J. Dairy Sci. 106
https://doi.org/10.3168/jds.2022-22041

Effect of supplementing rumen-protected methionine, lysine, and histidine to low-protein diets on the performance and nitrogen balance of dairy cows

T. Van den Bossche,[1] K. Goossens,[1] B. Ampe,[1] G. Haesaert,[2] J. De Sutter,[3] J. L. De Boever,[1] and L. Vandaele[1]*
[1]ILVO, 9090 Melle, East-Flanders, Belgium
[2]Bioscience Engineering, Ghent University, 9000 Gent, East-Flanders, Belgium
[3]Orffa Additives B.V., 4817 ZL Breda, Brabant, the Netherlands

ABSTRACT

Lowering the dietary protein content can reduce N excretions and NH_3 emissions from manure and increase milk N efficiency of dairy cows. However, milk yield (MY) and composition can be compromised due to AA deficiency. Methionine and Lys are known as first limiting EAA for dairy cows, and recently His is also mentioned as limiting, especially in grass-based or low-protein diets. To examine this, a trial was conducted with a 3-wk pre-experimental adaptation period (diet 16.5% crude protein), followed by a depletion period of 4 wk, in which 39 cows (average ± standard deviation: 116 ± 29.3 d in milk, 1.8 ± 1.2 lactations, 638 ± 73.2 kg of body weight, and 32.7 ± 5.75 kg MY/d) received a low-protein diet (CTRL) (14.5% crude protein). Then, taking into account parity, His plasma concentration, and MY, cows were randomly assigned to 1 of 3 treatment groups during the rumen-protected (RP) AA period of 7 wk; (1) CTRL; (2) CTRL + RP-Met + RP-Lys (MetLys); (3) CTRL + RP-Met + RP-Lys + RP-His (MetLysHis). Products were dosed, assuming requirements for digestible (d) Met, dLys, and dHis being, respectively, 2.4%, 7.0%, and 2.4% of intestinal digestible protein. In the cross-back period of 5 wk, all cows received the CTRL diet. During the last week of each period, a N balance was conducted by collecting total urine and spot samples of feces. Total feces production was calculated using the inert marker TiO_2. Statistical analysis was performed with a linear mixed model with cow as random effect and data of the last week of the pre-experimental period used as covariate for the animal performance variables. No effect of supplementing RP-Met and RP-Lys nor RP-Met, RP-Lys, and RP-His on feed intake, milk performance, or milk N efficiency was observed. However, the plasma AA profile indicated additional supply of dMet, dLys, and dHis. Nevertheless, evaluation of the AA uptake relative to the cow's requirements showed that most EAA (exclusive Arg and Thr) were limiting over the whole experiment. Only dHis was sufficiently supplemented during the RP-AA period due to an overestimation of the diet's dMet and dLys supply in the beginning of the trial. The numerically increased milk urea N and urinary N excretion when RP-Met, RP-Lys, and RP-His were added to the low-protein diet suggest an increased catabolism of the excess His.

Key words: low-protein diet, rumen-protected amino acid, histidine

INTRODUCTION

There are worldwide concerns about the greenhouse gas emissions, N excretions, and atmospheric NH_3 emissions of ruminants contributing to climate change and diverse environmental problems. Nitrogen losses in particular are a major cause of water pollution, eutrophication, and acidification, endangering our natural areas (Lapierre et al., 2007; Dijkstra et al., 2013; Mottet et al., 2018). Dairy cows have a rather poor milk N efficiency (**MNE**) of, on average, 24.7 ± 4.1% (Huhtanen and Hristov, 2009). The main driver for N loss is the dietary N intake, making low-protein diets a promising strategy to lower the N excretions and NH_3 emissions in cattle. Additionally, the high cost and ecological burden of dietary protein (e.g., soy-based products) has reinforced the interest to reduce protein levels in dairy cow diets (Sinclair et al., 2013; Hristov, 2019; Vandaele et al., 2019). Nevertheless, lowering the protein content of the diet may decrease DMI, milk yield (**MY**), milk quality, and fiber digestibility in the rumen (Lee et al., 2012; Sinclair et al., 2013; Giallongo et al., 2016). The negative MY response to lower dietary N intake can be partially related to a lower voluntary feed intake (Lee et al., 2012). However, in other experiments, there was no effect on MY for cows fed a low-protein diet (Colmenero and Broderick, 2006; Mutsvangwa et al., 2016).

Received March 21, 2022.
Accepted October 7, 2022.
*Corresponding author: leen.vandaele@ilvo.vlaanderen.be

Further, a deficiency in one or multiple EAA can occur when providing reduced protein levels, which limits milk protein synthesis in lactating dairy cows (Apelo et al., 2014). Methionine and Lys are known to be, respectively, the first and second limiting EAA in lactating dairy cows at different intensive production stages (NRC, 2001; Schwab and Broderick, 2017). Recently, His is mentioned as the third limiting AA, especially in grass-based or low-protein diets where microbial protein provides the major part of MP to the small intestine (Doepel et al., 2004; Schwab and Broderick, 2017; Appuhamy, 2019). In addition, His is stored in endogenous labile pools that are present as muscle carnosine and blood hemoglobin, which can mask short-term deficiencies of dietary His (Ouellet et al., 2014; Hristov, 2019; Lapierre et al., 2021).

The supplementation of the most limiting EAA in rumen-protected (**RP**) form to low-protein diets may be a successful strategy to prevent AA shortages. Overall, the use of RP-Met and RP-Lys is widely described in the literature, and multiple commercial products are available for dairy farmers. Also, the use and development of RP-His sources is gaining interest as several experiments showed that the supplementation of RP-His increased or tended to increase milk and milk protein yield (**MPY**), most likely partially through increasing the DMI. This effect will only prevail when RP-Met and RP-Lys are supplemented or adequately provided in the diet (Lee et al., 2012; Giallongo et al., 2016; Zang et al., 2019). Therefore, the objective of our experiment was to determine the effect of supplementing RP-His to RP-Met and RP-Lys in a low-protein diet on the animal performance (DMI, milk production, and composition), N balance, and plasma AA levels in high-producing Holstein cows. We hypothesized that additional supplementation of RP-His would increase MY, MPY, DMI, and MNE. Furthermore, plasma samples were collected during the experiment to look at changes of AA plasma levels and His labile pools over the periods with different dietary treatments.

MATERIALS AND METHODS

The trial was carried out at the experimental cattle farm of ILVO (Flanders Research Institute for Agricultural, Fisheries and Food, Melle, Belgium) in 2020. All animal handling and sampling procedures were approved by the ILVO Animal Ethics Committee (EC 2019/363).

Experimental Design and Treatments

The experimental design is represented in Figure 1. The experiment included 39 Holstein cows (22 primiparous, 17 multiparous; average ± SD: 32.7 ± 5.75 kg MY/d, 116 ± 29.3 DIM, 1.8 ± 1.2 lactations, 638 ± 73.2 kg of BW) and followed a design consisting of a control (= depletion period of 4 wk), treatment (= RP-AA treatment period of 7 wk) and again a control (= cross-back period of 5 wk) period. This experimental design makes it possible to correct for present differences between animals. The treatment effect can be evaluated based on the evolution of the milk production during lactation based on control periods before and after the treatment period. The depletion period was included to decrease labile endogenous pools of His (mainly blood hemoglobin and muscle carnosine), which could mask short-term deficiencies of dietary His induced in this experiment (Ouellet et al., 2014; Hristov, 2019; Lapierre et al., 2021). The trial was preceded by a 3-wk pre-experimental adaptation period (diet 16.5% CP) to adapt the cows to the animal group, milking installation, and feedstuffs. Furthermore, plasma and milk reference samples were collected during the last day of this pre-experimental period. Data of this last week was used as a covariate for the experimental variables regarding animal performance. However, this was not possible for some other variables (e.g., N balance data and % supply of the requirement), as no N balance was conducted in the pre-experimental period and the AA content of the feedstuffs was not analyzed in this period. The plasma reference sample was not used as a covariate but as a threshold value for the other plasma samples.

During the depletion period (4 wk), all cows received the low-protein diet (**CTRL**) (14.5% CP) described in Table 1. Then, taking into account parity, His plasma concentration, and MY, cows were randomly assigned to 1 of 3 treatment groups during the RP-AA period of 7 wk; (1) CTRL (n = 13); (2) CTRL supplemented with RP-Met and RP-Lys (**MetLys**, n = 13); (3) CTRL supplemented with RP-Met, RP-Lys, and RP-His (**MetLysHis**, n = 13). The sources of RP-Met, RP-Lys, and RP-His were Excential Rumenpass MET (Orffa Additives), AjiPro-L (Ajinomoto H&N), and an experimental RP-His product (Ajinomoto Co.), respectively. The content of digestible (**d**) AA was claimed to be 25%, 25%, and 20% for RP-Met, RP-Lys, and RP-His, respectively. The dAA intake from roughages and concentrates was estimated based on the Dutch intestinal digestible protein (**DVE**) system (Tamminga et al., 2007). The RP-AA supplements were provided to meet individual cow's needs, assuming requirements for dMet, dLys, and dHis being, respectively, 2.4%, 7.0%, and 2.4% of DVE (INRA, 2018). The minimum dose was 2.5 g dAA, and the average supply was 5.0 g dMet, 13.2 g dLys, and 9.2 g dHis for each RP-AA. In the final period of 5 wk

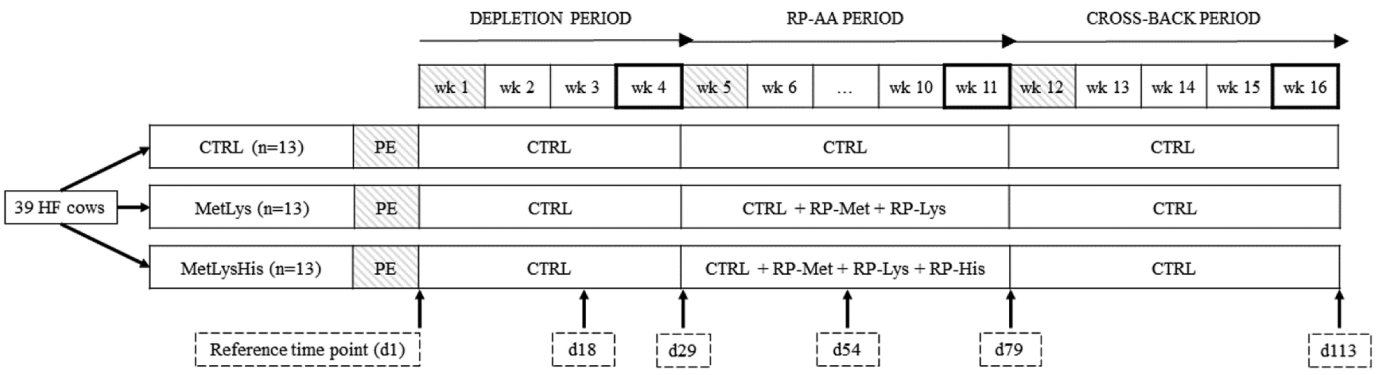

Figure 1. Overview of the experimental setup. All cows (n = 39) received a low-protein diet (CTRL; 14.5% CP) from wk 1 to 16. During the rumen-protected (RP) AA period (wk 5 to 11), the cows in the MetLys group received RP-Met (Excential Rumenpass MET, Orffa Additives) and RP-Lys (AjiPro-L, Ajinomoto H&N), and the cows in the MetLysHis group received RP-Met, RP-Lys, and RP-His (experimental RP-His product, Ajinomoto Co.). Shaded weeks were excluded from the statistical data analysis. Bold-rimmed weeks show when a N balance was conducted. The day time points show when plasma samples were collected. HF = Holstein Friesian; PE = pre-experimental adaptation period where a standard protein diet was fed (16.5% CP; = 3 wk).

(cross-back), all cows received the low-protein diet without supplementation of RP-AA (CTRL). During the last week of each period (wk 4, 11, and 16), a N balance was conducted, of which the data were processed separately. The first week of each period (wk 1, 5, and 12) was excluded for statistical data analysis, as it was considered a transition week.

Sample size was determined based on a data set including herd lactations from the past 2 yr. Three random groups were made, and for 2 groups, a treatment effect of +0.2 kg/d and +1 kg/d, respectively, was induced in the MY data. The statistical power analysis based on 1,000 simulations on this data set showed a power of 78% for the +1 kg/d treatment with 13 animals per group.

Diet

The basal diet was supplemented with concentrates in automatic feeders to meet individual cow's requirements of 105% NE_L, 100% DVE, and a CP level of 14.5%. The basal diet remained the same during the whole trial. The concentrate supplementation was lowered every 2 weeks with a fixed amount of 200 g for multiparous cows and 100 g for primiparous cows (from 7.0 ± 1.6 kg to 6.0 ± 1.6 kg), in function of the milk production curve (Table 1). The NE_L and DVE contents of the feedstuffs and the cow's requirements were calculated following Van Es (1978) and Tamminga et al. (2007), respectively. The dAA content of the feedstuffs was calculated from their analyzed AA profile following Van Duinkerken (1998). The requirements for the EAA (Met, Lys, His, Leu, Ile, Arg, Phe, Thr, and Val) were derived from INRA (2018). Based on the actual DMI, MY, and BW of the cows, the total supply, requirement, and thus also the fulfillment of the requirement (%) could be calculated for NE_L, DVE, and each EAA.

Table 1. Ingredients and chemical composition of the total low-protein diet (including concentrates) fed during the experiment[1]

Item	Value
Ingredient of total diet	
Prewilted grass silage	261
Corn silage	274
Pressed beet pulp	90
Corn meal	43
Rolled barley	14
Soybean meal	13
Chopped straw	12
Urea mix[2]	2
Balanced compound feed[3]	291
Chemical composition of total diet	
DM (g/kg)	427
CP	145
Crude fat	29
Crude ash	72
NDF	331
ADF	192
ADL	12
Starch	197
Sugars	43
NE_L[4] (MJ/kg DM)	7
DVE[5]	89
OEB[6]	0
Rumen fermentable OM	609

[1]The chemical composition (g/kg DM unless noted) was calculated based on the average intake by the cows, with exclusion of the pre-experimental adaptation period and the N balance weeks.

[2]15% rolled barley and 85% urea.

[3]Concentrates supplemented at automatic feeders to reach individual cow's requirements of 105% NE_L and 100% DVE.

[4]Van Es, 1978.

[5]DVE = true protein digested in the small intestine (Tamminga et al., 2007).

[6]OEB = degraded protein balance (Tamminga et al., 2007).

Sampling and Measurements

The cows were housed in a freestall with cubicles except for the N balance weeks, in which they were moved to the stanchion barn (wk 4, 11, and 16). In the freestall, cows had access to the roughage mixture at all times through Roughage Intake Control feed bins (Hokofarm Group BV) that were filled up 4 times a day (at 0700, 1100, 1600, and 1900 h) to ensure continuous feed supply. Concentrates were provided via standard in-parlor and out-of-parlor feed stations (DeLaval NV). Cows were milked twice a day in a herringbone milking parlor (DelPro Farm Manager). The BW of the cows was recorded on a daily basis during the entire experiment except for the N balance weeks (wk 4, 11, and 16). Individual milk samples were collected at 4 consecutive milkings in the last week of the pre-experimental adaptation period (as reference) and in wk 3, 5, 7, 9, 11, 13, 15, and 16 of the experiment. They were analyzed for milk protein, fat, lactose, and urea with Fourier-transform infrared spectroscopy (Lactoscope FTA-3.X, PerkinElmer). Milk composition for the weeks without samples was calculated from the measurements in the previous and the following week in the same period. For wk 2 and 4, the milk composition of wk 3 was used. Milk production was corrected for fat and protein content (**FPCMY**) with the following formula: FPCMY = MY × [0.337 + (0.116 × milk fat %) + (0.06 × milk protein %)] (Subnel et al., 1994). Feed efficiency was calculated as FPCMY (kg) divided by DMI (kg). The MNE was calculated with the formula:

$$\text{MNE} = \frac{\frac{\text{milk protein}\%}{100} \times \frac{\text{milk yield}(\text{kg})}{6.38}}{\frac{\text{CP intake}(\text{kg})}{6.25}}.$$

Feed samples (grass silage and maize silage) were taken every week and pooled per period or over the whole experiment. Their chemical composition was determined as described by De Boever et al. (2017). The NE_L content of the feeds was estimated with regression equations (De Boever, 1999) based on the chemical composition and the cellulase digestibility of the OM (De Boever et al., 1986), whereas the DVE content was estimated with regression equations based on chemical composition and protein solubility (J. L. De Boever, unpublished results). The feed samples were also analyzed for total AA profile (exclusive Tyr and Trp; Supplemental Table S1, https://doi.org/10.5281/zenodo.7509490, Van den Bossche, 2023e) with HPLC after acid hydrolysis. After oxidation, feed samples were hydrolyzed with hydrochloric acid and the hydrolysate was adjusted to pH 2.20. The AA were separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline) (EC, 1998).

Individual plasma samples were taken from the tail vein, always at 0900 h. The first plasma sample was taken at the end of the pre-experimental adaptation period on the first day of the experiment (d 1, reference), to determine baseline concentrations under standard feeding conditions (CP level of the complete standard diet 16.5%). Further, plasma samples were taken in wk 2 (d 18), wk 4 (d 29), wk 7 (d 54), wk 11 (d 79), and wk 16 (d 113) (Figure 1). The plasma samples from wk 4, 11, and 16 were taken on the last day of the depletion, RP-AA, and cross-back period, respectively. Taking multiple plasma samples during the experiment can provide valuable information on the changes in AA plasma levels, certainly in comparison with the reference (d 1). Blood heparin plasma was separated after centrifugation at 1,890 × *g* for 8 min at 21°C and analyzed for urea and complete AA profile. Urea in the samples was determined according to the manufacturer's instruction of the Urea Nitrogen2 assay (ARCHITECT c System, Abbott). To determine the AA profile, plasma samples (50 μL) were deproteinized by adding 100 μL of a 10% sulfosalicylic acid solution containing a 50 μ*M* internal standards mix. After vortexing, 50 μL of ultra-high-performance liquid chromatography-grade water was added. Centrifugation occurred for 10 min at 9,960 × *g* at 21°C. Derivatization of 10 μL of supernatant was performed according to the manufacturer's instructions of the AccQ-Tag kit of Waters. The AA were measured on an Acquity UPLC with QDA detector of Waters and quantified based on a 5-point calibration curve (Nguyen et al., 2018).

Nitrogen Balances

During the last week of each period (wk 4, 11, and 16), the cows were moved to the stanchion barn for 8 to 9 d to conduct a N balance (Figure 1). The roughage mixture and concentrates were fed in individual feed bins twice a day (at 0700 and 1900 h), and leftovers were weighed in the middle and at the end of the sampling week. Cows were milked twice a day with a milking cluster. The N balance can be calculated as the feed N input minus the milk N output, the urine N output and the feces N output. Therefore, MY and DMI were registered and urine and feces samples were collected during the last 6 d in the stanchion barn.

Urine was totally collected during 5 consecutive days. Each cow was provided with a Foley catheter (BARD), and urine was collected in a 50-L closed barrel. The pH

was maintained between 1.5 and 3.5 by gradual addition of H_2SO_4 (10%) via infusion drip sets. The pH was checked with a pH dip stick at 1300, 1700, and 0800 h. When pH exceeded 3.5, an additional, measured amount of H_2SO_4 (10%) was added. Between 0800 and 0900 h, the total volume of acidified urine (on average 92.5% urine + 7.5% H_2SO_4) was weighed and 2% was collected and pooled over 5 collection days. The samples of acidified urine were analyzed for N content according to ISO 5983–2 (2009) and a diluted sample (1/10) was analyzed for urinary urea (SAN++ Continuous Flow Analyzer, Skalar Analytical).

Ten feces samples of 100 g were taken from each cow directly from the rectum or during voluntary defecation at 10 different time points (0700, 0800, 0900, 1000, 1100, 1200, 1400, 1500, 1600, and 1700 h) spread over 6 d. These spot samples were pooled. Total feces production was estimated by the use of the inert marker titanium oxide (TiO_2; Hombitan FG, Venator). To do this, each cow was given 20 g of TiO_2 formulated in 1 kg of concentrate daily for 16 d, starting 10 d before the first d of fecal sampling in each period. Total fecal N was determined on the pooled sample for each period (ISO 5983–2, 2009). The content of TiO_2 in the concentrate and in the oven-dried feces was determined after grinding the samples through 1 mm (Myers et al., 2004), to estimate the total feces production.

Statistical Analysis

Daily data (BW, DMI, and MY) were averaged for each cow per week. To evaluate the effects of the treatments, all variables (BW, DMI, MY, FPCMY, MPY, % fat, % protein, % lactose, MUN, MNE, feed efficiency, plasma AA levels, total plasma EAA, and relative supplies of NE_L, DVE, and dAA) were averaged for each cow per treatment group (CTRL, MetLys, and MetLysHis) and period (depletion, RP-AA, and cross-back), with the exclusion of the first adaptation (wk 1, 5, and 12) and last N balance (wk 4, 11, and 16) wk of each period. The N balance variables [plasma urea N (**PUN**), MUN, urinary urea N (**UUN**), feed N intake, milk N output, urine N output, feces N output, and N balance] were an average of the last 5 d of each N balance period (wk 4, 11, and 16) per cow. All animal performance variables (BW, DMI, MY, FPCMY, MPY, % fat, % protein, % lactose, MUN, MNE, and feed efficiency), were analyzed including covariates of the pre-experimental period according to the following model:

$$Y_{ijk} = T_i + P_j + (TP)_{ij} + COV + C_k + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable; T_i is the fixed effect of the ith treatment group (i = CTRL, MetLys, MetLysHis); P_j is the fixed effect of the jth period (j = depletion, RP-AA, cross-back); $(TP)_{ij}$ is the interaction effect of the ith treatment group at jth period; COV is the effect of the covariate; C_k is the random effect of cow; ε_{ijk} is the residual error. The plasma AA variables were analyzed according to the following model:

$$Y_{ijk} = T_i + D_j + (TD)_{ij} + C_k + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable; T_i is the fixed effect of the ith treatment group (i = CTRL, MetLys, MetLysHis); D_j is the fixed effect of the jth day (j = 1, 18, 29, 54, 79, 113); $(TD)_{ij}$ is the interaction effect of the ith treatment group at the jth day; C_k is the random effect of cow; ε_{ijk} is the residual error. The relative supplies of NE_L, DVE, and dAA and the N balance variables were analyzed according to the following model:

$$Y_{ijk} = T_i + P_j + (TP)_{ij} + C_k + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable; T_i is the fixed effect of the ith treatment group (i = CTRL, MetLys, MetLysHis); P_j is the fixed effect of the jth period (j = depletion, RP-AA, cross-back); $(TP)_{ij}$ is the interaction effect of the ith treatment group at the jth period; C_k is the random effect of cow; ε_{ijk} is the residual error.

The results were presented as least squares means ± standard error of the means per period and treatment group. Pairwise comparisons between treatments within period or between periods within treatments were explored using a Tukey corrected post-hoc test. All statistical analyses were performed using the statistical software program R (version 4.0.4, www.r-project.org). The analyzed outcomes were assumed to be sufficiently normally distributed based on the graphical evaluation of the residuals of the model used (histogram and quantile-quantile plot). Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Animal Performance

The experiment did not include a positive control group (standard protein diet), so it is not possible to directly evaluate the effect of feeding the low-protein diet in comparison to a standard protein diet. However, in the pre-experimental adaptation period, all groups were fed a standard protein diet with 16.5% CP, and at the end of this period, milk and plasma reference

Table 2. Least squares means of BW, DMI, milk yield (MY), fat- and protein-corrected milk yield (FPCMY), milk protein yield (MPY), milk composition (milk fat, milk protein, milk lactose, and MUN), milk nitrogen efficiency (MNE), and feed efficiency for the low-protein diet (CTRL), MetLys, and MetLysHis groups in the RP-AA period of the trial

Item in RP-AA period	Group[1]			SEM	*P*-value[2]		
	CTRL	MetLys	MetLysHis		CTRL × MetLys	CTRL × MetLysHis	MetLys × MetLysHis
BW (kg)	655	657	662	5	0.99	0.67	0.47
DMI (kg/d)	23.0	22.8	22.5	0.3	0.93	0.62	0.83
Milk yield (kg/d)	30.6	30.8	30.7	0.6	0.95	>0.99	0.98
FPCMY[3] (kg/d)	32.3	32.5	32.6	0.7	0.98	0.96	>0.99
MPY[4] (g/d)	1,144	1,138	1,144	25	0.98	>0.99	0.98
Milk composition							
Milk fat (%)	4.34	4.35	4.41	0.08	>0.99	0.78	0.84
Milk protein (%)	3.75	3.74	3.77	0.03	0.55	0.42	0.97
Milk lactose (%)	4.69	4.69	4.70	0.02	0.98	0.85	0.94
MUN (mg/dL)	10.1	10.7	11.1	0.3	0.28	0.05	0.67
MNE[5] (%)	33.9	33.2	33.6	0.6	0.72	0.96	0.88
Feed efficiency[6]	1.41	1.42	1.44	0.03	0.97	0.66	0.80

[1]Rumen-protected (RP) AA [RP-Met (Excential Rumenpass MET, Orffa Additives); RP-Lys (AjiPro-L, Ajinomoto H&N); RP-His (experimental RP-His product, Ajinomoto Co.)] were provided in the RP-AA period only for the MetLys (n = 13; RP-Met + RP-Lys) and the MetLysHis (n = 13; RP-Met + RP-Lys + RP-His) groups. The low-protein diet control group (CTRL; n = 13) never received RP-AA.

[2]*P*-values for the pairwise comparison between groups within a period as determined by Tukey's pairwise comparison test. Means within a row within a period with $P \leq 0.05$ significantly differ and with $0.05 < P \leq 0.10$ tend to differ.

[3]Fat- and protein-corrected milk yield, calculated as FPCMY = milk yield × [0.337 + (0.116 × milk fat%) + (0.06 × milk protein%)].

[4]Milk protein yield, calculated as MPY = milk yield × milk protein% × 10.

[5]Calculated as [(milk protein/100) × milk yield/6.38]/(CP intake/6.25).

[6]Calculated as kilograms of FPCMY per kilogram of DMI.

samples (d 1) were taken from the cows (n = 39, 109 ± 29.3 DIM, 634 ± 76 kg BW, 34.1 ± 5.91 kg/d MY, 4.24 ± 0.542% milk fat, 3.52 ± 0.309% milk protein). The mean MNE was 30.4 ± 1.84%, and the MUN and PUN concentrations were 12.7 ± 1.44 mg/dL and 9.84 ± 1.52 mg/dL, respectively. Decreasing the CP intake by 2 percentage points in the depletion period (by changing the feedstuffs and composition of the diet) increased MNE to 36.1 ± 2.42% and lowered MUN content (9.01 ± 1.18 mg/dL) by 25% and PUN concentration (6.54 ± 1.09 mg/dL) by 34%, respectively. Milk yield decreased by 1.7 kg/d to 32.4 ± 5.8 kg/d.

Body weight, DMI, MY, FPCMY, MPY, milk composition, MNE, and feed efficiency for the RP-AA period and the pre-experimental, depletion, and cross-back periods are represented in Table 2 and Supplemental Table S2 (https://doi.org/10.5281/zenodo.7509506, Van den Bossche, 2023f), respectively. None of the above animal performance variables were different between the CTRL, MetLys, and MetLysHis groups in the pre-experimental, depletion, or cross-back periods (Supplemental Table S2). Body weight, DMI, MY, FPCMY, MPY, milk fat, milk protein, milk lactose, MNE, and feed efficiency were not affected by RP-AA supplementation (neither MetLys or MetLysHis) during the RP-AA period (Table 2). The MUN concentration of the MetLysHis group was higher ($P = 0.05$) in comparison to the CTRL group, but for the MetLys group, only a numerical difference ($P = 0.27$) with the CTRL group was found (Table 2).

Amino Acid Profile in Blood Plasma

The plasma AA concentrations at d 1 (reference), d 29 (last day of depletion period), d 79 (last day of RP-AA period), and d 113 (last day of cross-back period) are represented in Table 3. The variation for d 1, d 18, d 29, d 54, d 79, and d 113 of the Met, Lys, and His plasma levels are shown in Supplemental Figures S1 (https://doi.org/10.5281/zenodo.7509396, Van den Bossche, 2023a), S2 (https://doi.org/10.5281/zenodo.7509419, Van den Bossche, 2023b), and S3 (https://doi.org/10.5281/zenodo.7509447, Van den Bossche, 2023c), respectively. Methionine, Lys, and His plasma concentrations did not differ among treatment groups at the reference (d 1), at d 18 (depletion period), and at the end of the depletion (d 29) and cross-back (d 113) periods (Supplemental Figures S1, S2, and S3, and Table 3). On the last day of the RP-AA period (d 79), the Met and Lys plasma levels were higher ($P \leq 0.05$) for both the MetLys and the MetLysHis groups in comparison to the CTRL group (Supplemental Figures S1 and S2, and Table 3). The Met and Lys plasma concentrations were not different between the MetLys and MetLysHis group. In the middle of the RP-AA period (d 54), the Met plasma concentration

of the MetLys group tended ($P = 0.06$) to be higher compared with the CTRL group (Supplemental Figure S1), but no other differences in Met or Lys plasma levels were found (Supplemental Figures S1 and S2). The His plasma concentration of the MetLysHis group was higher at d 54 ($P = 0.02$) compared with the CTRL group and at d 79 ($P \leq 0.01$) compared with both the CTRL and MetLys group (Supplemental Figure S3 and Table 3). Thus, the supplementation of each RP-AA product (Met, Lys, and His) resulted in a significant response in plasma after 50 d in this study. Overall, feeding the low-protein diet and providing dHis for 85 to 90% of the cow's requirement lowered the His plasma level until it stabilized at 35 to 45 μM (based on the minimum values of the CTRL and MetLys groups) after a depletion of 4 to 5 wk.

Concerning the other EAA (Table 3), the plasma levels of Arg, Leu, and Val were higher ($P \leq 0.05$) at the reference (d 1) in comparison to the following time points (d 29, d 79, d 113), whereas for Ile, Phe, Thr, and Trp, the plasma concentrations were higher ($P \leq 0.05$) at the reference (d 1) but only in comparison to the last day of the cross-back period (d 113). This applies to all 3 treatment groups. The total EAA concentration (Table 3) was higher ($P \leq 0.05$) at the reference (d 1) in comparison to the end of the depletion (d 29), RP-AA (d 79), and cross-back periods (d 113) for all 3 treatment groups. Further, no significant differences among treatment groups were found within each period, except for Trp (Table 3). At the end of the depletion period (d 29), the CTRL group had a lower ($P \leq 0.05$) Trp plasma level compared with the MetLys and MetLysHis groups. The total EAA levels (Table 3) did not differ between treatment groups at every time point.

Nitrogen Balance

The results of the N balance variables (PUN concentration, UUN excretion, feed N intake, milk N output, urinary N output, and fecal N output) did not differ between treatment groups within each period, resulting in no differences for N balance either (Table 4 and Supplemental Figure S4, https://doi.org/10.5281/zenodo.7509480, Van den Bossche, 2023d). Generally, over all periods and treatments, the total N intake was 483 ± 56.1 g/d, of which 25.9% was lost through urine (125 ± 20.1 g/d), 35.2% through feces (170 ± 20.3 g/d), and 34.8% was used for milk protein (168 ± 28.2 g/d), resulting in a positive N balance of 4.1% of the total N intake or 20 ± 28 g/d. This surplus N can be assigned to other, less predominant N losses from sweat, hair, and scurf that were not included in the N balance.

Fulfillment of Energy, Protein, and Amino Acid Requirements

To evaluate the fulfillment of energy, DVE, dMet, dLys, and dHis requirements of the cows, the relative supply or % of the requirement was calculated and represented in Table 5. Net energy was adequately provided during the whole trial (102 to 109% of the requirement), whereas DVE was only provided between 92 and 100% of the requirement. For both variables, there were no significant differences between treatment groups within every period. When no RP-AA were supplemented, dMet (78 to 84% of the requirement), dLys (82 to 88% of the requirement), and dHis (86 to 93% of the requirement) were limiting, as expected. During the RP-AA period, the dMet and dLys supply as % of the requirement was not different between the MetLys and MetLysHis group. However, the dMet (91 to 93% of the requirement) and dLys (95 to 97% of the requirement) supply was still below the requirements of the cows in the MetLys and MetLysHis groups. The MetLysHis group showed a higher (111% of the requirement, $P \leq 0.001$) dHis supply in comparison to the CTRL and MetLys groups. The dHis supply as % of the requirement did not differ between the CTRL and MetLys groups. As for the other EAA (Supplemental Table S3, https://doi.org/10.5281/zenodo.7509514, Van den Bossche, 2023g), dLeu (85 to 92% of the requirement), dIle (90 to 97% of the requirement), dPhe (88 to 95% of the requirement), and dVal (93 to 101% of the requirement) were also limiting during the experiment, whereas dArg (132 to 142% of the requirement) and dThr (108 to 116% of the requirement) were adequately provided through the diet. So almost all EAA, except Arg and Thr, were limiting over the total experiment, and only dHis was adequately provided during the RP-AA period to the designated group.

DISCUSSION

Lowering the protein content of the diet can cause deficiencies in one or multiple EAA, limiting milk protein synthesis in lactating dairy cows (Sinclair et al., 2013; Apelo et al., 2014; Schwab and Broderick, 2017). Methionine and Lys are known as first and second limiting EAA, whereas His is mentioned as third limiting EAA, especially in grass-based or low-protein diets (Doepel et al., 2004; Appuhamy, 2019). Therefore, we hypothesized that the supplementation of RP-His in addition to RP-Met and RP-Lys would improve the cow's performance when fed a low-protein diet. However, in this study, the supplementation of RP-Met and RP-Lys or RP-Met, RP-Lys, and RP-His did not

Table 3. Least squares means of the plasma EAA levels (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; μmol/L), total EAA (μmol/L), and total NEAA (μmol/L) measured in the plasma samples taken on d 1 (reference), d 29 (last day of depletion period), d 79 (last day of RP-AA period), and d 113 (last day of cross-back period) for the 3 treatment groups [low-protein diet (CTRL), MetLys, and MetLysHis]

	Group[1]				*P*-value[2]		
Period and item	CTRL	MetLys	MetLysHis	SEM	CTRL × MetLys	CTRL × MetLysHis	MetLys × MetLysHis
Reference (d 1)							
Arg	118	115	107	5	0.89	0.36	0.61
His	57	56	58	4	0.99	0.97	0.92
Ile	137	141	133	6	0.90	0.88	0.62
Leu	154	157	150	7	0.94	0.93	0.75
Lys	98	98	95	5	>0.99	0.92	0.94
Met	23	25	24	1	0.48	0.69	0.94
Phe	52	55	55	2	0.60	0.63	>0.99
Thr	134	128	129	7	0.81	0.87	>0.99
Trp	54	55	56	2	0.91	0.78	0.96
Val	272	267	268	10	0.94	0.96	>0.99
Total EAA[3]	1,099	1,097	1,077	38	>0.99	0.91	0.93
Depletion period (d 29)							
Arg	68	69	74	5	0.99	0.78	0.69
His	37	44	40	4	0.37	0.84	0.72
Ile	122	127	123	6	0.80	0.98	0.90
Leu	120	128	125	7	0.66	0.84	0.95
Lys	70	77	79	5	0.51	0.39	0.98
Met	21	23	24	1	0.59	0.22	0.77
Phe	49	53	54	2	0.43	0.33	0.98
Thr	105	114	115	7	0.65	0.56	0.99
Trp	43	49	51	2	0.05	0.01	0.86
Val	202	212	212	10	0.76	0.75	>0.99
Total EAA[3]	837	896	897	38	0.50	0.50	>0.99
RP-AA period (d 79)							
Arg	77	82	83	5	0.77	0.72	>0.99
His	36	43	60	4	0.36	≤0.01	≤0.01
Ile	117	133	125	6	0.14	0.54	0.67
Leu	115	131	122	7	0.21	0.76	0.59
Lys	71	88	87	5	0.03	0.05	0.97
Met	21	26	26	1	0.01	≤0.01	0.94
Phe	47	53	50	2	0.09	0.43	0.67
Thr	113	111	112	7	0.98	0.98	>0.99
Trp	48	51	51	2	0.40	0.40	>0.99
Val	199	218	217	10	0.36	0.42	>0.99
Total EAA[3]	843	936	932	38	0.20	0.22	>0.99
Cross-back period (d 113)							
Arg	69	69	62	5	>0.99	0.63	0.61
His	41	44	42	4	0.79	0.96	0.92
Ile	107	107	94	6	>0.99	0.31	0.29
Leu	102	103	93	7	>0.99	0.62	0.53
Lys	70	65	60	5	0.72	0.23	0.67
Met	20	21	19	1	0.88	0.91	0.65
Phe	41	45	43	2	0.41	0.81	0.79
Thr	102	87	79	7	0.28	0.06	0.67
Trp	45	45	42	2	0.94	0.37	0.58
Val	181	179	181	10	0.98	0.75	0.84
Total EAA[3]	774	764	704	38	0.98	0.42	0.51

[1]Rumen-protected (RP) AA [RP-Met (Excential Rumenpass MET, Orffa Additives); RP-Lys (AjiPro-L, Ajinomoto H&N); RP-His (experimental RP-His product, Ajinomoto Co.)] were provided in the RP-AA period only for the MetLys (n = 13; RP-Met + RP-Lys) and the MetLysHis (n = 13; RP-Met + RP-Lys + RP-His) groups. The control group (CTRL; n = 13) never received RP-AA.

[2]*P*-values for the pairwise comparison between groups within a period as determined by Tukey's pairwise comparison test. Means within a row within a period with $P \leq 0.05$ significantly differ and with $0.05 < P \leq 0.10$ tend to differ.

[3]Total EAA = Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Trp + Val.

enhance animal performance or MNE of dairy cows fed a low-protein diet (14.5% CP).

Supplementing RP-His to a low-protein diet in addition to RP-Met and RP-Lys did not affect DMI in our experiment. This is in contrast to the reported positive effect of His supplementation on the DMI of high-producing lactating cows fed a MP deficient diet, sufficiently provided with Met and Lys, but being limiting in His (Lee et al., 2012; Giallongo et al., 2015, 2016, 2017). On the other hand, Zang et al. (2019) and Zang

Table 4. Least squares means of the N balance variables (PUN, UUN, feed N intake, milk N output, urine N output, feces N output, and N balance) for the low-protein diet (CTRL), MetLys, and MetLysHis groups measured during the last week of each period (depletion, RP-AA, and cross-back)

	Group[1]				*P*-value[2]		
Period and item	CTRL	MetLys	MetLysHis	SEM	CTRL × MetLys	CTRL × MetLysHis	MetLys × MetLysHis
Depletion period							
PUN[3] (mg/dL)	6.84	7.16	6.58	0.32	0.77	0.83	0.42
UUN[4] (g/d)	14.1	14.9	15.5	0.9	0.81	0.55	0.91
Feed N intake (g/d)	467	469	475	16	0.99	0.93	0.96
Milk N output (g/d)	174	170	180	8	0.92	0.89	0.66
Urine N output (g/d)	113	120	127	5	0.66	0.17	0.61
Feces N output (g/d)	184	173	169	5	0.37	0.14	0.85
N balance (g/d)	−5	6	−1	6	0.49	0.92	0.74
RP-AA period							
PUN (mg/dL)	6.97	7.26	7.16	0.32	0.80	0.91	0.97
UUN (g/d)	14.7	17.0	16.9	0.9	0.21	0.23	>0.99
Feed N intake (g/d)	496	486	495	16	0.89	>0.99	0.91
Milk N output (g/d)	174	163	177	8	0.61	0.95	0.43
Urine N output (g/d)	122	134	136	5	0.28	0.19	0.97
Feces N output (g/d)	175	172	168	5	0.89	0.65	0.91
N balance (g/d)	25	17	14	6	0.63	0.43	0.94
Cross-back period							
PUN (mg/dL)	6.92	7.33	7.04	0.32	0.64	0.96	0.80
UUN (g/d)	13.8	14.6	14.3	0.9	0.82	0.91	0.98
Feed N intake (g/d)	497	481	478	16	0.77	0.68	0.99
Milk N output (g/d)	164	154	154	8	0.63	0.60	>0.99
Urine N output (g/d)	123	125	125	5	0.96	0.96	>0.99
Feces N output (g/d)	168	161	158	5	0.72	0.47	0.92
N balance (g/d)	42	40	41	6	0.98	0.99	>0.99

[1]Rumen-protected (RP) AA [RP-Met (Excential Rumenpass MET, Orffa Additives); RP-Lys (AjiPro-L, Ajinomoto H&N); RP-His (experimental RP-His product, Ajinomoto Co.)] were provided in the RP-AA period only for the MetLys (n = 13; RP-Met + RP-Lys) and the MetLysHis (n = 13; RP-Met + RP-Lys + RP-His) groups. The control group (CTRL; n = 13) never received RP-AA.

[2]*P*-values for the pairwise comparison between groups within a period (Tukey's test). No significant differences nor tendencies between treatment groups within a period were determined ($P > 0.10$).

[3]PUN = plasma urea nitrogen.

[4]UUN = urinary urea nitrogen.

et al. (2021) reported no response of RP-His supplementation on DMI and ascribed this to the diets being not or only marginally deficient for MP. This explanation can be supported by the research of Räisänen et al. (2021a), who reported no effects of supplementing RP-His to a MP-adequate diet. However, in our study, DVE was only provided between 92 and 100% of the requirement (Tamminga et al., 2007), so an effect of supplementing RP-His could be expected. Further, BW did not differ between treatment groups, which can be seen as a logical consequence of the unaffected DMI.

Low-protein diets have the potential to decrease the environmental impact of milk production (Huhtanen and Hristov, 2009; Lee et al., 2015), but may also reduce MY (Lee et al., 2012; Giallongo et al., 2016; Hristov, 2019). In our experiment, MY decreased by 1.7 kg/d as a combined effect of lowering the CP content by 2%, changing the feedstuffs in the diet and the declining lactation curve of the cows. These combined effects cannot be dissociated from each other, so statistical evaluation of solely the effect of reducing the CP content is therefore not possible. Supplementing RP-Met and RP-Lys or RP-Met, RP-Lys, and RP-His to a low-protein diet did not affect milk performance (MY, FPCMY, MPY) in milk quality (fat, protein, and lactose), MNE, nor feed efficiency in our experiment. The latter is logical since no effect was found for the variables (MY, FPCMY, MPY, DMI, and N intake) determining the MNE and feed efficiency. However, additional N from AA supplementation could have even decreased the efficiencies of the MetLys and MetLysHis groups. The observations on milk performance are contradictory in the literature. In the long-term experiment (12 wk) of Lee et al. (2012), the DMI and MY decreased when feeding a MP deficient diet. The supplementation of RP-Lys and RP-Met to the MP deficient diet tended to increase DMI and MY, whereas the additional supplementation of RP-His further increased DMI and MY to a similar level of a MP-adequate diet. The RP-AA supplementation increased DMI, which in turn increased MY, whereas milk fat and true protein content remained unaffected. In the study of Giallongo et al. (2016), DMI, MY, and milk components (fat, protein, lactose) also decreased by feeding a MP deficient diet

Table 5. Least squares means of the NE_L, Dutch intestinal digestible protein (DVE), and digestible (d) AA (Met, Lys, and His) supply relative to the cow's requirements (%) for the low-protein diet (CTRL), MetLys, and MetLysHis groups in the RP-AA period of the trial[1]

	Group[2]				*P*-value[3]		
Period and item	CTRL	MetLys	MetLysHis	SEM	CTRL × MetLys	CTRL × MetLysHis	MetLys × MetLysHis
Depletion period							
NE_L[4]	102	102	102	2	0.99	0.97	0.99
DVE[5]	92.6	94.5	92.4	2.1	0.81	>0.99	0.77
dMet[6]	78.4	79.9	78.1	2.0	0.85	0.99	0.79
dLys[6]	82.3	83.9	82.1	1.9	0.83	>0.99	0.79
dHis[6]	86.3	88.0	86.2	2.0	0.80	>0.99	0.78
RP-AA period							
NE_L	109	109	108	2	0.99	0.96	0.92
DVE	98.1	99.8	95.9	2.1	0.84	0.75	0.41
dMet	83.5	93.1	90.8	2.0	<0.01	0.03	0.70
dLys	87.3	96.9	95.3	1.9	<0.01	0.01	0.83
dHis	91.1	92.6	111	2.0	0.86	<0.01	<0.01
Cross-back period							
NE_L	107	105	106	2	0.83	0.88	0.99
DVE	97.3	98.7	96.0	2.1	0.90	0.89	0.64
dMet	83.2	84.1	81.6	2.0	0.94	0.85	0.67
dLys	86.9	88.0	85.5	1.9	0.92	0.87	0.65
dHis	90.4	91.7	89.2	2.0	0.90	0.90	0.65

[1]Calculations based on the actual DMI, milk yield (MY), and BW of the cows during the experiment.

[2]Rumen-protected (RP) AA [RP-Met (Excential Rumenpass MET, Orffa Additives); RP-Lys (AjiPro-L, Ajinomoto H&N); RP-His (experimental RP-His product, Ajinomoto Co.)] were provided in the RP-AA period only for the MetLys (n = 13; RP-Met + RP-Lys) and the MetLysHis (n = 13; RP-Met + RP-Lys + RP-His) groups. The control group (CTRL; n = 13) never received RP-AA.

[3]*P*-values for the pairwise comparison between groups within a period as determined by Tukey's pairwise comparison test. Means within a row within a period with $P \leq 0.05$ significantly differ and with $0.05 < P \leq 0.10$ tend to differ.

[4]Van Es, 1978.

[5]DVE = true protein digested in the small intestine (Tamminga et al., 2007).

[6]The dAA contents of the feedstuffs were calculated from their analyzed AA profiles following Van Duinkerken (1998). The AA requirements were derived from INRA (2018).

compared with a MP-adequate diet. Supplementation of the MP deficient diet with RP-Met or RP-Lys did not affect DMI, whereas supplementation with RP-His (alone or in combination with RP-Met and RP-Lys) increased DMI to a similar extent than the MP-adequate diet. However, only supplementation of the MP deficient diet with all 3 RP-AA numerically increased MY but not to the same level as the MP-adequate diet. Also, Giallongo et al. (2017) reported a positive effect on MY by supplementing RP-His to a His-deficient diet, which provided sufficient Met and Lys. Similar results were found by Räisänen et al. (2021b) and Zang et al. (2019), even with diets being slightly deficient for Lys (92 to 97% of the requirement and 95 to 99% of the requirement, respectively). However, in the study of Giallongo et al. (2015), the diet was more deficient in Lys (86 to 90% of the requirement), and the supplementation of RP-His did not improve MY. In our experiment, His was the only RP-AA sufficiently provided during the RP-AA period (MetLysHis group, 111% of the requirement) as our diet (unintendedly) remained deficient for Met (91 to 93% of the requirement) and Lys (92 to 94% of the requirement) when supplemented with RP-Met and RP-Lys (and RP-His) (INRA, 2018). This is due to an overestimation of the dAA supply through the diet at the beginning of the experiment, leading to a greater AA deficiency than expected. In the first place, the estimated dAA content of the feedstuffs based on the Dutch DVE system (Tamminga et al., 2007) overestimated the actual dAA content determined by analysis after the experiment, especially the dMet and dLys content of the concentrates. Second, the actual roughage intake during the experiment was higher than the estimated roughage intake, which further reduced the dAA/DVE content of the ingested diet under the AA requirement thresholds. In addition, Leu, Ile, Phe, and Val were deficient for all cows during the whole experiment. From this, it seems that taking into account all EAA is important to obtain a general positive effect on animal performance.

Supplementing RP-Met, RP-Lys, and RP-His to the low-protein diet increased the MUN concentration in our experiment, whereas supplementation of RP-Met and RP-Lys only gives rise to a numerical difference. Supplementing RP-AA (Met and Lys or Met, Lys, and His) also numerically increased the UUN excretion. Despite research that has proven correlations between MUN, PUN, UUN, and N excretion (Spek et al., 2013;

Barros et al., 2019; Souza et al., 2021), PUN concentrations did not follow the same trend as MUN and UUN and were unaffected by RP-AA supplementation in our study. This is in line with the results of Lee et al. (2012) and Giallongo et al. (2016). The concentration of MUN was numerically greater for a MP deficient diet supplemented with RP-Met, RP-Lys, and RP-His compared with a MP deficient diet without supplementation, whereas PUN was not affected. In the study of Lee et al. (2012), the UUN excretion was not affected. Our diet remained deficient for Met (91 to 93% of the requirement) and Lys (92 to 94% of the requirement), despite supplementation, making His (111% of the requirement) the excess EAA for the MetLysHis group. Therefore, the higher MUN concentration suggests an increased catabolism of the excess His in respect to the other EAA (e.g., Met and Lys), and the additional N appeared in the urea N pool. This imbalance decreases the efficiency of AA utilization for milk protein synthesis (Doepel et al., 2004).

Decreasing plasma EAA concentrations can be directly linked to a linear decrease of the duodenal AA flow related to feed protein intake, as indicated in the meta-analysis of Patton et al. (2015). Feeding a low-protein diet decreased all EAA plasma levels during our experiment (compared with the reference, d 1), either rapidly (after 29 d, end of depletion period) for Lys, His, Arg, Leu, Val, and total EAA or slowly (after 113 d, end of cross-back period) for Ile, Phe, Thr, and Trp. However, the Met plasma concentration was not affected in the same way, as it remained unchanged for the CTRL group. The latter could be due to an already existing limited shortage for dMet before the start of the trial, as Met is known to be the first limiting EAA for dairy cows (NRC, 2001; Schwab and Broderick, 2017). Many other studies also report lower total EAA and individual EAA plasma levels while feeding a MP deficient diet. Lee et al. (2012) found a lower total EAA, Arg, His, Leu, Lys, Met, Phe, and Val plasma concentration [pooled sample from d 69 and d 83 (3 samples per day)] for cows fed a MP deficient diet compared with cows fed a MP-adequate diet. Giallongo et al. (2016) reported a lower plasma concentration for total EAA and Arg, His, Ile, Leu, Lys, Phe, and Val while feeding a MP deficient diet compared with a MP-adequate diet (plasma sample taken on d 35). Giallongo et al. (2017) reported lower His, Leu, Lys, and Val plasma levels for cows fed a His-deficient diet (+37 g/d MP) compared with cows fed a His adequate diet (+166 g/d MP). However, in contrast, Giallongo et al. (2015) found no lower plasma level for any EAA while feeding a MP deficient diet compared with a MP-adequate diet [pooled sample from d 38 and d 52 (3 samples per day)]. The variability in the literature may be explained by different diet compositions and feedstuffs, leading to variable EAA supplies.

For each RP-AA product (Met, Lys, and His), a significant response in plasma was observed after 50 d of supplementation. However, only a numerical difference in total EAA was observed for the MetLys ($P = 0.20$) and MetLysHis ($P = 0.22$) groups compared with the CTRL group during the RP-AA period. This can be explained by the rather limited proportion of Met and His in the total EAA plasma concentration. Similar effects of supplementing RP-Met, RP-Lys, and RP-His on plasma AA levels were reported by Lee et al. (2012), Giallongo et al. (2016) and Zang et al. (2021), also without affecting total EAA plasma concentration. Giallongo et al. (2017), Zang et al. (2019), and Räisänen et al. (2021b) reported a significant response of plasma His concentration to RP-His supplementation.

Overall, we observed a response in plasma concentration for each supplemented AA, while animal performance was unaffected. Possibly this could be explained by the N balance data, where N excretion variables were not significantly altered for the treatment groups. Nevertheless, the urine N output is numerically higher for the MetLys ($P = 0.28$) and MetLysHis ($P = 0.19$) groups compared with the CTRL group in the RP-AA period. In addition, the N losses through urine represent 28% and 27% of the total N intake, for the MetLys and MetLysHis groups, respectively, compared with 25% for the CTRL group. This is in line with the higher MUN concentration in the MetLys and MetLysHis groups, suggesting an increased breakdown of excessive AA, which would be His in this study (Doepel et al., 2004). In the study of Lee et al. (2012), N balance and N excretion variables were unaffected by supplementation of RP-AA (Met + Lys or Met + Lys + His), but a positive effect on DMI and MY was observed.

CONCLUSIONS

The plasma AA profile indicated additional supply of dMet, dLys, and dHis. However, supplementing RP-Met and RP-Lys or additionally also RP-His to a low-protein diet did not affect DMI, milk performance, or MNE. This is most probably related to the remaining deficiency for Met and Lys, despite supplementation, due to an overestimation of the dAA supply through the diet in the beginning of the experiment. Histidine was supplemented in sufficient quantity in contrast to other EAA such as Met and Lys. This imbalance decreases the efficiency of AA utilization for milk protein synthesis. The higher MUN concentration and numerically increased urinary N excretion when RP-Met, RP-Lys, and RP-His were added to the low-protein diet, suggest an increased catabolism of the excess His, and the ad-

ditional N appeared in the urea N pool. In conclusion, taking into account all EAA is important to obtain a general positive effect on animal performance.

ACKNOWLEDGMENTS

This research was funded by the government agency Flanders Innovation and Entrepreneurship (VLAIO, Belgium; HBC.2018.2222). The Excential Rumenpass MET product was provided by Orffa Additives (Breda, the Netherlands), the AjiPro-L by Ajinomoto H&N (Itasca, IL), and the experimental RP-His product by Ajinomoto Co. (Tokyo, Japan). Thanks to the staff of the ILVO (Flanders Research Institute for Agriculture, Fisheries and Food, Melle, Belgium) dairy farm for taking care of the animals, in particular the people who milked and fed the cows, the people of the feed mill, and those responsible for the overall functioning of the farm. Special thanks to Tilly Haertjens, Jorne Brouckaert, Björn Inghelbrecht, Sara Van Lembergen, Anja Van Havermaet, Ilke Van Hese, Joni Van Mullem, Maryline Lamérand, Sabrina Curial, and Nico Peiren (all of ILVO, Melle, Belgium) for their practical help during the experiment. Thanks also to the ILVO Animalab laboratory for all feed and milk analyses. The authors have not stated any conflicts of interest.

REFERENCES

Appuhamy, R. 2019. Beyond lysine and methionine: What have we learned about histidine? Four-State Dairy Nutrition & Management Conference. Dubuque, IA.

Arriola Apelo, S. I., A. L. Bell, K. Estes, J. Ropelewski, M. J. de Veth, and M. D. Hanigan. 2014. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. J. Dairy Sci. 97:5688–5699. https://doi.org/10.3168/jds.2013-7833.

Barros, T., K. F. Reed, J. J. Olmos Colmenero, and M. A. Wattiaux. 2019. Milk urea nitrogen as a predictor of urinary nitrogen and urea nitrogen excretions of late-lactation dairy cows fed nitrogen-limiting diets. J. Dairy Sci. 102:1601–1607. https://doi.org/10.3168/jds.2018-14551.

Colmenero, J. J. O., and G. A. Broderick. 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. J. Dairy Sci. 89:1704–1712. https://doi.org/10.3168/jds.S0022-0302(06)72238-X.

De Boever, J. 1999. Equations to predict digestibility and energy value of grass silages, maize silages, grass hays, compound feeds and raw materials for cattle. Pages 835–850 in Proc. Nutrition Abstracts and Reviews, Series B: Livestock Feeds and Feeding. CABI Publishing.

De Boever, J. L., K. Goossens, N. Peiren, J. Swanckaert, B. Ampe, D. Reheul, D. L. De Brabander, S. De Campeneere, and L. Vandaele. 2017. The effect of maize silage type on the performances and methane emission of dairy cattle. J. Anim. Physiol. Anim. Nutr. (Berl.) 101:e246–e256. https://doi.org/10.1111/jpn.12598.

De Boever, J. L., B. Cottyn, F. X. Buysse, F. W. Wainman, and J. M. Vanacker. 1986. The use of an enzymatic technique to predict digestibility, metabolizable and net energy of compound feedstuffs for ruminants. Anim. Feed Sci. Technol. 14:203–214. https://doi.org/10.1016/0377-8401(86)90093-3.

Dijkstra, J., C. Reynolds, E. Kebreab, A. Bannink, J. Ellis, J. France, and A. Van Vuuren. 2013. Challenges in ruminant nutrition: Towards minimal nitrogen losses in cattle. Pages 47–58 in Energy and Protein Metabolism and Nutrition in Sustainable Animal Production. Springer.

Doepel, L., D. Pacheco, J. J. Kennelly, M. Hanigan, I. López, and H. Lapierre. 2004. Milk protein synthesis as a function of amino acid supply. J. Dairy Sci. 87:1279–1297. https://doi.org/10.3168/jds.S0022-0302(04)73278-6.

EC. 1998. Determination of amino acids. Off. J. Eur. Commun. 257:16–23.

Giallongo, F., A. N. Hristov, J. Oh, T. Frederick, H. Weeks, J. Werner, H. Lapierre, R. A. Patton, A. Gehman, and C. Parys. 2015. Effects of slow-release urea and rumen-protected methionine and histidine on performance of dairy cows. J. Dairy Sci. 98:3292–3308. https://doi.org/10.3168/jds.2014-8791.

Giallongo, F., M. T. Harper, J. Oh, J. C. Lopes, H. Lapierre, R. A. Patton, C. Parys, I. Shinzato, and A. N. Hristov. 2016. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. J. Dairy Sci. 99:4437–4452. https://doi.org/10.3168/jds.2015-10822.

Giallongo, F., M. T. Harper, J. Oh, C. Parys, I. Shinzato, and A. N. Hristov. 2017. Histidine deficiency has a negative effect on lactational performance of dairy cows. J. Dairy Sci. 100:2784–2800. https://doi.org/10.3168/jds.2016-11992.

Hristov, A. N. 2019. Amino acid nutrition of dairy cows: Emphasis on histidine. Presentation at 20th Penn State Dairy Cattle Nutrition Workshop, Hershey, PA.

Huhtanen, P., and A. N. Hristov. 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. J. Dairy Sci. 92:3222–3232. https://doi.org/10.3168/jds.2008-1352.

INRA. 2018. INRA Feeding System for Ruminants. Wageningen Academic Publishers.

Lapierre, H., G. Lobley, D. Ouellet, L. Doepel, and D. Pacheco. 2007. Amino acid requirements for lactating dairy cows: Reconciling predictive models and biology. Pages 39–60 in Proc. Cornell Nutr. Conf. Dept. Anim. Sci. Cornell Univ., Ithaca, NY.

Lapierre, H., G. E. Lobley, and D. R. Ouellet. 2021. Histidine optimal supply in dairy cows through determination of a threshold efficiency. J. Dairy Sci. 104:1759–1776. https://doi.org/10.3168/jds.2020-19205.

Lee, C., F. Giallongo, A. Hristov, H. Lapierre, T. Cassidy, K. Heyler, G. Varga, and C. Parys. 2015. Effect of dietary protein level and rumen-protected amino acid supplementation on amino acid utilization for milk protein in lactating dairy cows. J. Dairy Sci. 98:1885–1902. https://doi.org/10.3168/jds.2014-8496.

Lee, C., A. N. Hristov, K. Heyler, T. Cassidy, H. Lapierre, G. Varga, and C. Parys. 2012. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. J. Dairy Sci. 95:5253–5268. https://doi.org/10.3168/jds.2012-5366.

Mottet, A., F. Teillard, and P. Boettcher., G. De' Besi, and B. Besbes. 2018. Review: Domestic herbivores and food security: Current contribution, trends and challenges for a sustainable development. Animal 12(Suppl. 2):s188–s198.

Mutsvangwa, T., K. L. Davies, J. J. McKinnon, and D. A. Christensen. 2016. Effects of dietary crude protein and rumen-degradable protein concentrations on urea recycling, nitrogen balance, omasal nutrient flow, and milk production in dairy cows. J. Dairy Sci. 99:6298–6310. https://doi.org/10.3168/jds.2016-10917.

Myers, W. D., P. A. Ludden, V. Nayigihugu, and B. W. Hess. 2004. A procedure for the preparation and quantitative analysis of samples for titanium dioxide. J. Anim. Sci. 82:179–183. https://doi.org/10.2527/2004.821179x.

Nguyen, H. A., Y. Su, J. Y. Zhang, A. Antanasijevic, M. Caffrey, A. M. Schalk, L. Liu, D. Rondelli, A. Oh, D. L. Mahmud, M. C. Bosland, A. Kajdacsy-Balla, S. Peirs, T. Lammens, V. Mondelaers, B. De Moerloose, S. Goossens, M. J. Schlicht, K. K. Kabirov, A. V. Lyubimov, B. J. Merrill, Y. Sauntharajah, P. Van Vlierberghe, and A. Lavie. 2018. A novel L-asparaginase with low L-glutaminase

coactivity is highly efficacious against both T- and B-cell acute lymphoblastic leukemias *in vivo*. Cancer Res. 78:1549–1560. https://doi.org/10.1158/0008-5472.CAN-17-2106.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. National Academies Press.

Ouellet, D., G. Lobley, and H. Lapierre. 2014. Histidine requirement of dairy cows determined by the indicator amino acid oxidation (AAO) technique. J. Dairy Sci. 97(Suppl. 1):757.

Räisänen, S. E., C. F. A. Lage, J. Oh, A. Melgar, K. Nedelkov, X. Chen, M. Miura, and A. N. Hristov. 2021a. Histidine dose-response effects on lactational performance and plasma amino acid concentrations in lactating dairy cows: 1. Metabolizable protein-adequate diet. J. Dairy Sci. 104:9902–9916.

Räisänen, S. E., C. F. A. Lage, M. E. Fetter, A. Melgar, A. M. Pelaez, H. A. Stefenoni, D. E. Wasson, S. F. Cueva, X. Zhu, M. Miura, and A. N. Hristov. 2021b. Histidine dose-response effects on lactational performance and plasma amino acid concentrations in lactating dairy cows: 2. Metabolizable protein-deficient diet. J. Dairy Sci. 104:9917–9930. https://doi.org/10.3168/jds.2021-20189.

Schwab, C. G., and G. A. Broderick. 2017. A 100-year review: Protein and amino acid nutrition in dairy cows. J. Dairy Sci. 100:10094–10112. https://doi.org/10.3168/jds.2017-13320.

Sinclair, K., P. Garnsworthy, G. Mann, and L. Sinclair. 2013. Reducing dietary protein in dairy cow diets: Implications for nitrogen utilization, milk production, welfare and fertility. Animal 8:1–13.

Souza, V., M. Aguilar, M. Van Amburgh, W. Nayananjalie, and M. Hanigan. 2021. Milk urea nitrogen variation explained by differences in urea transport into the gastrointestinal tract in lactating dairy cows. J. Dairy Sci. 104:6715–6726. https://doi.org/10.3168/jds.2020-19787.

Spek, J. W., J. Dijkstra, G. van Duinkerken, W. H. Hendriks, and A. Bannink. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. J. Dairy Sci. 96:4310–4322. https://doi.org/10.3168/jds.2012-6265.

Subnel, A. P. J., R. G. M. Meijer, W. M. van Straalen, and S. Tamminga. 1994. Efficiency of milk protein production in the DVE protein evaluation system. Livest. Prod. Sci. 40:215–224. https://doi.org/10.1016/0301-6226(94)90089-2.

Tamminga, S., G. Brandsma, G. van Duinkerken, A. van Vuuren, and M. Blok. 2007. Eiwitwaardering voor herkauwers; Het DVE-OEB 2007 systeem. Wageningen University.

Van den Bossche, T. 2023a. Supplemental Figure S1. Variation of the Met plasma concentration (μmol/L) over 6 time points in the experiment for each treatment group (CTRL, MetLys, MetLysHis). Zenodo. https://doi.org/10.5281/zenodo.7509396.

Van den Bossche, T. 2023b. Supplemental Figure S2. Variation of the Lys plasma concentration (μmol/L) over 6 time points in the experiment for each treatment group (CTRL, MetLys, MetLysHis). Zenodo. https://doi.org/10.5281/zenodo.7509419.

Van den Bossche, T. 2023c. Supplemental Figure S3. Variation of the His plasma concentration (μmol/L) over 6 time points in the experiment for each treatment group (CTRL, MetLys, MetLysHis). Zenodo. https://doi.org/10.5281/zenodo.7509447.

Van den Bossche, T. 2023d. Supplemental Figure S4. Nitrogen losses through feces, urine and milk as a percentage of the total N intake through feed (indicated by the red line), for each treatment group (CTRL, MetLys and MetLysHis) in each period (depletion, RP-AA, cross-back). Zenodo. https://doi.org/10.5281/zenodo.7509480.

Van den Bossche, T. 2023e. Supplemental Table S1. Total AA profile (exclusive Tyr and Trp) (g/kg DM) analysed with HPLC after acid hydrolysis (EC, 1998) for the feed stuffs used in the low protein diet during the experiment. [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7509490.

Van den Bossche, T. 2023f. Supplemental Table S2. Least square means of BW, DMI, milk yield (MY) and fat- and protein-corrected milk yield (FPCMY), milk protein yield (MPY), milk composition (milk fat, milk protein, milk lactose and MUN), milk N efficiency (MNE) and feed efficiency for the CTRL, MetLys and MetLysHis treatment groups in the pre-experimental, depletion and cross-back period. [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7509506.

Van den Bossche, T. 2023g. Supplemental Table S3. Least square means of the digestible (d) AA (Leu, Ile, Arg, Phe, Thr and Val) supply relative to the cow's requirements (%) for the CTRL, MetLys and MetLysHis groups in the depletion, RP-AA and cross-back period of the trial. Calculations based on the actual DMI, MY and BW of the cows during the experiment. [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7509514.

Van Duinkerken, G. 1998. Berekening van het gehalte aan darmverteerbaar methionine en lysine in voedermiddelen voor herkauwers. Unknown Publisher.

Van Es, A. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977-onwards in The Netherlands. Livest. Prod. Sci. 5:331–345. https://doi.org/10.1016/0301-6226(78)90029-5.

Vandaele, L., K. Goossens, J. De Boever, and S. De Campeneere. 2019. Several roads lead to Rome: About improving nitrogen efficiency in cattle. Pages 127–133 in EAAP Scientific Series. Wageningen Academic Publishers.

Zang, Y., L. H. P. Silva, Y. C. Geng, M. Ghelichkhan, N. L. Whitehouse, M. Miura, and A. F. Brito. 2021. Dietary starch level and rumen-protected methionine, lysine, and histidine: Effects on milk yield, nitrogen, and energy utilization in dairy cows fed diets low in metabolizable protein. J. Dairy Sci. 104:9784–9800. https://doi.org/10.3168/jds.2020-20094.

Zang, Y., L. Silva, M. Ghelichkhan, M. Miura, N. Whitehouse, M. Chizzotti, and A. Brito. 2019. Incremental amounts of rumen-protected histidine increase plasma and muscle histidine concentrations and milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 102:4138–4154. https://doi.org/10.3168/jds.2018-15780.

ORCIDS

T. Van den Bossche https://orcid.org/0000-0002-7004-6130
K. Goossens https://orcid.org/0000-0001-9064-0324
B. Ampe https://orcid.org/0000-0002-8223-2870
J. L. De Boever https://orcid.org/0000-0002-7073-630X
L. Vandaele https://orcid.org/0000-0002-4563-6796