Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet

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ABSTRACT

The objective of this experiment was to evaluate the effect of supplementing a metabolizable protein (MP)-deficient diet with rumen-protected (RP) Lys, Met, and specifically His on dairy cow performance. The experiment was conducted for 12 wk with 48 Holstein cows. Following a 2-wk covariate period, cows were blocked by DIM and milk yield and randomly assigned to 1 of 4 diets, based on corn silage and alfalfa haylage: control, MP-adequate diet (ADMP; MP balance: +9 g/d); MP-deficient diet (DMP; MP balance: −317 g/d); DMP supplemented with RPLys (AminoShure-L, Balchem Corp., New Hampton, NY) and RPMet (Mepron; Evonik Industries AG, Hanau, Germany; DMPLM); and DMPLM supplemented with an experimental RPHis preparation (DMPLMH). The analyzed crude protein content of the ADMP and DMP diets was 15.7 and 13.5 to 13.6%, respectively. The apparent total-tract digestibility of all measured nutrients, plasma urea-N, and urinary N excretion were decreased by the DMP diets compared with ADMP. Milk N secretion as a proportion of N intake was greater for the DMP diets compared with ADMP. Compared with ADMP, dry matter intake (DMI) tended to be lower for DMP, but was similar for DMPLM and DMPLMH (24.5, 23.0, 23.7, and 24.3 kg/d, respectively). Milk yield was decreased by DMP (35.2 kg/d), but was similar to ADMP (38.8 kg/d) for DMPLM and DMPLMH (36.9 and 38.5 kg/d, respectively), paralleling the trend in DMI. The National Research Council 2001 model underpredicted milk yield of the DMP cows by an average ($\pm$ SE) of 10.3 ± 0.75 kg/d. Milk fat and true protein content did not differ among treatments, but milk protein yield was increased by DMPLM and DMPLMH compared with DMP and was not different from ADMP. Plasma

essential amino acids (AA), Lys, and His were lower for DMP compared with ADMP. Supplementation of the DMP diets with RP AA increased plasma Lys, Met, and His. In conclusion, MP deficiency, approximately 15% below the National Research Council requirements from 2001, decreased DMI and milk yield in dairy cows. Supplementation of the MP-deficient diet with RPLys and RPMet diminished the difference in DMI and milk yield compared with ADMP and additional supplementation with RPHis eliminated it. As total-tract fiber digestibility was decreased with the DMP diets, but DMI tended to increase with RP AA supplementation, we propose that, similar to monogastric species, AA play a role in DMI regulation in dairy cows. Our data implicate His as a limiting AA in high-producing dairy cows fed corn silage- and alfalfa haylage-based diets, deficient in MP. The MP-deficient diets clearly increased milk N efficiency and decreased dramatically urinary N losses.

Key words: rumen-protected lysine, rumen-protected methionine, rumen-protected histidine, dairy cow

INTRODUCTION

The US dairy industry is under increasing pressure to more efficiently use resources and reduce environmental emissions of N, P, greenhouse gases, and volatile organic compounds (Place and Mitloehner, 2010). Nitrogen is one of the pollutants from animal operations, contributing to ground water nitrate, surface water eutrophication, and atmospheric ammonia and nitrous oxide emissions (US EPA, 2011). Diet manipulation is a critical mitigation strategy that will not only reduce N emissions, but can also be economically feasible for the producer (Hristov et al., 2011a, 2012). Reduction in urinary N excretion and manure ammonia emission is commonly reported for cows fed diets with decreased protein concentration (Külling et al., 2001; Olmos Colmenero and Broderick, 2006; Agle et al., 2010). In some studies, reduction in dietary CP had no effect on milk

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production (Leonardi et al., 2003; Olmos Colmenero and Broderick, 2006), but in others, milk production (Cyriac et al., 2008; Lee et al., 2011a) or milk protein concentration were decreased (Lee et al., 2012b). In such cases, supplementation with rumen-protected (RP) AA may be a successful strategy for alleviating the possible negative production effects of decreased dietary protein supply (Broderick et al., 2008). Indeed, recent meta-analyses have shown small but consistent increases in milk production and DMI with dietary addition of RP AA, particularly of RPMet when AA balance was positive (Patton, 2010; Robinson, 2010). The majority of the diets used in these analyses, however, appeared to be supplying MP in excess of requirements for the level of production of the cows (average CP from 16 to about 17% for average milk production of 31 to 33 kg/d), in which case production effects of the supplemental RP AA are unlikely.

Lee et al. (2012b) proposed that in situations when ruminally synthesized microbial protein provides the majority of MP supply to the small intestine (i.e., when RUP supply is low), His may limit milk production in dairy cows fed corn silage- and alfalfa haylage-based diets. Histidine has been identified as the first limiting AA in cows fed grass silage-based diets with low inclusion of plant protein supplements (Kim et al., 1999; Vanhatalo et al., 1999), but very little information exists on the importance of His in typical North American diets (Doelman et al., 2008). Lee et al. (2012b) reported a sharp decrease in plasma His concentrations in cows fed MP-deficient diets, which, in combination with the low concentration of His in microbial protein, led us to the conclusion that His may be a limiting AA in corn silage- and alfalfa haylage-based diets that are MP and RUP deficient.

Therefore, our goal with this experiment was to investigate the effect of RPHis supplementation on milk production and composition from dairy cows fed a MP-deficient diet balanced for Lys and Met. We hypothesized that in dietary situations, in which microbial protein synthesized in the rumen is the main source of MP, His may limit milk production and milk protein synthesis in high-producing dairy cows fed diets based on corn silage and alfalfa haylage.

MATERIALS AND METHODS

Animals involved in this experiment were cared for according to the guidelines of The Pennsylvania State University Animal Care and Use Committee (University Park; IACUC no. 35630). The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

The experiment was carried out at The Pennsylvania State University's Dairy Center as a randomized complete block design. Due to cow availability, the experiment was separated into 2 phases. Phase 1 (May to July 2011), involved 24 lactating Holstein cows (8 primiparous and 16 multiparous; average $\pm$ SD at the beginning of the trial: parity, 2 ± 1.0 lactations; milk yield, 40.2 ± 7.16 kg/d; DIM, 95 ± 40.3 d; BW, 605 ± 76 kg) blocked by parity, DIM, and milk yield into 6 blocks of 4 cows each (blocks 1 to 6). Phase 2 (August to November 2011) involved another group of 24 cows (11 primiparous and 13 multiparous; average $\pm$ SD at the beginning of the trial: parity, 2 ± 1.1 lactations; milk yield, 40.4 ± 7.18 kg/d; DIM, 54 ± 23.5 d; BW, 583 ± 66.3 kg) blocked as in phase 1 into 6 blocks of 4 cows each (blocks 7 to 12). In both phases, cows within block were randomly allocated to one of the following experimental diets: (1) MP-adequate diet (**ADMP**), meeting MP requirements of the cows according to NRC (2001); (2) MP-deficient diet (**DMP**) providing 87% of MP requirements according to NRC (2001); (3) DMP supplemented with 100 g/cow per day of RPLys and 30 g/cow per day of RPMet (**DMPLM**), providing 85% of MP requirements; (4) DMP supplemented with RPLys, RPMet, and 50 g/cow per day of RPHis (**DMPLMH**), providing 85% of MP requirements. Diets (Table 1) were formulated to meet the nutrient requirements, except MP for the DMP diets, of a lactating Holstein cow yielding 40 kg of milk/d, with 3.60% milk fat and 3.10% true protein at 26 kg of DMI/d, and 635 kg of BW (NRC, 2001). Sources of supplemental RPLys, RPMet, and RPHis were AminoShure-L (Balchem Corp., New Hampton, NY), Mepron (Evonik Industries AG, Hanau, Germany), and an experimental RPHis product (Balchem Corp.), respectively. Based on manufacturer specifications, the supplemental RP AA were expected to provide 24.3, 18.4, and 12.4 g/cow per day of digestible (**d**) Lys, dMet, and dHis, respectively (see Table 2, footnote). The application rates of RPLys and RPMet were determined assuming dLys and dMet requirements of 6.6 and 2.2% of MP (Schwab et al., 2005). The application rate of RPHis was estimated with the objective to match dHis supply with the ADMP diet (based on NRC, 2001). The supply of protein fractions and AA balances (Table 2) were estimated using NRC (2001) with individual cow information (actual DMI, milk yield, milk composition, and BW) during the experiment. Dietary composition was slightly modified in phase 2 of the trial (Table 1), mainly due to lower CP content of the alfalfa haylage (19.5 vs. 21.7% of DM, phase 2 and 1, respectively), to meet or exceed the MP requirements of cows receiving

the ADMP diet. Hence, the proportion of SoyPLUS (West Central Cooperative, Ralston, IA) was increased by 1 percentage unit for phase 2 of the trial, replacing corn silage.

The duration of the experiment was 12 wk, including a 2-wk covariate period, 2 wk of adaptation to the diets, and 8 wk of data collection (in wk 8, data were

collected in only 4 d; i.e., a total of 53 d of data collection). During the covariate period, all cows were fed the main herd diet, which contained similar feed ingredients to the ADMP and DMP diets, except SoyPLUS. The covariate period diet contained (% of DM): corn silage, 39.7; alfalfa haylage, 16.6; grass hay, 8.7; corn grain, ground, 7.6; bakery byproduct meal, 7.4; soy-

Table 1. Ingredient and chemical composition of the basal diets fed in the trial

Item	Diet ¹			
	Phase 1		Phase 2	
	ADMP	DMP	ADMP	DMP
Ingredient, % of DM				
Corn silage ²	40.7	40.7	39.7	39.7
Alfalfa haylage ³	16.7	16.6	16.7	16.6
Grass hay ⁴	5.8	5.8	5.8	5.8
Cottonseed, hulls	1.1	1.1	1.1	1.1
Forage	64.3	64.2	63.3	63.2
Corn grain, ground	5.7	11.7	5.7	11.7
Bakery by-product meal ⁵	7.4	7.4	7.4	7.4
Soybean seeds, whole, heated ⁶	5.5	6.6	5.5	6.6
Canola meal, mechanically extracted ⁷	5.0	3.0	5.0	3.0
SoyPLUS ⁸	5.0	0.0	6.0	1.0
Molasses ⁹	4.2	4.2	4.2	4.2
Mineral and vitamin premix ¹⁰	2.9	2.8	2.9	2.9
Composition, % of DM				
CP	15.7	13.6	15.7	13.5
RDP ¹¹	9.8	9.1	9.8	9.0
RUP ¹¹	5.9	4.4	5.9	4.5
NDF	29.5	30.0	29.3	29.0
ADF	19.0	19.4	20.4	19.5
Ether extract	4.8	4.7	4.1	4.5
NE _L , ¹¹ Mcal/kg	1.56	1.57	1.56	1.56
NE _L balance, ¹¹ Mcal/d	2	3	2	2
NFC	44.6	45.8	46.0	48.4
Ca	1.03	1.06	0.98	0.93
P	0.40	0.35	0.36	0.32

¹ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with rumen-protected (RP) Lys (AminoShure-L; Balchem Corp., New Hampton, NY) and RPMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RPMet, and RPHis.

²Corn silage was 30.7 and 42.9% DM and (DM basis) 7.8 and 7.6% CP and 42.1 and 35.5% NDF (phase 1 and 2, respectively).

³Alfalfa haylage was 49.4 and 45.4% DM and (DM basis) 21.7 and 19.5% CP and 41.4 and 43.0% NDF (phase 1 and 2, respectively).

⁴Grass hay contained (DM basis) 8.6 and 6.6% CP and 64 and 66% NDF (phase 1 and 2, respectively).

⁵Bakery by-product meal (Bakery Feeds Inc., Honey Brook, PA) contained (DM basis) 13.3 and 11.7% CP (phase 1 and 2, respectively) and 13.5% NDF (1 composite sample was analyzed for both experimental phases).

⁶Contained (DM basis) 40.2 and 40.1% CP (phases 1 and 2, respectively).

⁷Contained (DM basis) 39.2 and 43.3% CP (phases 1 and 2, respectively).

⁸SoyPLUS (West Central Cooperative, Ralston, IA) contained (DM basis) 44.9 and 45.3% CP (phases 1 and 2, respectively).

⁹Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.

¹⁰The premix contained (% as-is basis) trace mineral mix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA), 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

¹¹Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the trial.

Table 2. Rumen-protected (RP) AA supplementation and protein and AA balance¹ during the trial

Item	Diet ²			
	ADMP	DMP	DMPLM	DMPLMH
Supplementation of the basal diets, g/d				
AminoShure-L	—	—	100	100
Mepron	—	—	30	30
RPHis	—	—	—	50
Protein and AA supply, ^{3,4} g/d				
RDP and RUP				
RDP supply	2,420	2,108	2,166	2,219
RDP balance	−17	−192	−199	−199
RUP supply	1,490	1,034	1,081	1,100
RUP balance	12	−396	−464	−482
MP				
Requirements	2,647	2,395	2,522	2,583
Supply	2,656	2,079	2,152	2,198
Balance	9	−317	−370	−385
dLys				
Requirements ⁴	175	158	166	170
Supply from the diet	160	134	138	141
Supply from AminoShure-L ⁵	—	—	24	24
Balance	−15	−24	−5	−4
dMet				
Requirements ⁴	58	53	55	57
Supply from the diet	45	39	40	41
Supply from Mepron ⁵	—	—	18	18
Balance	−13	−14	3	2
dHis				
Requirements ⁴	58	53	55	57
Supply from the diet	54	44	46	47
Supply from RPHis ⁵	—	—	—	12
Balance	−5	−9	−10	1

¹Due to rounding, balance may not exactly match requirements and supply.

²ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with RPLys (AminoShure-L; Balchem Corp., New Hampton, NY) and RMPMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RMPMet, and RPHis.

³All values were estimated using NRC (2001) based on actual DMI, milk yield, milk composition, and BW of the cows throughout the trial; dLys, dMet, dHis = digestible Lys, Met, and His, respectively.

⁴Requirements of dLys and dMet were calculated as 6.6 and 2.2% (respectively) of MP requirements. Requirements of dHis were assumed as 2.2% of MP requirements (see Results section).

⁵Digestible Lys, Met, and His from AminoShure-L, Mepron, and RPHis, respectively, were estimated from Lys, Met, and His content and bioavailability data provided by the manufacturer or determined in the in situ experiment (for RPHis): 38% Lys and 64% bioavailability (AminoShure-L), 85% Met and 72% bioavailability (Mepron), and 40% His and 62% bioavailability (78% rumen bypass and assumed 80% intestinal digestibility; RPHis).

bean seeds, whole, heated, 6.8; canola meal, mechanically extracted, 5.7; molasses, 4.2; Optigen (Alltech Inc., Nicholasville, KY), 0.4; and mineral and vitamin premix, 2.8. The diet contained (% of DM): CP, 16.0; NDF, 32.8; and ether extract, 5.0. The experimental diets were fed as TMR, once daily at 0830 h ad libitum, targeting approximately 10% refusals. The rations were mixed every morning using a Kuhn Knight model 3142 Reel Auggie Mixer Wagon (Kuhn Knight Inc., Brodhead, WI). Immediately after the diets were delivered in the feed bunks, RPLys, RMPMet, and RPHis were top-dressed and mixed with a small portion of the diet. Visual observation confirmed that the RP AA were completely consumed.

Sampling and Measurements

Feed intake of individual cows (TMR offered and refused) was monitored daily during the entire experiment. Samples of TMR and refusals from each diet were collected twice weekly and composited (equal weight basis) by week and treatment. Samples were dried for 48 h at 65°C in a forced-air oven and ground in a Wiley Mill (A. H. Thomas Co., Philadelphia, PA) through a 1-mm sieve. Composited TMR samples were submitted to Cumberland Valley Analytical Services Inc. (Maugansville, MD) for wet chemistry analyses of CP, NDF, ADF, Ca, and P and estimated NE_L (details at http://www.foragelab.com/pdf/CVAS_Procedure_Refer-

ences.pdf, accessed March 5, 2012). Individual forage and concentrate ingredients were sampled biweekly and stored frozen at -20°C until analyzed. The CP content of the diets was estimated from N analysis of individual feed ingredients. Cows were milked twice daily at 0500 and 1700 h and the milk yield of individual cows was recorded. Feed efficiency was calculated by dividing milk yield by DMI. Milk yield, DMI, and feed efficiency data for 53 d (wk 5 through 11, plus 4 d of wk 12) were used in the statistical analysis.

Spot fecal (about 500 g) and urine (about 300 mL per sampling) samples were collected 7 times at 1000 and 1600 h on d 1; 0400, 1300, and 1900 h on d 2; and 0100 and 0800 h on d 3 of experimental wk 9 and 11 from the rectum and by massaging the vulva, respectively. About 400 g of fecal sample was dried at 65°C in a forced-air oven for 48 h and then composited (equal dry-weight basis) by cow and week. The composite fecal samples were analyzed for DM, OM, CP, NDF, and ADF. Dry matter and OM of feces were determined following the procedure of AOAC Int. (2000), and NDF and ADF were analyzed using an Ankom A200 fiber analyzer (Ankom Technology Corp., Macedon, NY) based on Van Soest et al. (1991), using amylase and sodium sulfide in the NDF analysis. A portion of each composite fecal sample was pulverized using a Mixer Mill MM 200 (Retsch GmbH, Haan, Germany) and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA); CP was calculated as $\text{N} \times 6.25$. Fecal and TMR samples were analyzed for indigestible NDF (as an intrinsic digestibility marker) according to Huhtanen et al. (1994), except 25- μm pore size filter bags (Ankom Technology Corp.) were used for the ruminal incubation. Total-tract apparent digestibility was calculated based on indigestible NDF concentration in TMR and feces (Schneider and Flatt, 1975). Aliquots of the urine samples were immediately acidified with 2 M H_2SO_4 to a pH <3.0 , diluted 1:10 with distilled water, and stored frozen at -20°C . These samples were composited by cow and week and analyzed for allantoin (Chen et al., 1992), uric acid (Stanbio Uric Acid Kit 1045; Stanbio Laboratory Inc., San Antonio, TX), urea-N (UUN; Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.), and creatinine (Stanbio Creatinine Kit 0400; Stanbio Laboratory Inc.). Another aliquot (80 μL) of the composited urine samples was freeze-dried and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer. Daily volume of excreted urine was estimated based on urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW (determined based on total urine collection samples from Hristov et al., 2011b). Estimated

urine output was used to calculate daily total N, UUN, and purine derivative (PD; allantoin and uric acid) excretions. Urinary PD excretion was used to estimate duodenal microbial N flow as described in Hristov et al. (2009). A ratio of purine N to total N in rumen microorganisms of 0.134 was assumed based on the data of Valadares et al. (1999).

Blood samples were collected from the tail vein or artery at 1400, 1800, and 2200 h on d 5 of wk 9 and 11. Blood plasma was immediately separated by centrifugation at $1,500 \times g$ for 15 min and frozen at -20°C until analyzed. Plasma samples were composited per cow and sampling week and analyzed for urea-N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.). Aliquots of individual cow plasma samples were composited by cow and submitted for plasma AA analysis to the University of Missouri-Columbia, Agricultural Experiment Station Chemical Laboratory (ESCL, Columbia; following the procedures of Deyl et al., 1986 and Fekkes, 1996).

Samples of whole ruminal contents from multiple sampling events from 2 previous experiments (Lee et al., 2011b; Tekippe et al., 2011) were composited (equal DM basis) per individual cow ($n = 14$) and used to analyze AA composition of bacterial protein. Bacterial pellets were isolated from whole ruminal contents through differential centrifugations as described elsewhere (Hristov and Broderick, 1996). This procedure recovers liquid-associated and loosely associated with feed particles bacteria. Amino acid analysis of the samples was performed by the ESCL using acid hydrolysis and performic acid oxidation for Met and Cys.

Milk samples were collected at 2 consecutive milkings (p.m. and a.m.) in wk 2 of the covariate period and wk 6, 8, 10, and 12 of the data-collection period. Milk samples were directly collected in tubes containing 2-bromo-2-nitropropane-1,3-diol and submitted to Dairy One laboratory (Pennsylvania DHIA, University Park, PA) for analysis of milk fat, true protein, lactose, and MUN using infrared spectroscopy (MilkoScan 4000; Foss Electric A/S, Hillerød, Denmark; AOAC, 1990, method no. 972.16). Milk composition data of consecutive milking samples were averaged according to milk weights and the average values were used in the statistical analysis (i.e., 4 observations per cow).

Cow BW was recorded throughout the entire experiment using AfiFarm 3.04E scale system (S.A.E. Afikim, Rehovot, Israel) while cows exited the milk parlor. For the statistical analysis, BW data collected during the data collection period (wk 5 to 12) were used. Body weight change was calculated by the difference between average BW during the first 2 wk and the last wk (wk 12) of the data collection period.

In Situ Disappearance of RPHis

Ruminal disappearance of the experimental RPHis product was tested using a standard *in situ* procedure (IACUC approval no. 34821) with 4 ruminally cannulated (10-cm rumen cannulas; Bar Diamond Inc., Parma, ID) Holstein cows (average $\pm$ SD: DIM, 96 ± 37 d; BW, 728 ± 59 kg; DMI, 24 ± 1.8 ; milk yield, 48 ± 6 kg/d) fed the covariate diet (see Animals and Experimental Design section). Eight grams of RPHis was put into each 5-cm $\times$ 10-cm, 50- μ m porosity polyester bag (Ankom Technology Corp.) and incubated in the rumen of the cow for 0 (washed but not incubated in the rumen), 6, 12, and 24 h. Bags were incubated in triplicate for each time period and cow and were inserted into the rumen simultaneously and removed sequentially. Incubation was repeated twice using different cows. Bags were washed by hand with cold tap water until the wash water was clear and dried in an oven at 50°C for 48 h. Bag residues were analyzed for His as specified in the previous sections (ESCL). The fractional rate of ruminal disappearance of His from RPHis was derived by plotting the natural logarithm of the fraction of His remaining in the bag over time and fitting the data to a linear regression model (SigmaPlot 10.0; Systat Software Inc., San Jose, CA; average $R^2 = 0.85 \pm 0.04$). Ruminal escape of His was estimated from His analysis of the original product and *in situ* bag residues, based on NRC (2001) protein degradability equations as His escape (%) = 100 – degradable His; and degradable His (%) = soluble His (%) + [100 – soluble His (%) $\times$ [rate of His disappearance $\div$ (rate of His disappearance + ruminal passage rate)]]. The average fractional rate of His disappearance was 0.007 ± 0.0025 h⁻¹. Passage rates were estimated individually for each cow based on actual DMI and BW of the cows according to NRC (2001); the average fractional rate of passage was 0.06 ± 0.002 h⁻¹.

Statistical Analysis

All data were analyzed using PROC MIXED of SAS (SAS Institute, 2003). Dry matter intake, milk yield, feed efficiency, BW, and milk composition data were analyzed with day as repeated measure assuming an AR(1) covariance structure. The following model was used:

$$Y_{ijkl} = \mu + B_i + \tau_j + B\tau_{ij} + D_k + \tau D_{jk} + T_l + \tau T_{jl} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, B_i is the block, τ_j is the j th treatment (diet),

$B\tau_{ij}$ is the block $\times$ treatment interaction, D_k is the day, τD_{jk} is the treatment $\times$ day interaction, T_l is the trial phase (phase 1 and 2), and τT_{jl} is the treatment $\times$ phase interaction, with the error term e_{ijkl} assumed to be normally distributed with mean = 0 and constant variance. Block and block $\times$ treatment effects were random, whereas all others were fixed.

Urinary PD excretion, microbial N flow, nutrient intakes, total-tract apparent digestibility, urinary and fecal N losses, and plasma urea-N (PUN) data were analyzed using the following model:

$$Y_{ijkl} = \mu + B_i + \tau_j + B\tau_{ij} + W_k + \tau W_{jk} + T_l + \tau T_{jl} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, B_i is the block, τ_j is the j th treatment, $B\tau_{ij}$ is the block $\times$ treatment interaction, W_k is the sampling week (wk 9 and 11), τW_{jk} is the treatment $\times$ week interaction, T_l is the trial phase (phase 1 and 2), and τT_{jl} is the treatment $\times$ phase interaction, with the error term e_{ijkl} assumed to be normally distributed with mean = 0 and constant variance. Block and block $\times$ treatment were random effects, whereas all others were fixed. Body weight change and plasma AA composition data were analyzed with the same model excluding sampling week and treatment $\times$ week interaction.

All data are presented as least squares means. Significant interactions are presented in the table footnotes. Significant differences among treatments were declared at $P \leq 0.05$. Differences at $0.05 < P \leq 0.10$ were considered a trend toward significance. When the main effect of treatment was significant, means were separated by pairwise *t*-test (pdiff option of PROC MIXED).

RESULTS

The slight adjustment in feed ingredients during phase 2 of the experiment did not significantly affect the chemical composition of the diets (Table 1). As planned, the DMP diets had lower CP and slightly higher NFC content compared with the ADMP diets (both experimental phases). All diets provided NE_L in excess of cow requirements.

Ruminal escape of His from the RPHis product was estimated (*in situ*) at $78 \pm 2.8\%$. This escape was used to calculate dHis supply in this experiment (see Table 2, footnote). It is worth noting that RPHis escape varied between experimental cows. Two of the cows (one in each of the 2 incubation events) had considerably greater *in situ* disappearance of RPHis, which resulted in His escape of about 72% (both cows had a similar DMI as percent of BW). The other 2 cows (one in each

incubation) had slower rate of in situ disappearance of RPHis, which resulted (again with similar DMI on a BW basis) in greater estimated escape of His (about 82%). This difference emphasizes animal-to-animal variability and the need for replication to derive accurate data with the in situ procedure. As indicated in the Table 2 footnote, intestinal digestibility of rumen bypass His was assumed at 80%.

Because it was estimated that protein and AA supply were similar between phase 1 and 2 of the trial, data were combined and average data are presented in Table 2. On average, DMP diets were estimated to be about 197 and 447 g/d deficient in RDP and RUP, respectively. The estimated RDP and RUP supply with the ADMP diet was close to requirements. The estimated supply of MP was adequate for the ADMP diet and, on average, 357 g below requirements for the DMP diets. Assuming that the milk production potential of the DMP cows was similar to that of the ADMP cows (i.e., their MP requirements would be similar), the DMP and DMPLM diets would be 568 and 495 g/d deficient in MP (based on the actual DMI of the cows, which was significantly or numerically decreased by both diets compared with the ADMP diet). For DMPLMH, the potential MP deficiency would be about 16% greater than the estimated deficiency based on actual milk production. According to NRC (2001), the unsupplemented diets were estimated to be 9 (ADMP) to 15% (DMP) dLys deficient, whereas the diets supplemented with RPLys were, on average, about 3% dLys deficient. The ADMP and DMP diets were similarly about 24% deficient in dMet (DMP cows had lower estimated dMet requirements due to lower milk protein production); the RPMet-supplemented diets supplied adequate amounts of dMet. Digestible His requirements were assumed to be similar to dMet requirements (i.e., 2.2% of MP; Schwab et al., 2005). This assumption was based on the similar concentration of His and Met in milk protein (5.5% of total EAA; NRC, 2001). Based on this assumption, the ADMP diet was estimated to be about 9% deficient and the DMP and DMPLM diets were about 18% deficient in dHis. Supplementation with RPHis resulted in slight excess of dHis over estimated requirements for the DMPLMH diet.

Nutrient intakes and digestibility during sampling weeks (wk 9 and 11) are shown in Table 3. As expected, CP intake (including N from supplemental RP AA) was lower ($P < 0.01$) for the DMP diets than the ADMP diet. Intakes of DM, OM, NDF, and ADF were similar among diets during the sampling weeks. It is noted that DMI for DMP and DMPLM was numerically lower compared with ADMP and DMPLMH during sampling weeks and reached a trend toward significance ($P = 0.06$) when data for the entire trial were analyzed (see

Table 5 and related discussion). Digestibility of all nutrients was decreased ($P < 0.01$) by the DMP diets compared with the ADMP diet. The difference varied from about 4 to 5% for DM and OM to about 10 to 11% for NDF and CP. An experimental phase $\times$ diet interaction was observed for NDF and ADF digestibility ($P = 0.03$ and <0.01 , respectively). In phase 1, NDF digestibility was numerically lower ($P = 0.14$ and 0.54) for DMP and DMPLMH, respectively, and lower ($P = 0.02$) for DMPLM compared with ADMP (40.5, 41.9, 39.0, and 42.8%, respectively). In phase 2, NDF digestibility was decreased ($P < 0.01$) for all DMP diets compared with the ADMP diet (34.6, 32.6, 33.5, and 40.6%, respectively). Total-tract digestibility of ADF was 39.5, 38.8, 35.8, and 40.3% in phase 1 and 37.7, 30.9, 30.5, and 29.0% in phase 2 for ADMP, DMP, DMPLM, and DMPLMH, respectively. In phase 1, ADF digestibility was lower ($P = 0.01$) for DMPLM compared with all other diets (DMP and DMPLMH were not different from ADMP; $P = 0.63$ and 0.60). In phase 2, ADF digestibility was decreased ($P < 0.01$) by all DMP diets compared with the ADMP diet. The reason for the large difference in ADF digestibility for the DMPLMH diet between phase 1 and 2 could not be determined and appeared to be a result of natural variability.

Milk true protein N secretion was not affected by diet, but was increased (16%; $P < 0.01$) as a proportion of N intake for all DMP diets compared with the ADMP diet (Table 4). Urinary N excretion was, on average, 36% lower ($P < 0.01$) and was decreased ($P < 0.01$) to about 17 to 19% of N intake for the DMP diets compared with the ADMP diet, which was 23% of N intake. Excretion of UUN was also reduced ($P < 0.01$) by the DMP diets compared with the ADMP diet. An experimental phase $\times$ diet interaction was observed for UUN excretion as a proportion of N intake ($P = 0.04$). Urinary urea-N excretion as a proportion of N intake was 67.2, 52.4, 51.6, and 54.2% in phase 1 and 77.1, 46.4, 40.7, and 43.4% in phase 2 for ADMP, DMP, DMPLM, and DMPLMH, respectively. In both experimental phases, this variable was decreased by the DMP diets compared with the ADMP diet ($P = 0.01$ to <0.01 in phase 1 and $P < 0.01$ in phase 2). Fecal N excretion was not affected by diet, but as proportion of N intake was increased ($P < 0.01$) by the DMP diets. Total N losses with feces and urine were lower ($P = 0.01$) for DMP and DMPLM compared with ADMP, but similar between DMPLMH and ADMP. Recovery of ingested N in excreta and milk true protein was greater ($P = 0.03$) for DMP and DMPLMH compared with ADMP. Urine output tended to be lower ($P = 0.09$) for the DMP diets, except for DMPLMH compared with ADMP. Urinary excretion of allantoin, uric acid,

Table 3. Effect of MP supply and rumen-protected (RP) AA supplementation on nutrients and total-tract apparent digestibility in dairy cows

Item	Diet ^{1,2}				SEM	P-value
	ADMP	DMP	DMPLM	DMPLMH		
Nutrient intake, ³ kg/d						
DM	24.9	22.9	23.6	24.2	1.18	0.59
OM	23.3	21.5	22.1	22.7	1.11	0.63
NDF	7.32	6.75	6.96	7.14	0.350	0.63
ADF	4.90	4.45	4.59	4.71	0.231	0.48
CP ⁴	3.90 ^a	3.09 ^b	3.24 ^b	3.37 ^b	0.165	<0.01
Apparent digestibility, %						
DM	67.2 ^a	65.1 ^b	64.1 ^{bc}	63.7 ^c	0.45	<0.01
OM	68.3 ^a	66.2 ^b	65.2 ^{bc}	65.0 ^c	0.44	<0.01
NDF ⁵	41.7 ^a	37.6 ^b	36.3 ^b	37.2 ^b	0.76	<0.01
ADF ⁶	38.6 ^a	34.9 ^b	33.1 ^b	34.7 ^b	0.74	<0.01
CP	62.5 ^a	57.2 ^b	56.8 ^b	55.3 ^b	0.79	<0.01

^{a-c}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with RPLys (AminoShure-L; Balchem Corp., New Hampton, NY) and RPLMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RPLMet, and RPHis.

²Least squares means; $n = 94$ (n represents number of observations used in the statistical analysis).

³Intake data are for experimental wk 9 and 11.

⁴Includes N from supplemental RP AA.

⁵Experimental phase $\times$ diet interaction ($P = 0.03$).

⁶Experimental phase $\times$ diet interaction ($P < 0.01$).

total PD, and the estimated microbial protein outflow from the rumen were not affected by diet. Blood PUN was, on average, 35% lower ($P < 0.01$) for the DMP diets compared with the ADMP diet. A sampling week $\times$ diet interaction ($P = 0.04$) was observed for PUN. Plasma urea-N concentrations were 10.9, 6.7, 7.5, and 6.6 mg/dL in wk 9 and 12.1, 6.9, 7.8, and 9.4 mg/dL in wk 11 for ADMP, DMP, DMPLM, and DMPLMH, respectively. In both sampling weeks, the DMP diets had lower ($P < 0.01$) PUN concentrations compared with the ADMP diet.

Dry matter intake over the entire data collection period (53 d) tended to be decreased ($P = 0.06$) by DMP, compared with ADMP and DMPLMH (mean comparisons: $P = 0.01$ and 0.04 , respectively; Table 5). These trends were in the same direction as the numerical trends in DMI observed for sampling wk 9 and 11 (Table 3). The DMP diet decreased ($P < 0.01$) milk production compared with ADMP and DMPLMH, most likely resulting from the decreased DMI with the former diet. Milk yield of the cows fed DMPLM tended to be lower ($P = 0.07$) than ADMP cows and higher ($P = 0.10$) than DMP cows. Feed efficiency, FCM feed efficiency, and milk fat percentage were not affected by diet, but milk fat yield tended to be lower ($P = 0.10$) for the DMP diets compared with the ADMP diet. Fat-corrected (3.5%) milk yield was decreased ($P = 0.03$) by DMP and DMPLM compared with ADMP and DMPLMH. Milk true protein content was not af-

ected by diet, but protein yield followed the trend ($P < 0.01$) in milk yield: lower for DMP compared with the other diets and similar between ADMP, DMPLM, and DMPLMH. Lactose yield followed the trend ($P < 0.01$) in milk yield. Milk NE_L was decreased ($P = 0.03$) by DMP and DMPLM compared with ADMP. All DMP diets decreased ($P < 0.01$) MUN compared with the ADMP diet. The concentration of MUN was numerically greater ($P = 0.12$), or tended to be greater ($P = 0.05$) for DMPLMH compared with DMP and DMPLM, respectively. Body weight or average BW change of the cows was not affected by diet.

Blood plasma Met concentration was increased ($P < 0.01$) by RPLMet supplementation (DMPLM and DMPLMH) compared with ADMP and DMP (Table 6). Plasma Lys concentration was decreased ($P = 0.03$) by DMP compared with ADMP, but supplementation with RPLys eliminated this effect and plasma Lys for DMPLM and DMPLMH was not different from ADMP. Rumen-protected His supplementation increased ($P < 0.01$) plasma His concentration of DMPLMH cows compared with the other DMP diets, but plasma His concentration still tended to be lower ($P = 0.06$) than that in ADMP cows. Blood plasma concentrations of several other EAA, namely Ile, Leu, Val, and Phe, and the concentration of EAA minus Lys, Met, and His were also decreased ($P = 0.02$ to <0.01) by DMP compared with ADMP. The concentration of total EAA was decreased ($P < 0.01$) by all DMP diets, compared with

Table 4. Effect of MP supply and rumen-protected (RP) AA supplementation on milk N secretion, urinary and fecal N excretion, urine purine derivative (PD) excretion, and plasma urea-N (PUN) concentration in dairy cows

Item	Diet ^{1,2}				SEM	P-value
	ADMP	DMP	DMPLM	DMPLMH		
N intake, ³ g/d	623 ^a	494 ^b	518 ^b	539 ^b	26.4	<0.01
N secretion and excretion, g/d						
Milk TPN ⁴	183	167	177	178	8.3	0.47
Urinary N	143 ^a	92 ^b	87 ^b	97 ^b	5.7	<0.01
Urinary urea-N (UUN), g/d	104 ^a	47 ^b	41 ^b	49 ^b	5.8	<0.01
UUN ÷ total urinary N, ⁵ %	72.2 ^a	49.4 ^b	46.1 ^b	48.8 ^b	2.68	<0.01
Fecal N	233	213	224	241	12.0	0.35
Total excreta N	376 ^a	305 ^b	311 ^b	338 ^{ab}	15.9	0.01
Total N in excreta and milk	559 ^a	472 ^b	489 ^b	516 ^{ab}	22.9	0.04
As proportion of N intake, %						
Milk TPN	29.4 ^b	34.2 ^a	34.4 ^a	33.6 ^a	0.99	<0.01
Urine N	23.1 ^a	19.0 ^b	16.9 ^b	18.2 ^b	0.83	<0.01
Fecal N	37.6 ^b	42.9 ^a	43.3 ^a	44.7 ^a	0.79	<0.01
Total excreta N	60.6	61.9	60.3	62.9	1.09	0.29
Total N in excreta and milk	90.0 ^b	96.2 ^a	94.7 ^{ab}	96.5 ^a	1.59	0.03
Urinary PD excretion						
Urine output, ⁶ kg/d	14.5	12.1	12.0	13.4	0.73	0.09
Allantoin, mmol/d	520	462	473	513	25.1	0.19
Uric acid, mmol/d	43.3	41.5	37.2	44.3	2.76	0.26
Total PD, mmol/d	563	504	510	557	27.3	0.20
Microbial N flow, g/d	383	338	343	377	19.7	0.19
PUN, ⁷ mg/dL	11.5 ^a	6.8 ^b	7.6 ^b	8.0 ^b	0.52	<0.01

^{a,b}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with RPLys (AminoShure-L; Balchem Corp., New Hampton, NY) and RPMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RPMet, and RPHis.

²Least squares means; $n = 94$ (n represents number of observations used in the statistical analysis).

³Includes N from supplemental RP AA.

⁴Milk true protein N (milk true protein ÷ 6.38).

⁵Experimental phase × diet interaction, $P = 0.04$.

⁶ADMP versus DMP ($P = 0.03$); ADMP versus DMPLM ($P = 0.03$).

⁷Sampling week × diet interaction ($P = 0.04$).

the ADMP diet. Plasma concentrations of Gly and Ser were increased ($P < 0.01$ and $= 0.01$, respectively) by DMP and DMPLM, but not by DMPLMH ($P = 0.10$ and 0.36 , respectively), compared with ADMP.

DISCUSSION

One of the notable effects in this study was the trend for depressed DMI with the DMP diet (on average, 1.5 kg/d throughout the 53 d of data collection). A recent meta-analysis reported increased feed intake and milk production with increasing dietary protein intake (Huhtanen and Hetta, 2012). This and the apparent reduction in total-tract digestibility, particularly fiber, suggest impaired rumen function due to RDP and ammonia deficiency. We have observed similar trends with low-CP, MP-deficient diets in our previous studies (Lee et al., 2011a). In both of these studies, total-tract NDF digestibility and DMI were decreased by the MP-deficient diets and in Lee et al. (2012b),

urinary purine derivatives excretion also tended to be decreased, similar to the numerical trend observed in the current experiment. Weigel et al. (1997) and Allen (2000) have suggested that RDP deficiency may depress DMI through inhibition of rumen function, although no statistical effect on DMI of dietary CP concentrations as low as 12 to 13% was observed by Olmos Colmenero and Broderick (2006) and Kröber et al. (2000).

Addition of RPLys and RPMet tended to increase DMI by 0.7 kg/d and the supplementation of all 3 RP AA (Lys, Met, and His) further increased it to the extent of being similar to DMI of the ADMP cows. Our hypothesis is that, in this study, AA supplementation increased DMI, which in turn increased milk yield. It is generally accepted that milk volume is regulated by lactose synthesis; as lactose concentration is relatively constant, increased lactose synthesis will increase milk yield (Schingoethe, 1996). On the other hand, milk protein synthesis is generally dependent on AA supply, unless supply exceeds requirements (Doepel et al., 2004).

Table 5. Effect of MP supply and rumen-protected (RP) AA supplementation on DMI, milk yield and composition, and BW in dairy cows

Item	Diet ^{1,2}				SEM	<i>P</i> -value
	ADMP	DMP	DMPLM	DMPLMH		
DMI, ^{3,4} kg/d	24.5	23.0	23.7	24.3	0.43	0.06
Milk yield, ³ kg/d	38.8 ^a	35.2 ^b	36.9 ^{ab}	38.5 ^a	0.74	<0.01
Milk ÷ DMI	1.59	1.56	1.57	1.59	0.032	0.89
Milk fat, %	3.50	3.51	3.32	3.30	0.117	0.44
Yield, ⁵ kg	1.34	1.20	1.21	1.23	0.045	0.10
3.5% FCM, kg	35.5 ^a	32.0 ^b	32.8 ^b	33.5 ^{ab}	0.86	0.04
3.5% FCM ÷ DMI	1.46	1.40	1.41	1.39	0.032	0.45
Milk true protein, %	2.98	2.94	2.99	3.03	0.030	0.23
Yield, kg/d	1.13 ^a	1.01 ^b	1.10 ^a	1.14 ^a	0.025	<0.01
Milk lactose, %	4.89	4.85	4.86	4.88	0.028	0.71
Yield, kg/d	1.87 ^a	1.67 ^b	1.78 ^{ab}	1.84 ^a	0.045	0.01
Milk NE _L , ⁶ Mcal/d	26.2 ^a	23.6 ^b	24.4 ^b	25.1 ^{ab}	0.61	0.03
MUN, mg/dL	13.0 ^a	10.3 ^{bc}	10.1 ^c	11.1 ^b	0.37	<0.01
BW, kg	599	591	602	597	5.0	0.43
BW change, kg	2.8	-4.8	9.1	1.5	6.16	0.46

^{a-c}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with RPLys (AminoShure-L; Balchem Corp., New Hampton, NY) and RPMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RPMet, and RPHis.

²Least squares means; DMI, $n = 2,258$; milk yield, $n = 2,106$; milk yield ÷ DMI, $n = 2,097$; BW, $n = 2,241$; BW change, $n = 48$; all others, $n = 168$ (n represents number of observations used in the statistical analysis).

³Averaged DMI and milk yield for the experimental period (53 d).

⁴ADMP versus DMP ($P = 0.01$); DMP versus DMPLMH ($P = 0.04$).

⁵ADMP versus DMP ($P = 0.03$); ADMP versus DMPLM ($P = 0.04$); ADMP versus DMPLMH ($P = 0.08$).

⁶Milk NE_L (Mcal/d) = kg of milk × (0.0929 × % fat + 0.0563 × % true protein + 0.0395 × % lactose) (NRC, 2001).

Milk protein concentration was not affected by RP AA supplementation in this trial. Therefore, our data suggest that milk yield was increased as a consequence of increased DMI. The increase in DMI was in spite of the apparent RDP deficiency (Oldham, 1984) with the DMP diets and indicates a potential physiological effect of AA on feed intake. In his discussion of DMI regulation, Allen (2000) concluded that little evidence exists that improved AA supply can affect DMI in dairy cows. Allen (2000) pointed out that AA deficiency has been shown to depress feed intake in nonruminant species (Harper et al., 1970), but that was unlikely in ruminants because AA composition of microbial protein synthesized in the rumen is relatively constant. Feed intake depression with protein- and AA-deficient diets is a well-known phenomenon in pigs and poultry (Henry, 1985; Picard et al., 1993) and some reports suggested short-term DMI depression in dairy cows as well (Weekes et al., 2006). In a literature review, Henry (1985) stated that feed intake and growth performance of pigs were depressed both by a severe deficiency in dietary limiting AA and by an excessive supply of total protein or some EAA. Growing pigs fed a 14% CP diet containing supplemental Lys (0.73% Lys) had feed intake and growth performance similar to pigs fed a 16% protein diet (0.77% Lys; Baker et al., 1975). Analogous

results were reported by Yen and Veum (1982) who observed feed intake and ADG with growing pigs fed a protein-deficient (13% CP) diet supplemented with Lys and Trp similar to pigs fed a 16% CP diet. Serotonin concentration in the posterior hypothalamus and dietary imbalance between Trp and less-limiting EAA have been suggested as contributing factors for the decreased feed intake in growing pigs (Henry et al., 1992). On the other hand, sufficient evidence exists that elevated blood plasma concentrations of AA that cannot be used for protein synthesis may depress feed intake in rats and monogastric farm species (Peng and Harper, 1970; Henry, 1985).

Two recent meta-analyses have examined the effect of RPLys and RPMet supplementation on feed intake (and production) in lactating dairy cows and generally reported no effect of these 2 AA on DMI (Patton, 2010; Robinson, 2010). In both analyses, the diets did not appear to be deficient in MP for the average milk production of the cows in the data sets. The assumption of a constant AA composition of rumen microbes (Allen, 2000) is not correct, as variability in bacteria and protozoa AA content has been reported (Czerkawski, 1976; Storm and Ørskov, 1983; Korhonen et al., 2002), perhaps partially due to different methodologies. Analysis of bacterial pellets isolated through differential cen-

Table 6. Effect of MP supply and rumen-protected (RP) AA supplementation on blood plasma AA composition ($\mu\text{mol/L}$) in dairy cows

Item	Diet ^{1,2}				SEM	P-value
	ADMP	DMP	DMPLM	DMPLMH		
Ala	249	253	262	277	12.7	0.40
Arg	76.3 ^a	59.9 ^b	64.6 ^b	66.2 ^b	2.72	<0.01
Asn	39.2	36.9	40.5	40.5	1.73	0.42
Asp	6.43	6.04	5.57	5.92	0.390	0.47
Gln	199	189	191	193	5.5	0.62
Glu ³	52.4	59.6	54.8	53.9	2.07	0.10
Gly	240 ^c	288 ^{ab}	318 ^a	274 ^{bc}	14.1	<0.01
His ⁴	48.4 ^a	25.9 ^b	25.8 ^b	41.1 ^a	2.70	<0.01
Ile	130 ^a	101 ^b	107 ^b	111 ^b	5.4	<0.01
Leu	144 ^a	111 ^b	112 ^b	120 ^b	5.1	<0.01
Lys	70.2 ^a	59.2 ^b	63.2 ^{ab}	67.9 ^a	2.69	0.03
Met	17.1 ^b	17.8 ^b	26.5 ^a	24.3 ^a	0.85	<0.01
Phe	46.3 ^a	42.3 ^b	40.0 ^b	41.6 ^b	1.39	0.02
Pro	81.6	71.4	79.4	77.3	3.15	0.14
Ser	74.0 ^c	83.6 ^{ab}	86.6 ^a	77.5 ^{bc}	3.06	0.01
Tau	36.7	33.8	37.1	39.2	2.95	0.61
Thr	85.4	85.2	93.7	99.5	5.79	0.25
Trp	39.6	37.7	37.6	35.4	1.99	0.50
Tyr	44.2	45.0	43.4	43.1	1.47	0.80
Val	249 ^a	191 ^b	198 ^b	208 ^b	8.7	<0.01
Carnosine ⁵	13.9	12.7	13.6	14.6	1.08	0.68
EAA ⁶	906 ^a	731 ^c	769 ^{bc}	814 ^b	27.5	<0.01
EAA – LMH ⁷	770 ^a	628 ^b	653 ^b	681 ^b	24.8	<0.01
Non-EAA	985	1,032	1,080	1,042	32.6	0.25

^{a-c}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹ADMP = diet adequate in MP; DMP = diet deficient in MP; DMPLM = DMP diet supplemented with RPLys (AminoShure-L; Balchem Corp., New Hampton, NY) and RPMet (Mepron; Evonik Industries AG, Hanau, Germany); DMPLMH = DMP diet supplemented with RPLys, RPMet, and RPHis.

²Least squares means; $n = 48$ (n represents number of observations used in the statistical analysis).

³ADMP versus DMP ($P = 0.02$); DMP versus DMPLMH ($P = 0.06$).

⁴ADMP versus DMPLMH ($P = 0.06$).

⁵Dipeptide (β -alanyl-L-histidine); anserine, another dipeptide (β -alanyl-N-methylhistidine), was not detected.

⁶Essential AA.

⁷EAA – (Lys + Met + His).

trifugations from whole rumen-content samples from previous experiments with cows fed diets similar to the diet fed in the current experiment showed about 27% lower concentration of His compared with Met (Table 7). Similarly, Schwab et al. (2005) and Huhtanen et al. (2002) reported about 25 to 30% lower His than Met concentration in bacterial protein and even lower His concentrations were reported by Czerkawski (1976; 50% lower than Met). The NRC (2001) protein model uses 2 studies compiling literature data (Storm and Ørskov, 1983; Clark et al., 1992) reporting Met:His ratios in ruminal bacteria of 1.30 and 1.14, respectively. In contrast, Patton et al. (2006) assumed a Met:His ratio in microbial protein of 0.96 (i.e., 1.68% Met and 1.75% His in microbial CP). According to NRC (2001), the concentration of both Met and His in milk protein EAA is 5.5%. Based on this, we assumed that MP should contain 2.2% His (similar to the recommended concentration of Met in MP; Schwab et al., 2005). Lapierre et al. (2008) suggested that His requirements should be

similar to Met requirements, but they also pointed out that the large variability in the recommended requirements for His (from 2.4 to 3.2% of MP) may be related to the potential contribution of carnosine, a dipeptide containing His, as a source of His. Based on these data, we concluded that diets deficient in RUP [i.e., when microbial protein is the most important (more so than with RUP-adequate diets) source of MP for the cow NRC, 2001], may not be supplying sufficient amounts of dHis due to the relatively low concentration of His compared with Met (and Lys) in microbial protein.

In the current study, increased postruminal supply of Lys, Met, and His (DMPLM and DMPLMH diets) tended to gradually increase DMI, which resulted in increased milk yield (either a trend or statistically significant). Milk protein yield was also increased, compared with the DMP diet, by both RPLys plus RPMet and by additional RPHis supplementation. The important role of His as a limiting AA in dairy cows fed grass silage-based diets has been demonstrated (Kim et al.,

Table 7. Crude protein concentration (% of DM) and individual AA composition (% of CP) of fluid-associated ruminal bacteria isolated from lactating Holstein dairy cows¹ (n = 14)

Item	Mean	SD
CP	40.5	3.70
AA		
Ala	5.72	0.208
Arg	3.40	0.304
Asn	8.77	0.332
Asp	0.80	0.061
Glu	9.10	0.497
Gly	4.23	0.208
His	1.01	0.291
Ile	4.24	0.231
Leu	5.75	0.242
Lys	3.86	0.213
Met	1.38	0.072
Phe	3.45	0.232
Pro	2.45	0.120
Ser	2.84	0.163
Tau	0.08	0.042
Thr	4.05	0.139
Trp	ND ²	ND
Tyr	0.13	0.020
Val	4.73	0.296
Hydroxylysine	0.10	0.018
Ornithine	0.09	0.024
Total	66.2	3.08

¹Bacterial samples isolated from whole ruminal content samples from 14 cows (Lee et al., 2011b; Tekippe et al., 2011).

²Not detected.

1999; Vanhatalo et al., 1999; Huhtanen et al., 2002). Earlier studies with corn grain and corn silage diets that had implicated Met as first limiting AA in dairy cows did not find any effect of intravenous infusion of His on milk production (Fisher, 1972). Later, Vanhatalo et al. (1999), Kim et al. (1999), and Rulquin and Pisulewski (2000) confirmed the status of His as the most important AA limiting milk production and milk protein synthesis in diets based on grass silage with low supplementation of protein feeds and low RUP content. Rulquin and Pisulewski (2000) proposed an optimum concentration of dietary His between 3.4 and 5.6% dHis in protein truly digested in the small intestine. Later studies by the Hannah group (Kim et al., 2000, 2001) were not conclusive regarding the role of His as first limiting AA, but in spite of some discrepancies, it can be assumed based on the existing literature that His is the first limiting AA in diets comprising grass silage providing relatively low RUP supply (Korhonen et al., 2000; Huhtanen et al., 2002). Attempts to investigate His as a first limiting AA in dairy cows fed corn silage- and alfalfa- (silage and hay) based diets had limited success (Doelman et al., 2008; Aines et al., 2010). In both of these experiments, however, dietary CP was relatively high (17.6 and 17.0%, respectively) and His was an unlikely candidate for limiting milk or milk protein production. Data from the current study con-

firmed that His is an important and potentially limiting AA in dairy cows under dietary conditions, in which RUP supply is limited and the role of microbial protein synthesized in the rumen as the primary source of MP is increased. An important difference between the current study and previous experiments investigating His supplementation is the duration of treatment. Most of the European data were derived from Latin square design post-ruminal infusion experiments and the studies of Doelman et al. (2008) and Aines et al. (2010) were also relatively short termed (7 and 28 d, respectively). Histidine deficiency may not become apparent in short-term trials. The carnosine mechanism (Lapierre et al., 2008; see earlier discussion) may be sufficient to maintain His supplies in short, Latin square, or cross-over experimental periods. In contrast to data from the current experiment and that of Lee et al. (2012b), plasma His concentrations were not different in cows fed ADMP and DMP diets in a Latin square design trial with 21-d experimental periods (37.4 and 40.6 $\mu\text{mol/L}$, respectively, $P = 0.89$; Lee et al., 2011b). In addition, urea recycling may have a significant effect on rumen function in a short-term study, but, to the best of our knowledge, its contribution to ruminal N supply in long-term experiments has not been investigated.

The increased plasma Lys and particularly Met concentration with DMPLM was expected and similar results have been reported for RPMet (Berthiaume et al., 2006; Preynat et al., 2009; Cabrita et al., 2011) and RPLys (Piepenbrink et al., 1996), although we did not find a significant increase in plasma Met or Lys with the same RPMet and RPLys products in our previous study (Lee et al., 2012b). Similar to Lee et al. (2012b), however, the DMP diet resulted in a significant decrease in plasma concentrations of several EAA, most notably His, Leu, Val, and Phe and total EAA (excluding supplemental Lys, Met, and His). The magnitude of reduction in His in this experiment (47%) was similar to the 43% reduction observed by Lee et al. (2012b). A similar reduction in plasma His, among other AA such as Arg, Ile, Leu, Lys, Val, and Phe, with low-protein diets have been reported by others (18 vs. 14% CP, corn silage- and alfalfa haylage-based diets, Piepenbrink et al., 1996; 12.5 vs. 17.5% CP, grass- and corn silage-based diets, Kröber et al., 2000). The response will depend on various factors, mainly diet composition and milk protein yield of the cows, and has not been consistently reported (e.g., Cabrita et al., 2011). As discussed by Lapierre et al. (2008), His may be unique among the EAA by having labile pools that provide a source of stored His during short periods of deficiency (i.e., intramuscular carnosine and circulating hemoglobin). Lapierre et al. (2008) estimated that the total body content of carnosine in a dairy cow would be

approximately 420 g of His, which could contribute substantially to His supply, especially in studies with short experimental periods. By the time plasma samples were collected in the current experiment (i.e., experimental wk 9 and 11), however, carnosine storage depots may have been depleted and carnosine concentration of composited plasma samples was not different among diets. Interestingly, the DMP diets, except DMPLMH, resulted in generally increased plasma concentration of the non-EAA, Gly, and Ser. This effect was not observed in our previous experiment (Lee et al., 2012b), but similar trends, at least for Gly, were reported for low-protein diets by others (Piepenbrink et al., 1996; Kim et al., 2000). The mechanism (or mechanisms) responsible for the increased plasma concentrations of these 2 AA with the DMP diets is not clear. Decreased oxidation of Met was associated with increased plasma Gly and Ser concentrations (Preynat et al., 2009) likely related to the combination of Ser to homocysteine in the Met oxidative pathway and such a decreased oxidation of Met may have occurred in the DMP diets. Alternatively, as both AA are involved in the synthesis of purine and pyrimidine bases (Wu, 2009), their increased concentration with the DMP diets may indicate decreased tissue nucleotide synthesis.

Dietary N input is the primary factor determining milk N efficiency (Huhtanen and Hristov, 2009) and cows fed the MP-deficient diets had enhanced milk N efficiency in the current study. The NRC (2001) protein model severely underpredicts milk response in cows fed MP-deficient diets (see Lee et al., 2012b). The current experiment was not an exception: milk yield for the DMP diets was underpredicted, on average, by 10.3 ± 0.75 kg/d (MP-allowable milk yield, 27.5 ± 1.03 kg/d; actual milk yield, 37.8 ± 1.36 kg/d). The dramatic reduction in urinary N and urea-N excretion with the DMP diets was expected based on our previous experience (Lee et al., 2011a, 2012b) and has been commonly reported in the literature for low-protein diets (Kebreab et al., 2002; Olmos Colmenero and Broderick, 2006; Cyriac et al., 2008). An apparent increase occurred in the relative proportion of fecal N compared with urinary N, decreasing the latter to less than 19% of the total excreta N. These trends are again commonly reported when dietary CP concentration is decreased (Lee et al., 2012b). The important implication of this shift in the route of N excretion is that manure ammonia emissions will also be expected to decrease due to the fact that urinary urea is the primary source of ammonium and consequently ammonia in cattle manure (Hristov et al., 2011a). The mitigating effect of decreasing dietary CP on ammonia emissions from manure has been clearly demonstrated by our group in experimental (Lee et al., 2012a) and field conditions (Hristov et al., 2012) and

by others (Külling et al., 2001; van der Stelt et al., 2008). Our previous work also indicated that decreasing urinary N excretion, through a reduction in dietary CP concentration, can potentially have a mitigating effect on nitrous oxide, a potent greenhouse gas, emitted from manure-amended soil (Lee et al., 2012a).

CONCLUSIONS

We observed a trend for increased DMI with RP AA supplementation, particularly when all 3 RP AA (Lys, Met, and His) were supplemented to a dairy cow diet predicted to be about 15% deficient in MP. All RP AA products had sufficient rumen protection to supply AA postruminally and intestinal digestibility to allow absorption, which was evident by the increased plasma Lys, Met, and His concentrations, compared with the unsupplemented diet. The increased DMI triggered milk and milk protein yield responses, particularly evident when all 3 RP AA were supplemented. As total-tract fiber digestibility was suppressed with all MP-deficient diets, we propose that, similar to monogastric species, AA play a role in DMI regulation in dairy cows. Analysis of rumen bacterial samples from unrelated experiments indicated about 27% lower His than Met concentration in bacterial protein. Microbial protein is an increasingly important source of AA for the cow when MP-deficient diets are fed and this report indicates that His may be a limiting AA in high-producing dairy cows fed corn silage- and alfalfa haylage-based diets deficient in MP. Our analysis indicates that the proportion of His in MP should be similar to that of Met, which, for practical purposes, is suggested to be 2.2%. The MP-deficient diets clearly increased milk N efficiency and decreased dramatically urinary N losses, which, based on previous observations, is expected to reduce manure ammonia and potentially nitrous oxide emissions. Thus, the combination of RP AA (Met, Lys, and His) has the potential to reduce these emissions while maintaining milk production and milk protein yield in dairy cows.

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