Amino acid efficiencies of utilization vary by different mechanisms in response to energy and protein supplies in dairy cows: Study at mammary-gland and whole-body levels

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ABSTRACT

Variations of mammary gland (MG) metabolism were studied in dairy cows in response to diets containing 2 levels of net energy of lactation [NE_L; 25.0 and 32.5 Mcal/d for low (LE) and high energy (HE), respectively], combined with 2 levels of metabolizable protein [MP, 1,266 and 2,254 g/d of protein digestible in the intestine for low (LP) and high protein (HP), respectively] in a 2 × 2 factorial arrangement. Four cows received 4 diets (LELP, HELP, LEHP, and HEHP) in a 4 × 4 Latin square design with 2-wk experimental periods. Milk production and feed intake were measured on the last 5 d of each period, whereas MG net uptake of AA was determined on d 13. Efficiencies were estimated as the sum of measured milk true protein yield (MPY) and of estimations of metabolic fecal and scurf proteins multiplied by their respective AA profile and divided by the estimated AA supply minus the AA endogenous urinary loss. The increased MPY in the HE compared with the LE diets (higher by 123 g/d) was accompanied by increased mammary plasma flow and MG uptake of the nonessential AA (NEAA) and the essential AA (EAA), except for branched-chain AA. In contrast, the increase in MPY (higher by 104 g/d) observed in the HP compared with the LP diets was linked to increased MG uptake of EAA without a change in mammary plasma flow and a decreased NEAA uptake. Because MG uptake of total AA-N was almost equal to cows' milk output on a nitrogen basis, these different mechanisms involve a large MG flexibility, with variable synthesis of NEAA. In addition, MP efficiency did not increase only through increased MPY in the HE compared with the LE diets but also through metabolic fecal protein, estimated to increase (by 65 g/d) with dry matter intake. The MPY increased in

the HP compared with the LP diets, but the increase was smaller than the calculated increase (greater by 993 g/d) in MP supply. The highest MG clearance rates of individual EAA could suggest that Met, His, and Lys were limiting in LP, and Met was the most limiting AA in HP. Interestingly, a similar hypothesis could be stated by analyzing estimated AA efficiencies. The highest efficiencies among EAA, observed for His in HELP and for Met with the other diets, could indicate that they were the most limiting AA in these respective diets, whereas other EAA (including Lys) efficiencies varied with MP efficiency. The MG metabolic flexibility with regard to individual AA utilization partially contributes to the anabolic fate of AA through MPY; however, other export proteins also contribute to variations in MP and AA efficiencies.

Key words: amino acid, efficiency, mammary gland, metabolism

INTRODUCTION

Increasing dietary N efficiency (milk N/N intake) for lactating dairy cows, without a detrimental effect on milk true protein yield (MPY), is an important challenge faced by dairy nutritionists. Indeed, correctly addressing this challenge has both economic and environmental effects. At the animal level, it is widely acknowledged that MPY increases with either NE_L or MP supplies (Broderick, 2003), and MP efficiency (MP_{eff}) decreases with increasing MP (Metcalf et al., 2008) but increases with increasing NE_L (Hanigan et al., 1998; Rius et al., 2010a). Overall, MP_{eff} depends on the partition of individual AA between their uses in the different proteins exported and their catabolism. The AA are the nutrients absorbed in the small intestine after MP hydrolysis and taken up by tissues and organs to synthesize all proteins, including milk proteins. Studying mammary gland (MG) metabolism will help us understand MPY responses through variations in MG uptake of AA used to support MPY or catabolized within the MG. For example, MG uptake of

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AA from group 2 (Ile, Leu, Lys, and Val) increased in response to increased MP supply (Raggio et al., 2004). Moreover, this increase was larger than the increase of group 2 AA in milk output, indicating increased MG use of these AA not related to their direct incorporation into MPY, suggesting intramammary catabolism. The effects of NE_L , or of the interaction between NE_L and MP ($E \times P$), on MG use of AA are less studied than the effects of MP supply alone, despite knowing that utilization of AA is an energy-dependent process. Post-ruminal infusion of glucose or ruminal infusion of propionate increased MPY and MG uptake of group 1 AA (His, Met, Phe, and Tyr) without a change in AA supply (Lemosquet et al., 2010a). Moreover, MG uptake of AA from group 2 did not change with these infusions. In most studies analyzing $E \times P$ effects on MG metabolism, treatments were infusions of energy sources such as starch, glucose (post-ruminally), or propionate (in rumen), or infusions of AA (in duodenum; e.g., Clark et al., 1977; Raggio et al., 2006a; Rius et al., 2010a; Nichols et al., 2016), but dietary changes could affect MG metabolism differently than infusions of a single substrate (Cantalapiedra-Hijar et al., 2015).

However, these changes in MG metabolism cannot totally explain the variations in MP_{eff} , because MP_{eff} depends on both the export of MPY and unproductive protein fractions such as metabolic fecal protein (INRA, 2018; Lapierre et al., 2016). The latter represents the second contributor of protein export after MPY. In addition, extramammary AA metabolism may also be modified, as reported by Raggio et al. (2004), who calculated individual AA efficiencies (AA_{eff}) as the ratio of individual AA in MPY relative to net portal absorption. These AA_{eff} decreased with increasing MP supply and were related to increased hepatic removal of Met, Phe, and Tyr (Raggio et al., 2004). These mechanisms could explain the variable AA_{eff} between AA when increasing AA supply (Doepel et al., 2004). These variations among AA have led to the calculation of individual EAA efficiencies (EAA_{eff}) rather than MP_{eff} (Arriola Apelo et al., 2014), using the pattern of calculation of MP_{eff} as already done in Lee et al. (2015). In addition, some MG metabolism parameters (e.g., uptake to milk output ratio of AA from group 2 or EAA clearance rate) can help to delineate which EAA are extensively catabolized or which have the lowest supplies (Arriola Apelo et al., 2014; Haque et al., 2015).

The present experiment aimed to describe MPY responses to variable NE_L and MP supplies occurring via dietary changes and to relate these responses to MG and extramammary uses of AA. We hypothesized that the increased MPY seen with both NE_L and MP supplies would occur through different mechanisms at

the MG level. We also hypothesized that MP_{eff} and AA_{eff} variations with NE_L and MP supplies could be explained by individual AA metabolism and were partially related to MG metabolism. Last, we hypothesized that different parameters (MG metabolism and individual AA_{eff}) could be used jointly to identify AA potentially in short supply.

MATERIALS AND METHODS

Cows and Surgery

The trial was carried out at the experimental farm of the Institut National de la Recherche Agronomique, Unité Mixte de Recherche, Physiologie, Environnement et Genétique pour l'Animal et les Systèmes d'Élevage (INRA UMR PEGASE 1348; Méjusse, 1.71°W, 48.11°N, Brittany, France) in 2007 in accordance with the National Legislation on Animal Care (certified by the French Ministry of Agriculture, Paris; Agreement Number C35-275-23). At 48 ± 16 DIM, 4 Holstein cows in their second lactation were surgically implanted with a permanent catheter in the left carotid artery according to Raggio et al. (2006b). For this surgery, protocols for analgesia and anesthesia were followed as described in Haque et al. (2015). To maintain catheter patency, twice every week 10 mL of blood were drawn by syringe, and then 5 mL of saline solution followed by 2 mL of heparin (5,000 IU/mL) were injected into the catheter. At the same visit, the exteriorization site of the catheter was cleaned with an alcohol and ether mixture, and iodinated alcohol was dripped onto the site. Betadine gel (Meda Pharma, Hoeilaart, Belgium) was then applied and the catheter protected by a bandage. The day before blood collection, using local anesthesia (injection of 2 mL of Lurocaine; Vetoquinol SA, Lure, France), another catheter (Nutricath S; inner diameter 1.02 mm, outer diameter 1.78 mm; Vygon, Ecouen, France) was temporarily implanted in the left subcutaneous abdominal vein.

Cows began the experiment at 86 ± 12 DIM. From 66 to 72 DIM, the cows averaged 593 ± 19 kg of BW, 23.0 ± 1.2 kg of DMI/d, and 37.2 ± 3.0 kg of milk/d. Milk true protein and fat concentrations were 29.8 ± 1.6 g/kg and 37.0 ± 1.4 g/kg, respectively. These production parameters were used as reference values. During the pre-experimental period and throughout the study, cows were housed in individual tiestalls and had ad libitum access to fresh water.

Experimental Design, Diets, Feeding, and Milking

Diets containing two levels of NE_L supply [low energy (**LE**) and high energy (**HE**)] combined with 2

levels of MP supply [low protein (**LP**) and high protein (**HP**)] were tested according to a 2×2 factorial arrangement. The cows received the 4 diet combinations (**LELP**, **HELP**, **LEHP**, and **HEHP**) in a 4×4 Latin square design, balanced for residual effects, with 2-wk experimental periods. During each period, the supplies of NE_L and MP [in g/d of protein digestible in the intestine (**PDI**) in the INRA system] in the **LELP** diet were adjusted to provide 70% of cows' NE_L and MP requirements (INRA, 1989). To maximize the difference between NE_L and MP levels, the **HEHP** diet was designed to provide 100% of the NE_L requirement and 125% of the MP requirement. Assuming no change in BW, the requirements were calculated for each cow in each period using milk yield and composition of the reference period, applying a lactation persistency of 98% per week, and assuming that milk protein and fat concentrations did not change. The 4 diets included corn silage, an energy concentrate, and formaldehyde-treated (**FT**) soybean meal. The chemical composition of the 3 major ingredients is detailed in Table 1, and complete diet composition is given in Table 2. The difference in the NE_L supply was obtained by changing the amount of feed offered (14.7 ± 0.77 vs. 19.5 ± 0.96 kg of DM/d for **LE** vs. **HE**, respectively) and by altering the proportion of energy concentrate or **FT** soybean meal (Table 2). A higher MP supply was achieved by substituting energy concentrate with **FT** soybean meal. We adjusted the quantities offered and the proportion of each ingredient of each diet to each cow's requirements at each period. The daily amount of corn silage offered averaged 12.5 ± 0.7 kg of DM in **LELP**, **HELP**, and **HEHP**. To maintain the NE_L supply in **LEHP** similar to **LELP**, we had to reduce the amount of both corn silage and energy concentrate (8.7 ± 0.8 kg of DM/d and 0.3 ± 0.2 kg of DM/d offered, respectively) and increase MP supply through additional **FT** soybean meal (4.9 ± 0.5 kg of DM for **LEHP** vs. 1.7 ± 1.2 kg of DM for the 3 other diets). Therefore, the percentage of concentrate (**FT** soybean meal, energy concentrate, and urea) averaged 13.8%, 32.3%, 36.5%, and 31.4% in **LELP**, **HELP**, **LEHP**, and **HEHP**, respectively. Lastly, an adequate rumen balance between soluble N and fermented organic matter (i.e., energy) was maintained because the calculated rumen protein balance was higher than the recommended -14.2 g/kg of DM (INRA, 2018) in the 4 diets (Table 2).

To minimize postprandial variations in plasma metabolite concentrations, feed access was limited to 1 or 2 h every 3 h starting at 0715 h. Silage was offered 3 times per day: 25% at 0715 h, 25% at 1315 h, and 50% of the daily allowance at 1915 h. Other ingredients (energy concentrate, **FT** soybean meal, urea, and mineral and vitamin premix) were offered every 3 h starting at

0715 h. During blood sampling days, corn silage was distributed in 5 portions: 12.5% at each of 4 feeding times (0715 h, 1015 h, 1315 h, and 1615 h) and 50% from 1715 h to 1915 h. For the first 7 d of each period, cows were milked twice daily at 0630 h and 1730 h, whereas for the last 7 d they were milked at 0630 h and 1830 h, with the udder halves of each cow milked separately.

Sampling and Laboratory Analyses

Feed. Every day, feed offered and orts (when present) were weighed. A sample of corn silage and orts were collected daily to determine DM concentration; they were dried at 80°C for 48 h. Dry matter of corn silage was used to adjust the quantity of wet feed offered, to maintain a constant amount of DM offered. Samples of concentrates were collected on a weekly basis to determine DM content. One pooled sample per feed ingredient was analyzed for chemical composition (Laboratoire de Développement et d'Analyses des Côtes d'Armor, Saint-Brieuc, France). Methods used were in accordance with those recommended by INRA (Baumont et al., 2018a). More specifically, the effective degradability coefficients for N (%) were calculated for a passage rate of 6% per hour, using the nylon bag technique (Michalet-Doreau et al., 1987) for the corn silage and energy concentrate. For energy concentrate and **FT** soybean meal, the coefficient was calculated from the enzymatic protein degradation, measured according to the method of Aufrère et al. (1991). Although the experiment was conducted in 2007, the dietary supplies of NE_L and MP were calculated using the most recent INRA equations (Baumont et al., 2018b), to better account for the digestive interactions between NE_L and MP than the previous INRA (1989) and to include the effects of DMI and percentage of concentration on microbial protein and the effect of DMI on RUP. In addition, the AA digestible in the intestine (**AADI**)—that is, the AA supply as % **PDI**—was estimated using the INRA (2018) feeding system; these calculations use a fixed AA microbial profile for the digestible microbial protein and the AA profiles of feed ingredients (Table 2) for the digestible RUP fractions from INRA (2018) feed tables. The **AADI** flow is the **AADI** multiplied by calculated MP supply.

Milk. On the last 5 d of each period, milk production was recorded from the left and right udder at each milking, and samples were collected separately to analyze true protein, fat, and SCC via infrared analysis (ISO 9622: ISO, 1999; Milkoscan, Foss Electric, Hillerød, Denmark). On d 13, at the evening milking, milk samples were collected from left-half udder, and sub-samples were frozen (-20°C) for further analysis.

Milk lactose content was measured according to Hurtaud et al. (1993), and total N was determined via the Kjeldahl method. The NPN and noncasein N contents in milk were also obtained using the Kjeldahl method, by mineralization with sulfuric acid at 95 to 97% and then distillation with boric acid at 50 g/L. Using the N, NPN, and noncasein N concentrations, we calculated true protein, CN, and whey protein proportions in milk protein.

Blood. On d 13 of each period, starting at 0700 h, 6 blood samples from each cow were collected at 2-h intervals simultaneously from the arterial and venous catheters. Blood was immediately put on ice and centrifuged for 10 min at $2,000 \times g$ to yield plasma. To determine AA concentration of each individual sample, 1.0 g of plasma was added to 0.2 g of an internal standard containing each AA to be analyzed, labeled with stable isotopes as described in Doepel and Lapierre (2010). The processed samples were stored at -80°C until analyzed for plasma AA concentrations by isotope

dilution using gas chromatography/mass spectrometry (model GC 6890-MS 5973, Agilent Technologies, Wilmington, DE), as previously described (Calder et al., 1999). In addition, 1-methyl-His, Arg, Cit, homocysteine, phosphoserine, Orn, and Tau were analyzed using an AA analyzer (Biotronic LCC 5000, Biotronic GmbH, Maintal, Germany). Plasma urea was assayed using a colorimetric enzymatic kit (urease and glutamate dehydrogenase, Urée UV Cinétique; Kone Diagnostics, Evry, France) in a multiparameter analyzer (Kone Instruments Corp., Espoo, Finland).

Calculations and Statistical Analyses

Calculations. Mammary plasma flow was determined using the Fick principle, with Phe + Tyr as internal markers (Cant et al., 1993). The Fick principle assumes that all MG uptake of Phe and Tyr is from free AA and that there is no other fate for their MG uptake but milk protein secretion. This assumption has been

Table 1. Chemical composition (g/kg of DM unless otherwise noted) of major feed ingredients

Item	Corn silage	Energy concentrate ¹	FT-SBM ²
DM, %	45	87	86
OM	968	962	917
CP	67	140	501
ADF	194	43	66
NDF	381	72	140
ADL	20	4	4
Crude cellulose	163	36	55
Starch	412	615	49
Ether extract	30	35	33
Fermentation products	61		
ED6_N, ³ %	72	92	34 ⁴
AA composition, ⁵ % of 16 AA excluding Cys and Trp (INRA, 2018)			
EAA			
Arg	2.1	9.1	7.7
His	2.9	2.7	2.8
Ile	4.4	4.5	4.8
Leu	13.6	7.6	8.1
Lys	4.5	7.8	6.6
Met	1.8	1.1	1.5
Phe	5.1	5.0	5.3
Thr	4.5	4.1	4.0
Val	6.3	5.1	5.1
NEAA			
Ala	9.0	4.8	4.6
Asx ⁶	8.6	12.9	12.0
Glx ⁶	14.7	17.6	18.9
Gly	4.8	4.8	4.4
Pro	8.1	4.5	5.3
Ser	5.5	5.0	5.0
Tyr	4.0	3.4	3.7

¹Concentrate contained, on DM basis, 54.2% peas, 38.2% cornstarch, 4.4% sugar molasses, 2.0% soy oil, and 1.2% NaCl.

²Formaldehyde-treated soybean meal contained 94.7% soybean meal, 4.2% sugar molasses, and 1.1% formol.

³Effective degradability of N in the rumen, calculated for a passage rate of 6%/h.

⁴Calculated using the enzymatic protein degradation (equal to 7.1%) according to Aufrère et al. (1991).

⁵Estimated using feed tables ($n \geq 48$ for each raw material) and equations 24.6 and 24.5 for concentrate (INRA, 2018).

⁶Called Asp and Glu in INRA (2018) feed tables.

Table 2. Composition and predicted nutritive values of experimental diets, using INRA (2018)

Item	Diet ¹			
	LELP	HELP	LEHP	HEHP
Corn silage, % of DM	83.2 ± 1.5	65.4 ± 4.0	60.4 ± 1.1	66.3 ± 3.8
Energy concentrate, ² % of DM	3.0 ± 2.2	30.5 ± 3.7	2.3 ± 1.1	11.9 ± 4.1
FT-soybean meal, ³ % of DM	9.7 ± 0.9	0.8 ± 0.7	33.9 ± 1.5	18.7 ± 1.8
Minerals and vitamins, ⁴ % of DM	1.9 ± 0.2	1.5 ± 0.0	2.0 ± 0.2	1.5 ± 0.2
Urea, ⁵ % of DM	1.1 ± 0.1	1.0 ± 0.1	0.3 ± 0.1	0.8 ± 0.3
Mineral feed, ⁶ % of DM	1.1 ± 0.1	0.8 ± 0.0	1.1 ± 0.1	0.8 ± 0.1
Quantity offered, kg of DM/d	14.8 ± 0.7	19.2 ± 0.3	14.5 ± 0.9	19.8 ± 1.4
CP, % of DM	13.9	11.8	22.2	17.6
MP, g/kg DM of PDI ⁷	87.6	66.0	154.5	116.7
PDIA, ⁸ g/kg of DM	39.1	16.4	106.2	68.9
RPB, ⁹ g/kg of DM	−0.3	2.1	14.1	6.9
NE _L , Mcal/kg of DM	1.7	1.7	1.8	1.7
AADI, ¹⁰ % PDI				
His	2.2	2.1	2.5	2.4
Leu	8.8	8.8	8.6	8.7
Lys	7.0	7.4	6.8	6.9
Met	1.9	2.0	1.6	1.7

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein. Mean ± SD.

²Concentrate contained, on DM basis, 54.2% peas, 38.2% cornstarch, 4.4% sugar molasses, 2.0% soy oil, and 1.2% NaCl.

³Formaldehyde-treated soybean meal contained 94.7% soybean meal, 4.2% sugar molasses, and 1.1% formol.

⁴Calcium (carbonate): 200 g/kg; phosphorus: 90 g/kg; magnesium: 40 g/kg; sodium: 20 g/kg; zinc (oxide and sulfate): 6,000 mg/kg; manganese (oxide and sulfate): 4,000 mg/kg; copper (oxide): 1,400 mg/kg; iodine (potassium iodide): 100 mg/kg; cobalt (carbonate): 50 mg/kg; selenium: 24 mg/kg; vitamin A: 500,000 IU/kg; vitamin D₃: 100,000 IU/kg; vitamin E: 2,200 mg/kg; vitamin B₁: 50 mg/kg (Turbomine Emeraude, Néolait, Yffiniac, France).

⁵Urea values were taken from INRA (2018) feed tables as CP = 2,870 g/kg of DM and all AA = 0% of total of 16 AA, excluding Cys and Trp.

⁶CaCO₃ contained calcium (38%) and magnesium (1.5%; Néolait).

⁷PDI = true protein digested in the small intestine supplied by RUP and rumen microbial protein (INRA, 2018), equivalent to MP.

⁸PDIA = true digestible RUP (INRA, 2018).

⁹RPB = rumen protein balance, difference between CP intake and sum of RUP + microbial CP + endogenous CP (INRA, 2018).

¹⁰AADI = AA digestible in the small intestine, estimated based on INRA (2018) feed tables and calculations.

previously verified, because no Phe or Tyr oxidation was detected when Phe supply was doubled through infusion of labeled Phe (Lemosquet et al., 2010b). The amount of Phe and Tyr exported in milk protein was calculated from the left udder MPY from the evening milking of d 13; the contribution of blood-borne protein (3.23%, adapted from Lapierre et al., 2016; sum of albumin 1.04%, IgG1 1.64%, IgGA 0.04%, IgG2 0.18%, and IgM 0.33%) was excluded so that only proteins synthesized within the MG from free plasma AA were considered. The AA composition of milk protein was estimated for each cow for each period from the proportion of measured CN and whey proteins relative to total true protein multiplied by the AA composition of each family of protein reported by Farrell et al. (2004), excluding the contribution of blood-borne protein. The CN distribution (% of CN) was 42.4% α_{s1} -casein, 9.3% α_{s2} -casein, 36.7% β -casein, and 11.6% κ -casein, and the whey protein distribution (% of whey protein)

was 72.8% α -lactalbumin and 27.2% β -lactoglobulin. Although total CN and whey protein proportions, expressed in percent of true protein, changed for each cow for each period as measured, lactoferrin and blood-borne protein concentrations were assumed to remain constant (percent of true protein: lactoferrin 0.21%; and blood-borne protein 3.23%, as detailed previously). The MG uptake of each AA was calculated by multiplying the AA arteriovenous (AV) plasma concentration difference by the mammary plasma flow and is reported in mmol of N/h. The mammary clearance rate (L/h) was calculated as MG uptake divided by venous concentration of each AA (Hanigan et al., 1998).

The estimated MP_{eff} was calculated as proposed in INRA (2018), as the sum of export proteins (i.e., measured MPY and estimated metabolic fecal and scurf proteins) divided by calculated MP supply minus estimated endogenous urinary losses. Because endogenous urinary losses are not protein per se but end products

Table 3. Amino acid composition of proteins used in the calculations¹

Item	Scurf	Endogenous urinary urea	Metabolic fecal protein	Milk, excluding BBP ^{2,3}	Milk, including BBP ^{2,4}
Arg	93.2	79.7	57.2	36.2 ± 0.7	37.0 ± 0.5
His	16.9	29.4	31.4	28.8 ± 0.3	28.8 ± 0.3
Ile	27.4	36.6	53.4	63.6 ± 0.3	62.8 ± 0.3
Leu	69.4	82.9	91.8	105.9 ± 1.6	106.2 ± 1.6
Lys	55.9	78.3	75.3	89.3 ± 1.2	89.1 ± 1.2
Met	13.7	23.3	17.0	30.3 ± 0.2	29.9 ± 0.2
Phe	36.6	44.7	53.4	51.6 ± 0.6	51.9 ± 0.6
Thr	40.6	49.0	74.3	45.7 ± 0.3	46.4 ± 0.3
Trp	7.7	11.0	18.7	16.6 ± 0.6	17.1 ± 0.6
Val	46.7	51.6	70.0	69.3 ± 0.4	69.2 ± 0.4
Ala	92.2	85.4	63.3	36.1 ± 0.7	36.6 ± 0.7
Asn				45.0 ± 0.5	44.5 ± 0.5
Asp				38.5 ± 1.7	39.1 ± 1.6
Asx	84.2	96.4	75.5		
Cys	28.8	18.6	35.3	9.5 ± 1.4	10.6 ± 1.3
Gln				96.3 ± 1.3	94.7 ± 1.3
Glu				129.9 ± 0.7	128.6 ± 0.7
Glx	147.4	158.2	156.9		
Gly	209.7	143.9	83.9	19.1 ± 0.1	19.8 ± 0.1
Pro	123.6	98.2	84.2	102.1 ± 2.6	100.9 ± 2.5
Ser	65.0	57.8	77.6	66.2 ± 1.0	66.4 ± 0.9
Tyr	26.5	31.2	47.0	58.4 ± 0.7	58.2 ± 0.6

¹Based on Lapierre et al. (2016), data expressed in g of anhydrous AA per kg of true protein.

²Average of 16 data points (cow × period).

³BBP = blood-borne proteins; for calculation of the mammary plasma flow and AA in milk protein output used for the calculation of mammary uptake:output ratio.

⁴For calculation of efficiency.

of AA metabolism, the MP_eff and AA_eff for this exportation are assumed to be 1 (INRA, 2018); they are not included in the protein output and are excluded from the protein supply. Although proposed by INRA (2018), we did not consider the potential variation of body reserves (protein mobilization or deposition) according to energy balance, because of the dichotomy created between NE_L and MP supplies related to the nature of the experimental diets. The estimation of individual AA_eff was calculated similarly, using the estimated AADI supply and the different protein outputs with their respective AA concentrations (Table 3). In terms of total N, endogenous urinary losses estimations are very similar between INRA (2018), at 50 mg of N/kg of BW, and Lapierre et al. (2016) at 53 mg of N/kg of BW. However, the latter defines an AA composition of endogenous urinary excretion based on the different urinary N fractions of this exportation and their AA precursors. We used the AA composition of the empty body to determine the endogenous urea excretion (Table 3). We considered His as the precursor for 3-methyl-His; Arg and Gly for creatinine and creatine; Asp, Glu, and Gly for purine derivatives; and Gly for hippuric acid. The AA composition of milk was estimated as described above for the calculation of MG metabolism but included the blood-borne protein (Table 3). Finally, the estimated catabolism, which ex-

presses the inefficiency in absolute numbers rather than as ratios of efficiencies, is calculated as supply minus all exportations (endogenous urinary losses, MPY, and metabolic fecal and scurf proteins).

Statistical Analyses. Intake and milk yields and composition from the last 5 d of each period were averaged and used for statistical analyses, whereas the daily means of the 6 blood samples collected on d 13 were used for mammary metabolism and associated calculations. The data were analyzed using the MIXED procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC), according to the following statistical model:

$$Y_{ijkl} = \mu + \text{cow}_i + \text{period}_j + \text{energy}_k + \text{protein}_l + (\text{energy} \times \text{protein})_{kl} + \varepsilon_{ijkl},$$

where μ is the mean; Y_{ijkl} is the dependent variable for the fixed effects of period_j (4 periods), energy_k (energy supply, LE or HE), protein_l (protein supply, LP or HP), and their interaction; and ε_{ijkl} is the residual error associated with ijkl observations. Cow was treated as a random effect. As the Latin square was balanced for residual effects, these effects were tested and found not significant ($P > 0.20$) for any of the 4 treatments, which means that there was no carryover effect. The results are presented as least squares means, with the highest

standard error of the means reported. Homogeneity of residues was verified. The significance level was set to $P \leq 0.05$ and the tendency to $0.05 < P \leq 0.10$. If the $E \times P$ interaction was significant, the SLICE procedure was used to test the effect of one factor within the other factor.

RESULTS

DMI and Supplies

Dry matter intakes (Table 4) were close to the quantities offered (Table 2) because refusals were very small (in kg of DM/d; LELP: 0.07; HELP: 0.36; LEHP: 0.09; HEHP: 0.27; not different from 0, $P > 0.05$, SEM = 0.26). As planned, DMI was greater for the HE diets than for the LE diets ($P < 0.01$, Table 4). The calculated NE_L supply was 30% greater with the HE diets than with the LE diets, whereas calculated MP supply was 78% greater in HP than in LP diets. According to the NRC system (NRC, 2001), NE_L supply increased by

26% (in Mcal/d; LELP: 23.4; HELP: 29.9; LEHP: 25.4; HEHP: 31.5) and MP supply by 81% (in g/d; LELP: 1,514; HELP: 1,396; LEHP: 2,651; HEHP: 2,632). With increasing NE_L supply (HE vs. LE diets), the estimated flow of digestible His decreased ($P = 0.03$), the estimated flow of digestible Arg tended to decrease ($P = 0.09$), and the estimated flow of digestible Thr and Met increased ($P \leq 0.04$), mainly in HP diets for Met ($E \times P$ interaction: $P = 0.08$; Table 4). With increasing MP supply (HP vs. LP diets), the digestible flows of all AA increased ($P < 0.01$) in variable proportions; Met flow increased by 52%, whereas flows of Arg and His were doubled.

Milk Production and Composition

During the last 5 d of each period, increasing the NE_L supply increased milk yield, MPY, and milk protein content by 13, 19, and 6%, respectively ($P \leq 0.03$, Table 5). Increasing the MP supply increased milk yield and MPY by 17 and 15%, respectively ($P < 0.01$), but did

Table 4. Dry matter intake, calculated protein and energy supplies, and estimated AA digestible flow in the intestine from the experimental diets

Item	Diet ¹				SEM	P-value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
DMI, kg/d	14.7	18.8	14.4	19.5	0.5	<0.01	0.22	0.02
N intake, ³ g/d	327	356	512	548	16	<0.01	<0.01	0.42
MP supply, g/d of PDI ⁴	1,289	1,243	2,232	2,276	78	0.98	<0.01	0.17
NE _L supply, Mcal/d ⁵	24.7	31.9	25.3	33.1	0.9	<0.01	<0.01	0.27
MP/NE _L , g of PDI/Mcal	52.2	39.0	88.2	68.8	0.9	<0.01	<0.01	<0.01
AADI ⁶ flow, g/d								
EAA								
Arg	64	59	122	120	4	0.09	<0.01	0.37
His	29	26	55	54	2	0.03	<0.01	0.17
Ile	65	65	108	112	4	0.15	<0.01	0.18
Leu	114	109	191	197	7	0.77	<0.01	0.08
Lys	91	92	153	158	5	0.16	<0.01	0.32
Met	24	25	36	39	1	<0.01	<0.01	0.08
Phe	66	63	115	117	4	0.87	<0.01	0.17
Thr	63	65	102	108	4	0.04	<0.01	0.16
Val	71	71	117	122	4	0.17	<0.01	0.14
NEAA								
Ala	85	85	137	144	8	0.98	<0.01	0.24
Asx ⁷	137	133	240	244	5	0.07	<0.01	0.12
Glx ⁷	189	171	355	351	13	0.06	<0.01	0.21
Gly	81	82	133	139	5	0.08	<0.01	0.18
Pro	59	53	105	105	4	0.10	<0.01	0.07
Ser	61	59	107	109	4	0.74	<0.01	0.16
Tyr	53	51	90	92	3	0.63	<0.01	0.17

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³N intake = CP intake/6.25.

⁴PDI = protein digested in the small intestine, supplied by RUP and rumen microbial protein (calculated by INRA, 2018), equivalent to MP.

⁵Calculated in INRA (2018).

⁶AADI = AA digestible in the small intestine (% PDI, estimated by INRA, 2018, equations and feed tables) × MP.

⁷Called Asp and Glu in INRA (2018) feed tables.

Table 5. Milk yield and milk composition from samples collected from the whole udder on the last 5 d and from left-half udder during the evening milking of d 13

Item	Diet ¹				SEM	<i>P</i> -value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Mean of the last 5 d								
Milk yield, kg/d	24.2	26.5	27.6	31.8	1.3	<0.01	<0.01	0.11
Milk protein yield, ³ g/d	654	757	736	891	43	<0.01	<0.01	0.16
Milk protein content, ³ g/kg	26.9	28.9	26.9	28.1	1.6	0.03	0.46	0.50
Fat content, g/kg	38.6	41.5	37.1	40.3	1.6	0.06	0.36	0.91
Left-udder milking on d 13 evening								
Milk yield, kg/12 h	5.63	6.20	6.50	7.45	0.33	<0.01	<0.01	0.34
Milk protein yield, ³ g/12 h	148	172	169	206	9	<0.01	<0.01	0.31
Milk protein content, ³ g/kg	26.3	27.9	26.4	27.7	1.5	0.03	0.91	0.69
Casein, % of TP	80.3	78.8	79.0	80.5	1.7	0.99	0.72	0.03
CP, ⁴ g/kg	27.6	28.8	28.6	29.6	1.5	0.09	0.17	0.83
NPN, % CP	6.70	4.74	8.74	7.18	0.54	<0.01	<0.01	0.58
Lactose content, g/kg	46.9	47.7	46.1	48.0	1.4	0.05	0.70	0.30

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²*P*-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³Expressed in true protein (TP).

⁴CP = total milk N × 6.39.

not affect milk protein content ($P = 0.46$). We observed similar increases in milk yield and milk composition in response to HE compared with LE diets or to HP compared with LP diets from the left udder evening milking on d 13 (used for MG uptake measurement). The proportion of NPN relative to CP decreased by 23% ($P < 0.01$) in HE compared with LE diets and increased by 39% in HP compared with LP diets ($P < 0.01$, Table 5). We observed an E × P interaction ($P = 0.03$) for the proportion of CN in true protein; increasing the NE_L supply tended to decrease CN with the LP diets ($P = 0.08$ from the slice test) but tended to increase CN with the HP diets ($P = 0.08$).

Amino Acid Mammary Metabolism

Plasma Arterial Concentrations. The arterial concentration of urea decreased in HE compared with LE diets ($P < 0.01$, Table 6) and increased in HP compared with LP diets ($P < 0.01$). Plasma arterial concentrations of total AA on nitrogen basis (**TAA-N**), EAA-N, and NEAA-N were not affected by the NE_L supply ($P > 0.10$, Table 6). Increasing the NE_L supply decreased (E × P interaction: $P = 0.04$) the arterial concentration of group 1 AA-N [sum of Met, Phe, Trp, and Tyr (Mephram, 1982) plus His] only in LP diets. For individual EAA, increasing the NE_L supply decreased ($P < 0.02$) concentrations of Arg and Phe and those of His (E × P interaction: $P = 0.03$) but only in LP diets. In contrast, concentrations of Thr and Ser increased (E × P interaction: $P \leq 0.04$) only in LP diets. Among NEAA, the HE diets increased Gly ($P <$

0.01), decreased Cit ($P = 0.03$), and tended ($P = 0.09$) to decrease Cys concentrations compared with those in the LE diets.

Increasing the MP supply increased ($P < 0.01$) arterial plasma concentration of TAA-N, EAA-N, group 2 AA-N, and branched-chain AA-N (**BCAA-N**). Group 1 AA-N also increased with HP diets, but the effect was greater with HE rather than with LE diets (E × P interaction: $P = 0.04$). In contrast, we found no MP effect ($P = 0.18$) on NEAA-N concentrations. Increasing the MP supply increased the arterial concentration of all EAA ($P < 0.01$) except Met ($P = 0.67$). Arterial concentration of Thr increased (E × P interaction: $P < 0.01$) in HP compared with LP diets in LE but not in HE diets. The increase in His arterial concentration with HP compared with LP diets was less pronounced in LE than in HE diets (E × P interaction: $P = 0.03$). Arterial concentrations were higher for Cit, Orn, Pro, and Tyr in HP diets than in LP diets ($P < 0.01$), lower for Gly ($P < 0.01$), and tended to be lower for Asp and Gln ($P \leq 0.07$).

Arteriovenous Concentration Differences. The AV difference of TAA-N and EAA-N decreased ($P \leq 0.02$, Table 7) and that of group 1 AA-N tended to decrease ($P = 0.06$) in HE compared with LE diets. Individually, increasing the NE_L supply decreased AV differences of Arg, Pro, and Ser ($P \leq 0.01$) and tended to decrease the AV difference of Ile ($P = 0.08$).

Increasing the MP supply increased ($P \leq 0.05$) the AV differences of TAA-N, EAA-N, group 2 AA-N, and BCAA-N, and tended to increase those of group 1 AA-N ($P = 0.10$). Increasing the MP supply increased

AV differences of Arg, Ile, Leu, and Phe ($P \leq 0.05$) and tended to increase that of Val ($P = 0.10$). Increasing the MP supply decreased the AV difference of Ala ($P < 0.01$) and increased the AV difference of Orn ($P = 0.04$).

Mammary Net Uptake of AA. Mammary plasma flow was greater ($P < 0.01$) in the HE diets than in the LE diets but was not affected by MP supply (Table 7).

Increasing the NE_L supply increased uptake of TAA-N, EAA-N, and group 1 AA-N ($P \leq 0.02$) and tended to increase uptake of group 2 AA-N and NEAA-N ($P \leq 0.08$; Table 8). With HE compared with LE diets, the uptake of Lys, Met, Phe, and Thr increased ($P \leq 0.04$), and the uptake of His tended to increase but only in the HEHP diet compared with the 3 other diets ($E \times P$ interaction: $P = 0.09$). Among the NEAA, the uptake

Table 6. Effects of NE_L and MP supplies on plasma arterial concentration of urea N and AA

Item	Diet ¹				SEM	P-value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Urea N, mM	9.5	3.4	17.8	13.3	0.8	<0.01	<0.01	0.32
Groups of AA, μM								
TAA-N ³	2,754	2,751	3,449	3,335	191	0.52	<0.01	0.53
EAA-N ⁴	1,214	1,145	1,979	1,815	153	0.11	<0.01	0.47
Group 1 AA-N ⁵	274	231	400	397	15	0.02	<0.01	0.04
Group 2 AA-N ⁶	573	592	1,112	1,007	162	0.59	<0.01	0.44
BCAA-N ⁷	419	437	912	805	144	0.59	<0.01	0.44
NEAA-N ⁸	1,540	1,607	1,470	1,520	124	0.30	0.18	0.88
Individual AA, μM								
Individual EAA								
Arg	84	67	106	92	5	0.02	<0.01	0.79
His	35.2	20.5	59.9	59.1	3.7	0.02	<0.01	0.03
Ile	145	158	252	237	46	0.97	<0.01	0.6
Leu	113	104	272	233	42	0.31	<0.01	0.51
Lys	77.3	77.4	100	101	12	0.88	<0.01	0.89
Met	16.4	17.5	16.9	17.6	0.9	0.23	0.67	0.75
Phe	44.6	41.4	60.0	53.5	3.1	0.04	<0.01	0.41
Thr	65	96	90	92	8	<0.01	<0.01	<0.01
Trp	37.2	34.9	48.3	50.2	3.4	0.94	<0.01	0.46
Val	159	175	388	335	57	0.58	<0.01	0.33
Individual NEAA								
Ala	167	177	157	171	20	0.29	0.44	0.85
Asn	76	58	60	70	14	0.67	0.80	0.16
Asp	28.5	32.4	24.2	23.1	3.4	0.67	0.07	0.46
Cit	1.69	1.26	2.44	2.09	0.15	0.03	<0.01	0.78
Cys	93	78	93	86	6	0.09	0.49	0.57
Gln	305	305	279	274	25	0.84	0.06	0.86
Glu	43.8	49.4	43.3	41.0	2.5	0.52	0.11	0.15
Gly	287	358	249	295	32	<0.01	<0.01	0.26
Orn	0.55	0.48	0.78	0.74	0.06	0.36	<0.01	0.82
Pro	69	66	105	97	8	0.32	<0.01	0.62
Ser	68	89	82	80	13	0.07	0.57	0.04
Tau	0.49	0.52	0.52	0.51	0.05	0.82	0.77	0.71
Tyr	32.7	40.7	46.9	48.1	3.5	0.13	<0.01	0.25
Other AA, peptides, and AA metabolites, ⁹ μM								
Homocysteine	0.210	0.143	0.133	0.175	0.040	0.66	0.45	0.10
Phosphoserine	0.34	0.28	0.23	0.30	0.07	0.92	0.48	0.33

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³TAA-N = total AA on nitrogen basis.

⁴EAA-N = sum of N from Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁵Group 1 AA-N = sum of N from His, Met, Phe, Trp, and Tyr.

⁶Group 2 AA-N = sum of N from Ile, Leu, Lys, and Val.

⁷BCAA-N = branched-chain AA on nitrogen basis; sum of N from Ile, Leu, and Val.

⁸NEAA-N = sum of N from Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tau, and Tyr.

⁹Other nitrogen derivatives measured with a plasma concentration higher than the limit of quantification.

Table 7. Effects of NE_L and MP supplies on mammary plasma flow and arteriovenous (AV) differences of AA

Item	Diet ¹				SEM	P-value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Mammary plasma flow, L/h	215	300	233	292	23	<0.01	0.78	0.44
AV differences between groups of AA, μmol/L								
TAA-N ³	611	515	694	594	57	0.01	0.03	0.94
EAA-N ⁴	401	335	506	431	33	0.02	<0.01	0.86
Group 1 AA-N ⁵	92	75	95	91	6	0.06	0.10	0.24
Group 2 AA-N ⁶	210	189	285	232	26	0.12	0.03	0.46
BCAA-N ⁷	117	101	183	134	23	0.15	0.05	0.43
NEAA-N ⁸	210	180	187	163	30	0.12	0.23	0.88
AV differences between individual AA, μmol of N/L								
Individual EAA								
Arg	23.2	16.4	29.8	25.9	1.6	<0.01	<0.01	0.19
His	11.7	8.5	10.7	11.0	0.9	0.17	0.43	0.10
Ile	34	29	52	36	6	0.08	0.05	0.29
Leu	48	41	71	56	7	0.12	0.02	0.53
Lys	46.4	44.1	50.9	49.0	3.1	0.48	0.14	0.95
Met	10.5	9.1	10.7	10.9	0.7	0.39	0.19	0.26
Phe	18.1	15.6	19.8	19.0	1.2	0.11	0.02	0.35
Thr	22.9	19.7	24.2	22.9	1.7	0.18	0.18	0.55
Trp	5.7	5.2	7.4	5.1	1.1	0.11	0.34	0.29
Val	36	31	61	42	10	0.26	0.10	0.48
Individual NEAA								
Ala	29.5	29.2	15.9	12.5	4.6	0.61	<0.01	0.67
Asn	19	9	5	9	10	0.71	0.40	0.40
Asp	-2.4	3.5	3.6	1.8	1.5	0.22	0.20	0.04
Cit	0.01	0.03	0.12	0.01	0.05	0.45	0.38	0.24
Cys	2.6	2.9	7.1	2.4	1.8	0.20	0.25	0.16
Gln	42	41	43	41	5	0.67	0.91	0.82
Glu	25.5	29.7	25.9	24.7	1.6	0.41	0.21	0.15
Gly	3.4	0.5	9.0	0.9	4.6	0.12	0.36	0.41
Orn	0.17	0.14	0.30	0.25	0.05	0.40	0.04	0.85
Pro	12.4	8.3	13.2	9.3	1.2	0.01	0.43	0.93
Ser	16.8	4.9	14.6	10.6	3.4	0.01	0.47	0.13
Tau	-0.066	-0.004	0.008	-0.013	0.046	0.67	0.51	0.40
Tyr	17.3	14.8	18.1	17.3	1.1	0.14	0.15	0.39

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³TAA-N = total AA on nitrogen basis.

⁴EAA-N = sum of N from Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁵Group 1 AA-N = sum of N from His, Met, Phe, Trp, and Tyr.

⁶Group 2 AA-N = sum of N from Ile, Leu, Lys, and Val.

⁷BCAA-N = branched-chain AA on nitrogen basis; sum of N from Ile, Leu, and Val.

⁸NEAA-N = sum of N from Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tau, and Tyr.

of Gln and Tyr increased ($P < 0.05$) with the HE diets compared with the LE diets, whereas the uptake of Glu increased to a greater extent in LP than in HP diets ($E \times P$ interaction: $P = 0.05$) with HE compared with LE diets.

With increasing MP supply, uptake of all groups of AA except NEAA-N increased ($P \leq 0.03$). Mammary uptake of most EAA (Arg, Ile, Leu, Lys, Phe, Thr, and Val) was lower ($P \leq 0.03$) with the LP diets than with the HP diets. Increasing the MP supply did not affect uptake of Trp ($P = 0.22$) and tended to increase

uptake of Met ($P = 0.10$). Uptake of His tended to differ among MP diets ($E \times P$ interaction: $P = 0.09$), with an increase only in HE diets. Uptake of NEAA decreased for Ala ($P < 0.01$), increased for Tyr ($P = 0.02$), and tended to increase for Orn ($P = 0.07$) in HP compared with LP diets.

Mammary Clearance Rate of AA. The mammary clearance rates of TAA-N increased ($P = 0.03$; Table 9) and group 1 AA-N tended to increase ($P = 0.09$) in HE compared with LE diets. The clearance rates of Phe increased ($P \leq 0.02$), whereas those of Thr

Table 8. Effects of NE_L and MP supplies on left-udder uptake of AA¹

AA	Diet ²				SEM	P-value ³		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Uptake of groups of AA, mmol/h								
TAA-N ⁴	133.1	152.2	161.5	177.0	8.4	0.01	<0.01	0.78
EAA-N ⁵	83.5	93.9	110.8	122.4	3.7	0.02	<0.01	0.86
Group 1 AA-N ⁶	17.4	18.8	18.6	23.3	1.3	0.02	0.03	0.14
Group 2 AA-N ⁷	45.0	54.7	63.5	67.4	3.1	0.07	<0.01	0.39
BCAA-N ⁸	25.1	29.0	40.2	38.8	3.1	0.70	0.01	0.43
NEAA-N ⁹	49.5	58.3	49.7	54.5	6.5	0.08	0.60	0.55
Uptake of individual AA, mmol of N/h								
Individual EAA								
Arg	19.9	19.0	27.3	30.1	1.8	0.62	<0.01	0.36
His	7.6	7.4	7.5	9.6	0.8	0.13	0.11	0.09
Ile	7.2	8.4	11.2	10.4	0.8	0.82	0.01	0.28
Leu	10.3	11.7	15.8	16.2	1.0	0.37	<0.01	0.64
Lys	20.0	25.7	23.3	28.5	1.4	<0.01	0.03	0.85
Met	2.27	2.61	2.47	3.17	0.21	0.04	0.10	0.39
Phe	3.88	4.50	4.51	5.53	0.22	<0.01	<0.01	0.22
Thr	4.93	5.69	5.51	6.64	0.32	<0.01	0.01	0.37
Trp	2.45	2.97	3.29	2.88	0.42	0.83	0.22	0.14
Val	7.6	8.9	13.2	12.2	1.6	0.94	0.03	0.49
Individual NEAA								
Ala	6.6	8.8	3.7	3.6	1.4	0.31	0.01	0.28
Asn	7	5	2	5	5	0.91	0.53	0.56
Asp	-0.6	1.1	1.0	0.5	0.4	0.26	0.30	0.05
Cit	0.00	0.05	0.15	0.03	0.07	0.55	0.35	0.26
Cys	0.51	0.84	1.48	0.68	0.34	0.48	0.23	0.11
Gln	17.8	23.0	19.5	23.8	2.0	0.05	0.55	0.82
Glu	5.5	8.9	5.9	7.2	0.5	<0.01	0.17	0.05
Gly	0.6	-0.1	1.7	0.2	0.9	0.13	0.31	0.53
Orn	0.078	0.081	0.152	0.155	0.033	0.94	0.07	1.00
Pro	2.67	2.41	2.95	2.72	0.22	0.21	0.14	0.96
Ser	3.6	1.1	3.5	3.0	0.9	0.12	0.30	0.25
Tau	-0.013	-0.005	0.002	-0.003	0.012	0.91	0.50	0.62
Tyr	3.74	4.27	4.14	5.03	0.25	0.01	0.02	0.37

¹Mammary net uptake of homocysteine and phosphoserine, shown in Table 6, were not significantly different from zero, so were not reported in this table.

²LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

³P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

⁴TAA-N = total AA on nitrogen basis.

⁵EAA-N = sum of N from Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁶Group 1 AA-N = sum of N from His, Met, Phe, Trp, and Tyr.

⁷Group 2 AA-N = sum of N from Ile, Leu, Lys, and Val.

⁸BCAA-N = branched-chain AA on nitrogen basis; sum of N from Ile, Leu, and Val.

⁹NEAA-N = sum of N from Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tau, and Tyr.

tended to decrease ($P \leq 0.09$) with increasing the NE_L supply. The HP compared with LP diets decreased ($P < 0.05$) the mammary clearance rates of EAA-N, group 1 AA-N and group 2 AA-N, and TAA-N ($P < 0.10$), and had no effect on the mammary clearance rate of NEAA-N. The clearance rates of His, Leu, Lys, Trp, and Val decreased ($P \leq 0.05$), whereas the clearance rate of Phe only tended to decrease ($P = 0.10$) in HP compared with LP diets.

AA Exported in Milk Protein and Ratio of Mammary Uptake to Milk Protein Output. The quantity of N exported in milk output as TAA-N (data

not shown; refer to Materials and Methods section for details of calculation) varied similarly to that of MPY ($P < 0.01$) in response to NE_L or MP supplies, because the CN content in the milk true protein varied minimally with treatments (Table 5). The TAA-N ratio of mammary uptake to milk protein output (**U:O ratio**) was not different from 1 in all 4 diets ($P > 0.26$; Table 9). The U:O ratio for group 1 AA-N was also not different from 1 for all diets ($P > 0.20$), but the U:O ratios for EAA-N, group 2 AA-N, and BCAA-N were higher than 1 ($P \leq 0.03$), whereas the ratio for NEAA-N was lower than 1 ($P \leq 0.01$).

Table 9. Effects of NE_L and MP supplies on clearance rates of groups of AA and uptake:output ratio (U:O) of AA

AA	Diet ¹				SEM	P-value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Clearance rate of groups of AA, L/h								
TAA-N ³	61	67	56	63	3	0.03	0.10	0.97
EAA-N ⁴	111	125	80	95	13	0.14	0.01	0.96
Group 1 AA-N ⁵	116	145	72	86	12	0.09	<0.01	0.51
Group 2 AA-N ⁶	158	155	90	105	30	0.73	0.01	0.64
BCAA-N ⁷	106	100	64	72	21	0.95	0.03	0.61
NEAA-N ⁸	32	36	33	34	3	0.22	0.74	0.46
Clearance rate of individual EAA, L/h								
Arg	82	96	91	118	11	0.12	0.20	0.57
His	183	309	50	68	56	0.12	<0.01	0.23
Ile	88	79	70	72	23	0.81	0.36	0.69
Leu	207	206	98	117	39	0.71	<0.01	0.66
Lys	425	451	265	312	76	0.35	0.01	0.78
Met	384	318	407	499	55	0.82	0.11	0.21
Phe	148	182	112	164	14	0.02	0.10	0.57
Thr	124	82	87	98	12	0.09	0.22	0.01
Trp	40	50	40	33	5	0.61	0.03	0.03
Val	73	71	43	48	13	0.87	0.03	0.69
U:O ratio of groups of AA								
TAA-N	0.98	0.97	1.04	0.95	0.04	0.15	0.59	0.24
EAA-N	1.30*	1.26*	1.51*	1.37*	0.05	0.07	<0.01	0.24
Group 1 AA-N	1.04	0.97	0.98	1.00	0.03	0.43	0.57	0.12
Group 2 AA-N	1.22*	1.27*	1.50*	1.32*	0.08	0.42	0.07	0.17
BCAA-N	1.12	1.11	1.57*	1.25	0.14	0.28	0.07	0.29
NEAA-N ⁹	0.69*	0.71*	0.62*	0.55*	0.07	0.61	0.05	0.44
U:O ratio of individual AA ¹⁰								
Individual EAA								
Arg	1.99*	1.66*	2.42*	2.21*	0.16	0.08	<0.01	0.63
His	1.13†	0.96	0.99	1.03	0.06	0.31	0.56	0.11
Ile	1.24	1.25	1.71*	1.32†	0.15	0.19	0.09	0.18
Leu	1.07	1.04	1.43*	1.23†	0.10	0.27	0.03	0.37
Lys	1.38*	1.51*	1.40*	1.42*	0.03	0.02	0.22	0.05
Met	0.93†	0.93†	0.89*	0.94	0.03	0.50	0.76	0.46
Thr	1.07*	1.07*	1.05†	1.04	0.02	0.84	0.39	0.76
Trp	1.27	1.29	1.48*	1.05	0.17	0.17	0.92	0.14
Val	1.08*	1.09*	1.64*	1.24*	0.20	0.38	0.14	0.35
Individual NEAA ¹¹								
Ala	1.32	1.52†	0.63	0.47†	0.23	0.93	<0.01	0.39
Asn	0.73	0.50	0.27	0.42	0.45	0.92	0.46	0.59
Asp	−0.12*	0.25*	0.24*	0.11*	0.11	0.28	0.34	0.06
Cys	0.53	0.78	1.34	0.50	0.34	0.41	0.45	0.15
Gln	1.11	1.25†	1.09	1.09	0.11	0.43	0.27	0.46
Glu	0.52*	0.74*	0.49*	0.49*	0.05	0.07	0.04	0.07
Gly	0.22*	−0.04*	0.48	0.01*	0.28	0.07	0.39	0.55
Pro	0.25*	0.20*	0.25*	0.18*	0.02	0.02	0.59	0.70
Ser	0.48*	0.10*	0.38*	0.27*	0.11	0.05	0.72	0.22

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³TAA-N = total AA on nitrogen basis.

⁴EAA-N = sum of N from Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁵Group 1 AA-N = sum of N from His, Met, Phe, Trp, and Tyr.

⁶Group 2 AA-N = sum of N from Ile, Leu, Lys, and Val.

⁷BCAA-N = branched-chain AA on nitrogen basis; sum of N from Ile, Leu, and Val.

⁸NEAA-N = sum of N from Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tau, and Tyr.

⁹For U:O ratio only, NEAA-N = sum of N from Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr.

¹⁰U:O ratio of (Phe + Tyr) is assumed to equal 1 for calculation of mammary plasma flow using the Fick principle; no statistical analyses were made on these calculated parameters.

¹¹Because Cit, Orn, and Tau have no output, their U:O ratio cannot be reported.

*U:O ratio different from 1 at $P \leq 0.05$.

†Tendency at $P \leq 0.10$.

We found no effect of NE_L or MP supplies on the TAA-N U:O ratio. The EAA-N U:O ratio tended to decrease ($P = 0.07$) in response to HE compared with LE diets, but those of group 1 AA-N, group 2 AA-N, and NEAA-N were not affected by the NE_L supply ($P > 0.41$). For Lys, the U:O ratio was affected differently by the NE_L supply ($E \times P$ interaction: $P = 0.05$): it increased in LP diets ($P < 0.01$) but was not affected in HP diets ($P = 0.59$). We did not observe any NE_L effect on the U:O ratio of the other EAA ($P > 0.18$) except a tendency for Arg ($P = 0.08$) that decreased in HE compared with LE diets. With increased NE_L supply, the U:O ratio of Pro and Ser decreased ($P < 0.05$), and that of Gly tended to decrease ($P = 0.07$), whereas that of Glu tended to increase ($E \times P$ interaction: $P = 0.07$) in response to HE compared with LE diets but only in LP diets ($P = 0.02$).

With HP compared with LP diets, the EAA-N U:O ratio increased ($P < 0.01$), that of group 2 AA-N and BCAA-N tended to increase ($P = 0.07$), and that of NEAA-N decreased ($P = 0.05$). Increasing the MP supply had no effect on U:O ratios of individual EAA except for Arg and Leu, which increased ($P \leq 0.03$), and for Ile, which tended to increase ($P = 0.09$). Increasing the MP supply decreased ($P \leq 0.04$) the U:O ratio of Ala and Glu.

Nitrogen Efficiency and Estimated Metabolizable Protein and AA Efficiencies. Nitrogen efficiency, which is the proportion of N intake recovered in milk, varied between 0.34 in HELP and 0.22 in LEHP. It increased by 13% in HE compared with LE diets ($P = 0.04$; Table 10) and decreased by 24% in response to HP compared with LP diets ($P < 0.01$). The estimated MP_{eff} (Table 10) showed great variation in response to the 4 diets, averaging in ascending order 0.44 (LEHP), 0.55 (HEHP), 0.76 (LELP), and 0.95 (HELP). With increasing NE_L supply, the estimated MP_{eff} increased by 0.15 but with an $E \times P$ interaction ($P < 0.01$): the increment of estimated MP_{eff} in response to HE compared with LE diets was higher in the LP diets (+0.19) compared with the HP diets (+0.10). Because endogenous urinary losses and scurf protein estimations are based on BW, TAA-N in these export proteins were not affected by the diets (2.1 mol N/d and 0.1 mol N/d, respectively). Moreover, TAA-N supply expressed in mol N/d had the same variations of AA supply as previously mentioned (Table 4). The same trends as MP_{eff} were observed for efficiency of TAA-N, because TAA-N differs from MP only by the exclusion of Cys and Trp ($P < 0.01$), and for all estimated individual EAA efficiencies ($P < 0.01$). An $E \times P$ interaction ($P < 0.01$) was observed for TAA-N and for the individual EAA efficiencies, except that the $E \times P$ interaction tended to be significant ($0.06 \leq P \leq 0.09$)

for the estimated efficiencies of Lys, Thr, and Val and was not significant ($P = 0.74$) for estimated Met_{eff}.

Contrary to the effect seen with increasing NE_L supply, increasing MP supply decreased estimated MP_{eff} by 0.36, and also decreased TAA-N and all estimated individual EAA efficiencies ($P < 0.01$), with the $E \times P$ interaction indicating that the decrease was greater in HE than in LE diets. No interaction ($P = 0.79$) was observed on the estimated catabolism of TAA-N.

For all individual EAA, we observed the highest estimated efficiencies with the HELP diet and the lowest with the LEHP diet, as also observed for MP. However, the magnitude of the differences varied between individual AA. The estimated efficiencies of Ile, Leu, Lys, Phe, Thr, and Val varied similarly to that of estimated MP_{eff} (from +19 to +24% with HE vs. LE diets and from -34 to -38% with HP vs. LP diets). In contrast, Arg and His had the highest variations of estimated efficiencies (+38% and +33% with HE vs. LE diets, and -62% and -46% with HP vs. LP diets for Arg and His, respectively). In contrast, Met showed the least variation (+13% with HE vs. LE diets and -27% with HP vs. LP diets). With the HELP diet, these 3 AA presented the highest estimated efficiencies compared with the other EAA; His_{eff} and Arg_{eff} were greater than 1 ($P \leq 0.05$), whereas Met_{eff} (0.95) tended to be less than 1. All the other estimated efficiencies of individual AA were less than 1 regardless of the diets ($P \leq 0.05$).

DISCUSSION

Milk Protein Yield Increased Through Different Mechanisms in Response to NE_L or MP Supplies

In the present trial, we created great variations in NE_L (+30%, between LE and HE diets) and in MP supplies (+78%, between LP and HP diets). These variations allowed analysis of the effects on AA metabolism of the extreme values of NE_L and MP supplies (from very low to high supplies) used to predict the MPY response in INRA (2018). However, we did not study the effects of gradual changes in NE_L or MP supplies. Increasing NE_L (between LE and HE diets) and MP supplies (between LP and HP diets) increased MPY similarly (19 and 15%, respectively) but through different mechanisms at the MG level, demonstrating the high metabolic flexibility of the MG. The increased MG uptake of TAA-N in response to HE compared with LE diets was explained by increased mammary plasma flow, whereas the AV differences of TAA-N and EAA-N decreased. Increasing the NE_L supply through increased DMI and different starch contents of the dietary concentrates, as performed in this trial, increased the mammary plasma

Table 10. Effects of NE_L and MP supplies on the partition of the TAA-N (total AA on nitrogen basis, mol of N/d) exported and on estimated efficiencies based on INRA (2018) and Lapierre et al. (2016)

Item	Diet ¹				SEM	P-value ²		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Nitrogen efficiency ³	0.312	0.333	0.224	0.253	0.008	<0.01	<0.01	0.40
Estimated MP_eff ⁴	0.769*	0.957*	0.447*	0.551*	0.010	<0.01	<0.01	<0.01
TAA-N ⁵								
Estimated supply	15.5	14.9	27.0	27.5	1.0	0.90	<0.01	0.19
Measured milk	7.3	8.4	8.2	9.9	0.5	<0.01	<0.01	0.15
Estimated MFP ⁶	2.21	2.91	2.06	3.03	0.10	<0.01	0.80	<0.01
Estimated catabolism ⁷	3.7	1.3	14.5	12.3	0.5	<0.01	<0.01	0.79
Estimated efficiency	0.720*	0.897*	0.417*	0.517*	0.008	<0.01	<0.01	<0.01
Efficiencies of individual EAA								
Arg	0.94	1.32*	0.37*	0.48*	0.02	<0.01	<0.01	<0.01
His	0.80*	1.08*	0.44*	0.57*	0.01	<0.01	<0.01	<0.01
Ile	0.68*	0.81*	0.45*	0.54*	0.01	<0.01	<0.01	0.07
Leu	0.67*	0.83*	0.43*	0.52*	0.01	<0.01	<0.01	<0.01
Lys	0.72*	0.85*	0.46*	0.55*	0.01	<0.01	<0.01	0.07
Met	0.85*	0.95†	0.62*	0.71*	0.02	<0.01	<0.01	0.74
Phe	0.60*	0.75*	0.37*	0.46*	0.01	<0.01	<0.01	0.01
Thr	0.61*	0.72*	0.40*	0.48*	0.01	<0.01	<0.01	0.09
Val	0.71*	0.85*	0.46*	0.56*	0.01	<0.01	<0.01	0.06

¹LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

²P-values correspond to the effect of energy supply (LE vs. HE), effect of protein supply (LP vs. HP), and interaction between energy and protein (E × P).

³Ratio of N in milk to N intake.

⁴MP_eff = MP efficiency.

⁵TAA-N = total AA on nitrogen basis; sum of N from Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val, Ala, Asx, Glx, Gly, Pro, Ser, and Tyr—i.e., total of 16 AA, excluding Cys and Trp.

⁶MFP = metabolic fecal protein.

⁷Estimated catabolism = supply – endogenous urinary losses – milk true protein yield – metabolic fecal protein – scurf.

*Different from 1 at $P \leq 0.05$.

†Tendency at $P \leq 0.10$.

flow, as previously observed when greater NE_L supply was achieved through post-ruminal glucose or ruminal propionic acid infusion (Raggio et al., 2006a; Lemosquet et al., 2010a). We cannot exclude the possibility that the increased mammary plasma flow might also be explained by increased DMI, as feed restriction decreased it (Lough et al., 1990; Guinard-Flament et al., 2006, 2007). However, such a restriction decreased both NE_L and MP supplies, contrary to the results of our trial. In contrast, in response to HP compared with LP diets, TAA-N uptake increased only through increased mammary AV differences in TAA-N, driven by EAA, with no change in mammary plasma flow.

The MG flexibility was also characterized by the MG uptake response to HE compared with LE diets, or HP compared with LP diets, which differed among AA. On the one hand, the uptake of group 1 AA-N increased in response to both HE compared with LE diets and HP compared with LP diets. In fact, the increment in MG uptake paralleled the increment in MPY for these AA, as indicated by the constant U:O ratio of group 1 AA-N. The latter also did not differ from 1,

indicating a stoichiometric transfer of the MG uptake of these AA to milk protein, as initially proposed by Mephram (1982) and previously reported (Lemosquet et al., 2010a; Lapierre et al., 2012). This also suggested no variation in intramammary metabolism of these AA with dietary changes. In addition, the mammary clearance rate of group 1 AA-N increased in response to HE compared with LE diets, suggesting an increase in mammary transport capacity and intracellular utilization of these AA (Hanigan et al., 1998). On the other hand, increasing the NE_L supply only tended to increase the uptake of group 2 AA-N, mainly driven by Lys because there was no effect on BCAA-N; in fact, Lys uptake represented the major contributor (50%) to the increased EAA-N uptake. In contrast, increasing MP supply increased uptake of group 2 AA-N and BCAA-N, and Arg, Leu, Ile, and Lys all contributed to the increased EAA-N uptake (33, 18, 11, and 11% respectively). Increasing the NE_L supply also tended to increase NEAA-N uptake, whereas increasing the MP supply did not change it. Overall, MG uptake variations of the AA groups in response to NE_L or MP

supplies are consistent with the literature either from meta-analysis for increased NE_L through increased propionic acid or intestinal glucose absorption (Lemosquet et al., 2010a) or MP supplies (Lapierre et al., 2012), or from individual experiments for NE_L or MP supplies (e.g., Raggio et al., 2006a; Rius et al., 2010b). However, increasing NE_L through rumen infusion of propionate or post-ruminal infusion of glucose did not increase uptake of AA from group 2 (Lemosquet et al., 2010a) but increased that of NEAA-N (Raggio et al., 2006a).

Finally, differences in intramammary metabolism also suggested high metabolic mammary flexibility. The U:O ratio of group 2 AA-N was always greater than 1, which indicates intramammary metabolism of these AA for functions other than supporting MPY. There is indeed a need for mammary synthesis of NEAA, for which the U:O ratio was always lower than 1. As the TAA-N U:O ratio was not different from 1 and was not affected by dietary changes, the N of group 2 AA was probably transaminated to NEAA synthesized within the mammary gland (Lapierre et al., 2003, 2009). In addition, the Arg U:O ratio was always higher than 1.5, as also previously reported (Doepel and Lapierre, 2010). This indicated that Arg could be used to support mammary NEAA de novo synthesis, namely Pro, whose U:O ratio was lower than 0.25. The excess uptake relative to MPY of AA from group 2 did not increase in HE compared with LE diets. Furthermore, the nonresponse of Arg uptake led to a decreased U:O ratio in HE compared with LE diets. In HELP, the increased Lys U:O ratio could be used to compensate for this decreased Arg U:O ratio and supply N for the mammary synthesis of NEAA such as Ser, whose U:O ratio decreased in HE compared with LE diets. This result was consistent with Lapierre et al. (2009), who observed mammary transfer of the N from Lys to NEAA, including Ala, Glu, and Ser. In contrast, in response to HP compared with LP diets, the increased excess of group 2 AA-N and Arg relative to output (U:O ratio: $P = 0.07$) was probably used to support de novo NEAA synthesis. In response to HP compared with LP diets, higher intramammary catabolism was suggested by the increased U:O ratio of group 2 AA-N. In fact, we can consider that the first step in catabolism of the group 2 AA occurred within the MG, because their N are transaminated to NEAA (Mephram, 1982). This is probably what occurs with Leu, which is deaminated to methyl oxopentanoate before oxidation. The oxidation to CO_2 of at least the first carbon of Leu increased with increased MP supply through post-ruminal CN infusion (Raggio et al., 2006a), which is proof of group 2 AA-N catabolism. Overall, MPY increased in response to NE_L and MP supplies through different mechanisms, consistent with the fact that no $E \times P$ interaction was observed in the

MPY responses. Consequently, the effects of NE_L and MP supplies were additive, as already reported (Clark et al., 1977; Broderick, 2003; Raggio et al., 2006a). In contrast, Brun-Lafleur et al. (2010) reported an $E \times P$ interaction, but their experiment was designed more specifically to detect such an interaction. These different mechanisms (variations of mammary plasma flow and AV differences, different increased uptakes of groups of AA, and changes in mammary metabolism) provided strong evidence of high metabolic mammary flexibility.

Different Partitions Explained Variations of Efficiency of Utilization of MP

Although independent increases in NE_L and MP supplies increased MPY by a similar magnitude, their respective effects on MP_eff was different. Because the estimated MP_eff and TAA-N_eff differed only by the exclusion of Cys and Trp from TAA, the same conclusions can be applied to these 2 efficiencies. Increasing the NE_L supply increased the estimated MP_eff, as previously reported by Hanigan et al. (1998) and Daniel et al. (2016) for CP and MP efficiencies, but to a higher extent in LP than in HP diets. Increasing the NE_L supply did not affect the calculated MP supply but increased MPY. In contrast, the estimated MP_eff decreased in HP compared with LP diets; this effect of MP supply has been well documented (Doepel et al., 2004; Metcalf et al., 2008; INRA, 2018). This decrease, observed in the present experiment, is the result of the increase in MP supply (+15%), as observed previously (Metcalf et al., 2008). These efficiencies were only estimated, with no direct measurement on MP or AA duodenal flow, consistent with previous studies (Metcalf et al., 2008; Daniel et al., 2016) that also estimated MP_eff or AA_eff and did not directly measure MP and AA duodenal flow. Nevertheless, the trends of efficiency variations were similar between the different feeding systems; we must keep in mind that the absolute variations of efficiency are valid only within the feeding system used to estimate both the supplies and the nonproductive functions. In the present experiment, we used the INRA (2018) feeding system because this new system revisited not only the supply but also the estimation of several protein exports. We cannot state that the absolute values of MP and AADI supplies or protein exported were completely accurate in the present experiment. However, rumen N degradation of each feed was determined (Table 1) to improve the accuracy of the prediction of MP supply. Nevertheless, the diet changes may not have achieved their full effect because of the short duration (2-wk period) for adaptation of the rumen microbial population. In addition, as

mentioned in our Materials and Methods section, the contributions of protein mobilization in MP supply or protein accretion in export proteins were not taken into account in our calculations of efficiencies. Determining protein accretion or mobilization through monitoring BW is unrealistic because of the short duration of the experimental periods. If we had used the exact INRA (2018) calculations, it would have involved including fat and subsequent protein mobilizations in 3 of the diets because of the predicted negative energy balance (in Mcal/d, LELP: -3.8; LEHP: -4.9; HEHP: -1.2). This scenario cannot be totally excluded in LE diets with the 2-wk duration of the experimental periods. In contrast, fat and protein accretion would have to be included in the HELP diet, linked to the predicted positive energy balance (1.0 Mcal/d) and the lowest plasma NEFA concentration (in μM , LELP: 170; HELP: 85; LEHP: 142; HEHP: 117; $P_E < 0.01$, $P_P = 0.87$, and $P_{E \times P} = 0.01$). However, in HELP, the efficiencies of MP, TAA-N and each EAA were higher than in the 3 other diets, and the efficiencies of 3 EAA (Arg, Met, His) were close to or even higher than 1, suggesting that protein mobilization might be the highest in this diet. Therefore, it would be unrealistic to assign a protein accretion value in this treatment, as proposed by INRA (2018). Interestingly, in the present experiment, the variations of MPY predicted (+125 g in HE vs. LE, and +99 g in HP vs. LP) using the INRA (2018) feeding system without taking account protein mobilization or accretion were close to the observed MPY (+129 g in HE vs. LE; +108 g in HP vs. LP). In fact, the estimation of MPY in INRA (2018) depends both on the calculated MP supply and on the estimation of other proteins exported; MP_{eff} is the ratio between both. Consequently, it was relevant to analyze the variations in MP and TAA-N efficiencies despite uncertainties as to supplies and protein exported, especially accretion.

Metabolic fecal protein and endogenous urinary loss, both considered export proteins, affect AA partitioning and all the estimated efficiencies. This supplementary information was needed to analyze the effect of NE_L supply, because the increment of efficiencies in HE compared with LE diets was the result of increased MPY and metabolic fecal protein, due to increased DMI. An increment of metabolic fecal protein with greater DMI was already proposed by Schneider (1934) and therefore was estimated for the HE diets (0.8 mol N/d), for which the DMI of the cows was 4.6 kg/d greater than with the LE diets. Increasing NE_L supply did not affect TAA-N supply, estimated scurf protein, or endogenous urinary loss (based on BW; Sauvant et al., 2015; Lapierre et al., 2016). The increased TAA-N_{eff} depended on the increased MPY and metabolic fecal protein, the latter representing about 64% of the mea-

sured increase in TAA-N output in milk (1.4 mol N/d). This also suggests a decreased catabolism of TAA-N in HE compared with LE diets, estimated by the difference between supply and export proteins (-2.3 mol N/d), assuming no change in BW and no pregnancy. The lower arterial concentration of urea, as well as the lower NPN concentration in milk with the HE compared with LE diets could also suggest decreased catabolism when energy supply increased, as observed by Jonker et al. (1998), who reported a positive relation between urinary urea excretion and plasma urea concentration. The increased plasma urea concentration in HP compared with LP diets suggests higher catabolism of MP, resulting in increased urinary urea excretion. Overall, taking account of metabolic fecal protein export, even if it is only an estimation, clearly showed that MP and TAA-N efficiencies increased more than the increased MPY in HE compared with LE diets, through increased DMI. This changes our understanding of MP and TAA-N efficiencies compared with the previous INRA (2007) calculation.

Several Parameters Suggested Variations of Individual Efficiencies Among EAA

Because individual EAA_{eff} varied differently with diets (Doepel et al., 2004), we opted to analyze whole-body metabolism of each EAA rather than the aggregation of AA as MP (Arriola Apelo et al., 2014). The differences between each EAA_{eff} result from differences in AA supply, whole-body utilization, or catabolism. Interestingly, some MG parameters could help to indicate which EAA were supplied in excess (e.g., increased U:O ratio above 1) or which EAA were more limiting relative to others (e.g., highest MG clearance rate of some EAA), the latter probably corresponding to the least-catabolized AA (Arriola Apelo et al., 2014). In our trial, the MG clearance rates of Lys, Met, His, and Leu were higher than those of the other EAA in LP diets (Table 9), suggesting these 4 AA could be the most limiting AA. However, the Lys U:O was significantly ($P \leq 0.05$) higher than 1, whereas Lys U:O ratios close to 1 were reported in treatments deficient in Lys (Guinard et al., 1994; Lapierre et al., 2012; Haque et al., 2015). This suggests that Lys transport capacity was increased in LP compared with HP diets, to provide N for NEAA synthesis, and Lys was not the first limiting AA in the present experiment. Moreover, Leu U:O increased in HP compared with LP diets and became higher than 1 in the former diets, which suggested that this AA was not limiting in HP diets. In addition, the tendency ($P = 0.11$) for higher mammary clearance of Met in HP compared with LP diets (453 vs. 351 L/h, respectively) also suggests a greater deficiency of Met supply than of

the other EAA supplies. This occurred when His, Leu, and Lys clearances were lower than the Met clearance rate as well as when these 3 clearance rates decreased (Table 9) in HP compared with LP diets. All of this could suggest that His and Met were the 2 first limiting AA in LP diets, whereas only Met was the first limiting AA in HP diets, as often observed when increasing MP supply (Broderick et al., 2008). In addition, this is also suggested by our estimated supplies of His and Met digestible in the intestine (Table 2) based on INRA (2018) AA feed tables, which were lower than the recommendations set at 2.4% of PDI ($\pm 0.2\%$ of PDI) for both His and Met. In contrast, the estimated supplies of Leu and Lys digestible in the intestine were at or above the recommendations set at 8.5% and 7.0% of PDI ($\pm 0.2\%$ of PDI; INRA, 2018), suggesting that these AA are less limited than Met and His.

Interestingly, a similar conclusion regarding the most limiting AA was also reached through the analysis of individual EAA_eff. In fact, efficiencies of most individual EAA (Table 10) varied following a pattern similar to TAA_eff, but Arg and His had the largest variations of efficiencies, and Met had the smallest. As for MG clearance, the estimated efficiencies of His and Met were the highest of the EAA in LP, and the estimated Met_eff was the highest in HP, if Arg_eff is excluded. In HELP, the efficiencies of these 3 AA were close to or higher than 1. As previously discussed, protein mobilization cannot be totally excluded with the 2-wk duration of the experimental period. This might be the case for all EAA if protein mobilization occurs in HELP. However, in HELP, the highest predicted energy balance and the lowest plasma NEFA concentrations observed did not indicate fat and subsequent protein mobilization. Concerning Arg, a semi-essential AA, Doepel et al. (2004) suggested a de novo synthesis of 30 g/d, which, in the current study, represents half of the estimated digestible flow of Arg for the LP diets. Moreover, the high estimated Arg contribution to endogenous urinary losses due to creatine and creatinine urinary excretion (174 and 528 mmol N/d, respectively), was roughly equal to the amount of Arg exported in milk in HEHP (760 mmol N/d). Although these data add to our knowledge of Arg metabolism, more information is needed on true Arg supply, including de novo synthesis, to better characterize Arg_eff. Histidine efficiency was higher than Met_eff and higher than 1 in HELP; this high efficiency could be achieved without impeding protein mobilization. Indeed, an alternative endogenous source of His may contribute to His supply: His-dipeptides in muscular intracellular pools (carnosine and anserine) and hemoglobin could be used as an endogenous His supply (Lee et al., 2012; Lapierre et al., 2014; Giallongo et al., 2015) but not taken up by the MG for MPY (Haquet et

al., 2015). This could lead to efficiency higher than 1 when only the digestible flow is included into the estimated AA supply. Indeed, at low MP supply, Raggio et al. (2004) observed mammary uptake of His larger than the post-liver supply, and this was observed only for His. Additionally, His plasma concentration was lower in cows fed HELP than in those fed LELP ($E \times P$ interaction: $P = 0.03$), which suggested a decreased His availability for other functions than MPY synthesis. If we hypothesize that the highest efficiencies define the most limiting AA, the limiting AA differed among diets and was probably His for HELP and Met for the 3 other diets. Overall, combining MG clearance rate and estimation of individual AA_eff improves our analysis of individual EAA utilization. Future research aimed at improving the models to predict protein exports (as metabolic fecal protein or endogenous urinary losses) and their associated AA composition would better define individual AA_eff or catabolism.

CONCLUSIONS

This experiment confirmed the high flexibility of the MG in contrasting nutritional situations. The increased MPY in response to wide variations of NE_L and MP, between low and high supplies, was associated with different changes in MG AA uptakes and metabolism. With increasing NE_L supplies, EAA and NEAA uptakes increased or tended to increase, through an increase in mammary plasma flow, with no indications of substantial change in intramammary metabolism for most AA. However, with increasing MP supply, mammary uptake of EAA increased, whereas uptake of NEAA decreased. The excess uptake relative to milk protein outputs of Arg and group 2 AA increased in HP compared with LP diets; these AA could be used for de novo NEAA MG synthesis. Despite the fact that increasing NE_L or MP supplies both increased MPY, these supplies respectively increased and decreased the estimated efficiencies. Increasing the NE_L supply increased the estimated MP_eff and individual AA_eff, not only through an increase in the partition of AA toward MPY, with no change in AA supply, but also through the increase in metabolic fecal protein (linked to the increased DMI). With increased MP supply, the estimated MP_eff and AA_eff decreased, because AA exported in MPY increased to a lesser extent than the increased supply. Measurements of individual AA flows in metabolic fecal protein, individual AA digestibilities, or tissue protein mobilization (such as 3-methyl His) are required to confirm our conclusions about AA_eff. Nevertheless, analyses of MG metabolism parameters (as the U:O ratios of AA from group 2 and the clearance rate of some EAA), combined with the estima-

tion of individual EAA_eff, could help determine the most limiting EAA, considering that the most limiting EAA has the highest efficiency. The most limiting AA was probably His in HELP and Met in the 3 other diets. The fact that some specific individual AA were involved in variations of MP_eff and in changes to MG metabolism suggests the possibility of developing a future feeding system that integrates individual AA_eff instead of only MP_eff. The great challenge will be, in future, to confirm these calculated variations of individual AA_eff using less-invasive methods.

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