Effects of rumen-protected methionine supplementation on the performance of high production dairy cows in the tropics

Valdir Chiogna Junior¹, Fernanda Lopes², Charles George Schwab³, Mateus Zucato Toledo⁴, Edgar Alain Collao-Saenz^{1*}

¹Department of Animal Science, Federal University of Jataí, GO, Brazil

²Adisseo South America, São Paulo, SP, Brazil.

³Schwab Consulting LLC, Boscobel, WI, USA

⁴Department of Dairy Science, University of Wisconsin, Madison, WI, USA

*Corresponding author

E-mail: edgar_collao_saenz@ufg.br

Abstract: Increasing methionine availability in dairy cow diets during the first third of lactation may enhance their performance and health. The aim of this study was to determine the effect of supplementing rumen-protected methionine (RPMet) in a lactation diet containing adequate protein and energy levels. Seventy-six multiparous Holstein cows (39.1 ± 6.8 kg of milk/d and 65 ± 28 DIM) were assigned to 1 of 2 dietary treatments (38/treatment) according to a randomized complete block design with a 2-wk (covariate) and 10-wk experimental period. Treatments were a basal diet (CON; 3.77 Lys:1Met); and CON + 14 g RPMet (2.97 Lys:1 Met). Individual milk samples were taken every 2 weeks to determine milk composition. Blood was collected from 24 cows on d+30 d to measure plasma AA levels. Body weight and body condition score (BCS) were measured at the beginning and the end of the experiment. The SM diet promoted higher milk yield (41.7 vs. 40.1 kg/d; $P = 0.026$), energy-corrected milk yield (41.0 vs. 38.0 kg/d; $P \leq 0.01$), milk protein yield (1.30 vs. 1.18 kg/d; $P \leq 0.01$), and milk fat yield (1.42 vs. 1.29 kg/d; $P = 0.02$). Higher milk protein (3.14% vs. 2.97%; $P \leq 0.01$) and

casein (2.39% vs. 2.28%; $P \leq 0.01$) and a trend ($P = 0.06$) for higher milk fat % (3.41% vs. 3.21%) were also observed. Both diets resulted in similar body weight, but CON-fed cows tended ($P = 0.08$) to have higher BCS. Higher plasma methionine levels were determined with SM compared with CON (29.6 vs. 18.4 μM ; $P < 0.01$), but lysine and histidine were not different. Dietary supplementation of RPM improved productive performance by increasing milk yield and milk components yields, suggesting better dietary AA utilization when Met levels are adjusted in Lys-adequate lactation diets.

Keywords: amino acids, milk protein, milk yield.

INTRODUCTION

Precision feeding is a valuable strategy to improve income-over-feed-cost in dairy production, and feeding AA-balanced diets may be an effective strategy to increase milk yield and milk components [1]. Dairy nutritionists are changing their focus from balancing rations for crude protein (CP) to rumen degradable protein (RDP) and more optimum concentrations of AA in rumen undegradable protein (RUP); the latter of which provides for more optimum concentrations of AA in metabolizable protein. These changes are being implemented to better meet but not exceed the protein requirements of ruminal microorganisms and the metabolizable AA requirements of the animals. The ultimate goal is to reduce the need for supplemental protein and reduce the risk of the most limiting AA limiting animal performance. The desired outcome is reduced feed costs, increased milk and milk component yields, improved dietary protein efficiency, and reduced environmental pollution [2].

Lysine (Lys) and methionine (Met) have been identified most frequently as the two most limiting AA for milk protein synthesis. This is not surprising given their low content in most feed proteins relative to their content in mixed rumen microorganisms and milk protein. In vitro studies using mammary epithelial cells have also shown the importance of Lys and Met concentrations in the medium, not only on maximal milk protein synthesis but also on AA transport and signal transduction pathways affecting expression of genes related to milk protein synthesis [3]. While the data indicated that both AA can activate the expression of genes involved in milk protein transcription and translation, peak concentrations of casein and cell proliferation rates were observed when the ratio of supplemental Lys and Met was ~3:1. The optimum concentrations for Lys and Met in metabolizable protein have been defined for different nutritional models [4, 5] and have proven useful for balancing diets for these two AA.

Methionine has long been recognized as being the first limiting AA for lactating dairy cows, particularly when fed high Lys, low Met-containing protein supplements such as blood meal and soybean meal. An effective approach to increase post-ruminal Met supply is feeding RPMet. Several studies have shown the beneficial effects of RPMet supplementation on sparing of dietary protein [6] and overall lactation performance of dairy cows when diets were based on typical North American ingredients dairy cow performance [6,7]. A recent focus has been on RPMet supplementation of transition cow diets. Met supplementation to achieve a Lys:Met ratio of approximately 2.9:1 as % of MP improved milk yield, which was partially attributed to an increase in dry matter intake (DMI) and possible better utilization of body lipid reserves [7]. Those authors also reported enhanced immune function and suggest that these results support the fine-tuning of EAA content as a percentage of MP requirement in diet formulation.

Methionine is the only AA which contains sulfur acting as a precursor for other sulfur-containing AA (SAA), such as cysteine, homocysteine, and taurine, which are important in methylation reactions. Methionine metabolism starts with its conversion into S-adenosylmethionine (SAM), which is a key cofactor of the Met intermediate metabolism used for methylation reactions. SAM is a methyl donor to a wide variety of acceptors, such as amino acid residues in proteins, DNA, RNA, and small molecules [8]. The dietary supplementation of rumen-protected methyl donors, such as RPMet, may allow meeting the requirements of cows at the peak of lactation, when the output of methylated compounds in the milk is high.

Most continuous feeding studies investigating the impacts of Met-supplementation of otherwise nutrient balanced diets have been carried out under research-controlled conditions during the transition period or beginning at the onset of

85 lactation. There are limited data on dietary Met supplementation around the peak of
 86 lactation under field conditions and its effects during the remaining of the lactation, and
 87 in particular when cows are maintained under tropical conditions. The hypothesis of
 88 this experiment was that supplementing a corn-based, soybean meal lactation diet with
 89 RPM to achieve the desired 3.0:1 Lys:Met ratio in MP [4] would improve the
 90 performance of lactating cows during the first half of lactation. The objectives were to
 91 measure milk yield and milk component responses, including peak milk yields, of early
 92 lactation dairy cows, by supplying the correct amount of supplemental RPMet to a
 93 basal diet that was otherwise formulated for high milk production. Plasma free AA were
 94 also measured to confirm that the RPMet supplement provided the intended supply of
 95 metabolizable Met.

MATERIALS AND METHODS

Animal Housing and Care

The experiment was conducted on a commercial dairy farm in the state of Goiás, in the central-western region of Brazil. All experimental procedures were approved by the Committee of Ethics on Animal Use (CEUA) of the Federal University of Goiás – Campus Jataí, under protocol number 024/17.

Cows were housed in a free-stall system, equipped with sand litter and a cooling system (sprinklers and fans) to provide good animal welfare conditions. Cows had free access to drinking water. One of two total mixed rations (TMR) were offered twice daily (at 06:00 and 15:00 h). Feed allowance was adjusted daily to ensure 5% feed refusals.

Experimental Design and Dietary Treatments

Seventy-six lactating multiparous Holstein cows were distributed evenly to one of two experimental diets (Table 1) according to a randomized complete block experimental design (38 blocks). Blocking criteria were milk yield (39.1 ± 6.8 SD kg) and days-in-milk (DIM) (65 ± 28 SD). At the beginning of the study, cows had an average of 2.75 ± 1 lactations, 645.4 ± 45.6 kg BW, and 3.3 ± 0.3 body condition score (BCS). The duration of the experiment was 12 weeks: the first two weeks were used for diet adaptation (covariate), and the following 10 weeks were considered the experimental period.

The two experimental diets contained equal nutrient levels, except for Met. The basal diet was formulated using the NRC (2001) model [4] to meet the cows' nutritional requirements for an average milk yield of (indicate what you used), an average BW of, and an average milk composition of 3.6% fat and 3.07% true protein. Based on a

predicted dry matter intake (DMI) of 26.3 kg/d, the control diet (CON) contained (kg/d, DM basis) 12.1 corn silage, 1.2 fresh grass, 4.01 soybean meal, 0.33 xylose-treated soybean meal, 4.55 high-moisture corn, 2.73 soybean hulls, 0.06 urea, 0.48 rumen-protected saturated fatty acids, and 0.84 mineral-vitamin premix. The Met-supplemented diet (SM) consisted of the basal diet plus 23 g/d of SMARTAMINE® M (Adisseo Inc., Antony, France) which provided 14 g/d of additional metabolizable Met. SMARTAMINE® M is a lipid/pH-sensitive polymer-protected product that contains 75% DL-Met with an assumed Met bioavailability value of 80% [9]. Smartamine M was pre-mixed with the mineral/vitamin premix, urea, treated soybean meal and saturated fat and then mixed with the remainder of the diet. The nutritional composition of the experimental diets is shown in Table 1, calculated according to the NRC (2001). Predicted flows of EAA to the small intestine (NRC, 2001) are shown in Table 2.

132 Table 1. Ingredient and nutritional composition of treatments diets

	Diet ¹	
	CON	SM
<i>Ingredient (% of DM)</i>	45.81	45.81
Corn silage ²		
Fresh grass (Tifton) ³	4.41	4.41
Soybean meal, solv. ⁴ , 46% CP	15.57	15.57
Soybean meal treated ⁵	1.25	1.25
Smartamine® M ⁶	-	0.09
High-moisture corn ⁷	17.28	17.28
Soybean hulls	10.36	10.36
Saturated fat supplemented ⁸	1.81	1.81
Urea	0.23	0.23
Mineral-vitamin premix ⁹	3.20	3.11
<i>Nutritional composition¹⁰, %</i>		
Crude Protein	16.7	16.7
RDP	10.3	10.3
RUP	6.4	6.4
NDF	30.8	30.8
Forage NDF	20.8	20.8
ADF	19.0	19.0
NFC	42.3	42.3
Starch	27.7	27.7
Ether extract	4.6	4.6
Net energy for lactation (NEI, Mcal/kg)	1.6	1.6
Ca	1.0	1.0
P	0.4	0.4
<i>Dietary Protein Balance¹⁰</i>		
RDP required, g/d	2595	2597
RDP supplied, g/d	2711	2715
RDP balance, g/d	116	118
RUP required, g/d	1539	1537
RUP supplied, g/d	1672	1688
MP balance, g/d	113	129

¹CON = Control diet; SM = CON + SM (0.09% of DMI)

² Corn silage = 33.5% DM, 7.98% CP, 33.3% starch, 39.5% NDF

³Fresh grass = 28.8% DM, 14.9% CP, 60.7% NDF

⁴Soybean meal = Soybean meal 46% CP Cargill® Inc.

⁵Soybean meal, treated = Soy Pass® BR Cargill® Inc.

⁶Smartamine® M = rumen-protected methionine, Adisseo Inc., Antony, France

⁷High moisture corn = 64.8% DM, 8.8% CP, 69% starch

⁸Saturated fat supplemented = Enerfat®, Kemim® Industries Inc., USA

⁹Mineral-vitamin premix = Precisão Nata® (Campo® Rações e Minerais); Mg 35 g/kg, Na 85 g/kg, Cl 80 g/kg, Ca 190 g/kg, P 24 g/kg, S 4,000 mg/kg, K 9,900 mg/kg, Cu 450 mg/kg, Zn 2,300 mg/kg, Fe 2,000 mg/kg, Mn 1,800 mg/kg, I 25 mg/kg, Co 30 mg/kg, Se 15 mg/kg, F 300 mg/kg, Vit. A 156,000 IU/kg, Vit. D 51,500 IU/kg, Vit. E 1,000 IU/kg, Biotin 35 mg/kg, Cr 20 mg/kg, Monensin 510 mg/kg, *Saccharomyces cerevisiae* 1.40x10⁵ CFU/kg.

¹⁰Predicted using NRC (2001), laboratory analyses of feeds and an average DM intake of 26.3kg/d.

Table 2. Predicted flows of EAA to the small intestine (NRC, 2001)¹

Amino acid	Diet ²					
	CON			SM		
	Flow (g/d)	DigAA flow (g/d)	% of MP	Flow (g/d)	DigAA flow (g/d)	% of MP
Arginine	169	141	4.79%	169	142	4.77%
Histidine	75	62	2.12%	75	62	2.10%
Isoleucine	173	142	4.81%	173	142	4.79%
Leucine	312	256	8.67%	312	256	8.62%
Lysine	228	188	6.37%	229	189	6.35%
Methionine	61	50	1.69%	75	64	2.14%
Phenylalanine	179	147	4.98%	179	148	4.97%
Threonine	171	140	4.73%	171	140	4.71%
Valine	194	158	5.37%	194	159	5.35%
Total	1562	1286	43.53%	1578	1301	43.8%

¹The NRC (2001) evaluation of diets was based on final DMI and production data and feed analysis.

²CON = Control diet, Lys:Met ratio 3.77:1; SM = CON + SM (0.09 % of DMI), Lys:Met ratio 2.97:1

Sampling and measurements

Feed allowance and refusal amounts were recorded daily to allow for calculating group feed intakes. Samples of the TMRs were collected twice weekly to determine DM content (%). Forages were analyzed for DM content every 15 days to adjust diet allowance.

Composite feedstuff samples (corn silage, fresh grass Tifton and high moisture corn) were analyzed by wet chemistry in the 3rLab (Lavras, MG, Brazil) for CP, neutral detergent fiber (NDF), acid detergent fiber (ADF), starch, ether extract (EE), ash, calcium (Ca), phosphorus (P) contents according to the methods of the AOAC International.

Milk samples were individually collected during the adaptation period and every two weeks until the end of the study. Samples were preserved in 2-bromo-2nitropropane-1,3-diol, and submitted to the milk lab of the University of São Paulo (Clínica do Leite, USP, Piracicaba, SP, Brazil), where infrared analyses of total solids

content, milk urea nitrogen (MUN), and somatic cell count (SCC) were performed (ISO 9622:2013/IDF 141:2013).

Cows were milked three times daily at 05:30, 12:00, and 20:00 h. Milk yield was individually recorded using a dairy management software (DairyPlan®). Energy-corrected milk yield (ECM) was calculated as $ECM = [(0.323 \times \text{kg milk}) + (12.82 \times \text{kg fat}) + (7.13 \times \text{kg true protein})]$. During the adaptation period and at the end of the experimental period, cows were individually weighed and their body condition scores determined after the second milking of the day.

Blood samples were collected from 12 cows on each treatment on day 30 of the experimental period ($DIM = 89 \pm 27$ SD) to measure plasma AA levels. Blood was collected from the tail vein at 13:00 h (6 hours after the 1st feeding). Blood plasma was immediately separated by centrifugation at $1.500 \times g$ for 15 min, aliquoted into 2-mL tubes, and frozen at -20°C until analyses at the Dairy Science Department, University of Wisconsin-Madison, USA. Briefly, plasma samples were combined with an internal standard (homoarginine, methionine- d_3 , and homophenylalanine) before deproteinization with 1 N perchloric acid (final concentration 0.5 N) and the supernatant collected and filtered through a $0.22 \mu\text{m}$ filter (Millex-GV filter; Millipore Sigma, Burlington, MA). Filtered samples were separated using a 50×3 mm column (length $\times$ i.d; mixed mode with normal phase plus ion exchange; Intrada Amino Acid, Imtakt Inc., OT, USA) and the Nexera-i LC-2040C (Shimadzu, Kyoto, Japan). Mobile phases were acetonitrile containing 0.1% of formic acid (A) and 100 mM ammonium formate in water (B), with 14% B gradient for 0-3 min, increasing to 100% B gradient for 3-10 min, followed by a decreased to 14% for 10-12 min and re-equilibrated with 14% B gradient for 12-16 min. The eluent was ionized using electrospray ionization and analyzed in

positive selected ion-monitoring mode using a Single quadrupole mass spectrometry (LCMS-2020; Shimadzu, Kyoto, Japan. Samples were analyzed in duplicates

Statistical Analysis

Data were analyzed according to a randomized complete block design using the MIXED procedure of SAS statistical package (version 9.4, SAS Institute Inc., Cary, NC, USA). The model included a covariate (data collected during a 2-wk adaptation period), block, treatment, week, and the relevant interactions as fixed effects, and cow and cow within block as random effects. Week of treatment was included as a repeated measure using the autoregressive of order 1 covariance structure to account for autocorrelated errors. Means were calculated using the least squares means statement, and treatment means were compared using Bonferroni t-test when overall treatment F-test was significant ($P < 0.05$). Interaction effects were partitioned using the SLICE options of SAS (version 9.4, SAS Institute Inc.). Statistical significance and trends were considered at $P \leq 0.05$ and $P > 0.05$ to $P \leq 0.10$, respectively. Plasma AA levels (μM) were analyzed and compared between treatments as described above but excluding week and treatment x week interaction.

$$Y_{ij} = \mu + c_i + T_j + e_{ij},$$

where Y_{ij} = dependent variable, μ = overall mean, c_i = random effect of cow ($i = 1$ to 24), T_j = fixed effect of treatment ($j = 1$ or 2), and e_{ij} = residual error.

RESULTS AND DISCUSSION

The typical recommendations for balancing dietary AA are made relative to total MP supply. The SM diet was formulated to ensure an adequate amount of Met in MP, relative to the predicted concentration of Lys in MP. The NRC [4] suggests the required predicted concentrations of Lys and Met in MP for maximal milk protein yield are 7.2% and 2.4%, respectively. As these amounts are difficult to achieve without both RPLys and Met supplementation, it is recommended that the first step to balancing diets for Lys and Met is to feed high-Lys protein supplements like blood and soybean meal and then supplement with RPMet to maintain their ratio in MP at 3:1 while trying to achieve practical levels in MP as close as possible to 6.6% and 2.2%, respectively [10]. In this experiment, the predicted concentrations of Lys and Met in MP in the CON diet were 6.37 and 1.69%, respectively. Prior evaluation of the CON diet with NRC (2001) indicated that 23 g/d of Smartamine M was needed to achieve a predicted Lys:Met ratio in MP of 3:1. The predicted concentrations of Lys and Met in MP in the SM diet were 6.35 and 2.14%, respectively.

The predicted ratios of metabolizable Met and Lys relative to predicted supplies of metabolizable energy (ME) were 1.17 g Met/Mcal ME and 3.05 g Lys/Mcal ME in the SM diet. These values are close to the suggested optimum values of 1.14 g Met/Mcal and 3.03 g Lys/Mcal [11]. Using the latest version of the CNCPS software [5], predicted concentrations of Lys and Met in MP were 6.57 and 2.45, respectively, yielding a Lys:Met ratio in MP of 2.7:1. The required predicted concentrations for Lys and Met in MP for maximal milk protein yields are 7.00 and 2.60%, respectively [5]. This provides for an optimal Lys/Met ratio in MP of 2.69/1.

In the present experiment, DMI was not affected ($P>0.05$) by treatment (26.2 kg/d for CON vs. 26.1 kg/d for SM). Previous studies on RPMet supplementation reported inconsistent DMI responses. While some studies did not find any effect on DMI [6,12,13,14,15], others observed an increase in DMI [7,16,17,18]. Most of the studies reporting an increase in DMI were initiated before or at the time of calving [7,16,17,18]. In these studies, the increases in DMI were partially attributed to reduced inflammation and oxidative stress, resulting in improvements in immunometabolic status and liver function [19,20]. Moreover, Zhou et al. [20] suggested that since Met serves as substrate for glutathione synthesis, the most abundant natural antioxidant, its higher availability has a positive immunomodulatory effect in dairy cows, particularly during periods of stress, such as the transition period. According to a meta-analysis of 35 studies [21], differences in the level of Met supplementation, presence of potentially co-limiting AA, length of feeding period, and stage of lactation may all affect feed intake and, therefore, confound DMI results.

Effects of dietary treatments on milk and milk component yields and milk composition are presented in Table 3. The SM diet promoted higher milk yield, ECM, FCM, milk protein percentage, milk protein yield, milk casein percentage, milk fat yield, and total solids yield, and tended ($P=0.06$) to increase milk fat percentage (Table 3) than the CON diet. Milk lactose content was lower (4.54% vs. 4.61, $P = 0.03$) for cows on SM versus CON; the same tendency for lower lactose percentage was observed in other experiment [22]. Cows on CON tended to present higher BCS (3.4 vs. 3.3; $P = 0.08$) compared with the SM cows although no BW differences were detected (656.5 vs. 648.5 kg; $P = 0.12$). There was no influence of dietary treatment on milk lactose yield, total solids percentage, MUN, or SCC.

Table 3. Effects of the dietary treatments on milk yield and composition in dairy cows.

Item	Diet ¹		SEM	P-value		
	CON	SM		Diet	week	Diet x week
Milk yield, kg/d	40.0	41.7	0.50	0.03	0.19	0.87
ECM ² , kg/d	38.0	41.0	0.67	<0.01	0.07	0.99
FCM ³ , 3.5%	38.3	41.1	0.77	0.01	0.14	0.97
Milk fat, %	3.21	3.41	0.07	0.06	0.85	0.99
Milk fat, kg/d	1.29	1.42	0.04	0.02	0.64	0.99
Milk protein, %	2.97	3.14	0.03	<0.01	<0.01	0.95
Milk protein, kg/d	1.19	1.30	0.01	<0.01	<0.01	0.95
Milk casein, %	2.28	2.39	0.03	<0.01	<0.01	0.96
Milk lactose, %	4.61	4.54	0.02	0.03	<0.01	0.83
Milk lactose, kg/d	1.85	1.89	0.03	0.26	0.02	0.80
Total solids, %	11.78	12.10	0.13	0.10	0.42	0.96
Total solids, kg/d	4.75	5.01	0.09	0.05	0.20	0.96
MUN, mg/dL	10.43	10.74	0.24	0.37	<0.01	<0.01
SCC, X10 ³	135.86	180.50	44.70	0.48	0.13	0.37

¹CON = Control diet; SM = CON + SM (0.09 % of DMI)

²Energy-corrected milk (kg/d) = [(0.323 x kg milk) + (12.82 x kg fat) + (7.13 x kg true protein)] (Hutjens, 2010).

³Fat-corrected milk (3.5%) = 0.4318 kg milk + 16.23 kg milk fat

Milk Yield

Higher milk yield ($P=0.03$), FCM ($P=0.01$) and ECM ($P<0.01$) were observed in the SM treatment. The mechanisms by which RPMet supplementation increases milk yield are not fully understood. However, considering mechanisms proposed by others who also observed improved lactation performance with RPMet supplementation, we suggest that the milk yield response in this experiment was due to increased efficiency of use of the other absorbed AA. There are two reasons for this suggestion. The first is that the predicted supply of ME for the achieved milk yield was not limiting, hence there was an opportunity for increased milk yield without increased feed intake. The second factor is that milk yield is a function of milk component synthesis, particularly lactose and protein, and increases in milk protein percentages by nutritional intervention alone are limited.

Figure 1 shows the lactation curves (average weekly milk yields), according to treatment during the 10-week experimental period. Most striking is how quickly the

281 cows responded to Met supplementation. This observation indicates that Met was not
282 only the first limiting AA, but also the most limiting nutrient, and that other aspects of
283 dairy farm management were not limiting animal performance.

284
285 Fig 1. Effect of dietary RPMet supplementation on milk yield compared with the
286 control diet. Values are expressed in average weekly milk yield (kg/d).

287
288 Several studies reported increases in milk yield when RPMet was supplemented
289 to periparturient cows [6,7,16,17,18], but only a few started supplementation at or
290 shortly after peak lactation. Such studies indicate that milk yield responses to Met
291 supplementation during that period is influenced by several factors, including Met
292 supply and source [6], adequacy of supplies of metabolizable Lys [23], MP and RDP
293 in the diet and stage of lactation [24].

294 Although Met is a glycogenic AA, and most of the glucose required for lactose
295 synthesis in the mammary gland is derived from gluconeogenesis [25], AA are not
296 prioritized for glucose production, and the quantitative contribution of EAA to

glycogenic carbon is very small [26]. Indeed, in cattle as well as other animals, Met has other metabolic priorities [27] for essential biological processes such as protein synthesis, DNA methylation, SAM-dependent trans-methylation reactions, polyamine formation, and synthesis of glutathione, phosphatidylcholine, and the non-essential AA cysteine [28]. It is assumed that the biological priority for these purposes exceeds Met utilization for hepatic gluconeogenesis [29]. Doepel and Lapierre [30] observed a positive response of the mammary gland of dairy cows to EAA infusion, increasing milk and milk protein yields, without a concurrent increase in glucose uptake, demonstrating the metabolic priority of milk production and the flexibility of the mammary gland to use various substrates (beta-hydroxybutyrate acid and lactate) when there are sufficient EAA to support it.

It has also been shown that cows supplemented with RPMet had a healthier metabolic status, which is associated with increased net energy efficiency, partially due to reduced energy requirements by the immune system [31]. The activation of the immune system increases glucose utilization. The inflammatory status of cows in early lactation cows may cause a reduction in milk synthesis as a strategy to spare glucose for the immune system [31].

Milk Components

Independently of week, RPMet supplementation increased milk protein percentage ($P<0.01$), milk protein yield ($P<0.01$), and milk casein percentage ($P<0.01$). Methionine may influence fractional protein synthesis rate [32]. When activated by Met, mTOR may increase the initiation rates of protein synthesis, which may explain why lactating dairy cows fed corn-based diets typically increase milk protein production

when supplemented with RPMet. Amino acids not only serve as direct precursors for protein synthesis, but also act as regulators of protein synthesis rate.

Although the amounts of absorbed EAA are important for milk protein synthesis, being absorbed in their correct balance is also essential for maximum yields of protein yield [33,34]. An in vitro experiment using bovine mammary cells indicated that the ratio of Lys to Met influences the expression of casein by bovine mammary epithelial cells [3] and, may explain the higher milk casein percentage observed in the present study. The peak of casein concentration in was observed at 3:1Met:Lys ratio, and appeared to be at least partially driven by the upregulation of mRNA expression and mTOR pathways [3]. Several studies evaluating RPMet supplementation to lactating cows reported increases in milk protein percentage [7,12,16,17], and, according to Schwab et al. [10], the magnitude of the increase is directly proportional to the adequacy of Met in metabolizable protein.

Cows fed the SM diet had a higher yield of milk fat ($P=0.02$), a result of higher milk yields and a trend of higher milk fat percentage ($P=0.06$) compared with cows fed the CON diet (Table 3). The effect of Met supplementation on milk fat synthesis, with resulting increases in milk fat percentage, warrants further investigation; however, some mechanisms have been proposed. Methionine is considered a lipotropic agent. Possibly, the most important roles of Met in the liver is to stimulate synthesis of very low-density lipoprotein (VLDL) and as a methyl-donor to enhance the synthesis of the methylated compound, S-adenosylmethionine (SAM) [35]. In turn, SAM can be used to produce phosphatidylcholine, which is the main component of the membrane that encloses VLDL, allowing their assembly and secretion from the liver [36]. The result is reduced accumulation of triacylglycerols (TAG) in the liver. About 40 to 60% of milk fat consists 18 and 16-carbon fatty acids, which are almost entirely derived from VLDL. In

the mammary gland, VLDL is anchored to the endothelium by the enzyme lipoprotein lipase (LPL), which then hydrolyzes TAG present in the lipoprotein nucleus to release fatty acids [37].

Oxidative stress status also appears to be influenced by Met availability. Methionine serves as substrate for glutathione synthesis via homocysteine produced in the Met cycle [35]. Glutathione is one of the most abundant natural antioxidants produced in the liver, and in rodents, a reduction in its synthesis promoted steatosis, mitochondrial damage, and marked increases in lipid peroxidation [38]. In addition, Met may also influence milk fat synthesis by upregulating lipogenic gene networks and changing the expression of key miRNA involved in the control of lipogenic balance. This indicates a potentially important role of EAA ratios and mTOR signaling pathways in the regulation of milk fat synthesis [39].

Cows fed the CON diet showed higher milk lactose percentage ($P = 0.03$) than the SM-fed group, but differences in milk lactose yield were not detected (Table 3), suggesting the dilution of this component in the higher milk yield obtained with the SM diet. The higher total solids yield ($P = 0.05$) of the SM-fed cows, but the lack of significant difference of total solids percentage relative to those fed the CON diet may be because of the observed lower lactose percentage obtained with the SM diet.

Plasma AA concentrations

Plasma free AA concentrations are reported in Table 4. There were significant effects of RPMet supplementation on plasma Met and alanine (Ala) concentrations ($P < 0.05$).

Table 4. Effect of dietary RPMet supplementation on plasma AA concentrations (μM/mL) of dairy cows¹.

	Diet ²		P-value
	CON	SM	
Arg	77.1 ± 4.8	86.0 ± 6.8	0.29
His	33.5 ± 3.8	31.7 ± 5.3	0.79
Ile	78.7 ± 5.2	70.0 ± 7.3	0.34
Leu	73.7 ± 7.5	70.0 ± 10.5	0.78
Lys	57.4 ± 4.5	64.7 ± 6.3	0.36
Met	18.4 ± 1.4	29.6 ± 1.9	<0.001
Phe	38.7 ± 1.6	39.0 ± 2.3	0.91
Thr	54.0 ± 3.6	62.1 ± 5.0	0.21
Trp	31.2 ± 1.7	32.3 ± 2.4	0.73
Val	220 ± 16.4	212 ± 22.9	0.76
Ala	206 ± 15.9	272 ± 22.3	0.03
Asn	24.0 ± 2.2	30.5 ± 3.1	0.10
Gln	95.4 ± 8.3	90.6 ± 11.6	0.74
Glu	63.4 ± 4.3	59.6 ± 6.0	0.61
Pro	72.9 ± 5.4	84.4 ± 7.5	0.23
Ser	104 ± 15.8	97.7 ± 22.2	0.80
Tyr	38.7 ± 4.4	39.1 ± 6.1	0.95

¹Data are presented as mean ± SEM; ² CON = Control diet; SM = CON + SM (0.09 % of DMI)

Dietary RPMet supplementation increased plasma Met concentrations by 61% (29.6 vs. 18.4 μM; $P < 0.001$). The increase in plasma Met concentrations was significantly higher than reported by others feeding similar amounts of supplemental Met from other RPMet supplements [22,40,41,42]. Increases in plasma free Met in “Met-deficient” Holstein cows duodenally infused with incremental amounts of DL- Met (g/d) were reported as 1.25 μM of plasma Met/g of Met infused [43,44]. Using the same approach, we found 11.2 μM of plasma Met increase for an intake of 17.25 g of fed DL-Met (0.65 μM of plasma Met/g of Met) and, a calculated estimate of Met bioavailability of 52% (0.65/1.25). This attests to the high bioavailability of Met in Smartamine M. The plasma Met value obtained with the CON diet is very similar to concentrations reported by others when cows are fed corn-based diets supplemented with high Lys protein supplements with RPMet supplementation [10,45,46]. Milk protein

concentration increased in 0,17% in response to a 23 g supplementation of a product with 75% of MET content (17.25 g MET), the change was 0.01% / g of consumed Met. This value was almost the double of the 0,0047% /g reported in other experiment with the same product [40] suggesting that other variables in the diet, environment or cows can interact with this response.

Similarly, RPMet supplementation increased ($P = 0.03$) plasma Ala level in 32% (206.3 μM vs 271.6; $P = 0.03$). There are evidences of increased contribution of Ala to liver glucose synthesis at beginning of lactation. Since Ala is an essential glucogenic AA, and hepatic gluconeogenesis from Ala is greater than from other AA, the higher Met levels may have improved hepatic metabolism sufficiently to increase Ala release from the muscle into the bloodstream [47]. There was no influence of dietary treatment on plasma levels of the other AA.

CONCLUSIONS

We conclude that Met was the most limiting nutrient in this experiment, and that balancing the ration for a more ideal Lys/Met ratio of absorbed AA was of significant benefit to the cows. These findings suggest that, when the diet contains more optimal adequate Lys levels, Met supplementation to achieve the correct Met to Lys ratio improves AA utilization. The best performance appeared to be associated with plasma methionine concentration close to 30 μM /mL.

Overall, the results of this experiment underscore the benefits of balancing for a more ideal profile of absorbed AA in lactating dairy cows on milk and milk component yields and overall production efficiency.

The increase in plasma Met concentrations indicate high bioavailability and stability of the rumen protected MET product used in this experiment even when mixed with other feeds or wet TMR.

REFERENCES

1. Zanton GI. Protein and amino acid nutrition. In Beede DK, editor. Large Dairy Herd Management. Third edition. Champaign IL: American Dairy Science Association; 2017. pp. 625-637.
2. Schwab CG, Broderick GA. A 100-Year Review: Protein and amino acid nutrition in dairy cows. J Dairy Sci. 2017; 100:10094–112.
3. Nan X, Bu D, Li X, Wang J, Wei H, Hu H, et al. Ratio of lysine to methionine alters expression of genes involved in milk protein transcription and translation and mTOR phosphorylation in bovine mammary cells. Physiol Genomics. 2014; 46: 268–275.
4. NRC. Nutrient Requirements of Dairy Cattle. 7th ed. Washington DC: National Research Council National Academy Press; 2001.
5. Amburgh ME Van, Collao-Saenz EA, Higgs RJ, Ross DA, Recktenwald EB, Raffrenato E, et al. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J Dairy Sci. 2015; 98: 6361–6380.
6. Chen ZH, Broderick GA, Luchini ND, Sloan BK, Devillard E. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows¹. J Dairy Sci. 2011; 94:1978–1988.
7. Osorio JS, Ji P, Drackley JK, Luchini D, Looor JJ. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci. 2013; 96:6248–6263.
8. Brosnan JT, Brosnan ME. The sulfur-containing amino acids: An overview. The Journal of Nutrition. 2006; 136(Suppl.):1636S–1640S.
9. Rulquin H, Kowalczyk J. Development of a method for measuring lysine and methionine bioavailability in rumen-protected products for cattle. J Anim Feed Sci. 2003; 12:465–474.
10. Schwab CG, Ordway RS, Whitehouse, NL. Amino acid balancing in the context of MP and RUP requirements. Proc Four-State Applied Nutrition and management Conference. 2003. Pages 10-25.
11. Higgs, RJ, Van Amburgh ME. Evolution of the CNCPS-Development of V7. Proc. Cornell Nutrition Conference; 2016. Syracuse, NY. Cornell University, Pages 125-146
12. Ordway RS, Boucher SE, Whitehouse NL, Schwab CG, Sloan BK. Effects of providing two forms of supplemental methionine to periparturient Holstein

- 450 dairy cows on feed intake and lactational performance. J Dairy Sci. 2009;
451 92:5154–5166.
- 452 13. Preynat A, Lapierre H, Thivierge MC, Palin MF, Matte JJ, Desrochers A, et
453 al. Influence of methionine supply on the response of lactational performance
454 of dairy cows to supplementary folic acid and vitamin B12. J Dairy Sci. 2009;
455 92:1685–1695.
- 456 14. Lee C, Giallongo F, Hristov AN, Lapierre H, Cassidy TW, Heyler KS, et al.
457 Effect of dietary protein level and rumen-protected amino acid
458 supplementation on amino acid utilization for milk protein in lactating dairy
459 cows. J Dairy Sci. 2015; 98:1885–1902.
- 460 15. Giallongo F, Harper MT, Oh J, Lopes JC, Lapierre H, Patton RA, et al. Effects
461 of rumen-protected methionine, lysine, and histidine on lactation
462 performance of dairy cows. J Dairy Sci. 2016; 99:4437–4452.
- 463 16. Sun F, Cao Y, Cai C, Li S, Yu C, Yao J. Regulation of nutritional metabolism
464 in transition dairy cows: Energy homeostasis and health in response to post-
465 ruminal choline and methionine. PLoS One. 2016;11:1–27.
- 466 17. Zhou Z, Bulgari O, Vailati-Riboni M, Trevisi E, Ballou MA, Cardoso FC, et al.
467 Rumen-protected methionine compared with rumen-protected choline
468 improves immunometabolic status in dairy cows during the periparturient period.
469 J Dairy Sci. 2016; 99: 8956–8969.
- 470 18. Batistel F, Arroyo JM, Bellingeri A, Wang L, Saremi B, Parys C, et al. Ethyl-
471 cellulose rumen-protected methionine enhances performance during the
472 periparturient period and early lactation in Holstein dairy cows. J Dairy Sci.
473 2017; 100: 7455-7467
- 474 19. Osorio JS, Trevisi E, Ji P, Drackley JK, Luchini D, Bertoni G, et al. Biomarkers
475 of inflammation, metabolism, and oxidative stress in blood, liver, and milk
476 reveal a better immunometabolic status in periparturient cows supplemented with
477 Smartamine M or MetaSmart. J Dairy Sci. 2014; 97:7437–50.
- 478 20. Zhou Z, Vailati-Riboni M, Trevisi E, Drackley JK, Luchini DN, Looor JJ. Better
479 postparturient performance in dairy cows supplemented with rumen-protected
480 methionine compared with choline during the periparturient period. J Dairy Sci.
481 2016; 99: 8716–8732.
- 482 21. Patton R a. Effect of rumen-protected methionine on feed intake, milk
483 production, true milk protein concentration, and true milk protein yield, and
484 the factors that influence these effects: A meta-analysis. J Dairy Sci. 2010;
485 93: 2105–2118.
- 486 22. Lee C, Lobos NE, Weiss WP. Effects of supplementing rumen-protected
487 lysine and methionine during prepartum and postpartum periods on
488 performance of dairy cows. J Dairy Sci. 2019; 110:11026-11039.
- 489 23. Socha MT, Putnam DE, Garthwaite BD, Whitehouse NL, Kierstead NA,
490 Schwab CG, et al. Improving Intestinal Amino Acid Supply of Pre- and
491 Postpartum Dairy Cows with Rumen-Protected Methionine and Lysine. J
492 Dairy Sci. 2005; 88:1113–1126.
- 493 24. Schwab CG, Bozak CK, Whitehouse NL, Olson VM. Amino Acid Limitation
494 and Flow to the Duodenum at Four Stages of Lactation. 2. Extent of Lysine

- 495 Limitation. J Dairy Sci. 1992; 75:3503–3518.
- 496 25. Aschenbach JR, Kristensen NB, Donkin SS, Hammon HM, Penner GB.
497 Gluconeogenesis in dairy cows: The secret of making sweet milk from sour
498 dough. IUBMB Life. 2010; 62:869–877.
- 499 26. Larsen M, Kristensen NB. Precursors for liver gluconeogenesis in
500 periparturient dairy cows. Animal. 2013; 7:1640–1650.
- 501 27. Galindo C, Larsen M, Ouellet DR, Maxin G, Pellerin D, Lapierre H. Abomasal
502 amino acid infusion in postpartum dairy cows: Effect on whole-body,
503 splanchnic, and mammary glucose metabolism. J Dairy Sci. 2015; 98:7962–
504 7974.
- 505 28. Osorio JS, Ji P, Drackley JK, Luchini D, Loor JJ. Smartamine M and
506 Metasart supplementation during the periparturient period alter hepatic
507 expression of gene networks in 1-carbon metabolism, inflammation, oxidative
508 stress, and the growth hormone-insulin-like growth factor 1 axis pathway. J.
509 Dairy Sci. 2014; 97:7451-7464.
- 510 29. Zhang Q, Bertics SJ, Luchini ND, White HM. The effect of increasing
511 concentrations of DL-methionine and 2-hydroxy-4-(methylthio) butanoic acid
512 on hepatic genes controlling methionine regeneration and gluconeogenesis.
513 J Dairy Sci. 2016; 99:8451–8460.
- 514 30. Doepel L, Lapierre H. Changes in production and mammary metabolism of
515 dairy cows in response to essential and nonessential amino acid infusions. J
516 Dairy Sci. 2010; 93:3264–3274.
- 517 31. Kvidera SK, Horst EA, Abuajamieh M, Mayorga EJ, Fernandez MVS,
518 Baumgard LH. Glucose requirements of an activated immune system in
519 lactating Holstein cows. J Dairy Sci. 2017; 100:2360–2374.
- 520 32. Appuhamy JADRN, Knoebel NA, Nayananjali WAD, Escobar J, Hanigan
521 MD. Isoleucine and Leucine Independently Regulate mTOR Signaling and
522 Protein Synthesis in MAC-T Cells and Bovine Mammary Tissue Slices. The
523 Journal of Nutrition. 2012; 142:484-491.
- 524 33. Haque MN, Guinard-Flament J, Lamberton P, Mustière C, Lemosquet S.
525 Changes in mammary metabolism in response to the provision of an ideal
526 amino acid profile at 2 levels of metabolizable protein supply in dairy cows:
527 Consequences on efficiency. J Dairy Sci. 2015; 98:3951–3968.
- 528 34. Gao HN, Zhao SG, Zheng N, Zhang YD, Wang SS, Zhou XQ, et al.
529 Combination of histidine, lysine, methionine, and leucine promotes β -casein
530 synthesis via the mechanistic target of rapamycin signaling pathway in bovine
531 mammary epithelial cells. J Dairy Sci. 2017; 100:7696–7709.
- 532 35. Martinov M V., Vitvitsky VM, Banerjee R, Ataullakhanov FI. The logic of the
533 hepatic methionine metabolic cycle. Biochim Biophys Acta - Proteins
534 Proteomics. 2010; 1804:89–96.
- 535 36. Zom RLG, van Baal J, Goselink RMA, Bakker JA, de Veth MJ, van Vuuren
536 AM. Effect of rumen-protected choline on performance, blood metabolites,
537 and hepatic triacylglycerols of periparturient dairy cattle. J Dairy Sci. 2011;
538 94:4016–27.
- 539 37. Bionaz M, Loor JJ. Gene networks driving bovine milk fat synthesis. BioMed

- Cent. 2008; 9:366.
38. Chen Y, Yang Y, Miller ML, Shen D, Shertzer HG, Stringer KF, Wang B, Schneider SN, Nebert DW, Dalton TP. Hepatocyte-specific Gc/c deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. *Hepatology*. 2007; 45:1118-1128.
39. Li S, Hosseini A, Danes M, Jacometo C, Liu J, Looor JJ. Essential amino acid ratios and mTOR affect lipogenic gene networks and miRNA expression in bovine mammary epithelial cells. *J Anim Sci Biotechnol*. 2016; 7:44-54.
40. Flemming AJ, Estes KA, Choi H, Barton BA, Zimmerman CA, Hanigan MD. Assessing bioavailability of ruminally protected methionine and lysine prototypes. *J Dairy Sci*. 2019; 102: 4014-4024.
41. Lee C, Hristov AN, Cassidy TW, Heyler KS, Lapierre H, Varga GA et al. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. *J Dairy Sci*. 2012; 95:6042–6056.
42. Giallongo F, Harper MT, Oh J, Lopes JC, Lapierre H, Patton RA, et al. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. *J Dairy Sci*. 2016; 99: 4437–4452.
43. Whitehouse NL, Schwab CG, Tylutki T, Sloan BK. Optimal lysine and methionine concentrations for milk protein production as determined with the latest versions of Dairy NRC 2001 and AMTS.Cattle. *J. Dairy Sci*. 2013; 96 (E-Suppl. 1): 253.
44. Whitehouse, N. L., C. G. Schwab, M. C. Blais, A. F. Brito, and B. K. Sloan. 2014. Evaluating the plasma free amino acid dose–response method to determine the content of metabolizable methionine in a rumen-protected methionine supplement. *J. Dairy Sci*. 2014; 97 (E-Suppl. 1):763
45. Nichols K, Laar, H van, Bannink A, Dijkstra J. Mammary gland utilization of amino acids and energy metabolites differs when dairy cow rations are isoenergetically supplemented with protein and fat. *J Dairy Sci*. 2019; 102:1160-1175.
46. Zang Y, Samii SS, Phipps ZC, Tager LR, McFadden JW, Krause KM. Comparative effects of multiple sources of rumen-protected methionine on milk production and serum amino acid levels in mid-lactation dairy cows. *The professional Animal Scientist*; 2017; 33: 692-699.
47. Rodwell VW, Bender DA, Bothan KM, Kennely PJ, Weil PA. *Bioquímica ilustrada de Harper*. 30ª ed. Porto Alegre: AMGH; 2017.