 543	1 593	26.6	0.035	0.539	0.233
Concentration in milk (g/kg)								
Fat	45.5	39.9	41.4	40.8	0.80	0.002	0.261	0.625
Protein	31.7	32.1	31.6	30.3	0.41	0.446	0.066	0.077
Lactose	45.2	44.9	44.6	45.0	0.20	0.163	0.602	0.303
Urea (mg/100 ml)	16.9	28.1	27.4	28.1	1.26	<0.001	0.807	0.714
Production efficiency								
ME[2]	0.625	0.588	0.580	0.616	0.0091	0.027	0.402	0.030
MP[3]	0.533	0.518	0.507	0.526	0.0066	0.091	0.875	0.096
NUE[4]	328	283	277	281	3.8	<0.001	0.416	0.518

[1] ECM = Energy corrected milk.
[2] Energy utilization using metabolizable energy intake based on feed values multiplied by feed intake and applying the Luke (2021) correction equation.
[3] Metabolizable protein use efficiency.
[4] NUE = N use efficiency (g/kg) = N excreted in milk (g)//feed N intake (kg).

Table 5
Rumen fermentation of dairy cows fed grass silage-based diets supplemented with concentrate mixtures without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL).

	Protein supplementation				SEM	*P-value*		
Item	CON	RSM	FB	BL		CON vs RSM + FB + BL	RSM vs FB + BL	FB vs BL
pH	6.35	6.50	6.45	6.32	0.04	0.174	0.053	0.064
Ammonia N (mmol/l)	4.20	7.54	8.13	9.32	0.399	<0.001	0.052	0.081
Total acids (mmol/l)	117	117	117	120	1.4	0.700	0.374	0.221
Proportions of volatile fatty acids in the rumen fluid (mmol/mol)								
Acetic acid	652	654	657	646	5.6	0.948	0.794	0.223
Propionic acid	160	176	165	179	4.3	0.033	0.500	0.064
Butyric acid	140	123	127	126	4.7	0.034	0.585	0.799
Isobutyric acid	8.9	9.6	10.3	9.4	0.26	0.032	0.455	0.049
Isovaleric acid	14.0	13.0	14.3	13.5	1.17	0.787	0.531	0.659
Valeric acid	15.0	15.4	15.4	16.2	0.48	0.277	0.545	0.287
Caproic acid	10.4	9.6	10.5	10.1	1.23	0.824	0.648	0.856

Cornell fractions (R^2 = 0.99, n = 3). The crude fat concentration was numerically highest in the concentrate including RSM and that of starch in the concentrate with FB. It must be noted that there is variability in the quality of different feed batches within feed types and this should be taken into account when comparing results from different experiments.

As concentrate mixtures were designed to be isonitrogenous, the quantity of each protein supplement included in the diet varied between treatments. The differences in the quality and quantity of protein supplements caused minor differences in the composition of the concentrate mixtures, but they were not balanced to prevent possible confounding effects of variable concentrations of other dietary components. The difference in e.g. starch content in diet DM ranged from 0.15 to 0.21 which was considered unlikely to bias the results.

The amount of protein supplement DM given daily was 2.64, 3.21 and 2.84 kg for RSM, FB and BL, respectively, resulting in a CP supply of 0.985, 0.921 and 0.914 kg for the three supplements. These amounts are within practical levels and not exceeding amounts used e.g. in Puhakka et al. (2016) and Rinne et al. (2015). Johnston et al. (2019) used as much as 8.4 kg FB per cow per day without complications. The proportion of concentrate feeds in the diet was on average 0.46, and NDF originating from forage 0.29 of total diet DM, which should have ensured proper rumen function supported by the relatively high average rumen pH observed (6.40).

Physiological responses

Protein supplementation has rarely affected rumen fermentation except for higher ruminal ammonia concentration (Ahvenjärvi et al., 1999; Puhakka et al., 2016; Rinne et al., 2015). It is however interesting to note that the proportion of butyrate was higher for CON than for the other treatments, but the reason for that is unclear. It may be linked with the clearly higher milk fat concentration on CON diet compared with the protein supplemented diets.

According to the Finnish protein feeding system (Luke, 2021), even CON provided an adequate amount of rumen degradable protein (RDP) into the rumen as the PBV intake was 222 g/day equalling to a surplus of 10 g of rumen degradable protein per kg DM intake, which is not consistent with negative apparent rumen N digestibilities. The digesta flow to the lower tract may have been overestimated in the current experiments as the feeding schedule was not evenly distributed over the 24-hour cycle. However, there was a close relationship between the PBV values calculated according to Luke (2021) and those derived from the *in vivo* experiment (R^2 = 0.89, n = 4).

The numerical value for rumen ammonia N concentration on CON was 4.20 mmol/l, and although clearly lower than for the protein supplemented diets (on average 8.33 mmol/l), it should be adequate for microbial protein synthesis (Hoover, 1986). The dietary CP concentration of 139 g/kg DM is exactly the same as indi-

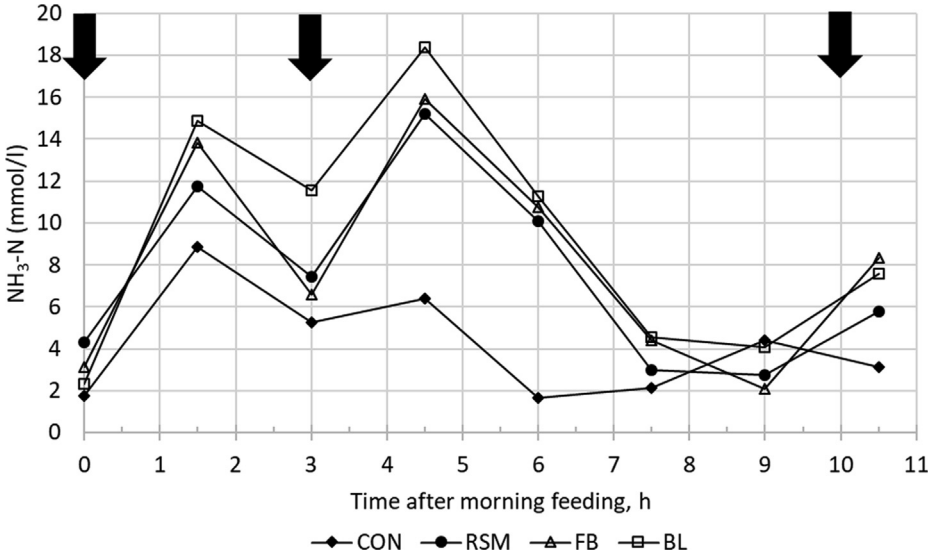

Fig. 1. Diurnal variation in ruminal ammonia N concentration when cows were fed a control diet without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL). The black arrows indicate the times of concentrate feed delivery.

Table 6
Nitrogen (N), organic matter (OM) and NDF digestion of dairy cows fed grass silage-based diets supplemented with concentrate mixtures without protein supplement (CON) or including rapeseed meal (RSM), faba bean (FB) or blue lupin (BL).

	Protein supplementation				SEM	*P-value*		
Item	CON	RSM	FB	BL		CON vs RSM + FB + BL	RSM vs FB + BL	FB vs BL
N intake (g/day)	502	631	621	602	4.9	<0.001	0.016	0.036
Diet N concentration (g/kg DM)	22.3	26.7	26.4	26.8	0.06	<0.001	0.450	0.002
Omasal canal flow per day								
Total non-ammonia N (g)	642	705	697	630	18.9	0.157	0.120	0.045
Microbial N (g)	463	473	501	443	10.6	0.464	0.920	0.008
Non-ammonia non-microbial N (g)	179	232	196	187	10.9	0.088	0.023	0.574
OM (kg)	12.0	12.5	12.0	11.0	0.33	0.791	0.048	0.072
NDF (kg)	5.14	5.22	4.80	4.86	0.134	0.283	0.058	0.765
Microbial N efficiency[1]	33.2	32.9	33.3	30.7	0.98	0.454	0.455	0.113
Excreted in faeces per day								
N (g)	175	189	191	178	5.0	0.102	0.500	0.103
OM (kg)	6.06	6.15	6.01	5.70	91.7	0.352	0.037	0.055
NDF (kg)	3.91	3.97	3.70	3.63	76.5	0.163	0.016	0.539
N excreted in urine (g/day)	148	222	227	222	2.9	<0.001	0.460	0.315
Proportion of N excreted in urine	0.292	0.350	0.365	0.369	0.0050	<0.001	0.0374	0.542
N balance	15.1	42.2	30.8	33.2	5.51	0.019	0.181	0.769
N digestibility (g/g)								
Apparent ruminal	−0.310	−0.146	−0.151	−0.080	0.0288	0.002	0.415	0.135
True ruminal	0.646	0.632	0.687	0.689	0.0178	0.292	0.042	0.923
Apparent total	0.653	0.701	0.691	0.726	0.006	<0.001	0.757	0.151
OM digestibility (g/g)								
Apparent ruminal	0.427	0.426	0.449	0.470	0.0124	0.191	0.069	0.280
True ruminal	0.670	0.659	0.693	0.695	0.0101	0.326	0.028	0.892
Apparent total	0.710	0.717	0.724	0.726	0.0031	0.012	0.098	0.596
NDF digestibility (g/g)								
Ruminal	0.397	0.439	0.455	0.444	0.0118	0.012	0.500	0.553
Total	0.544	0.574	0.578	0.586	0.0067	0.004	0.365	0.418
Total pdNDF[2] digestibility (g/g)	0.677	0.725	0.693	0.694	0.0066	0.011	0.009	0.893
Total starch digestibility (g/g)	0.978	0.973	0.976	0.966	0.0018	0.021	0.451	0.009

[1] Microbial N synthesized in the rumen (g/kg OM truly digested).
[2] pdNDF = potentially digestible NDF.

cated for zero rumen protein balance in the meta-analysis of Sauvant and Nozière (2016). There was a clear diurnal variation in rumen ammonia N concentrations during the daytime sampling cycle, and the peak was higher on protein supplemented diets compared to CON (Fig. 1). The minimum value reached on CON was 1.66 mmol/l.

However, microbial protein production did not increase in absolute terms nor when expressed relative to OM truly digested when cows were given the protein supplemented diets similarly as found by Ahvenjärvi et al. (1999), Korhonen et al. (2002) and Rinne et al. (2015).

It is possible that the ruminal ammonia N concentration was limiting fibre digestion resulting in the significantly lower OM and NDF digestibility on CON compared to protein supplemented diets in accordance with Nousiainen et al. (2009). The reasons may be linked to improved nutrition of rumen microbes and/or

protein supplements having an intrinsically higher digestibility than the feed components they replace in the diet. Significantly higher pdNDF digestibility for the diets supplemented with protein suggests that fibre digestibility was associated with improved conditions for rumen microbes rather than the intrinsic characteristics of feed ingredients.

The true ruminal CP digestibility of RSM protein was numerically lower than that of FB and BL, and the increased by-pass protein from RSM may be a major factor contributing to the higher milk protein production of RSM compared with FB and BL supplemented diets. The Cornell protein fraction analysis was in line with the *in vivo* results showing also clearly lower rumen degradability in RSM compared to the grain legumes.

The majority of protein available for the dairy cow originates from the microbial protein synthesized in the rumen, although with increasing total MP supply, the proportion of microbial protein declines (Hristov et al., 2019) indicating that high yielding dairy cows are increasingly dependent on feed protein bypassing rumen. The proportion of microbial N from total non-ammonia N flowing to the omasum in this study was 0.72 for control and 0.67, 0.72 and 0.70 for RSM, FB and BL, respectively. The corresponding values in Rinne et al. (2015) were 0.76 for the control diet and 0.66 and 0.60 for low and high levels of protein supplementation, respectively.

The microbial N flow was significantly lower for BL than for FB, which may be related to the higher fat and lower starch concentration of it, and thus lower availability of energy for rumen microbes. The higher flow of microbial N to the omasal canal on FB diet compensated for the lower feed-originating N resulting in similar total non-ammonia N flow to the duodenum in RSM and FB. However, evidence from a study by Stefański et al. (2020) with ^{15}N labelled RSM indicates that distinction between microbial and non-microbial protein may not be as unequivocal as regarded thus far as rumen microbes adsorb soluble feed protein rapidly from rumen fluid. At least part of this adsorbed protein may escape the rumen associated with microbial protein but without extensive ruminal metabolism (Stefański et al., 2020).

Both microbial N flow and efficiency of microbial N synthesis were numerically high compared to e.g. the meta-analysis by Sauvant and Nozière (2016). Also the N balance was rather high while the proportion of NDF digestion in rumen (on average 0.76) seems low. These results are probably at least partly due to the unrepresentative sampling as samples were only collected during the daytime when the cow activity and delivery of feeds were greater, thus increasing the average digesta flow to the lower digestive tract. This unrecommendable practice should however not have affected the comparisons between the experimental diets.

The numerically higher histidine concentration in the arterial plasma of RSM-fed cows may have contributed to the higher protein production responses (results not shown) as histidine has been identified as the first limiting amino acid on grass silage and small grain cereal based diets (Vanhatalo et al., 1999, Korhonen et al., 2000). Lamminen et al. (2019) also reported higher arterial histidine concentration of RSM than FB fed dairy cows.

Production responses

The differences in feed intake may be caused by several factors including improved diet digestibility and the increased "pull effect" as higher and more balanced amino acid supply may promote milk protein synthesis and thus increase the energy requirement of the cows. However, increased DMI in response to RSM and FB supplementation induced increases in milk and lactose production but no increases in ECM production due to decreased milk fat concentrations. The lower DMI of BL compared to FB is difficult to explain and would need further studies to be confirmed. Milk protein output increased by 0.085, 0.044 and 0.027 when RSM, FB and BL were compared to CON, which can be considered relatively modest increases.

The marginal efficiency (amount of additional feed protein captured in milk protein) was 0.110, 0.062 and 0.045 to RSM, FB and BL, respectively. The marginal efficiencies calculated in a meta-analysis by Huhtanen et al. (2011) were 0.136 for RSM and 0.098 for soya bean meal showing that the benefits of protein supplementation were somewhat lower in the current experiment. The marginal efficiency to MP use was on average 0.35, which is higher than the value 0.19 reported by Daniel et al. (2016), but the calculation basis of MP differed between the studies. The small milk production response to protein supplementation may have been caused by the relatively high ME and MP concentration of the grass silage which elicited high milk production even without any protein supplement, although responses to protein or energy supplements are often not related to the quality of the basal forage (Rinne et al., 1999).

For ECM production, there were not even numerical responses to protein supplementation when compared to CON due to the higher milk fat concentration in CON compared to protein supplemented diets. Lack of milk production responses to increasing levels of FB supplementation was also reported by Puhakka et al. (2016) and Ramin et al. (2017). Puhakka et al. (2017) found increased milk production with BL when compared to a control diet, but the response to BL was lower than for RSM. It can be argued that from ecological sustainability point of view, high quality protein supplementation of dairy cow diets is questionable (Leiber, 2014). It must however be noted that we used an experimental design with short 3-week periods, and the effects of particularly the diet without protein supplementation would need to be studied using long-term experiments as well.

Milk urea concentration contributes to milk CP content, which is used to describe milk protein content in experiments as well as in dairy industry. However, urea N does not have value e.g. in cheese production nor in human nutrition. If dairy cow diets differ clearly in milk urea concentration, it may be worthwhile to correct the milk protein content for it to make fair comparisons. In the current experiment, this means a reduction in milk protein concentration of 0.5 g/kg for CON and of 0.8 g/kg for the protein supplemented diets. For daily protein production, this results in a 17 g reduction for CON and 29 g reduction for the protein supplemented diets in daily milk protein production.

Nitrogen use efficiency

Improving NUE decreases the negative environmental impacts of excreted N such as surface water eutrophication, groundwater nitrification, emissions of nitrous oxide and ammonia to the atmosphere. Generally, NUE in milk production ranges between 250 and 300 g/kg N, but it is highly sensitive to the CP concentration of the diet (Huhtanen et al., 2008b). The NUE in the current experiment was 280 g/kg averaged over all diets, which is close to the average value of 277 g/kg derived from the meta-analysis by Huhtanen et al. (2008b).

Protein supplementation decreased NUE but the type of protein feed did not affect it. Incremental increases in dietary CP concentration seem to inevitably lead to a diminishing efficiency in partitioning N towards milk protein synthesis (Huhtanen et al., 2008b). With decreasing NUE, also the route of N excretion shifts from faeces to urine (Huhtanen et al., 2008b) which was also obvious in our case as the proportion of N excreted in urine increased from 0.29 on CON to 0.36 averaged over the protein supplemented diets.

Decreasing the diet CP concentration of dairy cows is the most effective way to improve NUE. Other attempts to improve NUE such as decreasing the ruminal degradation of protein by process-

ing or using different types of protein supplements have failed to show clear benefits *in vivo* even if the ruminal protein degradability values based on *in situ* measurements have changed (Huhtanen, 2019). There is however a limit to how low it is sensible to go in CP concentration of the diets. According to the Finnish protein evaluation system (Luke, 2021), PBV above zero in dairy cow diets is recommended to meet the N requirements of the rumen microbes. In the current experiment, PBV was positive even in CON suggesting that ruminal RDP supply did not limit microbial protein synthesis.

The current study supports the evidence that RSM is an excellent protein supplement for dairy cows. An additional benefit of RSM is that it is not human-edible meaning that livestock is needed to convert this by-product from plant oil industry into human-edible forms while faba beans and lupin seeds can be used directly in human diets.

Conclusions

All protein supplements increased milk protein production compared to control, but on average only for 5% with low marginal efficiency of converting feed protein into milk protein. Rapeseed meal showed some benefits compared to the grain legumes in greater milk protein production and smaller rumen degradation of protein. The nitrogen use efficiency decreased in response to protein supplementation pointing out that economic and environmental consequences of protein feeding need to be carefully considered.

Ethics approval

National Ethics Committee (Hämeenlinna, Finland) approved the experimental procedures of the animal experimentation in accordance with the guidelines established by the European Community Council Directive 2010/63/EU.

Data and model availability statement

None of the data were deposited in an official repository, but is available upon request.

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Declaration of interest

The authors declare no conflicts of interest.

Acknowledgements

None.

Financial support statement

The Strategic Research Program of the Academy of Finland (Grant no293045 and 314243; Novel protein Sources of Food Security) provided funding for reporting this work. Teagasc Walsh Fellowship funding for B.G. is gratefully acknowledged.

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