Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows

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ABSTRACT

When fed to meet the metabolizable protein requirements of the National Research Council, dairy cows consume an excess of N, resulting in approximately 75% of dietary N being lost to the environment as urine and feces. Reductions in environmental N release could be attained through an improvement in N efficiency. The objective of this study was to determine if the predicted reduction in milk yield associated with feeding a low-protein diet to lactating dairy cows could be avoided by dietary supplementation with 1 or more ruminally protected (RP) AA. Fourteen multiparous and 10 primiparous Holstein cows, and 24 multiparous Holstein × Jersey crossbred cows were used in a Youden square design consisting of 8 treatments and 3 periods. The 8 dietary treatments were (1) a standard diet containing 17% crude protein [CP; positive control (PC)], (2) a 15% CP diet [negative control (NC)], (3) NC plus RP Met (+M), (4) NC plus RP Lys (+K), (5) NC plus RP Leu (+L), (6) NC plus RP Met and Lys (+MK), (7) NC plus RP Met and Leu (+ML), and (8) NC plus RP Met, Lys, and Leu (+MKL). Dry matter intake was not affected by treatment. Crude protein intake was lower for NC and RP AA treatments compared with the PC treatment. No detrimental effect was detected of the low-CP diet alone or in combination with AA supplementation on milk and fat yield. However, milk protein yield decreased for NC and +MKL diets, and lactose yield decreased for the +MKL compared with the PC diet. Milk urea N concentrations were lower for all diets, suggesting that greater N efficiency was achieved by feeding the low-protein diet. Minimal effects of treatments on arterial plasma essential AA concentrations were detected, with only Ile and Val being significantly lower in the NC than in the PC diet. Phosphorylation ratios of signaling proteins known to

regulate mRNA translation were not affected by treatments. This study highlights the limitations of requirement models aggregated at the protein level and the use of fixed postabsorptive efficiency to calculate milk protein requirements. Milk protein synthesis regulation by signaling pathways in vivo is still poorly understood. **Key words:** nitrogen efficiency, rumen-protected essential amino acid, milk protein

INTRODUCTION

Dairy cows have requirements for specific EAA during lactation. However, current N requirements (NRC, 2001) are expressed in terms of MP rather than individual EAA. By feeding to meet MP requirements, animals are likely overfed several EAA to ensure meeting key EAA requirements across a range of diets, which leads to poor N efficiency. Approximately 25% of the N consumed by dairy cows is converted into milk protein, whereas the remaining N is excreted in urine and feces (Tamminga, 1992; Chase, 1994; Hristov et al., 2004). Feeding animals low-protein diets is one way to improve N efficiency (Kalscheur et al., 2006), but the practice is typically associated with decreased production, which is economically disadvantageous to producers. The production losses observed when MP supply is reduced are likely associated with a deficiency of 1 or several EAA, and supplementation of those deficient EAA may maintain production. Improving N efficiency by feeding reduced-protein diets and supplementation with only a few EAA should reduce N excretion to the environment and, depending on the cost of the reformulated diet with supplemental EAA, may be more economical.

Defining EAA requirements for dairy cows is especially difficult compared with monogastrics because of the use of a variety of feed products and the alteration of nutrients in the rumen by microorganisms (Lapierre et al., 2006). A better understanding of EAA requirements is needed to ensure successful application of low-protein diets. Lysine and Met have been generally accepted to be the main EAA limiting milk protein synthesis from corn-based diets (Clark, 1975; Schwab et al., 1976; NRC,

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2001). These observations have led to extensive research evaluating the supplementation with rumen-protected (RP) Lys and Met products. Observed responses indicate that cows fed both RP Met and Lys may increase milk protein yield depending on dietary CP supply (Robinson et al., 1998; Patton, 2010).

In addition to Met and Lys, dairy cows may respond to Leu supplementation. Leucine has been shown to increase protein synthesis rates in skeletal muscle of growing pigs (Escobar et al., 2006) and milk protein synthesis rates in mammary tissue slices (Appuhamy et al., 2012). Amino acids, in particular Leu, increase phosphorylation of mammalian target of rapamycin (mTOR), which controls protein synthesis rates (Wang and Proud, 2006; Appuhamy et al., 2012). Therefore, including RP Leu in dairy cow rations may result in increased milk protein synthesis through the activation of the mTOR pathway. Duodenal infusions of 40 g of Leu/d to corn-based diets improved both milk protein content and yield on diets limiting in Lys or Met (Rulquin and Pisulewski, 2006). However, jugular infusion of Ile, Leu, and Val to cows fed an MP-deficient diet (16% CP), but sufficient in Lys and Met, did not further increase milk protein yield (Appuhamy et al., 2011b).

The objectives of this study were to (1) determine the degree of reduction in milk production when MP supply is reduced by 15%; (2) determine whether the reduction in milk production associated with feeding an MP-deficient diet could be alleviated by supplementation of RP Met, Lys, Leu, or combinations of the RP AA; (3) determine the effects of feeding RP Lys and Met on cell signaling proteins associated with the mTOR pathway; and (4) to assess the effect of MP-deficient diets supplemented with RP AA on N efficiency of dairy cows.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (Blacksburg). Fourteen multiparous and 10 primiparous Holstein cows, and 24 multiparous Holstein $\times$ Jersey crossbred cows (189 ± 79 DIM; 566 ± 50 kg BW) were used in a Youden square design consisting of 8 treatments and 3 periods. Cows were randomly assigned to treatments. Experimental periods lasted 15 d and consisted of 10 d of diet adaptation and 5 d of sample collection. Cows were housed in a freestall barn with Calan Broadbent individual animal feeders (American Calan Inc., Northwood, NH) and free access to water. Four cows were removed from the study for reasons unassociated with the treatments.

Treatments

The 8 dietary treatments were (1) a standard diet containing 17% CP [positive control (PC)] formulated to meet requirements of a lactating cow producing 35 kg/d, (2) a 15% CP diet [negative control (NC)], (3) NC diet plus RP Met (+M), (4) NC diet plus RP Lys (+K), (5) NC diet plus RP Leu (+L), (6) NC diet plus RP Met and Lys (+MK), (7) NC diet plus RP Met and Leu (+ML), and (8) NC diet plus RP Met, Lys, and Leu (+MKL). Final diets contained 46% forage and 54% concentrate, and 75% of the diet ingredients were corn based (Tables 1 and 2). Three concentrate mixes were used in the experiment. A moderate-fat grain with high CP was used for the PC diet, a moderate-fat grain with low CP was used for the NC diet, and a low-fat grain with low CP was used in conjunction with the moderate-fat, low-CP mix to formulate the diets supplemented with AA. The moderate-fat low-CP and the low-fat low-CP concentrates were mixed in varying proportions to formulate the +AA diets so that dietary fat was similar across treatments. Ingredient compositions of the experimental diets are presented in Table 3. Diets were mixed as a TMR, provided in quantities to maintain between 5 and 10% refusals, and delivered to the Calan gates once daily by 1400 h. Rumen-protected AA were provided at 0.7 g of RP DL-Met/kg of DM (70.6% Met in DM; Balchem Corp., New Hampton, NY), 2.1 g of RP L-Lys/kg of DM (32.1% Lys in DM; AminoShure-L; Balchem Corp.), and 7.8 g of RP L-Leu/kg of DM (53.9% Leu in DM; source: Ajinomoto Co. Inc., Tokyo, Japan, protected by Balchem Corp.). These amounts were estimated to be adequate to achieve absorbed individual AA supplies that were equivalent to the PC diet.

Sample Collection and Analysis

Feed offered andorts were recorded daily to determine DMI. Dry matter content was determined weekly on the major components of the TMR (grain mixes, alfalfa haylage, and corn silage) and used to adjust the ration to maintain the targeted DM inclusion rates. Samples of the TMR and forage mix were collected on d 11, 13, and 15 of each period. Feed samples were stored at -20°C for later analysis. Individual feed samples were thawed and dried at 55°C in a forced-air oven for 48 h to determine DM concentrations. Dried samples were ground in a Wiley mill through a 1-mm screen (Arthur H. Thomas Co., Philadelphia, PA), and subsequently composited by diet and period. Composited samples were submitted to Dairyland Laboratories Inc. (Arcadia, WI) for nutrient analyses.

Table 1. Concentrate formulation (% of DM)

Item	Grain mix ¹		
	HP	LPLF	LP
Corn grain (ground, dry)	43.92	63.91	60.80
Corn dry distillers grains with solubles	14.90	15.66	14.90
Soybean meal (solvent; 48% CP)	21.09	6.86	6.52
Protected soybean meal	3.22	0.00	0.00
Soybean hulls	11.68	9.49	9.03
Urea	0.00	1.68	1.60
Tallow	3.22	0.00	4.83
Calcium carbonate	0.89	0.95	0.91
Dicalcium phosphate	0.00	0.34	0.33
Sodium bicarbonate	0.36	0.37	0.36
Salt	0.60	0.63	0.60
0.06% Selenium premix	0.05	0.06	0.05
Trace mineral mix	0.02	0.02	0.02
Trace vitamin premix	0.02	0.02	0.02
Vitamin E (60,000 IU)	0.02	0.02	0.02

¹HP = high protein; LPLF = low protein, low fat; LP = low protein.

Cows were milked twice per day at 0300 and 1300 h. Milk weights were recorded at each milking and BW were recorded as cows exited the milking parlor following milking. Milk samples were taken on d 11, 12, and 13 of each period at the morning and afternoon milkings. Milk samples were analyzed following each sampling by the United DHIA laboratory (Blacksburg, VA) for true protein, fat, lactose, and SCC by mid-infrared spectroscopy using a Fossomatic 4000 Combi infrared analyzer (Eden Prairie, MN) and for MUN using a modification of the Berthelot procedure (ChemSpec 150 Analyzer; Bentley Instruments Inc., Chaska, MN).

Blood samples were taken from the coccygeal vessels following the afternoon milking on d 13, 14, and 15. Blood samples were collected into 5-mL sodium heparin blood-collection tubes, placed on ice, and centrifuged at

3,000 × *g* for 5 min at 4°C to collect plasma, which was stored at −20°C until analysis. Plasma samples were pooled by period for each cow and assessed for AA concentration using GC-MS as described by El-Kadi et al. (2006).

Mammary biopsies were taken once from a rear quarter of every cow on the PC, NC, and +MK diets (*n* = 18) after the afternoon milking on either d 16 or 17 of the third period. The +MK treatment was chosen, as it had the largest numerical difference in milk yield from the NC treatment after the second period when we had to make a decision on which cows to biopsy. Prior to biopsies, cows were restrained in a standing position in a headlock. Cows were administered an epidural block by injection of 2% lidocaine-HCl at the sacrococcygeal site. The entry point of the rear quarter was washed

Table 2. Observed chemical composition (% of DM) of major feed ingredients used in the experimental diets

Item	Corn silage	Haylage	Grain mix ¹		
			HP	LPLF	LP
DM, %	31.49	54.03	89.98	90.36	89.80
Ingredient					
NDF	36.08	43.02	18.49	16.28	17.37
ADF	20.37	35.41	9.86	7.84	8.51
CP	7.90	18.78	21.26	19.71	18.75
Fat	3.52	2.92	6.91	4.95	9.19
NFC	49.68	25.39	49.16	55.13	51.32
Lignin	2.92	8.94	1.21	1.19	0.96
Starch	36.79	1.14	33.74	45.38	45.11
Sugar	2.13	4.22	6.56	3.67	3.06
Mineral					
Ca	0.19	1.18	0.64	0.65	0.51
P	0.23	0.32	0.49	0.47	0.48
Mg	0.16	0.32	0.23	0.20	0.19
K	1.16	2.98	1.10	0.81	0.76
S	0.10	0.24	0.27	0.22	0.20

¹HP = high protein; LPLF = low protein, low fat; LP = low protein.

Table 3. Ingredient composition, NRC (2001)-predicted NE_L and MP diet content, and NRC (2001)-predicted NE_L and MP allowable (allow.) milk based on observed DMI of the experimental diets

Item	Experimental diet ¹							
	PC	NC	+M	+K	+L	+MK	+ML	+MKL
Ingredient, % of DM								
Corn silage	35.38	35.38	35.36	35.31	35.10	35.28	35.08	35.00
Haylage	9.75	9.75	9.74	9.73	9.67	9.72	9.66	9.64
Corn grain (ground, dry)	24.10	33.36	33.35	33.36	33.32	33.35	33.32	33.32
Corn dry distillers grain with solubles	8.18	8.18	8.17	8.18	8.17	8.17	8.16	8.16
Soybean meal (solvent; 48% CP)	11.57	3.58	3.58	3.58	3.57	3.58	3.57	3.57
Protected soybean meal	1.77	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Soybean hulls	6.41	4.96	4.96	4.96	4.95	4.96	4.95	4.95
Urea	0	0.88	0.88	0.88	0.88	0.88	0.88	0.88
Tallow	1.77	2.65	2.62	2.53	2.27	2.50	2.24	2.13
Calcium carbonate	0.49	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	0.00	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Sodium bicarbonate	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Salt	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
0.06% Selenium premix	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Trace mineral mix	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Trace vitamin premix	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin E (60,000 IU)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
RP Leu	0.00	0.00	0.00	0.00	0.78	0.00	0.78	0.78
RP Lys	0.00	0.00	0.00	0.21	0.00	0.21	0.00	0.21
RP Met	0.00	0.00	0.07	0.00	0.00	0.07	0.07	0.07
Protein and energy content								
RDP, % of CP	58.8	67.3	66.6	67.1	66.2	67.0	66.1	65.9
RUP, % of CP	41.2	32.7	33.4	32.9	33.8	33.0	33.9	34.1
NE _L , Mcal/kg of DM	1.65	1.66	1.65	1.66	1.68	1.67	1.68	1.69
NE _L (allow. milk), kg/d	37.2	34.6	37.5	36.6	35.2	34.8	35.5	33.8
MP, g/kg of DM	120	102	102	105	105	102	106	105
MP (allow. milk), kg/d	36.2	26.9	28.7	27.6	27.7	26.9	28.9	27.8

¹PC = positive control (high-CP) diet; NC = negative control (low-CP) diet; +M = NC diet + rumen-protected (RP) methionine; +K = NC diet + RP lysine; +L = NC diet + RP leucine; +MK = NC diet + RP methionine and lysine; +ML = NC diet + RP methionine and leucine; +MKL = NC diet + RP methionine, lysine, and leucine.

sequentially with alcohol, iodine, and alcohol. Approximately 100 mg of tissue was collected by biopsy from different locations through a common entry point using a Magnum Core Biopsy System (Bard Medical Division, Covington, GA) equipped with a 12-gauge needle. A subsample of the tissue (~20 mg) was immediately homogenized in the presence of protease and phosphatase inhibitors, centrifuged at $16 \times g$ for 10 min at 4°C to remove insoluble material, and the supernatant was boiled in Laemmli SDS sample buffer (Laemmli, 1970) as described by Escobar et al. (2006). The remaining tissue was snap frozen in liquid N. Following collection and processing, samples were transported to the laboratory and stored at –80°C for later analysis.

Ruminal Degradation and Intestinal Digestion of Protected EAA

Twenty-four samples (1.5 g) of each RP EAA (Leu, Lys, and Met) were placed in 5 × 5-cm, 50-μm-pore-size bags (Ankom Technology Corp., Macedon NY) and bags were heat sealed. Rossi et al. (2003) observed that after 8 h of rumen incubation, losses of RP AA were

similar to those predicted by the effective degradability equation developed by Sniffen et al. (1992). Thus, bags were incubated for 8 h in the rumen of 2 ruminally cannulated dairy heifers. Four samples per EAA were subjected to only rumen degradation to assess N loss in the rumen. After rumen incubation, the other 20 samples per EAA were soaked for 1 h in HCl (pH 2.4) and then 2 h in HCl (pH 2.4) solution supplemented with 100 mg of pepsin/L (Ali et al., 2012). Four of the pepsin-digested samples per EAA were washed and frozen for analysis. Sixteen samples per EAA were placed in the duodenal cannula of 2 fistulated steers at a rate of 3 every half hour, and recovered in feces. Recovered bags were washed in a series of 3 cold-water baths then placed into a salad spinner to remove excess water. Samples were freeze-dried and weighed to determine DM recovery and analyzed for total N via combustion analysis.

Cell Signaling Analysis

An aliquot (20 μL) of the biopsy supernatant was separated by gel electrophoresis (Laemmli, 1970) and

transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., Hercules, CA). Membranes were blocked for 1 h with StartingBlock [Tris-buffered saline (TBS)] Blocking Buffer (Thermo Scientific, Rockford, IL), and probed with primary antibodies against the phosphorylated and total forms of the signaling proteins of interest. Proteins of interest included α -tubulin, ribosomal protein S6 (**rpS6**; Ser235/236), p70 S6 kinase (**S6K1**; Thr389), eukaryotic initiation factor 4E binding protein 1 (Thr37/46), eukaryotic initiation factor 2 α subunit (**eIF2 α** ; Ser51), and eukaryotic elongation factor 2 (Thr56). Primary antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA) and consisted of monoclonal rabbit, polyclonal rabbit, or monoclonal mouse antibodies. For rpS6, S6K1, and eIF2 α , primary antibodies for the total and phosphorylated forms were from different species, allowing simultaneous probing for both protein forms using secondary antibodies (goat anti-mouse and goat anti-rabbit; LI-COR Biosciences, Lincoln, NE) with different fluorescing compounds. Proteins were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences). α -Tubulin was used as a covariate for loading differences. For eukaryotic initiation factor 4E binding protein 1 and eukaryotic elongation factor 2, the phosphorylated form of the protein was quantified first and then stripped for 1 h using Gentle ReView Stripping Buffer (Amresco LLC, Solon, OH), and reprobed to determine the total form. Bands were quantified using Odyssey software (version 3; LI-COR Biosciences).

Statistical Analysis

Data were analyzed with SAS (version 9.3; SAS Institute Inc., Cary, NC). The effects of dietary treatment were assessed with the following model:

$$Y_{ijklm} = \mu + T_i + P_j + B_k + R_l + (T_i \times P_j) + (T_i \times B_k) + (T_i \times R_l) + \beta_{ijkl}(B_k \times R_l) + \varepsilon_{ijklm},$$

where Y_{ijklm} = the dependent variable, μ = the population mean of Y , T_i = the effect of treatment, P_j = the effect of period, B_k = the effect of breed, R_l = the effect of parity, β_{ijkl} = the random effect of cow, and ε_{ijklm} = the residual error. Interactions were removed from the model when they were not significant, but all main effects were left in the model regardless of significance. For cell signaling data, period was removed from the model and cow was the experimental unit without repeated measure. Values are reported as least squares means, except for the EAA protection data where means and standard deviations are reported. The P -

values for pairwise comparisons against PC and NC diets were adjusted by the Bonferroni method. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Ingredient Composition of Experimental Diets

The chemical composition of feed ingredients used in the experimental diets is presented in Table 2, and the chemical composition of the experimental diets is presented in Table 4. Acid detergent fiber and NDF were lower and NFC was higher for all treatments than the NRC-formulated values. This resulted in NFC and starch concentrations that were greater than intended, which, in combination with the high fat content of the rations, could have contributed to milk fat content below normal concentrations (Table 5). However, the cows did not exhibit signs of ruminal acidosis. Crude protein contents were lower for all treatments compared with the PC treatment, as expected, although they tended to be above the target level of 15% CP. Starch content was higher for all treatments compared with the PC treatment, and the same was observed for NFC, except for the NC and +L treatments. Despite inclusion of a low-fat concentrate mix to correct for fat content in RP EAA, all EAA-supplemented treatments, except +MKL, had higher levels of fat compared with the PC treatment. As a result, predicted TDN was higher for RP EAA-supplemented treatments compared with the PC treatment. In addition, +M, +MK, and +ML treatments had greater predicted TDN than the NC treatment.

Predicted EAA Supply

The ruminal degradation and intestinal digestion of RP AA are presented in Table 6. Duodenal EAA flows for the experimental diets as predicted by the NRC (2001) model are presented in Table 7. Estimates for duodenal flow of Leu, Lys, and Met for the PC treatment were 8.90, 6.15, and 1.80% of MP, respectively, and estimates for the NC treatment were 9.30, 6.28, and 1.97% of MP, respectively. Estimates for both Lys and Met appeared to be limiting for the PC and NC treatments, as values were lower than recommended to obtain maximal milk protein synthesis: 7.2% of MP for Lys and 2.4% of MP for Met (NRC, 2001). Leucine was not deficient in either treatment, as the level of Leu required for optimal milk protein synthesis is 8.9% of MP (Rulquin and Pisulewski, 2006), although the NC treatment certainly provided less Leu per day. Supplementation of RP Leu, Lys, and Met increased digestible flow of each EAA, which resulted in flows of 12.0% Leu, 6.6% Lys, and 2.1% Met of MP for the +L, +K, and

Table 4. Least squares means of chemical composition (% of DM) of the experimental diets¹

Item	Experimental diet ²								SEM	P-value
	PC	NC	+M	+K	+L	+MK	+ML	+MKL		
DM, %	51.6	50.6	52.5	51.4	51.1	51.9	52.7	50.7	1.3	0.9
Ingredient										
CP	16.88	15.02*	15.23*	15.14*	15.39*	15.05*	15.49*	15.43*	0.24	0.001
NDF	27.65	26.84	21.88*†	24.70*	25.88	24.02*	23.14*†	25.32	0.62	<0.001
ADF	17.60	16.22	13.99*	14.70*	16.15	15.51	15.08	15.69	0.60	0.023
Lignin	3.23	2.77	2.72	2.74	2.80	2.16*	2.26	2.22*	0.21	0.025
NFC	45.76	48.00	52.11*†	49.75*	48.10	50.22*	50.62*	48.82	0.71	<0.001
Starch	28.40	33.75*	37.73*†	36.17*	34.18*	36.10*	35.83*	35.29*	0.78	<0.001
Sugar	3.93	2.98	3.18	3.30	2.68	3.10	2.87	3.32	0.68	0.9
Fat	5.40	6.15	7.08*	6.65*	6.70*	6.82*	6.94*	6.56	0.25	0.005
Ash	5.15	4.74	4.45*	4.51*	4.69	4.63	4.57*	4.64	0.12	0.022
TDN	77.66	79.85	83.71*†	81.73*	81.00*	82.76*†	83.36*†	82.13*	0.57	<0.001
NDICP ³	3.02	2.92	5.39	5.02	4.44	3.68	4.35	3.10	0.64	0.09
ADICP ⁴	1.81	1.69	1.62	2.44	2.43	1.67	1.47	0.85	0.23	0.004
Mineral										
Ca	0.587	0.583	0.563	0.513	0.553	0.590	0.593	0.557	0.023	0.3
P	0.3800	0.3767	0.3733	0.3700	0.3633	0.3667	0.3667	0.3633	0.0074	0.7
Mg	0.2267	0.2067	0.1933*	0.1967*	0.1933*	0.1900*	0.1867*	0.1967*	0.0051	0.001
K	1.330	1.123*	1.063*	1.073*	1.160*	1.137*	1.110*	1.177*	0.020	<0.001
S	0.2133	0.1800*	0.1933	0.1800*	0.1767*	0.1967	0.1933	0.1867*	0.0054	0.004

¹Samples were pooled and analyzed by period.²PC = positive control (high-CP) diet; NC = negative control (low-CP) diet; +M = NC diet + rumen-protected (RP) methionine; +K = NC diet + RP lysine; +L = NC diet + RP leucine; +MK = NC diet + RP methionine and lysine; +ML = NC diet + RP methionine and leucine; +MKL = NC diet + RP methionine, lysine, and leucine.³Neutral detergent-insoluble CP.⁴Acid detergent-insoluble CP.**P* < 0.05 for Bonferroni-adjusted pairwise comparisons versus PC.†*P* < 0.05 for Bonferroni-adjusted pairwise comparisons versus NC.**Table 5.** Least squares means of intake, milk production and composition, BW, and N efficiency for lactating dairy cows fed experimental diets

Item	Experimental diet ¹								SEM	P-value
	PC	NC	+M	+K	+L	+MK	+ML	+MKL		
Intake, kg/d										
DM	20.77	19.95	20.93	20.03	19.46	19.66	19.93	19.50	0.91	0.4
CP	3.51	2.99*	3.18	3.04*	2.99*	2.95*	3.08*	3.01*	0.15	<0.001
NDF	5.71	5.34	4.55*†	4.99*	5.07*	4.76*	4.66*†	4.91*	0.23	<0.001
ADF	3.64	3.24*	2.89*	2.96*	3.17*	3.08*	3.02*	3.06*	0.15	<0.001
Milk production										
Milk yield, kg/d	34.3	32.3	33.4	33.2	33.1	32.5	32.2	31.2	1.6	0.19
True protein, kg/d	1.103	1.012*	1.047	1.050	1.048	1.031	1.027	0.980*	0.040	0.016
True protein, %	3.264	3.291	3.225	3.245	3.236	3.241	3.235	3.282	0.070	0.8
Fat, kg/d	1.058	1.020	1.029	0.968	0.994	1.019	1.016	1.020	0.057	0.7
Fat, %	3.12	3.31	3.14	2.95	3.07	3.18	3.23	3.41	0.13	0.042
Lactose, kg/d	1.644	1.505	1.570	1.573	1.571	1.553	1.549	1.469*	0.079	0.045
Lactose, %	4.829	4.797	4.789	4.827	4.815	4.815	4.834	4.799	0.040	0.6
ECM, ² kg/d	33.3	31.6	32.1	31.4	31.7	31.8	31.7	30.9	1.5	0.5
SCC, ³ × 1,000 cells/mL	173.6	129.3	216.2	110.9	110.9	193.8	145.2	186.8	1.3	0.4
MUN, mg/dL	11.78	9.72*	10.07*	9.91*	9.89*	9.69*	10.44*	10.55*	0.35	<0.001
ADG, kg	-2.3	-0.1	-1.3	-0.1	-0.2	-1.7	0.7	0.6	2.3	0.9
N efficiency, ⁴ %	32.8	34.7	33.0	35.1	35.1	35.0	34.0	33.3	1.4	0.6

¹PC = positive control (high-CP) diet; NC = negative control (low-CP) diet; +M = NC diet + rumen-protected (RP) methionine; +K = NC diet + RP lysine; +L = NC diet + RP leucine; +MK = NC diet + RP methionine and lysine; +ML = NC diet + RP methionine and leucine; +MKL = NC diet + RP methionine, lysine and leucine.²Energy-corrected milk = milk yield × 0.327 + milk protein yield × 7.65 + milk fat yield × 12.95.³Somatic cell counts were logarithmically transformed for the statistical analysis.⁴Nitrogen efficiency = true protein (kg/d)/CP intake (kg/d).**P* < 0.05 for Bonferroni-adjusted pairwise comparisons versus PC.†*P* < 0.05 for Bonferroni-adjusted pairwise comparisons versus NC.

Table 6. Cumulative N losses (%) after ruminal incubation, acid and intestinal (mobile bag) digestion, and mobile bag transit time (h)

AA	Ruminal incubation	SD	Acid digestion	SD	Total tract	SD	Transit time (h)	SD
Leu	23.62	0.71	69.93	3.87	98.29	0.40	42.05	0.35
Lys	31.32	0.87	92.05	1.24	97.87	0.21	41.96	2.45
Met	54.57	2.31	83.30	2.39	99.37	0.10	43.14	2.33

+M treatments, respectively. Thus supplementation of Lys and Met increased predicted duodenal supply of Met and Lys to values that were closer to target values established by the NRC (2001). It is worth noting that transit time of mobile bags in the intestine was significantly longer than expected. This could result in an overestimation of digestible EAA supply on the RP EAA treatments.

DM and Nutrient Intake

Dry matter intake was not affected by treatment ($P > 0.05$; Table 5). Crude protein and ADF intake were lower for NC and RP AA treatments compared with the PC treatment ($P < 0.001$). Neutral detergent fiber intake was lower for RP AA-supplemented treatments compared with the PC treatment ($P < 0.001$), and it was lower for +M and +ML treatments compared with the NC treatment ($P < 0.001$).

Milk Yield and Composition

Milk yield did not differ among treatments, nor did the percentage protein and lactose in milk ($P > 0.05$; Table 5). Despite the lack of significant differences in the abovementioned variables, the numerical differences did indicate true protein yields (MPY) that were significantly reduced for NC and +MKL treatments, and lactose yields that were reduced for the +MKL compared with the PC treatment. The NRC model predicted a reduction in MPY between the PC and NC diets for the observed intake levels of 29% (303 g/d). However, only an 8% (91 g/d) difference in MPY was observed between PC and NC diets. The NRC (2001) assumes an MP milk protein efficiency (i.e., MPY/[MP supply – MP for maintenance]) for nonpregnant, nongrowing dairy cows of 67%, whereas the observed efficiency for the NC diet based on MP predicted supply and maintenance was 77%. Furthermore, if the NRC (2001) assumption of a fixed MP efficiency were correct, the cows fed the PC diet should exhibit the same efficiency as those fed the NC diet, but it was 63 or 14% lower than for the NC diet. These data suggest that the efficiency for converting MP to milk protein is variable (curvilinear response) as previously observed

(Whitelaw et al., 1986; Hanigan et al., 1998). If that is the case, MP efficiency for milk protein should be represented by the tangent of the milk protein response curve, after subtraction of maintenance, rather than by the linear slope. An approximation to the instantaneous MP efficiency is provided by the marginal efficiency between NC and PC, which is the difference in MPY between treatments (91 g/d) divided by the difference in MP supply (469 g/d). This calculation yields a marginal MP efficiency for milk protein of 19%, similar to that observed by Whitelaw et al. (1986) with abomasal infusion of casein to early-lactation dairy cows. The difference between predicted and observed marginal efficiencies draws into question the accuracy of the assumed value of 67% efficiency as well as the maintenance requirement. These observations have economic and environmental implications associated with reducing the supply of one of the most expensive dietary fractions (St-Pierre, 2012).

Supplementation with RP EAA partially reversed the effect of reduced MP supply, yielding milk protein levels similar to those of both PC and NC diets. Even Leu supplementation (+L), which was not predicted to have an effect on MPY (NRC, 2001; Rulquin and Pisulewski, 2006), resulted in MPY response similar to those observed in the PC diet. The +K and +L diets yielded marginal efficiencies of MP for milk protein greater than 100% and numerically increased the average (fixed) MP efficiency.

The NRC (2001) does not accommodate additive EAA effects such that if the diet is limiting in 1 EAA (e.g., Met), supplementation of only that AA may cause a response (i.e., single limiting AA theory). Thus, if NC diets were Lys deficient, by the single limiting AA theory, they should have responded only to treatments supplemented with RP Lys. However, low-CP diets supplemented with RP Leu and Met also resulted in MPY similar to those observed in cows fed PC diets. The results observed in response to the +L diet cannot be explained by increased MP supply, and they are in agreement with results observed in mammary tissue slices (Appuhamy et al., 2012; Arriola Apelo et al., 2014b). Treatments supplemented with multiple RP AA resulted in a numerically lower response than observed with individual RP AA additions.

Table 7. Duodenal flows of digestible EAA as predicted by NRC (2001) from the experimental diets

EAA	Experimental diet ¹											
	PC		NC		+M		+K		+L		+MK	
	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %
Arg	140	4.68	114	4.58	119	4.56	115	4.56	112	4.48	112	4.55
His	68	2.26	57	2.28	59	2.27	57	2.26	54	2.16	56	2.25
Ile	143	4.68	122	4.85	127	4.82	123	4.83	119	4.70	120	4.81
Leu	271	8.90	234	9.30	243	9.25	234	9.23	290 ²	11.99	228	9.20
Lys	188	6.15	158	6.28	164	6.23	167 ³	6.64	156	6.17	164 ³	6.64
Met	55	1.80	49	1.97	56 ⁴	2.14	50	1.95	48	1.89	53 ⁴	2.15
Phe	152	4.98	126	5.00	131	4.98	126	4.98	124	4.92	124	4.98
Thr	143	4.68	123	4.89	128	4.85	124	4.86	119	4.71	121	4.84
Val	164	5.35	138	5.47	144	5.44	139	5.45	135	5.34	136	5.44

¹PC = positive control (high-CP) diet; NC = negative control (low-CP) diet; +M = NC + rumen-protected (RP) Met; +K = NC + RP Lys; +L = NC + RP Leu; +MK = NC + RP Met and Lys; +ML = NC + RP Met and Leu; +MKL = NC + RP Met, Lys, and Leu.

²Rumen-protected Leu was supplemented at 7.8 g/kg of DM with 53.9% AA content, 23.6% rumen degradability (8-h retention time), and 98.3% digestibility (intestinal digestibility could be overestimated).

³Rumen-protected Lys was supplemented at 2.1 g/kg of DM with 32.1% AA content, 31.3% rumen degradability (8-h retention time), and 97.9% digestibility (intestinal digestibility could be overestimated).

⁴Rumen-protected Met was supplemented at 0.7 g/kg of DM with 70.6% AA content, 54.6% rumen degradability (8-h retention time), and 99.4% digestibility (intestinal digestibility could be overestimated).

Using a deletion approach, Weekes et al. (2006) observed that removing Met, Lys, or His, but not branched-chain AA (BCAA), from an EAA mix infused (1.1 kg/d) into dairy cows consuming a 9% CP diet and producing 21 kg of milk/d decreased MPY. In agreement with observations of Weekes et al. (2006), jugular infusion of Met (12 g/d) and Lys (21 g/d) to lactating cows producing 53 kg of milk/d and consuming a 16% CP diet (MP deficient) increased MPY, but no further response was observed when BCAA were added to the infusate (Appuhamy et al., 2011b). Dietary CP and DMI were similar between the study of Appuhamy et al. (2011b) and the current study; however, milk yield was about 20 kg/d higher in the earlier work. Similarly, RP Met (30 g/d) and Lys (100 g/d) supplemented to lactating cows producing 35 kg of milk/d and consuming a more severe MP-deficient diet (87% of requirement; 13.5% CP) increased MPY to similar values of the MP-sufficient diet (Lee et al., 2012). These studies are in agreement with our observations suggesting general responses to Met and Lys supplementation in many North American diets. In addition, the study of Weekes et al. (2006) suggests independent effects between these 2 AA, which is in agreement with the observation in the current study. On the other hand, the results reported by Appuhamy et al., (2011b) and Weekes et al. (2006), in addition to Rulquin and Pisulewski (2006), contradict the response observed with the +L diet in the current study regarding Leu requirements. However, MP supply levels relative to MPY could explain those differences and, in that case, they would suggest a positive interaction between Leu and other EAA.

No significant increases in milk fat content or yield were detected in this study. These results are in agreement with previous studies where increasing dietary CP or supplementing with Lys, Met, or BCAA had no effect on milk fat yield (Korhonen et al., 2002; Appuhamy et al., 2011b; Haque et al., 2012). Nevertheless, the lack of effect of CP or AA on milk fat is not universal (Robinson et al., 1995; Socha et al., 2005).

MUN and N Efficiency

Milk urea N and N efficiency results are presented in Table 5. Jonker et al. (1998) demonstrated that MUN was a useful predictor of N excretion and utilization efficiency for dairy cows. Milk urea N was significantly reduced for all treatments compared with the PC treatment. In accordance with Broderick et al. (2008), these results suggest animals fed the NC diet or any of the RP AA-supplemented diets exhibited increased N efficiency. It is certainly possible that treatments with higher energy supply resulted in increased muscle de-

Table 8. Concentrations of free AA in plasma of cows fed experimental diets

AA	Experimental diet ¹								SEM	P-value
	PC	NC	+M	+K	+L	+MK	+ML	+MKL		
EAA, μM										
Arg	138	120	128	139	134	132	130	133	10	0.7
His	51.4	47.6	49.8	52.2	50.4	48.5	50.0	48.2	1.8	0.19
Ile	117.2	93.1*	98.1	109.4	100.4	105.5	107.6	107.3	5.8	0.03
Leu	180.9	160.8	180.5	189.2	181.3	176.8	194.0	183.6	8.9	0.09
Lys	88.7	78.9	82.9	87.4	84.1	85.1	86.9	83.5	4.4	0.6
Met	25.4	26.8	27.8	28.7	27.6	28.3	28.4	27.6	1.2	0.4
Phe	49.0	43.6	45.6	48.3	45.8	47.9	49.0	47.7	1.6	0.08
Thr	99.9	96.9	96.9	103.7	102.9	102.3	101.3	99.0	4.6	0.8
Val	270	221*	231	258	245	245	261	251	13	0.03
NEAA, μM										
Ala	270	269	288	292	282	289	290	269	12	0.3
Asp	94.35	94.15	93.40	95.50	94.00	94.08	94.01	94.64	0.68	0.4
Glu	100.8	105.7	104.8	112.3	105.4	109.8	109.6	106.6	4.0	0.3
Gly	394	489	457	463	482	445	429	421	29	0.10
Pro	111.8	109.5	118.9	123.5	119.7	114.2	113.6	111.3	4.6	0.13
Ser	99.6	106.1	108.1	112.3	115.1	108.9	107.9	95.4	5.1	0.05
Tyr	53.2	46.1	49.6	51.7	48.7	48.6	53.6	47.2	2.5	0.18

¹PC = positive control (high-CP) diet; NC = negative control (low-CP) diet; +M = NC diet + rumen-protected (RP) methionine; +K = NC diet + RP lysine; +L = NC diet + RP leucine; +MK = NC diet + RP methionine and lysine; +ML = NC diet + RP methionine and leucine; +MKL = NC diet + RP methionine, lysine and leucine.

* $P < 0.05$ for Bonferroni-adjusted pairwise comparisons versus PC.

position. However, energy was not limiting for any of the treatments, and reducing AA supply should have reduced rather than increased muscle protein synthesis (Escobar et al., 2006). Nitrogen efficiency values were not different among treatments. Kalscheur et al. (2006) observed a significant increase in N efficiency of dairy cows from 28.2 to 36.5% when dietary CP was decreased from 17.1 to 12.3%. Ipharraguerre and Clark (2005) also observed an increase in N efficiency from 25 to 33% when CP was reduced from 18.7 to 14.8%. As noted before, it is likely that the marginal change in efficiency with 11% change (16.88 vs. 15.02%) in the percentage CP in the diet is too small to be detected, and markers such as MUN may be more sensitive.

Plasma Concentration of AA

Plasma concentrations of NEAA were largely unaffected by treatment (Table 8). Plasma concentration of Ile and Val were significantly reduced in the NC diet. Plasma concentrations of the other EAA were not affected by treatment. Based on classical methods of interpretation of blood AA concentrations (Almquist, 1954), the lack of change in plasma concentrations of EAA, in addition to the reduction in MPY and MUN, suggests that the NC diet was deficient for several of the EAA, so a proportion of those extra EAA supplied was used for milk protein synthesis. Otherwise, plasma concentrations of EAA would have increased with extra supply. However, as discussed above, the results for

MPY suggest a very low marginal efficiency of MP for milk protein (19%).

Cell Signaling Proteins

The effect of dietary treatments on signaling proteins from mammary biopsy samples are presented in Table 9. Representative Western immunoblotting images for the phosphorylated forms of signaling proteins are shown in Figure 1, and representative total protein images are shown in Figure 2. The relative abundance of phosphorylated and total signaling proteins in the mTOR pathway were not affected by treatments. In short-term studies, casein and starch infusion for 36 h increased mTOR phosphorylation in mammary glands of cows fed 15.2% CP (Rius et al., 2010). Furthermore, feed-deprived cows for 22 h responded to 9-h infusion of Met and Lys, or Leu alone by increasing phosphorylation of S6K1 and rpS6 (Toerien et al., 2010). In vitro, addition of EAA, Ile, or Leu alone to EAA-starved tissue slices from dairy cows significantly increased phosphorylation of mTOR (Appuhamy et al., 2011a; Arriola Apelo et al., 2014a). Removing either Ile or Leu from the culture media decreased mTOR phosphorylation (Appuhamy et al., 2012). However, very little is known of the chronic responses of the mTOR pathway to EAA in bovine mammary tissue.

Phosphorylation of eIF2 α was also not affected by treatments. Few reports exist of EAA effects on eIF2 α phosphorylation in mammary tissue. Appuhamy et

Figure 1. Representative Western blot images of the phosphorylated forms (P) of analyzed signaling proteins from mammary biopsies. PC = positive control (a standard diet containing 17% CP); NC = negative control (a 15% CP diet); +MK = the NC diet plus RP Met and Lys; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; 4EBP1 = eukaryotic initiation factor 4E binding protein 1; eEF2 = eukaryotic elongation factor 2; eIF2 α = eukaryotic initiation factor 2 α subunit.

al. (2012) observed a significant effect on eIF2 α phosphorylation in mammary tissue slices only when all the EAA were completely removed from the media. In mammary alveolar (MAC-T) cells, addition of His, Phe, and Val to an EAA-depleted medium reduced eIF2 α phosphorylation (Arriola et al., 2010). A 9-h in vivo infusion of His reduced eIF2 α phosphorylation, but infusion of all EAA, Leu, or Met and Lys had no effect (Toerien et al., 2010).

Table 9. Effects of experimental diets on the relative abundance of signaling proteins in mammary biopsies

P/T ^{1,2}	Experimental diet ³			P-value
	PC	NC	+MK	
S6K1	1.00	0.81	1.04	0.8
rpS6	1.00	1.12	0.98	1.0
4EBP1	1.00	1.15	1.08	0.8
eEF2	1.00	0.99	0.95	1.0
eIF2 α	1.00	1.53	1.16	0.6

¹Phosphorylated (P) to total (T) ratio of signaling proteins standardized to positive control (PC) treatment.

²S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; 4EBP1 = eukaryotic initiation factor 4E binding protein 1; eEF2 = eukaryotic elongation factor 2; eIF2 α = eukaryotic initiation factor 2 α subunit.

³PC = high-CP diet; NC = negative control (low-CP) diet; +MK = NC diet + RP methionine and lysine.

Figure 2. Representative Western blot images of the total forms (T) of analyzed signaling proteins from mammary biopsies. PC = positive control (a standard diet containing 17% CP); NC = negative control (a 15% CP diet); +MK = the NC diet plus RP Met and Lys; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; 4EBP1 = eukaryotic initiation factor 4E binding protein 1; eEF2 = eukaryotic elongation factor 2; eIF2 α = eukaryotic initiation factor 2 α subunit.

CONCLUSIONS

This study highlights the limitations of using a fixed postabsorptive efficiency of MP for milk protein synthesis, and aggregating individual AA requirements into only 1 fraction (MP). As a consequence of fixed efficiency, milk protein responses close to requirements are overpredicted, which may lead to overfeeding of N, given the expectation of large production responses. Using a variable efficiency would alter the dose-response relationship above 15% CP, resulting in a reduced return for the extra MP. Including individual EAA requirements in absolute terms would allow reducing current dietary CP levels and supplementing with specific EAA known to stimulate milk protein synthesis, giving maximum N postabsorptive efficiency for milk protein.

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