J. Dairy Sci. 102:4014–4024
https://doi.org/10.3168/jds.2018-14667

Assessing bioavailability of ruminally protected methionine and lysine prototypes

A. J. Fleming,[1]* K. A. Estes,[1]* H. Choi,[1] B. A. Barton,[2] C. A. Zimmerman,[2] and M. D. Hanigan[1]†
[1]Department of Dairy Science, Virginia Tech, Blacksburg 24061
[2]Balchem Corporation, New Hampton, NY 10958

ABSTRACT

Met and Lys are essential AA that can limit lactational performance in dairy cattle fed protein-sufficient diets. Thus, there is industry demand for ruminally protected (RP) sources of Met and Lys. One method of providing ruminal protection for Met and Lys is lipid encapsulation. The objective of this work was to assess 3 lipid-encapsulated Met prototypes (P1, P2, and P3) and 1 Lys prototype (P4) to determine ruminal protection, small intestine absorption (experiment 1), and animal production responses (experiment 2). Ruminal protection was estimated from 8-h in situ retention during ruminal incubation and intestinal absorption from plasma appearance after an abomasal bolus of the in situ retentate. Blood samples were collected over time to determine plasma Met and Lys concentration responses compared with unprotected Lys and Met infused abomasally. The prototypes were not exposed to the total diet or subjected to typical feed handling methods before evaluation. The bioavailability of P1, P2, and P3 Met prototypes was found to be 14, 21, and 18% of the initial AA material, respectively. The RP-Lys prototype had a bioavailability of 45%. To evaluate production responses, 20 Holstein cows were randomly assigned to 2 trials (n = 10 each) in a replicated Latin square design with 14-d periods. The base diet was predicted to be deficient in metabolizable Met (−14.8 g/d) and Lys (−16.1 g/d) per the Cornell Net Carbohydrate and Protein System (version 6.55). In the Met trial, the base diet was supplemented with RP-Lys to meet Lys requirements, and treatments were as follows: no added RP-Met (NCM), NCM plus Smartamine M (SM; Adisseo, Alpharetta, GA), and NCM plus P1, P2, or P3 at 148% of the Met content of SM. In the Lys trial, the base diet was supplemented with RP-Met to meet the Met requirement, and treatments were as follows: no added Lys (NCL), NCL plus AjiProL (AL; Ajinomoto Heartland Inc., Chicago, IL), and NCL plus P4 at 55, 78, or 102% of the reported absorbed Lys in AL. All products were top dressed on the diet without prior mixing or extended exposure to the rest of the diet. Milk protein concentration significantly increased when diets were supplemented with P2, P3, or SM (3.12, 3.12, and 3.11%, respectively) compared with NCM (3.02%). Only P1 (3.04%) was significantly lower than SM. Prototype P2 had the greatest numerical milk protein output response among the 3 RP-Met prototypes, suggesting that it may have had the greatest efficacy when supplemented into these rations. There was a numerical milk protein concentration response to AL and a linear increase in milk protein concentration for P4. The P4 and AL treatments resulted in comparable milk protein production regardless of P4 dose.
Key words: milk production, methionine, lysine, abomasal infusion

INTRODUCTION

Increasing dietary MP concentrations leads to increased milk production when MP is below requirements (Kalscheur et al., 1999; Broderick, 2003). However, the true requirements are for individual AA rather than the aggregated MP. Therefore, it is possible to have a diet sufficient in MP but limiting in 1 or more EAA. To avoid this problem, it is not unusual for diets to be formulated to exceed MP requirements to ensure maximum milk production (Colmenero and Broderick, 2006). However, feeding excess MP leads to decreased N efficiency, causing increased manure N content. This can lead to increased ammonia emissions and surface water eutrophication (Chiou et al., 1995; St-Pierre and Thraen, 1999; US EPA, 2011). Due to negative environmental and economic effects, there has been increased interest in precision feeding of AA (making sure AA needs are met without overfeeding). Supplementing rumen-protected (**RP**) AA, specifically Met and Lys, in low-MP diets is one way to optimize milk production and N losses (Arriola Apelo et al., 2014b).

Received February 28, 2018.
Accepted January 11, 2019.
*These authors contributed equally to this work.
†Corresponding author: mhanigan@vt.edu

Methionine and Lys have been identified as AA that can limit synthesis of milk and milk protein in dairy cows fed North American diets (Schwab et al., 1992; NRC, 2001). Positive milk production responses in dairy cows supplemented with absorbable Met and Lys sources have been extensively studied (Rulquin et al., 1993; Stern et al., 1994; Overton et al., 1996). The NRC (2001) concluded that to achieve optimal milk production, metabolizable Met and Lys should be at least 2.2 and 7.2% of MP, respectively, when MP is fed at requirements. Several approaches have been evaluated to achieve the desired Met:Lys concentrations, but these values are difficult to achieve unless one uses supplemental RP-Met and RP-Lys to selectively increase concentrations.

For supplemental Met and Lys to elicit biological responses, they must be protected from ruminal degradation yet be available for absorption from the small intestine. Free AA are almost completely degraded in the rumen (Chalupa, 1975). Thus, they must be protected from ruminal degradation. Various approaches to protecting AA from ruminal degradation have been developed, including lipid encapsulation (Papas and Wu, 1997; NRC, 2001). Some studies have shown that cows fed RP-Met and RP-Lys products had increased milk protein yield when fed diets low in CP or MP supply. Thus, supplementing low-CP diets with RP-Met and RP-Lys can be a successful strategy to avoid the potential negative effects of an MP deficiency (Dinn et al., 1998; Broderick et al., 2008; Wang et al., 2010; Hristov and Giallongo, 2014). Protected forms of Met and Lys in place of excess CP allows for precision feeding, which could lead to increased animal performance and nitrogen efficiency.

The objective of this study was to evaluate 3 lipid-encapsulated Met prototypes and 1 lipid-encapsulated Lys prototype for ruminal protection, intestinal release, and production responses in lactating cows fed a diet deficient in metabolizable Met and Lys. It is important to note that this work does not consider the potential negative effects of feed handling, mixing, and delivery on prototype efficacy.

MATERIALS AND METHODS

In the first experiment, the efficacy of ruminal protection of Met and Lys prototypes was assessed using the combination of an in situ incubation and intestinal absorption using plasma appearance responses after an abomasal bolus. The second experiment assessed lactational responses to provision of each of the prototypes when supplemented to a diet deficient in either metabolizable Met or metabolizable Lys. All experimental procedures were reviewed and approved by the Virginia Tech Institutional Animal Care and Use Committee (Blacksburg). Prototypes were provided by Balchem Corporation (New Hampton, NY).

Experiment 1

Animals and Diet. Two nonlactating, Holstein × Jersey crossbred cows fitted with rumen cannulas were used in the study. Cows were housed in individual metabolism stalls for the duration of the experiment. The stalls were equipped with rubber matting, individual feed bunks, and automatic water bowls and were bedded with wood shavings. Cows were fed a standard lactating cow ration balanced for more than 40 L of milk/d (16.5% CP), and thus AA and energy supply exceeded daily requirements. The animals were adapted to the diet for 2 wk before the trial. The diet was fed once daily during the adaptation period and 6 times daily (4-h intervals) from 24 h before the start of ruminal incubations until completion of the trial. Feeding was restricted to 95% of ad libitum intake starting 12 h before the first period to help reduce diurnal variation in plasma AA.

Design and Treatments. The experiment was conducted as a crossover design with 6 periods of 1 d each. The RP-Met and RP-Lys prototypes used for both experiments were manufactured and provided by Balchem Corporation. Each prototype was an encapsulate consisting of an AA core and a lipid coating. The encapsulation technology used for both the RP-Met and RP-Lys prototypes was similar to that of the commercially available AminoShure-M product (Balchem Corporation). Each cow received a different treatment each day, and treatments were assigned to balance carryover effects.

The fat coating used on these prototypes was similar to that used on AminoShure-L, provided by Balchem Corporation, which was tested by Ji et al. (2016) for feed and mixing stability. Thus, it is assumed that these prototypes would have a level of protection against feed moisture and mixing similar to that reported by Ji et al. (2016).

Ruminal Protection. The prototypes were initially analyzed for N content. The 3 Met prototypes contained 68% Met (**P1**), 69% Met (**P2**), and 70% Met (**P3**), and the 1 Lys prototype contained 42% Lys (**P4**). Ruminal protection of each prototype was assessed by 8-h in situ ruminal exposure of the product. Approximately 15 g of the Met (P1, P2, or P3) or 76 g of Lys (P4) was incubated in 10-cm × 20-cm Dacron bags (1 bag per product, 50-μm porosity; Ankom Technology, Macedon, NY). Following incubation, bags were rinsed 3 times in fresh cold water, excess moisture was removed using a salad spinner, stored at −20°C, and subsequently freeze

dried. Dried residue and a subsample of the source material were ground and analyzed for N content by combustion using a Vario El Cube analyzer (Elementar, Langenselbold, Germany). Ruminal protection was estimated as the proportion of the initial product N that remained after 8 h of incubation.

Bioavailability. To determine bioavailability, approximately 40 g of Met (61.5 g of P1, 60.6 g of P2, or 57.1 g of P3) or 128 g of Lys (306 g of P4) was evenly distributed into four 10-cm × 20-cm Dacron bags (4 bags per product, 50-μm porosity; Ankom Technology) and ruminally incubated for 8 h. Upon removal, each bag was briefly hand rinsed in cold water, and the residue of the 4 bags was aggregated, suspended in 3 L of water, and bolus infused into the abomasum via a small-bore Tygon infusion line (Saint-Gobains, Paris, France) containing a distal flange. The lines were introduced into the abomasum via the reticular omasal orifice as previously described by Spires et al. (1975). Control infusions consisted of 30.0 g of unprotected Met (100% DL-Met) or 112.5 g of unprotected lysine (100% Lys-HCl) infused without prior rumen incubation. The dosage of unprotected AA was less than that contained in the products to reflect anticipated product absorption resulting from losses during ruminal incubation and less than 100% intestinal digestibility. The infusions each required 10 to 15 min. After the infusion, the line was flushed with an additional 100 mL of water to ensure complete delivery of the dose.

Blood was sampled from an indwelling jugular catheter at −4, −2, 0, 0.33, 0.67, 1, 2, 4, 6, 8, 10, 12, and 16 h relative to the completion of the abomasal infusion. Samples were placed on ice until all samples in a period had been collected and transported to the laboratory on ice, and plasma was prepared by centrifugation at 1,665 × *g* at 4°C for 12 min. Plasma was stored in polypropylene tubes at −20°C until analysis. Plasma samples were deproteinized using sulfosalicylic acid (8% wt/vol) and centrifugation, spiked with a ^{13}C-labeled mix of AA, desalted by ion exchange chromatography (BioRad Resin AG 50-X, 100–200 mesh; Bio-Rad, Hercules, CA), and eluted using ammonium hydroxide (2 *N*) into salinized glassware as described by Calder et al. (1999). Desalted samples were freeze dried and subsequently derivatized with *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide in acetonitrile. Samples were analyzed for isotopic enrichment by GC-MS, and the resulting data were used to calculate AA concentrations by isotope dilution as described by Calder et al. (1999).

Plasma appearance of the crystalline and prototype AA was estimated from the area under the plasma AA concentrations curves (**AUC**) with respect to time. The AUC was numerically estimated using the trapezoidal rule after subtraction of the mean of the preinfusion concentrations from all values. There was no evidence that plasma concentrations did not return to baseline by the end of the 24-h period. The AUC was expressed per gram of AA infused, and the ratio of the prototype AUC over the crystalline AA AUC (per gram of AA infused) represented the infused AA appearance in plasma relative to the abomasally infused crystalline AA, which is assumed to be 100% absorbed. Results were expressed as a mean of the 2 independent observations.

Experiment 2

Animals and Diets. Twenty Holstein cows with average DIM of 85 ± 6.2 at the start of the trial were selected for the trial. Animals were housed in a freestall barn equipped with a Calan gate feeding system (American Calan Inc., Northwood, NH) to obtain individual feed intakes and refusals. Cows were trained and acclimated to the gates for 14 d before the start of the experiment. Cows were milked twice daily at approximately 0100 and 1300 h, and milk yields were recorded. Animals were weighed automatically as they exited the parlor via walk-through scales in the exit alleys.

Before the start of the experiment, samples of corn silage and alfalfa haylage were analyzed at Cumberland Valley Analytical Services (Hagerstown, MD) using near-infrared reflectance spectroscopy analysis for proximate analyses and wet chemistry for minerals (Ca, P, Mg, K, Fe, Mn, Zn, Cu), and the resulting values were used to finalize diet formulation. The base diet was formulated to be deficient in both Met (−14.8 g/d) and Lys (−16.1 g/d) while meeting NRC (2001) energy requirements using the Cornell Net Carbohydrate and Protein System (version 6.55; http://blogs.cornell.edu/cncps/purchase/). Diet composition is provided in Tables 1 and 2. The diet was fed ad libitum to all cows once daily to achieve greater than 5% refusals. Offered and refused feed weights were recorded daily.

Design and Treatments. The Lys and Met feeding experiments were conducted simultaneously using a replicated 5 × 5 Latin square design with 2 squares for each AA. The squares were arranged with 5 periods of 14 d, 5 treatments, and 5 cows. The treatments were assigned a sequence number, and each animal was randomly assigned to 1 of the 5 treatments in period 1. Animals progressed through the remainder of the treatments according to the sequence number in the following periods. Treatments were not balanced for carryover.

The first 10 d of each period were used for diet adaptation, and the last 4 d were used for sampling. Intakes were determined daily during the experiment and aver-

Table 1. Formulated ingredient and chemical composition of the basal TMR

Item	% of DM
Ingredient	
Corn silage, kernel processed	33.9
Legume hay, alfalfa	16.9
Brewers grain, wet	5.10
Corn grain, ground	16.1
Soybean meal, protected, SoyPlus[1]	5.36
Soybean meal, 48% CP	4.58
Citrus pulp, dried	4.50
Wheat middlings	3.39
Soybean hulls, ground	3.25
Molasses, sugarcane	1.30
Energy Booster Mag[2]	0.84
Megalac[3]	0.66
Urea	0.27
ProvAAl AAdvantage[4]	0.70
Mineral–vitamin mix[5]	3.12
Nutrient composition	
CP	16.8
Soluble CP	5.41
RDP 3× level	9.84
aNDFom[6]	29.3
ADF	19.3
NFC	41.9
WSC[7]	5.25
Starch	26.8
Soluble fiber	7.61
Ether extract	4.89
Total fatty acids	4.08
Ash	7.67
Ca	0.85
P	0.37

[1]Landus Cooperative, Ralston, IA.

[2]Dry fat supplement manufactured by Milk Specialties Global (Eden Prairie, MN).

[3]Rumen bypass fat marketed by Church & Dwight Co. Inc. (Princeton, NJ).

[4]Animal protein supplement manufactured by Perdue AgriBusiness LLC (Binghamton, NY).

[5]Contained (DM basis) 26.9% ground limestone, 20.2% sodium sesquicarbonate, 12.5% salt, 8.33% potassium magnesium sulfate, 6.73% potassium carbonate, 6.41% OmniGen-AF (Phibro Animal Health, Teaneck, NJ), 4.81% dicalcium phosphate, 4.81% XP Yeast Culture (Diamond V, Cedar Rapids, IA), 4.16% magnesium oxide, 0.96% vitamin E 132,159 IU/kg, 0.64% Availa 4 (Zinpro Corporation, Eden Prairie, MN), 0.96 vitamin ADE premix, 0.96% trace mineral premix, 0.64% organic selenium 600 mg/kg, 0.64% selenium 100, and 0.32% Rumensin 200 g/kg (Elanco Animal Health, Greenfield, IN).

[6]Ash-free NDF.

[7]Water-soluble carbohydrate.

aged by week, and the amount of RP-AA supplement added to the diet was calculated for each cow based on observed DMI in the previous week. All RP-AA were from the same batch as those used in experiment 1.

For the Met trial, the base diet was supplemented with 32.2 g of RP-Lys [80.5 g of AjiProL–Gen 2 (**AL**), 40% Lys; Ajinomoto Heartland Inc., Chicago, IL], which, based on the manufacturer's specifications, increased MP Lys to 7.16% of MP in an attempt to ensure that the diet was not deficient in Lys. This diet served as the negative control (**NCM**) for the Met trial. The remaining treatments were as follows: NCM supplemented with 24.7 g of Smartamine M (**SM**; 18.5 g of Met at 75% Met; Adisseo, Alpharetta, GA) and NCM supplemented with 40.3 g of P1 (27.4 g of Met), 39.7 g of P2 (27.4 g of Met), or 39.2 g of P3 (27.4 g of Met), resulting in Met intakes that were 139% of the Met intake from SM. The SM supplementation level was chosen to provide 14.8 g of absorbed Met, thus alleviating the predicted deficiency.

Treatments for the Lys trial were as follows: the base diet supplemented with 24.7 g of SM (18.5 g of Met) to ensure that the base diet was not deficient in Met, resulting in a MP Met concentration of 2.53% of MP (**NCL**); NCL supplemented with 80.5 g of AL (32.2 g of Lys; Ajinomoto Heartland Inc.); and NCL supplemented with 41.5 g (17.4 g of Lys), 59.3 g (24.9 g of Lys), or 77.2 g (32.4 g of Lys) of P4, resulting in 55, 78, or 102% of the Lys feeding rate of AL. All RP-AA were top dressed onto the base ration and gently mixed within the upper portion of the TMR twice daily. Refusals were collected on d 11 to 14 of each period, weighed once daily at approximately 1730 h, and frozen until composited.

Feed Sampling and Analysis. Samples of alfalfa hay and the grain mix were collected once weekly, whereas samples of corn silage, wet brewers grain, and the TMR ration were collected 3 times weekly, and a subsample was used for DM determination (55°C for 48 h). The remainder was frozen and subsequently composited by period. The total ration was adjusted weekly for fluctuations in ingredient DM content. Orts samples were collected on d 11 to 14 of each period and frozen until composited by cow and period. A sample of each batch of the grain mix (delivered on d 0 and 51 of the trial) and samples of the major feed ingredients [corn silage, alfalfa hay, soybean meal protected, soybean meal (48% CP), corn grain, and ProvAAI Aadvantage (Perdue Agribusiness, Salisbury, MD)] from each period were sent to Midwest Labs (Omaha, NE) for AA analysis. The data from those analyses are presented in Table 3.

Milk Sampling Analysis. Milk samples were collected on d 11 to 14 of each period and were analyzed for fat, lactose, true protein, MUN, and SCC by United DHIA (Radford, VA). Milk yield and composition were used to calculate ECM (Luo et al., 2017):

$$\text{ECM} = 12.95 \times \text{milk fat (kg/d)} + 7.65 \times \text{milk protein (kg/d)} + 0.327 \times \text{milk yield (kg/d)}.$$

Statistical Analysis. Data were analyzed using a linear mixed-effects model function of the LME4 pack-

Table 2. Basal diet summary of energy, protein, and AA balance based on Cornell Net Carbohydrate and Protein System (version 6.55; http://blogs.cornell.edu/cncps/purchase/)

Dietary variable	Supply	Balance	Requirement[1]	Summary
Formulated diet[2]				
ME, Mcal/d	69.4	−2.65	96.3	46.6 kg of allowable milk
ME, Mcal/kg	2.60			
ME of allowable ECM				46.4 kg of allowable milk
NE_L, Mcal/kg	1.675			
MP, g/d	3,055	−66.0	97.9	47.5 kg of allowable milk
MP, % of DMI	11.41			
Metabolizable Met, g	62.40	−14.8	80.8	2.04% of MP
Metabolizable Lys, g	202.7	−16.1	92.6	6.63% of MP
Total EAA, g/d	1,441	−29.4	98.0	47.18% of MP
Actual diet[3]				
ME, Mcal/d	76.3	5.63	108.0	52.3 kg of allowable milk
ME, Mcal/kg	2.58			
ME of allowable ECM				52.4 kg of allowable milk
NE_L, Mcal/kg	1.66			
MP, g/d	3,407	250.2	107.9	52.6 kg of allowable milk
MP, % of DMI	11.52			
Metabolizable Met, g	68.9	−8.4	89.1	2.02% of MP
Metabolizable Lys, g	224.1	3.9	101.8	6.58% of MP
Total EAA, g/d	1,600	118.7	108.0	6.98% of MP

[1]Percentage of required.

[2]Formulated for Holstein cows weighing approximately 669 kg at 85 DIM, consuming 26.8 kg of DM/d, and producing 49 kg of milk/d.

[3]Formulated based on observed mean values for cows when assigned to the negative control diets: BW = 669 kg, DIM = 85, DMI = 29.6 kg/d, milk production = 47 kg/d, milk fat = 3.61%, and milk protein = 3.04%.

age in the R statistical software program (version 3.2.2; R Core Team, 2015). The Met trial was analyzed as a replicated 5 × 5 Latin square design with period and treatment as fixed effects and square and cow nested within square as random effects:

$$Y_{ijkl} = \mu + P_i + T_j + S_k + C(S)_{kl} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the population mean of Y, P_i is the fixed effect of the ith period (i = 1 to 5), T_j is the fixed effect of the jth treatment (j = 1 to 5), S_k is the random effect of the kth square (k = 1 or 2), $C(S)_{kl}$ is the random effect of cow nested within square, and e_{ijkl} is the residual error.

The Lys trial was analyzed as a mix of discrete and continuous effects:

Table 3. Amino acid composition of individual feed ingredients (% of total AA)

Item	Grain mix	Corn silage	Alfalfa hay	Soybean meal, protected	Soybean meal, 48% CP	Corn grain	ProvAAI Advantage[1]
Arginine	1.25	0.18	1.03	3.28	4.22	0.51	2.78
Histidine	0.61	0.13	0.45	1.35	1.52	0.27	6.62
Isoleucine	0.77	0.25	1.00	1.85	2.34	0.30	0.59
Leucine	1.70	0.72	1.86	3.80	5.01	0.86	12.7
Lysine	0.98	0.22	1.12	2.81	3.48	0.31	7.61
Methionine	0.34	0.15	0.39	0.60	0.98	0.23	0.92
Phenylalanine	0.96	0.29	1.13	2.44	2.59	0.40	4.05
Tryptophan	0.26	0.04	0.40	0.67	0.87	0.09	1.69
Tyrosine	0.73	0.23	0.84	1.90	2.24	0.36	1.64
Valine	1.02	0.35	1.19	2.35	2.63	0.41	8.44
Alanine	1.05	0.57	1.21	2.15	2.38	0.57	7.64
Aspartic acid	2.05	0.48	2.69	5.88	7.31	0.60	11.3
Cysteine	0.36	0.11	0.29	0.64	0.83	0.17	1.03
Glutamic acid	3.29	0.87	2.27	9.10	11.5	1.36	9.21
Glycine	0.89	0.31	1.07	1.86	1.74	0.34	3.88
Proline	1.18	0.59	1.39	2.48	2.92	0.70	2.81
Serine	0.98	0.24	1.00	2.50	2.85	0.38	3.16
Threonine	0.77	0.24	0.99	2.02	2.29	0.30	2.24
CP	21.6	7.84	24.8	47.3	54.4	8.57	84.2

[1]Animal protein supplement manufactured by Perdue AgriBusiness LLC (Binghamton, NY).

Table 4. Ruminal protection and plasma appearance of rumen-protected (RP) Met and RP-Lys prototypes in dairy cows[1]

Prototype (P)	N remaining,[2] % of prototype N	Plasma appearance, % of N infused	AA bioavailability, % of prototype N
RP-Met			
P1	83.5	17.2	14.4
P2	83.9	25.5	21.2
P3	85.3	20.7	17.6
RP-Lys			
P4	106.3	42.1	44.7

[1]Values are a mean of 2 observations.

[2]N remaining after 8-h ruminal incubation.

$$Y_{ikl} = \mu + P_i + AL + dose + S_k + C(S)_{kl} + e_{ikl},$$

where Y_{ikl} is the dependent variable, μ is the population mean of Y, AL represents the discrete effect of AL supplementation, P_i represents the effect of the ith period, dose represents the continuous effect of P4 supplementation rate, S_k represents the random effect of the kth square (k = 1 or 2), and $C(S)_{kl}$ represents the random effect of cow nested within square.

Milk composition, daily feed intake, and production data were averaged over the last 4 d of each period, and those averages were used for statistical analyses. Significance of response estimates was tested using ANOVA, and differences between treatments were detected using the difflsmeans function of the lmerTest package (Kuznetsova et al., 2017).

RESULTS AND DISCUSSION

Ruminal Protection and Intestinal Absorption of the RP-AA by Pulse Dose

In situ, small intestinal absorption, and overall AA bioavailability (the net blood appearance after ruminal incubation expressed as a percentage of the original AA) results for the 4 prototypes are presented in Table 4. The 3 Met prototypes had similar 8-h ruminal protections of 83.5, 83.9, and 85.3% of N remaining for P1, P2, and P3, respectively, indicating effective ruminal protection when not subjected to the effects of diet mixing or exposure. Eight-hour ruminal recovery of N from P4 was 106%, indicating that some residual microbial or feed contamination occurred. Such contamination may have occurred for P1, P2, and P3 as well, which would result in an overestimate of their ruminal protection as well. Despite this limitation, all the prototypes appeared to be well protected from ruminal degradation. We have previously screened several such prototypes and have observed ruminal protection as low as 41%, indicating that contamination probably accounts for a small percentage of the overall ruminal recovery. The 8-h time point was chosen because it may reflect the actual time these products reside in the rumen. Other studies have performed single time point (4.5 h) or time series (0–96 h) incubations to estimate rumen degradation of RP-AA (Berthiaume et al., 2000; Koenig and Rode, 2001). Residence times of these RP-AA can be estimated based on particle size, density of the product, ruminal outflow rate, and time series incubations; however, the actual residence time or passage rate of these RP-AA have not been determined (Koenig and Rode, 2001). This assumption should have a minimal effect on the intestinal digestibility estimates because they are expressed relative to the infused material, but they will affect the overall estimates of bioavailability because it is a summative calculation.

The abomasal bolus method has previously been used to determine the availability of several RP-Met products (Faivre et al., 2013). An increase in plasma Met and Lys concentrations above baseline was observed after each infusion herein, indicating that the encapsulated AA were at least partially absorbed (Figure 1; plasma Lys concentrations only). Intestinal absorption was observed to be 17.2, 25.5, and 20.7% of the abomasally dosed N for P1, P2, and P3, respectively, and 42.1% of the Lys contained in the P4 infusion.

When combined with estimates of ruminal losses, estimated bioavailabilities (percentage of consumed AA that is absorbed; ruminal release × absorption) of the P1, P2, P3, and P4 prototypes were 14, 21, 18, and 45%, respectively. These results suggest that P2 and P4 were the most efficacious in delivering Met and Lys to the animal, although the efficiency of delivery of the Met prototypes was relatively low. It should be noted that there was no prior exposure of these prototypes to mechanical mixing, extended exposure to the base diet, and exposure to the act of eating, and thus availability in a commercial setting may be lower or greater depending on the effect of such factors. Although these estimates are partially in situ based, the absorption of

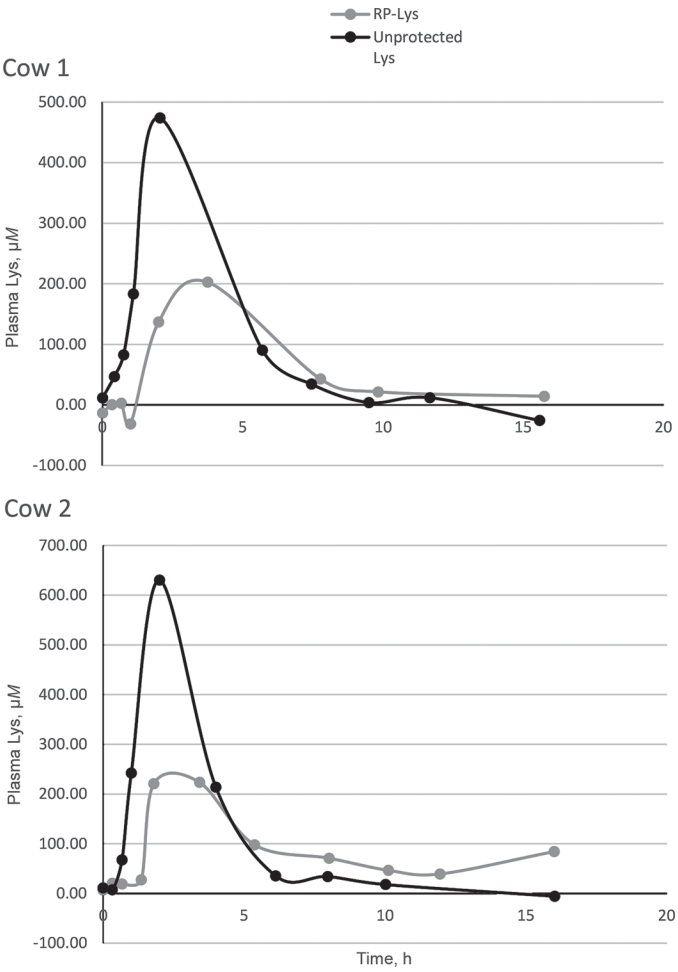

Figure 1. Observed plasma Lys concentrations in each of 2 cows following an abomasal bolus of the residual rumen-protected (RP) Lys prototype after 8 h of ruminal incubation or unprotected Lys.

P4 was within the bioavailability range of other commercially available Lys products that were tested in an in vivo model (Whitehouse et al., 2017).

Plasma appearance of Met was highest for P2 at 25%, followed by P3 at 21% and P1 at 17%. All 3 of the Met prototypes appeared to have similar or lower intestinal digestibilities compared with those obtained for commercially available RP-Met supplements tested in other in situ experiments (Berthiaume et al., 2000; Koenig and Rode, 2001). One might expect plasma appearance to be lower than absorption due to gut and splanchnic first pass use (Maxin et al., 2013); however, the loss should be similar for the infused crystalline AA and thus removed by difference when appearance of the prototype is expressed as a percentage of the crystalline AA appearance.

Animal Production and Milk Protein Responses

Results from the RP-Lys trial are presented in Table 5. There was a numerical milk protein concentration response to the addition of AL as expected given the design of the diets. However, the diets as consumed by the animals were not predicted to be limiting according to Cornell Net Carbohydrate and Protein System version 6.5 (Table 2), which may partially explain the small response to AL. However, there was a significant linear milk protein concentration increase in response to increasing P4 that exceeded the AL response, indicating that the animals were indeed deficient in Lys and the model predictions were inaccurate. The linear response to P4 supports the assessment of its efficacy in delivering Lys to plasma in the first trial.

Milk yield and composition data for the RP-Met trial are summarized in Table 6. Of the production variables, only milk protein concentration was significantly affected by treatment. Milk protein concentration

Table 5. Effects of supplemental AjiProL (AL; Ajinomoto Heartland Inc., Chicago, IL) or rumen-protected lysine prototype on milk yield and composition in lactating cows

Item	NCL[1]	NCL + AL	Dose[2]	*P*-value of AL	*P*-value of dose
DMI, kg/d	27.7	27.2	0.004	0.635	0.871
Milk yield, kg/d	53.6	53.4	0.0009	0.844	0.965
Milk fat, %	3.29	3.17	−0.0003	0.345	0.323
Milk fat, kg/d	1.74	1.68	−0.001	0.407	0.337
Milk protein, %	2.86	2.89	0.002	0.481	0.025
Milk protein, kg/d	1.53	1.54	0.0009	0.774	0.304
Milk lactose, %	4.80	4.82	0.0004	0.614	0.638
Milk lactose, kg/d	2.56	2.58	0.0002	0.733	0.873
MUN, mg/dL	9.33	9.36	−0.0008	0.955	0.956
SCC, cells/mL	129	85.4	−0.534	0.451	0.747
ECM, kg/d	51.56	50.96	−0.015	0.659	0.694

[1]Negative control ration supplemented with Smartamine M (Adisseo, Alpharetta, GA).
[2]Kilograms or percent per gram of Lys in the rumen-protected lysine prototype.

Table 6. Effects of supplemental RP-Met[1] on milk yield and composition in lactating cows

Item	Treatment[2]					SEM
	NCM	NCM + SM	NCM + P1	NCM + P2	NCM + P3	
DMI, kg/d	28.6	29.1	28.5	28.5	28.6	2.1
Milk yield, kg/d	45.2	45.7	45.0	45.4	45.2	1.4
Milk fat, %	3.62	3.50	3.74	3.64	3.51	0.29
Milk fat, kg/d	1.62	1.59	1.68	1.64	1.59	0.12
Milk protein, %	3.02[a]	3.11[b]	3.04[a]	3.12[b]	3.12[b]	0.05
Milk protein, kg/d	1.36	1.42	1.36	1.42	1.41	0.05
Milk lactose, %	4.84	4.83	4.79	4.83	4.85	0.12
Milk lactose, kg/d	2.18	2.21	2.16	2.19	2.19	0.07
MUN, mg/dL	11.0	11.2	11.9	11.3	11.4	1.19
SCC, cells/mL	187	202	170	261	174	50.2
ECM yield, kg/d	46.1	46.4	46.9	47.0	46.1	1.65

[a,b]Means within a row with different superscripts are significantly different ($P < 0.05$).

[1]RP-Met = rumen-protected Met prototype (P1, P2, and P3).

[2]NCM = negative control diet supplemented with AjiProL (Ajinomoto Heartland Inc., Chicago, IL); NCM + SM = NCM supplemented with Smartamine M (Adisseo, Alpharetta, GA); NCM + P1 = NCM supplemented with RP-Met P1; NCM + P2 = NCM supplemented with RP-Met P2; NCM + P3 = NCM supplemented with RP-Met P3.

significantly increased when diets were supplemented with SM, P2, or P3 compared with NCM, whereas P1 supplementation did not significantly affect milk protein. The SM responses were expected given dietary Met concentrations in the NCM diet and the known efficacious delivery of Met to plasma from that product. Graulet et al. (2005) reported Met bioavailability for SM of 74.4% based on plasma appearance. This agreed with values reported in other studies (Schwab, 1995; Robert and Williams, 1997; Rulquin and Kowalczyk, 2003), suggesting that a large percentage of Met from SM is transported to blood for animal use. Leonardi et al. (2003) also reported that RP-Met caused increases in milk protein content for cows fed high- and low-CP diets with no significant change in milk yield and milk protein production. Others have reported positive milk yield and milk fat percentage responses (Ordway et al., 2009) as well as positive milk protein concentration responses to RP-Met supplementation (Yang et al., 2010). The significant increases in milk protein concentrations with P2 and P3 demonstrate that both prototypes delivered Met to the animal.

Using the milk protein concentration responses from the lactation trial and the manufacturer's values for SM and AL, one can estimate bioavailability values for each prototype relative to SM or AL.

The manufacturer-stated Lys content for AL is 40% of DM with a bioavailability of 50% of the Lys content (Ajinomoto Heartland Inc.). Given the mean AL feeding rate of 32.2 g of Lys/d, the absorbed Lys supply provided by AL would have been 16.1 g/d according to manufacturer specifications. The linear response in milk protein concentration to P4 was 0.002%/g of consumed Lys. Given that slope and assuming the numeric response to AL was true, 35.4 g of P4 prototype containing 15 g of Lys would be required to achieve the same milk protein response as for AL. This equates to a bioavailability for P4 over 100%. Recovery above 100% is, of course, not possible. Thus, the stated bioavailability of AL may be too high or the small numeric response to AL is not representative of the true response. Because the response to P4 was linear with no evidence of nonlinearity and the AL response fell within the middle of the P4 response range, the calculated relative values should be valid.

Literature (Schwab, 1995; Graulet et al., 2005) and manufacturer values for SM indicate a Met content of 75% of DM and 80% bioavailability. Although milk protein percentage did not significantly differ between the SM treatment and P2 or P3 treatments, the numerical differences were 0.01 percentage units greater than for SM. Using the Met content in SM of 75%, bioavailability of 80%, and a feeding rate of 25.57 g of SM/d, the SM treatment should have supplied 15.3 g of bioavailable Met. The change in milk protein concentration was 0.0047%/g of consumed Met. Because the milk protein response for P2 and P3 was not statistically different compared with SM, one might assume that they supplied approximately 15.3 g of bioavailable Met, which represents a bioavailability of 69%. However, if one uses the observed milk protein responses for each, at P2 and P3 intakes of 28.2 and 27.8 g, the milk protein responses were 0.0051 and 0.0045%/g, respectively. These equate to bioavailabilities of 87% for P2 and 77% for P3 if SM has a bioavailability of 80%. These values suggest that both prototypes completely released the Met remaining from ruminal incubation in the intestine. Although it did not elicit a significant milk protein response, the

bioavailability of P1 was approximately 16% based on the numerical milk protein responses and the assumed bioavailability of SM.

The calculated bioavailabilities of P2 and P3 are approximately 3 times that estimated from the ruminal incubation and pulse dose method. Similarly, the estimate of bioavailability for the P4 prototype from the pulse dose trial (experiment 1) was 44.7%, suggesting that it was no better than AL, whereas the milk protein responses indicated that P4 had twice the bioavailability of AL. Recognizing that both the AL and prototype bioavailabilities have error of estimation, with the former subject to greater errors due to less replication, it is possible that the estimates of difference between AL and the Lys prototype is less than observed herein.

Method Comparison

It is unclear what may have caused the large discrepancy between the milk protein and the plasma appearance methods of assessing Met bioavailability. There was variation among the results between the 2 animals used for the plasma appearance method; however, the results from the 2 animals were much closer to each other than to the responses calculated from milk protein. Thus, the addition of another observation would likely not resolve the difference between the methods.

A potential pitfall for the pulse dose approach is the possibility that the pulse dose elicits a regulatory response that increases rates of catabolism. Given that the AUC was much greater for the unprotected control infusions than for the prototypes, the recovery of the unprotected control may have been less than that of the prototypes, which would have led to overestimates of their bioavailabilities. Thus, this also seems unlikely to be the cause of the difference.

Another potential source of the difference is the efficacy of delivery of the product to the small intestine during dosing. Although a flange is used to help retain the infusion line within the abomasum, it does not provide an effective barrier to reflux from the abomasum into the omasum. Even though the products have a specific gravity greater than 1, it is still theoretically possible that a portion of the infused product is refluxed back into the omasum and rumen during or after infusion, where it would be subject to degradation without absorption. Such occurrence would potentially explain the low estimates of bioavailability from the pulse dose technique compared with the feeding trial. However, the encapsulates are particulates, and it is difficult to envision significant reflux of particulate matter through the omasal and ruminal-reticular orifices given the rate of flux of liquid through those orifices. Additionally, the unprotected Met is infused through the same infusion line. Given that it is already in solution, it would be even more prone to such reflux if it were significant.

Finally, one must also consider the artificial nature of the ruminal incubations. The Dacron bags would at least partially protect the enclosed material from abrasion, and they may reduce degradative action. One would anticipate such potential actions to reduce ruminal activity rather than increase it. However, it is possible that abrasion and mechanical force within the rumen may create additional surface area without releasing the AA. The former perhaps leads to better emulsification in the intestine, thus yielding higher intestinal digestibility. Of the potential sources of difference, this latter explanation seems most likely to explain the low estimates of Met encapsulate bioavailability when assessed by the pulse dose method.

There are also some potential pitfalls with the milk protein response approach that could have affected the results and may contribute to the large discrepancy between the 2 methods of assessing AA bioavailability. One possibility is that the cows assessed for milk protein responses were not as deficient in Met or Lys as initially predicted, which is supported by model assessments of the diets using the trial observations. Clearly, the cows were deficient given the observed milk protein responses. However, the modeling work suggests that the deficiency was less than that provided by the AA sources. According to the classical rectilinear response model, a portion of the additional absorbed AA would have elicited a response, whereas the AA provided in excess of requirements would have elicited no response. For example, if the deficiency was 10 g of absorbed Met, provision of 10, 15, or 20 g of Met would all yield the same protein response, resulting in declining apparent bioavailability as the dose increased. However, there is ample evidence at the tissue and animal group levels that responses should follow saturation kinetics (Whitelaw et al., 1986; Hanigan et al., 1998; Arriola Apelo et al., 2014a; Liu et al., 2017). Even if individual animal responses were rectilinear, the group response would still be curvilinear due to varying individual animal requirements and thus varying proportions of the added supply exceeding requirements for each animal. For example, if the requirements for 3 animals were 10, 11, and 12 g/d, group supplies of 9, 10, 11, and 12 g/d would yield a curvilinear group response. Thus, regardless of whether the response is curved or rectilinear at the animal level, it should be curvilinear at the group level, which is consistent with mammary tissue responses to varying medium Ile, Leu, Met, or Thr concentrations within the in vivo range (Arriola Apelo et al., 2014b). Fitting a rectilinear model to such data will result in requirement estimates that are well below the maximum response range, ensuring ad-

ditional responses well above the apparent requirement. Thus, responses to Met supplementation greater than calculated requirements might be expected, but with potentially declining marginal efficiency.

The numerically greater milk protein concentration responses to P2 and P3 compared with SM suggest that supplementation of the latter did not exceed the maximum response of the group, as suggested by the model results, and thus the observed response is not truncated. One cannot determine whether P2 and P3 supplementation exceeded the maximum responses, but if so, the calculated bioavailabilities would be under estimates. It is important to stress that the P2 and P3 responses were only numerically greater than SM, and thus we cannot rule out with high certainty that supplementation of all 3 did not exceed the maximum responses. This potential problem could have been avoided if a full dose–response curve relating absorbed Met and milk protein had been developed so that the maximum response was established and the curvature in the response surface was explicitly accommodated.

Because multiple doses of the Lys prototype were used for the production trial and found to generate a linear response in milk protein concentrations, with the upper range exceeding that of the numerical response to AL (Table 5), the prior concerns for Met are negated for the Lys trial. However, the single point estimate for AL is defined with considerable variation, which is intrinsic to the comparison among the 2 products. If the AL response was an underestimate, then the 2 products may be more similar than indicated by the data herein.

From these observations, it seems logical to conclude that the pulse dose method may be used to rank lipid encapsulates correctly and may provide the correct relative differences among products, but it apparently underestimates bioavailability. It is important to note that both experimental approaches for bioavailability assessment used reference products and prototypes that were not fully exposed to the feed and feed processing as would occur on farm. Additional work is required to determine feed stability with these prototypes and could provide further knowledge of the effects of prior exposure to the diet on ruminal stability and intestinal availability.

CONCLUSIONS

All of the Met and Lys prototypes were well protected from ruminal degradation with 8-h in situ retentions of 83% or greater. Overall, the lactation trial and bioavailability trial ranked the Met prototypes similarly in their effectiveness to supply absorbable AA. Both trials suggested that the Met prototype P1 had the lowest bioavailability compared with the other Met prototypes. Numerically, P2 had the highest bioavailability and milk protein content response. The prototype P3 elicited a similar milk protein content response as P2, but P3 had a lower bioavailability measured by milk protein response and plasma appearance than P2. Bioavailability by plasma appearance for all prototypes was low compared with apparent effects on milk protein concentration in the lactation trial, suggesting that the former underestimates the true bioavailability or that the latter overestimated the true bioavailability.

ACKNOWLEDGMENTS

Funding for the work, including support of K. Estes, was provided by Balchem Corporation (New Hampton, NY). B. Barton and C. Zimmerman were employees of Balchem Corporation at the time this study was conducted.

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