Effects of jugular infused methionine, lysine, and histidine as a group or leucine and isoleucine as a group on production and metabolism in lactating dairy cows

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ABSTRACT

Essential AA (EAA), particularly leucine, isoleucine, methionine, and histidine, possess signaling properties for promoting cellular anabolic metabolism, whereas methionine, lysine, and histidine are considered also to be substrate limiting AA. The objective of this study was to evaluate production responses to supplementation of 2 AA groups in a 2×2 factorial design. Eight cows (99 ± 18 days in milk) were assigned to 4 jugular infusion treatments consisting of saline (CON), methionine plus lysine plus histidine (MKH), isoleucine plus leucine (IL), or MKH plus IL, in a replicated 4×4 Latin square design. Periods were 18 d in length, comprising 8 d of rest followed by 10 d of jugular infusion. Daily infusion amounts were 21 g of methionine, 38 g of lysine, 20 g of histidine, 50 g of leucine, and 22 g of isoleucine. Cows were ad libitum fed a common diet consisting of 15.2% crude protein and 1.61 Mcal/kg NE_L on a dry matter basis that was predicted to meet rumen degradable protein requirements but was 15% deficient in metabolizable protein. Milk and energy-corrected milk yields increased by 2.3 kg/d and 1.9 kg/d, respectively, with infused IL, and no change was observed for MKH. Milk protein concentration increased by 0.13 percentage units for MKH, whereas milk protein yield increased for both MKH and IL by 84 g/d and 64 g/d, respectively. The milk protein yield increase for MKH+IL was 145 g/d versus CON. Gross feed efficiency tended to increase with IL infusion, and N efficiency tended to increase with MKH infusion. Aggregate arterial EAA concentrations less Met, Lys, and His declined by 7.2% in response to MKH infusion. Arterial EAA less Ile and Leu also declined by 6.2% in response to IL infusion. Net total AA (TAA) and EAA

uptake by the udder tended to increase in response to MKH infusion, whereas mammary blood flow increased in response to IL infusion, but TAA and EAA net uptakes were unaffected. Apparent udder affinity increased for TAA and EAA less Met, Lys, and His in response to MKH infusion, whereas affinity for EAA less Ile and Leu increased for IL infusion. Venous Met and Leu concentrations increased by 192% and 35% from the MKH and IL infusions, respectively, compared with CON, which indicates that intracellular concentration of these EAA changed substantially. Increases in milk protein yield were observed from 2 groups of amino acids independently and additively, which contradicts the single limiting amino acid theory that a single EAA will limit milk protein yield.

Key words: milk protein, methionine, lysine, histidine, leucine, isoleucine

INTRODUCTION

Improving our ability to manipulate milk protein yield to increase profitability and N efficiency, the latter being quite low at 25% (Hristov et al., 2004), is critical for human food supply security and industry sustainability. Milk protein yield is determined by AA availability and enzyme catalytic potential, with the former considered to be the major regulator of milk protein synthesis.

Mitchell and Block (1946) formulated a conceptual framework using the assumption that the most limiting AA regulates synthesis, and addition of other AA will have no effect. The most limiting EAA for milk protein production are considered to be Met, Lys, and possibly His. Milk protein responses to Met are well documented and fairly consistent, and responses to Lys and His are somewhat more variable but have often been observed (Schwab et al., 1976; Lee et al., 2012; Giallongo et al., 2017). However, the conceptual framework of Mitchell and Block (1946) can yield variable and unpredictable

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responses (Hanigan et al., 2000). For example, in diets less than 15% CP, supplementation of Met and Lys resulted in no overall benefit in fat-corrected milk yield and only a small benefit in milk protein yield across 16 experiments (Sinclair et al., 2014). Greater understanding of the mechanisms mediating the effects of Met, Lys, and His on milk protein yield, and interactions with other factors, is needed to better predict responses to supplementation of these EAA.

Net AA uptake by the udder as a proportion of arterial daily AA influx is quite low, having been shown to be 22% (Hanigan et al., 2004). This leaves a significant proportion of arterial AA available for splanchnic tissue metabolism. Given such a low udder capture of AA, a logical strategy for improving N efficiency is to increase intracellular demand via increased protein synthesis rates, which would increase the uptake of AA from arterial supplies and decrease AA available for splanchnic metabolism. Changes in phosphorylation of translation factors related to protein synthesis rate and milk protein yield have been observed in vitro and in a few in vivo trials (Rius et al., 2010; Toerien et al., 2010; Arriola Apelo et al., 2014c). Mechanistically, Leu appears to be the most potent of the EAA for activating protein translation. This occurs through Leu binding to Sestrin2 in the cytoplasm, which disrupts the GATOR2-Sestrin2 bond (Saxton et al., 2016). Methionine and Arg mechanistically have also been linked to mTOR regulation (Sabatini, 2017).

Evidence that supplementing Leu or the branched-chain AA (**BCAA**) increases milk protein yield or phosphorylation of translation factors is not well documented, particularly in longer-term studies (>5 d; Arriola Apelo et al., 2014a; Doelman et al., 2015b; Nichols et al., 2017). Infusion of BCAA has not increased milk protein yield in several studies (Korhonen et al., 2002; Appuhamy et al., 2011; Curtis et al., 2018). Several reasons may exist for the lack of a response to BCAA: the BCAA were not limiting, inappropriate BCAA infusion amounts, other EAA were more limiting, BCAA caused transport competition for EAA uptake, or BCAA stimulated increased nonmammary tissue use of EAA. Experiments using removal of BCAA or Leu from treatment EAA infusate as a model have shown negative effects on milk protein yield (Rulquin and Pisulewski, 2006; Doelman et al., 2015b; Tian et al., 2017), whereas others have shown no response (Weekes et al., 2006).

We propose several treatment variants from previous studies to better assess responses of lactating cows. First, infusion of only Leu and Ile instead of all BCAA, since only Leu and Ile are known to possess strong mTOR signaling properties. Second, infusion amounts that are similar to replacing the observed Leu and Ile

supplies in typical industry diets and that are large enough to elicit a change in blood plasma concentrations. Last, utilizing high-producing dairy cows (approximately 50 kg/d milk production) might also yield different results than the previously mentioned studies. Regarding AA transport, Val competes for cellular uptake with Leu and Ile by using System L transporters (Shennan and Boyd, 2014). Although speculative, addition of Val to BCAA infusates may counteract the benefits of Leu and Ile. This hypothesis regarding Val is also supported by a recent meta-analysis that found a negative milk protein response to increasing Val dietary supplies (Hanigan et al., 2018).

We hypothesized that supplementation of Met, Lys, and His as a group and Ile and Leu as a group would independently increase milk protein yield and that these effects would be additive. The objectives of this study were to determine milk yield and milk component responses to these 2 groups of EAA in dairy cows (>50 kg milk/d) and to observe changes in metabolism that may mediate changes in milk protein yield.

MATERIALS AND METHODS

Experimental Design, Animals, and Feeding Management

All animal procedures were approved by the Virginia Tech Animal Care and Use Committee. Eight Holstein cows in second lactation were blocked by DIM (2 blocks) and randomly assigned to 1 of 4 treatment sequences in a 2×2 factorial arrangement in 2 orthogonally replicated 4×4 Latin squares. For 1 of the blocks, cows were biopsied for muscle and mammary tissue on d 18 ($n = 4$ cows) for assessment of post-translation modification of proteins by proteomics, whereas the cows in the other block were infused in the jugular with isotopically labeled algae ($n = 4$ cows) to measure bidirectional transport of AA in the mammary gland. The data related to tissue biopsies and isotope tracing of metabolism are not presented herein and will be presented in future publications. Treatments were jugular infusion of saline (**CON**); methionine, lysine, and histidine (**MKH**); isoleucine and leucine (**IL**); and the combination of MKH and IL (**MKH⁺IL**). Before the experiment, all cows were fed the experimental diet (Table 1, Table 2) for 28 d for adaptation. Period length was 18. At the beginning of the experiment (i.e., before the adaptation period), animals averaged 71 DIM (18 d SD). Cows were housed in a 12-unit freestall pen for the adaptation period and the first 8 d of each period. On d 8 of each period, cows were moved to individual metabolism stalls and housed until d 18 of each period. Cows were fed a TMR ad libitum twice daily in the

metabolism stalls, with a target minimum refusal of 5%, and milked twice daily at 0100 and 1230 h. Starting at 1200 h on d 15, cows were fed every 2 h for 36 h at 100% of the observed previous 7-d DMI average to standardize feed intake for blood flow and isotope infusion measurements. Milk, feed intake, and body weight were recorded daily. The corn silage was from plants containing the brown midrib 3 gene and was sourced from a single silage bag. Corn silage DM was assayed 5 times per period in duplicate by drying for 24 h at 100°C. Dietary adjustments were made when DM changed by more than 2 units. Refusals and TMR DM were assayed 3 times per period by drying for 24 h at 100°C. Corn silage, alfalfa hay, corn grain, wet brewers grain, grain mix, and TMR were sampled 3 times per period, composited by period, and stored at -20°C. Ingredients were assayed for DM using a forced-air oven at 55°C for 48 h and then sent to Cumberland Valley Analytical Services (Waynesboro, PA) for analysis of CP, ADF, NDF on an OM basis, lignin, in vitro (IV)-NDF digestibility 30 h, IV-NDF 120 h, IV-NDF 240 h, acid detergent insoluble CP, neutral detergent insoluble CP, soluble CP, ammonia, starch, IV-Starch 7 h, ether extract, fatty acids, ash, minerals, and fermentation [Cumberland Valley Analytical NIR2 (near-infrared spectroscopy) package; Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17082>].

Cows were fed a common diet during the entire study, including the adaptation period, which was formulated to meet requirements for energy, rumen-degradable protein, minerals, and vitamins, but to be deficient in metabolizable protein for a 680-kg Holstein cow producing 47 kg of milk containing 3.60% fat and 3.10% protein (Table 1, Table 2). The amount of jugular-infused amino acids for the MKH (21 g/d of Met, 20 g/d of His, and 38 g/d of Lys) and IL (22 g/d of Ile and 50 g/d of Leu) treatments was estimated based on meta-analytical work in progress, predictions from the NDS Professional ration formulation software (version 6.55; RUM&N, NDS Professional, Reggio Nell'Emilia, Emilia-Romagna, Italy), the amounts of each AA contained in a typical diet containing heat-treated soybean meal, blood meal, and rumen-protected methionine, and the recommended supply relative to metabolizable energy supply (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-17082>; Van Amburgh et al., 2015). The ratio of Lys to Met supplied from the diet plus infusate was targeted at 2.65 to 1, whereas the ratio of Leu to Ile was targeted to be similar to what is observed in true milk protein (1.67 to 1; Lapierre et al., 2012). Leucine and Ile are metabolized with similar efficiencies from absorbed protein supplies (Myers et al., 2018); hence, the targeted ratio. The amount of Leu

Table 1. Average ingredient composition of the experimental diet (% of DM)

Item	Mean	SD
Corn silage	38.1	2.4
Alfalfa hay	9.37	1.2
Wet brewers	9.26	0.81
Corn grain	20.2	4.4
Soybean meal	7.43	0.48
Citrus pulp, dry	6.39	0.41
Soybean hulls, ground	4.04	0.26
Palmitic enriched fat supplement ¹	1.12	0.070
Sodium bicarbonate ²	0.943	0.061
Calcium carbonate	0.600	0.039
Potassium carbonate ³	0.495	0.032
Molasses	0.314	0.020
Dicalcium phosphate	0.419	0.027
Urea	0.210	0.014
Sodium bentonite ⁴	0.191	0.012
Omnigen ⁵	0.171	0.011
Salt	0.181	0.012
Magnesium oxide	0.171	0.011
Potassium chloride red	0.162	0.010
Magnesium potassium sulfate	0.0861	0.0064
Flow matrix ⁶	0.0480	0.0033
Trace minerals and vitamins ⁷	0.0672	0.0041

¹Palmit 80, Global Agri-Trade Corp., Long Beach, CA.

²Church and Dwight Co. Inc., Princeton, NJ.

³DCAD PLUS, Church and Dwight Co. Inc.

⁴AB-20, Prince Agri Products Inc., Quincy, IL.

⁵Prince Agri Products Inc.

⁶PMI Nutritional Additives, Arden Hills, MN.

⁷Contained 221 mg of Mn, 140 mg of Cu, 2 mg of Fe, 397 mg of Zn, and 15 mg of Co from Availa 4 (Zinpro Corp., Eden Prairie, MN), 16 mg of I (calcium iodate), 7.3 mg of Se (Sel-Plex 2000, Alltech Inc., Nicholasville, KY), 131 kIU of vitamin A, 30 kIU of vitamin D, and 550 IU of vitamin E.

and Ile infused was limited by the solubility of Leu in 3 L of saline.

Jugular Infusions and Catheterizations

Pharmaceutical-grade L-methionine, L-histidine, L-lysine-HCl, L-isoleucine, and L-leucine were obtained from ACP Chemicals (Toronto, ON, Canada). Infusates were prepared approximately 1 to 3 d before jugular infusion by dissolving 21 g of methionine, 20 g of histidine, and 47.5 g of lysine-HCl in 3 L of 0.9% saline for the MKH treatment; 22 g of isoleucine and 50 g of leucine in 3 L of 0.9% saline for the IL treatment; and 21 g of methionine, 20 g of histidine, 47.5 g of lysine-HCl, 22 g of isoleucine, and 50 g of leucine in 3 L of 0.9% saline for the MKH+IL treatment. The 0.9% saline was pharmaceutical-grade (MWI Animal Health, Boise, ID). Treatment solutions were sterile-filtered through 0.22- μ m membrane filters (Sterivex, Millipore, Billerica, MA) and stored at 4°C until the day of infusion. Additionally, 2 g of ¹³C-labeled AA derived from

algae ($U-^{13}C$, 97 to 99% enriched, Cambridge Isotope Laboratories, Andover, MA) were dissolved in 1 L of 0.9% saline, sterile-filtered through 0.22- μ m membrane filters, and jugular infused for 5 h on d 16 of each period for 1 block of cows ($n = 4$).

On d 8 of each period, cows were fitted with a jugular catheter (90 cm $\times$ 2.03 mm internal diameter microcatheter, Braintree Scientific Inc., Braintree, MA) for jugular infusion of the treatment solutions. Catheter placement alternated sides by period. Catheters were checked every 4 h for efficacy and infection at the site of insertion and were removed on d 18. Treatment and isotope solutions were jugular infused at 135 mL/h and 167 mL/h, respectively, using clinical infusion pumps (model RF-5000, Abott Lifecare, San Antonio, TX). Treatment and isotope solutions bags were gravimetrically weighed before and after infusion. On d 16, at 0800 h, during periods 2 to 4, cows were fitted with a subcutaneous mammary abdominal vein catheter (13 cm $\times$ 14 gauge, Jorvet, Loveland, CO) for venous sampling, and this catheter was removed on d 16 at 2400 h.

Sample Collection and Analysis

Milk samples were collected twice daily on d 7 before treatment infusions and then from d 9 to 18 during the treatment infusions at 0100 and 1230 h, stored at 4°C, and analyzed within 72 h for fat, true protein, lactose, SCC, and MUN concentrations with a CombiFoss FT

+ Fourier transform infrared analyzer (Foss Analytics, Hillerød, Denmark) by United DHIA (Radford, VA). On d 16, 1 block of cows ($n = 4$) were frequently milked in their metabolism stalls for measurement of isotope enrichment in milk protein at approximately 1500, 1700, and 1900 h, or 2, 4, and 6 h relative to the start of the infusion. To ensure complete milk removal, 5 cc of oxytocin was administered intramuscularly. Blood was sampled twice daily, at approximately 0800 and 1800 h, from the coccygeal vein via venipuncture into potassium EDTA and sodium heparin vacutainers (Becton Dickinson, Rutherford, NJ) on d 7, 9, and 12. On d 16, blood was again sampled from the coccygeal vein and the subcutaneous mammary abdominal vein by venipuncture in period 1 and by an indwelling catheter during periods 2 to 4 at approximately 1300, 1500, 1700, 1900, 2100, and 2300 h for 1 block of cows ($n = 6$ time points). For the 2nd block of cows, blood was sampled at the additional times of 1330, 1400, 1430, 1600, and 1800 ($n = 11$ time points) for isotope enrichment of plasma AA. The coccygeal vein sampling was considered arterial (Emery et al., 1965; Hanigan et al., 1991) and will be referenced as arterial blood herein, despite the fact that the blood sampling origin could be arterial or venous. Upon sampling, blood samples were placed on ice and then centrifuged (<6 h from first blood sample) at $1,600 \times g$ for 15 min. The resulting plasma was stored in polypropylene tubes at $-20^{\circ}C$ until further analysis. Tissue samples from the longissimus dorsi and the rear quarter of the udder were biopsied (average = 542 mg, SD = 139; Toerien et al., 2010; Daley et al., 2018) on d 18, immediately following the 1230 milking on the tissue biopsy-designated block of cows ($n = 4$). Biopsies of udder tissue were alternated from the right to left rear quarters and vice versa in successive periods.

Approximately 0.50 mL of blood plasma were gravimetrically sampled and acid deproteinized using 10% sulfosalicylic acid and centrifuged at $16,000 \times g$ for 20 min at 4°C. The resulting supernatant (0.25 mL) was gravimetrically combined with a mixture of $U-^{13}C, ^{15}N$ AA derived from algae (Lot #16824, Product #CNLM-452) supplemented with $[^{15}N_3, 99\%$ enriched] His (Lot #15858, Product #NLM-1513), $[^{15}N, 98\%$ enriched] Cys (Lot #14674, Product #NLM-2295), $[^{15}N, 99\%$ enriched] Thr (Lot #20756, Product #NLM-742), [methyl- $^{13}C_3, 99\%$ enriched] Met (Lot #24031, Product #CLM-206), [Amide- $^{15}N, 98\%$ enriched] Asn (Lot #12319, Product #NLM-120), and [Amide- $^{15}N, 98\%$ enriched] Gln (Lot #15592, Product #NLM-557) and subsequently desalted via ion chromatography (AG 50W-X8 resin, Bio-Rad Life Science, Hercules, CA). All isotopes were purchased from Cambridge Isotope Laboratories Inc., Tewksbury, MA. Desalted

Table 2. Nutrient composition of basal diet (% of DM)

Item	Mean	SD
CP	15.2	0.45
RDP ¹	9.98	—
RUP ¹	5.21	—
NDF	28.5	1.0
uNDF ²	9.74	—
peNDF ³	18.3	—
Starch	31.1	2.2
Sugar	4.87	—
Fat	4.89	0.081
PUFA	2.47	—
Ca	0.691	0.052
P	0.363	0.011
K	1.36	0.073
Mg	0.301	0.021
NE _L , ¹ Mcal/kg	1.69	—
NE _L , ⁴ Mcal/kg	1.60	—
MP, ¹ % DM	9.91	—

¹Calculated using NDS Professional ration formulation software (version 6.55; RUM&N, NDS Professional, Reggio nell'Emilia, Emilia-Romagna, Italy) and observed DMI, feed composition, and period average BW.

²uNDF = undigestible NDF.

³peNDF = physically effective NDF.

⁴Calculated using NRC (2001) and observed DMI, feed composition, and period average BW.

samples were freeze-dried, solubilized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ) and converted to *N*-(*tert*-butyldimethyl) AA derivatives by incubation for 1 h at 70°C in *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (Selectra-SIL; UCT Inc., Bristol, PA). The derivatized AA were separated using gas chromatography (Trace GC Ultra; Thermo Fisher Scientific, Waltham, MA) and quantified via mass spectrometry (DSQII; Thermo Fisher Scientific; Calder et al., 1999). Calibration curves for the labeled AA mixture were gravimetrically determined using an AA standard (AAS18; Sigma-Aldrich, St. Louis, MO) supplemented with L-Asn, L-Gln, L-Trp, and L-Cys (Sigma-Aldrich).

Statistical Analysis and Calculations

Metabolizable protein and EAA supplies were estimated using NDS Professional ration formulation software (version 6.55) and NRC (2001) nutrition models (Table 3). Model inputs were based on average dietary nutrient composition (Table 2) and observed least square treatment means of DMI and milk production (Table 4). Efficiency of converting MP and EAA supplies into milk protein were calculated. Metabolizable protein and EAA supplies and maintenance MP requirements were predicted using the NRC (2001) model. Maintenance MP requirements were subtracted from MP supply to yield MP available for lactation. Milk protein yield was then divided by the MP available for lactation to yield a milk protein efficiency from MP for lactation. Metabolizable protein requirements for growth and pregnancy were not considered, as cows did not gain nor lose BW. The EAA profile used for maintenance and milk protein was assumed to be similar. Overall efficiency was also calculated as milk AA yield (g/d) divided by predicted MP-AA supply (g/d). Marginal efficiency was calculated by dividing the difference in milk AA yield for the respective treatment (MKH, IL, or MKH⁺IL) compared with CON by the treatment amount of infused AA. Energy-corrected milk yield was calculated as follows: ECM = [(12.86 × kg of fat) + (7.04 × kg of protein) + (0.3246 × kg of milk)]; NRC (2001). Milk AA output was calculated using true milk protein with a 3.37% correction for blood-derived proteins and the AA composition of milk protein (Lapierre et al., 2012). Mammary plasma flow (MPF) was estimated using the Fick principle, with Phe and Tyr as the internal markers (Cant et al., 1993). Milk Phe and Tyr yield (μmol/h) were determined from milk protein yield using the average 12-h period (1300 to 0100 h) from d 15 to 17 of each period. Blood arterial and venous Phe and Tyr concentrations were sampled 6 (biopsy block of cows, n = 4) or 11 times (isotope block of cows, n = 4) on d 16 from 0130 to 1130 h and

analyzed individually, which should improve precision compared with pooling samples (Lapierre et al., 2012). Net uptake of AA by the mammary gland (μmol/h) was calculated as arterial-venous difference multiplied by MPF. Clearance of AA by the udder was calculated as net uptake divided by venous concentration (Cant et al., 2016).

One cow during period 3 was diagnosed with clinical mastitis, and data for that cow in periods 3 and 4 were discarded. Another cow was diagnosed with clinical mastitis during period 2, and data for only that period were discarded. Data were analyzed with a mixed model using the “lmer” package in R (version 3.4.3; R Core Team, 2017) for all data except repeated measures data. We used SAS version 9.4 (SAS Institute, Cary, NC, 2010) to examine repeated measures. Milk production, milk composition, and DMI of treatments were analyzed by day, whereas arterial AA, net AA uptake, AA clearance, and ratio of AA uptake to milk protein output were analyzed by experimental period. The following model was used for analyzing data summarized by period:

$$Y_{ijklm} = \mu + MKH_i + IL_j + MKH^+IL_{ij} + Block_k + Period_L + Cow(Block)_{m(k)} + e_{ijklm},$$

where Y_{ijklm} = the dependent variable, μ = population mean of Y , MKH_i = fixed effect of methionine, lysine, and histidine infusion (df = 1), IL_j = fixed effect of isoleucine and leucine infusion (df = 1), MKH^+IL_{ij} = interaction of MKH_i and IL_j (df = 1), $Block_k$ = random effect of block (df = 1), $Period_L$ = random effect of period (df = 3), and $Cow(Block)_{m(k)}$ = random effect of cow nested within block (df = 6). Main effects and interactions were declared significant at $P \leq 0.05$ and trends at $P \leq 0.10$, and denominator degrees of freedom for all tests were adjusted using the Kenward-Roger option. Residual error was evaluated for homogeneity of error and for outliers; if studentized residuals exceeded 3, the sample was removed from statistical analysis. Post-hoc means-separation testing was conducted only on significant main effects, using the “lsmeansLT” package in R, with degrees of freedom adjusted using the Kenward-Roger option. When the interaction was significant, only the interaction effects were considered during mean-separation tests.

For the data analyzed by day, the following model was constructed using the MIXED procedure of SAS version 9.4 (SAS Institute), which included the previously described model with the addition of repeated fixed effect day (9 df) and all 2-way and 3-way interactions with the other fixed effects. The covariance structure was autoregressive (1) and was selected from the

available covariance structures using the lowest Bayesian information criterion. When the treatment $\times$ day interaction was significant ($P \leq 0.10$), the SLICE option was used to determine on which days of the period ($P \leq 0.10$) treatment means would be separated using Fisher's protected least significant difference test ($P \leq 0.10$). No treatment $\times$ day, 2-way, or 3-way interactions were found to be significant ($P > 0.20$) and thus are not reported or discussed. All results reported in this paper are least squares means of the treatment effects, whereas main-effect means are listed in Supplemental Tables S3, S4, S5, S6, S7, and S8 (<https://doi.org/10.3168/jds.2019-17082>).

RESULTS AND DISCUSSION

The objective of this study was to test the hypothesis that multiple EAA can independently increase

milk protein yield in lactating dairy cows. The MKH was designed to represent industry and literature recommendations for EAA supplementation, and the resulting treatment achieved ratios and supply balances considered optimal for maximizing milk protein yield (NRC, 2001; Van Amburgh et al., 2015; Table 3; Supplemental Table S2, <https://doi.org/10.3168/jds.2019-17082>). The factorial requirement model of CNCPS (v. 6.55) predicted deficiencies for these EAA (-19 g/d Met, -47 g/d Lys, and -6 g/d His in CON). The IL objective was to evaluate the hypothesis that signaling properties of these specific EAA could increase milk protein yield. The factorial requirement model of CNCPS (v. 6.55) suggested that these EAA were deficient (-24 g/d of Ile and -87 g/d of Leu in CON), and supplementation with IL reduced this deficiency (Table 3; Supplemental Table S2).

Table 3. Metabolizable protein supplies as predicted by NRC (2001) given observed DMI, infused AA, milk and component yields, and BW, expressed as percentage of metabolizable protein and supply relative to calculated AA requirement

Item	Treatment ¹			
	CON	MKH	IL	MKH ⁺ IL
MP allowable milk, kg/d	37.0	38.0	39.6	39.6
MP, g/d	2599	2704	2689	2801
MP balance, g/d	-478	-468	-451	-513
RDP balance, g/d	-25	-23	-22	-23
RUP balance, g/d	-574	-656	-680	-797
Histidine				
Calculated requirement, ² g/d	68	70	69	73
Diet supply, g/d	57	57	57	58
Jugular infusion, g/d	0	20	0	20
Balance, g/d	-11	7	-12	5
Percent of MP	2.19	2.85	2.12	2.78
Isoleucine				
Diet supply, g/d	126	127	127	128
Jugular infusion, g/d	0	0	22	22
Percent of MP	4.85	4.70	5.54	5.36
Leucine				
Diet supply, g/d	231	233	233	236
Jugular infusion, g/d	0	0	50	50
Percent of MP	8.89	8.62	10.52	10.21
Lysine				
Calculated requirement, ² g/d	203	209	207	219
Diet supply, g/d	166	168	168	169
Jugular infusion, g/d	0	38	0	38
Balance, g/d	-37	-3	-39	-12
Percent of MP	6.39	7.62	6.25	7.39
Methionine				
Calculated requirement, ² g/d	68	70	69	73
Diet supply, g/d	49	49	49	50
Jugular infusion, g/d	0	21	0	21
Balance, g/d	-19	0	-20	-2
Percent of MP	1.89	2.59	1.82	2.53

¹CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH⁺IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

²Calculated requirements for His, Lys, and Met were assessed as 2.2, 6.6, and 2.2%, respectively, of MP requirements.

Cows in second lactation were selected, to minimize the issue of leaky external pudic vein valves, which involves nonmammary blood entering the subcutaneous abdominal venous blood drainage of mammary tissue (Bequette et al., 1999). This issue becomes more prevalent in older cows—that is, third lactation and higher (Bequette et al., 1999). To avoid any uncertainty with respect to the bioavailability of ruminally protected AA, the supplemented AA were provided by jugular infusion (Vyas and Erdman, 2009). Additionally, jugular infusion eliminates uncertainty regarding digestion and gastrointestinal metabolism of AA when supplying AA via post-ruminal infusions. Gastrointestinal metabolism has been demonstrated to vary by individual amino acid (Hanigan et al., 2004; Estes et al., 2018). Feeding rumen-protected AA instead of jugular infusion may result in slightly different results than those obtained herein. The NRC (2001) does not account for gastrointestinal catabolism varying by EAA, only for catabolism of the aggregate; therefore, even if we formulated for metabolizable supplies of the treatment EAA, supplies available for metabolism would have been slightly different.

Experimental Diet and Nutrient Supply

Composition of the basal diet is provided in Table 1, and nutrient composition of forages and major in-

gredients are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17082>). Dietary mixing variation was greater than expected for corn grain, which resulted in a 21.9% coefficient of variation on a diet DM basis (Table 1). This was primarily due to an employee ingredient-loading accuracy issue. The mixing variation affected all treatments similarly. This mixing variation could be considered problematic by some; however, experiments with high-producing cows that were subjected to extreme dietary variation have shown no major effects on cow performance (McBeth et al., 2013; Yoder et al., 2013).

Dietary nutrient concentrations are listed in Table 2. Predicted protein and AA supplies by the NRC 2001 are provided in Table 3, and predictions by CNCPS (v. 6.55) are listed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-17082>). The experimental diet was nearly adequate in rumen-degradable protein (NRC, 2001), which achieved our objective of not hindering ruminal nutrient digestion or intake (Table 3). The MP deficit predicted by the NRC (2001) and CNCPS (v. 6.55) models was 14 to 15%, or 10 to 11 kg of milk, for all treatments (Table 3; Supplemental Table S2). The underprediction might reflect inaccurate supply estimates, particularly microbial protein, protein mobilization from body stores, maintenance requirement overestimation, or error in the marginal efficiency of metabolizable protein conversion to milk protein.

Table 4. Effect of AA infusions on intake, milk production parameters, nutrient efficiency, and BW (least-square treatment means)

Item	Treatment ¹				SEM	Effect (<i>P</i> -value)		
	CON	MKH	IL	MKH ⁺ IL		MKH	IL	MKH × IL
Intake								
DMI, kg/d	25.3	25.6	25.5	25.8	0.41	0.45	0.31	0.90
Dietary CP, kg/d	3.85	3.87	3.88	3.92	0.06	0.45	0.31	0.90
Infused AA, g/d	0	78.9	71.9	150.8	0.04	<0.001	<0.001	0.53
Total CP, kg/d	3.85	3.96	3.95	4.06	0.06	0.01	0.01	0.99
Milk production								
Milk, kg/d	47.3	47.8	49.5	50.3	0.96	0.31	0.001	0.86
ECM, ² kg/d	47.2	48.3	49.1	50.1	1.03	0.14	<0.01	0.93
Milk protein %	3.10	3.19	3.04	3.21	0.04	<0.001	0.45	0.09
Milk protein, g/d	1,458	1,517	1,498	1,603	32	<0.001	<0.01	0.28
Milk fat %	3.58	3.55	3.55	3.42	0.11	0.29	0.28	0.48
Milk fat, g/d	1,675	1,716	1,744	1,741	56	0.60	0.22	0.56
Lactose, %	5.04	4.99	5.05	4.97	0.03	<0.001	0.67	0.48
Lactose, g/d	2,389	2,388	2,505	2,508	53	0.98	<0.01	0.97
MUN, mg/dL	8.17	8.68	8.50	8.40	0.53	0.55	0.94	0.39
Efficiency								
Nitrogen efficiency ³	38.1	38.1	38.3	39.6	0.01	0.09	0.19	0.18
GFE ⁴	1.85	1.87	1.93	1.91	0.06	0.99	0.07	0.68
BW, kg/d	668	672	675	677	15.5	0.52	0.24	0.97
BW change, kg/period	−13.2	−13.5	−14.0	−8.46	9.94	0.69	0.76	0.66

¹CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH⁺IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

²ECM calculated as (12.82 × kg of fat) + (7.13 × kg of protein) + (0.323 × kg of milk).

³Protein efficiency calculated as milk protein yield (kg/d)/total CP intake (kg/d).

⁴GFE = gross feed efficiency, calculated as ECM yield (kg/d)/DMI (kg/d).

Model underprediction of protein-allowable milk at levels similar to those found in this study (approximately 11 kg of milk) has previously been observed (Doelman et al., 2015a,b; Doepel et al., 2016), but not at the level of milk production in this study, which averaged 48.7 kg/d, approximately 20 kg/d higher than the mentioned studies. Metabolizable supplies of Met, Lys, and His for CON were below calculated requirements (NRC, 2001; Table 3) and less than optimal supplies relative to metabolizable energy (CNCPS v. 6.55; Table 3; Supplemental Table S2). Metabolizable Ile and Leu for CON deficits were 16 and 31% of requirements, according to CNCPS v. 6.55 (Supplemental Table S2).

Intake

Dry matter and dietary CP intakes were not affected by treatment (Table 4). The jugular treatment infusions increased total CP intake for MKH ($P = 0.01$) and IL ($P = 0.01$), with no interaction of the main effects. Histidine supplementation has previously been shown to increase feed intake (Lee et al., 2012; Giallongo et al., 2017), whereas methionine supplementation, if excessive, can decrease intake (Satter et al., 1975; Robinson et al., 2000). Numerically, intake increased in response to MKH, but counteracting effects of potentially excess Met may have negated any benefit from His supplementation. Another potential reason for the lack of a DMI response to supplemental His is that the trial was short-term (18-d periods), as His deficiency takes several weeks to occur due to mobilization from plasma hemoglobin and muscle carnosine (Lapierre et al., 2008; Lee et al., 2012). This may have buffered His deficiency in this trial. Infusions of Ile and Leu were not expected to change intake, given previous findings (Korhonen et al., 2002; Weekes et al., 2006; Appuhamy et al., 2011).

Milk Components

Milk component concentrations and yields are shown in Table 4 and Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17082>). Milk protein yield increased by 5.5 and 4.3% for MKH ($P < 0.01$) and IL ($P < 0.01$), respectively (Table 4). Milk protein concentration increased for MKH ($P < 0.01$) but was unchanged for IL (Table 4). We found no interaction between MKH and IL, indicating that milk protein yield can be stimulated independently and additively by EAA. The milk protein yield increase from provision of additional Met, Lys, and His supplementation was expected (Lee et al., 2012; Giallongo et al., 2017). The increase in

milk protein yield by IL independent of MKH agrees with in vitro findings (Appuhamy et al., 2012; Arriola Apelo et al., 2014c) but disagrees with previous in vivo trials (Huhtanen et al., 2002; Appuhamy et al., 2011; Arriola Apelo et al., 2014a). The observation also disagrees with the hypothesis that the maximal milk protein yield is capped solely by a single first-limiting EAA. Methionine, Lys, or His were, as a group, limiting EAA, given the observed positive response herein; however, despite this limitation, milk protein yield also increased with Ile and Leu supplementation (Table 4).

Regarding the other milk components, we found no treatment effect on milk fat yield or concentration (Table 4). Lactose concentration was decreased for MKH ($P < 0.01$) and unchanged by IL (Table 4). Lactose yield was increased for IL ($P < 0.01$) but unchanged by MKH (Table 4). Milk fat yield is usually not affected by supplementation of the classical limiting EAA, Met, Lys, and His, either as a group or individually (Appuhamy et al., 2011; Lee et al., 2012). The increase in lactose yield for IL correlates with the increased milk yield observed for IL.

Milk Yield

Milk production is shown in Table 4 and Supplemental Figure S1. The IL treatment increased milk and ECM yields by 2.3 ($P < 0.01$) and 1.9 kg/d ($P = 0.01$), respectively. No effect was observed for MKH or for the interaction (Table 4). Increased milk or ECM yields from supplementation of Ile or Leu have not been previously observed (Korhonen et al., 2002; Arriola Apelo et al., 2014a; Doelman et al., 2015b). However, a recent meta-analysis ($n = 258$ treatment means) indicated positive milk yield responses to Leu (Lean et al., 2018). Based on that meta-regression equation, the change in milk yield from CON to IL was predicted to be 1.5 kg/d, which was close to the observed response. Decreased activation of the mTORC1 pathway when Leu and Ile are deficient has been observed for both in vitro and in some in vivo studies (Arriola Apelo et al., 2014b; Doelman et al., 2015b; Liu et al., 2017). Decreased mTORC1 activation due to Leu and Ile deficiency might lead to decreased mammary tissue anabolic activity and milk protein yield. The increased protein yield observed with IL treatment ($P < 0.01$) may have increased α -lactalbumin, thereby driving the lactose disaccharide formation of galactose and glucose via allosteric activation of galactosyl transferase. Increased lactose yield propels greater milk yield. However, this latter theory of protein yield driving milk yield did not occur for MKH, only for IL.

The increased milk and protein yield for IL differ from previous studies. Given the complexity of mTOR regulation, perhaps the variations in cow responses to Ile and Leu supplementation are not surprising. The anabolic signaling of mTORC1 is regulated by a multitude of factors, including oxygen, glucose, AA, energy, and insulin (Sabatini, 2017), as well as being inhibited by the integrated stress pathway via ATF4—that is, amino acid and glucose deficiency, endoplasmic reticulum stress (unfolded proteins), and oxidative stress (Baird and Wek, 2012; Wolfson and Sabatini, 2017). Hence, variation in cow responses to the strategy of manipulating mTORC1 seems likely. Intracellular Leu and, to a lesser extent, Ile bind to Sestrin2 triggering GATOR2 to activate mTORC1, which then phosphorylates key initiation factors controlling rates of translation and protein synthesis (Saxton et al., 2016; Wolfson et al., 2016). Activation of mTORC1 by Leu or Ile can be inhibited by Sestrin2 concentrations. For example, high levels of Sestrin2 expression can completely blunt Leu activation despite increasing Leu intracellular concentrations (Wolfson and Sabatini, 2017). Sestrin2 expression is regulated by the transcription factor ATF4, and activation of this transcription factor can arise from a variety of factors, including long-term AA starvation and endoplasmic reticulum stress via phosphorylation of eIF2a (Baird and Wek, 2012). Milk fat depression via *trans*-10,*cis*-12 conjugated linoleic acid causes endoplasmic reticulum stress and inhibition of SREBP1 in mouse adipocytes, which have been shown to be related to phosphorylation of eIF2a and increased expression of ATF4 (LaRosa et al., 2007). Hence, although the link is speculative, severe milk fat depression might block anabolic activation of mTORC1 via Sestrin2. In 2 previous studies (Appuhamy et al., 2011; Arriola Apelo et al., 2014a), BCAA or individual Leu supplementation failed to increase milk protein yield, but in those studies the average treatment milk fat concentrations were 2.46% and 3.07%, both indicative of severe milk fat depression (Bauman et al., 2011). Additionally, milk fat depression (2.86 and 2.82%) as well as very low CP diets (11.2% DM, 12.4% DM), coupled with relatively low milk production given DIM (30.1 kg/d at 105 DIM and 33.9 kg/d at 78 DIM) in 2 recent studies that evaluated mTORC1 signaling, may explain the lack of mTORC1 signaling responses to the subtraction or provision of Leu (Doelman et al., 2015b; Nichols et al., 2017). In summary, other factors might block the effects of supplementing translation stimulatory EAA (e.g., Leu, Ile), thereby imposing multivariate complexity on inference.

The lack of response to MKH in milk and ECM yields is consistent with some studies, particularly short-term studies and meta-analyses (Appuhamy et al., 2011; Sin-

clair et al., 2014; Zanton et al., 2014) but inconsistent with others (Noftsgger and St-Pierre, 2003; St-Pierre and Sylvester, 2005). The mechanism for mTORC1 activation by Lys or His appears to be less potent or nonexistent compared with Ile and Leu (Appuhamy et al., 2012). Methionine does activate mTORC1 via Sestrin2 binding, although with much lower affinity than leucine (Wolfson et al., 2016) or via SAMTOR via 1-carbon metabolism (Gu et al., 2017). Another reason for the lack of milk and ECM responses to MKH is that these EAA are perhaps not as deficient or limiting as nutrition models would imply (NRC, 2001). Hence, the modest response to MKH in milk protein yield and concentration might reflect that these EAA were not significantly deficient.

Efficiency

Nutrient efficiency, MUN, and BW measurements are shown in Table 4. Body weight change and ending weights were unaffected. Gross feed efficiency tended to increase (+3.2%) with IL ($P = 0.07$). Greater partitioning of nutrients to mammary gland or enhanced anabolic activity of the udder (e.g., rBST supplementation) have been observed to increase feed efficiency. If our hypothesis is accurate and IL promotes more anabolic activity, then the latter may explain the increase in gross feed efficiency. Nitrogen efficiency also tended to increase for MKH ($P = 0.09$), and the difference is observed in the main-effect treatment means (Supplemental Table S3, <https://doi.org/10.3168/jds.2019-17082>). Overall, the average N efficiency across treatments was 38.5%, which is substantially higher than the average efficiency of 25% for lower-producing lactating dairy cows fed conventional diets (Hristov et al., 2004) but similar to the results of a previous jugular infusion trial (Appuhamy et al., 2011). Milk urea nitrogen was unaffected and below typical concentrations, indicative of high N efficiency, averaging 8.44 mg/dL. The lack of treatment effects on MUN suggests that the increased N intake ($P < 0.01$) from the infusions likely offset the gain in milk protein yield and reduced catabolism of AA that were not infused. This has also been found in previous studies (Appuhamy et al., 2011; Lee et al., 2012; Giallongo et al., 2016).

Arterial Plasma AA

The arterial plasma AA concentrations are shown in Table 5. The infused EAA all increased as expected ($P < 0.03$; Table 5, Supplemental Figures S2 and S3, <https://doi.org/10.3168/jds.2019-17082>). This differs from the results of some previous jugular infusion trials, in which Leu and Ile concentrations did not in-

crease in response to Leu and Ile infusions (Appuhamy et al., 2011; Kassube et al., 2017). Total AA, EAA, and NEAA arterial concentrations were unaffected. Individually, Leu ($P = 0.03$), Phe ($P < 0.01$), Thr ($P < 0.01$), and Val ($P = 0.06$) all declined or tended to decline in arterial concentration in response to MKH, which was likely driven by increased demand by mammary tissue in support of greater milk protein yield. For IL, only Val declined ($P = 0.06$), and this was likely also driven by increased demand by mammary tissue. Previously, jugular infusions of Met and Lys increased milk protein yield but did not contribute to a decline in plasma concentrations of other EAA except for Ile (Appuhamy et al., 2011; Kassube et al., 2017). However, dietary CP (16.0%) and MUN (12.5 mg/dL) were much higher for these prior studies than for this study, which may have buffered changes in the concentrations of those other EAA.

For NEAA, Gly ($P = 0.02$), Ser ($P = 0.12$), and Tyr ($P < 0.01$) arterial plasma concentrations decreased for MKH, and none were changed individually for IL. This

is not usually observed with cows supplemented with Met, Lys, and His (Lee et al., 2012; Giallongo et al., 2016); however, the high level of protein yield, coupled with a 14% deficit in MP in this experiment, might result in a limited supply of AA in general—hence the drawdown of Gly, Ser, and Tyr. The Tyr venous concentration fell 81% in response to MKH (Supplemental Table S5, <https://doi.org/10.3168/jds.2019-17082>). Minimal synthesis of Tyr from Phe occurs in the mammary gland, observed to be 3 to 5% in lactating goats (Bequette et al., 1999), so increased protein yield likely drove much greater extraction of Tyr from the arterial blood.

Mammary Uptake of AA

Mammary plasma flow and net AA uptake by the udder are shown in Table 6. Mammary plasma flow increased by 24% ($P = 0.01$) in response to IL, whereas plasma flow numerically decreased by 9.5% ($P = 0.22$) for MKH. Mammary plasma flow is highly correlated

Table 5. Arterial AA plasma concentrations, μM (least squares treatment means)¹

Item	Treatment ²				SEM	Effect (P -value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH $\times$ IL
Arg	80.8	87.6	81.8	83.7	4.6	0.20	0.66	0.45
His	64.7	84.8	63.4	84.0	3.0	<0.001	0.65	0.93
Ile	127.3	137.1	158.2	136.3	9.6	0.32	0.02	0.02
Leu	201.2	189.1	267.8	239.3	16.9	0.03	<0.001	0.33
Lys	85.2	121.4	87.6	109.6	5.9	<0.001	0.20	0.05
Met	23.6	45.9	23.6	43.4	2.2	<0.001	0.52	0.52
Phe	46.2	44.5	48.2	42.5	2.0	<0.01	0.98	0.11
Thr	99.0	89.0	95.4	83.2	4.9	<0.001	0.10	0.69
Val	285.6	269.5	263.9	231.8	22.2	0.06	0.03	0.51
Ala	265.9	270.8	270.7	271.4	20.5	0.76	0.78	0.82
Asn	22.9	23.0	23.3	21.4	2.2	0.53	0.68	0.48
Asp	13.8	14.3	13.0	13.5	1.4	0.59	0.43	0.98
Gln	146.0	143.3	152.0	146.1	11.1	0.23	0.23	0.65
Glu	109.9	104.8	106.3	108.4	9.5	0.68	0.99	0.31
Gly	291.8	252.0	280.3	257.0	31.7	0.02	0.79	0.49
Pro	108.6	109.3	109.0	110.3	5.6	0.77	0.83	0.92
Ser	90.4	84.9	91.7	84.3	6.5	0.12	0.93	0.797
Tyr	35.4	31.8	35.3	27.9	2.7	<0.01	0.22	0.24
TAA ³	2,103	2,109	2,167	2,115	57.8	0.61	0.45	0.53
EAA ⁴	1,023	1,078	1,087	1,060	55.8	0.67	0.50	0.21
BCAA ⁵	623	596	688	608	48.8	0.06	0.18	0.34
EAA-MKH ⁶	849	817	912	817	50.8	0.05	0.32	0.30
EAA-IL ⁷	685	749	664	682	29.5	0.03	0.02	0.21
NEAA ⁸	1,089	1,041	1,080	1,046	54.5	0.19	0.95	0.81

¹Data represent average AA concentrations sampled from the coccygeal vessels for d 9, 12, and 16 of the experimental period.

²CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

³TAA = total AA (EAA + NEAA).

⁴EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

⁵BCAA = branched-chain AA: Ile, Leu, and Val.

⁶EAA-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

⁷EAA-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

⁸NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Table 6. Mammary plasma flow (MPF) and net uptake of AA (least squares treatment means)¹

Item	Treatment ²				SEM	Effect (<i>P</i> -value)		
	CON	MKH	IL	MKH ⁺ IL		MKH	IL	MKH × IL
MPF, L/h	807	665	928	905	86.7	0.22	0.01	0.37
Net mammary uptake, mmol/h								
Arg	29.0	29.6	34.4	33.5	3.4	0.93	0.02	0.71
His	9.7	9.2	12.1	11.9	1.3	0.74	0.04	0.9
Ile	35.4	40.5	46.2	44.0	4.1	0.65	0.04	0.25
Leu	59.8	58.1	74.8	74.4	5.1	0.75	<0.001	0.85
Lys	38.7	50.4	38.6	53.0	3.5	<0.001	0.52	0.48
Met	12.1	13.8	11.6	12.7	1.6	0.22	0.51	0.78
Phe	18.9	19.5	19.8	21.2	0.9	0.02	<0.01	0.25
Thr	23.5	23.9	25.0	23.4	2.0	0.69	0.74	0.52
Val	52.6	46.0	52.4	51.0	5.6	0.31	0.54	0.50
Ala	32.3	31.4	32.5	38.2	10.4	0.72	0.62	0.62
Asn	7.0	8.8	8.5	9.3	1.0	0.12	0.23	0.59
Asp	4.4	4.3	3.4	3.5	1.1	0.95	0.38	0.91
Gln	27.3	30.4	29.6	33.9	3.3	0.12	0.22	0.8
Glu	37.2	28.7	40.6	38.6	5.1	0.21	0.12	0.43
Gly	10.6	14.7	20.8	24.7	5.9	0.43	0.06	0.98
Pro	17.2	15.5	16.9	19.5	2.9	0.85	0.44	0.37
Ser	18.2	22.2	18.9	24.8	3.0	<0.01	0.30	0.53
Tyr	18.2	19.7	18.8	20.1	0.7	<0.01	0.28	0.91
TAA ³	450	466	493	520	34.2	0.41	0.09	0.84
EAA ⁴	278	291	314	318	18.2	0.44	0.01	0.70
BCAA ⁵	148	145	173	163	12.3	0.44	0.02	0.69
EAA-MKH ⁶	219	217	253	241	15.5	0.55	0.02	0.65
EAA-IL ⁷	182	192	193	206	11.9	0.11	0.10	0.81
NEAA ⁸	172	176	177	203	24.0	0.46	0.42	0.56

¹Data represent arterial and venous measurements ($n = 6$ to 11 samples per cow) taken on d 16.

²CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH⁺IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

³TAA = total AA (EAA + NEAA).

⁴EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

⁵BCAA = branched-chain AA: Ile, Leu, and Val.

⁶EAA-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

⁷EAA-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

⁸NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

with milk yield (Prosser et al., 1996); hence, the 2.3 kg/d milk yield response to IL correlates with increased MPF. Two potential reasons for the increased MPF exist: (1) greater anabolic activity associated with stimulation of mTORC1 that results in ADP buildup and release into the interstitium, which triggers nitric oxide synthesis from arginine (Cieslar et al., 2014), or (2) a shortage of 1 or more individual EAA (Cant et al., 2018), acting through an unknown mechanism. Using the regression equation of Purdie et al. (2009), MPF was predicted to increase by 111 L/h for the IL treatment, given the observed milk yield change. The observed increase was 180 L/h. Plasma flow stimulation has been observed in response to deficiencies in His, Lys, and Thr (Bequette et al., 2000; Doepel et al., 2016; Guo et al., 2017). This appears to be a mechanism to maintain an adequate supply of nutrients to the udder for maintenance of milk production even under deficient conditions. Clearly MKH and IL stimu-

lated mammary activity, which may have altered ADP concentrations. Plasma Thr concentrations also tended to decline ($P = 0.10$), which may have been the trigger for a local MPF response. Whether the change in MPF was caused by increased metabolism or by shortage of an EAA remains to be elucidated. However, this study demonstrated the plasticity of the mammary gland to facilitate increased milk protein yield without supplementation of the classic limiting EAA.

Net uptake of several EAA increased in response to the treatments likely to support greater protein yield (Table 6). Amino acid substrates for greater yield must originate from either reduced catabolism or accretion of protein in the mammary gland or from greater uptake from arterial blood. Despite the 9.5% numerical decline in MPF in response to MKH, increased net uptake of Lys ($P < 0.01$), Phe ($P = 0.02$), Asn ($P = 0.12$), Gln ($P = 0.12$), Ser ($P < 0.01$), and Tyr ($P < 0.01$) occurred. Venous concentration of Lys increased 32% ($P < 0.01$)

in response to MKH (Supplemental Table S5, <https://doi.org/10.3168/jds.2019-17082>), which led to net udder uptake increasing by 32% ($P < 0.01$; Supplemental Table S6, <https://doi.org/10.3168/jds.2019-17082>). The Lys uptake-to-output ratio increased from 1.05 to 1.32 for MKH (Supplemental Table S7, <https://doi.org/10.3168/jds.2019-17082>), and the marginal transfer efficiency of the extra Lys into milk protein Lys was only 13%. The nitrogen in Lys can be used for synthesis of Gln, Glu, Asp, Asn, Ser, and Ala (Lapierre et al., 2009). It has been shown that Lys net uptake to milk protein output ratios approaching 1 result in reduced milk protein yield (Lapierre et al., 2009; Guo et al., 2017). Thus, some Lys catabolism is apparently obligatory, and one cannot assume that all EAA with uptake-to-output ratios greater than 1 are not deficient. Uptake of Phe and Tyr increased for MKH (5.2 and 7.6%, respectively), which closely matched the milk protein response of 5.5%. Methionine uptake numerically increased by 12.7% ($P = 0.22$), which is greater than the milk protein response for MKH. Histidine net uptake was unchanged ($P = 0.74$), as was the net uptake-to-output ratio ($P = 0.54$) for MKH. Histidine is unique in that a significant portion of His supply can originate from peptides (carnosine, anserine; Lapierre et al., 2008). Plasma His could have been used by muscle to synthesize these peptides, and the peptides released to plasma for use by mammary tissue to synthesize milk protein (Lapierre et al., 2012). Histidine catabolism by mammary tissue could also have been reduced. The latter should be reflected in uptake-to-output ratios, but His is subject to greater analytical variation (Lapierre et al., 2012). The increase or numerical increase in net uptake of the other NEAA for MKH—that is, Ser ($P < 0.01$), Asn ($P = 0.12$), and Gln ($P = 0.12$)—might reflect the increased demand to match the milk protein response or diminished catabolism of EAA for synthesis of NEAA due to reduction in EAA availability. For example, Arg and BCAA net uptake did not change for MKH, and these EAA serve as precursors for Gln, Ser, and Asn (Roets et al., 1979, 1983; Rubert-Alemán et al., 1999). The uptake-to-output ratios of Leu ($P = 0.14$) and Val ($P = 0.10$) tended to decline, indicating less catabolism by mammary tissue.

For IL, increased net uptake of Arg ($P = 0.02$), His ($P = 0.04$), Ile ($P = 0.04$), Leu ($P < 0.01$), and Phe ($P < 0.01$) was observed (Table 6). Net uptake of Ile and Leu increased by 19 and 27%, respectively, for IL (Supplemental Table S6, <https://doi.org/10.3168/jds.2019-17082>), which compares closely to the 29.5% and 34.6% increases in venous concentrations of Ile and Leu (Supplemental Table S5, <https://doi.org/10.3168/jds.2019-17082>). Greater capture of these EAA likely

increased intracellular concentrations, which should facilitate greater activation of the mTORC1 pathway and substrate for NEAA synthesis, the latter negating the need for greater uptake of Asn, Gln, and Ser, as observed with MKH. Despite a 30- μ M drop in Val arterial concentration ($P = 0.03$), net uptake increased numerically ($P = 0.54$), driven by a higher Val clearance rate ($P = 0.09$) and greater MPF. The lack of response in Met and Tyr uptake is difficult to explain, as synthesis by the mammary gland is minimal to nonexistent for these AA, so how greater milk protein yield resulted without greater uptake is perplexing. In goats, as much as 18% of milk protein Met and 14% of milk protein Tyr may be derived from peptide uptake by the udder (Bequette et al., 1999). However, these values are overestimated, as pointed out by Hanigan et al. (1998b), due to very slow exchange with body protein pools, but one cannot rule out contributions from peptides containing Met and Tyr. Overall, the aggregate of total amino acids (TAA; $P = 0.09$), EAA ($P = 0.01$), and BCAA ($P = 0.02$) all increased or tended to increase in net uptake for IL. The net uptake AA responses in general match with expectations given changes in MPF that altogether would support greater protein yield and demonstrate the metabolic flexibility of the udder for securing AA.

Mammary AA Clearance Rates

Mammary AA clearance rates are listed in Table 7. Clearance of TAA increased ($P = 0.02$) with MKH but did not change for IL (Table 7). Assuming mammary demand is a determinant of transport activity, there may not have been a need for additional transport activity given the increase in TAA supply driven by increased MPF with IL. Histidine and Met clearance rates dropped by 31% ($P = 0.01$) and 64% ($P < 0.01$), respectively, with MKH. These EAA generally are not taken up in excess when supply increases above mammary demand for milk protein (Lapierre et al., 2012), so the dramatic decrease in clearance likely indicates that these EAA were oversupplied with MKH. Clearance rates of Phe ($P < 0.01$), Val ($P = 0.02$), Gly ($P = 0.01$), and Ser ($P = 0.07$) all increased or tended to increase for MKH, which reflects greater affinity for extracting these AA from extracellular space and should reduce their availability for splanchnic catabolism. Clearance of Lys (which was also infused) did not change despite greater supplies ($P = 0.95$). This lack of change in clearance of Lys reflects varying proportions of Lys destined for catabolism or other non-milk protein metabolic demand sinks in the udder. Clearance of BCAA ($P < 0.01$) and EAA other than Met,

Table 7. Mammary clearance rates (L/h) of AA (least squares treatment means)¹

Item	Treatment ²				SEM	Effect (<i>P</i> -value)		
	CON	MKH	IL	MKH ⁺ IL		MKH	IL	MKH × IL
Arg	768	703	905	883	133	0.65	0.11	0.82
His	195	136	271	187	27.6	0.01	0.02	0.61
Ile	451	545	430	476	90.4	0.33	0.53	0.74
Leu	545	658	418	529	109	0.20	0.15	0.99
Lys	1,219	1,084	1,193	1,313	164	0.95	0.42	0.31
Met	1,592	624	1,868	635	321	<0.001	0.43	0.46
Phe	880	1,155	818	1,367	121	<0.001	0.30	0.06
Thr	394	495	425	528	66.0	0.11	0.60	0.99
Val	243	230	214	346	34.3	0.02	0.09	<0.01
Ala	180	150	168	221	60.5	0.73	0.40	0.23
Asn	727	902	718	956	189	0.16	0.88	0.84
Asp	740	807	605	678	269	0.76	0.56	0.99
Gln	273	345	280	328	57.5	0.12	0.91	0.75
Glu	706	554	639	639	137	0.48	0.93	0.48
Gly	49	69	39	118	28.4	0.01	0.26	0.10
Pro	219	195	238	287	55.2	0.79	0.26	0.44
Ser	347	506	503	707	144.0	0.07	0.07	0.81
Tyr	−989	−334	4,349	185	1,978	0.31	0.10	0.17
TAA ³	308	334	287	404	36.2	0.02	0.37	0.11
EAA ⁴	430	454	444	528	50.1	0.22	0.31	0.48
BCAA ⁵	362	401	289	446	51.0	<0.01	0.64	0.06
EAA-MKH ⁶	401	446	413	509	53.9	0.14	0.42	0.57
EAA-IL ⁷	411	416	465	541	43.8	0.31	0.03	0.37
NEAA ⁸	226	241	243	307	44.4	0.26	0.24	0.47

¹Data represent arterial and venous measurements ($n = 6$ to 11 samples per cow) taken on d 16.

²CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH⁺IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

³TAA = total AA (EAA + NEAA).

⁴EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

⁵BCAA = branched-chain AA: Ile, Leu, and Val.

⁶EAA-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

⁷EAA-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

⁸NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

Lys, and His ($P = 0.14$) increased in response to MKH, which again reflects greater pull from mammary tissue for these AA.

Similar increases in clearance rates of noninfused AA were also observed with Arg ($P = 0.11$), His ($P = 0.02$), Val ($P = 0.09$), Ser ($P = 0.07$), and the EAA other than Ile and Leu ($P = 0.03$) with IL treatment infusion, again to support the greater milk protein yield (Table 7). Clearance rates of Ile and Leu did not change despite increased concentrations associated with the treatment infusions. The latter likely reflects varying proportions of Ile and Leu being catabolized or utilized for non-milk protein functions in the udder.

Ratio of Mammary AA Uptake to Milk AA Output

The ratios of mammary AA uptake to milk protein output are provided in Table 8. The capture of extracted TAA into milk protein averaged 90% across treatments, which agrees precisely with previous findings of Cant et al. (1993; Table 8). The capture of extracted His,

Met, Phe, and Tyr averaged close to 100%, whereas Arg, Ile, Leu, Lys, Thr, and Val were much lower. Of the infused EAA for MKH, only Lys increased in the uptake-to-output ratio, as is usually observed (Lapierre et al., 2009). The uptake-to-output ratio declined for BCAA ($P = 0.07$) and the sum of EAA minus Met, Lys, and His, which means less catabolism or transamination in the udder, which might explain the decline in lactose concentration because of a reduction in cellular energy status. The ratio for one NEAA, Glu ($P < 0.01$), declined, whereas the ratio for Ser increased ($P = 0.02$). Lysine is used to synthesize Glu (Lapierre et al., 2009), and the 31.7% increase in net Lys uptake likely reduced demand for extracellular Glu, given a much larger Lys precursor pool to synthesize Glu. In contrast, Ser intracellular synthesis may have decreased due to reduced intracellular BCAA with MKH (Roets et al., 1979; Roets et al., 1983), thereby necessitating greater uptake ($P < 0.01$) to support milk synthesis. These changes are reflected in a higher uptake-to-output ratio for Ser ($P = 0.07$). The Ser ratio was not

Table 8. Effect of AA infusions on ratio of mammary AA uptake to milk protein output (least squares treatment means)¹

Item	Treatment ²					Effect (<i>P</i> -value)		
	CON	MKH	IL	MKH ⁺ IL	SEM	MKH	IL	MKH × IL
Arg	2.28	2.21	2.41	2.36	0.25	0.48	0.15	0.91
His	0.85	0.78	1.02	0.96	0.11	0.54	0.10	0.97
Ile	1.25	1.35	1.54	1.38	0.13	0.76	0.14	0.23
Leu	1.26	1.16	1.51	1.41	0.08	0.14	<0.01	0.98
Lys	1.08	1.32	1.03	1.31	0.08	<0.001	0.60	0.72
Met	0.97	1.07	0.93	0.96	0.12	0.49	0.39	0.71
Phe	1.00	0.98	1.01	1.01	0.02	0.30	0.07	0.57
Thr	1.00	0.98	1.02	0.90	0.07	0.26	0.66	0.46
Val	1.47	1.23	1.40	1.28	0.14	0.10	0.94	0.57
Ala	1.36	1.31	1.32	1.48	0.41	0.82	0.80	0.68
Asn	0.35	0.42	0.41	0.43	0.04	0.27	0.42	0.52
Asp	0.26	0.24	0.19	0.19	0.06	0.84	0.24	0.84
Gln	0.67	0.71	0.70	0.75	0.07	0.39	0.50	0.92
Glu	0.70	0.57	0.65	0.53	0.04	<0.01	0.13	0.83
Gly	0.66	0.91	1.23	1.44	0.35	0.44	0.08	0.94
Pro	0.31	0.27	0.29	0.32	0.05	0.90	0.74	0.39
Ser	0.47	0.55	0.48	0.58	0.08	0.02	0.62	0.75
Tyr	0.94	0.96	0.92	0.93	0.02	0.30	0.07	0.57
TAA ³	0.88	0.87	0.92	0.92	0.05	0.89	0.36	0.96
EAA ⁴	1.23	1.22	1.33	1.26	0.07	0.42	0.14	0.48
BCAA ⁵	1.33	1.23	1.48	1.31	0.09	0.07	0.11	0.58
EAA-MKH ⁶	1.32	1.24	1.45	1.29	0.08	0.06	0.15	0.48
EAA-IL ⁷	1.21	1.22	1.24	1.23	0.07	0.98	0.62	0.86
NEAA ⁸	0.61	0.59	0.59	0.65	0.07	0.73	0.73	0.59

¹Data represent arterial and venous measurements ($n = 6$ to 11 samples per cow) taken on d 16 and average milk AA yield from d 15 to 17, between 1300 and 0100 h.

²CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH⁺IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

³TAA = total AA (EAA + NEAA).

⁴EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

⁵BCAA = branched-chain AA: Ile, Leu, and Val.

⁶EAA-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

⁷EAA-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

⁸NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

affected by IL ($P = 0.62$) despite a similar increase in milk protein yield; however, Leu and Ile net flux to the udder increased significantly, thereby providing substrate for Ser synthesis.

The ratio of Leu uptake to milk protein increased 21% ($P < 0.01$) for IL (Table 8). This change is usually observed when higher amounts of Leu are present in arterial blood (Bequette et al., 1996). However, increased Ile supply with IL did not change the uptake-to-output ratio, although it was numerically higher ($P = 0.14$). The ratio of Gly uptake to milk protein also tended to increase ($P = 0.08$) for IL, which likely reflects reduced Gly intracellular synthesis. This might have occurred because the substrates for Gly synthesis are primarily Thr (Verbeke et al., 1972) and Val (Roets et al., 1979), and both of these declined in arterial concentration ($P = 0.10$ and $P = 0.03$) and were unchanged in net uptake, hence less availability for Gly synthesis. In general, changes in ratios of AA uptake to output were as expected, in that changes in arterial supply translated

to changes in flux and proportion catabolized by the udder.

Conversion Efficiency of AA to Milk Protein

The efficiencies of converting metabolizable AA supplies into milk protein are listed in Table 9. The observed efficiency of conversion of predicted absorbed TAA to milk protein averaged 84.3% across treatments after accounting for maintenance requirements (Table 9). The underprediction of milk yield by 10 to 11 kg/d by the NRC (2001) and CNCPS v. 6.55 models is likely caused by efficiency that is too low (unlikely) or maintenance costs that are too high. Efficiencies of AA greater than 100% (i.e., His, Ile, Lys, Met, and Val) are not possible. This indicates either that the profile of absorbed AA utilized for maintenance is different from that utilized for milk protein production, or that AA were being mobilized from a source we had not considered (e.g., muscle tissue). From the perspective of

Table 9. Effect of AA infusions on MP for lactation (lact), overall, and marginal efficiencies for conversion of total and individual EAA to milk protein¹

Item	Treatment ²											
	CON			MKH		IL		MKH + IL				
	MP lact ³	Overall ⁴		MP lact ³	Overall ⁴	Marginal ⁵	MP lact ³	Overall ⁴	Marginal ⁵	MP lact ³	Overall ⁴	Marginal ⁵
TAA	0.85	0.56		0.84	0.56	0.55	0.83	0.56	0.43	0.85	0.57	0.77
Arg	0.67	0.44		0.68	0.46	—	0.67	0.45	—	0.70	0.48	—
His	1.12	0.74		0.87	0.57	0.09	1.14	0.76	—	0.88	0.59	0.20
Ile	1.09	0.72		1.13	0.74	—	0.93	0.62	0.11	0.98	0.66	0.37
Leu	0.99	0.65		1.02	0.67	—	0.82	0.55	0.08	0.85	0.58	0.27
Lys	1.17	0.77		0.98	0.64	0.13	1.17	0.78	—	1.00	0.68	0.31
Met	1.37	0.90		1.00	0.66	0.09	1.39	0.93	—	1.01	0.68	0.20
Phe	0.88	0.58		0.91	0.60	—	0.89	0.59	—	0.92	0.63	—
Thr	0.82	0.54		0.85	0.56	—	0.82	0.55	—	0.85	0.58	—
Val	1.09	0.72		1.12	0.73	—	1.09	0.73	—	1.14	0.77	—

¹Data represent LSM of intake, infusion, and milk production. TAA = total AA.²CON = infused 3 L of 0.9% saline; MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; IL = infused 22 g/d of Ile and 50 g/d of Leu; MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.³Metabolizable protein for lactation efficiency calculated as milk protein yield-AA/(predicted MP-AA supply – predicted MP-AA maintenance).⁴Overall efficiency calculated as milk protein yield-AA/predicted MP-AA supply.⁵Marginal efficiency calculated as change in milk protein yield-AA compared with the control/infused amount of the treatment AA.

overall efficiency (milk protein yield to MP supply), the observed efficiencies for the aggregate and individual EAA seem more appropriate. Indeed, the overall individual EAA efficiencies for the 3 treatment means are similar to what has previously been derived from model development literature data sets (Van Amburgh et al., 2015; Martineau et al., 2016). The total AA marginal efficiency of the treatments was much higher than that commonly observed for infused EAA or casein (15 to 25%; Hanigan et al., 1998a; Doelman et al., 2015a), ranging from 43 to 77%. The marginal efficiencies of MKH and IL alone were less than overall efficiency, implying declining responses as supply increases, whereas the combination of MKH and IL increased marginal efficiency above the overall efficiency, implying that all AA as a sum were used more efficiently for milk protein. That the marginal efficiency did not improve for MKH, which involved commonly described limiting EAA, implies that one or more of those EAA were oversupplied or that other EAA were co-limiting. Our observations that infusion of other EAA (i.e., Ile and Leu) doubled the marginal efficiency of supplemental Met, Lys, and His implies that consideration for other EAA beyond the AA currently viewed as limiting may be necessary when formulating diets in lactating dairy cows.

CONCLUSIONS

Supplementing 2 groups of EAA, (1) Met, Lys, and His and (2) Ile and Leu, independently and additively increased milk protein yield in high-producing dairy cows. These outcomes suggest we may need to move beyond the single-limiting AA theory that is often encompassed in a barrel-stave visual for understanding regulation of milk protein yield. Increased supplies of Ile and Leu increased milk and ECM yields in dairy cows, which appeared to be facilitated by increased mammary plasma flow. Increased milk protein yield by these 2 groups of AA was assisted by changes in arterial AA concentrations, mammary plasma flow, and udder clearance rates of AA, which yielded altered net uptake. We also found changes in the proportion of AA catabolized and used for milk protein, which demonstrated plasticity in mammary protein metabolism. In summary, other EAA beyond Met, Lys, and His may be critical in optimizing dairy cattle performance when formulating lactating cow diets based on these results.

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