Lactational performance of dairy cows in response to supplementing *N*-acetyl-L-methionine as source of rumen-protected methionine

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ABSTRACT

The objective of this experiment was to evaluate the effects of supplementing a rumen-protected source of Met, *N*-acetyl-L-methionine (NALM), on lactational performance and nitrogen metabolism in early- to mid-lactation dairy cows. Sixty multiparous Holstein dairy cows in early lactation (27 ± 4.3 d in milk, SD) were assigned to 4 treatments in a randomized complete block design. Cows were blocked by actual milk yield. Treatments were as follows: (1) no NALM (control); (2) 15 g/d of NALM (NALM15); (3) 30 g/d of NALM (NALM30); and (4) 45 g/d of NALM (NALM45). Diets were formulated using a Cornell Net Carbohydrate and Protein System (CNCPS) v.6.5 model software to meet or exceed nutritional requirements of lactating dairy cows producing 42 kg/d of milk and to undersupply metabolizable Met (control) or supply incremental amounts of NALM. The digestible Met (dMet) supply for control, NALM15, NALM30, and NALM45 were 54.7, 59.8, 64.7, and 72.2 g/d, respectively. The supply of dMet was 88, 94, 104, and 115% of dMet requirement for control, NALM15, NALM30, and NALM45, respectively. Milk yield data were collected, dry matter intake (DMI) was measured daily, and milk samples were collected twice per week for 22 wk. Blood, ruminal fluid, urine, and fecal samples were collected during the covariate period and during wk 4, 8, and 16. Data were analyzed using the GLIMMIX procedure of SAS (SAS Institute) using covariates in the model for all variables except body weight. Linear, quadratic, and cubic contrasts were also tested. Treatments did not affect DMI, milk yield, and milk component concentration and

yield; however, feed efficiency expressed as milk yield per DMI and 3.5% fat-corrected milk per DMI were quadratically affected, with greater response observed for NALM15 and NALM30 compared with control. Acetate proportion linearly increased, whereas propionate proportion linearly decreased with NALM supplementation. Blood urea nitrogen linearly decreased with NALM supplementation. Total plasma essential AA concentrations were quadratically affected, as greater values were observed for control and NALM45 than other treatments. Plasma Met concentration was quadratically affected as lower levels were observed with NALM15, whereas Met concentrations increased with NALM45 compared with control. Nitrogen utilization efficiency and apparent total-tract nutrient digestibility were not affected by treatment. Supplementation of NALM at 15 or 30 g/head per day resulted in the greatest improvements in feed efficiency without affecting N metabolism of early- to mid-lactation dairy cows.

Key words: amino acid profile, feed efficiency, metabolizable methionine, *N*-acetyl-L-methionine

INTRODUCTION

Diet formulation on a CP basis has been shown to have several shortcomings over the past decades (Schwab and Broderick, 2017). In addition, the growing concerns about N pollution by dairy cows have led researchers to focus on nutrient management for more efficient utilization of available resources and reduction of environmental emissions of nitrous oxide and volatile organic compounds such as NH₃ (Place and Mitloehner, 2010). Thus, diet formulations for dairy cows have moved away from balancing CP requirements toward formulations based on providing ruminal NH₃ and AA for ruminal and post-ruminal metabolism (Schwab and Broderick, 2017).

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Nitrogen is one of the major pollutants from dairy production, as dietary sources of N in lactation diets are used inefficiently by dairy cows (Noftsker et al., 2005). Low N efficiency results in greater excretion of N in manure rather than in milk, increasing environmental N excretion and reducing farm profitability (Noftsker et al., 2005). For optimizing N efficiency, and subsequently lactational performance, balancing rations for individual AA has been the most promising strategy (Noftsker and St-Pierre, 2003; Lee et al., 2012). Furthermore, milk protein synthesis is limited by the AA that is in shortest supply relative to the AA requirements (Schwab et al., 1976). The goal for optimizing N efficiency is to maximize microbial protein synthesis in the rumen and supplement additional rumen-protected AA to meet the requirement for milk protein synthesis (Schwab et al., 1976). Hence, balancing AA profile of dairy cattle diets is imperative, as it enhances milk protein synthesis, improves N efficiency, and reduces N excretion.

Supplemental dietary AA should be provided in rumen-protected forms to prevent microbial or chemical degradation in the rumen and increase availability at the absorption sites in the small intestine (Lapierre et al., 2006). *N*-Acetyl-L-Met (**NALM**) is a Met derivative produced by acetylation of Met and excess acetic acid. Rotruck and Boggs (1975) showed greater bioavailability of Met along with greater Met incorporation in tissue proteins, supporting growth in rats fed with NALM when compared with only L-Met. *N*-Acetyl-L-Met supplementation increased milk fat synthesis and feed efficiency in early-lactation (53 ± 10 DIM, SD) dairy cows (Fagundes et al., 2018). Liang et al. (2019) observed greater milk yield with NALM supplementation in mid-lactation dairy cows (167 ± 13 DIM); however, no effects were observed on feed efficiency and milk fat synthesis. Although rumen-protected Met (**RPM**) supplementation in dairy cow diets have been extensively evaluated (Schwab, 1996; Schwab and Broderick, 2017), we are still lacking studies evaluating the long-term efficacy of RPM on production performance of lactating dairy cows. In addition, not many studies have investigated the effectiveness of using NALM as source of RPM. Thus, the objective of the current study was to assess the effects of supplementing incremental amounts of NALM on lactational performance and N balance of early- to mid-lactation dairy cows fed diets with adequate amounts of MP (average MP balance; +120.6 g/d) but deficient in metabolizable Met (average Met balance; -8.9 g/d). We hypothesized that providing adequate amounts of metabolizable Met to diets deficient in the nutrient would increase milk protein synthesis and N efficiency by lactating dairy cows.

MATERIALS AND METHODS

The dairy cows used in this study were cared for in accordance to guidelines approved by the University of Florida Institutional Animal Care and Use Committee.

Cows, Treatments, and Experimental Design

Sixty lactating, multiparous (parity 2–5) Holstein dairy cows in early lactation (27 ± 4.3 DIM) were blocked by actual milk yield in a randomized complete block design and assigned to 1 of 4 treatments as follows: (1) no NALM (control); (2) 15 g/d of NALM (**NALM15**); (3) 30 g/d of NALM (**NALM30**); and (4) 45 g/d of NALM (**NALM45**) in a continuous lactation trial for 26 wk [1 wk of training to access feed from Calan gates (American Calan Inc.), 1 wk of a covariate period, 2 wk of adaptation to NALM supplementation, and 22 wk of data collection]. Cows were housed in a freestall, open-sided, sand-bedded barn fitted with Calan gates for individual feeding with 2 rows of fans (1 fan every 6 linear meters) and misters with low-pressure nozzles for cooling the cows once ambient temperature reached 21.1°C. Data on the prevailing temperature and humidity during the trial were collected from the Florida Automated Weather Network (<http://fawn.ifas.ufl.edu/>). During the experiment, the temperature averaged 18.7°C and ranged from 3.8 to 31.2°C, whereas relative humidity averaged 81.6% and ranged from 71 to 87%. The sand in beds was cleaned once per day and topped up twice per week. Water was provided ad libitum from an automatic paddle water bowl system. Manure in the areas behind the feed bunks was scraped and flushed out twice daily by an automated flushing system.

N-Acetyl-L-Met (CJ CheilJedang) had 78.4% Met concentration with ruminal escape rate of 67% and 80% absorption coefficient from small intestine; the supply of digestible Met from NALM was assumed to be 42% from the dose supplemented (Met content $\times$ rumen bypass rate $\times$ absorption rate in the small intestine). Hence, adding 15, 30, and 45 g/d of NALM as top-dress onto corresponding experimental diets can provide 6.30, 12.6, and 18.9 g/d of metabolizable Met, respectively. The basal diet was formulated to meet or exceed most nutrient requirements (except metabolizable Met) of lactating dairy cows weighing 670 kg of BW, producing 42 kg of milk/d with 3.50% milk fat and 2.90% milk true protein, averaging 100 DIM, and having expected daily DMI of 24.0 kg. Diets were formulated using NDS Professional (RUM&N) based on Cornell Net Carbohydrate and Protein System equations (CNCPS v.6.5) such that the ration was adequate in meeting energy

and protein requirements but deficient in metabolizable Met (Table 1). The basal diet was MP adequate but Met deficient (-8.9 g/d) and was fed as a TMR twice daily (0600 and 1200 h) using a Calan data ranger (American Calan Inc.). *N*-Acetyl-L-Met was used as a source of RPM and was top-dressed daily along with the morning feeding on the TMR. We prepared 200 g of top-dress for the control group by mixing ground corn (80%) and dried molasses (20%). The top-dress for treatment groups was prepared by replacing ground corn with increasing proportions of NALM to provide 15, 30, and 45 g/cow per day of RPM for NALM15, NALM30, and NALM45, respectively; however, the total amount of top-dress remained similar for all treatments (200 g).

Feed Intake and Total-tract Apparent Digestibility

The TMR offered was weighed daily for each cow. Orts were collected and weighed every morning before feeding. Cows were fed ad libitum (108% of the previous day's DMI). Representative samples (approximately 400 g) of silage, TMR, and orts were collected daily and dried at 55°C for 48 h in a forced-air oven for TMR adjustment and DMI calculation. For digestibility measurements, TMR and individual orts samples were collected for 3 d during the covariate period and at wk 4, 8, and 16 of the experiment. Spot fecal samples were collected (4 samples/cow per day) for 3 d during covariate period and wk 4, 8, and 16. Fecal samples were collected at 0500, 0900, 1300, and 1700 on d 1, 0600, 1000, 1400, and 1800 on d 2, and 0700, 1100, 1500, and 1900 on d 3. Samples were dried at 55°C for 48 h in a forced-air oven, ground to pass through the 1-mm screen of a Wiley mill, and dried in oven overnight at 105°C for DM estimation. Samples were ashed at 550°C for 5 h (AOAC International, 2000; method 942.05) for OM estimation. The concentration of N was determined by rapid combustion using a micro elemental N analyzer (Vario Micro Cube, Elementar Analysensysteme GmbH), following AOAC International method 992.15 (AOAC International, 1995), and CP values were calculated by multiplying N concentrations by 6.25. Amylase-NDF and ADF concentrations were sequentially analyzed using a fiber analyzer (200/220, Ankom Technology) using methodology based on Van Soest et al. (1991). Sodium sulfite along with heat-stable amylase (Type XI-A from *Bacillus subtilis*; Sigma-Aldrich) was used for the determination of NDF concentration. Ether extract was measured using a fat analyzer (XT20, Ankom Technology; AOAC International, 2000; method 2003.05). Indigestible NDF (iNDF) was used as an internal marker for fecal output estimation (Huhtanen

et al., 1994). For the estimation of iNDF, 0.7 g of TMR, orts, and fecal samples were weighed in Ankom F57 filter bags (Ankom Technology) and incubated in 2 ruminally cannulated lactating Holstein cows for 288 h. Bags were washed and analyzed for NDF as previously described. Fecal output (kg of DM/d) was calculated as follows: iNDF intake (kg/d)/iNDF% feces, where iNDF intake (kg/d) = [iNDF% TMR $\times$ TMR offered (kg/d)] $-$ [iNDF% orts $\times$ orts (kg/d)]. Total-tract apparent digestibility (%) was calculated for all nutrients analyzed as follows: $100 - \{[\text{nutrient\% feces} \times \text{fecal output (kg/d)}] / [\text{nutrient intake (kg/d)}]\}$, where nutrient intake (kg/d) = [nutrient% TMR $\times$ TMR offered (kg/d)] $-$ [nutrient% orts $\times$ orts (kg/d)].

Milk Production, Milk Composition, and BW

Cows were milked twice daily at 1100 and 2300 h. Milk yield was recorded daily by the Afikim milking system (AfiLab mini laboratory, Afimilk Ltd.). Morning and evening milk samples were collected once weekly during the entire experiment. Milk samples were analyzed for milk fat, protein, lactose, SCC, and MUN at Eastern Laboratory Services (Medina, OH). Milk fat and protein yield and feed efficiency were calculated for each cow individually. Similarly, 3.5% FCM and ECM were calculated using the following equations:

$$3.5\% \text{ FCM} = [0.4324 \times \text{milk yield (kg/d)}] + [16.216 \times \text{fat yield (kg/d)}];$$

$$\text{ECM} = 0.327 \times \text{milk yield (kg/d)} + 12.95 \times \text{fat yield (kg/d)} + 7.2 \times \text{protein yield (kg/d)}.$$

Body weight was recorded twice daily after milking (Afiweight scale, Afimilk Ltd.) and averaged over week. Body weight gain (kg/d) for each cow was calculated as the slope of the linear regression of weekly BW over time.

Blood Chemistry and Plasma AA Profile

Blood samples were collected once on d 5 during the covariate period and wk 4, 8, and 16 approximately 2 h after the morning feeding from the coccygeal vein into evacuated tubes containing sodium heparin anticoagulant. Tubes were centrifuged at $3,000 \times g$ for 20 min at 4°C , and plasma samples were stored at -20°C for further analysis. Concentrations of nonesterified fatty acids (NEFA; NEFA-C kit; Wako Diagnostics Inc.; as modified by Johnson and Peters, 1993), BHB (Wako

Autokit 3-HB; Wako Diagnostics Inc.), and glucose (Glucose [Oxidase] Liquid Reagent Set, TECO Diagnostics Inc.) in plasma were determined in duplicate. Plasma AA profile and BUN were analyzed from 40

randomly selected cows (10 from each treatment) at Experimental Station Chemical Laboratories, University of Missouri (Columbia, MO) using the automated ion-exchange chromatography method (Hitachi L-

Table 1. Ingredient and chemical composition of the experimental diets fed to dairy cows during early and mid lactation (n = 32)

Item	Experimental diet ¹			
	Control	NALM15	NALM30	NALM45
Ingredient, % of DM				
Corn silage	41.9	41.9	41.9	41.9
Corn grain, ground shelled	18.2	18.2	18.2	18.2
Soybean meal, 44%	15.4	15.4	15.4	15.4
Whole cottonseed	8.20	8.20	8.20	8.20
Canola meal	3.90	3.90	3.90	3.90
Citrus pulp	6.32	6.32	6.32	6.32
Energy booster ²	1.76	1.76	1.76	1.76
Ca montmorillonite clay ³	0.39	0.39	0.39	0.39
Mineral and vitamin mix ⁴	3.92	3.92	3.92	3.92
Top-dressed, g/d				
Corn grain	160	145	130	115
Molasses	40.0	40.0	40.0	40.0
NALM ⁵	0	15.0	30.0	45.0
Chemical composition, % of DM $\pm$ SD				
DM, % as fed	54.3 $\pm$ 5.34	—	—	—
OM	96.4 $\pm$ 0.38	—	—	—
CP	17.5 $\pm$ 0.58	—	—	—
NDF	33.6 $\pm$ 3.24	—	—	—
ADF	16.8 $\pm$ 1.33	—	—	—
Starch	25.0 $\pm$ 0.69	—	—	—
NE _L , ⁶ Mcal/kg	1.66	—	—	—
Protein supply, ⁷ g/d				
MP supply	2,754	2,689	2,612	2,676
MP from bacteria, g/d	1,296	1,275	1,249	1,271
MP from RUP, g/d	1,458	1,414	1,362	1,405
MP requirements	2,553	2,593	2,538	2,565
MP balance	201	96.2	73.8	111.2
Lys and Met balance, ⁷ g/d				
dLys requirement	177.2	180.4	176.9	178.5
dLys supplied by the diet	193.5	189.2	184.1	188.4
Lys balance	16.3	8.90	7.2	9.9
dMet requirement	61.9	63.2	62.1	62.6
dMet supplied by the diet	54.7	53.5	52.1	53.3
dMet from Met derivative	0	+6.30	+12.6	+18.9
Met balance	-7.20	-3.40	+2.60	+9.60
dLys:dMet	3.54:1	3.16:1	2.85:1	2.61:1

¹Control = 0 g/d of *N*-acetyl-L-Met (NALM); NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.

²Prilled fatty acids (Milk Specialties Global).

³Novasil Plus (BASF Corp.).

⁴Formulated to contain (as-is): 29.4% of sodium bicarbonate, 22.4% of calcium carbonate, 15.8% of potassium chloride, 13.5% of monocalcium phosphate, 8.68% of magnesium oxide, 6.57% of NaCl, 1.10% of Cellulo-Gest, 1.05% of ClariFly (0.67%), 0.41% of selenium yeast, 0.27% of vitamin E (50%; 227,000 IU/lb), Intellibond vital 4-0.25, 0.17% of biotin (2%), 0.07% of vitamin A, 0.01% of vitamin D3, 0.005% of calcium iodate, 0.005% of cobalt carbonate, and 0.20% of Rumensin (Elanco Animal Health).

⁵A developmental NALM product (CJ CheilJedang, Seoul, Korea) was the source of rumen-protected Met, and it contained 78.0% Met.

⁶Estimated using NDS Professional (RUM&N) based on Cornell Net Carbohydrate and Protein System equations (CNCPS v.6.5).

⁷Protein supply and Lys and Met balance were adjusted using NDS professional (RUM&N) program using actual DMI observed for different treatments during the experiment. dLys = digestible Lys; and dMet = digestible Met.

8500A), as previously described by Le Boucher et al. (1997).

Ruminal Fluid VFA and $\text{NH}_3\text{-N}$

Ruminal fluid was collected from noncannulated cows in the covariate period and during wk 4, 8, and 16 approximately 2 h after the morning feeding. A stomach tube was used to pump fluid out of the rumen into a vessel. The first 200 mL of fluid pumped out was discarded, and the subsequent 200 mL was filtered with 4 layers of cheese cloth. Ruminal fluid (40 mL) was transferred to 50-mL centrifuge tubes, acidified with 0.4 mL of 50% H_2SO_4 solution (vol/vol), and centrifuged at $7,000 \times g$ for 15 min at 4°C , and the supernatant was stored at -20°C for further analysis. Volatile fatty acids were analyzed using a Merck Hitachi Elite LaChrome HPLC system (L2400, Hitachi) and a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories). Briefly, the column was used in an isocratic elution system containing 0.015 M sulfuric acid (H_2SO_4) in the mobile phase of HPLC with a UV detector (wavelength 210 nm; L2400, Hitachi) and a flow rate of 0.7 mL/min at 46°C . Ammonia-N was measured using an autoanalyzer (Technicon) that quantifies N by a colorimetric method adapted from Noel and Hambleton (1976).

Nitrogen Balance and Microbial Protein Production

Nitrogen balance was calculated using N intake and the fecal, urine, and milk N excretions. Spot urine samples were collected twice daily (a.m. and p.m.) for 2 consecutive days during the covariate period and wk 4, 8, and 16. Urine samples were filtered with 2 layers of cheese cloth to remove contaminants such as hair, diluted (1:4) with 0.036 N H_2SO_4 standardized to pH below 2 with 50% H_2SO_4 solution (vol/vol), and frozen at -20°C for further analysis. Concentrated urine samples were stored at -20°C as back-up samples for further analysis. Urine N was analyzed by digesting 1 mL of concentrated urine with a digestion mixture of 1.5 g of 9:1 $\text{K}_2\text{SO}_4\text{:CuSO}_4$ and 4.5 mL of H_2SO_4 using the modified aluminum block digestion procedure of Gallaher et al. (1975). Tubes were set in a digestion block (120-tube Martin Machine Magnum Series) and digested at 375°C for 4 h. Digested urine samples were cooled overnight before addition of 75 mL of deionized water. Nitrogen in the digested samples was determined by semiautomated colorimetry using an Alpkem Rapidflow Analyzer II according to Hambleton (1977). The absorbance of the ammonia-salicylate complex was measured at 660 nm. Urinary urea N was analyzed using a kit (Urea-N Colorimetric Detection Kit, Arbor Assays).

Creatinine concentration in undiluted samples was measured using a colorimetric assay (Creatinine Urinary Detection Kit, Arbor Assays). Total urine output was calculated using urinary excretion of creatinine equivalent to 29 mg/kg of BW per day (Valadares et al., 1999). Uric acid was measured using a colorimetric assay kit (Infinity Uric Acid Liquid Stable Reagent, Thermo Scientific), and allantoin was estimated using a method based on Chen and Gomes (1992) adapted to a 96-well plate reader. Total purine derivatives (PD) were calculated as the sum of uric acid and allantoin excreted in urine. Endogenous PD excretion was calculated from BW as $0.385 \text{ mmol/BW}^{0.75}$. Daily absorption of microbial purines was calculated as total PD excretion – endogenous PD. Microbial N flow (g/d) was estimated using daily absorption of microbial purines as $(\text{microbial purine absorption} \times 70)/(0.116 \times 0.83 \times 1,000)$ according to Chen and Gomes (1992).

Statistical Analysis

Data were analyzed using the GLIMMIX procedure of SAS (version 9.4; SAS Institute). All variables, except BW gain, were analyzed including covariates according to the following model:

$$Y_{ijklm} = \mu + T_i + W_j + (TW)_{ij} + B_k + \text{Cov}_l + C(T)_{im} + E_{ijklm},$$

where Y is the dependent variable; T_i is the fixed effect of the i th treatment ($i = 1, 2, 3, 4$); W_j is the fixed effect of j th sampling week ($j = 1, 2, 3, \dots, 22$); $(TW)_{ij}$ is the interaction effect of i th treatment at j th level; B_k is the random effect of k th block ($k = 1, 2, 3, 4, 5, \dots, 15$); COV_l is the effect of covariate; $C(T)_{im}$ is the random effect of cow nested within treatment; and E_{ijklm} is the residual error. Errors within cows across weeks, which are repeated measures due to sequential sampling of milk, DMI, and blood, were modeled by using the Akaike information criteria covariance structure (unstructured, compound symmetry, first-order autoregressive) with the lowest Bayesian information criterion. Linear, quadratic, and cubic contrasts were tested. Significance was declared at $P \leq 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$.

RESULTS

The ingredient and chemical composition of experimental diets are presented in Table 1. The basal diet provided adequate MP; however, it was deficient in Met (-8.9 g/d ; Table 1). Based on actual DMI and ingredient AA composition, supplementation of NALM

allowed Met balances of -7.20 , -3.40 , 2.60 , and 9.60 g/cow per day for control, NALM15, NALM30, and NALM45, respectively. Methionine balance estimates were based on CNCPS v.6.5. The ratio of digestible Lys to Met was 3.54:1, 3.16:1, 2.85:1, and 2.61:1 for the respective treatments (Table 1).

Supplementing NALM had no effects on DMI, milk yield, 3.5% FCM yield, and ECM yield (Table 2). However, feed efficiency expressed as milk yield per unit of DMI showed quadratic tendency with greater values observed for NALM30 compared with the control ($P = 0.06$). Feed efficiency was increased quadratically when expressed as 3.5% FCM per unit of DMI ($P = 0.04$; Table 2). Milk components including fat, protein, and lactose concentration averaged 3.43, 3.05, and 4.73%, respectively, and were unaffected by NALM supplementation. Similarly, no NALM effects were observed on yields of milk fat, milk protein, milk lactose, MUN concentration, and SCC. In contrast, quadratic effects were observed on BW gain with NALM supplementation, with lower gain observed for NALM15 and NALM30 compared with control and NALM45 ($P < 0.02$). No NALM $\times$ week interaction was observed for the production parameters.

Concentrations of $\text{NH}_3\text{-N}$ and total VFA and pH were not influenced by NALM supplementation (Table 3). However, molar proportion of ruminal acetate linearly increased ($P = 0.04$), whereas that of ruminal propionate linearly decreased ($P = 0.02$) with NALM supplementation, resulting in linear increase of acetate-

to-propionate ratio due to NALM supplementation. Ruminal butyrate proportion tended to increase linearly ($P = 0.09$), whereas no effects were observed on molar proportion of iso-butyrate and iso-valerate with NALM supplementation.

Plasma glucose and BHB concentrations averaged 3.89 mM and 0.63 μM , respectively, and were not influenced by NALM supplementation (Table 4). However, BUN concentration linearly decreased ($P = 0.02$) and treatment $\times$ sampling week interaction was significant (Supplemental Figure S1, <https://doi.org/10.7910/DVN/RCSH1J>), and significant quadratic and cubic effects were observed with NEFA concentration ($P = 0.03$; quadratic and cubic effect).

Quadratic effects were observed on total EAA with lower values observed for NALM15 and NALM30 compared with other treatments ($P < 0.01$; Table 4), whereas total NEAA concentration was not influenced by NALM supplementation. Individual EAA concentrations including Arg, His, Lys, Met, Phe, and Trp were quadratically affected with lower concentration observed for NALM15 and NALM30 ($P \leq 0.01$). Both quadratic ($P < 0.01$) and cubic effects ($P = 0.05$) were observed for Leu with lower concentration observed for NALM15. In addition, treatment $\times$ week interactions were observed for Arg (Supplemental Figure S2), Lys (Supplemental Figure S3), and Tyr (Supplemental Figure S4, <https://doi.org/10.7910/DVN/RCSH1J>). When expressed as percentage of total EAA, plasma Met linearly increased ($P < 0.01$), whereas plasma Lys

Table 2. Effects of N-acetyl-L-Met (NALM) supplementation on DMI, milk production and composition, and BW gain in dairy cows during early and mid lactation¹

Item	Treatment ²					Contrast ³		
	Control	NALM15	NALM30	NALM45	SEM	L	Q	C
DMI, kg/d	24.6	24.1	23.5	24.0	0.53	0.29	0.36	0.64
Milk yield, kg/d	38.4	39.7	39.6	39.5	1.20	0.56	0.56	0.77
Milk yield/DMI	1.55	1.66	1.75	1.64	0.06	0.16	0.06	0.43
3.5% FCM, ⁴ kg/d	38.0	40.1	38.5	37.5	1.40	0.65	0.29	0.49
3.5% FCM/DMI	1.54	1.66	1.68	1.57	0.06	0.65	0.04	0.83
ECM, ⁵ kg/d	38.1	39.9	38.5	39.0	1.58	0.86	0.69	0.47
ECM/DMI	1.54	1.65	1.69	1.62	0.06	0.30	0.15	0.92
Fat, %	3.54	3.54	3.31	3.30	0.14	0.13	0.95	0.47
True protein, %	3.08	3.00	3.04	3.06	0.25	0.25	0.28	0.41
Lactose, %	4.76	4.71	4.75	4.69	0.04	0.28	0.91	0.22
MUN, mg/dL	11.6	11.6	11.0	11.3	0.40	0.47	0.74	0.39
Fat, kg/d	1.32	1.40	1.31	1.27	0.06	0.39	0.35	0.45
True protein, kg/d	1.18	1.18	1.18	1.21	0.10	0.24	0.41	0.69
Lactose, kg/d	1.83	1.88	1.88	1.86	0.06	0.73	0.63	0.93
Log SCC	2.15	2.07	1.99	2.03	0.11	0.40	0.58	0.82
BW gain, kg/d	0.82	0.51	0.73	0.85	0.10	0.39	0.02	0.23

¹Early and mid lactation = cows were evaluated during a period of 22 wk, n = 15 animals/treatment.

²Control = 0 g/d of NALM; NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.

³L = linear; Q = quadratic; C = cubic effect of NALM. Treatment and treatment by sampling week interaction were not significant.

⁴3.5%FCM = $[0.4324 \times \text{milk yield (kg/d)}] + [16.216 \times \text{fat yield (kg/d)}]$.

⁵ECM = $0.327 \times \text{milk yield (kg/d)} + 12.95 \times \text{fat yield (kg/d)} + 7.2 \times \text{protein yield (kg/d)}$.

Table 3. Effects of *N*-acetyl-L-Met (NALM) supplementation on ruminal fermentation parameter estimates in dairy cows during early and mid lactation¹

Item	Treatment ²				SEM	Contrast ³		
	Control	NALM15	NALM30	NALM45		L	Q	C
Rumen pH	6.43	6.32	6.23	6.33	0.07	0.17	0.10	0.52
Ammonia-N, mg/dL	8.44	7.19	8.11	8.69	0.84	0.64	0.26	0.50
Total VFA, mM	104.5	106.8	107.8	106.9	6.89	0.73	0.77	0.98
Individual VFA, mol/100 mol								
Acetate (A)	57.1	56.4	56.8	59.1	0.81	0.04	0.03	0.75
Propionate (P)	31.3	31.8	30.4	29.1	0.82	0.02	0.19	0.54
Butyrate	9.73	10.1	10.2	10.7	0.43	0.09	0.89	0.92
Isobutyrate	1.84	1.50	2.02	1.49	0.33	0.72	0.78	0.17
Isovalerate	0.10	0.18	0.17	0.14	0.21	0.23	0.24	0.40
A:P	1.91	1.82	1.94	2.13	0.07	<0.01	0.03	0.60

¹Samples were collected during the covariate period and wk 4, 8, and 16; n = 15 animals/treatment.

²Control = 0 g/d of NALM; NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.

³L = linear; Q = quadratic; C = cubic effect of NALM. Treatment and treatment by sampling week interaction were not significant (except for acetate).

quadratically decreased with NALM15 and NALM30 ($P = 0.02$).

Quadratic effects of NALM supplementation were observed for Asn, Gln, and Tyr ($P \leq 0.05$; Table 4), and a tendency was observed for Pro concentration ($P = 0.06$; quadratic effect), with consistently lower concentrations of AA observed for NALM15 and NALM30. The plasma concentrations of 1-methyl-histidine linearly decreased ($P = 0.02$), whereas quadratic effects were observed for ornithine ($P = 0.02$), citrulline ($P = 0.09$), and hydroxylysine ($P = 0.04$) concentrations with NALM supplementation.

No effects of NALM supplementation were observed on N intake, milk protein N, and milk total N (Table 5). Similarly, no effects were observed on urine parameters including uric acid, allantoin, PD, and urine N ($P > 0.05$); however, quadratic tendency was observed for urea N when presented as percent of urine total N ($P = 0.08$), as greater values were observed for NALM15 and NALM30 compared with other treatments. Microbial N flow was not influenced by treatments; however, numerical linear improvement was observed with NALM supplementation ($P = 0.16$). Fecal and manure N excretions were not affected by NALM supplementation; however, cubic tendency was observed on fecal N per unit of N intake ($P = 0.06$). In addition, no NALM effects were observed on apparent total-tract digestibilities of DM, OM, CP, NDF, ADF, and ether extract ($P \geq 0.10$; Table 6).

DISCUSSION

Previous studies have investigated the efficacy of RPM supplementation using MP-adequate and MP-deficient

diets (Schwab et al., 1976; Lee et al., 2012; Fagundes et al., 2018). Based on CNCPS v.6.5, the control diet used in this study was adequate in MP but deficient in metabolizable Met (-7.20 g/d). The MP was adequate for all NALM-supplemented groups; however, Met balance was negative for NALM15 (-3.40 g/d), and positive for NALM30 ($+2.60$ g/d) and NALM45 ($+9.60$ g/d).

Production Performance

The lack of effects of NALM on DMI is in agreement with earlier studies evaluating NALM or other forms of RPM in lactating dairy cows (Leonardi et al., 2003; Chen et al., 2011); however, some studies reported greater DMI with RPM supplementation in the transition period (Osorio et al., 2013; Zhou et al., 2017). In a meta-analytical study, Zanton et al. (2014) reported that DMI responses were dependent on the source of Met supplemented; nevertheless, DMI in ruminants is primarily attributed to gut fill and distension as well as hepatic oxidation of fuels such as propionate, lactate, and NEFA (Allen et al., 2009). Because diets in the present study only differed in levels of metabolizable Met, the lack of treatment effects on DMI was expected.

Milk yields of 3.5% FCM and ECM were not affected by NALM supplementation, and the results probably reflected lack of NALM effects on DMI and milk components. Fagundes et al. (2018) reported similar effects on milk yield with NALM supplementation in diets adequate or deficient in MP; however, 3.5% FCM and ECM yields tended to increase with 30 g of NALM, regardless of the dietary MP status (Fagundes et al., 2018). Liang et al. (2019) reported a quadratic effect on yields of milk, 3.5% FCM, and ECM, as greater

production was observed with 30 g of NALM/d when compared with no NALM supplementation. It is difficult to explain inconsistent responses on the milk production measures. The nutrient composition of the

experimental diets across studies were different. For example, basal diets in the present study had greater concentrations of CP (17.5% vs 15.2%) and starch (25% vs. 18%) compared with those used by Fagundes

Table 4. Effects of *N*-acetyl-L-Met (NALM) supplementation on BUN, nonesterified fatty acids (NEFA), and plasma AA profiles in dairy cows during early and mid lactation¹

Item	Treatment ²				SEM	Contrast ³		
	Control	NALM15	NALM30	NALM45		L	Q	C
Glucose, mM	3.80	3.84	3.89	4.04	0.12	0.11	0.64	0.18
BUN, mg/dL	15.1	14.2	13.4	13.8	0.58	0.02	0.14	0.54
NEFA, μ M	101.0	140.6	116.7	119.1	13.95	0.50	0.03	0.03
BHB, μ M	0.64	0.63	0.62	0.63	0.03	0.79	0.81	0.74
EAA, μ M								
Arg	101.4	88.5	90.2	104.9	3.59	0.40	<0.01	0.90
His	60.6	49.4	52.2	56.1	1.47	0.33	<0.01	0.24
Ile	132.6	122.1	119.9	111.3	4.44	0.26	0.01	0.08
Leu	179.5	145.0	157.6	162.1	6.41	0.15	<0.01	0.05
Lys	100.3	79.0	84.6	95.4	4.34	0.65	<0.01	0.26
Met	22.4	19.5	21.4	24.3	1.07	0.13	0.01	0.43
Phe	46.8	41.8	41.4	43.9	1.31	0.11	<0.01	0.74
Thr	111.3	98.2	100.0	102.5	5.01	0.28	0.13	0.53
Trp	32.4	29.5	30.3	31.1	1.19	0.45	0.04	0.34
Val	312.4	258.8	283.5	274.9	10.89	0.05	0.03	0.02
Total EAA	1,099.8	921.1	981.5	1,017.5	30.88	0.19	<0.01	0.16
Met/EAA, %	2.05	2.13	2.19	2.38	0.09	<0.01	0.55	0.67
Lys/EAA, %	9.12	8.55	8.56	9.37	0.29	0.53	0.02	0.91
NEAA, μ M								
Ala	243.8	240.1	230.4	245.5	10.16	0.92	0.36	0.50
Asn	40.3	35.8	34.9	38.6	1.87	0.48	0.04	0.91
Asp	13.9	12.8	13.2	14.2	0.76	0.72	0.12	0.73
Cys	0.32	0.33	0.34	0.31	0.02	0.73	0.31	0.62
Glu	77.6	75.5	75.3	74.3	3.80	0.55	0.89	0.87
Gln	227.1	183.0	195.7	197.9	7.53	0.04	<0.01	0.05
Gly	274.0	324.8	296.5	312.3	17.50	0.28	0.32	0.12
Pro	96.9	84.2	87.1	88.6	3.63	0.18	0.06	0.30
Ser	92.5	90.1	87.6	94.0	3.45	0.90	0.21	0.56
Tyr	49.3	38.6	41.3	44.6	2.29	0.28	<0.01	0.22
Total NEAA	1,115.5	1,085.1	1,062.2	1,110.0	30.1	0.77	0.20	0.64
Others, μ M								
α -Amino-adipic acid	7.24	7.16	7.74	6.03	0.69	0.31	0.24	0.32
α -Amino-n-butyric acid	8.74	8.01	8.80	8.56	0.55	0.96	0.71	0.28
β -Alanine	1.11	0.94	0.90	0.99	0.12	0.38	0.25	0.97
γ -Amino-butyric acid	1.43	1.67	1.16	1.34	0.22	0.43	0.89	0.15
1-Methyl-histidine	13.1	11.4	11.4	11.1	0.51	0.02	0.19	0.40
3-Methyl-histidine	2.81	2.97	2.75	2.65	0.17	0.38	0.44	0.53
Carnosine	12.5	11.3	10.6	12.2	0.76	0.59	0.03	0.58
Citrulline	101.2	84.9	90.2	91.9	5.19	0.34	0.09	0.29
Cystathionine-allocystathionine	1.81	1.65	1.53	1.81	0.08	0.72	<0.01	0.33
Ethanalamine	2.90	2.84	3.0	2.75	0.21	0.73	0.59	0.44
Homocystine	3.67	4.08	3.18	3.31	0.31	0.16	0.65	0.10
Hydroxylysine	1.32	1.70	1.53	1.49	0.10	0.45	0.04	0.14
Hydroxyproline	11.9	12.6	12.1	12.2	0.79	0.93	0.69	0.59
Ornithine	58.2	48.0	52.5	55.6	2.95	0.78	0.02	0.20
Phosphoserine	7.37	8.24	9.55	9.17	0.98	0.09	0.47	0.58
Sarcosine	19.5	20.1	21.2	21.4	0.86	0.09	0.79	0.73
Taurine	56.0	52.4	52.9	59.6	3.66	0.48	0.17	0.90
Total others	310.8	280.0	291.1	302.2	10.33	0.75	0.05	0.37
Total	2,526.1	2,286.2	2,334.8	2,429.7	55.32	0.34	<0.01	0.33

¹Samples were collected during the covariate period and wk 4, 8, and 16; n = 10 animals/treatment for AA and BUN, otherwise n = 15.

²Control = 0 g/d of NALM; NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.

³L = linear; Q = quadratic; C = cubic effect of NALM. Treatment and treatment by sampling week interaction were not significant except for Arg, Lys, Tyr, and BUN.

Table 5. Effects of *N*-acetyl-L-Met (NALM) supplementation on N metabolism in dairy cows during early and mid lactation¹

Item	Treatment ²					Contrast ³		
	Control	NALM15	NALM30	NALM45	SEM	L	Q	C
N intake, g/d	573.6	574.7	576.8	591.2	26.75	0.45	0.69	0.88
Milk protein N, g/d	192.5	198.6	197.9	197.8	4.89	0.48	0.53	0.73
Milk protein N, % of N intake	34.3	35.2	36.0	34.3	1.75	0.83	0.24	0.61
Milk total N, g/d	196.8	203.1	201.9	202.1	13.13	0.85	0.77	0.71
Milk total N, % of N intake	35.0	36.0	36.7	35.1	1.78	0.85	0.23	0.65
Urinary excretion								
Urine volume, L/d	26.8	27.7	28.8	29.6	2.10	0.33	0.98	0.97
Urine total N, g/d	243.0	238.1	240.2	273.3	18.1	0.12	0.15	0.68
Urine total N, % of N intake	43.2	40.6	42.8	47.7	3.60	0.15	0.11	0.84
Urine urea N, g/d	135.4	145.1	144.9	148	11.39	0.48	0.77	0.80
Urea N, % of urine total N	57.7	63.3	62.8	58.9	2.96	0.80	0.08	0.82
Allantoin, mmol/d	302.1	316.6	329.7	321.1	17.59	0.34	0.48	0.78
Uric acid, mmol/d	61.9	62.8	67.9	67.3	5.28	0.39	0.88	0.66
Purine derivatives, mmol/d	364.7	379.7	408.3	388.9	20.84	0.27	0.39	0.49
Microbial N flow, g/d	265.3	277.8	309.2	292.2	19.46	0.16	0.39	0.38
Fecal N excretion								
Fecal N, g/d	200.9	189.4	203.3	207.9	7.75	0.28	0.27	0.29
Fecal N/N intake, %	35.2	33.2	35.7	35.7	1.79	0.27	0.20	0.06
Manure N, g/d	444.1	432.0	442.6	478.3	22.32	0.12	0.14	0.98

¹Samples were collected during the covariate period and wk 4, 8, and 16, n = 15 animals/treatment.²Control = 0 g/d of NALM; NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.³L = linear; Q = quadratic; C = cubic effect of NALM. Treatment and treatment by sampling week interaction were not significant.

et al. (2018) and Liang et al. (2019). Although the differences in starch and CP concentration may not affect production parameters because diets were balanced for MP, different standards for diet formulation (CNCPS vs. National Research Council) may have resulted in differences in estimation of MP adequacy. The NRC estimates microbial protein synthesis based on TDN, whereas CNCPS uses fermentable carbohydrates for the estimation of microbial protein and subsequently MP (NRC, 2001; Tedeschi et al., 2015).

Despite inconsistent milk yield responses, NALM supplementation increased feed efficiency in previous studies. Liang et al. (2019) observed a tendency for greater feed efficiency, expressed as FCM/DMI, for cows supplemented with 30 g of NALM. Similarly, Fa-

gundes et al. (2018) and Osorio et al. (2013) reported greater 3.5% FCM/DMI and ECM/DMI for cows supplemented with RPM sources. Meanwhile, cows supplemented with NALM had greater NEFA concentrations, probably originating from adipose tissue mobilization because BW gain was lower for cows provided 15 and 30 g/d of NALM. We speculated that the improvement in feed efficiency may have been attributed to greater plasma NEFA concentrations observed with NALM supplementation. Plasma NEFA from adipose tissue mobilization (Bauman and Currie, 1980) can be oxidized or re-esterified to triacylglycerol (**TAG**; Vazquez-Añon et al., 1994) in the liver; however, factors that regulate the flux of NEFA between re-esterification and oxidation are not well understood (Drackley, 1999).

Table 6. Effects of *N*-acetyl-L-Met (NALM) supplementation on total-tract nutrient digestibility in dairy cows during early and mid lactation¹

Item	Treatment ²					Contrast ³		
	Control	NALM15	NALM30	NALM45	SEM	L	Q	C
DM, %	65.3	66.0	64.6	65.4	0.72	0.72	0.93	0.21
OM, %	68.1	68.8	67.0	68.2	0.85	0.63	0.74	0.12
CP, %	64.7	66.4	63.8	64.6	1.68	0.50	0.62	0.14
NDF, %	42.3	43.2	40.8	42.0	1.16	0.43	0.81	0.16
ADF, %	35.8	37.5	36.0	36.4	1.50	0.95	0.51	0.35
Ether extract, %	78.5	79.2	78.2	78.7	0.43	0.73	0.80	0.20

¹Samples were collected during the covariate period and wk 4, 8, and 16; n = 15 animals/treatment.²Control = 0 g/d of NALM; NALM15 = 15 g/d of NALM; NALM30 = 30 g/d of NALM; NALM45 = 45 g/d of NALM.³L = linear; Q = quadratic; C = cubic effect of NALM. Treatment and treatment by sampling week interaction were not significant.

Although liver is prone to greater NEFA esterification and TAG deposition during the early postpartum period, animals were not used in this study until 3 wk postcalving. In addition, lack of effects on milk fat concentration and yield in this study suggested that greater NEFA levels may not have been used by mammary gland as a source of long-chain fatty acids (FA) for milk fat synthesis. Hepatic oxidation of NEFA may have provided energy for the increase in feed efficiency in the NALM-supplemented group. Lysine and Met are precursors of carnitine, a compound that participates in the transport of medium- and long-chain FA into the mitochondria for β -oxidation (Drackley, 1999). Although carnitine levels were not measured in this study, we can speculate that Met utilization for carnitine synthesis may have occurred, thus channeling FA toward oxidation and not re-esterification to TAG.

The most common and expected responses with RPM supplementation in dairy cows are the increases in yield and concentration of milk protein (Rulquin et al., 1993; Vyas and Erdman, 2009; Patton, 2010). However, effects on milk protein synthesis were not consistent in NALM studies (Fagundes et al., 2018; Liang et al., 2019) or in studies supplementing other sources of RPM (Kröber et al., 2001; Younge et al., 2001; Davidson et al., 2008). Fagundes et al. (2018) reported increased milk fat synthesis with NALM, and the effects were attributed to the role of Met in one-carbon metabolism, resulting in greater FA delivery and synthesis in the mammary gland. The lack of effects on milk protein were attributed to partitioning of supplemented NALM toward FA synthesis (Fagundes et al., 2018). Liang et al. (2019) observed no changes in milk components and yields with NALM supplementation, and it was speculated that NALM was partitioned toward protein anabolic reactions in the liver instead of protein synthesis in the mammary gland, as observed by greater plasma protein and globulin concentrations when cows were supplemented with 30 g of NALM. The effects of RPM have not been very consistent across studies and are dependent on dietary CP, AA availability in the small intestine, and intestinal digestibility of the rumen undegraded protein fraction of diet (Cabrita et al., 2011). We can speculate that the lack of NALM effects in this study could be due to changes in NALM bioavailability, with incremental doses resulting in variability in the actual amounts of Met delivered to the intestinal absorptive site. Koenig and Rode (2001) reported 22 and 34% intestinal bioavailability of RPM orally dosed at 20 and 63 g/d, respectively, suggesting lower bioavailability at increased dose of supplementation. In addition, the deficiency of other AA cannot be ruled out because the idea of multiple limiting AA

rather than 1 or 2 limiting AA has been widely accepted in lactating cows (Hanigan et al., 1998; Lapierre et al., 2020).

Nitrogen and AA Metabolism

Dietary manipulations including feeding rumen-protected AA are often used for maximizing N efficiency in dairy cows (Noftsger and St-Pierre, 2003; Lee et al., 2012) at the same time as decreasing N excretion in the environment (Place and Mitloehner, 2010). Noftsger and St-Pierre (2003), Sylvester et al. (2003), and Noftsger et al. (2005) reported greater milk N secretion with RPM supplementation. Furthermore, with RPM supplementation, N-utilization efficiency increased due to a reduction in dietary CP, lower urine N excretion, and greater milk N secretion (Noftsger and St-Pierre, 2003). However, in the present study, milk protein yield and N metabolism were not affected by NALM supplementation. This is consistent with lack of NALM effects on total PD and microbial N flow because total PD can be used as indirect biomarker for microbial protein synthesis in the rumen and absorption in the small intestine (Chen and Gomes, 1992). Total urine volume was estimated in this study using spot urine samples collected twice per day for 2 d during the covariate period and wk 4, 8, and 16 of the experimental duration. The method of spot sampling was selected based on Broderick (2003), Olmos Colmenero and Broderick (2006), and Tebbe and Weiss (2018). However, we acknowledge that recent studies have optimized spot-sampling procedures (Lee et al., 2019), where 12 and 6 sampling time points were considered more optimal compared with 4 and 2 time points. The frequency of spot-sample collection may have affected total urine volume in this study; however, it may not have affected urine N concentration as sampling frequency had no effects on urine N in the previous study (Lee et al., 2019). Furthermore, based on negative N balance and high urinary N excretion in the current experiment, there is a possibility that we overestimated urinary N excretion.

Urea is the major nitrogenous end product of protein metabolism, AA deamination, and systemic protein turnover (Schwab and Broderick, 2017). Blood urea N concentration is related to microbial protein synthesis, as greater concentration may indicate lower incorporation of ruminal $\text{NH}_3\text{-N}$ into microbial protein and consequently greater assimilation to BUN in the liver (Moharrery, 2004). In addition, BUN has been positively correlated with urinary N excretion and is considered a proxy for N-utilization efficiency (Kauffman and St-Pierre, 2001). Because conversion of $\text{NH}_3\text{-N}$ to BUN in the liver consumes energy (Moharrery, 2004;

Neto et al., 2015), we can speculate that higher BUN concentrations may have lowered feed efficiency of cows fed the control compared with those fed NALM diets. Similarly, lower BUN in NALM-supplemented groups may also indicate improved N-utilization efficiency (Broderick and Clayton, 1997; Olmos Colmenero and Broderick, 2006).

Plasma Met concentration can serve as an indicator of effectiveness of RPM supplementation, as abomasal and duodenal Met infusions have been shown to increase blood and plasma Met concentrations (Seymour et al., 1990; Pisulewski et al., 1996). Similarly, dietary supplementation with RPM has shown greater plasma Met concentrations (Blum et al., 1999; Lee et al., 2015; Liang et al., 2019), whereas others did not observe any changes (Noftsker et al., 2005; St-Pierre and Sylvester, 2005; Arriola Apelo et al., 2014b). The quadratic effect observed in the present study for plasma Met concentration was unexpected because metabolizable Met supply linearly increased with NALM treatments. These results should be interpreted with caution because plasma AA can vary greatly throughout the day, and multiple collections over time with more sensitive approaches, such as the dose-response technique, may provide precise and accurate changes in plasma AA profiles (Whitehouse et al., 2017). Nevertheless, lower plasma Met may be attributed to greater utilization of metabolizable Met supplemented at 15 and 30 g/d in pathways such as carnitine synthesis, which may have improved oxidation of long-chain FA for energy production and consequently increased feed efficiency in these 2 groups, as previously discussed.

Nonessential AA are presumably not limiting for milk protein synthesis (Schwab and Broderick, 2017); however, due to their role in several metabolic pathways, NEAA and other related metabolites can be used as physiological biomarkers for N homeostasis. Citrulline and ornithine are intermediates of the urea cycle in the liver (Curis et al., 2005; Breuillard et al., 2015). In the current study, lower plasma citrulline concentrations along with the tendency for lower ornithine concentrations with NALM supplementation agree with the lower BUN concentration with NALM-supplemented cows.

Ruminal Fermentation

Rumen-protected AA are expected to be inert or have minimal effects on ruminal fermentation, and this explains lack of treatment effects on pH, $\text{NH}_3\text{-N}$, and total VFA concentrations as observed in a previous study (Fagundes et al., 2018). However, NALM is partially degraded in the rumen (approximately 33%; Windschitl and Stern, 1988; Fagundes et al., 2018), and

acetyl cleavage during NALM degradation may have resulted in the linear increase in molar proportion of acetate and concomitant reduction in propionate with NALM supplementation in the present study. Aminoacylase-1, an enzyme that can break down NALM and release Met and an acetyl group, is present in bovine epithelial cells (Wu et al., 2012). This is an important trait considering that NALM must be hydrolyzed to free Met and acetate in the small intestine to be absorbed (Baxter et al., 2001). However, several microbes such as bacteria and archaea can synthesize aminoacylases (Sakanyan et al., 1993; Wakayama et al., 1996, 1998). Story et al. (2001) proposed a catabolic pathway for N-acetylated proteins by *Pyrococcus furiosus* where the final metabolite, N-acetyl-Met, is converted to Met and acetate by the microbial aminoacylase. Although this mechanism was not described in ruminal microbes, it is possible that increased acetate is a consequence of the partial hydrolysis of NALM by microbial aminoacylases.

Digestibility of Nutrients

To the best of our knowledge, no published studies have reported the effects of NALM supplementation on apparent total-tract digestibility of nutrients. However, in a study testing 3 different sources of Met supplementation [2-hydroxy-4-(methylthio)-butanoic acid, isopropyl-2-hydroxy-4-(methylthio)-butanoic acid, and DL-Met] providing 9 g of ruminally available Met, authors reported greater ruminal OM and NDF digestibilities, whereas no effect on total-tract OM digestibility was observed (Noftsker et al., 2005). Some RPM sources such as 2-hydroxy-4-(methylthio)-butanoic acid and isopropyl-2-hydroxy-4-(methylthio)-butanoic acid are partially degraded in the rumen and can supply additional Met to rumen microbes, potentially promoting the growth of fibrolytic bacteria and improving fiber digestion. Potential effects of NALM supplementation on nutrient digestibility in the rumen may have been masked by postruminal compensatory digestion. Hence, further research is still needed to confirm these findings.

CONCLUSIONS

N-Acetyl-L-Met supplementation did not affect yields of milk and milk components and N-utilization efficiency; however, feed efficiency expressed as milk yield/DMI, and 3.5%FCM/DMI was increased with NALM supplemented at 15 and 30 g/d. The present study validated the effects of long-term NALM supplementation on improving feed efficiency, and the results are probably attributed to greater fat mobilization.

Nevertheless, further research is required to investigate the intermediary mechanism involved. Linear decrease in BUN with NALM supplementation may suggest greater N efficiency of NALM-supplemented cows. Supplementation of NALM, particularly in metabolizable Met-deficient diets, in early- to mid-lactation diets, is an effective approach for improvement in feed efficiency.

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