Effect of protein level and methionine supplementation on dairy cows during the transition period

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ABSTRACT

Cows experience a significant negative protein balance during the first 30 d of lactation. Given the functional effects of AA on health, especially in challenging periods such as calving, higher levels of protein and specific AA in the diet may act to improve health and feed intake. The response of dairy cows to 3 protein supplementation strategies during the transition period and through the first 45 d in milk was evaluated. The final data set had 39 Holstein cows blocked based on parity (primiparous vs. multiparous) and expected calving and randomly assigned within each block to one of 3 dietary treatments: low protein (LP), high protein (HP), or high protein plus rumen-protected methionine (HPM). Treatments were offered from d -18 ± 5 to 45 d relative to parturition. Pre- and postpartum diets were formulated for high metabolizable protein (MP) supply from soybean meal, and HP and HPM provided higher MP balance than LP. Preplanned contrasts were LP versus HP+HPM and HP versus HPM. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Cows fed HP and HPM had greater fry matter intake (DMI) prepartum than LP ($+2$ kg/d), and there was a trend for greater DMI with HPM than with HP ($+1.6$ kg/d). Body weight and condition score before and after calving did not differ among treatments. High protein (HP and HPM) tended to increase milk yield during the first 45 d of lactation ($+1.75$ kg/d), increased milk lactose content and urea-N in milk and plasma, tended to increase blood BHB 14 d postpartum, and tended to reduce milk/DMI compared with LP. Blood concentrations of calcium at calving and of glucose, and nonesterified fatty acids pre- and postpartum did not differ. High protein induced lower concentration of plasma IL-1 at calving and lowered blood lymphocytes 21 d postpartum, suggestive of a reduced inflammatory status compared with LP. The concentrations of IL-10, tumor necrosis factor alpha, and other hemogram variables did not differ among treatments. Addition of rumen-protected methionine to the HP diet did not alter milk yield but increased fat and total solids concentrations. The rumen-protected methionine had no effect on blood metabolites and immunity markers, with the exception of increased pre-partum insulin concentrations. The data indicate that dairy cows around calving respond positively to an increase in the supply of MP and to rumen-protected methionine supplementation of the HP diet by increasing intake and improving immune status.
Key words: dry matter intake, blood markers, milk fat, immune system

INTRODUCTION

The onset of lactation markedly increases nutrient requirements of dairy cows (Bell et al., 1995). The concomitant reduction in DMI around calving predisposes transition cows to a negative nutrient balance (Pickett et al., 2003). Negative energy balance and its health-related consequences have been extensively studied over the last decades, resulting in several dietary recommendations for the transition period regarding energy (Mann et al., 2015). On the other hand, the protein negative balance during transition has received less attention. High-yielding dairy cows may mobilize as much as 1 kg of tissue protein/d from skeletal muscle during the first 7 to 10 d of lactation to meet AA requirements (Bell et al., 2000). Additionally, protein mobilization starts before parturition, likely to meet amino acid requirements for growth of fetus, uterus,

and mammary tissue (Bell et al., 2000). In fact, these authors estimated that NRC (2001) underestimates the MP requirements of the late-pregnant dry cows.

Amino acid mobilization from muscle protein during the transition period is confirmed both by ultrasound imaging (Van der Drift et al., 2012) and by the ratio between plasma concentrations of 3-methylhistidine and creatinine (Pires et al., 2013). Moreover, the magnitude of this protein mobilization seems to be related to the amount of muscle mass before calving (McCabe and Boerman, 2020). Prepartum dietary protein levels influence protein partitioning for tissue accretion among carcass, organs, and mammary gland. Late-pregnant ewes fed 8, 12, or 16% CP diets showed carcass-protein deposition only with the higher protein diet (McNeill et al., 1997). Therefore, protein supplementation strategies capable of increasing MP supply have the potential to increase muscle mass before calving, improving the labile protein reserves in maternal tissues that can be used by the animal after calving. Moreover, increasing MP supply by abomasally infusing casein or AA during the first 30 d of lactation greatly improved milk yield (Larsen et al., 2014, 2015), suggesting that during that period the mammary gland is very responsive to extra protein supply.

Some AA have functional roles that may help the cow's transition through the onset of lactation. Metabolic challenges during the transition period can result in reduced hepatic function, along with increased inflammation and oxidative stress (Osorio et al., 2014). Depressed immune function compromises the ability of cows to resist disease, especially during the challenges associated with parturition. Methionine is often the most limiting AA for milk protein synthesis (Schwab et al., 1976), as well as a precursor of important antioxidants and a methyl donor for synthesis of choline and carnitine, both of which are involved in lipid metabolism (Chandler and White, 2017). Previous studies have reported the beneficial effects of rumen-protected Met (**RPMet**) on the performance of dairy cows when fed during the entire transition period (Osorio et al., 2013; Zhou et al., 2016b) and through peak lactation (Batistel et al., 2017). Positive responses have also been reported in health-related variables to RPMet supplementation during the transition period (Osorio et al., 2014; Zhou et al., 2016a; Vailati-Riboni et al., 2017). Blood, milk, and liver biomarkers have indicated that at least part of the effect of Met supplementation on milk production is due to improved immune status (Zhou et al., 2016a). Whether this positive effect on performance and immunity in those studies is a direct effect of Met or an indirect effect of increased DMI is not clear.

Even though both protein supply and Met supplementation have shown positive effects during the transition period and early lactation, these 2 factors have not been combined experimentally as a feeding strategy during this period. Our hypothesis is that greater MP, specifically balanced for Met, is beneficial to support the transition to lactation. Therefore, the objective of this study was to examine the effect of 3 protein feeding strategies during the close-up period through early lactation on intake, milk yield, and health-related variables.

MATERIALS AND METHODS

Animals and Diets

The experiment was conducted in a tie-stall barn at the Better Nature Research Center, located in Ijaci, Minas Gerais, Brazil. Protocols for this study were approved by the University of Lavras Committee for Animal Use in Research, under protocol 049/2016. All cows were in apparent good health at the beginning of the study. Although 46 cows started the experiment, 7 were removed due to reasons unrelated to the treatments (injured leg, injury in right radial nerve, abortion, twin birth, clinical mastitis, death, and unexplained agalactia). Therefore, 39 cows (13 primiparous and 26 multiparous) completed the study and were used in the statistical analysis. All cows calved between January 20, 2016, and August 9, 2016, and the entire experimental period lasted 264 d.

Cows were blocked based on parity (primiparous vs. multiparous) and expected day of calving, and within each block, were randomly assigned to one of 3 treatments. Experimental diets were offered at d 259 of gestation (18 ± 5 SD d prepartum) and continued through 45 d postpartum. Pre- and postpartum diets were formulated (NRC, 2001) to contain 2 levels of MP, referred as low protein (**LP**) and high protein (**HP**), as well as an HP diet with RPMet supplementation (**HPM**; Table 1). Within each treatment, the number of primiparous and multiparous cows were, respectively: LP (5 and 8), HP (4 and 9), HPM (4 and 9). Main ingredients used to formulate the experimental diets were corn silage (49.6% NDF, 19.1% starch, 8.6% CP), oat hay (62.8% NDF, 2.1% starch, 7.2% CP), soybean meal (15.0% NDF, 2.4% starch, 49.0% CP), high RUP soybean meal (10.8% NDF, 0.6% starch, 49.1% CP), corn grain (9.0% NDF, 74.8% starch, 9.2% CP), whole cottonseed (39.6% NDF, 0.4% starch, 20.3% CP), and citrus pulp (23.9% NDF, 4.3% starch, 7.3% CP). The LP diets were formulated to represent an adequate protein diet based on NRC (2001) recommendations with

Table 1. Composition of pre- and postpartum diets and model predicted nutrient balance during the experiment (NRC, 2001) on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM)

Item	Prepartum			Postpartum		
	LP	HP	HPM	LP	HP	HPM
Ingredient, % of DM						
Corn silage	66.4	66.3	66.3	45.1	45.1	45.1
Oat hay	4.5	4.5	4.5	3.8	3.8	3.8
Soybean meal	8.2			17.9	9.1	9.1
High RUP soybean meal[1]		13.2	13.2		13.3	13.3
Finely ground mature corn	15.8	10.9	10.8	14.5	14.5	14.5
Whole cottonseeds				8.1	8.1	8.1
Citrus pulp				7.8	3.4	3.3
Urea	0.7	0.7	0.7			
Limestone				0.9	0.9	0.9
Sodium bicarbonate				0.8	0.8	0.8
Magnesium oxide				0.3	0.3	0.3
Salt				0.2	0.2	0.2
Prepartum premix[2]	4.4	4.4	4.4			
Postpartum premix[3]				0.4	0.4	0.4
Rumen-protected Met[4]			0.09			0.13
CP	13.8	15.6	15.8	16.3	18.4	18.6
NDF	39.3	39.7	39.5	32.4	32.8	32.5
Ether extract	4.1	4.2	4.3	4.6	4.8	4.9
Starch	24.6	20.8	20.7	20.4	20.1	20.1
Ash	10.3	10.5	10.4	8.8	8.8	8.8
NFC[5]	32.5	30.0	29.9	37.9	35.2	35.2
RDP balance, g/d	−25	45	63	19	121	143
RDP supplied, % of required	98	103	105	101	106	108
MP balance, g/d	262	496	585	−269	−66	−17
MP supplied, % of required	135	164	172	86	97	99
NE_L balance, Mcal/d	5.3	7.7	9.3	−4.1	−2.9	−2.1
NE_L supplied, % of required	140	159	171	87	91	94
Lys, % of MP	7.11	6.88	6.85	6.51	6.28	6.26
Met, % of MP	2.04	1.95	2.36	1.79	1.74	2.10
Lys/Met	3.48	3.53	2.90	3.64	3.61	2.98
DCAD, mEq/kg of DM	−204	−178	−178	—	—	—

[1]SoypassBR (Cargill Nutrição Animal).

[2]30 g/kg Ca; 14 g/kg P; 50 g/kg Mg; 40 g/kg S; 15 g/kg Na; 95 g/kg Cl; 12 mg/kg Co; 250 mg/kg Cu; 500 mg/kg Mn; 1,600 mg/kg Zn; 6 mg/kg Se; 20 mg/kg I; 132.000 UI/kg vit A; 36.000 UI/kg vit D; 3.000 UI/kg vit E; 400 mg/kg monensin; 250 g/kg calcium salts of soybean fatty acids (Megalac E, Vaccinar Indústria e Comércio Ltda).

[3]200 g/kg Ca; 156 g/kg P; 35 g/kg S; 31g/kg Mg; 150 mg/kg Co; 2,000 mg/kg Cu; 5,000 mg/kg Mn; 11,900 mg/kg Zn; 82 mg/kg Se; 200 mg/kg I; 1,000 KUI/kg vit A; 220 KUI/kg vit D; 6,200 UI/kg vit E.

[4]Mepron (Evonik).

[5]NFC was calculated by difference [100 − (%NDF + %CP + %Fat + %Ash)].

CP concentration representative of what is routinely used in many commercial farms and with soybean meal as the major CP source. For the HP diet, the goal was to create a big enough difference without getting out of practical level. High-protein treatments (HP and HPM) had higher MP balance compared with the LP diet, due to the increase in RUP from slowly degradable soybean meal (SoypassBR, Cargill).

The HPM diet was adjusted to obtain a formulated 3:1 ratio of Lys:Met using a commercial RPMet source (Mepron, Evonik). Mepron was assumed to contain 85% DL-Met, with 80% of rumen escape (Overton et al., 1996) and 90% of intestinal digestibility (Schwab, 1995). Therefore, for each 10 g of Mepron added to the diet, the estimated metabolizable Met was 6.1 g. Values for dLys, dMet, and Lys to Met ratios are presented in Table 1.

Cows were individually fed a TMR offered in equal proportions at 0700 and 1300 h, allowing for 5 to 10% orts. The dose of Mepron was weighed using a precision scale and premixed with a small amount of concentrate before being added to the batch for mixing.

Data and Sample Collection and Analysis

Dry matter intake was measured daily. Diet nutrient composition was determined from weekly feed samples that were composited by daily subsamples. Similarly, orts were sampled daily and composited weekly per cow. Composite samples were oven-dried at 55°C for 72

h and then ground through a Wiley mill fitted with a 1-mm sieve screen (Arthur H. Thomas Co.). The DM concentration was determined by drying at 100°C for 24 h, and CP was determined by micro-Kjeldahl analysis (AOAC International, 1990). Ether extract (**EE**) was analyzed following the Randall method (AOAC method 2003.05) with submersion in petroleum ether (AOAC International, 2012). Ash content was analyzed by incineration at 550°C for 8 h. The ash-free NDF was determined by filtration in porous crucibles with addition of thermostable amylase and sodium sulfite (Van Soest et al., 1991). The NFC fraction was calculated as: $NFC = 100 - (CP + EE + ASH + NDF)$. Starch content was analyzed enzymatically according to Hall (2009). The values for NEL, RUP, RDP, MP, Met, and Lys were predicted using the NRC (2001).

Cows were milked 3 times/d starting at 0500, 1300, and 2000 h, and milk yield was recorded at every milking. Milk samples were collected twice weekly from each milking and mixed in proportion to the milk yield. Samples were analyzed for fat, protein, lactose, total solids, SCC, and MUN by mid-infrared analysis (Bentley Instruments Inc.) in a commercial laboratory (Centralized Laboratory of the Parana State Holstein Breeders Association). Energy secreted in milk (**MILK E**, Mcal/d) was calculated as: $[(0.0929 \times \% \text{ Fat}) + (0.0547 \times \% \text{ Protein}) + (0.0395 \times \% \text{ Lactose})] \times$ kg of milk (NRC, 2001). Energy corrected milk (kg/d) was calculated as: MILK E/0.70 (assuming 0.70 Mcal/kg for milk with 3.7% fat, 3.2% protein, and 4.6% lactose). Feed efficiency was calculated by dividing milk yield by DMI and ECM by DMI.

The SCC was log-transformed to a linear scale from 0 to 9 (**Linear SCC**) in which scores represented the following values of SCC ($\times$ 1,000 cells/mL): 12.5 for SCC score 0; 25 for SCC score 1; 50 for SCC score 2; 100 for SCC score 3; 200 for SCC score 4; 400 for SCC score 5; 800 for SCC score 6; 1,600 for SCC score 7; 3,200 for SCC score 8; and 6,400 for SCC score 9. The SCC score was calculated from the natural logarithm of the measured SCC (1,000 cells/mL) and the above-metioned SCC value of each score: $\text{SCC score} = -3.6438 + 1.4427 \times \text{Ln(SCC)}$. Negative values were rounded to zero.

Body condition score was evaluated using a 1 to 5 scale according to Wildman et al. (1982) by 3 independent evaluators on d -18 ± 5 and -8 ± 5 prepartum, at calving (d 0), and at d 7, 14, 21, and 28 postpartum, and scores were averaged for each cow. Body weight was measured immediately after the morning milking on the same day as BCS. Udder edema was scored on d 0, 7, and 14 postpartum on a 1 to 9 scale (no edema to severe edema) according to Tucker et al. (1992). Urine

pH was measured on d -8 ± 5 prepartum with a pH meter on 3 samples obtained at 1000, 1400, and 1800 h.

Blood Collection and Analysis of Metabolites

All blood samples were collected from a coccygeal vessel. For the analysis that needed plasma separation, samples were centrifuged at 2,000 $\times$ g for 10 min at room temperature, and plasma was frozen at -20°C until analysis. For glucose analysis, samples were taken with vacuum tubes containing EDTA and potassium fluoride on d -8 ± 5 prepartum and 7, 14, and 21 postpartum immediately before (T0) and 12 h after (T12) the first daily feeding (T0). Plasma glucose was analyzed by a colorimetric enzymatic method without deproteinization in an auto biochemistry analyzer (HumaStar 300SR).

Plasma total calcium concentration was analyzed by o-cresolphthalein-complexone method with an auto biochemistry analyzer (HumaStar 300, HUMAN) on samples collected immediately after calving and 24 h after calving in vacuum tubes containing sodium heparin. For the evaluation of humoral immune responses, IL-1, IL-10, and tumor necrosis factor alpha (**TNFα**) plasma concentrations were also analyzed with a commercial kit (Bovine kit; NeoBiolab) for samples collected on the day of calving. Cellular immune response was evaluated by hemogram in a commercial laboratory (Laboratório Santa Cecília) within 1 h of blood sampling for samples collected into tubes containing EDTA at 12 h after feeding on d 21 after calving.

Plasma urea nitrogen (**PUN**) was analyzed with a commercial kit (Urea 500, Doles Reagents Laboratories) on samples taken into EDTA tubes at 0 h before and 2, 6, 9, 12, and 18 h after the first morning feeding on d -8 ± 5 prepartum and d 7 and 21 postpartum.

Samples taken on d -8 ± 5 prepartum and d 7, 14 and 21 postpartum, 12 h after the first feeding, were analyzed for serum BHB, nonesterified fatty acids (**NEFA**), and insulin concentration (tubes with clot activator). An auto biochemistry analyzer was used for BHB (kit RB1007) and NEFA (kit FA 115) analysis (Bioclin BS-200E). Insulin was analyzed with a multi-species radioimmunoassay kit (Millipore, Cat. # XL-85K).

Statistical Analysis

Feed intake, BW, and BCS data were analyzed separately for prepartum and postpartum periods. Data were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute Inc.) with repeated measures according to the following model: $Y_{ijk} = \mu + B_i + A_j +$

$T_k + A \times T + e_{ijk}$. Where: μ = the overall mean, B_i = the random effect of the block, A_j = the fixed effect of the treatment (j = LP, HP, HPM), T_k = the fixed effect of time (j = days, weeks, hours after morning feeding), $A \times T$ = the interaction of A_j and T_k, and $e_{ij}k$ = the residual error. The mean square for the effect of cow nested within treatments was the whole plot error term to test the treatment effect. The best covariance structure was defined by the Akaike's information criterion among first-order autoregressive, unstructured, and compound symmetry. The effect of dietary protein level was assessed by the contrast of LP versus HP and HPM, whereas the effect of RPMet supplementation was tested by the contrast of HP versus HPM. Statistical significance and trends were considered at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

Figure 1. Dry matter intake pre- and postpartum on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM). SEM: Prepartum: LP = 0.648, HP = 0.646, HPM = 0.663; postpartum: LP = 0.743, HP = 0.752, HPM = 0.772.

RESULTS

Data from the prepartum period are presented in Table 2. Cows fed the HP and HPM diets consumed 2.0 kg/d more feed than LP-fed cows during the prepartum period ($P < 0.01$). Additionally, there was a tendency for greater DMI for cows fed HPM compared with HP (+1.6 kg/d; $P = 0.07$). There was an effect of day of gestation and day of lactation on DMI (Figure 1) but no treatment × day effect for DMI in either the pre- or postpartum periods. Prepartum BW, BCS, and urine pH did not differ ($P \geq 0.78$).

Postpartum intake and performance data are in Table 3. There was a trend for greater protein supply in HP and HPM to increase milk (+1.75 kg/d; $P = 0.10$) and lactose (+115 g/d; $P = 0.07$) yields. Daily milk yield is shown in Figure 2. Feed efficiency, as kg milk/kg DMI, tended to respond to protein level (LP > HP+HPM; $P = 0.08$) and to RPMet supplementation (HP > HPM; $P = 0.08$). However, feed efficiency calculated as ECM/kg DMI (ECM/DMI) was not affected. Likewise, lactose concentration increased with greater protein supply (LP < HP+HPM; $P = 0.03$) and tended to
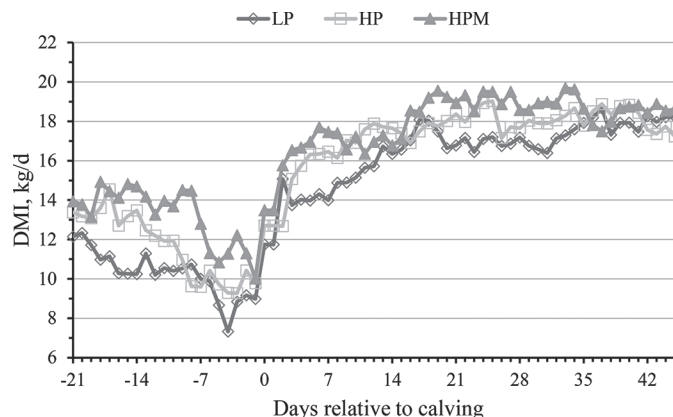
increase with RPMet supplementation (HP < HPM; $P = 0.07$). Supplementation of RPMet increased milk fat ($P = 0.04$) and total solids concentrations ($P = 0.02$). Greater protein supply increased MUN compared with LP ($P < 0.01$). Body weight, BCS, linear SCC, and udder edema did not differ.

Pre- and postpartum blood metabolites are reported in Table 4. Prepartum serum insulin concentration was increased by RPMet (+6.6 µU/mL relative to HP; $P = 0.05$). Prepartum concentrations of PUN, glucose, NEFA, and BHB did not differ ($P \geq 0.12$). On the other hand, high protein increased PUN on d 21 postpartum ($P < 0.01$) and tended to increase BHB on d 14 ($P = 0.10$). Postpartum concentrations of glucose, insulin, and NEFA and calcium at calving were not affected by the treatments ($P \geq 0.19$).

Immune health-related blood variables are presented in Table 5. The HP diets decreased IL-1 at calving ($P = 0.03$) and lymphocytes 21 d postpartum ($P < 0.01$) relative to LP. Interleukin-10, TNFα, erythrocytes, hemoglobin, hematocrit, neutrophils, eosinophils, mono-

Table 2. Prepartum DMI, BW, BCS, and urine pH on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM), n = 13/treatment

	Treatment				P-value[1]			
Item	LP	HP	HPM	SEM	D	Trt × D	LP vs. HP+HPM	HP vs HPM
DMI,[2] kg/d	10.4	11.6	13.2	0.7	<0.01	0.26	<0.01	0.07
BW,[3] kg	675	663	658	19	0.44	0.88	0.51	0.83
BCS,[3] 1 to 5	3.33	3.40	3.44	0.12	0.64	0.80	0.49	0.78
Urine pH[4]	6.96	7.07	7.12	0.14	—	—	0.42	0.80

[1]Treatment and d effects, interaction, and contrasts.

[2]Daily for 18 ± 5 d.

[3]D −18 ± 5 and −8 ± 5 relative to parturition.

[4]D −8 ± 5 relative to parturition.

Table 3. Postpartum DMI, lactation performance, feed efficiency, MUN, BW, BCS, linear SCC, and udder edema score (Tucker et al., 1992) on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM); n = 13/treatment

Item	Treatment				P-value[1]			
	LP	HP	HPM	SEM	Time	Trt × Time	LP vs HP+HPM	HP vs HPM
DMI,[2] kg/d	16.6	17.5	18.3	0.8	<0.01	0.57	0.14	0.48
Milk,[2] kg/d	31.2	33.7	32.2	0.8	<0.01	0.78	0.10	0.19
ECM,[3] kg/d	27.4	28.7	29.1	1.3	<0.01	0.47	0.23	0.76
Fat,[3] kg/d	0.97	0.94	1.00	0.06	<0.01	0.45	0.98	0.47
Fat,[3] %	3.15	2.92	3.31	0.13	<0.01	0.79	0.84	0.04
Protein,[3] kg/d	0.94	0.97	0.97	0.05	0.27	0.91	0.54	0.89
Protein,[3] %	3.24	3.05	3.31	0.14	<0.01	0.99	0.74	0.15
Lactose,[3] kg/d	1.33	1.48	1.42	0.07	<0.01	0.75	0.07	0.42
Lactose,[3] %	4.29	4.40	4.55	0.07	<0.01	0.39	0.03	0.07
Solids,[3] kg/d	3.49	3.72	3.68	0.17	<0.01	0.73	0.18	0.79
Solids,[3] %	11.60	11.32	12.10	0.24	<0.01	0.98	0.69	0.02
Milk/DMI[2]	2.01	1.96	1.82	0.10	0.98	0.48	0.08	0.08
ECM/DMI[3]	1.79	1.67	1.68	0.11	0.41	0.10	0.37	0.97
MUN,[3] mg/dL	15.7	19.4	18.3	0.9	0.68	<0.01	<0.01	0.19
BW,[4] kg	610	596	593	19	0.58	0.88	0.48	0.92
BCS,[4] 1–5	2.99	2.99	3.05	0.08	0.62	0.76	0.70	0.55
Ln SCC,[3,5] 0–9	3.05	2.83	2.69	0.36	0.95	0.87	0.49	0.75
Edema,[6] 1–9	1.64	1.95	1.55	0.31	0.49	0.57	0.92	0.23

[1]Treatment and time (day or week) effects, interaction, and contrasts.
[2]Daily for 45 d.
[3]Weekly for 45 d.
[4]D 0, 7, 14, 21, and 28 postpartum.
[5]Equivalency: 3.05 = 103,000 cells/mL; 2.83 = 89,000 cells/mL; 2.69 = 81,000 cells/mL.
[6]D 0, 7, and 14 postpartum.

cytes, and platelets were similar among treatments ($P \geq 0.13$).

DISCUSSION

The main hypothesis of the present study was that transition cows would benefit from greater supply of MP, in particular balanced with RPMet. The current estimates of prepartum MP requirements do not ac-
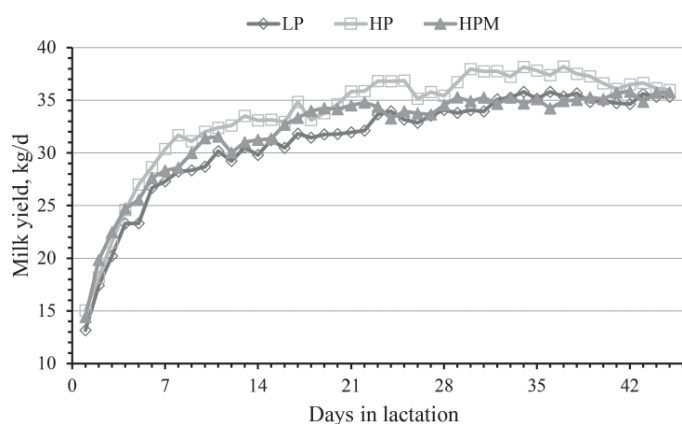

Figure 2. Milk yield on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM). SEM: LP = 1.295, HP = 1.294, HPM = 1.309.

count for mammary tissue accretion (NRC, 2001). Therefore, feeding protein in excess to the requirement during the prepartum period might promote mammary growth, as well as N retention (Putnam and Varga, 1998) and carcass N accretion (McNeill et al., 1997). A major source of AA for milk production in the postpartum period is body protein (Larsen and Kristensen, 2009), thus a greater muscle mass at calving increases the pool of AA available for mobilization with the onset of lactation. Additionally, greater postruminal supply of MP postpartum expressively increased milk and milk protein yields during the first 30 d of lactation (Larsen et al., 2014, 2015), demonstrating the responsiveness of the mammary gland to protein at the beginning of lactation. Moreover, increased peripartum protein has been associated with better peripartum health (Curtis et al., 1985). Specifically, the supplementation of RPMet has improved health and performance of dairy cows during the transition period (Osorio et al., 2013, 2014; Zhou et al., 2016a,b; Batistel et al., 2017). Taken together, these results led us to develop the 3 different protein feeding strategies for the transition period, initiated at 18 (±5) d before parturition and tested in this study.

All pre- and postpartum diets supplied RDP close to the requirement (ranged from 98 to 108%) to optimize rumen function without excess of ammonia. The pre-

Table 4. Blood metabolites prepartum (d -8 ± 5 relative to parturition) and postpartum (d 7, 14, 21) on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM), n = 13/treatment

| Item | Treatment | | | | P-value[1] | | | |
	LP	HP	HPM	SEM	H	Trt × H	LP vs. HP and HPM	HP vs. HPM
Insulin,[2] U/mL								
d −8	6.97	6.24	12.83	2.44	—	—	0.37	0.05
d 7	4.64	3.76	4.92	0.98	—	—	0.81	0.41
d 14	5.12	5.71	5.76	1.34	—	—	0.70	0.98
d 21	5.43	5.36	5.62	1.97	—	—	0.52	0.33
Glucose,[3] mg/dL								
d −8	69.8	63.2	66.0	5.16	0.67	0.90	0.36	0.70
d 7	60.5	56.9	57.7	2.5	0.83	0.60	0.31	0.83
d 14	56.6	55.2	54.9	2.7	0.26	0.73	0.65	0.92
d 21	58.7	59.3	57.4	2.8	0.11	0.56	0.91	0.63
Plasma urea-N,[4] mg/dL								
d −8	20.6	23.4	22.2	1.0	<0.01	0.87	0.12	0.41
d 7	20.9	24.3	23.9	1.7	<0.01	0.72	0.14	0.87
d 21	21.6	27.5	25.4	1.5	<0.01	0.44	0.01	0.36
NEFA,[5] mM								
d −8	0.09	0.11	0.09	0.01	—	—	0.92	0.37
d 7	0.35	0.42	0.32	0.06	—	—	0.79	0.19
d 14	0.26	0.25	0.30	0.05	—	—	0.76	0.42
d 21	0.21	0.18	0.21	0.04	—	—	0.74	0.57
BHB,[2] mM								
d −8	0.64	0.57	0.56	0.06	—	—	0.32	0.86
d 7	0.80	0.96	1.18	0.20	—	—	0.18	0.36
d 14	0.83	1.41	1.36	0.30	—	—	0.09	0.88
d 21	0.96	1.13	1.45	1.13	—	—	0.30	0.36
Ca at calving,[6] mg/dL	8.6	8.7	8.2	0.4	0.43	0.76	0.44	0.83

[1]Treatment (Trt) and hour (H) effects, interaction, and contrasts.
[2]12 h after morning feeding.
[3]0 and 12 h after morning feeding.
[4]0, 2, 6, 9, 12, and 18 h after morning feeding.
[5]NEFA = nonesterified fatty acids; 12 h after morning feeding.
[6]0 and 24 h relative to parturition.

partum MP supply was 1,001, 1,274, and 1,399 g/d for LP, HP, and HPM, respectively, due to differences in CP content but also in intake among treatments. A recent meta-analysis (Husnain and Santos, 2019) showed that primiparous cows benefited from increased prepartum MP supply up to 1,100 g/d, improving subsequent lactation performance. However, the benefits were not observed for multiparous cows, and the authors con-

Table 5. Immunity markers at calving and hemogram 21 d postpartum on treatments low protein (LP), high protein (HP), and high protein plus rumen-protected Met (HPM), n = 13/treatment

| Item | Treatment | | | | P-value[1] | |
	LP	HP	HPM	SEM	LP vs. HP and HPM	HP vs. HPM
IL-1, pg/mL	376.8	288.3	217.9	43.9	0.03	0.30
IL-10, pg/mL	44.9	44.3	48.8	31.7	0.57	0.16
TNFα,[2] pg/mL	835.9	643.7	735.2	131.7	0.36	0.63
Erythrocytes, 10^6/μL	5.4	5.2	5.2	0.18	0.32	0.88
Hemoglobin, g/dL	8.9	8.6	8.5	0.25	0.26	0.73
Hematocrit, %	26.0	25.5	24.9	0.71	0.39	0.59
Leukocytes, 10^3/μL	21.9	15.5	19.3	2.50	0.14	0.28
Neutrophils, 10^3/μL	4.2	7.0	4.2	1.28	0.32	0.18
Eosinophils, 10^3/μL	0.79	0.48	0.32	0.21	0.13	0.59
Monocytes, 10^3/μL	0.73	0.66	0.55	0.21	0.61	0.70
Lymphocytes, 10^3/μL	17.5	9.7	11.2	2.06	<0.01	0.63
Platelets, 10^3/μL	244	255	249	41	0.25	0.32

[1]Treatment effect and contrasts.
[2]TNFα = tumor necrosis factor alpha.

cluded that there was no advantage of supplying more than 800 g/d of prepartum MP (Husnain and Santos, 2019).

Nonetheless, in our study, both greater protein supply and RPMet supplementation had a positive effect on DMI. Adachi et al. (2006) reported a positive effect of increasing diet CP (12–15%) on DMI for primiparous but not for multiparous cows. Likewise, Park et al. (2002) fed 5 levels of CP (9.7–16.2%) to multiparous dairy cows for the last 4 wk of dry period with no effect on DMI. In fact, Husnain and Santos' meta-analysis confirmed this parity effect, because the increased DMI with greater protein supply was only observed for the primiparous cows. Unfortunately, we did not have enough statistical power to test the interaction between parity and treatments.

The 15% increase in DMI due to RPMet (relative to HP diet, $P = 0.07$) is comparable to changes in DMI observed by Batistel et al. (2017; +1.2 kg/d) and Zhou et al. (2016b; +1.1 kg/d), when they added RPMet to pre-partum diets with, respectively, 15.7 and 14.5% CP. On the other hand, neither Lee et al. (2019) nor Potts et al. (2020) observed any effect of RPMet on prepartum DMI of cows fed the supplement with RPMet during the close-up period. Interestingly, the studies that did observe positive effects of RPMet (current study; Zhou et al., 2016b; Batistel et al., 2017) provided a greater MP supplied (around 1,300 g/d) than the ones that did not (Lee et al., 2019; Potts et al., 2020; MP supply around 900 g/d).

Earlier research has shown a positive correlation between prepartum DMI and postpartum DMI and milk yield (Bertics et al., 1992; Mashek and Grummer, 2003). However, we did not confirm such correlation, because postpartum DMI was not different among treatments. On the other hand, milk yield tended to be higher for HP diets relative to LP, which agrees with the prepartum DMI.

Besides the amount of feed consumed during the close-up period, the change in DMI from −3 to −1 wk relative to calving appears to be of importance as well. In fact, the change in DMI during prepartum period is a better predictor of NEFA concentration and liver triglyceride at d 1 after calving (although not for post-fresh DMI and milk yield) than total prepartum DMI (Mashek and Grummer, 2003). In the present study, the decrease in DMI from wk −3 to −1 was 20% for LP and HPM and 27% for HP, all smaller than the 30% observed for 699 cows from 16 experiments at 8 universities (Hayirli et al., 2002). Regardless of the size of reduction in prepartum DMI, postpartum NEFA concentrations were unaffected and remained lower than potentially problematic thresholds (0.6–0.7 mmol/L; Ospina et al., 2013). On the other hand, HPM cows had postpartum

BHB levels on d 14 and 21 of lactation slightly greater than 1.2 mM, which may be indicative of subclinical ketosis (Oetzel, 2007). Therefore, the trend for increased prepartum DMI observed with HPM did not change fatty acid mobilization postpartum, as inferred from the variables measured in the current trial. Additionally, lipid metabolism might also have been affected by the greater prepartum insulin levels in cows fed HPM. The same response was observed by Liang et al. (2019), in which the same RPMet product elicited an increase in plasma insulin 10 d before calving. The mechanisms behind this effect are unclear, as it may be a direct effect of the Met on insulin secretion or an effect of the greater intestinal flow of nutrients due to greater intake.

Cows fed HP diets had a trend for increased BHB on d 14 (1.38 vs. 0.83, respectively). Although this may be linked to higher milk production, increased blood BHB has been observed with abomasal casein infusion in transition cows but not increased hepatic BHB flux (Galindo et al., 2015). Conversely, the uptake of BHB by mammary tissue is elevated with postruminal protein supply (Guinard and Rulquin, 1994). We did not observe ketosis during the trial that would warrant clinical intervention despite elevated BHB. Future research is encouraged to evaluate the potential for greater resilience to elevated BHB in transition cows with increased postruminal protein supply.

The increase in milk yield with greater protein supply was brought about by an increase in lactose yield, which has been reported in several studies (Clark et al., 1977; Lemosquet et al., 2009a; Doepel and Lapierre, 2010; Galindo et al., 2011). Excess glucogenic AA (Met, His, and Val) can potentially spare glucose and increase its availability to the mammary gland (Lemosquet et al., 2007). The greater MUN concentration in the HP treatments may indicate higher AA catabolism for gluconeogenesis or for PDV oxidation (Larsen et al., 2014). The increase in milk and lactose yields confirms the mammary gland responsiveness to postruminal protein supply observed previously (Larsen et al., 2014, 2015). Because we began feeding more protein before calving, and this increased prepartum DMI, we cannot know precisely address if it was a direct effect of AA supply (from the postpartum diet and from body AA pool that might have increased during close-up), or an indirect effect of greater prepartum DMI, as this relationship has been previously reported (Mashek and Grummer, 2003).

The lack of a milk protein response with greater dietary protein supply is intriguing. One possibility would be that the increase in AA supply was not enough to elicit a response. However, both MUN and postpartum PUN increased, indicating excess of protein. Another option

would be that AA were being preferentially oxidized to produce more energy for milk production. However, even though most AA are glucogenic, the majority of the glucose synthesized during early lactation is from lactate-derived carbons (Larsen and Kristensen, 2013). A third explanation would be the limitation of specific AA. Even though we used high RUP soybean meal, which presumably has an adequate (but not perfectly balanced) AA profile, the high urea levels in plasma and milk are a result of AA catabolism. However, as discussed later, the addition of RPMet, the most likely AA to be limiting, to the HP diet did not result in more milk or milk protein. Therefore, it might also be that cows did not need the extra protein (as suggested by both Adachi et al., 2006 and Husnain and Santos, 2019 for multiparous cows), and the increase in milk yield was indeed due to the greater prepartum DMI.

As mentioned before, based on current AA recommendation for dairy cows, Met was predicted to limit performance in the postpartum HP diet (dMet was 1.74% of MP). Despite this, and disagreeing with previous research that reported improved performance (Osorio et al., 2013; Zhou et al., 2016b; Batistel et al., 2017) with RPMet during transition, the results we observed rejected our hypothesis that balancing the diet for Met would lead to greater performance. Nonetheless, such positive responses were not always observed. Potts et al. (2020) and Lee et al. (2019) reported no effect of supplementing RPMet during the transition period on milk yield or postpartum DMI. There are many possible reasons for the difference in response between these 2 groups of studies. The method of administration of the RPMet (top-dress vs. mixed in the TMR) could affect the way absorbed Met is metabolized, because top-dressing increases the probability that cows will consume the supplement all at once and promotes a high peak of plasma Met without the other amino acids. However, Zhou et al. (2016b), Batistel et al. (2017), and Potts et al. (2020) all used top-dress, whereas our study and the one by Lee et al. (2019) mixed in the concentrate, not corresponding to the positive or neutral responses observed. It is also possible that the amount of RPMet supplemented did not elicit a large enough change in plasma Met to stimulate protein synthesis. In fact, Lee et al. (2019) reported no changes in plasma Met with the supplementation of RPMet. This alternative, however, cannot be verified in the present study or in Potts et al. (2020), because neither of the studies analyzed plasma concentrations of AA.

We compared diet characteristics of these 6 studies (Osorio et al., 2013; Zhou et al., 2016b; Batistel et al., 2017; Lee et al., 2019; Potts et al., 2020, and the present study) to evaluate if they had differences

that could group the studies with positive or neutral responses to RPMet (data not shown). The CP content of the diet was 17.2 to 17.6% for the 3 studies with positive responses, whereas for the other 3, it varied between 16 and 18.6%. However, because of differences in DMI across the studies, daily supply of MP, dMet of the basal diet, and the ratio between total dMet/NEl concentration greatly varied and did not help explaining the responses. For instance, the lowest values for these 3 measures was for Osorio et al. (2013), that reported positive responses. One difference between the 2 groups of studies is milk yield. Cows from the 3 studies that did see an effect of RPMet produced between 36 and 44 kg/d, whereas cows from the other 3 studies produced from 32 to 36 kg/d. If we calculate average feed efficiency (milk/DMI) for each study, the difference between the 2 groups is even more evident. Feed efficiency of group with positive responses varied from 2.3 to 2.6, whereas for the other group ranged from 1.8 to 1.9. Therefore, the differences in response could have been due to productive potential of the cows in each study. Alternatively, there is also a chance that other AA were limiting milk protein synthesis in the 3 studies with no response, because the feed ingredients used in the diets varied (whereas in the other 3, ingredients were very similar because they are all from the same research group). However, no study reported diet AA composition and, therefore, there is no way to verify this hypothesis.

Methionine supplementation to the HP diet increased milk total solids concentration. However, this was not a result of increased protein concentration, but rather lactose content and fat content relative to HP. Increases in milk fat yield and content are not uncommon with RPMet supplementation (Patton, 2010; Chen et al., 2011; Zanton et al., 2014). Same response was also consistently observed when RPMet was fed during the transition period (Osorio et al., 2013; Zhou et al., 2016b, Potts et al., 2020). Recent studies have showed regulatory effects of amino acids on lipid metabolism (Li et al., 2016). More specifically, Met has been shown to activate transcription factors that ultimately enhance milk fat synthesis (Qi et al., 2018; Li et al., 2019).

Amino acids play an important functional role in modulation of immune system. For instance, Met is a precursor of glutathione (Martinov et al., 2010), an important antioxidant. In fact, previous research has shown health-related benefits of RPMet supplementation during the periparturient period (Coleman et al., 2020). Improvements in concentration of plasma biomarkers of inflammation in dairy cows in response to Met, such as reduced IL-1B and haptoglobin and increased albumin (Osorio et al., 2014; Sun et al., 2016;

Zhou et al., 2016a; Batistel et al., 2018), as well as in biomarkers of oxidative stress (Sun et al., 2016; Batistel et al., 2018) have been reported previously.

In this study we evaluated the cytokines IL-1, TNF-α, and IL-10, which participate in pro- and anti-inflammatory cytokine activities around calving. The IL-1 is an important inflammatory cytokine that has been widely characterized in mammals and has been reported as a pleiotropic cytokine because of its diverse functions (Yang et al., 2017). A low concentration of IL-1 postpartum in cows fed HP diets has been reported as an indicator of good health status (Vailati-Riboni et al., 2017) and reduced environmental health challenge, which suggest that the use of HP level may be a viable way of alleviating a detrimental response to the immune system, acting as an immunomodulatory in the prevention of nonspecific inflammatory responses.

The innate immune system responds to inflammation, infection and injury by leukocyte recruitment (Schmidt et al., 2013) that involves IL-1, production by cell types, such as macrophages, monocytes, and synovial lining cells, and induction of inflammation by synovial cells, endothelial cells, lymphocytes, and macrophages (Dinarello, 1996). Inflamed tissues have a very well-regulated strategy to recruit leukocytes, including TNF-α, IL-1, LTB4, IL-6, CXCL1, and CXCL2, which will modulate endothelium function, enhancing their adhesiveness and the contact time with circulating leukocytes (Peres et al., 2016). We observed a trend for reduction in IL-1 on the day of calving in response to feeding HP diets prepartum compared with the LP treatment group. Likewise, blood lymphocyte concentration was lower in cows fed the HP diets compared with diet LP. The reduced levels of IL-1 and lack of difference in TNF-α and IL-10 concentrations suggests a quiescent innate immune response and no active recruitment of lymphocytes during this study. However, there was also no anti-inflammatory response because IL-10 did not increase between treatments. Further studies with a controlled immune challenge environment are needed to determine the role of prepartum protein supply and Met status on innate immunity and lymphocyte activation in dairy cows.

CONCLUSIONS

Increasing CP content of diets from 14 to 16% with high RUP soybean meal during the prepartum period increased prepartum DMI. Feeding HP diets also tended to decrease the concentration of IL1, suggesting reduced basal inflammatory status. In addition, cows fed HP diets tended to have increased average daily milk yield by 1.7 kg, compared with a low-protein diet, as well as increased milk lactose content and MUN.

Postpartum insulin, plasma glucose, NEFA, IL10, and TNFα concentrations were not responsive to protein supplementation strategies. The inclusion of RPMet to the HP diet increased milk fat and total solids content and insulin concentration prepartum, and had no effect on milk yield, DMI, or markers of health status in blood. Taken together, these data point to the beneficial effect of increased MP supply from high RUP soybean meal and to RPMet supplementation on dairy cows during the periparturient period.

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