Effects of rumen-protected methionine and other essential amino acid supplementation on milk and milk component yields in lactating Holstein cows

K. Zhao, W. Liu, X. Y. Lin, Z. Y. Hu, Z. G. Yan, Y. Wang, K. R. Shi, G. M. Liu, and Z. H. Wang*

Ruminant Nutrition and Physiology Laboratory, College of Animal Science and Technology, Shandong Agricultural University, Taian 271018, P. R. China

ABSTRACT

Objectives of this study were to investigate the effects of supplementing rumen-protected methionine (RP-Met), threonine (RP-Thr), isoleucine (RP-Ile), and leucine (RP-Leu) individually or jointly to a low-protein diet, on the performance of lactating dairy cows, as well as to determine the effects of these amino acids (AA) on the mammalian target of rapamycin (mTOR) *in vivo*. Ten lactating Holstein cows were randomly allocated to a repeated 5×5 Latin square experiment with five 19-d periods. Treatments were high-protein diet (16% crude protein, positive control; HP), low-protein diet (12% crude protein, negative control; LP), LP plus RP-Met (LPM), LP plus RP-Met and RP-Thr (LPMT), and LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu (LPMTIL). The dry matter intakes (DMI) of the LP, LPM, and LPMT diets were lower than that of the HP diet, whereas the DMI of the LPMTIL diet was intermediate between the HP diet and the other LP diets. Supplementing RP-Met to the LP diet increased the yields of milk and milk protein, increased the content of milk urea N, and tended to increase milk N efficiency. Co-supplementation of RP-Thr with RP-Met resulted in no further milk production increase. Co-supplementation of all 4 rumen-protected amino acids (RP-AA) increased milk and lactose yields to the level of the HP diet and tended to increase milk protein yield compared with the LPMT diet. We found no significant differences in the contents and yields of milk components between the LPMTIL and HP diets except for a lower milk urea N content in the LPMTIL diet. Venous concentrations of the measured AA were similar across the LP and LP diets supplemented with RP-AA. Relative to levels of the HP diet, LP diets had higher venous concentrations of Met and Gly and tended to have higher Phe concentration and lower con-

centrations of Val and BCAA. The LPMTIL diet had higher venous concentrations of Arg, Lys, Met, Phe, and Glu, and a lower Val concentration. Phosphorylation status of the measured mTOR components in LPM and LPMT treatments were similar to those in the LP treatment but phosphorylation status of mTOR and eIF4E-binding protein 1 (4eBP1) in LPMTIL treatment were higher. The phosphorylation rates of eukaryotic elongation factor 2 (eEF2) in the 4 LP and LP plus RP-AA diets were higher than that of the HP diet. Overall, results of the present study supported the concept that under the relatively short time of this experiment, supplementing RP-AA, which are believed to stimulate the mTOR signal pathway, can lead to increased milk protein yield. This increase appears to be due to increased DMI, greater mTOR signaling, and greater eEF2 activity.

Key words: rumen-protected amino acid, nitrogen efficiency, milk protein, dairy cow

INTRODUCTION

Although it varies greatly, the conversion efficiency of dietary nitrogen into milk protein in lactating cows is relatively low, about 25%, with the remaining ingested dietary N excreted through feces and urine (Spears et al., 2003; Nadeau et al., 2007). Although efficiency could be readily improved by decreasing the dietary CP level, this practice will generally sacrifice the yields of milk and milk protein (Lee et al., 2012a; Broderick et al., 2009). Supplementing individual AA to balance the profile of dietary AA is widely used in the modern swine and poultry industries, and conversion efficiency of dietary N is generally 40% or higher in these animals (Nahm, 2002). Although this may be a promising technique, previous efforts at supplementing dairy cows with individual AA have produced inconsistent results. In response to individual AA supplementation, some studies observed positive responses in milk protein yield (Berthiaume et al., 2006; Appuhamy et al., 2011; Haque et al., 2012), but no responses or negative

Received September 17, 2018.

Accepted May 2, 2019.

*Corresponding author: zhwang@sdaa.edu.cn

responses were observed in others (Socha et al., 2005; Benefield et al., 2009; Doelman et al., 2015). However, no ideal AA recommendations except for Met and Lys have yet been established for lactating cows as they have in growing animals.

Previous studies revealed that some AA, apart from the substrates for protein synthesis, may act as signals to regulate protein synthesis through intracellular signal pathways. Leucine has been reported to stimulate protein synthesis through the mammalian target of rapamycin complex 1 (**mTORC1**) pathway in skeletal muscle (Anthony et al., 2002). The mTORC1 protein complex functions as a nutrient sensor to control protein synthesis (Jewell et al., 2013). The AA may moderate mammalian target of rapamycin (**mTOR**) activity at multiple sites (Tomé, 2018). General AA sensing by the tRNA charge and mTOR was described by Semba et al. (2016). Nutrient deficiency may lead to autophagy, and the mTORC1 regulates autophagy initiation by affecting both complexes of Unc51-like autophagy-activating kinase 1 and vacuolar protein sorting 34 (He et al., 2018). If deficiency of an AA depresses the DMI of lactating cows, the observed variation in mTOR activity might be an effect of nutrient deficiency rather than a weakened direct signal of that specific AA to mTOR. Both in vitro and in vivo studies have been employed to screen the essential AA that may modulate mammary mTOR activity. In vitro studies using bovine mammary epithelial cells (MAC-T) and mammary tissue slices have revealed that essential AA regulate both the initiation and elongation of mRNA translation independent of insulin (Appuhamy et al., 2011), and isoleucine and leucine regulate mTOR signaling independently (Appuhamy et al., 2012). Isoleucine and threonine positively affected mTOR phosphorylation, whereas Ile and Leu linearly decreased eukaryotic elongation factor 2 (**eEF2**) phosphorylation in slices of bovine mammary tissue (Arriola Apelo et al., 2014). It has been demonstrated in lactating mice that individual supplementation of Ile, Leu, Thr, or Met to a 15% CP diet promoted litter weight gain in mouse pups (Liu et al., 2017). Aims of the present study were to investigate (1) whether dietary supplementation of rumen-protected (**RP**) Leu, Ile, Met, and Thr could promote milk protein synthesis in lactating cows; and (2) whether the effects of these AA on milk protein synthesis were additive or not.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

Ten healthy multiparous lactating Holstein cows (575 ± 46 kg of BW; 110 ± 26 DIM) were randomly assigned

to 1 of the 5 groups in a repeated 5×5 Latin square experiment, balanced for carryover effects, with five 19-d periods followed by 2 d of rest. Cows received 1 of the 5 experimental diets in each period, which were as follows: high-protein diet (16% CP, positive control; **HP**), low-protein diet (12% CP, negative control; **LP**), LP diet supplemented with RP-Met (**LPM**), LP diet supplemented with RP-Met and RP-Thr (**LPMT**), and LP diet supplemented with RP-Met, RP-Thr, RP-Ile, and RP-Leu (**LPMTIL**). All the RP-AA were commercially purchased (KDQ Technology Co. Ltd., Hangzhou, China), and the amount of each was supplemented to the LP diet to match the amount supplied by the HP diet. Ingredients and compositions of the experimental diets are listed in Table 1. Diets were prepared as TMR and delivered 3 times daily at 0200, 1000, and 1800 h. Daily amounts delivered allowed for 10% refusal. All the cows were kept in individual stalls, allowed ad libitum intake and free access to water, and milked 3 times daily at 0100, 0900, and 1700 h. Duodenal flow of each essential AA was estimated according to NRC (2001) (Table 2). The LP diet was balanced by substituting 9% of the soybean meal in the HP diet for an equal amount of flaked corn (Table 1).

Ruminal escape of the 4 RP-AA (i.e., RP-Met, RP-Thr, RP-Ile, and RP-Leu) was determined by an in situ experiment as described by Lee et al. (2012a), with 2 ruminally cannulated Holstein cows (575 ± 46 kg of BW; 110 ± 26 DIM; 30 ± 2.3 kg/d milk yield; 24 ± 1.9 kg/d DMI). About 6 g of each individual RP-AA per bag was incubated in the rumen of the cows for 0 (washed but not inserted in the rumen), 2, 4, 8, 12, and 24 h. Nylon bags were inserted sequentially at specified time points and removed simultaneously from the rumen. The RP-Met, RP-Thr, RP-Ile, and RP-Leu products and residues of each in the in situ bag were analyzed for Met, Thr, Ile, and Leu content, respectively, as determined by an automatic AA analyzer (type 835-50, Hitachi, Tokyo, Japan). Ruminal escapes of Met, Thr, Ile, and Leu were calculated using the method of Ørskov and McDonald (1979). Passage rate was 0.055 h^{-1} , based on actual DMI and BW of the cows according to NRC (2001). The intestinal digestibility of each EAA was estimated by a 3-step in vitro procedure described by Calsamiglia and Stern (1995).

Sampling and Analysis

The amount of TMR offered and residues for each cow was recorded on daily basis. Samples of fresh TMR and residues were collected in the last 6 d of each period and stored at -20°C . The TMR and the refusals were pooled together by treatment and period basis for the determination of the content of DM (AOAC, 1990),

Table 1. Ingredients and composition of the experimental diets

Item	Diet ¹				
	LP	LPM	LPMT	LPMTIL	HP
Ingredient, % of DM					
Corn grain, ground	2.45	2.45	2.45	2.45	2.45
Steam-flaked corn	30.1	30.1	30.1	30.1	21.1
Soybean meal	2.69	2.69	2.69	2.69	11.69
Corn silage	31.7	31.7	31.7	31.7	31.7
Wheat bran	2.96	2.96	2.96	2.96	2.96
Cottonseed	3.18	3.18	3.18	3.18	3.18
Oaten hay	2.01	2.01	2.01	2.01	2.01
Alfalfa hay	18.4	18.4	18.4	18.4	18.4
Brewers grains	3.82	3.82	3.82	3.82	3.82
Vegetable fat	0.65	0.65	0.65	0.65	0.65
Premix ²	2.01	2.01	2.01	2.01	2.01
RP-Met, ³ g/d	—	7.8	7.8	7.8	—
RP-Thr, ⁴ g/d	—	—	54.8	54.8	—
RP-Leu, ⁵ g/d	—	—	—	45.9	—
RP-Ile, ⁶ g/d	—	—	—	29.7	—
Composition, g/kg DM					
CP ⁷	127	127	127	127	161
RDP ⁸	70.9	70.9	70.9	70.9	96.7
RUP ⁸	56.4	56.4	56.4	56.4	63.8
MP ⁸	90.9	90.9	90.9	90.9	114.3
NE _L , ⁸ Mcal/kg	1.65	1.65	1.65	1.65	1.65
Ether extract	76.7	76.7	76.7	76.7	74
Ash	49.2	49.2	49.2	49.2	53.2
NFC ⁹	397	397	397	397	341
ADF	191	191	191	191	195
NDF	330	330	330	330	334

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus rumen-protected (RP) Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

²Premix contains: vitamin A, 160 kIU/kg; vitamin D₃, 30 kIU/kg; vitamin E, 300 mg/kg; vitamin K₃, 30 mg/kg; P, 80 mg/kg; Mn, 550 mg/kg; Zn, 750 mg/kg; NaCl, 80 mg/kg; Ca, 200 mg/kg; Cu, 400 mg/kg; and Fe, 1,800 mg/kg.

³The RP-Met product contains 65.56% methionine and has 82% bioavailability (90.9% rumen escape and 90.4% intestinal digestibility).

⁴The RP-Thr product contains 31.78% threonine and has 73% bioavailability (86.2% rumen escape and 84.8% intestinal digestibility).

⁵The RP-Leu product contains 70.04% leucine and has 77% bioavailability (89.4% rumen escape and 86.1% intestinal digestibility).

⁶The RP-Ile product contains 65.4% isoleucine and has 80% bioavailability (88.9% rumen escape and 89.8% intestinal digestibility).

⁷Contributions of supplemented RP-AA to dietary CP content were not included. Including the contribution of supplemented RP-AA, CP content of the LPM, LPMT, and LPMTIL diets was 128.2, 128.9, and 131.0 g/kg of DM, respectively.

⁸Calculated data.

⁹NFC = 100 - (% NDF + % CP + % ether extract + % ash).

OM (Yang and Beauchemin, 2007), and CP (according to the Kjeldahl method; AOAC, 1990). The NDF and ADF contents were determined as described by Van Soest et al. (1991), using amylase and sodium sulphite. The NDF and ADF contents included residual ash. The nutrients in the refused feed were subtracted from the nutrients in the TMR to determine the nutrients consumed, as shown in Table 3.

Milk yield was recorded at each milking. Milk samples for each cow were collected at d 14 and 15 in each period and stored at -20°C for later analysis of milk composition by an automated near-infrared milk

analyzer (Foss-600, Foss Analytics, Hillerød, Denmark). The calculation of milk component yields was based on milk sampled in d 14 and 15 in each period. Each sampling day, milk was sampled at each milking time. Daily milk samples were pooled together according to milk produced in each milking time. Daily milk component yields were calculated by multiplying the content of a specific component measured by the daily milk yield. Daily yield of that specific component in a period was the average of the successive 2-d measurements.

Fecal and urine samples were collected at d 16, 17, and 18 in each period, according to the procedures of

Table 2. Duodenal flows of digestible EAA as predicted by NRC (2001)

EAA	Diet ^{1,2}									
	LP		LPM ³		LPMT		LPMTIL		HP	
	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %	AA flow, g/d	MP, %
Arg	114.9	4.60	114.9	4.47	114.9	4.44	114.9	4.36	137.3	5.04
His	56.8	2.27	56.8	2.21	56.8	2.20	56.8	2.16	65.3	2.40
Phe	124.8	4.99	124.8	4.86	124.8	4.83	124.8	4.74	146.6	5.40
Ile	119.4	4.77	119.4	4.65	119.4	4.62	137.4	5.21	137.4	5.05
Leu	227.3	9.09	227.3	8.84	227.3	8.79	258.2	9.78	258.2	9.48
Lys	152.9	6.12	152.9	5.95	152.9	5.91	152.9	5.80	176.7	6.49
Met	47.2	1.89	52.2	2.03	52.2	2.02	52.2	1.98	52.2	1.92
Thr	120.6	4.82	120.6	4.69	136.7	5.28	136.7	5.19	136.7	5.02
Val	136.4	5.46	136.4	5.31	136.4	5.27	136.4	5.18	156.4	5.74

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus rumen-protected (RP) Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

²MP contents of the HP, LP, LPM, LPMT, and LPMTIL diets were 2,722, 2,565, 2,570, 2,586, and 2,635 g/d, respectively.

³Ratios of specific RP-EAA to MP in the 3 supplemented diets were calculated as follows: (content of individual EAA in the HP diet)/[content of MP in LP diet + (content of individual EAA in the HP diet – content of that EAA in the LP diet)].

Lee et al. (2012a). Samples were kept at -20°C for later analysis. Fresh fecal samples were air-dried at 60°C and ground through a 1-mm Wiley mill screen before storage. Conventional methods for feed samples were used to determine chemical compositions of fecal and urine samples. Contents of creatinine and urea in urine samples were determined via picric acid colorimetry and the diacetymonoxime method, respectively, using commercially available kits (JC Tech Co. Ltd., Nanjing, China). The volume of urine excreted daily was estimated using the measured creatinine concentration in the urine, assuming a creatinine excretion rate of 29 mg/kg of the cow's BW (Valadares et al., 1999). Estimated urine output was used to calculate daily total N, and urea-N (UUN) excretions in urine. Acid-insoluble ash (AIA) was used as an intrinsic digestibility marker

to estimate total-tract apparent digestibility and fecal N output. Total-tract apparent digestibility was calculated based on AIA concentration in TMR and feces. Nitrogen balance was calculated as [N intake – (milk N + urine N + fecal N)].

Ten milliliters of blood was sampled from the tail blood vessels of each cow after morning and afternoon milkings and collected in heparinized vacuum tubes on d 18 of each experimental period. The collected sample was immediately immersed in ice water and centrifuged at $3,500 \times g$ for 15 min at 4°C . Separate plasma samples were kept frozen at -20°C until later analysis. The separated plasma samples were pooled by period for each cow upon analysis. Free AA contents were determined by an automatic AA analyzer (type 835-50, Hitachi); contents of urea nitrogen, alanine aminotransferase,

Table 3. Effects of dietary protein level and rumen-protected (RP) AA supplementation on nutrient intake and apparent digestibility in lactating Holstein cows

	Diet ¹						
Item	LP	LPM	LPMT	LPMTIL	HP	SEM	<i>P</i> -value
Nutrient intake, kg/d							
DM	22.9 ^b	22.5 ^b	22.1 ^b	23.8 ^{ab}	25.6 ^a	0.8	0.02
OM	20.9 ^b	20.6 ^b	20.2 ^b	22.1 ^{ab}	23.7 ^a	0.9	0.02
CP	2.91 ^b	2.93 ^b	2.88 ^b	3.17 ^b	4.28 ^a	0.11	<0.01
NDF	7.24 ^b	6.95 ^b	6.68 ^b	7.63 ^b	8.82 ^a	0.38	<0.01
ADF	4.82	4.77	4.62	5.11	5.24	0.21	0.16
Apparent digestibility, %							
DM	56.9	57.6	55.0	57.1	61.1	1.7	0.18
OM	59.8	60.5	58.1	60.1	63.5	1.6	0.21
CP	61.9	61.9	57.7	59.9	63.4	1.9	0.25
NDF	38.0	38.0	34.9	38.8	41.8	2.4	0.41
ADF	29.2	33.5	30.2	31.3	33.2	2.3	0.65

^{a,b}Means within a row with different superscript letters are significantly different.

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

aspartate aminotransferase, albumin, total protein, and triacylglycerol were determined by an automatic biochemical analyzer (type 7020, Hitachi); concentration of insulin-like growth factor-I, insulin, glucagon, prolactin, and growth hormone were determined by radioimmunoassay methods using commercially available kits (JD Biochemical Engineering Co. Ltd., Tianjin, China).

Mammary biopsies were performed on the last day (d 19) in each experimental period. After local injection of lidocaine hydrochloride and skin incision, about 0.2 g of mammary tissue was sampled from one of the hind quarters from each cow using a 16-cm MG 1522 Bard Magnum core biopsy instrument (Bard Medical Division, Covington, GA). The sample was immediately washed with ice-cold sterilized saline, frozen, and stored in liquid nitrogen for later analysis using Western blotting as described previously by Liu et al. (2017). Briefly, 40 µg of protein was loaded in each well, separated via SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in blocking buffer (P0023B, Beyotime, Jiangsu, China) at room temperature for 2 h and probed for phosphorylated forms of mTOR (Ser2448), 4eBP1 (Thr37/46), S6K1 (Thr389), eEF2 (Thr56), eIF2 α (Ser51), and AKT (Ser473; Cell Signaling Technology, Danvers, MA). After primary antibody incubating, the membranes were washed using Tris buffered saline Tween (TBST) and incubated with anti-rabbit secondary antibodies (Beyotime). Relative abundance of each protein was determined by chemiluminescence through a BeyoECL Plus kit (Beyotime). Relative band densities were determined by a FUSION Fx imaging system (Vilber Lourmat, Marne-la-Vallée, France) and associated software. After determination of the relative abundance of phosphorylated proteins, the stripped membranes were subsequently probed for total mTOR, 4eBP1, S6K1, eEF2, eIF2 α , and AKT (Cell Signaling Technology).

Statistics

Data were subjected to the mixed model procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC) for variance analysis, using the following statistical model:

$$Y = \mu + S_i + C_{j(i)} + P_k + T_l + e_{ijkl},$$

where μ = overall mean; S_i = fixed effect of square ($i = 1$ to 2); $C_{j(i)}$ = random effect of cow within square ($j = 1$ to 5); P_k = fixed effect of period within square ($k = 1$ to 5); T_l = fixed effect of treatment ($l = 1$ to 5); and e_{ijkl} = random residual error, assumed to be normally

distributed. All data are presented as least squares means. Means separation tests were conducted using the PDIFF procedure of SAS. The PDIFF was only used when there was a significant F -test for treatment. Effects of the factors were declared significant at $P \leq 0.05$ unless otherwise noted, and trends were recognized at $0.05 < P \leq 0.10$.

RESULTS

Predicted EAA Supply

For the 4 supplemented AA, duodenal flow of Met, Thr, Ile, and Leu represented 1.92, 5.02, 5.05, and 9.48% of MP, respectively, for the HP diet and 1.89, 4.82, 4.77, and 9.09% of MP, respectively, for the LP diet. Supplementation of RP-Met, Thr, Ile, or Leu to the LP diet elevated their respective duodenal flows to 2.03, 5.28, 5.21, and 9.78% of MP, respectively. The Met requirement recommended by NRC (2001) and the requirements of Thr, Ile, and Leu recommended by Doepel et al. (2004) are 2.4, 5.0, 5.3, and 8.9% of MP, respectively. In all 5 experimental diets, therefore, Met contents were below the NRC requirement recommendation. It is worth noting that ruminal escape of the 4 RP-AA (RP-Met, RP-Thr, RP-Ile, and RP-Leu) was previously determined via an in situ experiment that ended at 24 h (Lee et al., 2012a). However, 24 h is not long enough to assess the extent of N disappearance in feedstuffs known to be sources of RUP (Aldrich et al., 1996). Therefore, a nonlinear program may not adequately fit the extent of degradation.

Milk Yield and Composition

The LP diet decreased the yields of milk, milk protein, and lactose ($P < 0.01$) relative to HP but had no significant effect on milk fat yield (Table 4). Milk component concentrations were generally not affected by treatments except for that of MUN, which was decreased by low-protein feeding and elevated by supplementing RP-AA to the LP diet ($P < 0.01$). Supplementing RP-Met to the LP diet increased the yields of milk and milk protein ($P < 0.05$). Co-supplementation of RP-Thr with RP-Met resulted in no further increase in milk and milk components yields. Co-supplementation of all the 4 RP-AA (Met, Thr, Ile, and Leu) further increased the yields of milk and lactose ($P < 0.05$). Addition of all 4 RP-AA increased milk protein yield to such an extent that we found no statistical difference between the HP and LPMTIL diets for milk protein yield (Table 4). Dietary treatments had no significant effects on feed efficiency.

Nutrient Intake and Apparent Digestibility

Low-protein feeding decreased ad libitum DMI ($P < 0.05$, Table 3). Supplementing RP-Met or RP-Met plus RP-Thr to the LP diet failed to affect DMI ($P > 0.05$). Supplementing all 4 RP-AA to the LP diet elevated DMI to a similar level to that of the HP diet (during the entire data-collection period of the trial, i.e., the last 6 d of each period). Daily OM intake varied similarly to DMI by treatments. We found no effect on daily ADF intake among dietary treatments. Similarly, there was no difference in daily CP and NDF intakes among the 4 LP and RP-AA-supplemented diets, but they were all lower than those of the HP diet ($P < 0.05$). On average, dietary CP intake for the LP, LPM, LPMT, and LPMTIL diets was 1.37, 1.35, 1.40, and 1.11 kg/d less than that of the HP diet, respectively. Apparent digestibility of the measured nutrients was not affected by treatments.

Nitrogen Balance

Dietary nitrogen intakes of the 4 LP and LP plus RP-AA diets were lower than that of the HP diet ($P < 0.01$); however, the difference among LP diets was insignificant (Table 5). Nitrogen secretion and excretion in the 4 LP diets were also lower than those of the HP diet ($P < 0.05$), except that milk N secretion and fecal N excretion in the LPMTIL diet were not different from those of the HP diet. The proportion of fecal N to intake N was different among treatments. The proportions of urine N to intake N of the 4 LP and LP plus RP-AA diets were also lower, and the proportions

of milk N to intake N of the 4 diets were higher than those of the HP diet ($P < 0.05$). The proportions of urine to MP-intake N of the 4 LP and LP plus RP-AA diets were also lower, and the proportions of milk N to MP-intake N of the 4 diets were higher than those of the HP diet ($P < 0.05$).

Circulating AA

Venous concentrations of the measured AA were generally unaffected by treatments, except for Met, Val, Glu, and Gly (Table 6). Low-protein feeding increased venous concentration of Met and decreased venous Val ($P < 0.05$). Supplementation of RP-AA to the LP diet did not change venous concentrations of these 2 amino acids ($P > 0.05$). Low-protein feeding tended to increase venous concentrations of Glu and Gly.

Circulating Metabolites and Hormones

Venous urea-N concentration of the LP diet was lower than that of the HP diet ($P < 0.01$; Table 7). Supplementing RP-Met or RP-Met plus RP-Thr did not affect venous urea-N concentration of the LP diet. The venous urea-N concentration was elevated by supplementing all 4 RP-AA to the LP diet but was still lower than that of the HP diet ($P < 0.05$). Venous concentrations of the other metabolites and hormones measured were not affected by treatment except for elevated alanine aminotransferase (ALT) concentration in the LPM and LPMT diets relative to those of the HP and LP diets ($P < 0.05$).

Table 4. Effects of dietary protein level and rumen-protected (RP) AA supplementation on milk yield and composition in lactating Holstein cows

Item	Diet ¹					SEM	P-value
	LP	LPM	LPMT	LPMTIL	HP		
Milk yield, kg/d	28.2 ^c	29.4 ^b	29.4 ^b	30.6 ^a	31.5 ^a	1.4	<0.01
ECM, ² kg/d	29.1	31.0	30.5	32.2	31.9	1.3	0.07
Milk protein, %	3.48	3.65	3.58	3.64	3.60	0.09	0.44
kg/d	0.98 ^c	1.06 ^b	1.05 ^b	1.11 ^{ab}	1.13 ^a	0.04	<0.01
Milk fat, %	3.95	4.16	4.02	4.14	3.83	0.21	0.75
kg/d	1.12	1.21	1.17	1.25	1.19	0.07	0.62
Lactose, %	5.25	5.20	5.22	5.23	5.23	0.03	0.57
kg/d	1.49 ^b	1.53 ^b	1.53 ^b	1.60 ^a	1.65 ^a	0.08	<0.01
Total solids, %	12.8	13.1	12.9	13.1	12.7	0.3	0.59
MUN, mg/dL	9.9 ^c	11.2 ^b	11.0 ^b	11.6 ^b	14.9 ^a	0.4	<0.01
Feed efficiency, ³ kg/kg	1.23	1.31	1.35	1.29	1.24	0.07	0.28

^{a,b}Means within a row with different superscript letters are significantly different.

¹HP = 16% CP diet; LP = 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

²Energy-corrected milk, calculated according to Sjaunja et al. (1990): milk yield (kg/d) $\times$ [(38.3 $\times$ % fat $\times$ 10 + 24.2 $\times$ % true protein $\times$ 10 + 16.54 $\times$ % lactose $\times$ 10 + 20.7) $\div$ 3,140].

³Milk yield/DMI.

Table 5. Effects of dietary protein level and rumen-protected (RP) AA supplementation on nitrogen balance in lactating Holstein cows

Item	Diet ¹					SEM	P-value
	LP	LPM	LPMT	LPMTIL	HP		
N intake, g/d	469 ^b	469 ^b	462 ^b	509 ^b	684 ^a	18	<0.01
N secreted and excreted, g/d							
Milk N	157 ^c	167 ^b	170 ^b	178 ^{ab}	181 ^a	7	<0.01
Urine N	94.8 ^b	100.3 ^b	95.6 ^b	113.0 ^b	193.4 ^a	6.7	<0.01
Urinary urea-N (UUN), g/d	68.4 ^c	83.0 ^{bc}	82.5 ^{bc}	93.4 ^b	159.1 ^a	6.6	<0.01
UUN/total urinary N, %	74.7	83.2	86.3	82.6	81.8	3.6	0.12
Fecal N	181 ^b	178 ^b	195 ^b	206 ^{ab}	251 ^a	13	<0.01
N-balance	40.6 ^{ab}	23.9 ^b	6.6 ^b	16.4 ^b	63.1 ^a	13.1	0.04
As percentage of N intake, %							
Milk protein N ²	32.9 ^a	36.0 ^a	36.0 ^a	34.3 ^a	25.9 ^b	1.5	<0.01
Urine N	20.5 ^b	21.5 ^b	20.9 ^b	22.6 ^b	28.5 ^a	1.3	<0.01
Fecal N	38.1	38.1	42.3	40.1	36.6	1.9	0.25
As percentage of MP-N intake, %							
Milk N	45.8 ^b	51.0 ^{ab}	51.3 ^a	48.1 ^b	38.2 ^c	2.2	<0.01
Urine N	28.6 ^b	30.6 ^b	29.7 ^b	31.8 ^b	42.0 ^a	2.0	<0.01

^{a-c}Means within a row with different superscript letters are significantly different.

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

²Nitrogen use efficiency.

Phosphorylation Status of Cell-Signaling Proteins

The ratio of phosphorylated to total protein was determined for several mTOR components and protein kinase B (**AKT**; Table 8). Phosphorylation of mTOR and eIF4E binding protein 1 (**4eBP1**) were decreased by the LP diet relative to the HP diet ($P < 0.01$). These decreases were recovered by co-supplementation

of the 4 RP-AA but not by supplementing RP-Met or RP-Met plus RP-Thr. Although the LP diet promoted the phosphorylation of eEF2 compared with the HP diet, the response was not reversed by supplementing RP-AA to the LP diet. Phosphorylation of ribosomal protein S6 kinase 1 (**S6K1**), eukaryotic initiation factor 2 α subunit (**eIF2 α**), and AKT were not affected by treatments ($P > 0.05$; Figure 1).

Table 6. Effects of dietary protein level and rumen-protected (RP) AA supplementation on concentrations of AA (μM) in venous plasma of lactating Holstein cows

Item	Diet ¹					SEM	P-value
	LP	LPM	LPMT	LPMTIL	HP		
EAA							
Arg	54.2	59.5	60.1	65.4	56.1	3.5	0.20
His	41.0	42.4	39.3	42.6	44.6	4.0	0.69
Ile	98.8	99.0	103.4	101.9	104.0	4.2	0.80
Leu	122	121	127	135	132	6	0.20
Lys	66.1	66.9	67.6	73.2	61.5	3.5	0.26
Met	22.9 ^a	24.5 ^a	25.3 ^a	25.1 ^a	18.3 ^b	1.1	<0.01
Phe	39.7	37.3	40.2	40.8	36.1	1.5	0.13
Thr	80.1	86.5	86.7	89.5	82.6	6.1	0.48
Val	211 ^b	213 ^b	217 ^b	215 ^b	247 ^a	10	0.04
BCAA	432	433	447	453	482	19	0.22
Total EAA	736	751	766	789	782	28	0.64
NEAA							
Ala	228	240	240	238	222	12	0.48
Asp	5.57	5.83	5.83	6.14	5.78	0.36	0.73
Cys	3.78	4.13	3.23	3.41	3.89	0.60	0.83
Glu	50.6 ^a	56.1 ^b	53.7 ^{ab}	56.5 ^b	50.3 ^a	2.7	0.05
Gly	303 ^b	309 ^b	290 ^{ab}	285 ^{ab}	256 ^a	19	0.06
Ser	75.0	76.7	74.4	75.3	68.6	3.8	0.36
Tyr	50.3	48.3	48.5	50.8	45.5	3.2	0.72

^{a,b}Means within a row with different superscript letters are significantly different.

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile and RP-Leu.

Table 7. Effects of dietary protein level and rumen-protected (RP) AA supplementation on venous concentrations of metabolites and hormones in lactating Holstein cows

Item	Diet ¹					SEM	P-value
	LP	LPM	LPMT	LPMTIL	HP		
ALT, ² U/L	26.0 ^c	29.3 ^{ab}	30.5 ^a	28.4 ^{abc}	27.0 ^{bc}	1.4	0.04
AST, ³ U/L	82.1	87.4	90.0	88.2	78.2	7.1	0.38
Albumin, g/L	29.8	31.2	30.8	30.9	30.4	0.7	0.53
Total protein, g/L	72.9	78.3	76.7	77.4	75.4	2.2	0.20
Urea-N, mmol/L	2.63 ^c	2.90 ^c	2.94 ^c	3.53 ^b	5.55 ^a	0.19	<0.01
Glucose, mmol/L	3.50	3.54	3.50	3.51	3.59	0.07	0.87
TG, ⁴ mmol/L	0.13	0.12	0.12	0.13	0.11	0.01	0.35
Insulin, uIU/mL	3.16	3.16	3.52	4.52	3.69	0.83	0.76
Glucagon, pg/mL	130	124	135	130	133	7	0.65
IGF-I, ng/mL	36.9	32.2	29.2	33.8	31.4	2.6	0.19
PRL, ⁵ ng/mL	1.15	1.49	2.05	1.26	1.64	0.27	0.16
Growth hormone, ng/mL	1.42	1.26	1.25	1.44	1.19	0.14	0.60

^{a-c}Means within a row with different superscript letters are significantly different.

¹HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

²ALT = alanine aminotransferase.

³AST = aspartate aminotransferase.

⁴TG = triacylglycerol.

⁵PRL = prolactin.

DISCUSSION

Experimental Diets and DMI

The aim of the present study was to investigate whether MP levels of lactating cows could be reduced by the addition of “signal” AA without sacrificing milk production. Due to the inevitable variations in compositions of the experimental diets, factors other than “signal” AA might also play a part in any observed variations in milk protein yield by treatment. The LP diet was balanced by substituting 9% of soybean meal in the HP diet for an equal amount of steam-flaked corn, which resulted in elevated starch levels in the LP and LP plus RP-AA diets during this experiment. Elevated dietary starch has been reported to promote

milk and milk protein yields (Boerman et al., 2015). Dietary protein level has been related positively to milk protein yields of lactating cows (Haque et al., 2012; Owens and Sapienza, 2014). However, the effect was not consistently observed in previous studies (Colmenero and Broderick, 2006; Lee et al., 2012b), and some authors have attributed this inconsistency to differences in dietary RDP contents across experiments (Weigel et al., 1997; Lee et al., 2012a). The DMI of the HP diet was higher than that of the LP diet in the present study (Table 3). The NE_L concentration was not changed, so NE_L intake must have been increased for HP, simply because of the difference in DMI. Increasing the energy supply has been shown to stimulate milk protein synthesis (Hanigan et al., 1998a). Therefore, the elevated DMI of the HP diet may have also increased

Table 8. Effects of dietary protein level and rumen-protected (RP) AA supplementation on phosphorylation (p) of cell signaling proteins in mammary tissue of lactating Holstein cows

Item ¹	Diet ²					SEM	P-value
	LP	LPM	LPMT	LPMTIL	HP		
p-mTOR/mTOR	0.42 ^b	0.41 ^b	0.44 ^b	0.81 ^a	0.66 ^a	0.07	<0.01
p-4eBP1/4eBP1	0.39 ^b	0.39 ^b	0.24 ^b	0.63 ^a	0.76 ^a	0.07	<0.01
p-S6K1/S6K1	0.68	0.96	0.65	0.46	0.62	0.14	0.17
p-eEF2/eEF2	0.73 ^a	0.83 ^a	0.88 ^a	0.68 ^a	0.40 ^b	0.08	0.02
p-eIF2α/eIF2α	0.67	0.46	0.50	0.43	0.57	0.15	0.14
p-AKT/AKT	0.60	0.72	0.55	0.59	0.57	0.07	0.52

^{a,b}Means within a row with different superscript letters are significantly different.

¹mTOR = mammalian target of rapamycin; 4eBP1 = eIF4E binding protein 1; S6K1 = ribosomal protein S6 kinase 1; eEF2 = eukaryotic elongation factor 2; eIF2α = eukaryotic initiation factor 2; AKT = protein kinase B.

²HP = high-protein, 16% CP diet; LP = low-protein, 12% CP diet; LPM = LP plus RP-Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu.

milk protein yield. The length of the experimental periods might be of concern. There is a time-dependent DMI with different CP, such that the variation in DMI was greater when the experiment was conducted for more than 1 mo (Faverdin et al., 2003). If periods were lengthened, the difference in DMI might be widened between the HP treatment and the LPMTIL treatment, meaning that the data do not seem to support using such low dietary CP and AA supplementation in this study. The spot sampling in the present study may not be as accurate or precise as total urine collection (Spek et al., 2013) because mobilization of body tissue protein biases the creatinine basis for predicting urine output. The 19-d periods followed by 2 d of rest may be too short to reach a nadir for mobilization in terms of spot sampling, so it might be better if periods are lengthened to 1 mo in future studies.

A further consideration is the variation in DMI caused by RP-AA supplementation. Previous studies have found that the effect of RP-AA supplementation was variable (Patton, 2010), had no effect (Korhonen et al., 2002; Weekes et al., 2006; Giallongo et al., 2015), or

DMI was increased because of RP-AA supplementation (Lee et al., 2012a). Previous authors have attributed this inconsistency variously to sources of RP-AA (Zanton et al., 2014), extent of individual EAA deficiency, presence of co-limiting AA (Meijer et al., 1993; Patton, 2010), excessive supplementation (Robinson et al., 2000), and decreased palatability (Benefield et al., 2009). Fortunately, DMI and intakes of measured nutrients were not affected by the 4 LP and RP-AA supplemented diets in the present study (Table 4), which allowed these treatments to be compared on similar CP and energy intake bases.

The predicted duodenal Met supplied by the LP diet (1.89% of MP) was lower than recommended by NRC (2001; see the Materials and Methods section). The positive effect of RP-Met supplementation on milk protein yield may be therefore be due to a mitigation of Met deficiency in the LP diet. The NRC (2001) did not recommend requirements for Thr, Leu, or Ile. However, predicated duodenal Thr, Leu, and Ile flows of the LP diets were lower than the recommendations of Doepel et al. (2004). Although RP-Leu and RP-Ile supplemen-

Figure 1. Representative immunoblots of phosphorylated (P) and total (T) forms of signaling proteins from mammary tissue of lactating Holstein cows. HP = 16% CP diet; LP = 12% CP diet; LPM = LP plus rumen-protected (RP) Met; LPMT = LP plus RP-Met and RP-Thr; LPMTIL = LP plus RP-Met, RP-Thr, RP-Ile, and RP-Leu; mTOR = mammalian target of rapamycin; 4eBP1 = eIF4E binding protein 1; S6K1 = ribosomal protein S6 kinase 1; eEF2 = eukaryotic elongation factor 2; eIF2α = eukaryotic initiation factor 2; AKT = protein kinase B.

tation to the LPMT diet resulted in a further increase in milk protein yield, it was not enhanced by RP-Thr supplementation to the LPM diet.

Milk Yield, Compositions, and N Balance

The reduced milk protein yield caused by the LP diet was partially recovered by RP-Met supplementation but was not enhanced by the addition of RP-Thr (Table 3). The reduction of milk protein due to feeding the LP diet was alleviated by feeding all 4 RP-AA. Thus, according to the single limiting AA theory, these results could be reasonably interpreted as Met being first limiting and either Ile or Leu being second limiting in the LP diet, but it was not easy to discern why and how they were limited, or whether an interaction between the added AA caused this result. Although the addition of Thr to LPM did not increase milk or milk protein yield, it is not clear whether the addition of Thr is required for the complete restoration of milk protein production when all the selected AA were added; this, as well as any dose dependencies, should be the subject of further studies. Supplementation of RP-Met (LPM) or RP-Met plus RP-Thr (LPMT) to the LP diet resulted in negligible variations in cows' DMI and CP intake (Table 4). The elevated milk protein associated with the LPM and LPMT diets may reasonably be attributed to a mass action of the supplemented Met. For the LPMTIL treatment, DMI and CP intakes were 0.87 kg/d and 260 g/d, respectively, higher than that of the LP diet, which, with the observed milk N efficiency of 0.34, contributed 88 of the 130 g of increased milk protein produced.

Feeding LP diets would generally improve the conversion efficiency of dietary N into milk protein in lactating cows (Broderick et al., 2008; Kalscheur et al., 2006). The phenomenon was consistently observed in the present study, as indicated by the higher conversion efficiencies of the 4 LP and LP plus RP-AA diets compared with that of the LP diet ($P < 0.05$; Table 5). Treatments had no significant effects on dietary N digestibility (Table 5), which implies that the improvement in dietary N efficiency was mainly due to a shifted participation of absorbed AA toward milk protein synthesis. Although the underlying mechanism is a topic worth further investigation, one of the possibilities may relate to the differences between the liver and the mammary gland in affinities for extracellular AA. The liver is the major site of ureagenesis in the body (Lobley et al., 1996) and is often considered the tissue responsible for AA catabolism. Although the liver removes substantial amounts of AA, this is not equally observed for all EAA. The BCAA (Leu, Ile, and Val) and ly-

sine show limited removal by the liver and are more available for use by the mammary gland. A modeling analysis based on published literature revealed that liver removal parameters estimated for EAA were 2- to 3-fold lower than those of the mammary gland (Hanigan et al., 1998b). The absorbed AA undergo multiple passes through the liver with a small amount extracted on each pass, which results in a 15-min availability window to peripheral tissues for those AA catabolized almost exclusively by the liver, as confirmed by direct bolus injections in sheep (Lobley and Lapierre, 2003). A decreased supply of Met has been reported to increase its mammary affinity (Guo et al., 2017), which implies that more of the absorbed Met is directed into the mammary gland during the availability window. A variable supply of AA is compensated by mammary blood flow and mammary affinity, often resulting in no significant change. Therefore, an increase in DMI resulting from greater energy supply may not cause greater splanchnic clearance and a resultant increase in protein output.

mTORC1 Signaling in the Mammary Gland

Transcription and translation are activated by mTORC1 through phosphorylation of 4eBP1, eIF4E, and S6K1. The LP diet decreased the phosphorylation of mTOR and 4eBP1 in the present study, implying a changed mTOR signaling pathway ($P < 0.05$; Table 8). The decreased phosphorylation was not improved by supplementation of RP-Met or RP-Thr but was fully recovered by supplementation of either RP-Ile or RP-Leu or both. These results were generally consistent with those of several previous *in vitro* studies. The phosphorylation of mTORC1 substrates was substantially decreased in the bovine mammary gland by removing all the essential AA from the medium (Burgos et al., 2010). However, some studies have revealed that mechanisms other than phosphorylation of mTORC1 are responsible for long-term nutritional effects on milk protein yield (Cant et al., 2018). Removal of either Ile or Leu reduced mTOR phosphorylation and the fractional synthesis rate of casein in bovine mammary tissue (Appahamy et al., 2012). The modeling work of Castro et al. (2016) indicated that mTOR may be sensitive to Met, Thr, Ile, and Leu, and this signal appeared to be transduced by mTOR into greater protein synthesis. The results of the present study support these postulations, with the exception of leaving the role of Thr undefined.

Activated mTORC1 will phosphorylate translation inhibitor 4eBP1, releasing free eIF4E to form complex with other eukaryotic translation initiation factors (4G,

4A, and 4B) to remove hairpin loops in the 5' untranslated regions of mRNA to start translation. The ratio of phosphorylated 4eBP1 was lower for the LP diet than for the HP diet ($P < 0.05$, Table 8), which implies the contribution of 4eBP1 to decreased milk protein yield in the LP diet. Phosphorylation of 4eBP1 was not improved by supplementation of RP-Met and RP-Thr, although milk protein yields of the LPM and LPMT diets were higher than that of the LP diet. Consistent with full recovery of milk protein yield, 4eBP1 phosphorylation of the LPMTIL diet was restored to the level of the HP diet.

Activated S6K1 promotes protein synthesis through the recruitment of the upstream regulator S6K1 to eIF3, which can phosphorylate eIF4B (Holz et al., 2005), and activated AKT enhances the activity of mTORC1 by suppressing the negative regulation of the tuberous sclerosis complex (TSC1/2) on mTORC1. Although eIF2 is not a member of the mTOR signal pathway, phosphorylation of its α subunit inhibits protein synthesis. Treatments in the present study had no significant effects on the phosphorylation of these 3 proteins. The elongation factor eEF2 is an essential factor for protein synthesis and is also a downstream protein of the mTOR signal pathway. Low-protein diets activated the protein, as indicated by higher phosphorylation ratios in the LP and LP plus RP-AA diets than that of the HP diet (Table 8). Phosphorylation status of the eEF2 factor was elevated by LP diets in the present study, which implies that this was a negative modulator of milk protein synthesis when AA supply was limited.

CONCLUSIONS

In agreement with our hypothesis, supplementation of selected EAA to the LP diet recovered the lost milk caused by feeding LP alone. Further, under the conditions of the present study, supplementation of Met alone partially restored the loss of milk protein production; however, only when Met, Thr, Ile, and Leu were supplemented together was the decreased milk protein restored. Low-protein diets appeared to decrease milk protein yield by depressing DMI and reduce the phosphorylation status of mTOR in the mammary gland. The effects on DMI and mTOR appeared to be restored upon supplementation of all 4 EAA.

ACKNOWLEDGMENTS

This work was funded by the Natural Science Foundation of China (31572427, Beijing), the Modern Agriculture Research System of China (CARS-37, Beijing), and the Modern Agriculture Research System of Shandong Province (Jinan, China).

REFERENCES

- Aldrich, J. M., L. A. Holden, L. D. Muller, and G. A. Varga. 1996. Rumen availabilities of nonstructural carbohydrate and protein estimated from in situ incubation of ingredients versus diets. *Anim. Feed Sci. Technol.* 63:257–271.
- Anthony, J. C., C. H. Lang, S. J. Crozier, T. G. Anthony, D. A. MacLeam, S. R. Kimball, and L. S. Jefferson. 2002. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. *Am. J. Physiol. Endocrinol. Metab.* 282:E1092–E1101.
- AOAC. 1990. Official Methods of Analysis. Vol. I. 15th ed. Association of Official Analytical Chemists, Arlington, VA.
- Appuhamy, J. A., A. L. Bell, W. A. Nayananjali, J. Escobar, and M. D. Hanigan. 2011. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 141:1209–1215.
- Appuhamy, J. A., N. A. Knoebel, W. A. Nayananjali, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 142:484–491.
- Arriola Apelo, S. I., L. M. Singer, X. Y. Lin, M. L. McGilliard, N. R. St-Pierre, and M. D. Hanigan. 2014. Isoleucine, leucine, methionine, and threonine effects on mammalian target of rapamycin signaling in mammary tissue. *J. Dairy Sci.* 97:1047–1056.
- Benefield, B. C., R. A. Patton, M. J. Stevenson, and T. R. Overton. 2009. Evaluation of rumen-protected methionine sources and period length on performance of lactating dairy cows within Latin squares. *J. Dairy Sci.* 92:4448–4455.
- Berthiaume, R., M. C. Thivierge, R. A. Patton, P. Dubreuil, M. Stevenson, B. W. McBride, and H. Lapierre. 2006. Effect of ruminally protected methionine on splanchnic metabolism of amino acids in lactating dairy cows. *J. Dairy Sci.* 89:1621–1634.
- Boerman, J. P., S. B. Potts, M. J. VandeHaar, M. S. Allen, and A. L. Lock. 2015. Milk production responses to a change in dietary starch concentration vary by production level in dairy cattle. *J. Dairy Sci.* 98:4698–4706.
- Broderick, G. A., M. J. Stevenson, and R. A. Patton. 2009. Effect of dietary concentration and degradability on response to rumen-protected methionine in lactating dairy cows. *J. Dairy Sci.* 92:2719–2728.
- Broderick, G. A., M. J. Stevenson, R. A. Patton, N. E. Lobos, and J. J. Olmos Colmenero. 2008. Effect of supplementing rumen-protected methionine on production and nitrogen excretion in lactating dairy cows. *J. Dairy Sci.* 91:1092–1102.
- Burgos, S. A., M. Da, and J. P. Cant. 2010. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J. Dairy Sci.* 93:153–161.
- Calsamiglia, S., and M. D. Stern. 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. *J. Anim. Sci.* 73:1495–1465.
- Cant, J. P., J. J. M. Kim, S. R. Cieslar, and J. Doelmant. 2018. Symposium review: Amino acid uptake by the mammary glands: Where does the control lie? *J. Dairy Sci.* 101:5655–5666.
- Castro, J. J., S. A. Apelo, J. A. D. R. N. Appuhamy, and M. D. Hanigan. 2016. Development of a model describing regulation of casein synthesis by the mammalian target of rapamycin (mTOR) signaling pathway in response to insulin, amino acids, and acetate. *J. Dairy Sci.* 99:6714–6736.
- Colmenero, J. J., and G. A. Broderick. 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. *J. Dairy Sci.* 89:1704–1712.
- Doelman, J., R. V. Curtis, M. Carson, J. J. Kim, J. A. Metcalf, and J. P. Cant. 2015. Essential amino acid infusions stimulate mammary expression of eukaryotic initiation factor 2B ϵ but milk protein yield is not increased during an imbalance. *J. Dairy Sci.* 98:4499–4508.
- Doepel, L., D. Pacheco, J. J. Kennelly, M. D. Hanigan, I. F. Lopez, and H. Lapierre. 2004. Milk protein synthesis as a function of amino acid supply. *J. Dairy Sci.* 87:1279–1297.

- Faverdin, P., D. M'hamed, M. Rico-Gomez, and R. Verite. 2003. La nutrition azotée influence l'ingestion chez la vache laitière. *Prod. Anim.* 16:27–37.
- Giallongo, F., A. N. Hristov, J. Oh, T. Frederick, H. Weeks, J. Werner, H. Lapierre, R. A. Patton, A. Gehman, and C. Parys. 2015. Effects of slow-release urea and rumen-protected methionine and histidine on performance of dairy cows. *J. Dairy Sci.* 98:3292–3308.
- Guo, C. L., Y. T. Li, X. Y. Lin, M. D. Hangian, Z. G. Yan, Z. Y. Hu, Q. L. Hou, F. G. Jiang, and Z. H. Wang. 2017. Effects of graded removal of lysine from an intravenously infused amino acid mixture on lactation performance and mammary amino acid metabolism in lactating goats. *J. Dairy Sci.* 100:4552–4564.
- Hanigan, M. D., J. P. Cant, D. C. Weakley, and J. L. Beckett. 1998a. An evaluation of post-absorptive protein and amino acid metabolism in the lactating dairy cow. *J. Dairy Sci.* 81:3385–3401.
- Hanigan, M. D., J. France, D. Wray-Cahen, D. E. Beever, G. E. Lobley, L. Reutzel, and N. E. Smith. 1998b. Alternative models for analyses of liver and mammary transorgan metabolite extraction data. *Br. J. Nutr.* 79:63–78.
- Haque, M. N., H. Rulquin, A. Andrade, P. Faverdin, J. L. Peyraud, and S. Lemosquet. 2012. Milk protein synthesis in response to the provision of an “ideal” amino acid profile at 2 levels of metabolizable protein supply in dairy cows. *J. Dairy Sci.* 95:5876–5887.
- He, L., J. Zhang, J. Zhao, N. Ma, S. W. Kim, S. Qiao, and X. Ma. 2018. Autophagy: The last defense against cellular nutritional stress. *Adv. Nutr.* 9:493–504.
- Holz, M. K., B. A. Ballif, S. P. Gygi, and J. Blenis. 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* 123:569–580.
- Jewell, J. L., R. C. Russell, and K. L. Guan. 2013. Amino acid signaling upstream of mTOR. *Nat. Rev. Mol. Cell Biol.* 14:133–139.
- Kalscheur, K. F., R. L. Baldwin VI, B. P. Glenn, and R. A. Kohn. 2006. Milk production of dairy cows fed differing concentrations of rumen-degraded protein. *J. Dairy Sci.* 89:249–259.
- Korhonen, M., A. Vanhatalo, and P. Huhtanen. 2002. Evaluation of isoleucine, leucine, and valine as a second-limiting amino acid for milk production in dairy cows fed grass silage diet. *J. Dairy Sci.* 85:1533–1545.
- Lee, C., A. N. Hristov, T. W. Cassidy, K. S. Heyler, H. Lapierre, G. A. Varga, M. J. de Veth, R. A. Patton, and C. Parys. 2012a. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. *J. Dairy Sci.* 95:6042–6056.
- Lee, C., A. N. Hristov, K. S. Heyler, T. W. Cassidy, H. Lapierre, G. A. Varga, and C. Parys. 2012b. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. *J. Dairy Sci.* 95:5253–5268.
- Liu, G. M., M. D. Hanigan, X. Y. Lin, K. Zhao, F. G. Jiang, Y. Wang, Z. Y. Hu, and Z. H. Wang. 2017. Methionine, leucine, isoleucine, or threonine effects on mammary cell signaling and pup growth in lactating mice. *J. Dairy Sci.* 100:4038–4050.
- Lobley, G. E., and H. Lapierre. 2003. Post-absorptive metabolism of amino acids. Pages 737–756 in *Progress in Research on Energy and Protein Metabolism*. W. B. Souffrant and C. C. Metges, ed. Publication no. 109. European Association for Animal Production, Rome, Italy.
- Lobley, G. E., P. J. Weijjs, A. Connell, A. G. Calder, D. S. Brown, and E. Milne. 1996. The fate of absorbed and exogenous ammonia as influenced by forage or forage-concentrate diets in growing sheep. *Br. J. Nutr.* 76:231–248.
- Meijer, G. A., J. Van der Meulen, and A. M. Van Vuuren. 1993. Glutamine is a potentially limiting amino acid for milk production in dairy cows: A hypothesis. *Metabolism* 42:358–364.
- Nadeau, E., J. E. Englund, and A. H. Gustafsson. 2007. Nitrogen efficiency of dairy cows as affected by diet and milk yield. *Livest. Sci.* 111:45–56.
- Nahm, K. H. 2002. Efficient feed nutrient utilization to reduce pollutants in poultry and swine manure. *Crit. Rev. Environ. Sci. Technol.* 32:1–16.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. National Academy of Science, Washington, DC.
- Ørskov, E. R., and I. McDonald. 1979. The estimate of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J. Agric. Sci.* 92:499–503.
- Owens, F. N., and D. A. Sapienza. 2014. Invited review: Applied protein nutrition of ruminants—Current status and future directions. *Prof. Anim. Sci.* 30:150–179.
- Patton, R. A. 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. *J. Dairy Sci.* 93:2105–2118.
- Robinson, P. H., W. Chalupa, C. J. Sniffen, W. E. Julien, H. Sato, T. Fujeda, T. Ueda, and H. Suzuki. 2000. Influence of abomasal infusion of high levels of lysine or methionine, or both, on ruminal fermentation, eating behavior, and performance of lactating dairy cows. *J. Anim. Sci.* 78:1067–1077.
- Semba, R. D., I. Trehan, M. Gonzalez-Freire, K. Kraemer, R. Moaddel, M. Ordiz, L. Ferrucci, and M. J. Manary. 2016. Perspective: The potential role of essential amino acid and the mechanistic target of rapamycin complex 1 (mTORC1) pathway in the pathogenesis of child stunting. *Adv. Nutr.* 7:853–865.
- Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setälä. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. *Proc. 27th Session Int. Comm. Breeding Product. Milk Anim.*, Paris, France. Wageningen Acad. Publ., Wageningen, the Netherlands.
- Socha, M. T., D. E. Putnam, B. D. Garthwaite, N. L. Whitehouse, N. A. Kierstead, C. G. Schwab, G. A. Ducharme, and J. C. Robert. 2005. Improving intestinal amino acid supply of pre- and postpartum dairy cows with rumen-protected methionine and lysine. *J. Dairy Sci.* 88:1113–1126.
- Spears, R. A., R. A. Kohn, and A. J. Young. 2003. Whole-farm nitrogen balance on western dairy farms. *J. Dairy Sci.* 86:4178–4186.
- Spek, J. W., J. Dijkstra, G. van Duinkerken, W. H. Hendriks, and A. Bannink. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. *J. Dairy Sci.* 96:4310–4322.
- Tomé, D. 2018. 90th anniversary commentary: The mTORC1 complex—A central player in the control and regulation of amino acid sufficiency. *J. Nutr.* 148:1678–1682.
- Valadares, R. F., G. A. Broderick, S. C. Valadares Filho, and M. K. Clayton. 1999. Effect of replacing alfalfa silage with high moisture corn on ruminal protein synthesis estimated from excretion of total purine derivatives. *J. Dairy Sci.* 82:2686–2696.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharide in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597.
- Weekes, T. L., P. H. Luimes, and J. P. Cant. 2006. Responses to amino acid imbalances and deficiencies in lactating dairy cows. *J. Dairy Sci.* 89:2177–2187.
- Weigel, D. J., J. P. Elliott, and J. H. Clark. 1997. Effects of amount and ruminal degradability of protein on nutrient digestibility and production by cows fed tallow. *J. Dairy Sci.* 80:1150–1159.
- Yang, W. Z., and K. A. Beauchemin. 2007. Altering physically effective fiber intake through forage proportion and particle length: Chewing and ruminal pH. *J. Dairy Sci.* 90:2826–2838.
- Zanton, G. I., G. R. Bowman, M. Vazquez-Anon, and L. M. Rode. 2014. Meta-analysis of lactation performance in dairy cows receiving supplemental dietary methionine sources or postruminal infusion of methionine. *J. Dairy Sci.* 97:7085–7101.