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## Synthesis of milk components involves different mammary metabolism adaptations in response to net energy and protein supplies in dairy cows

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### ABSTRACT

Net energy for lactation (NE<sub>L</sub>) and metabolizable protein (MP) are the 2 main nutritional forces that drive synthesis of milk components. This study investigated mammary-gland metabolism in dairy cows in response to variations in the supply of NE<sub>L</sub> and MP. Four Holstein dairy cows were randomly assigned to a 4 × 4 Latin square design, in which each experimental period consisted of 14 d of dietary treatment. The diets provided 2 levels of NE<sub>L</sub> (low energy, 25.0 Mcal/d vs. high energy, 32.5 Mcal/d) and 2 levels of MP (low protein, 1,266 g/d vs. high protein, 2,254 g/d of protein digestible in the intestine) in a 2 × 2 factorial arrangement. Performance and dry matter intake (DMI) were measured during the last 5 d of each period, and the mammary net balance was measured on d 13 by collecting 6 sets of blood samples from the left carotid artery and left mammary vein. Mammary plasma flow was measured according to the Fick principle for Phe and Tyr. The mammary net balance of carbon equaled the uptake of nutrients expressed as carbon minus the output of lactose, fatty acids (FA) synthesized in the mammary gland, AA of milk protein, and glycerol-3P from triglyceride on d 13. Milk, lactose, fat, and protein yields increased when NE<sub>L</sub> and MP supplies increased. However, increasing the NE<sub>L</sub> supply increased FA synthesis more than increasing the protein supply did. In addition, FA secretion increased more than lactose secretion when the NE<sub>L</sub> supply increased. Increasing the NE<sub>L</sub> supply increased the left half-udder uptake of all major energy-yielding nutrients by increasing mammary plasma flow. However, nutrient uptake increased more than milk output did, which in turn increased carbon dioxide output. This increase in nutrient oxidation by the mammary gland decreased the mammary efficiency

of nutrients utilization when the NE<sub>L</sub> supply increased. Increasing MP supply tended to increase glucose uptake through mammary clearance and increased mammary AA uptake with no change in mammary plasma flow. In addition, the protein supply did not change the mammary uptake of acetate or β-hydroxybutyrate. The increase in milk-component secretions in response to either NE<sub>L</sub> or MP supplies occurred through different metabolic adaptations (increase in mammary plasma flow vs. clearances, respectively). These results suggest that the nutrient use by the mammary gland is highly flexible, which helps in maintaining milk and milk-component yields even with limiting nutrient supplies.

**Key words:** lactose, fatty acid, protein, nutrient uptake, efficiency

### INTRODUCTION

Decreasing the MP supply greatly increases MP efficiency (Vérité and Delaby, 2000; Socha et al., 2005; INRA, 2018) by decreasing urea excretion and nitrogen waste (Raggio et al., 2006; Lee et al., 2012). However, decreasing the MP supply below a certain threshold (95 g/kg of DM of protein digestible in the intestine; INRA, 2018) is not recommended because it decreases yields of milk and milk components. On the other hand, increasing NE<sub>L</sub> supply can increase the efficiency of utilization of MP, without altering MP supply (Omphalius et al., 2020), indicating it is a main nutritional factor driving synthesis of milk components. These 2 factors are used to predict milk yield and composition in the INRA (2018) feeding system. Indeed, increasing the MP or NE<sub>L</sub> supplies increases milk, lactose, and fat yields following curvilinear responses, as described in the meta-analysis of Daniel et al. (2016). However, increasing the NE<sub>L</sub> supply induces a different pattern of increased nutrient absorption than increasing the MP does (VFA, intestinal glucose, and fat vs. AA; Sutton, 1985; Erasmus et al., 1994), which suggests that different metabolic adaptations, especially in the mammary

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gland, increase the 3 major milk components when only the MP or NE<sub>L</sub> supply increases.

The response of mammary metabolism to greatly increasing the supplies of both NE<sub>L</sub> and MP in diets via feeding level has been studied; however, effects of the NE<sub>L</sub> supply could not be distinguished from those of the MP supply (Guinard-Flament et al., 2007). To our knowledge, mammary responses to increased NE<sub>L</sub> supply independent of the MP supply through dietary treatments has not been reported yet. In most other studies of effects of increasing the NE<sub>L</sub> supply on the mammary net balance, only one nutrient was administered, via digestive infusion (Lemosquet et al., 2009a; Nichols et al., 2019a; Danes et al., 2020), which usually increased secretion of the milk component that corresponded to the precursor. For example, postrumen glucose infusion increased milk-lactose secretion (Rigout et al., 2002b), whereas acetate infusion (Danes et al., 2020) increased fatty acid (FA) synthesis. In comparison, digestive infusion of a given nutrient supply does not change other nutrient supplies, which could induce different mammary responses than does increasing the dietary NE<sub>L</sub> supply, which increases the availability of several energetic nutrients.

We therefore hypothesized that changing the NE<sub>L</sub> supply through dietary treatments can change the mammary uptake of several energetic nutrients. Increasing the NE<sub>L</sub> supply could then induce different mammary metabolic adaptations than those observed with infusions of only propionate or glucose. In addition, studies of the net mammary-gland uptake (MGU) of nutrients did not report the uptake of all major nutrients, except for a few studies (e.g., Lemosquet et al., 2009a; Nichols et al., 2019a). For example, studies that increased the MP supply reported mainly MGU of AA (e.g., Doeppel and Lapierre, 2010), whereas studies that infused propionate or postrumen glucose did not measure AA uptake, which made it difficult to understand the increased fat and lactose yields observed when the MP supply increased. Some studies have, however, reported the effects of energy-yielding nutrients (glucose, propionate, acetate, BHB, and dietary FA) on MGU of AA (Nichols et al., 2019a).

The aim of the present study was to analyze mammary metabolism in response to increased NE<sub>L</sub> or MP supplies to understand the changes in milk-component secretions. This study was based on the same experiment as that reported by Omphalius et al. (2019), which focused on AA metabolism. In the present experiment, we analyzed mammary use of all major nutrients, including energy-yielding nutrients and the sum of AA, and related these nutrients to milk secretion of lactose, FA, and protein on a carbon basis. We hypothesized that the effects of increased MP or NE<sub>L</sub> supplies on

lactose, fat, and protein yields occurred through different mammary metabolic adaptations.

## MATERIALS AND METHODS

### Cows

The experiment was performed in 2007 at IE PL INRAE Dairy Nutrition and Physiology (INRAE, 2021; Installation Expérimentale de Production Laitière, Institut National de La Recherche Agricolture, Alimentation et Environnement, IE PL, Le Rheu, France), in accordance with National Legislation on Animal Care (certified by the French Ministry of Agriculture; agreement no. C35–275–23). The experiment is described in detail by Omphalius et al. (2019). Briefly, 4 second-lactation Holstein cows were equipped with a permanent catheter implanted in the left carotid artery at  $48 \pm 16$  DIM, and they began the experiment at  $86 \pm 12$  DIM. The cows were housed in individual tiestalls and had ad libitum access to fresh water.

### Treatments and Experimental Design

Two levels of NE<sub>L</sub> supply, low energy (LE) and high energy (HE), were combined with 2 levels of MP supply, low protein (LP) and high protein (HP) in a  $2 \times 2$  factorial arrangement to create 4 treatments (LELP, HELP, LEHP, and HEHP), which were tested in a  $4 \times 4$  Latin square design balanced for residual effects with 14-d experimental periods.

The MP supply was initially designed to create the maximum variation of MP through diets as done with digestive infusions (Galindo et al., 2011). Diets were therefore formulated to provide 70% and 125% of MP requirements in the LP and HP treatments, respectively, according to the INRA (1989) feeding system, for a cow producing 33 kg of milk/d, with milk-protein and fat concentrations of 29.5 and 36.5 g/kg, respectively. For NE<sub>L</sub> supply, it was not possible to create the same range of variations due to intake limitations; therefore, diets were formulated to provide 70% and 100% of NE<sub>L</sub> requirements in the LE and HE treatments, according to the INRA (1989) feeding system. The LELP, HELP, LEHP, and HEHP diets, which contained corn silage, an energy concentrate, formaldehyde-treated (FT) soybean meal, vitamins, minerals, and urea, were offered in restricted amounts (14.8, 19.2, 14.5, and 19.8 kg of DM/d, respectively). The chemical composition of the 4 diets and the predicted nutritional values of the treatments based on the INRA (2018) feeding system varied (Table 1). The amount of NE<sub>L</sub> supplied in the LE and HE treatments was determined by the amount of feed offered (Table 2). The proportion of concentrate was

**Table 1.** Composition and nutritional values of diets (mean  $\pm$  SD)

Item <sup>2</sup>	Diet <sup>1</sup>			
	LELP	HELP	LEHP	HEHP
Concentrate, <sup>3</sup> %	16.7 $\pm$ 1.5	34.6 $\pm$ 4.0	39.6 $\pm$ 1.1	33.7 $\pm$ 3.8
Energy concentrate <sup>4</sup>	3.0 $\pm$ 2.17	30.5 $\pm$ 3.68	2.3 $\pm$ 1.06	11.9 $\pm$ 4.11
FT-SBM <sup>5</sup>	9.7 $\pm$ 0.91	0.8 $\pm$ 0.69	33.9 $\pm$ 1.5	18.7 $\pm$ 1.83
Minerals and vitamins <sup>6</sup>	1.9 $\pm$ 0.15	1.5 $\pm$ 0.03	2.0 $\pm$ 0.17	1.5 $\pm$ 0.2
Urea	1.1 $\pm$ 0.06	1.0 $\pm$ 0.08	0.3 $\pm$ 0.08	0.8 $\pm$ 0.26
Calcium carbonate <sup>7</sup>	1.0 $\pm$ 0.09	0.8 $\pm$ 0.02	1.1 $\pm$ 0.1	0.8 $\pm$ 0.09
NDF, g/kg of DM	333	272	280	288
Starch, g/kg of DM	366	457	280	356
Ether extract, g/kg of DM	31	31	35	33
OM, g/kg of DM	932	943	915	933
OMd, <sup>8</sup> %	73.1	72.3	74.4	71.9
NE <sub>L</sub> , Mcal/kg of DM	1.7	1.7	1.8	1.7
MP, g/kg of DM of PDI <sup>9</sup>	87.6	66.0	154.5	116.7

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Items listed are given as percent of DM, unless otherwise noted.

<sup>3</sup>Sum of energy concentrate and formaldehyde-treated soybean meal (FT-SBM), minerals and vitamins, and urea.

<sup>4</sup>Energy concentrate contained, on DM basis, 54.2% peas, 38.2% cornstarch, 4.4% sugar molasses, 2.0% soy oil, and 1.2% NaCl.

<sup>5</sup>The FT-SBM contained 94.7% soybean meal, 4.2% sugar molasses, and 1.1% formol.

<sup>6</sup>Calcium, 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 B<sub>1</sub>, 50 mg/kg (Turbomine Emeraude, Néolait, Yffiniac, France).

<sup>7</sup>CaCO<sub>3</sub> contained calcium (38%) and magnesium (1.5%; Néolait, Yffiniac, France).

<sup>8</sup>Organic matter digestibility.

<sup>9</sup>PDI = true protein digested in the small intestine supplied by RUP and rumen microbial protein (INRA, 2018), equivalent to MP.

lower in LELP than in the other 3 treatments (Table 1). The MP supply was increased differently in the LE and HE diets; FT soybean meal replaced the maize silage used in the LELP diet to make the LEHP diets, and the energy concentrate increased in the HEHP diet (18.7%  $\pm$  1.8% of DM offered) compared with the HELP diet (0.8%  $\pm$  0.7% of DM offered). Feed access was limited to short periods (1 h every 3 h starting at 0715 h over 24 h) to minimize postprandial variations in plasma metabolite concentrations. Corn silage was distributed 3 times per d: 25% of the daily allowance at 0715 h, 25% at 1315 h, and 50% at 1915 h, except on the days that blood was sampled, when it was distributed 5 times per day: 12.5% of the daily allowance each at 0715 h, 1015 h, 1315 h, and 1615 h, and 50% from 1715 h to 1915 h. The other ingredients (i.e., energy concentrate, FT soybean meal, urea, and the vitamin and mineral premix) were offered every 3 h starting at 0715 h.

## Analysis

**Feed.** The feed offered and refused was weighed daily. Corn silage DM was determined daily to adjust the quantity of wet feed offered. A pooled sample per feed was analyzed for chemical composition and enzymatic rumen degradability throughout the experiment (LDA, Saint-Brieuc, France). The methods used were in accordance with those recommended by INRA (2018).

**Milk.** The cows were milked twice daily at 0630 h and 1730 h during the first 7 d of each period and at 0630 h and 1830 h during the last 7 d of each period. During the last 5 d, the left and right halves of each cow's udder were milked separately; for each half-udder, milk yield was recorded, and milk-protein and milk-fat concentrations were measured at each milking via infrared analysis (Milkoscan, Foss Electric, Hillerød, Denmark). After the evening milking on d 13, samples of the milk from the left half-udder were frozen until being analyzed for lactose and FA. Lactose in milk was analyzed through a spectrophotometry technique using a multiparameter analyzer (KONE Instruments Corporation, Espoo, Finland) and a kit ( $\beta$ -galactosidase,  $\beta$ -galactose dehydrogenase; lactose/D-galactose kit; Roche Diagnostics, Meylan, France). Milk individual

**Table 2.** Effects of NE<sub>L</sub> and MP supplies on DMI, predicted nutrient intake on the last 5 d of each experimental period

Item	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
DMI, kg/d	14.7	18.8	14.4	19.5	0.54	<0.01	0.22	0.02
NE <sub>L</sub> intake, Mcal/d	24.7	31.9	25.3	33.1	0.94	<0.01	<0.01	0.27
MP intake, g/d of PDI <sup>3</sup>	1,289	1,243	2,232	2,276	78.0	0.98	<0.01	0.17
Starch intake, g/d	5,380	8,612	4,037	6,923	187.2	<0.01	<0.01	0.06
NDF intake, g/d	4,895	5,121	4,043	5,608	158.9	<0.01	<0.01	<0.01
Ether extract intake, g/d	456	584	505	643	17.8	<0.01	<0.01	0.32
Intestinal glucose, <sup>4</sup> g/d	835	1,432	643	1,207	51.9	<0.01	<0.01	0.56
Rumen acetate, <sup>4</sup> g/d	2,277	2,709	1,987	2,579	54.9	<0.01	<0.01	<0.01
Rumen propionate, <sup>4</sup> g/d	980	1,366	899	1,306	39.0	<0.01	<0.01	0.41
Rumen butyrate, <sup>4</sup> g/d	645	848	593	799	20.8	<0.01	<0.01	0.74
NE <sub>L</sub> balance, Mcal/d	-3.1	1.7	-4.3	-0.5	0.27	<0.01	<0.01	<0.01

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>PDI = true protein digested in the small intestine supplied by RUP and rumen microbial protein (INRA, 2018), equivalent to MP.

<sup>4</sup>Available intestinal glucose, and ruminal acetate, propionate, and butyrate as predicted with INRA (2018).

FA were analyzed by GC using a GIRA 1600 chromatograph (GIRA, Morlaàs, France) according to Couvreur et al. (2006).

**Blood and Plasma.** On d 13, 6 sets of blood samples were collected from the left carotid artery and the left mammary vein every 2 h, starting after milking at 0700 h. Blood was collected in 2- and 7.5-mL syringes to determine blood gases and other parameters, respectively (heparin, S-Monovette, Sarstedt, Nümbrecht, Germany). Blood gases (O<sub>2</sub> and CO<sub>2</sub>) were determined immediately using a blood gas and mineral analyzer (ABL 625 OSM 3, Radiometer, Copenhagen, Denmark) with a specific oximeter module for bovines (OSM 3). Hematocrit was assayed after ultracentrifugation (5 min, 14,810 × g, 20°C) in capillary tubes. The remaining blood syringes were immediately placed on ice and centrifuged for 10 min at 2,000 × g at 4°C to yield plasma. A subsample of plasma was deproteinized with 2 volumes of perchloric acid (0.6 M) to measure acetate and lactate. Plasma and deproteinized plasma were stored at -20°C until analysis. Plasma nonesterified FA (NEFA), glucose, urea, and BHB were analyzed using a multiparameter analyzer (KONE Instruments Corporation). Enzymatic kits were used to determine NEFA (acetyl-CoA synthase, acyl-CoA oxidase and peroxidase; NEFA C Wako kit; Oxoid S.A., Dardilly, France), glucose (hexokinase; GLUC HK 07 3672, Roche Diagnostics, Meylan, France), urea (urease and glutamate dehydrogenase; Urée UV Cinétique, KONE Diagnostics, Evry, France), and BHB (BHB dehydrogenase; Sigma, Saint-Quentin-Fallavier, France). The kit used to analyze triglycerides (TG; i.e., lipase, glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase; GPO PAP, Biotrol, Biomerieux, Lyon, France)

quantified the sum of free glycerol and triacylglycerol. L-Lactate was analyzed using lactate oxidase and peroxidase (Lactate-PAP 61192, bioMerieux SA, Marcy-l'Etoile, France), and acetate was analyzed by using the acetyl-CoA synthetase, citrate synthetase, malate dehydrogenase method (Sigma). Plasma Phe and Tyr were measured by isotopic dilution, as described by Omphalius et al. (2019).

**Calculations.** The energy content of milk was calculated using the following equation of Sjaunja et al. (1990):

$$\text{energy (kcal/L)} = 38.30 \times \text{fat (g/kg)} \\ + 24.20 \times \text{protein (g/kg)} + 783.2.$$

Averaged milk-protein and fat concentrations of the whole udder on the last 5 d (Table 3) were calculated as the sum of milk-protein or fat yields measured at each milking for each half-udder divided by the sum of corresponding milk yields. We assumed that milk-lactose concentration on the last 5 d was the same as the concentration on the left half-udder on the evening of d 13 to calculate the mean of whole-udder lactose yield during the last 5 d of each experimental period.

Digestible AA (i.e., MP) and glucose absorbed in the intestine, as well as absorbed acetate, propionate, and butyrate from the rumen, were predicted according to INRA (2018) based on the equations of Nozière et al. (2011) for VFA and Offner and Sauvant (2004) for intestinal glucose using measured intake. The MGU of nutrients was calculated by multiplying arteriovenous difference measured in the left half-udder by the mammary plasma flow (MPF) determined according to the



**Table 3.** Effects of NE<sub>L</sub> and MP supplies on milk and milk components yields and concentrations on the last 5 d of each experimental period and from the left half-udder during the evening milking of d 13

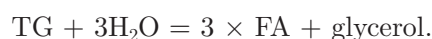
Item	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Mean of the last 5 d								
Milk yield, kg/d	24.2	26.5	27.6	31.8	1.26	<0.01	<0.01	0.11
Yield, g/d								
Solids <sup>3</sup>	2,726	3,118	3,033	3,677	150.9	<0.01	<0.01	0.06
Lactose	1,138	1,268	1,283	1,532	87.6	<0.01	<0.01	0.09
Fat	935	1,093	1,014	1,255	50.6	<0.01	0.07	0.21
True protein	654	757	736	891	42.4	<0.01	<0.01	0.16
Milk energy, Mcal/d	15.9	18.9	17.8	21.4	0.72	<0.01	<0.01	0.14
Left udder milking on d 13 evening								
Milk yield, kg/12 h	5.6	6.2	6.5	7.5	0.33	<0.01	<0.01	0.34
Yield, g/12 h								
Solids <sup>3</sup>	629	720	716	857	36.1	<0.01	<0.01	0.15
Lactose	264	289	301	353	20.3	0.01	<0.01	0.20
Fat	216	260	246	299	13.0	<0.01	<0.01	0.32
True protein	148	172	169	206	9.0	<0.01	<0.01	0.31
Concentrations, g/kg								
Lactose	46.9	47.7	46.1	48.0	1.44	0.05	0.70	0.30
Fat	38.5	42.3	38.2	40.3	1.35	0.05	0.37	0.54
True protein	26.3	27.9	26.4	27.7	1.54	0.03	0.91	0.69

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Milk solids = lactose yield + fat yield + protein yield.

Fick principle applied to Phe and Tyr, as described by Omphalius et al. (2019). Oxygen consumption and CO<sub>2</sub> production were calculated using mammary blood flow estimated from MPF and hematocrit as in Lemosquet et al. (2009b). The mammary clearance rate (L/h) was calculated as mammary-gland uptake divided by venous concentration of each metabolite to represent tissue affinity to substrate (Hanigan et al., 1998). The mammary net balance of carbon was calculated as described by Lemosquet et al. (2009b) using principles similar to those adopted by Waghorn and Baldwin (1984) and Hanigan and Baldwin (1994). Briefly, all nutrients taken up and secreted in milk and blood CO<sub>2</sub> were expressed in millimoles per hour of carbon. The MGU of TG was used to estimate the MGU of glycerol taken up because the analysis measured total glycerol. Fatty acid yields for carbon balance were calculated as described in Lemosquet et al. (2009b) using fat yield of the left half-udder during the d 13 evening milking and FA composition of this milk (Table 4 and Supplemental Table S1; <https://doi.org/10.5281/zenodo.8366232>; Anger et al., 2023), based on the hypotheses that (1) all the FA in milk are TG (Palmquist et al., 1969) and (2) each TG has the following composition:



$$\text{Then FA (mol/12 h)} = \frac{\text{FA (\%)} \times \text{fat (g/12 h)}}{\text{MW}_{\text{FA}} + \frac{1}{3} \times \text{MW}_{\text{glycerol}} - \text{MW}_{\text{H}_2\text{O}}},$$

where MW represented molecular weight of FA, glycerol, and H<sub>2</sub>O, respectively. However, the MGU of long-chain FA and secretion of preformed FA in milk were excluded, as reported by Waghorn (1982), Waghorn and Baldwin (1984), and Lemosquet et al. (2009b). Therefore, it was assumed that all FA from C<sub>4</sub> to C<sub>12</sub>, 85% of C<sub>14</sub> and C<sub>15</sub>, and 60% of C<sub>16</sub> and C<sub>17</sub> were synthesized in the mammary gland based on carbon isotope injections of acetate and butyrate (Palmquist et al., 1969; Waghorn and Baldwin, 1984). Preformed FA (Table 4) were then hypothesized to correspond to the sum of 15% of C<sub>14</sub> and C<sub>15</sub>, 40% of C<sub>16</sub> and C<sub>17</sub>, and all C<sub>18</sub> and higher carbon chain length FA (Table 4). Preformed FA were assumed not to be oxidized because tracer studies of the mammary gland of fed goats detected no appreciable catabolism (Annison et al., 1967). Carbon output in milk equaled the sum of lactose, AA, synthesized FA, and synthesized glycerol as in Lemosquet et al. (2009b). Total carbon output equaled the carbon output in milk plus CO<sub>2</sub>. The output of AA carbon in milk was calculated using the same principle as that used by Omphalius et al. (2019).

**Table 4.** Effect of NE<sub>L</sub> and MP supplies on milk fatty acid yield measured on the left half-udder in dairy cows

Sum, <sup>3</sup> g/12 h	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
C <sub>4</sub> to C <sub>8</sub>	15.8	19.9	18.5	23.1	1.44	<0.01	<0.01	0.69
C <sub>10</sub> to C <sub>14</sub>	33.3	42.4	37.1	50.8	2.77	<0.01	0.04	0.35
C <sub>16:1</sub> and C <sub>16:0</sub>	77.5	100.7	80.3	106.9	4.55	<0.01	0.05	0.39
All C <sub>18</sub>	72.1	75.6	90.6	93.4	7.13	0.53	<0.01	0.95
Synthesized FA <sup>4</sup>	104	133	113	150	6.4	<0.01	0.02	0.33
Preformed FA <sup>4</sup>	112	127	133	149	8.6	0.01	<0.01	0.87

<sup>1</sup>LELP = low energy and low protein; HELP = high energy and low protein; LEHP = low energy and high protein; HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Calculated using the fatty acid percentage measured on the morning of d 13 for each period and the average milk fat yield measured in the evening milking of the second week of each period.

<sup>4</sup>Milk synthesized fatty acids within the mammary gland [(C<sub>4</sub> to C<sub>13</sub>) × 1 + (C<sub>14</sub> + C<sub>15</sub>) × 0.85 + (C<sub>16</sub> + C<sub>17</sub>) × 0.60] and preformed fatty acids [(C<sub>14</sub> + C<sub>15</sub>) × 0.15 + (C<sub>16</sub> + C<sub>17</sub>) × 0.40 + (C ≥ C<sub>18</sub>) × 1].

## Statistical Analyses

Intake, milk yield, and milk composition of the last 5 d of each period were averaged as described in the “Calculations” section and used for statistical analyses. Daily means of the 6 blood samples collected on d 13 were used for mammary metabolism and associated calculations. Data were analyzed using the MIXED procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC) according to the following statistical model:

$$Y_{ijkl} = \mu + cow_i + period_j + energy_k + protein_l + (energy \times protein)_{kl} + \varepsilon_{ijkl}$$

where  $\mu$  is the mean;  $Y_{ijkl}$  is the dependent variable for the fixed effects of  $period_j$  (4 periods),  $energy_k$  is the energy supply (LE or HE),  $protein_l$  is the protein supply (LP or HP),  $cow_i$  is a random effect, and  $\varepsilon_{ijkl}$  is the residual error associated with observations  $ijkl$ . Because 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 indicates that there was no carryover effect. The results were assessed as least squares means  $\pm$  the largest standard error. The homogeneity of residues was verified. Differences were considered significant at  $P \leq 0.05$  and trends for  $0.05 < P \leq 0.10$ . If the energy × protein interaction was significant or tended to be significant, the SLICE procedure of SAS was used to test the effect of one factor within the other factor.

## RESULTS

### DMI, Nutrient Supplies, and Milk Production and Composition

**DMI and Nutritional Values.** The NE<sub>L</sub> supply was higher with the HE treatment than with the LE treatment (+30%;  $P < 0.01$ ) due to higher DMI (+4.6 kg/d; Table 2). The NE<sub>L</sub> supply was also slightly higher with the HP treatment than the LP treatment (+3%,  $P < 0.01$ ). When the NE<sub>L</sub> supply increased, the higher DMI and change in diet composition increased starch (+3,059 g/d; +65%; Table 2), NDF (+896 g/d; +20%), and ether extract (**EE**; +133 g/d; +28%) supplies ( $P < 0.01$ ), but with a trend for an energy × protein interaction ( $P = 0.06$ ) for starch and a significant energy × protein interaction for NDF ( $P < 0.01$ ). When the NE<sub>L</sub> supply increased, starch intake tended to increase more with the HP than LP treatment (energy effect: +71% vs. +60%, respectively;  $P < 0.01$  from the 2 SLICE tests) due to changes in composition of LP versus HP diets, and NDF intake increased significantly more with the HP treatment (+39%;  $P < 0.01$ ) than with the LP treatment (+5%;  $P < 0.01$ ).

As expected, the MP supply was higher with the HP treatment than with the LP treatment (+968 g/d of protein digestible in the intestine; +78%,  $P < 0.01$ ; Table 2). Compared with the LP treatments, replacing corn silage and energy concentrate with FT soybean meal in the HP treatments decreased mainly the supply of starch (−3,032 g/d; −22%) and, to a lesser extent, NDF (−365 g/d; −4%), and slightly increased EE (+108 g/d; +10%), with some interactions. When the MP supply increased, starch intake tended to decrease (energy × protein interaction:  $P = 0.06$ ) more with the LE treatment (−25%;  $P < 0.01$ ) than HE treatment (−20%;  $P < 0.01$ ) because of changes in diet composition, as described in “Materials and Methods.” When the MP supply increased, NDF intake decreased with the LE treatment (−17%,  $P < 0.01$ ) but increased with the HE treatment (+10%,  $P < 0.01$ ).

**Predicted Nutrient Supplies.** Increasing the NE<sub>L</sub> supply increased the estimated amounts of digestible glucose in the intestine and acetate, propionate, and butyrate absorbed from the rumen (Table 2;  $P < 0.01$ ) by 79%, 24%, 42%, and 33% respectively, with an energy  $\times$  protein interaction ( $P < 0.01$ ) for acetate. When the NE<sub>L</sub> supply increased, the predicted absorbed acetate from the rumen increased more with the HP treatment (+30%,  $P < 0.01$ ) than with the LP treatment (+19%,  $P < 0.01$ ). Conversely, when the MP supply increased, predicted digestible glucose and estimated absorption of acetate, propionate, and butyrate decreased by 18%, 8%, 6%, and 7%, respectively ( $P < 0.01$ ), with an energy  $\times$  protein interaction ( $P < 0.01$ ) for acetate. The SLICE test showed that when the MP supply increased, the predicted absorbed acetate decreased more with the LE treatment (−13%,  $P < 0.01$ ) than with the HE treatment (−5%,  $P < 0.01$ ).

**Milk Production and Composition During the Last 5 d of Each Period.** During the last 5 d of each period, milk, fat, and protein yields were higher with the HE treatment than the LE treatment (Table 3: +13%, +20%, and +19%, respectively;  $P < 0.01$ ). Milk and protein yields were also higher with the HP treatment (+17% and +15%, respectively;  $P < 0.01$ ), and fat yield tended to be higher (+12%,  $P = 0.07$ ). When the NE<sub>L</sub> supply increased, lactose yield increased (+16%,  $P < 0.01$ ), but tended to increase more (energy  $\times$  protein interaction:  $P = 0.09$ ) with the HP treatment (+19%,  $P < 0.02$ ) than with the LP treatment (+11%,  $P < 0.01$ ). When the MP supply increased, lactose yield also increased (+16%,  $P < 0.01$ ), but increased more with the HE treatment (+21%,  $P < 0.01$ ) than with the LE treatment (+13%,  $P < 0.01$ ). Energy in milk (Table 3) was higher when the NE<sub>L</sub> supply increased (+18%,  $P < 0.01$ ) and when the MP supply increased (+15%,  $P < 0.01$ ).

**Milk Production and Composition During Mammary Net-Balance Measurement.** Milk solids yields (Table 3) were higher with the HE treatment than the LE treatment and with the HP treatment than the LP treatment (+17% and +17%, respectively;  $P < 0.01$ ). Milk, lactose, fat, and protein yields from the left half-udder were higher with the HE treatment than the LE treatment (+13%, +13%, +21%, and +19%, respectively;  $P \leq 0.01$ ) and with the HP treatment than the LP treatment (+18%, +18%, +15%, and +17%, respectively;  $P \leq 0.01$ ). Milk lactose, fat, and protein concentrations were higher only with the HE treatment than the LE treatment (+3%, +8%, and +6%, respectively,  $P \leq 0.05$ ).

**Milk Fatty Acids.** The sum of C<sub>4</sub> to C<sub>8</sub> (Table 4) was higher with the HE treatment than the LE treatment (+4.4 g/12 h;  $P < 0.01$ ) and with the HP treatment

than the LP treatment (+2.9 g/12 h;  $P < 0.01$ ). The sum of C<sub>10</sub> to C<sub>14</sub> was higher with the HE treatment than the LE treatment (+11.4 g/12 h,  $P < 0.01$ ) and with the HP treatment than the LP treatment (+6.1 g/12 h,  $P = 0.04$ ). The sum of all C<sub>16</sub> was higher with the HE treatment than the LE treatment (+24.9 g/12 h,  $P < 0.01$ ) and with the HP treatment than the LP treatment (+4.5 g/12 h,  $P < 0.05$ ). The sum of all C<sub>18</sub> was higher with the HP treatment than the LP treatment (+18.2 g/12 h,  $P < 0.01$ ) but did not change when the NE<sub>L</sub> supply increased. These results led to a greater increase in synthesized FA (+30%) than in preformed FA (+13%) when the NE<sub>L</sub> supply increased. In contrast, preformed FA (+18%) increased more than synthesized FA (+11%) when the MP supply increased. Details on FA concentrations are presented in Supplemental Table S1.

### Circulating Metabolites

**Arterial Concentrations.** Arterial glucose did not differ among treatments (Table 5), but arterial plasma concentrations of acetate and BHB indicated an energy  $\times$  protein interaction ( $P = 0.01$ ) or a trend for an interaction ( $P < 0.07$ ), respectively. When the NE<sub>L</sub> supply increased, arterial plasma concentrations of acetate and BHB were higher only with the HP treatment (+34%,  $P = 0.02$ ; +44%  $P < 0.05$ , respectively). When the MP supply increased, the arterial plasma concentration of acetate was higher only with the HE treatment (+27%,  $P = 0.03$ ). The arterial plasma concentration of NEFA was lower with the HE treatment than the LE treatment (−35%,  $P = 0.04$ ). The arterial plasma concentration of TG was higher with the HP treatment than the LP treatment (+9%,  $P = 0.04$ ). The arterial blood concentration of O<sub>2</sub> was lower with the HE treatment than the LE treatment (−2.8%,  $P = 0.02$ ) but higher with the HP treatment than the LP treatment (+2.5%,  $P = 0.04$ ). The arterial blood concentration of CO<sub>2</sub> tended to be lower (−3.1%,  $P = 0.07$ ) with the HP treatment than the LP treatment.

**Mammary Clearance Rate.** The mammary clearance rate of plasma glucose (Table 6) increased with the HE treatment compared to the LE treatment (+37%,  $P = 0.01$ ) and tended to be higher with the HP treatment than the LP treatment (+21%,  $P = 0.09$ ). Mammary clearance rate of acetate was higher with the HE treatment than the LE treatment (+50%,  $P = 0.04$ ). Mammary clearance rate of NEFA was not significantly different from 0, except for the HELP treatment (which was negative). We observed a tendency for an energy  $\times$  protein interaction ( $P < 0.09$ ) in the mammary clearance rate of TG: increasing the MP supply increased it with the HE treatment ( $P = 0.02$  from the SLICE



**Table 5.** Effects of NE<sub>L</sub> and MP supplies on arterial concentrations of metabolites

Item	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Plasma								
Glucose, mM	3.21	3.20	3.32	3.14	0.082	0.27	0.77	0.33
Lactate, mM	0.252	0.230	0.222	0.225	0.0423	0.66	0.45	0.58
Acetate, mM	2.52	2.16	2.04	2.75	0.276	0.28	0.72	0.01
BHB, mM	1.95	1.76	1.54	2.22	0.462	0.26	0.89	0.07
NEFA, <sup>3</sup> μM	170	85	143	117	35.0	0.04	0.93	0.21
TG, <sup>4</sup> μM	60.2	54.5	62.9	62.6	3.15	0.19	0.04	0.23
Blood								
O <sub>2</sub> , mM	5.28	5.12	5.40	5.26	0.126	0.02	0.04	0.81
CO <sub>2</sub> , mM	28.3	27.4	27.0	27.0	0.523	0.29	0.07	0.29

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Nonesterified fatty acids.

<sup>4</sup>Triglycerides.

test). Blood O<sub>2</sub> clearance rate was higher in the HE treatment compared with the LE treatment (+49%,  $P = 0.02$ ). Mammary arteriovenous differences are presented in Supplemental Table S2 (<https://doi.org/10.5281/zenodo.8366232>; Anger et al., 2023).

**Mammary Net Uptake.** Mammary plasma and blood flows (Table 7) were higher with the HE treatment than the LE treatment (+32% and +31%, respectively;  $P < 0.01$ ). Glucose MGU (Table 7) was higher with the HE treatment than the LE treatment (+30%,  $P < 0.01$ ) and tended to be higher with the HP treatment than the LP treatment (+16%,  $P = 0.07$ ). Lactate MGU did not differ significantly from zero with any treatment ( $t$ -test). Acetate and BHB MGU were higher with the HE treatment than the LE treatment (+47%,  $P < 0.01$ ; +28%,  $P = 0.05$ , respectively) but did not differ with

MP supply. Left half-udder O<sub>2</sub> MGU (Table 7) was higher with the HE treatment than the LE treatment (+31%  $P < 0.01$ ) and tended to be higher with the HP treatment than the LP treatment (+16%,  $P < 0.10$ ). Mammary net output of CO<sub>2</sub> (negative net uptake, Table 7) was higher with the HE treatment than the LE treatment (+50%,  $P < 0.01$ ). The respiratory quotient (Table 7) was higher with the HE treatment than the LE treatment (+15%,  $P < 0.01$ ) but was lower with the HP treatment compared with the LP treatment (−8%,  $P = 0.05$ ). Mammary NEFA uptake was not different from zero ( $P > 0.10$ ). We observed an energy × protein interaction ( $P = 0.03$ ) for TG MGU: when the NE<sub>L</sub> supply increased, the TG MGU was higher with the HP treatment than the LP treatment (+30%,  $P = 0.01$ ), and when the MP supply increased, the TG

**Table 6.** Effects of NE<sub>L</sub> and MP supplies on clearance rate of metabolites

Item	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Plasma clearance rate, L/h								
Glucose	68	84	73	110	9.4	0.01	0.09	0.21
Acetate	281	536	407	498	93.0	0.04	0.53	0.26
BHB	81	100	99	108	24.9	0.41	0.44	0.75
NEFA <sup>3</sup>	31*	−54	35*	4*	21.3	<0.01	0.06	0.09
TG <sup>4</sup>	139	132	144	189	16.2	0.19	0.05	0.09
Blood clearance rate, L/h								
O <sub>2</sub>	128	159	137	204	17.7	0.02	0.13	0.29

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Nonesterified fatty acids.

<sup>4</sup>Triglycerides.

\*Not different from zero ( $P > 0.1$ ).

**Table 7.** Effects of NE<sub>L</sub> and MP supplies on mammary uptake of metabolites

Item	Diet <sup>1</sup>					P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP	SEM	Energy	Protein	E × P
MPF <sup>3</sup>	215	300	233	292	23.0	<0.01	0.78	0.44
MBF <sup>4</sup>	290	400	317	393	31.0	<0.01	0.65	0.46
Plasma, mmol/h								
Glucose	165	206	183	248	18.5	<0.01	0.07	0.42
Lactate	-0.5*	-7.8*	4.3*	2.0*	3.53	0.18	0.06	0.46
Acetate	294	409	288	444	35.4	<0.01	0.61	0.48
BHB	91	125	94	113	12.4	0.05	0.68	0.54
NEFA <sup>5</sup>	5.09*	-5.38*	4.56*	1.24*	3.011	<0.01	0.08	0.05
TG <sup>6</sup>	5.06	4.88	5.52	7.15	0.527	0.07	<0.01	0.03
Blood, mmol/h								
O <sub>2</sub>	465	576	509	698	45.2	0.01	0.10	0.40
CO <sub>2</sub>	-637	-922	-663	-1,025	73.6	<0.01	0.32	0.54
RQ <sup>7</sup>	1.38	1.61	1.30	1.46	0.056	<0.01	0.05	0.51

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Mammary plasma flow (L/h).

<sup>4</sup>Mammary blood flow (L/h).

<sup>5</sup>Nonesterified fatty acids.

<sup>6</sup>Triglycerides.

<sup>7</sup>Respiratory quotient (CO<sub>2</sub> release:O<sub>2</sub> uptake).

\*Not different from zero ( $P > 0.1$ ).

MGU was higher with the HE treatment than the LE treatment (+47%,  $P < 0.01$ ).

#### **Nutrient Partitioning Toward the Left Half-Udder and Left Half-Udder Carbon Balance.**

The percentage of MGU of glucose relative to predicted absorbed propionate plus intestinal glucose tended to be lower with the HE treatment than the LE treatment (40.3% vs. 33.7%;  $P = 0.07$ ) on a carbon basis (Table 8). This percentage was higher with the HP treatment than the LP treatment (42.0% vs. 32.0%;  $P = 0.01$ ). The ratio of the MGU of acetate and BHB to predicted absorbed acetate and butyrate, respectively, did not differ among the treatments.

Left half-udder MGU of total carbon (Table 8) was higher with the HE treatment than with LE treatment (+29%,  $P < 0.01$ ). Total carbon output (including CO<sub>2</sub>) was higher with the HE treatment than the LE treatment (+27%;  $P < 0.01$ ) and with the HP treatment compared with the LP treatment (+14%,  $P < 0.01$ ). Overall, the balance between mammary carbon uptake and output did not differ significantly from zero among the treatments.

Outputs of carbon in milk components synthesized by the mammary gland (i.e., lactose + AA in protein + synthesized FA) were higher with the HE treatment than the LE treatment (+20%,  $P < 0.01$ ) and with the HP treatment compared with the LP treatment (Table 8; +16%,  $P < 0.01$ ). On a carbon basis (Table 8), synthesized FA represented a higher percentage

of synthesized output in milk with the HE treatment (28.5%) than with the LE (26.2%) treatment ( $P < 0.01$ ), whereas carbon of lactose represented a lower percentage of synthesized output of carbon in milk with the HE treatment (38.2%) compared with the LE (40.3%) treatment ( $P < 0.01$ ). Synthesized FA represented a lower percentage of synthesized output in milk with the LP treatment (27.9%) than with the HP (26.8%) treatment ( $P = 0.04$ ) on a carbon basis. The total AA percentage of carbon output in milk (29.2%) did not differ among the treatments.

The balance between carbon uptake as glucose and carbon output as lactose was higher with the HE treatment than the LE treatment (+82%,  $P = 0.03$ ), and the ratio of lactose to MGU of glucose (on a carbon basis, Table 8) tended to be lower with the HE treatment compared with the LE treatment (65.6% vs. 76.4%, respectively;  $P = 0.07$ ). The balance, on a carbon basis, between glycerol MGU from TG and glycerol-3P in milk fat (i.e., synthesized glycerol) was negative for the 4 treatments, suggesting that MGU of glucose could serve to synthesize glycerol-3P. These negative balances were smaller in the HE treatment than the LE treatment (-15%;  $P < 0.01$ ) and with the HP treatment than the LP treatment (-8%). The balance between carbon of glucose taken up and the sum of carbon outputs from lactose and synthesized glycerol-3P (an estimate of glucose used in the pentose phosphate pathway, tricarboxylic acid cycle carbon

**Table 8.** Effects of NE<sub>L</sub> and MP supplies on mammary balance and partitions of carbons on the left half-udder during the evening milking of d 13

Carbon	Diet <sup>1</sup>				SEM	P-value <sup>2</sup>		
	LELP	HELP	LEHP	HEHP		Energy	Protein	E × P
Left udder uptake: digestive precursors, <sup>3</sup> %								
Glucose: (C3 + Int. GLC) <sup>4</sup>	35.1	28.9	45.5	38.5	3.31	0.07	0.01	0.40
Acetate + BHB: (C2 + C4) <sup>5</sup>	34.0	37.7	38.2	40.7	3.86	0.35	0.81	0.85
Mammary net balance								
Uptake, <sup>6</sup> mmol/h of carbon	2,447	3,120	2,694	3,520	228.0	<0.01	0.12	0.68
Glucose	990	1,237	1,098	1,487	111.0	<0.01	0.07	0.42
Lactate	-1*	-23*	13*	6*	10.6	0.18	0.06	0.46
AA	490	576	615	667	29.1	0.01	<0.01	0.39
EAA	327	378	447	479	18.4	0.05	<0.01	0.60
NEAA	163	198	168	188	15.4	<0.01	0.77	0.31
BHB	366	499	375	452	49.6	0.05	0.68	0.54
Acetate	588	817	577	888	70.9	<0.01	0.61	0.48
Glycerol <sup>7</sup>	15	15	17	22	1.6	0.07	<0.01	0.03
Output, <sup>8</sup> mmol/h of carbon	2,465	3,049	2,716	3,545	153.2	<0.01	0.01	0.29
Output in milk <sup>9</sup>	1,827	2,127	2,053	2,520	103.2	<0.01	<0.01	0.17
Lactose	735	802	837	980	56.1	<0.01	<0.01	0.15
Synthesized fatty acids <sup>10</sup>	486	619	526	700	29.3	<0.01	0.02	0.12
Glycerol-3P <sup>11</sup>	74	90	85	104	4.5	<0.01	<0.01	0.25
AA	532	615	606	736	32.2	<0.01	<0.01	0.38
EAA	275	319	314	381	16.8	<0.01	<0.01	0.32
NEAA	257	296	292	356	15.5	<0.01	<0.01	0.29
CO <sub>2</sub>	637	922	663	1,025	73.6	<0.01	0.32	0.54
Percentage of output in milk, %								
Lactose	40.3	37.7	40.4	38.7	1.28	<0.01	0.37	0.41
Synthesized fatty acids <sup>10</sup>	26.6	29.2	25.8	27.9	0.41	<0.01	0.04	0.53
Glycerol-3P <sup>11</sup>	4.1	4.2	4.1	4.1	0.07	0.27	0.72	0.32
AA	29.0	28.9	29.7	29.3	1.32	0.78	0.50	0.87
Balance, mmol/h of carbon	-17*	71*	-22*	-25*	114.7	0.71	0.66	0.69
Glucose – lactose	255	435	262	508	84.4	0.03	0.62	0.68
Synthesized glycerol-3P <sup>12</sup>	-59	-76	-68	-83	3.3	<0.01	<0.01	0.53
Glucose – (lactose – synthesized G3P) <sup>13</sup>	315	510	329	590	84.7	0.02	0.56	0.68
AA balance	-42*	-39*	9*	-70	26.8	0.15	0.67	0.13
EAA	52	59	133	98	17.8	0.47	0.02	0.28
NEAA	-94	-98	-124	-168	14.4	0.08	<0.01	0.13
(Acetate + BHB) – synthesized FA <sup>14</sup>	468	696	426	640	102.0	0.04	0.58	0.93
Output:uptake, <sup>15</sup> %								
Lactose:glucose	75.5	64.7	77.2	66.4	5.50	0.07	0.74	1.00
Synthesized fatty acids: (acetate + BHB) <sup>16</sup>	52.0	47.2	57.6	53.6	4.54	0.25	0.14	0.92

<sup>1</sup>LELP = low energy and low protein, HELP = high energy and low protein, LEHP = low energy and high protein, HEHP = high energy and high protein.

<sup>2</sup>Probability values correspond to the effect of NE<sub>L</sub> supply (LE vs. HE), effect of MP supply (LP vs. HP), and interaction between energy (E) and protein (P): E × P.

<sup>3</sup>Percentage of nutrients taken up by the left udder relative to estimated absorbed digestive precursors from Table 2 (all expressed in millimoles of carbon).

<sup>4</sup>Left-udder uptake of glucose: (rumen propionate + intestinal glucose).

<sup>5</sup>Sum of left-udder uptakes of (acetate + BHB):(rumen acetate + butyrate).

<sup>6</sup>Sum of carbon from glucose, lactate, glycerol from free glycerol plus glycerol liberated from triglycerides, acetate, BHB, and all AA uptakes.

<sup>7</sup>Glycerol from triglycerides analyses (i.e., free glycerol + glycerol of triglycerides).

<sup>8</sup>Sum of carbon outputs in milk plus blood CO<sub>2</sub> production.

<sup>9</sup>Output in milk (i.e., sum of carbon from milk lactose plus milk protein plus milk glycerol-3P in milk triglycerides plus fatty acids within the mammary gland).

<sup>10</sup>Milk fatty acids synthesized within the mammary gland [(C<sub>4</sub> to C<sub>13</sub>) × 1 + (C<sub>14</sub> + C<sub>15</sub>) × 0.85 + (C<sub>16</sub> + C<sub>17</sub>) × 0.60].

<sup>11</sup>Milk glycerol-3P in triglycerides.

<sup>12</sup>Glycerol uptake – glycerol-3P output in milk (i.e., glycerol-3P synthesized within the mammary gland).

<sup>13</sup>Carbon from glucose uptake minus lactose and glycerol-3P synthesized. See text for details.

<sup>14</sup>Carbon from BHB + acetate uptakes – fatty acids (FA) synthesized.

<sup>15</sup>Percentage of output on uptake (mmol of C/mmol of C × 100).

<sup>16</sup>Synthesized fatty acids (acetate + BHB uptakes).

\*Not different from zero ( $P > 0.1$ ).

and isocitrate dehydrogenase pathway) was also higher (+71%,  $P < 0.02$ ) with the HE treatment than the LE treatment. The balance between BHB and acetate uptake and synthesized FA was higher with the HE treatment than the LE treatment (+49%,  $P = 0.04$ ), but the ratio of output in milk (i.e., synthesized FA) to their MGU did not differ, on a carbon basis. The balances between glucose uptake and lactose output and between BHB and acetate uptake and synthesized FA, on a carbon basis, did not differ with the MP supply. The carbon balance between total AA uptake and milk-protein output did not differ significantly from zero, except for the HEHP treatment ( $-70$  mmol/h per half-udder,  $P = 0.04$ ). The carbon balance between EAA uptake and output was higher with the HP treatment than the LP treatment (+108%,  $P = 0.02$ ). In parallel, the carbon balance between NEAA uptake and output was negative and tended to be lower with the HE treatment than the LE treatment ( $-22%$ ,  $P = 0.08$ ) and be lower with the HP treatment compared with the LP treatment ( $-51%$ ,  $P < 0.01$ ).

## DISCUSSION

The aim of this experiment was to study the response of mammary metabolism to increased  $NE_L$  or MP supply obtained through dietary treatments to explain the variations in milk yield and composition observed. We chose to modify  $NE_L$  and MP supplies through dietary treatments because our INRA feeding system mainly predicted milk yield in response to increased  $NE_L$  and MP supplies. This approach was original compared with most of mammary net-balance studies (Lemosquet et al., 2009b; Nichols et al., 2019a; Danes et al., 2020) that modified the  $NE_L$  or MP supply through digestive infusions of one energetic nutrient or a mixture of AA, respectively. A limitation of this experiment is that we were unable to create increments of similar magnitude for  $NE_L$  (+30%) and MP supplies (+78%). Also, the feed offered was restricted to achieve low-energy supply and differences between treatments. Therefore, we could not dissociate the effect of increased DMI from increased  $NE_L$ . When increasing  $NE_L$  supply, the changes in diet composition accompanied by the increase in DMI increased starch (+75%) and EE (+28%) supplies and were estimated to increase the absorption of several nutrients: intestinal absorption of glucose (+79%, on a carbon basis) and rumen absorption of VFA (+31%). The MP supply (+78%), increased by changing the diet composition, mainly resulted in increased the supply of individual AA (+78%; Omphalius et al., 2019); however, it also slightly increased the  $NE_L$  supply (+3%) by increasing the EE supply (+10%). The increased MP supply was also accompanied by a decrease in predicted

intestinal absorption of glucose ( $-18%$ ) and predicted rumen absorption of VFA ( $-7%$ , mainly acetate) because the 2 HP diets contained less starch and NDF than the 2 LP diets. Nevertheless, increasing the  $NE_L$  and MP supplies in the present study increased milk, lactose, fat, and protein yields, as usually reported (Daniel et al., 2016).

This approach differed from experiments that increased the MP supply via postpartum infusions of CN or mixtures of AA (Raggio et al., 2006; Rius et al., 2010a; Nichols et al., 2019c), which did not change the supply of energy-yielding nutrients. The changes in several energetic nutrients when increasing the  $NE_L$  supply and in the supplies of nutrients other than AA when increasing the MP supply could explain some of the differences in milk secretion and mammary metabolism observed between the present study and the mammary net-balance literature using one specific nutrient infusion. These differences are taken into account in this discussion. Finally, the effects of  $NE_L$  and MP supplies on milk yield and composition were mainly additive, as expected (Daniel et al., 2016); however our experimental design, with a limited number of cows due to mammary net-balance measurements, was less powerful at detecting interactions than that used by Brun-Lafleur et al. (2010). The potential additivity will not be discussed. Therefore, we first discuss effects of an increased  $NE_L$  supply on milk production, milk composition, and mammary metabolism before discussing effects of increased MP supply. Finally, we discuss the efficiencies of milk synthesis when increasing either the  $NE_L$  or the MP supplies.

### Responses to Increasing the $NE_L$ Supply

**Increasing the  $NE_L$  Supply Increased Lactose Yield and Synthesized FA.** Increasing the  $NE_L$  supply increased protein, lactose, and fat yields; increased fat yields were the result of increasing FA synthesized in the mammary gland (+33.0 g/12 h per half-udder, Table 4) and by increasing preformed FA in milk (+15.5 g/12 h per half-udder). The estimated increase in absorption of precursors (VFA and glucose; Table 2) when the  $NE_L$  supply increased likely increased the lactose yield and synthesized FA. Experiments with postpartum infusion of glucose or rumen infusion of propionate led to an increase in milk and lactose yields (meta-analysis of Rigout et al., 2003), but these infusions decreased milk fat yield (Rigout et al., 2002a; Lemosquet et al., 2009b), mainly via  $C_{18}$  FA, because they did not change FA synthesis in the mammary gland. In contrast, experiments with acetate (Urrutia and Harvatine, 2017; Danes et al., 2020) or butyrate infusions (Huhtanen et al., 1993) increased milk fat yield (Danes et al., 2020).



Overall, in the present study, the estimated increased ruminal absorption of acetate and butyrate supports the increase in milk fat yield.

In contrast, the increase in milk protein yield in response to increasing the  $NE_L$  supply was the result of metabolic adaptations, because the digestive absorption of precursors of milk protein (i.e., individual AA) did not increase when the  $NE_L$  supply increased, except for a slight increase in Met supply (Omphalius et al., 2019). Therefore, increasing the  $NE_L$  supply increased the efficiency with which absorbed EAA were used (Omphalius et al., 2019). Increasing the  $NE_L$  supply could have directly or indirectly stimulated, via hormones such as insulin or insulin-like growth factor 1 (Rigout et al., 2002a; Lemosquet et al., 2009a), the signaling pathways of mammary protein synthesis, including enzymes or constitutive proteins (Raggio et al., 2006). Genes involved in the different pathways of protein synthesis ( $\alpha$ -lactalbumin,  $\kappa$ -CN, acetyl-CoA carboxylase) were modified when DMI was modified in dairy cows (Boutinaud et al., 2019), although increasing  $NE_L$  supply did not generally affect expression of milk-protein genes (Cant et al., 2018), whereas glucose infusion reduced CN2 expression (Nichols et al., 2020). Increasing  $NE_L$  supply could also upregulate cell proliferation compared with cell death as observed when DMI was modified in dairy cows (Boutinaud et al., 2019) or when glucose availability was modified in vitro (Wang et al., 2019). These mechanisms could explain the simultaneous increase in milk lactose, fat, and protein yields (Toerien et al., 2010; Rius et al., 2010a). All this suggests that the increase in the supply of energy-yielding nutrients is associated with molecular and cellular adaptations to increase simultaneously lactose, protein yield, and synthesized FA when increasing the  $NE_L$  supply.

**Mammary Net Uptake of Nutrients Increased with  $NE_L$  Through MPF.** We observed no increase in the arterial plasma concentration of glucose, the main precursor of lactose, despite an increase in the prediction of absorption of available precursors of glucose and glucose itself. Plasma concentrations reflect the equilibrium between digestive absorption and metabolic use. This lack of increase in plasma arterial concentration of glucose was consistent with results of studies that increased the amount of feed supplied (Guinard-Flament et al., 2007) but was not consistent with studies that infused propionate in the rumen or infused postrumen glucose (Lemosquet et al., 2009b, 2010; Rius et al., 2010a). This lack of increase could be the result of an increase in plasma insulin as is often observed when increasing feeding level (Leduc et al., 2021) but not always observed when increasing only one energetic nutrient by infusions (Lemosquet et al., 2009a,b). Rulquin et al. (1983) also observed no in-

crease in arterial plasma glucose when infusing a mixture of VFA. Arterial plasma concentrations of acetate and BHB, the main precursors of synthesized FA, increased or tended to increase only when the  $NE_L$  supply increased with the HP treatments (energy  $\times$  protein interaction). Predicted ruminal acetate absorption in response to increased  $NE_L$  also increased more with the HP treatment than with the LP treatment (Table 2; energy  $\times$  protein interaction). However, the MGU of acetate and BHB significantly increased when the  $NE_L$  supply increased, with no interaction, as observed when increasing feeding levels (Guinard-Flament et al., 2007). In contrast, experiments with digestive infusions of glucose or propionate decreased or tended to decrease the MGU of plasma BHB (Rigout et al., 2002a; Lemosquet et al., 2009b; Nichols et al., 2019a). These differences in MGU of plasma BHB between our experiment and infusions of glucose and propionate seemed to be linked to the different changes in arterial supply. Overall, increasing the  $NE_L$  supply increased the MGU of all main energy-yielding nutrients (glucose, acetate, and BHB) as the prediction of their digestive precursors (VFA and intestinal glucose) supplies increased.

The increase in MPF, when the  $NE_L$  supply increased, mainly explained the increase in MGU of energy-yielding nutrients and of total EAA and NEAA (Table 8; further details on individual AA in Omphalius et al., 2019). This increase in MGU increased the yields of the 3 main milk components. As Omphalius et al. (2019) discussed, the MPF may have increased because of an increase in the  $NE_L$  supply (Raggio et al., 2006) or an increase in DMI (Lough et al., 1990; Guinard-Flament et al., 2007), or both. However, as discussed, MPF was observed to increase with glucose and propionate infusions (Rigout et al., 2002a; Lemosquet et al., 2009b; Nichols et al., 2019a), without any increase in  $NE_L$  or DMI. In contrast, increasing lipogenic nutrient supplies, such as acetate infusion (Danes et al., 2020), or fat via palm-oil infusion (Nichols et al., 2019a,b), did not increase the MPF. In the present study, the increase in MPF was likely due to the increase in starch supply, and consequent increment of estimated glucose and propionate absorption, and not to the predicted increase in other nutrients such as acetate and butyrate or fat (i.e., increase in EE). In terms of mechanism, because insulin and insulin-like growth factor 1 are known to regulate MPF (Prosser et al., 1996; Cant et al., 2018), the observed effect of increased  $NE_L$  supply on MPF may be linked to an increase in these circulating hormones, often reported when DMI varies (Leduc et al., 2021).

**All Milk Components Increased When the  $NE_L$  Supply Increased, but Not to the Same Extent.** The increase in MPF could not explain all changes

in milk-component secretion observed when the  $NE_L$  supply increased. Increasing the  $NE_L$  supply increased milk fat yield (+21%) and synthesized FA (+30%) more than it increased lactose (+13%) output (in g/h for a half-udder on a carbon basis). Synthesized FA represented a higher percentage of the carbon output in milk (i.e., lactose + AA in protein + synthesized FA) with the HE treatment compared with the LE treatment. In contrast, lactose output represented a smaller percentage of the carbon output in milk with the HE treatment compared with the LE treatment. In previous studies, lactose increased more than milk fat yield, but the increase in  $NE_L$  supply was smaller (+11% in Rius et al., 2010b; +14% in Laroche et al., 2022) than that in the present study (+30%). However, Brun-Lafleur et al. (2010) reported a linear increase in milk fat yield in response to increasing the  $NE_L$  supply, whereas milk yield increased according to a curvilinear response. This could explain why, in the current experiment with an increment of  $NE_L$  supply by +30%, milk fat yield increased more than lactose yield. In terms of mechanism, the possible increase in mammary tissue affinity to glucose and acetate observed through the increased clearances of glucose and acetate did not explain the higher increase in milk fatty-acid synthesis compared with lactose secretion. It is well known that acetate uptake mainly depends on arterial availability, which is less true for glucose (Miller et al., 1991). The larger increase in synthesized FA compared with lactose output in the present study suggested that FA synthesis could be a buffer metabolic pathway to control the excess  $NE_L$  supply, as suggested by Guinard et al. (2011). This suggested that despite a close increase in MGU of glucose (+318 mmol/h of carbon per half-udder) compared with MGU of acetate plus BHB (+375 mmol/h of carbon), lactose synthesis seemed to be more limited than FA synthesis. Glucose could be used in metabolic pathways other than lactose synthesis, including the pentose phosphate pathway to synthesize NADPH necessary to sustain FA syntheses.

**Increasing the  $NE_L$  Supply Changed Nutrient Partitioning.** The mammary gland did not use all of the nutrients available from the increased  $NE_L$  in the HE treatment. The balance of  $NE_L$  increased when the  $NE_L$  supply increased because the energy exported in milk (+19%; Table 3) increased less than the  $NE_L$  supply did (+30%). The changes in partitioning could also explain the differences in increased synthesis of milk lactose and fat, as well as protein yield. The MGU of glucose tended to increase proportionally less than the prediction of digestive absorption of propionate and glucose, whereas the MGU of acetate plus BHB increased proportionally to their predicted supply. As discussed above, this change in extramammary partition could

be linked to higher plasma insulin as observed when increasing feeding level (Leduc et al., 2021). Increasing the  $NE_L$  supply also changed the intramammary partitioning of glucose because the MGU of glucose tended to increase more than lactose output did, as indicated by the ratio of lactose to the MGU of glucose. In contrast, synthesized FA increased in parallel with the increase in the MGU of acetate plus BHB. These results suggest a change in the partitioning of glucose use in the whole body and udder but not for acetate or BHB, which could also explain the smaller increase in lactose output than in synthesized FA output. In fact, glucose is required for NADPH synthesis, which is necessary for FA syntheses (as previously discussed), glycerol-3P for TG synthesis and also to furnish ATP through oxidation (Lemosquet et al., 2009b).

### **Mammary Responses to Increasing the MP Supply**

**Increasing the MP Supply Increased Lactose and Fat Yields.** Increasing the MP supply increased milk lactose yield by 18%, milk fat yield by 15%, and milk protein yield by 17% for the evening milking of mammary net balance, as reported previously (Omphalius et al., 2019). Increasing MP supply has been reported to increase lactose and milk fat yields in meta-analyses of dietary treatments (Daniel et al., 2016) or with CN infusions in mammary net-balance studies (Lemosquet et al., 2010; Daniel et al., 2016). In more recent mammary net-balance experiments, milk lactose and fat did not increase in some studies (Rius et al., 2010a,b) but did increase in others (Nichols et al., 2019a,c). The trend for increased glucose MGU allowed lactose yield to increase in the present study despite the decrease in predicted propionate and glucose digestive absorption, which suggests a shift in the partitioning of glucose toward the mammary gland. An increase in the whole-body glucose rate of appearance with increased AA supply (Lemosquet et al., 2009b; Galindo et al., 2011; Nichols et al., 2022) could also explain this trend. In the left half-udder, this trend was not the result of a change in MPF, as reported in the meta-analysis of Lemosquet et al. (2010), but mammary glucose clearance tended to increase. The mammary gland tended to increase the MGU of glucose despite the decrease in the starch supply and estimated intestinal glucose to support the increases in milk lactose in response to increased MP supply. We observed no change in intramammary glucose partitioning when the MP supply increased (Table 8), in agreement with Galindo et al. (2011) but unlike Lemosquet et al. (2009b) and Nichols et al. (2019a). Danes et al. (2020) observed significant changes in the MGU of glucose depending on the combination of nutrients supplied (acetate vs. glucose or

CN; glucose vs. CN plus glucose). This suggests that the MGU of glucose could depend on the supply and MGU of other nutrients.

**Preformed FA in Milk Increased More Than Synthesized FA.** The increase in milk fat yield increased preformed FA and synthesized FA in the mammary gland in response to increased MP supply (Table 4), but the increase was larger for preformed FA than for synthesized FA, unlike in the studies of Hurtaud et al. (1993) and Nichols et al. (2019b). The increase in milk fat yield from preformed FA could not be due to higher fat mobilization with the HP treatment compared with the LP treatment because the MGU of TG increased when the MP supply increased only for the HE treatments (energy  $\times$  protein interactions). In addition, the arterial plasma concentration of NEFA did not increase, whereas that of TG increased when the MP supply increased. Another explanation for this increase in preformed FA in the mammary gland was the higher EE (+108 g/d) with the HP treatment compared with the LP treatment, as observed by Nichols et al. (2019c) as palm oil increased, which increased the MGU of long-chain FA and their milk outputs. The increase in synthesized FA when the MP supply increased was not related to an increase in MGU of BHB, unlike what was reported in most studies (Lemosquet et al., 2009a, 2010; Nichols et al., 2019a). As expected, MGU of acetate did not change (Lemosquet et al., 2010; Nichols et al., 2019b). The mammary carbon balance remained equilibrated in response to the increased MP supply, suggesting that the slight increase in synthesized FA was possible with no change in MGU of BHBA and acetate. This absence of an increase in MGU of acetate and BHBA in response to increasing MP supply could also explain why the increases in milk fat yield and synthesized FA were smaller when the MP supply increased than when the  $NE_L$  supply did.

### Efficiencies in Lactose and Fat Syntheses

**Increasing the MP Supply Increased Nutrient-Use Efficiency.** Increasing the MP supply increased milk solids despite the predicted decreases in the digestive absorptions of glucose and VFA. The efficiency of utilization of predicted digestive nutrients in milk-solid secretion was higher when MP supply increased than when  $NE_L$  supply increased, although MP efficiency decreased greatly (Omphalius et al., 2019). Increasing the MP and  $NE_L$  supplies increased the predicted digestive supplies (VFA + glucose + total AA) by 517 and 1,689 g/d, respectively, and milk solids by 433 and 518 g/d, respectively. However, the extent of the gain of efficiency with increasing MP supply depended on the accuracy of the predictions of nutrient absorbed. The

INRA (2018) system predicted a decrease in glucose and propionate absorbed, because starch flux decreased between LP and HP treatments. Concerning the prediction of VFA molar percentages, and therefore those of acetate and butyrate, they presented relative low root mean square errors compared with other models (Nozière et al., 2011). In addition, this gain in efficiency was also observed in response to CN infusion compared with propionate infusion that did not reduce the intake of energy nutrients. When the MP supply increased, MGU of EAA increased (for individual AA see Omphalius et al., 2019), and MGU of glucose tended to increase (Raggio et al., 2004; Lemosquet et al., 2010; Nichols et al., 2019b). This occurred with no change in mammary MPF, unlike when the  $NE_L$  supply increased, but only through increases or tendencies to increase in mammary clearance rates (for glucose, TG precursors of preformed FA and EAA reported in Omphalius et al., 2019). To summarize, without an increase in energy precursor absorption when the MP supply increased, partitioning of glucose, TG, and EAA seemed to change because of increased MGU through increased mammary affinity to these nutrients. Based on the literature, stimulating mammary protein synthesis and signaling pathways (Rius et al., 2010b; Arriola Apelo et al., 2014; Nichols et al., 2016) could increase all 3 syntheses when MP and  $NE_L$  supplies increase, as discussed for the  $NE_L$  supply. In addition, the duration of treatments was long enough for mechanisms other than protein-signaling pathways to appear, such as endothelium reticulum biogenesis (Cant et al., 2018; Nichols et al., 2020) to sustain all milk-components synthesis.

**Decreased Nutrient Efficiencies When  $NE_L$  Supply Increased Because of an Increase in Mammary Oxidation of Nutrients.** We hypothesized no change in the partitioning of energy-yielding nutrients in the mammary gland when the MP supply increased because the estimated amount of carbon from acetate and BHBA available for oxidation and NEAA synthesis (i.e., differences between MGU of acetate + BHB – synthesized FA) and the estimated amount of carbon of glucose available for the pentose phosphate pathway, tricarboxylic acid cycle, and isocitrate dehydrogenase pathways (i.e., the differences between [glucose minus (lactose and glycerol-3P)] did not change (Table 8). In addition, mammary  $CO_2$  output did not change when the MP supply increased. In contrast, the decrease in mammary efficiency of utilization of nutrients observed when the  $NE_L$  supply increased also likely occurred through an increase in glucose, acetate, and BHB oxidation in the mammary gland. The  $CO_2$  output in the blood increased because the remaining glucose, acetate, and BHB were available for pathways other than lac-



tose and FA synthesis. Mammary blood CO<sub>2</sub> output increased more than MGU of O<sub>2</sub>, which significantly increased the respiratory quotient; this was consistent with an increase in the synthesis of FA from acetate, in which O<sub>2</sub>-rich nutrients are transformed into O<sub>2</sub>-poor nutrients (Thivierge et al., 2002). Last, AA carbon balances did not differ significantly from zero (except with HEHP treatment), which suggests that on a net basis little carbon from the AA taken up could be used for syntheses other than for milk protein. However, the carbon of branched-chain AA contributed to FA synthesis at least in vitro (Roets et al., 1983). Overall, increasing the NE<sub>L</sub> or MP supply increased milk lactose, fat, and protein yields through different changes in MGU and mammary metabolism. The increase in mammary efficiency of energy-yielding nutrients observed when the MP supply increased was due to the increase in secretion of the 3 components in milk, with an increase in only the MGU of EAA (Omphalius et al., 2019) and glucose. These selective MGU increases were the result of increased arteriovenous difference, with no change in MPF. The decreased efficiency as NE<sub>L</sub> increased was due to a change in partitioning toward tissues other than the mammary gland and higher nutrient oxidation in the mammary gland.

## CONCLUSIONS

Increasing the NE<sub>L</sub> and MP supplies increased milk, lactose, fat, and protein yields, but not to the same extent. Increasing the NE<sub>L</sub> supply increased milk fat more than lactose yields and induced different mammary metabolic adaptations than increasing the MP supply. Increasing the NE<sub>L</sub> supply increased most of the MGU of nutrients via increased MPF. Increasing the MP supply tended to increase or increased only the MGU of glucose and AA through their clearances. This experiment confirmed the high mammary metabolic flexibility, which increased its uptakes of nutrients through 2 mechanisms: blood flow and tissue affinity. However, increasing the NE<sub>L</sub> supply increased mammary oxidation of nutrients, contrasting with the higher mammary efficiency of utilization of energy-yielding nutrients with increasing the MP supply. These results also suggest that the stimulation of protein synthesis could regulate the MGU of energetic nutrients and their utilization in milk syntheses and oxidation.

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