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Effect of increasing dietary protein with constant lysine:methionine ratio on production and omasal flow of nonammonia nitrogen in lactating dairy cows

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ABSTRACT

Eight lactating cows were fed 4 diets in which dietary crude protein (CP) was increased in steps of approximately 2 percentage units from 11 to 17% of DM by replacing high-moisture corn with soybean meal supplemented with rumen-protected Met to maintain a Lys:Met ratio of 3:1 in metabolizable protein. Trial design was a replicated 4 × 4 Latin square; experimental periods lasted 28 d, with data and sample collection being performed during wk 3 and 4 of each period. Digesta samples were collected from the rumen as well as the omasum to measure metabolite concentrations and ruminal outflow of N fractions using infusion of ^{15}N-enriched ammonia to quantify microbial nonammonia N (NAN) and nonmicrobial NAN. Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc.). There were linear increases in the yields of milk and true protein and concentration of milk urea N, and a linear decrease in N efficiency, with increasing dietary CP. Apparent ruminal and total-tract N digestibility increased linearly with increasing dietary CP, but estimated true total-tract N digestibility was not affected. Apparent digestibility of the other macronutrients was not influenced by diet. Ruminal ammonia, total AA and peptides, and branched-chain VFA also increased linearly with dietary CP. The ^{15}N enrichment of liquid- and particle-associated microbes linearly declined with increasing dietary CP due to decreasing ^{15}N enrichment of the ammonia pool. Although no effect of dietary CP on nonmicrobial NAN flow was detected, total NAN flow increased linearly from 525 g/d at 11% CP to 637 g/d at 17% CP due to the linear increase in microbial NAN flow from 406 g/d at 11% CP to 482 g/d at 17% CP. Under the conditions of this study, when dietary CP was increased by adding soybean meal supplemented with rumen-protected Met, improved milk and protein yields were driven not by RUP supply but by increased ruminal outflow of microbial protein.
Key words: dietary crude protein, lysine:methionine ratio, omasal flow of microbial protein

INTRODUCTION

Sustainable animal diets are needed to reduce N and P excretion, diminish methane emissions, and improve environmental sustainability of livestock production (Makkar and Ankers, 2014). Increasing N efficiency in dairy cows without loss of milk yield is best achieved by minimizing the total amount of CP fed to the animal while supplying adequate metabolizable AA (Broderick, 2003; Reynolds et al., 2018). Ruminants obtain their MP from microbial protein and RUP that flow to the abomasum; these sources must be digested and absorbed as free AA from the small intestine to be utilized by the animal (Burroughs et al., 1975). Methionine is known to be the essential AA most often limiting for dairy cows, particularly when soybean meal (**SBM**) is the principal supplemental protein in the diet (Noftsger et al., 2005; Chen et al., 2011). This is especially important where milk is sold for both volume and component value (Vasconcelos et al., 2006; Abbasi et al., 2018; Burke et al., 2018). Supplementation of SBM-based diets with rumen-protected Met (**RPM**) will improve Met:Lys ratio in MP (NRC, 2001) and consequently reduce catabolism of absorbed AA and decrease blood urea N; this may also be valuable for improving the reproductive performance of dairy animals (Rhoads et al., 2006). Thus, AA balance is one of the most significant factors for improving N utilization. Supplementation with rumen-protected limiting AA to raise MP quality may decrease N losses, feed costs,

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and environmental pollution without adverse effects on animal performance (Lee et al., 2012; Sinclair et al., 2014; Zanton et al., 2014).

Earlier, we observed that about 16% CP in typical lactation diets was adequate to support milk and true protein yield in dairy cows (Broderick, 2003; Olmos Colmenero and Broderick, 2006b), although Kalscheur et al. (1999) and Wu and Satter (2000) found that about 17% CP was needed to optimize protein yield in early lactation. Microbial CP contributes more than 60% of the NAN leaving the rumen in dairy cows (Olmos Colmenero and Broderick, 2006b; Giallongo et al., 2015, 2016). However, decreasing dietary CP could depress production by both reducing RUP supply and providing insufficient RDP to optimize microbial protein formation and microbial fiber digestion in the rumen (Reynal and Broderick, 2005; Olmos Colmenero and Broderick, 2006a). In a companion study to the current trial, we observed linear increases in yields of milk and milk components in response to increasing dietary CP, whereas yields of milk, true protein, and ECM were not increased above 13% CP in cows fed diets with increasing SBM CP supplemented with RPM (Nursoy et al., 2018). We hypothesized that these production responses were driven mainly by increased RUP supplied by SBM supplemented with RPM rather than by depressed ruminal microbial digestion and protein formation. Therefore, the objective of the present study was to quantify the effects of dietary CP content from SBM, supplemented with RPM to maintain a Lys:Met ratio of 3.0 (NRC, 2001), on ruminal outflows of microbial and dietary NAN to explain our earlier production findings and to possibly identify an optimal CP concentration to maximize microbial protein yield.

MATERIALS AND METHODS

Animals and Diets

Care and handling of the animals, including ruminal cannulation, was conducted as outlined in the guidelines of the University of Wisconsin institutional animal care and use committee. Eight multiparous Holstein cows fitted with permanent 10-cm ruminal cannulas (Bar Diamond Inc.) with mean (±SD) parity 2.6 ± 0.75, 623 ± 54 kg of BW, 91 ± 37 DIM, and 48 ± 5 kg of milk/d at the beginning of the trial were blocked by DIM into two 4 × 4 Latin squares with 4-wk periods. Cows were fed ad libitum a TMR formulated with rolled corn silage, alfalfa silage, ground high-moisture and dry corn, solvent-extracted SBM, soyhulls, minerals, and vitamins to contain 11, 13, 15, and 17% CP (DM basis). Diets were supplemented with rumen-protected DL-Met (Mepron, Evonik Corp.) formulated to provide 4.4, 7.8, 10.0, and 12.0 g/d of absorbed Met (assuming 85% chemical DL-Met content and 72% bioavailability; Lee et al., 2012) at 25 kg/d of DMI. Based on the NRC (2001) model, these amounts were estimated to provide a Lys:Met ratio of about 3:1 in the MP in all diets. Other details on diet preparation, including both the control and RPM premixes, were described by Nursoy et al. (2018). Compositions of the principal feed ingredients are given in Table 1, and compositions of the experimental diets actually fed during the trial (based on daily as-fed weights and weekly mean DM contents of each ingredient mixed into the TMR) are given in Table 2.

All cows were injected with recombinant bST (500 mg of Posilac; Elanco Animal Health) beginning about

Table 1. Composition of principal dietary ingredients[1]

	Corn silage		Alfalfa silage		HMSC[2]		Soybean meal	
Component	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
DM, % as fed	39.1	1.69	39.3	2.24	84.0	1.57	89.5	0.17
CP, % of DM	6.1	0.38	20.9	0.31	7.7	0.22	52.6	0.63
Ash, % of DM	4.2	0.09	9.8	0.28	1.4	0.06	7.5	0.16
OM, % of DM	95.8	0.09	90.2	0.28	98.6	0.06	92.5	0.16
NDF, % of DM	40.9	1.49	42.4	0.66	8.8	0.23	9.8	0.60
ADF, % of DM	20.5	0.68	31.8	0.73	1.8	0.06	5.2	0.31
NFC,[3] % of DM	49.7	1.35	29.4	0.77	83.1	0.42	31.7	0.64
NDIN, % of total N	14.2	0.70	11.6	0.71	13.3	0.84	3.0	0.16
ADIN, % of total N	1.6	0.16	4.0	0.17	0.0	0.25	0.0	0.02
Fraction B2,[4] % of total N	12.7	0.57	8.4	0.91	13.3	0.73	2.9	0.15
NPN, % of total N	49.5	4.7	52.5	8.0	—	—	—	—
Ammonia N, % of total N	1.9	0.2	4.4	0.8	—	—	—	—
pH	4.06	0.04	4.46	0.55	—	—	—	—

[1]Adapted from Nursoy et al. (2018).

[2]High-moisture shelled corn.

[3]Calculated as OM − CP − NDF + NDIN × 6.25.

[4]Calculated as NDIN − ADIN (Higgs et al., 2015).

60 DIM; injections were synchronized such that animals received a full dose on d 1 and at 14-d intervals throughout the trial. Cows were housed in a tiestall barn and had water ad libitum during the trial. Diets were offered once daily at 1000 h; orts were collected and weights were recorded the next day at 0900 h. Feeding rate was adjusted daily to yield orts of about 5 to 10% of intake. Weekly composites of corn silage, alfalfa silage, and high-moisture shelled corn, plus the 4 different TMR and orts, were obtained from daily subsamples of about 0.5 kg of each material and stored at −20°C. Weekly samples of SBM, soyhulls, and the 2 premixes were also collected and stored at room temperature.

Each 28-d experimental period consisted of 14 d for adaptation to the diet and 14 d for data and sample collection. Cows were milked twice daily at 0500 and 1700 h, and milk yield was recorded at each milking in all experimental periods. Milk samples from both p.m. and a.m. milkings were collected on d 17 to 18 and 24 to 25 of each period. At collection, all milk samples were preserved with 2-bromo-2-nitropropane-1,3-diol. Individual p.m. and a.m. milk samples were analyzed for fat, true protein, lactose, SNF, and MUN by infrared

Table 2. Ingredients and composition of the experimental diets[1]

	Dietary CP, % of DM			
Item	11	13	15	17
Ingredient, % of DM				
Rolled corn silage	50.5	50.5	50.5	50.5
Alfalfa silage	10.4	10.4	10.4	10.4
High-moisture shelled corn	26.0	21.5	17.1	12.8
Ground shelled corn	1.01	1.03	1.05	1.06
Solvent soybean meal	5.6	10.0	14.3	18.5
Soyhulls	4.0	4.0	4.0	4.0
Molasses	0.02	0.02	0.02	0.02
RPM product[2]	0.029	0.052	0.067	0.079
Calcium sulfate	1.37	1.38	1.38	1.38
Biophos[3]	0.22	0.22	0.22	0.22
Sodium chloride	0.18	0.18	0.18	0.18
Magnesium oxide/sulfate	0.51	0.51	0.51	0.51
Vitamins and trace minerals[4]	0.13	0.13	0.13	0.13
Composition				
CP, % of DM	10.9	12.9	14.8	16.8
Soybean meal CP, % of total CP	27	41	51	58
Ash, % of DM	4.2	4.4	4.7	4.9
NDF, % of DM	30.3	30.4	30.5	30.5
ADF, % of DM	16.1	16.3	16.4	16.6
NDIN, % of total N	11.2	9.7	8.6	7.7
ADIN, % of total N	1.6	1.3	1.1	1.0
Fraction B2,[5] % of total N	9.7	8.4	7.4	6.7
Ether extract, % of DM	3.2	3.1	3.0	2.9
NFC,[6] % of DM	51.4	49.2	47.0	44.9
Starch, % of DM	30.6	28.5	26.4	24.4
NE_L,[6] Mcal/kg of DM	1.50	1.52	1.53	1.55
NE_L-allowable milk,[6] kg/d	37	37	38	38
MP-allowable milk,[6] kg/d	18	25	30	31
MP,[6] g/d	1,792	2,087	2,294	2,380
Lys in MP,[6] g/d	115	140	156	161
Met in MP,[6] g/d	33	38	40	40
RPM (Mepron),[7] g/d	4.4	7.8	10.0	12.0
Total Met supply, g/d	37	46	50	52
Lys:Met ratio (without RPM)[6]	3.5	3.7	3.9	4.0
Lys:Met ratio (total)[7]	3.07	3.06	3.12	3.10

[1]Adapted from Nursoy et al. (2018).

[2]RPM = rumen-protected Met (Mepron; Degussa Corp.).

[3]IMC-Agrico.

[4]Provided (per kg of DM): 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6,440 IU of vitamin A, 2,000 IU of vitamin D, 16 IU of vitamin E, and 12 mg of monensin.

[5]Calculated as NDIN − ADIN (Higgs et al., 2015).

[6]Computed according to the NRC (2001) model.

[7]Computed assuming RPM product contains 60% bioavailable Met by weight (Lee et al., 2012) at DMI = 25 kg/d.

analysis (AgSource) with a Foss FT6000 (Foss North America Inc.) using AOAC International (2012). Milk composition data (concentrations and yields), weighted for the corresponding p.m. and a.m. milk yields, were computed for each test day, and these values were used to compute average period yields. Apparent N efficiency (assuming no retention or mobilization of body N) was also computed for each cow by dividing the period mean for milk N secretion (milk true protein/6.38) by mean N intake. For computation of BW change, BW of all cows was measured on 3 consecutive days just before beginning the experiment and at the end of each period.

Omasal Sampling and Analysis

Omasal sampling was performed during the last week of each period using the techniques developed by Huhtanen et al. (1997) and Ahvenjärvi et al. (2000), as adapted by Reynal and Broderick (2005), to quantify digesta flow from the rumen. Indigestible NDF (Huhtanen et al., 1994), $YbCl_3$ (Siddons et al., 1985), and co-EDTA (Udén et al., 1980), which are mainly associated with the large particle, small particle, and fluid phases of digesta, respectively, were used as flow markers at the omasal canal. Indigestible NDF was determined in large particles, small particles, and TMR but not in fluid phases (Ahvenjärvi et al., 2000), as follows. Samples (0.35 g) were weighed into duplicate 5 × 10-cm Dacron bags with 6-μm pore size (Sefar America Inc.), incubated in the rumens of 2 cows for 12 d, rinsed with water, and then subjected to the NDF analysis described below. The external microbial marker ^{15}N was used to quantify microbial NAN flow from the rumen. The triple marker technique (Armentano and Russell, 1985; France and Siddons, 1986) was used to determine the proportions with which to recombine the 3 phases to produce the omasal true digesta. Before marker infusion began, whole ruminal contents were taken from each cow to determine background ^{15}N abundance. The mean of the 32 observations for background ^{15}N abundance was 0.36815% of N. Cobalt-EDTA, $YbCl_3$, and $^{15}NH_4SO_4$ containing 10% atom excess ^{15}N (Isotec) were dissolved in distilled water and continuously infused into the rumen at rates of 2.3 g of Co, 3.1 g of Yb, and 70 mg of ^{15}N/d. Markers were continuously infused from d 20 at 0800 h to d 26 (2200 h) using a peristaltic pump (AutoAnalyzer II, Technicon Corp.). After 86 h of infusion, omasal samples were collected at twelve 2-h intervals over a 3-d period to represent the 24-h day. Sampling protocols, including confirming that sample tubes were correctly positioned in the omasal canal, sampling times and volumes, sample processing, isolation of liquid-associated bacteria (**LAB**) and particle-associated bacteria (**PAB**), digesta marker analyses, and preparation of omasal true digesta, were as described by Reynal and Broderick (2005) and Brito et al. (2007), except ammonia was not isolated for determination of ^{15}N enrichment. Samples of omasal true digesta were analyzed for total N, DM, ash, OM, NDF, ADF, NDIN, and ADIN as detailed below for feed samples. Samples of omasal true digesta and isolated bacteria were treated with K_2CO_3 (Brito et al., 2007) to remove residual ammonia and analyzed for total N (equivalent to NAN) and for ^{15}N abundance using a Costech 4010 elemental analyzer (Costech Analytical Technologies Inc.) interfaced to a Thermo-Finnigan Delta-Plus Advantage isotope ratio mass spectrometer (Thermo-Electron GmbH). Equations used to compute flows of nutrients of dietary and microbial origin and extents of ruminal digestion were detailed by Brito et al. (2007) and Robles Jimenez et al. (2020).

Fecal and Ruminal Sampling

On d 28 of each period, spot fecal samples were collected 6 h before and 6 h after feeding and stored at −20°C until analyzed. Fecal samples were dried for 72 h at 60°C, ground through a 1-mm screen (Wiley mill, Arthur H. Thomas), and composited on an equal DM basis to obtain 1 fecal sample/cow per period. On d 27 to 28 of each period, about 100 to 200 mL of fluid digesta was collected from 4 locations in the ventral rumen at 0 (just before feeding), 1, 2, 4, 6, 8, 12, 18, and 24 h after feeding through the ruminal cannulas using the probe described by Olmos Colmenero and Broderick (2006b). At each sampling, mixed fluid digesta was strained through 2 layers of cheesecloth and pH was measured immediately in strained fluid using a glass electrode. Two 10-mL aliquots of ruminal fluid were then preserved in scintillation vials by addition of 0.2 mL of 50% H_2SO_4 and stored at −20°C. The remaining fluid and digesta were returned to the rumen. Just before analysis, samples were thawed and centrifuged (15,300 × *g* for 20 min at 4°C), and flow-injection analyses (Lachat QuikChem 8000) were applied to supernatants to determine ammonia using a phenol-hypochlorite method (Broderick et al., 2004). Supernatants were also analyzed using the *o*-phthaldialdehyde reaction (Roth, 1971) for total AA by fluorescence and for total AA plus oligopeptide concentrations by absorbance (Colombini et al., 2011). Leucine was the standard in both *o*-phthaldialdehyde assays, and total AA and total AA plus oligopeptides are reported as Leu equivalents. The other ruminal samples were thawed and centrifuged (28,000 × *g* for 30 min at 4°C) before VFA determination using the GLC method described by Broderick et al. (2015). This method did

not resolve isovalerate and 2-methyl butyrate. Time-weighted means were computed for all ruminal traits (pH and concentrations of ammonia, free AA, free AA plus peptides, and individual and total VFA) using the following equation:

$$\begin{aligned}\text{Time-weighted mean} = [&\text{avg}(0\text{–}1\text{ h}) + \text{avg}(1\text{–}2\text{ h}) \\ &+ 2 \times \text{avg}(2\text{–}4\text{ h}) + 2 \times \text{avg}(4\text{–}6\text{ h}) + 2 \\ &\times \text{avg}(6\text{–}8\text{ h}) + 4 \times \text{avg}(8\text{–}12\text{ h}) + 6 \\ &\times \text{avg}(12\text{–}18\text{ h}) + 6 \times \text{avg}(18\text{–}24\text{ h})]/24,\end{aligned}$$

where avg(0–2 h) . . . avg(18–24 h) represent the mean value for each ruminal trait observed at each of the sampling times listed.

Laboratory Analyses

Dry matter contents of samples of weekly composites and individual feed samples were determined by drying at 60°C (forced-air oven) for 48 h. The 60°C DM contents of dietary ingredients were used weekly to adjust as-fed compositions of the TMR. Dry matter intake was computed based on the 60°C DM contents of the TMR and orts. These samples were ground to pass a 1-mm Wiley mill screen (Arthur H. Thomas) and analyzed later for total N (Leco FP-2000 Nitrogen Analyzer, Leco Instruments Inc.), absolute DM, ash, and OM (AOAC, 1980); sequentially for NDF, ADF, and ADIN using heat-stable α-amylase and Na_2SO_3 (Van Soest et al., 1991; Hintz et al., 1996); for indigestible ADF (ADF remaining after a 12-d in situ incubation; Huhtanen et al., 1994), NDIN, and ADIN (N fraction in NDF and ADF residue, respectively); and for NDIN without use of Na_2SO_3 (Licitra et al., 1996). These assays were also applied to samples of omasal true digesta to determine total N, DM, ash, OM, NDF, ADF, NDIN, and ADIN. The TMR composites were analyzed for total fat (method 920.39, AOAC International, 1997; Dairyland Laboratories, Arcadia, WI) and starch (Hall et al., 1999; T. K. M. Webster, West Virginia University, Morgantown). Dietary content and intake of MP, Lys and Met in MP, and Lys:Met ratio were computed according to the NRC (2001) model. Fecal composites were analyzed for DM, ash, OM, NDF, ADF, total N, and indigestible ADF using the assays described above for feeds. Indigestible ADF was used as an internal marker to estimate apparent nutrient digestibility and fecal N output (Cochran et al., 1986). Metabolic fecal N, estimated as 4.8 g/kg of DMI (NRC, 2001), and fecal N output were used to compute true N digestibility as described earlier (Broderick, 2018).

Statistical Analysis

Data were analyzed as a replicated 4 × 4 Latin square using the mixed procedures of SAS (SAS Institute Inc., 2013). Although repeated measures analysis allows assessing correlation between observations and can reduce the error variance, according to Gliner et al. (2002), this approach is not recommended in situations in which a lasting treatment effect is anticipated. Thus, single mean observations were computed for each production trait for each cow over the last 2 wk of each period, as was done in the earlier published parallel study (Nursoy et al., 2018). Single means (Gliner et al., 2002) also were computed for each cow for results on apparent total-tract digestibility, omasal flows, apparent ruminal digestibility, and time-weighted mean ruminal metabolite concentrations. Model sums of squares from the data were separated into overall mean, cow (within square), square, period, and overall error. All variables were considered fixed except cow (within square) and overall error, which were considered random. Orthogonal contrasts were used to test for linear, quadratic, and cubic effects of dietary CP concentration. No significant cubic effects were detected. Least squares means are reported for all statistical analyses; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Feed Quality and Diet Composition

The chemical composition of the ingredients used in the TMR is given in Table 1. Concentrations of DM, CP, NDF, and ADF in alfalfa and corn silages and high-moisture corn indicated that these feedstuffs were of typical composition (NRC, 2001). The NPN in corn silage and alfalfa silage represented 49.5 and 52.5% of total N, respectively; NPN normally accounts for 45 to 55% of total N in alfalfa silage (Broderick, 1995). Very low concentrations of ammonia N and low pH in both alfalfa and corn silages indicated that these feeds were well preserved (McDonald et al., 1991). Concentrations of ADIN in the 2 silages also were low (1.6 and 4.0% of total N for alfalfa and corn silages, respectively), and, on average, no ADIN was detected in the high-moisture corn and SBM, indicating essentially no heat damage to these major ingredients fed in this study.

Composition of the 4 diets fed in the trial is given in Table 2. Soybean meal, which was added at the expense of high-moisture shelled corn, increased from about 5.6 to 18.5% of dietary DM. Actual CP concentrations ranged from 10.9 to 16.8% (DM basis),

Table 3. Production of ruminally cannulated lactating cows fed diets with increasing CP content but with a constant estimated Lys:Met ratio of 3:1

	Dietary CP, % of DM					Probability[2]		
Item	11	13	15	17	SEM[1]	Lin	Quad	Cubic
DMI, kg/d	23.7	24.0	24.3	24.7	1.34	0.29	0.95	0.97
N intake, g/d	414	495	576	653	30.0	<0.01	0.90	0.94
BW change, kg/d	0.16	0.65	0.47	0.14	0.20	0.76	0.06	0.54
Milk, kg/d	36.6	38.8	39.9	39.2	2.53	0.01	0.06	0.79
Milk/DMI	1.56	1.62	1.66	1.59	0.090	0.52	0.16	0.73
ECM, kg/d	35.0	38.5	35.6	35.7	2.58	0.89	0.23	0.14
ECM/DMI	1.48	1.56	1.48	1.44	0.073	0.45	0.35	0.49
Fat, %	4.01	4.20	3.60	3.55	0.393	0.10	0.65	0.28
Fat, kg/d	1.42	1.59	1.36	1.39	0.136	0.44	0.45	0.14
True protein, %	3.06	3.11	3.21	3.15	0.097	0.06	0.16	0.22
True protein, kg/d	1.10	1.18	1.23	1.20	0.066	0.01	0.04	0.70
Lactose, %	4.75	4.74	4.66	4.67	0.110	0.25	0.82	0.53
Lactose, kg/d	1.75	1.83	1.82	1.81	0.139	0.43	0.29	0.67
SNF, %	8.75	8.77	8.80	8.76	0.150	0.82	0.44	0.67
SNF, kg/d	3.18	3.37	3.42	3.36	0.224	0.08	0.10	0.90
MUN, mg/dL	5.3	7.6	9.1	13.5	1.00	<0.01	0.18	0.27
Milk N/N intake, %	42.1	37.5	33.8	28.8	1.49	<0.01	0.87	0.67

[1]Standard error of the LSM.

[2]Probability of significant effects of linear (Lin), quadratic (Quad), and cubic effects of dietary CP concentration.

which were slightly below the target values of 11.0 to 17.0% CP. These dietary alterations had little effect on NDF, ADF, and ADIN content; however, NDIN and N fraction B2 (as a proportion of total CP), as well as starch and NFC (as a proportion of DM), declined with increasing SBM inclusion. Compared with the diets of 11 and 13% CP, estimated NE_L-allowable milk (kg/d) computed using NRC (2001) increased by 1 kg/d with increased dietary CP for the 15 and 17% CP diets because NRC (2001) assigns greater NE_L density to SBM than to high-moisture corn. Without RPM supplementation (Table 2), Lys:Met ratios ranged from 3.5 to 4.0: thus, RPM supplementation was necessary to obtain appropriate Lys:Met ratios in all 4 diets (NRC, 2001). Assuming that bioavailability of chemical Met in the RPM source was 72% (Lee et al., 2012), RPM supplementation improved Lys:Met ratio to approximately 3:1 in all diets, slightly greater than the target value of 3:0.

Production Performance of Dairy Cows

Lactation performance results are given in Table 3. As was observed in our earlier study (Nursoy et al., 2018), yields of milk and true protein were linearly increased by dietary CP, and there was a quadratic response in milk true protein yield. Moreover, quadratic trends were detected for milk and SNF yields and milk true protein content to linearly increase and for milk fat content to linearly decrease in dietary CP. Milk yields observed in this trial were substantially greater than the MP-allowable milk yields predicted by the NRC (2001) model (Table 2) on all of the dietary treatments except 11% CP. The quadratic trend for BW gain should perhaps be discounted because the current study was done as a small, short-term reversal trial. Intake, milk/DMI, ECM/DMI, milk content of lactose and SNF, and lactose yield all were apparently not influenced by dietary CP content.

Concentration of MUN increased linearly and, conversely, milk N/N intake declined linearly, in response to increasing N intake and CP concentration of the diet (Table 3). Milk urea N typically increases and N efficiency decreases as CP intake increases in dairy cows (Broderick and Clayton, 1997; Nousiainen et al., 2004). Overall, production responses in the present trial closely coincided with those reported by Nursoy et al. (2018) in the parallel study, which was conducted using 36 dairy cows (versus 8 cows in the current trial) that showed lower milk yield (35.3 kg/d) on 11% CP but similar yield on the other 3 CP levels (average = 39.4 kg/d). However, Nursoy et al. (2018) also detected differences in DMI, milk/DMI, ECM, and ECM/DMI, all of which were lower on 11% CP versus the 13 to 17% dietary CP levels. These differences likely were due to fewer animals being used in the current study: the standard error of the mean for milk and true protein yields were, respectively, 1.03 and 0.035 in the earlier trial of Nursoy et al. (2018) versus 2.53 and 0.066 in the current study.

Regarding the quadratic response in true protein yield to increasing dietary CP, true protein yield was numeri-

cally greatest at 15% dietary CP; mean true protein yield averaged 1.20 kg/d on 13 to 17% CP versus 1.10 kg/d on 11% CP (Table 3). However, in the parallel trial, Nursoy et al. (2018) found that true protein yield was lower on 13% versus 17% dietary CP, with yield at 15% CP being intermediate. Olmos Colmenero and Broderick (2006b) detected quadratic responses in milk yield and milk true protein at mathematic maxima of, respectively, 16.7 and 17.1% CP with increasing dietary SBM, but with greater dietary proportions of alfalfa silage, lower proportions of corn silage, and notably without RPM supplementation. Lapierre et al. (2016) considered that the response of milk protein yield to MP and metabolizable AA is best described as quadratic rather than by a "broken-stick" model (NRC, 2001). As in the current study, Olmos Colmenero and Broderick (2006b) found a highly significant linear decline in milk N/N intake with increasing dietary CP.

By design, N intake increased linearly with elevated dietary CP concentration, but, as mentioned, there was no effect on DMI. Feed intake is a major driver of milk and component yield by providing energy for the animal as well as for stimulating microbial digestion and protein formation in the rumen. Previously, we found that feed intake was decreased at lower dietary CP content (Broderick, 2003). However, several reports have shown that DMI was unaffected by dietary CP concentration (Kalscheur et al., 1999; Olmos Colmenero and Broderick, 2006b; Lee et al., 2012). Adequate feed intake would be necessary to maintain milk and component yield to take advantage of the improved N utilization efficiency found on lower CP diets. Calsamiglia et al. (2010) summarized US data and found that the difference between the lowest and highest quartiles of FCM production differed by more than 3 kg of DMI/d. Lapierre et al. (2016) integrated energy and protein intake by describing MP and metabolizable AA requirements per unit of NE_L consumed. In a large meta-analysis of literature data from both North Europe and North America, Huhtanen and Hristov (2009) showed that dietary CP concentration was the most important factor influencing milk N/N intakes in dairy cows. However, adequate dietary CP is required to maintain functions other than milk production alone. Reynolds et al. (2018) summarized results collected over 3 lactations showing that milk and component yields were similar at 16% versus 18% dietary CP but that the greater milk N/N intakes at 14% dietary CP came at the expense of reduced reproductive efficiency and reduced longevity.

Ruminal and Total-Tract Digestibility

Apparent ruminal and total-tract digestibilities observed in the trial are given in Table 4. Intake of DM during omasal sampling averaged 24.0 kg/d versus 24.2 kg/d during the remainder of the trial, indicating that feed intake was not affected by the omasal sampling process ($P = 0.75$). Although ruminal NDF digestion was similar across diets, a quadratic effect of dietary CP content on apparent ruminal DM digestibility, and trends for quadratic effects on apparent ruminal digestibility of OM and ADF, were detected; it is unclear why these occurred. Apparent total-tract digestibility of NDF and ADF were not influenced by dietary CP; these results indicated that ruminal NDF and ADF digestibility accounted for, respectively, about 95 and 98% of total-tract digestibility. In the companion trial, Nursoy et al. (2018) observed that total-tract NDF digestibility was lower at 11% CP than 13% dietary CP. Although total-tract fiber digestibility observed in the current study appeared to be relatively low, Lee et al. (2012) found total-tract NDF and ADF digestibilities ranging from, respectively, 36 to 42% and 33 to 39%. Chen et al. (2011) reported a mean NDF digestibility of 50% for cows consuming a 60% forage diet, but with a 35:25 DM ratio of corn silage to alfalfa silage, and Faciola and Broderick (2014) observed NDF and ADF digestibilities of 44 and 49% when cows were fed the control diet (i.e., with no added lipid) with a 10:50 ratio of corn silage to alfalfa silage DM. The DM ratio of corn silage to alfalfa silage was 50.5:10.4 in the diets fed in the current trial (Table 2). Lopes et al. (2015) found that total-tract NDF digestibility increased from 38% on a diet with 56% corn silage DM to 44% on a diet with 55% alfalfa silage DM; in vivo rates of ruminal digestion of digestible NDF were 3.4%/h (56% corn silage DM) and 7.1%/h (55% alfalfa silage DM). Potts et al. (2017) found that the NDF digestibilities reported in the *Journal of Dairy Science* have declined 0.17 percentage unit/yr from 1970 to 2014, an effect these authors attributed to increasing DMI elevating passage rates; mean total-tract NDF digestibility in 2014 was 44.1%. Previously, we observed quadratic effects of dietary CP on total-tract NDF and ADF digestibility (Olmos Colmenero and Broderick, 2006b).

Apparent ruminal N digestibility was negative in all cases but, as expected, showed a linear response of becoming less negative as dietary CP increased. In ruminants, apparent extent of CP digestion in the rumen, expressed on the basis of dietary CP intake, becomes less negative with increasing dietary CP content because blood urea concentrations are elevated, resulting in increasing amounts of urea N being recycled to the rumen (Egan, 1965). As mentioned, MUN increased linearly with dietary CP in the current study (Table 3), and because blood urea parallels MUN (Broderick and Clayton, 1997; Marini and Van Amburgh, 2003), blood urea was presumed to be elevated. Santos et al. (1984)

found that CP flow at the duodenum was less than CP intake on 4 different protein sources, with diets containing greater proportions of RUP being more negative. When feeding diets containing 13.5 to 19.4% CP, Olmos Colmenero and Broderick (2006a) observed apparent negative CP ruminal digestibilities on all diets except that containing 19.4% CP, on which apparent CP disappearing from the rumen was approximately equal to CP consumed. A quadratic effect of CP on apparent ruminal N digestibility was also detected. As anticipated, apparent total-tract N digestibility increased linearly with increasing CP content of the diet (Table 4). This typically occurs because of dilution of metabolic fecal N with elevated dietary CP (Nousiainen et al., 2009). True total-tract N digestibility was computed assuming that metabolic fecal N equaled 4.8 g/kg of DMI (NRC, 2001); true total-tract N digestibilities estimated in this way averaged 86% and were not different among diets.

Ruminal N Metabolism

Effects of increasing dietary CP on ruminal pH and metabolite concentrations are given in Table 5. There was a quadratic effect of dietary CP on ruminal pH, with lower pH values observed at intermediate dietary CP concentrations. This followed the trend for a quadratic effect of increasing CP content on total VFA, with concentrations being elevated at intermediate CP levels. Other major effects observed were linear responses to dietary CP of ruminal concentrations of all metabolites deriving from RDP: ammonia N, total AA, total AA plus peptides, and molar proportions of isovalerate + 2-methylbutyrate and total branched-chain VFA. The ammonia N concentrations in the 4 diets were within the typical range of 1.3 to 28.9 mg/100 mL reported in lactating dairy cows (Kang-Meznarich and Broderick, 1980; Broderick et al., 1981). Branched-chain VFA are formed from microbial catabolism of the branched-chain AA in RDP (El-Shazly, 1952). Increasing ruminal concentrations of these compounds with increasing dietary CP and their linear accumulation indicate that ruminal protein degradation provided an adequate and improving supply of ammonia N, and branched-chain VFA was available for microbial protein synthesis.

Enrichments of ruminal microbial NAN and nutrient outflows from the rumen are reported in Table 6. The ^{15}N enrichments of both LAB and PAB were highest for the 11% CP diet, declining linearly as CP was increased. Mean ^{15}N enrichments (atom % excess) were 0.040 in LAB and 0.037 in PAB in this trial. Using ^{15}N ammonia to label ruminal microbes and techniques for omasal sampling and isolation of bacteria similar to those used in the present study, previous ^{15}N enrichments observed for LAB and PAB averaged, respectively, 0.042 and 0.039 atom % excess (Brito et al., 2007) and 0.041 and 0.036 atom % excess (Reynal et al., 2005). That the

Table 4. Intake and apparent ruminal and total-tract digestibility in ruminally cannulated lactating cows fed diets with increasing CP content but with constant estimated Lys:Met ratio of 3:1

	Dietary CP, % of DM					Probability[2]		
Item	11	13	15	17	SEM[1]	Lin	Quad	Cubic
DMI, kg/d								
Wk 3–4 mean	23.7	24.0	24.3	24.7	1.34	0.29	0.95	0.97
During omasal sampling	23.0	24.0	24.6	24.2	1.28	0.21	0.18	0.91
Apparent ruminal digestibilty,[3] %								
DM	26.6	28.9	31.5	25.3	2.18	0.84	0.02	0.25
OM	44.6	46.5	48.0	44.0	1.81	0.99	0.07	0.46
NDF	39.4	41.5	41.5	38.3	2.34	0.70	0.19	0.90
ADF	39.1	43.9	43.6	39.5	2.57	0.92	0.06	0.90
N	−67.0	−38.7	−13.8	−23.2	5.91	<0.01	<0.01	0.21
Apparent total-tract digestibility,[4] %								
DM	67.4	69.2	68.8	68.1	1.14	0.74	0.25	0.69
OM	69.0	70.8	70.6	69.8	1.07	0.56	0.16	0.71
NDF	40.6	44.1	43.3	41.8	1.98	0.71	0.12	0.61
ADF	39.7	43.7	43.6	41.9	2.35	0.44	0.13	0.75
N	56.9	64.8	65.5	69.3	1.99	<0.01	0.30	0.23
Estimated true total-tract digestibility,[5] %								
N	84.4	88.0	85.8	87.5	2.00	0.42	0.62	0.26

[1]Standard error of the LSM.

[2]Probability of significant effects of linear (Lin), quadratic (Quad), and cubic effects of dietary CP concentration.

[3]Estimated with omasal sampling using triple marker techniques.

[4]Estimated with spot fecal sampling using indigestible ADF as internal marker.

[5]Estimated with spot fecal sampling using indigestible ADF as internal marker and assuming 4.8 g of metabolic fecal N excreted/kg of DMI (NRC, 2001).

Table 5. Ruminal pH and metabolite concentrations in ruminally cannulated lactating cows fed diets with increasing CP content but with constant estimated Lys:Met ratio of 3:1[1]

	Dietary CP, % of DM					Probability[3]		
Item	11	13	15	17	SEM[2]	Lin	Quad	Cubic
pH	6.33	6.13	6.13	6.25	0.076	0.12	0.01	0.80
Ammonia N, mg/dL	3.9	5.9	8.0	12.2	0.87	<0.01	0.16	0.55
Total AA, m*M*	1.6	2.1	1.9	2.0	0.15	0.02	0.42	0.24
Total AA + peptides,[4] m*M*	3.1	3.9	3.8	3.8	0.23	0.01	0.28	0.30
Total VFA, m*M*	85.5	91.8	93.3	90.2	3.07	0.22	0.10	0.97
Molar proportions, mol/100 mol								
Acetate	58.8	58.4	59.2	59.7	2.15	0.34	0.61	0.74
Propionate	25.8	26.1	25.3	23.6	2.36	0.21	0.48	0.98
Butyrate	12.3	12.6	12.5	13.1	0.43	0.27	0.78	0.87
Isobutyrate	0.89	0.94	0.89	0.99	0.045	0.26	0.65	0.57
Isovalerate + 2-methyl butyrate	2.23	1.98	2.13	2.62	0.114	0.02	0.01	0.24
BCVFA[5]	3.13	2.93	3.03	3.61	0.121	0.02	0.01	0.87
Valerate	2.20	2.47	2.52	2.29	0.685	0.79	0.43	0.69

[1]Adapted from Nursoy et al. (2018).

[2]Standard error of the LSM.

[3]Probability of significant effects of linear (Lin), quadratic (Quad), and cubic effects of dietary CP concentration.

[4]Total AA plus small oligopeptides determined using *o*-phthaldialdehyde (OPA) absorbance assay (see Fecal and Ruminal Sampling section in text for details).

[5]Branched-chain VFA (isobutyrate + isovalerate + 2-methyl butyrate).

greatest ^{15}N enrichment of microbes was observed with the lowest CP level is not surprising because of the linear increase in ruminal ammonia concentration with dietary CP (Table 5), and ^{15}N enrichment of the ammonia pool likely will decline substantially with the increased ammonia concentrations formed at greatest protein degradation due to increased dietary CP input. Differences in chemical composition of the LAB and PAB microbial fractions have been reported previously (Craig et al., 1987; Martin et al., 1994). Lower enrichment of PAB compared with LAB probably results from greater direct utilization of amino N by organisms attached to particulate matter. Differences in ^{15}N enrichments between LAB and PAB are why sampling both of these bacterial pools is necessary to more accurately quantify bacterial protein flows.

Table 6. Microbial ^{15}N enrichment and ruminal outflows measured using omasal sampling in ruminally cannulated lactating cows fed diets with increasing CP content but with constant estimated Lys:Met ratio of 3:1

	Dietary CP, % of DM					Probability[3]		
Item[1]	11	13	15	17	SEM[2]	Lin	Quad	Cubic
LAB ^{15}N,[4] A%E	0.052	0.038	0.038	0.033	0.0027	<0.01	0.09	0.08
PAB ^{15}N,[4] A%E	0.044	0.038	0.036	0.030	0.0026	<0.01	0.83	0.38
DM flow, kg/d	16.5	17.1	16.7	18.0	0.89	0.02	0.28	0.11
OM flow, kg/d	11.7	12.1	11.9	12.5	0.61	0.08	0.56	0.23
Microbial OM flow, kg/d	5.14	5.25	5.31	5.68	0.476	0.12	0.59	0.73
Nonmicrobial OM flow, kg/d	6.61	6.83	6.57	6.84	0.325	0.64	0.91	0.28
Microbial NAN flow, g/d	406	437	451	482	39.7	0.02	0.99	0.72
Nonmicrobial NAN flow, g/d	120	180	147	155	23.5	0.50	0.28	0.21
Total NAN flow, g/d	525	617	597	637	40.0	<0.01	0.25	0.10
RUP, % of CP intake	32.8	39.1	27.4	27.1	5.57	0.26	0.57	0.25
RDP, % of CP intake	67.2	60.9	72.7	72.9	5.57	0.26	0.57	0.25
OMTDR, kg/d	15.0	15.8	16.4	15.7	1.11	0.31	0.20	0.62
OMTDR, %	68.5	69.5	71.2	69.1	1.81	0.49	0.19	0.42
Microbial efficiency, g of NAN/kg of OMTDR	27.9	27.6	27.8	31.0	1.67	0.14	0.24	0.70

[1]LAB = liquid-associated bacteria; A%E = atom percent excess of ^{15}N; PAB = particle-associated bacteria; OMTDR = OM truly digested in the rumen.

[2]Standard error of the LSM.

[3]Probability of significant effects of linear (Lin), quadratic (Quad), and cubic effects of dietary CP concentration.

[4]Microbial ^{15}N enrichment of LAB and PAB.

Ruminal outflow of DM increased linearly with dietary CP, whereas ruminal outflow of OM (i.e., excluding mineral matter) paralleled DM flow and numeric differences in DMI but only tended to increase linearly with dietary CP content. No effects were detected for microbial and nonmicrobial OM flow from the rumen, but there was a linear increase in total NAN flow from 525 g/d at 11% dietary CP to 637 g/d at 17% dietary CP. This increase was driven by the linear increase in microbial NAN flow occurring with increasing dietary CP content, which was likely stimulated by increased supply of protein degradation products with elevated CP (Table 5).

No differences among treatments were detected in the quantity of nonmicrobial NAN flow and RUP and RDP as proportions of CP intake (Table 6). That nonmicrobial NAN flows, from which RUP is computed, were not different when SBM CP increased from 27 to 58% of total dietary CP was surprising. However, nonmicrobial NAN measurements in this trial were quite variable; for example, estimated nonmicrobial NAN flow declined from 180 to 147 g/d between diets containing 13 and 15% CP and 41 and 51% SBM CP in total dietary CP. The fact that ruminal concentrations of all metabolites deriving from RDP increased linearly with increasing dietary CP content (Table 5) indicated that there were no inadvertent mix-ups of dietary treatments in this study. These results also suggest that increased RUP flow did not explain production responses in the current study and those reported earlier (Nursoy et al., 2018). Contrary to these findings, several trials have shown that NAN flow to the small intestine increased with elevated dietary CP due to greater ruminal escape of dietary protein, with microbial NAN flow being largely unaffected (Cunningham et al., 1996; Korhonen et al., 2002; Reynal and Broderick, 2003).

Synthesis of microbial protein is an energy-dependent process (Hoover and Stokes, 1991). However, in the current study, no effect of increasing RDP supply on microbial efficiency was detected, possibly because of a constant supply of energy availability for microbial fermentation. Although high-moisture corn was reduced from 26 to 13% of dietary DM as CP increased from 11 to 17% (Table 2), the amount and dietary content of OM truly digested in the rumen (i.e., ruminally digested OM corrected for proportion of microbial OM) was not different among diets (Table 6). Similar to the present study, Christensen et al. (1993), Cunningham et al. (1996), Korhonen et al. (2002), and Reynal and Broderick (2003) all reported no increase in microbial efficiency with increasing the CP content of the diet.

The linear improvements in milk and true protein yield in the current trial, and in yields of milk and milk components in our earlier study (Nursoy et al., 2018), appeared to have resulted from the linear increase in total NAN flow deriving from the linear increase in microbial NAN flow from the rumen (Table 6). Expressed as a percentage of CP intake, RUP and RDP were similar among diets, with RDP averaging 68% and RUP averaging 32% across diets. A portion of the RUP estimated in this way will derive from endogenous NAN flow. The NRC (2001) estimate of endogenous NAN flow at the duodenum is 1.9 g/kg of DMI; however, Ørskov et al. (1986) found that endogenous NAN flow at the rumen was only 43% of that at the abomasum. Estimating endogenous NAN flow in omasal contents as 0.43 × 1.9 g/kg of DMI = 0.82 g/kg of DMI, endogenous NAN flows in the present trial would have averaged 20 g/d and not different among diets because DMI was similar. That greater RUP flows were not detected also likely occurred because SBM CP, which increased from 27 to 58% of total dietary CP, is a highly degradable protein source (NRC, 2001). Korhonen et al. (2002) and Reynal and Broderick (2003) reported that, when feeding SBM as the main protein supplement, flows of microbial NAN and dietary NAN were, respectively, 77 and 23% of total NAN for 11% dietary CP and 75 and 25% of total NAN for 17% dietary CP. On the contrary, Cunningham et al. (1996) reported that the proportion of microbial NAN in total NAN flow fell from 73 to 65 and 53% when SBM content of the diet increased from 9.9 to 11.0 and 14.6% of dietary DM, respectively. Stern et al. (1983) observed that the proportion of bacterial NAN in total NAN flow also declined from 54 to 42% when dietary CP content was increased from 13.1 to 22.9% by increasing corn gluten meal from 3.5 to 38.0% of dietary DM.

Our findings that milk and component yield responded to microbial protein flow rather than RUP supply led us to reject the hypothesis that the linear improvements in milk and true protein production observed in both of our trials resulted from increased RUP supply. This result emphasizes the need for optimizing microbial protein synthesis in the rumen of lactating dairy cows. It is noted that RPM also increased with the increase in dietary CP in the present study (Table 2). However, microbial CP also has greater Met content than SBM CP (NRC, 2001), and these production responses may be attributed to the increased MP supply from microbial NAN with its already adequate Met content.

CONCLUSIONS

Four levels of dietary CP from supplemental SBM were fed to dairy cows with RPM added to maintain a Lys:Met ratio of 3.1 in MP. Increasing CP of the diet resulted in linear increases in milk and true protein

yields, paralleling production responses reported in a larger companion lactation trial. Increasing dietary CP also increased omasal flow of microbial and total NAN but not estimated RUP flows. The results of this study also indicated that the NRC (2001) model underpredicted MP-allowable milk yield. Responses in milk and component yields observed in the companion trial as well as the present study derived from linear increases in ruminal microbial NAN flow, rather than RUP flow, in response to dietary CP. Thus, we rejected our hypothesis that improved production traits observed in our trials resulted from increased supply of RUP from dietary SBM supplemented with RPM. Our results emphasize the importance of the microbial protein contribution to MP requirements of lactating cows, and future research should help identify methods for optimizing ruminal microbial protein synthesis on low-CP diets.

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