Milk production and efficiency of utilization of nitrogen, metabolizable protein, and amino acids are affected by protein and energy supplies in dairy cows fed alfalfa-based diets

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ABSTRACT

Alfalfa has a lower fiber digestibility and a greater concentration of degradable protein than grasses. Dairy cows could benefit from an increased digestibility of alfalfa fibers, or from a better match between nitrogen and energy supplies in the rumen. Alfalfa cultivars with improved fiber digestibility represent an opportunity to increase milk production, but no independent studies have tested these cultivars under the agroclimatic conditions of Canada. Moreover, decreasing metabolizable protein (MP) supply could increase N use efficiency while decreasing environmental impact, but it is often associated with a decrease in milk protein yield, possibly caused by a reduced supply of essential AA. This study evaluated the performance of dairy cows fed diets based on a regular or a reduced-lignin alfalfa cultivar and measured the effect of energy levels at low MP supply when digestible His (dHis), Lys (dLys), and Met (dMet) requirements were met. Eight Holstein cows were used in a double 4 × 4 Latin square design, each square representing an alfalfa cultivar. Within each square, 4 diets were tested: the control diet was formulated for an adequate supply of MP and energy (AMP_AE), whereas the 3 other diets were formulated to be deficient in MP (DMP; formulated to meet 90% of the MP requirement) with deficient (94% of requirement: DMP_DE), adequate (99% of requirement: DMP_AE), or excess energy supply (104% of requirement: DMP_EE). Alfalfa cultivars had no significant effect on all measured parameters. As compared with cows receiving AMP_AE, the dry matter intake of cows fed

DMP_AE and DMP_EE was not significantly different but decreased for cows fed DMP_DE. The AMP_AE diet provided 103% of MP and 108% of NE_L requirements whereas DMP_DE, DMP_AE, and DMP_EE diets provided 84, 87, and 87% of MP and 94, 101, and 107% of NE_L requirements, respectively. In contrast to design, feeding DMP_EE resulted in a similar energy supply compared with AMP_AE, although MP supply has been effectively reduced. This resulted in a maintained milk and milk component yields and improved the efficiency of utilization of N, MP, and essential AA. The DMP diets decreased total N excretion, whereas DMP_AE and DMP_EE diets also decreased milk urea-N concentration. Reducing MP supply without negative effects on dairy cow performance is possible when energy, dHis, dLys, and dMet requirements are met. This could reduce N excretion and decrease the environmental impact of milk production.

Key words: amino acid, histidine, rumen-protected lysine, rumen-protected methionine, fatty acid

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most widely grown forage legume in Canada (Statistics Canada, 2021). Legumes have a lower fiber concentration than grasses, but their fibers are generally less digestible (Hoffman et al., 1998). Based on intake potential and energy availability, increasing digestibility of legume cell walls would be beneficial for ruminants (Jung and Allen, 1995). In addition, alfalfa has a high CP concentration, but a large portion of this CP is either NPN or true protein highly degradable in the rumen (NRC, 2001). Utilization of these highly degradable CP by rumen microorganisms could be enhanced by a greater availability of energy substrates in the rumen. Therefore, increasing the digestibility of legume cell walls would

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also be beneficial for ruminants by increasing rumen microbial protein synthesis (Jung and Allen, 1995).

Improving fiber digestibility would be an opportunity to increase profitability and decrease environmental impacts for the dairy sector (Hatfield et al., 1999). According to a meta-analysis (Oba and Allen, 1999), an increase of 1 percentage unit of *in vitro* or *in situ* NDF digestibility (**NDFd**) is associated with a 0.25 kg/d increase in 4% FCM. Several alfalfa cultivars with enhanced digestibility have recently been commercialized in North America. Given the lack of independent studies testing these cultivars under the agroclimatic conditions of Canada, our first objective was to verify the potential benefits of using an alfalfa cultivar with improved digestibility in dairy rations.

Increasing prices of feed ingredients and the need to reduce environmental impact of dairy production have stimulated interest in reducing CP concentration in dairy rations (e.g., Lee et al., 2012b). Dairy cows are regularly fed diets providing excess protein, whereas milk production and composition could be maintained, or even improved, with reduced CP content of the diets (Broderick, 2018). Reducing dietary CP concentration could enhance profitability by improving N efficiency from diet to milk while reducing N excretion in the environment (Holt et al., 2013). Although current protein requirements of dairy cows are expressed in MP rather than CP (e.g., NRC, 2001), cows fed diets providing the required MP supply might be overfed in some EAA and underfed in others, resulting in a poor N utilization efficiency (Arriola Apelo et al., 2014). A decrease in MP supply is often associated with a reduced milk protein yield (**MPY**), probably because requirements for one or many EAA are not met (Arriola Apelo et al., 2014). Therefore, it is of utmost importance to consider EAA supply when formulating low-MP dairy rations.

Methionine and Lys are usually considered as first limiting AA for milk protein production in cows fed typical North American dairy diets (NRC, 2001). However, because microbial protein has a lower His content than milk and feed ingredients (NRC, 2001; Sok et al., 2017), it may become a limiting AA for milk production and MPY in diets supplying a high proportion of MP from microbial protein, as is the case when low-MP diets are fed to dairy cows (Huhtanen et al., 2002; Lee et al., 2012c; Giallongo et al., 2016).

Effects of energy and MP supplies on MPY have been reported to be additive and independent (Broderick, 2003; Raggio et al., 2006; Omphalius et al., 2019a), except in one study (Brun-Lafleur et al., 2010). Increased energy intake or postruminal glucose infusion led to an increased efficiency use of MP, whereas an increased MP supply decreased it (Omphalius et al., 2019a,

2020). Maintaining an adequate supply of EAA with a reduced supply of MP combined with an increased energy supply might further increase MP efficiency use and MPY.

Therefore, the objectives of the current study were (1) to compare the performance of dairy cows fed a diet based on a regular alfalfa cultivar to those fed a diet based on a cultivar selected for its reduced lignin concentration; and (2) to determine the effect of decreasing MP supply but maintaining adequate His, Lys, and Met supplies at different energy levels on cow performance and efficiency of N utilization. We hypothesized that cows fed an alfalfa cultivar selected for its reduced lignin concentration would have a greater DMI and produce more milk. We also hypothesized that, for a same energy supply, cows fed a MP-deficient diet with adequate His, Lys, and Met supplies would have a comparable MPY than cows fed a MP-adequate diet, and that increasing dietary energy content at low MP but adequate His, Lys, and Met supplies would lead to a further increment of MPY and efficiency of N utilization.

MATERIALS AND METHODS

Haylage Production

Two alfalfa cultivars were seeded on an area of 16 ha at the Université Laval's agronomic research station in Saint-Augustin-de-Desmaures (46°43'26"N, 71°30'1"W). Four fields were sown with both cultivars; to avoid contamination between them, some parts of the fields were not harvested. A regular alfalfa cultivar ('Calypso', Sollio Cooperative Group; as control) and an alfalfa cultivar selected for its reduced lignin concentration ('Boost' HG, Quality Seeds) were used for the experiment (Table 1).

In the first postseeding year, the first cut of alfalfa was harvested on June 19, 2018, at the early-bloom stage of development with a disc mower (Krone Easy Cut 9140 HP, Krone NA Inc.). Forages were wilted in the field until they reached about 45% of DM to promote preservation without the addition of inoculants (Coblentz et al., 2016). Forages were then harvested as large rectangular bales with a roto-cut press (Kuhn LSB 890 D, Kuhn) and plastic coated with a wrapper (Anderson SB-780, Groupe Anderson Inc.).

Cows, Diets, and Experimental Design

The experiment was carried out at the Centre de Recherche en Sciences Animales de Deschambault (QC, Canada) from March to May 2019. All procedures in-

volving dairy cows were approved by the animal care committee of Université Laval in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009). We used 8 multiparous Holstein dairy cows, averaging at the initiation of the trial (mean $\pm$ SD) 117 ± 42 DIM, 764 ± 79 kg of BW, and 40.9 ± 4.8 kg of milk/d, housed in a tiestall barn. Parity of the cows was on average 3.5 (25% second lactation, 25% third lactation, 50% greater than third lactation). Animals were distributed in a double 4×4 Latin square design balanced for residual effects, with 21-d periods.

Cows were assigned to each Latin square ensuring that the average milk yield and DIM was equivalent between squares. Cows of the first square (41.9 ± 5.3 kg of milk/d; 109 ± 41 DIM; mean $\pm$ SD) were fed haylage made with the regular alfalfa cultivar, whereas those of the second square (39.9 ± 4.9 kg of milk/d; 124 ± 47 DIM) were fed haylage made with the reduced-lignin alfalfa cultivar. In each square, cows were randomly assigned to treatments according to a 4×4 Latin square design. Energy and protein requirements were based on milk production and intake measured 3 d before the beginning of the experiment. Considering the data collected during this pre-experimental period, diets were formulated to meet the nutritional requirements of a 764-kg lactating cow, eating 27.2 kg DM/d, and producing 40.9 kg milk/d with a concentration of milk fat and true protein of 3.90% and 3.17%, respectively. Treatments were 4 diets: the control diet supplied

adequate levels of MP and energy (**AMP_AE**; i.e., 100% of requirements; NRC, 2001), whereas the 3 other diets were MP deficient (**DMP**), formulated to meet 90% of MP requirement (NRC, 2001) with deficient (94% of requirement: **DMP_DE**), adequate (99% of requirement: **DMP_AE**), or excess supply of energy (104% of requirement; **DMP_EE**). Metabolizable protein requirement was estimated at 2,895 g/d and the 3 DMP diets were estimated to be 276 g MP/d deficient. Energy requirement was estimated at 41.5 Mcal NE_L/d and supply of NE_L was estimated to be 41.5, 39.2, 41.1, and 43.0 Mcal/d for AMP_AE, DMP_DE, DMP_AE, and DMP_EE diets, respectively. As the analyses did not indicate any difference in chemical composition and digestibility between alfalfa haylages (Table 1), the average nutrient composition of both alfalfa haylages was used to formulate diets. Requirements of Met and Lys were estimated at 2.38% and 6.95% of MP supply from the AMP_AE diet, respectively (Whitehouse et al., 2010a,b); these amounts in grams per day were maintained in the DMP diets. As a consequence, His, Lys, and Met supplies expressed as percentages of MP were higher for DMP treatments. Because metabolisms of His and Met are similar in splanchnic and mammary gland tissue (Lapierre et al., 2012) and because these AA have a similar concentration in milk protein (Lapierre et al., 2020), His requirement was assumed to be similar to that of Met. Rumen-protected (**RP**) AA were used to ensure a sufficient supply of Lys and Met. Sources of supplemental RP-Lys and RP-Met were Aji-Pro-L 3rd Generation (Ajinomoto Heartland Inc.) and Smartamine M (Adisseo NA), respectively. The AjiPro-L supplement contained 41% of Lys with a bioavailability of 30% (information from feed manufacturer). The Smartamine M supplement contained 75% of Met with a bioavailability of 71% (information from feed manufacturer). Blood meal (Agribands Purina Canada Inc.) was used as a His source. Therefore, requirements of digestible His (**dHis**) were estimated at 69 g/d with all diets providing 67 g/d; requirements of digestible Lys (**dLys**) were estimated at 201 g/d with all diets providing 205 g/d; and requirements of digestible Met (**dMet**) were estimated at 69 g/d with all diets providing 67 g/d. Experimental diets were formulated to meet the requirements for all other nutrients according to NRC (2001). The supply of RDP was ensured to be adequate with urea being added to the DMP diets.

Diets were mixed using a Rovibec Supermix RTM 530 (Rovibec Agrisolutions) and fed as a TMR once daily at 0930 h. Diets were pushed in front of the cows at least 6 times daily. The RP AA supplements and urea were top-dressed and mixed with 100 g of ground corn 2 h after the TMR feeding. Visual observation

Table 1. Chemical composition of alfalfa haylages

Chemical component ¹	Alfalfa	
	Cultivar Calypso ²	Cultivar Boost HG ³
DM (% as fed)	42.9	43.6
OM	90.2	89.5
CP	18.1	16.8
aNDF ⁴	43.7	44.9
ADF	37.4	37.7
ADL	8.5	8.6
IVTD ⁵	77.9	76.7
48-h NDF ⁶ (% aNDF)	49.5	48.2
uNDF ₂₄₀ ⁷	21.2	22.5
Ether extract	2.7	2.6
Ca	1.30	1.35
P	0.27	0.29

¹Percent of DM, unless specified.

²Haylage made with the control cultivar (Sollio Cooperative Group).

³Haylage made with the reduced-lignin cultivar (Quality Seeds).

⁴Neutral detergent fiber assayed with a heat-stable amylase and sodium sulfite.

⁵In vitro true digestibility.

⁶NDF digestibility.

⁷Undigested NDF estimated via 240-h in vitro fermentation.

confirmed that top-dress was completely consumed. Cows had free access to drinking water at all time. During the experiment, DM concentration of forages were determined twice a week to adjust composition of diets on an as-fed basis. The amount of feed offered was adjusted daily to ensure 10% refusals on an as-fed basis.

Measurements and Sampling

Refusals were weighed daily just before feeding. Samples of TMR, refusals, and ingredients were collected from d 18 to 21 of each period, composited by cow within period (TMR and refusals) or by period (ingredients), and stored at -20°C for further analysis. The BCS were determined accordingly to Edmonson et al. (1989) at 1340 h on d 19 and 20, whereas BW of cows were recorded at 1340 h on d 15 to 17.

Total excreted feces were collected and weighed daily (1100 h) from d 18 to 21. Daily samples representing 0.5% of the total amount excreted, on a fresh basis, were frozen at -20°C and further pooled by cow within period before analysis. For urine collection, catheters (24 mm, 75-mL balloon, Bardex Lubricath Foley Catheters, Bard Medical Division) were inserted into the bladder of each cow on d 17 and were connected to a plastic container using a polyvinyl chloride tubing at 1100 h the next day. Then, 200 mL of diluted sulfuric acid (50:50) was added to the container 3 times daily to prevent ammonia volatilization. Total urine was weighed and sampled daily (1100 h) from d 18 to 21. Density was determined by weighing 500 mL of urine. Two subsamples of urine were collected, one pure and one diluted (1:4, wt/wt) for purine derivatives (PD) and creatinine analysis. All samples were immediately frozen at -20°C and further pooled by cow within period for subsequent analyses.

Cows were milked twice daily at 0715 and 1640 h. Milk yield was measured using calibrated Tru-Test milk meters (Datamars). Milk samples were collected at each milking for the last 6 milkings from d 18 p.m. to d 21 a.m. Samples from the p.m. milking were stored at 4°C until they were mixed with samples from the next a.m. milking, proportionally to milk yield. Composited samples were stored at 4°C with 2-bromo-2-nitropropane-1,3-diol until further determination of milk fat, CP, lactose, and MUN. Additional milk samples were collected and stored at -20°C without preservative, and further pooled by cow within period for subsequent NPN and fatty acid analyses.

On d 20, blood samples were taken from the tail of all cows before a.m. milking (0650 h) and 4 h after feeding (1330 h). Samples were collected into heparinized

and EDTA vacutainer tubes (Becton Dickinson), and immediately put on ice. Tubes were then centrifuged at $1,800 \times g$ for 15 min at 4°C to collect plasma, of which different aliquots were stored at -20 and -80°C until chemical analyses.

Chemical Analyses

Feed, Feces, and Urine. Dry matter concentration of ingredients, TMR, and refusals was determined by freeze-drying. All these dried samples were then ground to 1 mm using a Wiley mill (model 3, Arthur H. Thomas Co.). After air acclimatization, concentrations of analytical DM and ash in dried and ground samples were determined using a thermogravimetric analyzer (Leco model TGA 701, Leco Corp.). The concentration of neutral detergent insoluble fiber assayed with a heat-stable α -amylase and sodium sulfite inclusive of residual ash (**aNDF**) was determined following Mertens (2002). The ADF and ADL concentration was determined following AOAC (1990). All fiber extractions were done using the Ankom filter bag (F57; 25 μm porosity) technique (Ankom 2000 Fiber Analyzer). Samples were mineralized using a mixture of sulfuric and selenious acids as described by Isaac and Johnson (1976). This extract was used to determine total N (method 13-107-6-2-D; Lachat Instruments, 2019) using a Lachat QuikChem8000 flow injection analysis system (Zellweger Analytics Inc., Lachat Instruments Division). Concentrations of P and Ca were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 4300DV, Perkin Elmer). Concentration of CP was calculated as total N $\times$ 6.25. Ether extract was determined according to the method of AOAC International (2005).

The in vitro true digestibility (**IVTD**) of DM was measured using the method of Goering and Van Soest (1970) based on a 48-h incubation with buffered rumen fluid followed by an aNDF determination of the postdigestion residues. The rumen fluid incubation was performed with Ankom F57 filter bags with an Ankom DaisyII incubator, using the batch incubation procedures outlined by Ankom Technology Corp. Rumen fluid was obtained from a lactating, ruminally fistulated dairy cow that was offered a diet of 42% corn silage, 38% mixed grass/legume silage, 2% soybean meal, and 18% concentrate mix. The diet was formulated to meet the nutritional requirements of a 670-kg lactating cow producing 9,760 kg of milk per year. The IVTD (% DM) was calculated as follows: $\text{IVTD} = [1 - (\text{postdigestion dry weight following aNDF wash/predigestion dry weight})] \times 100$. The in vitro NDF digestibility (NDFd; % aNDF) was calculated as follows: $\text{NDFd} =$

$[1 - (\text{postdigestion dry weight following aNDF wash} / \text{predigestion dry weight of aNDF})] \times 100$.

The *in vitro* NDF digestibility at 240 h (NDFd₂₄₀) was performed using procedures previously described for NDFd, but with a 240-h incubation time. The unavailable NDF (uNDF₂₄₀) fraction was calculated as $\text{uNDF}_{240} = (100 - \text{NDFd}_{240}) \times \text{aNDF} / 100$. To determine the fatty acid composition of TMR, direct transesterification was first performed, and FAME were then extracted following the method described by Jenkins (2010) with modifications. In the modified procedure, incubation in 3 mL of HCl 10% in methanol (vol/vol) was performed at 50°C for 30 min after a prior incubation in 3 mL of sodium methoxide (0.5 M in methanol) at 70°C for 60 min.

Fecal samples were freeze-dried and ground to pass a 1-mm screen using a Wiley mill (model 3, Arthur M. Thomas Co.). Feces were then analyzed for DM, analytical DM, aNDF, ADF, uNDF₂₄₀, N, and ash using procedures previously described for feed samples. Apparent digestibility of nutrients was calculated by subtracting daily excretion in feces from their daily intake.

Urinary analysis of N was performed using procedures previously described for feed samples. Urea-N was analyzed with an automatic analyzer (Technicon Autoanalyzer II, Technicon Instruments Corporation) as previously described by Huntington (1984). Creatinine and PD (allantoin and uric acid) were measured with a HPLC (1260 Infinity HPLC, Agilent Technologies) method adapted from George et al. (2006).

Milk. Milk samples with preservative were analyzed by infrared spectroscopy (Foss MilkoScan FT 6000) for fat, CP, lactose, and MUN at Lactanet (Dairy Production Center of Expertise, Québec and Atlantic, Sainte-Anne-de-Bellevue, QC, Canada). Samples without preservative were analyzed for NPN and fatty acid composition. The NPN was obtained by precipitation with trichloroacetic acid, with a final concentration of 12%; it was then analyzed with the Kjeldahl method and subtracted (as total N $\times$ 6.38) from CP to obtain true protein. Milk fatty acid composition was determined according to the procedure described by Boivin et al. (2013).

Plasma. For plasma AA analysis, 1-g aliquots of plasma were added to 0.2 g of an internal AA standard labeled with stable isotopes as described in Doepel and Lapierre (2010). Labeled AA (95–99 atom%) were supplied by CDN Isotopes for His, Leu, Lys, Met, and Phe and by Cambridge Isotope Laboratories for the other AA. Plasma AA concentrations were measured by isotope dilution using GC-MS (Calder et al., 1999) and *N*-(tert-butylidimethyl) AA derivative. Urea-N

was analyzed using procedures previously described for urine. Using commercial kits, plasma was analyzed for concentrations of nonesterified fatty acids [NEFA; Wako HR series NEFA-HR (2) Kit, FUJIFILM Wako Diagnostics USA] and glucose (PGO assay, Sigma-Aldrich).

Calculations and Statistical Analyses

Whole-body efficiency was calculated following the principles proposed by INRA (INRA, 2018) for MP and used later (Lapierre et al., 2020; Omphalius et al., 2020) for individual EAA. Briefly, this efficiency is calculated as the sum of true protein or EAA in exported proteins; that is, milk true protein yield, scurf, and metabolic fecal protein divided by MP or EAA supply (minus endogenous urinary loss). Metabolic fecal and endogenous urinary losses were calculated based on Lapierre et al. (2020), whereas MP and EAA supply were estimated using NRC (2001), but the duodenal endogenous contribution was excluded.

The alfalfa cultivar was the main plot factor and within each forage source, the 4 dietary treatments (subplots) were distributed in replicate Latin square fashion. No data were excluded from the analysis. Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc.) according to the following model:

$$Y_{ijkl} = \mu + S_j + C(S)_{lj} + T_i + P(S)_{kj} + (T \times S)_{ij} + \varepsilon_{ijkl},$$

where Y_{ijkl} = individual observation, μ = overall mean, S_j = fixed effect of square ($j = 1$ to 2), $C(S)_{lj}$ = random effect of cow ($l = 1$ to 4) within square, T_i = fixed effect of treatment ($i = 1$ to 4), $P(S)_{kj}$ = fixed effect of period ($k = 1$ to 4) within square, $(T \times S)_{ij}$ = effect of the interaction between treatment and square, and ε_{ijkl} = residual error term. Differences between treatments were declared at $P \leq 0.05$, and tendencies from $0.05 < P \leq 0.10$. When the treatment effect was significant, means were separated using the PDIF option with Tukey's adjustment.

Plasma urea-N, glucose, NEFA, and AA concentrations were initially analyzed as repeated measures according to the following model:

$$Y_{ijklm} = \mu + S_j + C(S)_{lj} + T_i + P(S)_{kj} + (T \times S)_{ij} + H_m + (T \times H)_{im} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the individual observation, H_m is the effect of sampling time ($m = 0650$ and 1330 h), $(T \times H)$

γ_{im} is the effect of the interaction between treatment and time of sampling, and ϵ_{ijklm} is the residual error term. Cow was considered as a random effect. The subject of the repeated measurements was cow (period $\times$ treatment), and the spatial covariance structure SP(POW) was used to estimate covariances. Because there was no sampling time $\times$ treatment interactions, mean values for plasma urea-N, glucose, NEFA, and AA concentrations were combined for statistical analysis according to the first model previously described. When the treatment effect was significant, means were separated using the PDIFF option with Tukey's adjustment.

RESULTS AND DISCUSSION

Effect of Alfalfa Haylages Cultivars

Chemical composition of alfalfa haylages was not affected by cultivars as illustrated by similar values for aNDF, ADF, ADL, IVTD, in vitro 48-h NDFd, and uNDF₂₄₀ (Table 1). Consequently, no significant effect of the alfalfa cultivars or interaction with dietary treatments was observed on the different parameters measured in this experiment, including apparent digestibility of nutrients. Further research is therefore needed with alfalfa cultivars that show a tangible difference in their digestibility to assess the in vivo effect of a more digestible alfalfa cultivar grown in Canada on the performance of dairy cattle. Many factors not controlled in our study may have prevented the improved alfalfa cultivar to express its enhanced digestibility potential. Air temperature, water stress, and even location can alter digestibility of alfalfa (Vough and Marten, 1971; Julier and Huyghe, 1997; Lauzon et al., 2019). Using in situ evaluation, it has also been reported that alfalfa cultivars selected for improved digestibility were not always more digestible than regular cultivars (Grev et al., 2017). Given the absence of significant effects of alfalfa cultivar, it will not be further discussed, and results will be presented for the 4 diets only.

Dietary Contrasts: Realized vs. Objective

As planned, all DMP diets had lower CP concentrations than AMP_AE diet (Table 2). To achieve this, soybean meal was removed, and ground corn was partially replaced with corn starch in DMP diets. Also, concentrations of NE_L estimated using NRC (2001) were as expected: AMP_AE and DMP_AE diets had equivalent NE_L, whereas DMP_DE and DMP_EE diets had, respectively, lower and higher NE_L concentration than the AMP_AE diet. The concentration of NE_L in DMP_AE and DMP_EE diets was adjusted with Ca

salts of long-chain fatty acids, whereas chopped wheat straw was used for energy dilution in the DMP_DE diet. Consequently, DMP_DE diet had greater concentrations of aNDF and ADF, and lower NFC concentration compared with AMP_AE diet. In contrast, due to a greater proportion of corn starch, DMP_AE and DMP_EE diets had lower concentrations of aNDF and ADF, and higher NFC concentration than the AMP_AE diet.

Protein fractions, MP, AA, and energy balance were calculated according to NRC (2001). Requirements were based on DMI, BW, milk yield, and milk composition of cows receiving AMP_AE treatment, assuming that these reflected the potential of the cows. Protein and energy supplies were based on observations for each treatment (i.e., individual intake; Table 3). Assuming that the milk production potential of the DMP-fed cows was similar to that of the AMP_AE-fed cows, DMP_DE, DMP_AE, and DMP_EE diets provided, respectively, 84, 87, and 87% of MP requirement. The AMP_AE diet slightly supplied (3%) above the MP requirement. The DMP_DE, DMP_AE and DMP_EE diets, respectively, supplied 94, 101, and 107% of NE_L requirements, whereas the AMP_AE diet supplied 108% of NE_L requirements. There was no estimated RDP deficiency for cows fed AMP_AE, but cows fed DMP_DE, DMP_AE, and DMP_EE diets had estimated RDP deficiencies of 15, 10, and 7%, respectively. The RDP deficiencies in DMP diets occurred despite the inclusion of urea into these diets. According to estimated requirements in g/d, AA balance was adequate for dHis, dLys, and dMet in AMP_AE-fed cows. In DMP_DE-, DMP_AE-, and DMP_EE-fed cows, AA deficiencies were estimated at 8, 7, and 5% for dHis, 3, 1, and 0% for dLys, and 7, 3, and 5% for dMet, respectively. Because of the lower DMI in deficient-MP compared with the adequate-MP diet (only numerical for DMP_AE and DMP_EE diets), only DMP_EE-fed cows had a similar supply and positive NE_L balance than that of AMP_AE-fed cows (+2.8 Mcal/d). Lower than expected DMI, and lower than expected CP concentration of ingredients could explain the differences between estimations from NRC (2001) and cow responses, which resulted in lower than predicted MP, RDP, RUP, and EAA (dHis, dLys, and dMet) balances.

Animal Performance

As compared with cows receiving AMP_AE, the DMI of cows fed DMP_AE and DMP_EE was not statistically different but was reduced for cows fed DMP_DE (Table 4). Cows fed the DMP diets had, however, a similar DMI. The decrease observed with the DMP_DE

Table 2. Ingredients and chemical composition of experimental diets

Item	Diet ¹			
	AMP_AE	DMP_DE	DMP_AE	DMP_EE
Ingredient, % of DM				
Alfalfa silage	51.0	51.0	51.0	51.0
Corn silage	17.0	17.0	17.0	17.0
Ground corn	20.3	8.1	12.6	9.7
Corn starch	0.0	9.8	13.6	13.6
Wheat straw	0.0	8.9	0.0	0.0
Soybean meal	7.6	0.0	0.0	0.0
Megalac ²	0.7	0.0	0.8	3.5
Blood meal ³	1.1	2.6	2.4	2.4
Mineral and vitamin mix ⁴	1.8	1.8	1.8	1.8
Water	0.5	0.8	0.8	0.9
Urea	0.0	0.2	0.3	0.4
Rumen-protected Lys ⁵	0.3	0.4	0.4	0.3
Rumen-protected Met ⁶	0.1	0.1	0.1	0.1
Analyzed composition ⁷				
DM (% as fed)	46.5	47.6	47.5	47.6
OM	91.3	91.9	92.0	91.6
CP	16.2	14.1	14.3	14.3
aNDF ⁸	36.6	40.5	33.9	34.0
ADF	28.9	31.6	26.8	26.8
ADL	6.0	6.4	5.5	5.4
IVTD ⁹	81.9	77.8	82.2	82.6
48-h NDFd ¹⁰ (% of aNDF)	50.8	45.6	48.1	49.3
uNDF ₂₄₀ ¹¹	16.3	19.3	15.7	15.7
Ether extract ¹²	3.3	2.4	3.2	5.3
Ca	1.14	1.03	1.09	1.22
P	0.40	0.31	0.33	0.33
Fatty acids, mg/g of DM				
12:0	0.11	0.10	0.11	0.22
14:0	0.23	0.20	0.24	0.42
14:1	0.05	0.05	0.06	0.06
16:0	5.88	3.48	5.48	11.86
16:1	0.06	0.06	0.07	0.07
18:0	0.81	0.56	0.77	1.39
<i>cis</i> -9 18:1	3.99	1.65	3.35	8.13
<i>cis</i> -11 18:1	0.15	0.10	0.16	0.25
<i>n</i> -6 18:2	6.96	4.46	5.50	6.37
<i>n</i> -3 18:3	3.73	3.58	3.71	3.71
20:0	0.20	0.17	0.18	0.23
Total	22.17	14.40	19.63	32.72
Calculated composition ^{7,13}				
RDP	10.5	8.9	9.2	9.4
RUP	6.0	5.4	5.1	5.0
NE _L , Mcal/kg of DM	1.53	1.44	1.51	1.59
NFC	43.1	41.0	47.3	44.9

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Megalac (Church and Dwight Co. Inc.).

³Agribands Purina Canada Inc.

⁴Contained (per kg of DM): Ca = 131.6 g/kg; P = 84.6 g/kg; Mg = 90 g/kg; Zn = 2,820 g/kg; Cu = 0.564 g/kg; Mn = 1,880 g/kg; vitamin A = 282 kIU/kg; vitamin D = 94 kIU/kg; vitamin E = 1,410 kIU/kg.

⁵AjiPro-L 3rd Generation: 30% bioavailability = 41% Lys, RUP: 80.5%, intestinal digestibility: 90.5%, from feed manufacturer Ajinomoto Heartland Inc.

⁶Smartamine M: 71% bioavailability = 75% Met, RUP: 100%, intestinal digestibility: 95%, from feed manufacturer Adisseo NA.

⁷Percent of DM, unless specified.

⁸Neutral detergent fiber assayed with a heat-stable amylase and sodium sulfite.

⁹In vitro true digestibility of DM.

¹⁰NDF digestibility.

¹¹Undigested NDF estimated via 240-h in vitro fermentation.

¹²Calculated from the ether extract concentration of each ingredient.

¹³Estimated based on NRC (2001).

Table 3. Estimated protein, digestible EAA (dEAA), and energy balance¹ in dairy cows

Item	Diet ²			
	AMP_AE	DMP_DE	DMP_AE	DMP_EE
Protein and dEAA balance, ³ g/d				
MP				
Requirements ⁴	2,582	2,582	2,582	2,582
Supply ⁵	2,666	2,180	2,234	2,255
Balance	84	-402	-348	-327
RDP and RUP				
RDP supply ⁵	2,751	2,135	2,263	2,341
RDP balance ⁴	242	-374	-246	-168
RUP supply ⁵	1,543	1,278	1,245	1,222
RUP balance ⁴	110	-155	-188	-211
dHis				
Requirements ^{4,6}	61	61	61	61
Supply ⁵	61	56	57	58
Balance	0	-5	-4	-3
dLys				
Requirements ^{4,6}	179	179	179	179
Supply ⁵	195	174	178	180
Balance	16	-5	-1	1
dMet				
Requirements ^{4,6}	61	61	61	61
Supply ⁵	64	57	59	58
Balance	3	-4	-2	-3
Other dEAA supply ⁵				
dArg	122	94	98	99
dIle	124	95	99	101
dLeu	232	195	202	204
dPhe	134	112	115	116
dThr	128	106	110	112
dVal	149	129	133	136
Energy balance, ³ Mcal/d				
NE _L				
Requirements ⁴	37.1	37.1	37.1	37.1
Supply ⁵	39.9	34.8	37.6	39.7
Balance	2.8	-2.3	0.5	2.6

¹All values estimated using NRC (2001).

²Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

³Due to rounding, balance may not exactly match requirements and supply.

⁴Requirements based on DMI, BW, milk yield, and milk composition of cows receiving AMP_AE treatment, assuming that these reflected the potential of the cows.

⁵Supply based on observations within each treatment.

⁶Requirements of digestible His (dHis), digestible Lys (dLys), and digestible Met (dMet) were calculated as 2.38%, 6.95%, and 2.38% of the MP requirement, respectively.

diet is probably related to its high aNDF and ADF concentrations. Indeed, forage NDF is a key driver of DMI (Sauvant et al., 2014). In addition, a low protein supplementation has been reported to decrease feed intake (Vérité and Delaby, 2000; Huhtanen and Hetta, 2012; Owens et al., 2014). A low protein supply can result in a RDP deficiency, as observed in the DMP diets. This deficiency could have depressed ruminal functions, which in turn could lower DMI (Allen, 2000). This hypothesis is supported by the tendency for a decreased apparent digestibility of CP (Table 5) as well as the decreased estimates of microbial N flow with

the DMP_DE diet and intermediate values observed with the 2 other DMP diets (Table 6), as compared with the AMP_AE diet. Although in our study no effect of treatment on NDF digestibility was observed, several studies have reported a significant or numerical decrease of total-tract NDF digestibility and DMI with RDP deficient diets (Lee et al., 2011, 2012a,c). Two of these studies also noted a trend or a numerical decrease in urinary PD excretion (Lee et al., 2012b,c), suggesting a decreased microbial synthesis. On the other hand, Boucher et al. (2007) did not notice an increase in DMI or fiber digestibility when RDP supply was above re-

Table 4. Effect of MP and energy supplies on DMI, milk yield, milk composition, and BW in dairy cows

Item	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
DMI, kg/d	26.0 ^a	23.9 ^b	24.5 ^{ab}	24.7 ^{ab}	0.93	0.04
Milk yield, kg/d						
Actual	33.6 ^a	28.6 ^b	30.2 ^b	34.7 ^a	1.84	<0.01
ECM ²	33.8 ^a	29.0 ^b	30.5 ^b	34.7 ^a	2.09	<0.01
Feed efficiency, ³ kg/kg						
Actual	1.29 ^{ab}	1.20 ^b	1.23 ^b	1.41 ^a	0.046	<0.01
ECM	1.30 ^b	1.21 ^c	1.24 ^{bc}	1.41 ^a	0.057	<0.01
Milk composition, %						
Fat	4.16	4.28	4.19	4.21	0.162	0.79
True protein	3.31 ^{ab}	3.26 ^{ab}	3.33 ^a	3.17 ^b	0.051	0.04
Lactose	4.47	4.45	4.45	4.45	0.063	0.91
Milk component yield, kg/d						
Fat	1.40 ^a	1.22 ^b	1.27 ^b	1.46 ^a	0.100	<0.01
True protein	1.11 ^a	0.93 ^b	1.00 ^{ab}	1.10 ^a	0.059	<0.01
Lactose	1.50 ^a	1.27 ^b	1.35 ^b	1.54 ^a	0.085	<0.01
MUN, mg/dL	16.2 ^a	14.8 ^a	10.5 ^b	11.0 ^b	0.56	<0.01

^{a-c}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Energy-corrected milk (kg/d) = $[23.8 \times (\text{protein yield in kg/d})] + [38.9 \times (\text{fat yield in kg/d})] + [16.3 \times (\text{lactose yield in kg/d})] \div 3.14$ (Madsen et al., 2008).

³Milk yield/DMI.

quirements. However, diets fed in this latter study had a low proportion of forages. As suggested by Giallongo et al. (2016), a high proportion of forage seems to exacerbate negative effects of RDP-deficient diets. In addition to its effect on rumen function, additional protein supply may have a metabolic effect in stimulating DMI. Indeed, Martineau et al. (2016) reported that postrumen casein infusion affected DMI differently depending on MP balance in dairy cows. They observed that for cows with a negative MP balance, postrumen casein

infusion increased DMI, whereas it decreased DMI for cows with a positive MP balance.

Compared with AMP_AE, milk yields (actual and ECM) as well as milk fat and lactose yields decreased in the DMP_DE- and DMP_AE-fed cows, but not in the DMP_EE-fed cows. Only DMP_DE cows had a lower milk true protein yield than that of AMP_AE cows and DMP_EE cows, with DMP_AE cows having an intermediate value. The NE_L supply did not differ between AMP_AE and DMP_EE; in DMP_EE,

Table 5. Effect of MP and energy supplies on intake and total-tract apparent digestibility of nutrients in dairy cows

Item	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
Nutrient intake, kg/d						
DM	26.0 ^a	23.9 ^b	24.5 ^{ab}	24.7 ^{ab}	0.93	0.04
OM	23.7	22.0	22.6	22.7	0.86	0.07
aNDF ²	9.59 ^a	9.65 ^a	8.36 ^b	8.51 ^b	0.323	<0.01
ADF	7.55 ^a	7.53 ^a	6.58 ^b	6.72 ^b	0.236	<0.01
CP	4.21 ^a	3.40 ^b	3.52 ^b	3.49 ^b	0.139	<0.01
Apparent digestibility, %						
DM	64.8 ^{ab}	61.8 ^b	65.7 ^a	65.2 ^a	0.75	0.01
OM	65.9 ^{ab}	63.3 ^b	67.2 ^a	67.1 ^a	0.66	<0.01
aNDF	49.2	45.2	46.5	46.6	1.73	0.45
ADF	53.0	48.3	50.6	50.0	1.49	0.19
CP	65.8	61.7	62.7	64.0	1.18	0.06

^{a,b}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Neutral detergent fiber assayed with a heat-stable amylase and sodium sulfite.

Table 6. Effect of MP and energy supplies on N input and output, and urinary purine derivative (PD) excretion in dairy cows

Item	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
N intake, g/d	674 ^a	544 ^b	564 ^b	559 ^b	22.3	<0.01
N secretion and excretion, g/d						
Milk TPN ²	173 ^a	145 ^b	156 ^{ab}	172 ^a	9.2	<0.01
Fecal N	231 ^a	208 ^{ab}	209 ^{ab}	200 ^b	12.4	0.03
Urinary N	233 ^a	156 ^b	149 ^b	152 ^b	8.8	<0.01
Urinary urea-N	185 ^a	104 ^b	102 ^b	101 ^b	7.2	<0.01
As % of total urinary N	79.5 ^a	66.7 ^b	67.7 ^b	66.2 ^b	1.98	<0.01
Total excreta N	463 ^a	364 ^b	358 ^b	352 ^b	19.3	<0.01
As proportion of N excretion, %						
Fecal N	49.9 ^b	57.1 ^a	58.4 ^a	56.7 ^a	1.18	<0.01
Urine N	50.1 ^a	42.9 ^b	41.6 ^b	43.3 ^b	1.18	<0.01
As proportion of N intake, %						
Milk TPN	25.6 ^b	26.7 ^b	27.8 ^b	31.1 ^a	0.95	<0.01
Fecal N	34.2	38.3	37.3	36.0	1.18	0.06
Urine N	34.5 ^a	28.6 ^b	26.6 ^b	27.5 ^b	1.01	<0.01
Total excreta N	68.7	66.9	63.8	63.4	1.56	0.05
Urine output, kg/d	30.6 ^a	21.6 ^b	21.7 ^b	22.0 ^b	1.05	<0.01
Urinary PD excretion, mmol/d						
Allantoin	424 ^a	353 ^b	390 ^{ab}	386 ^{ab}	21.3	<0.01
Uric acid	61.4 ^a	43.7 ^b	55.2 ^a	54.7 ^a	3.32	<0.01
Total PD	485 ^a	397 ^b	445 ^{ab}	441 ^{ab}	22.6	<0.01
Microbial N flow, ³ g/d	396 ^a	317 ^b	359 ^{ab}	360 ^{ab}	19.5	<0.01

^{a,b}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Milk true protein N (milk true protein $\div$ 6.38).

³Estimated based on urinary PD excretion (Chen and Gomes, 1992).

however, MP supply was 13% lower than predicted requirements, but dHis and dMet were only 5% below requirements whereas dLys met requirements. Therefore, despite a lower MP supply, with supplies of dHis, dLys, and dMet at or close to the requirements, MPY was maintained when the energy supply was equivalent to the level of the AMP_AE diet. This supports the hypothesis that the requirements of dairy cows are associated with the EAA and not the MP supply per se. As demonstrated by Lee et al. (2012c), decreasing MP supply at levels below predicted requirements does not negatively affect milk yield and MPY when His, Lys, and Met supplies are adequate, as long as energy intake is sufficient. In contrast, Giallongo et al. (2016) failed to increase milk yield with dietary supplementation of a MP-deficient diet with the combination of RP-His, RP-Lys, and RP-Met, but did increase MPY with supplementation of the combined 3 RP AA. Our results are consistent with those of Omphalius et al. (2019b), where MPY was dependent on both energy and EAA supplies. Our observations also suggest that MPY does not rely solely on MP supply: indeed, we did not observe that a decrease in MP supply reduced MPY, as long as the supplies of energy and of His, Lys, and Met did not decrease substantially below requirements. The most limiting EAA with typical North

American dairy diets are often considered to be His, Lys, and Met (Schwab and Broderick, 2017). Indeed, Robinson (2010) observed in his meta-analysis that a RP-Lys and Met combination increased milk and milk energy yields, milk protein concentration, feed use efficiency, and N use efficiency. With DMP diets based on corn silage and alfalfa fed to high-producing dairy cows, a combination of rumen-protected His, Lys, and Met was sufficient to maintain milk and milk component yields to the level observed with the MP-adequate diet (Lee et al., 2012c). In line with other observations (Lee et al., 2012c; Giallongo et al., 2015, 2016), MP allowable milk from the NRC (2001) model was severely underestimated in DMP diets (-2.1 , -2.9 , and -7.0 kg of milk/d, for DMP_DE, DMP_AE, and DMP_EE, respectively), indicating that the use of a fixed efficiency of utilization of MP does not fit reality and needs revision (Doepel et al., 2004). Efficiency of utilization of MP will be discussed in detail in a following section. Actual feed efficiency was higher for cows fed DMP_EE diet than for cows fed DMP_DE and DMP_AE diets, whereas AMP_AE-fed cows had an intermediate value. The ECM feed efficiency was greater for cows receiving DMP_EE diet in comparison with cows receiving other treatments. The high energy concentration in DMP_EE diet explains this observation.

Fat and lactose concentrations in milk were not affected by dietary treatments (Table 4). However, probably due to a dilution effect, true protein concentration in milk from DMP_EE-fed cows was lower from that of DMP_AE-fed cows, whereas AMP_AE- and DMP_DE-fed cows had intermediate values. The MUN concentration was lower when cows were fed DMP_AE or DMP_EE diets in comparison with AMP_AE. Despite a reduced N intake compared with AMP_AE, the DMP_DE diet did not allow a reduction in MUN, probably because of a lack in fermentable energy in the rumen as indicated by the lower PD urinary excretion, or a lack of digestible energy, which impaired the utilization of absorbed AA.

Apparent Digestibility, N Input and Output, MP, and EAA Efficiency of Utilization

Despite the effect of dietary treatments on DMI, only a trend was observed for intake of OM (Table 5). Intakes of fibers (i.e., aNDF and ADF) were lower when cows consumed DMP_AE and DMP_EE diets than when cows received AMP_AE and DMP_DE diets. As expected, CP intake was lower when cows were fed DMP diets compared with AMP_AE diet.

Total-tract apparent digestibilities of aNDF and ADF were not significantly affected by dietary treatments. However, we noticed a tendency in apparent digestibility of CP among diets. The amplitude of the RDP deficiency in DMP diets was probably too small to have a significant effect on apparent digestibility of aNDF and ADF, even though it seems to have affected microbial N flow. Also, a lack of statistical power may have prevented the observation of a significant effect since standard deviation relative to mean values were greater for aNDF and ADF digestibilities compared with other nutrients. Numerically, aNDF and ADF apparent digestibilities are lower with DMP diets compared with AMP_AE-fed cows, which is consistent with estimated microbial N flow. Moreover, predicted NDF digestibilities (NRC, 2001) changed little among treatments (maximum variation of 0.7 unit of %). In accordance with concentrations of aNDF, ADF, and NFC in diets, apparent digestibilities of DM and OM were greater with DMP_AE and DMP_EE diets when compared with DMP_DE diet, whereas AMP_AE had an intermediate value.

Intake of N was reduced with DMP diets (Table 6), similarly to CP intake described earlier. Fecal N excretion was reduced when cows were fed DMP_EE compared with AMP_AE; cows fed DMP_DE and DMP_AE diets had intermediate fecal N excretion. Fecal N as proportion of N intake, however, was not

affected by dietary treatments but tended to be higher in DMP diets. Urinary N excretion decreased by an average of 35% when cows were fed the DMP diets compared with AMP_AE (Table 6). The DMP diets also reduced urinary N excretion in proportion of N intake. Similarly, excretion of urinary urea-N was reduced by an average of 45% with DMP diets and the DMP diets reduced urinary urea-N excretion as a percentage of urinary N as well. These results have important implications, because N from urinary urea is rapidly transformed into ammonium, then volatilized as ammonia into the environment (Hristov et al., 2011). Ammonia is a molecule causing several environmental and health problems (Hristov et al., 2011). The positive effect of decreasing dietary CP concentration on ammonia emissions from manure is well documented (Külling et al., 2001; Lee et al., 2012a; Hristov et al., 2015). Some authors also suggested that a reduced dietary CP concentration could have mitigating effects on emission of nitrous oxide, a greenhouse gas (Külling et al., 2001; Lee et al., 2012a). Moreover, ammonia and nitrous oxide emissions represent a loss of fertilizing power of manure (Hristov et al., 2011). Total N in excreta, on a daily basis, decreased by an average of 23% with the DMP diets, and as proportion of N intake, total N in excreta tended to be lower with the DMP diets. When decreasing MP supply but balancing dairy diets for His, Lys, and Met, Lee et al. (2012c) reported a decreased urinary excretion of N and urea-N. Moreover, Giallongo et al. (2015) also reported a significant decrease in excretion of N and urea-N from urine when reducing MP supply below estimated dairy cow requirements. However, these authors did not observe a significant reduction in total N excretion, probably because of a limited effect on total N intake and no reduction of fecal N with decreased MP supply.

Daily urine output was lower when cows were fed the DMP diets (−29%). Urinary excretions of allantoin, uric acid, and total PD were affected by treatments. Whereas feeding DMP_DE resulted in a reduction of these compounds in comparison with AMP_AE, feeding DMP_AE or DMP_EE did not result in significant reductions. Other authors who have lowered the RDP supply below requirements have also observed a tendency for a reduction or a numerical decrease in urinary PD excretion (Gressley and Armentano, 2007; Lee et al., 2012b,c), probably related to a depression of ruminal functions. Therefore, when compared with the other diets, only DMP_DE diet reduced microbial N flow estimated from urinary PD excretion. As discussed earlier, microbial N flow values of cows receiving DMP_AE or DMP_EE were intermediate: lower than those of AMP_AE-fed cows but higher than DMP_DE-

Table 7. Effect of MP and energy supplies on the estimated efficiency¹ of use of individual EAA and MP in dairy cows

Item	Diet ²				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
MP	0.62 ^c	0.68 ^b	0.69 ^b	0.74 ^a	0.016	<0.01
His	0.74 ^{ab}	0.71 ^b	0.73 ^{ab}	0.77 ^a	0.018	0.02
Ile	0.66 ^c	0.75 ^b	0.75 ^b	0.81 ^a	0.020	<0.01
Leu	0.64 ^b	0.66 ^b	0.67 ^b	0.72 ^a	0.018	<0.01
Lys	0.63 ^b	0.61 ^b	0.62 ^b	0.67 ^a	0.017	<0.01
Met	0.61 ^b	0.58 ^b	0.60 ^b	0.66 ^a	0.018	<0.01
Phe	0.56 ^b	0.59 ^b	0.60 ^b	0.64 ^a	0.015	<0.01
Thr	0.59 ^c	0.63 ^b	0.63 ^{ab}	0.67 ^a	0.015	<0.01
Val	0.65 ^b	0.65 ^b	0.66 ^{ab}	0.70 ^a	0.017	<0.01

^{a-c}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Efficiency of MP and individual AA was calculated according to Omphalius et al. (2020): (milk protein + metabolic fecal protein + scurf)/(supply – endogenous urinary loss).

²Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

fed cows. Microbial N flow calculated based on PD excretion were on average 157 g/d greater than estimates from NRC (2001), but trends in changes were similar to estimations.

As a proportion of N intake, N in milk reflecting milk N efficiency was greater when cows were fed DMP_EE diet compared with all other diets (Table 6). Based on a meta-analysis, CP concentration and intake have been determined as the most important dietary factors influencing milk N efficiency (Huhtanen and Hristov, 2009). In addition, it is recognized that increasing energy supply increases milk N efficiency (NorFor, 2011; INRA 2018). However, the effect of the type of energy on milk N efficiency is not yet completely understood (Nichols et al., 2018, 2019). In a study of Omphalius et al. (2020), higher energy intake had increased MPY through increased mammary use of Group 2 EAA (Ile, Leu, Lys, and Val) taken up by the mammary gland to support MPY and reduced catabolism of EAA, which allowed the increment of milk N efficiency at a similar EAA supply. In contrast, when N intake was increased, MPY increased to a lesser extent than N intake (Omphalius et al., 2020), reducing milk N efficiency. The improved milk N efficiency when cows were fed the DMP_EE diet suggests that the sustainability of dairy farms may be improved when reducing MP supply at a constant NE_L supply, given the potential decreased usage of protein supplement for similar lactation performance.

Although milk N efficiency is interesting to give a global picture of the partition of N to milk at the whole body level, more detailed efficiency of utilization of digested protein and absorbed EAA is needed to help improving formulation of dairy rations. Effi-

ciency of utilization of MP and EAA are presented in Table 7. Compared with AMP_AE-fed cows, feeding DMP_EE diet increased whole-body efficiencies of MP and EAA, except for His (Table 7); this is explained by the fact that, in the latter diet, the supply of MP and EAA decreased but MPY did not. At the moment there are no reference values that we should target for this relatively new concept of EAA efficiency, but we can compare our values with means of a meta-analysis reported by Lapierre et al. (2020). The Lys and Met efficiencies were relatively low, and even in the DMP_EE diet, they did not increase above the mean values of 0.67 and 0.71, respectively, reported by Lapierre et al. (2020). This would indicate that their supplies were sufficient. The His efficiency was relatively high especially for DMP_EE-fed cows, but still slightly lower than the mean of 0.78 (Lapierre et al., 2020). Lapierre et al. (2021) recently reported that an efficiency of 0.77 should be targeted for His, above which His supply might be in shortage. Compared with the 0.61 mean reported by Lapierre et al. (2020), Ile efficiency was high, especially in the DMP diets: this indicates that we need to pay attention to all EAA when feeding low-MP diets. Limiting EAA may depend on the ingredients composing the diet. The effect of a higher energy supply on the efficiency of EAA in a DMP diet compared with an adequate energy and MP diet remains, unfortunately, an unanswered question. Although it was one of the objectives of the study, due to the numerically lower DMI in DMP_EE- than AMP_AE-fed cows, it could not be assessed. Maintaining a high DMI with a low-MP supply seems to be a challenge, hence the importance of having a diet that is concentrated in energy.

Table 8. Effect of MP and energy supplies on plasma metabolite concentrations in dairy cows

Item	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
Glucose, mM	3.54	3.41	3.62	3.44	0.087	0.34
NEFA, ² μ M	98.5	164.5	134.8	153.3	26.16	0.33
Urea N, mM	12.1 ^a	11.0 ^{ab}	8.3 ^c	9.0 ^{bc}	0.64	<0.01

^{a-c}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Nonesterified fatty acids.

Plasma Metabolites and AA Concentrations, and Milk Fatty Acid Composition

Concentrations of plasma glucose and NEFA were not affected by treatments (Table 8). In previous experiments, MP deficiency did not affect plasma glucose concentration (e.g., Giallongo et al., 2015, 2016). However, Urdl et al. (2015) reported a positive correlation between energy balance and plasma glucose in postpartum cows. Variation in energy balance in our study was probably not sufficient for differences to occur in plasma glucose. Similar to MUN, plasma urea-N concentrations of cows fed DMP_AE or DMP_EE were lower in comparison with AMP_AE-fed cows, whereas feeding DMP_DE did not allow a significant reduction. This observation is in agreement with the calculation of the MP use efficiency. Indeed, the pattern of plasma

urea concentrations is normally the reverse of estimated efficiencies of MP (Omphalius et al., 2020).

Feeding DMP diets did not decrease plasma His, Lys, and Met concentrations compared with the AMP diet (Table 9), which indicate that we were able to provide an equivalent level of these EAA within all treatments. Indeed, even though MP supply with DMP diets averaged only 83% of the supply from the AMP diet, the predicted digestible flows of His, Lys, and Met with DMP diets averaged, respectively, 93, 91, and 91% of the supply from the AMP diet. Predicted digestible flows of the other EAA with the DMP diets averaged between 80 and 89% of the supply from the AMP diet (Table 3). Diets did not affect the plasma concentration of the other EAA except for the branched-chain AA (BCAA). The DMP_AE-fed cows had lower plasma concentrations when compared with AMP_AE-fed

Table 9. Effect of MP and energy supplies on plasma AA concentrations (μ M) in dairy cows

AA	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
Ala	268	251	251	260	12.6	0.33
Asp	8.2	8.1	7.8	7.9	0.79	0.98
Cys	103	105	106	100	3.4	0.35
Gln	271	265	284	289	13.1	0.56
Glu	67.2	65.6	72.6	69.7	3.80	0.20
Gly	285 ^b	259 ^b	302 ^{ab}	351 ^a	14.8	<0.01
His	50.9	53.7	52.3	51.0	2.31	0.81
Ile	114 ^a	88.9 ^b	83.4 ^b	100 ^{ab}	6.37	<0.01
Leu	134 ^a	112 ^{ab}	109 ^b	112 ^{ab}	8.2	0.03
Lys	89.4	85.1	80.8	85.1	4.16	0.50
Met	39.6	40.1	44.4	40.5	1.98	0.30
Phe	44.0	41.6	44.1	41.2	2.06	0.52
Pro	81.7 ^a	67.9 ^b	70.6 ^b	71.0 ^b	2.80	<0.01
Ser	84.4	75.6	80.1	80.8	4.19	0.43
Thr	100	84.8	90.6	97.1	6.81	0.07
Trp	60.8	55.3	53.1	56.6	2.34	0.17
Tyr	47.3	38.3	40.3	42.6	2.47	0.06
Val	230 ^a	211 ^{ab}	190 ^b	210 ^{ab}	13.0	0.04

^{a,b}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

Table 10. Effect of MP and energy supplies on milk fatty acid composition (g/100 g of total fatty acid) in dairy cows

Fatty acid	Diet ¹				SEM	P-value
	AMP_AE	DMP_DE	DMP_AE	DMP_EE		
4:0	2.85	2.62	2.51	2.80	0.172	0.50
6:0	2.20	1.96	1.90	1.88	0.105	0.18
8:0	1.28 ^a	1.11 ^{ab}	1.10 ^{ab}	1.01 ^b	0.056	0.04
10:0	3.32 ^a	2.87 ^{ab}	2.91 ^{ab}	2.40 ^b	0.145	<0.01
12:0	3.93 ^a	3.49 ^a	3.64 ^a	2.80 ^b	0.172	<0.01
iso 13:0	0.04 ^a	0.03 ^{ab}	0.03 ^{ab}	0.03 ^b	0.003	0.03
anteiso 13:0	0.02	0.02	0.02	0.02	0.002	0.75
12:1	0.10 ^a	0.09 ^a	0.10 ^a	0.07 ^b	0.006	<0.01
13:0	0.12 ^a	0.11 ^{ab}	0.13 ^a	0.08 ^b	0.011	0.02
iso 14:0	0.13 ^b	0.16 ^a	0.11 ^b	0.11 ^b	0.010	<0.01
14:0	11.82 ^a	11.52 ^a	11.58 ^a	9.72 ^b	0.310	<0.01
cis-9 14:1	0.95 ^a	1.03 ^a	0.99 ^a	0.81 ^b	0.073	<0.01
iso 15:0	0.19 ^a	0.18 ^a	0.15 ^b	0.13 ^b	0.009	<0.01
anteiso 15:0	0.41 ^{ab}	0.44 ^a	0.41 ^{ab}	0.35 ^b	0.016	0.01
15:0	1.26 ^a	1.31 ^a	1.38 ^a	0.94 ^b	0.079	<0.01
iso 16:0	0.25	0.31	0.25	0.28	0.021	0.05
16:0	30.37	32.37	32.70	31.69	0.625	0.09
cis-9 16:1	1.09 ^c	1.47 ^a	1.30 ^b	1.24 ^{bc}	0.057	<0.01
iso 17:0	0.21 ^{ab}	0.23 ^a	0.19 ^{ab}	0.17 ^b	0.011	<0.01
anteiso 17:0	0.33 ^b	0.39 ^a	0.35 ^{ab}	0.32 ^b	0.013	<0.01
17:0	0.48 ^{bc}	0.56 ^a	0.54 ^{ab}	0.42 ^c	0.022	<0.01
cis-9 17:1	0.16 ^c	0.24 ^a	0.20 ^b	0.16 ^c	0.011	<0.01
iso 18:0	0.02 ^b	0.03 ^a	0.02 ^b	0.02 ^b	0.001	<0.01
18:0	6.61 ^a	5.68 ^b	5.89 ^{ab}	6.35 ^{ab}	0.220	0.04
trans-4 18:1	0.02 ^b	0.02 ^b	0.02 ^b	0.03 ^a	0.001	<0.01
trans-5 18:1	0.02 ^b	0.01 ^c	0.02 ^b	0.03 ^a	0.001	<0.01
trans-6–8 18:1	0.19 ^b	0.14 ^c	0.19 ^b	0.30 ^a	0.011	<0.01
trans-9 18:1	0.15 ^b	0.13 ^b	0.16 ^b	0.23 ^a	0.010	<0.01
trans-10 18:1	0.21 ^b	0.15 ^b	0.21 ^b	0.35 ^a	0.020	<0.01
trans-11 18:1	0.43 ^{ab}	0.42 ^{ab}	0.38 ^b	0.47 ^a	0.032	0.05
trans-12 18:1	0.20 ^{ab}	0.16 ^b	0.24 ^{ab}	0.29 ^a	0.024	0.01
trans-13–14 18:1	0.42	0.32	0.49	0.53	0.063	0.15
trans-15 18:1	0.26 ^{ab}	0.22 ^b	0.25 ^{ab}	0.31 ^a	0.015	0.01
trans-16 18:1	0.20 ^a	0.16 ^b	0.20 ^a	0.21 ^a	0.009	<0.01
cis-6–8 18:1	0.12 ^{ab}	0.09 ^b	0.10 ^{ab}	0.18 ^a	0.021	0.04
cis-9–10 18:1	12.02 ^b	12.64 ^b	12.05 ^b	15.76 ^a	0.594	<0.01
cis-11 18:1	0.40	0.50	0.50	0.49	0.037	0.11
cis-12 18:1	0.21 ^{ab}	0.18 ^b	0.20 ^b	0.25 ^a	0.013	<0.01
cis-13 18:1	0.04 ^b	0.07 ^a	0.05 ^{ab}	0.06 ^a	0.007	0.01
cis-14 18:1	0.03 ^b	0.03 ^b	0.03 ^b	0.04 ^a	0.003	<0.01
cis-15 18:1	0.05 ^{ab}	0.04 ^b	0.05 ^{ab}	0.06 ^a	0.005	0.02
cis-9, 12 18:2	1.36 ^{ab}	1.10 ^c	1.18 ^{bc}	1.52 ^a	0.062	<0.01
cis-9,trans-11 18:2	0.21 ^b	0.22 ^{ab}	0.19 ^b	0.26 ^a	0.017	<0.01
trans-10,cis-12 18:2	0.01	0.01	0.01	0.01	0.001	0.63
cis-9, 12, 15 18:3	0.38	0.38	0.37	0.38	0.016	0.94
Glycerol	12.52	12.37	12.33	12.24	0.090	0.24
Sum						
De novo fatty acids ²	23.94 ^a	22.41 ^a	22.53 ^a	18.95 ^b	0.798	<0.01
C16 fatty acids	31.46	33.84	34.00	32.93	0.634	0.05
Preformed fatty acids ³	25.27 ^b	24.40 ^b	24.51 ^b	29.82 ^a	0.990	<0.01

^{a-c}Within a row, means without a common superscript letter differ ($P \leq 0.05$).

¹Diets formulated to meet: 100% of MP and energy requirement = AMP_AE; 90% of MP requirement and 94% of energy requirement = DMP_DE; 90% of MP requirement and 99% of energy requirement = DMP_AE; 90% of MP requirement and 104% of energy requirement = DMP_EE.

²Sum of straight even-chain fatty acids from C6 to C14.

³Sum of odd- and branched-chain fatty acids, and all fatty acids with a carbon chain length of 17 or more.

cows, although plasma BCAA concentrations were not different between cows receiving the DMP diets (Table 9). From this observation, 2 points can be discussed. First, the BCAA showed the largest numerical decrease in their plasma concentrations among the EAA despite

a similar decrease in their supply with the DMP rations. This might be related to the fact that the BCAA are barely removed by the liver, and therefore, any change in their supply is more reflected in their peripheral concentrations than for the EAA removed sub-

stantially by the liver such as Phe and Trp (Lapierre et al., 2012). Second, the DMP_AE diet had the highest NFC concentration, and plasma glucose concentration was numerically higher with cows receiving this treatment. Increased glucose supply, often related to higher plasma glucose concentration, has been reported to decrease plasma BCAA concentrations (Nichols et al., 2016), which might explain this observation. Among the NEAA, only plasma Gly and Pro concentrations were affected by the reduction of MP supply. Cows receiving DMP diets had lower plasma Pro concentration when compared with AMP_AE-fed cows. This might be related to the lower Arg digestible flow with the MP diets, Arg being an important precursor of Pro into milk protein mammary synthesis. On the opposite, plasma Gly concentration was increased when cows received DMP_EE diets in comparison with AMP_AE or DMP_DE; DMP_AE-fed cows had an intermediate value. Lee et al. (2012c) also observed an increased plasma concentration of Gly with low-MP diets. However, mechanisms involved with this observation are not clear (Lee et al., 2012c).

When compared with all other diets, milk concentration of most de novo fatty acids was reduced whereas the sum of preformed fatty acids was increased when cows were fed DMP_EE (Table 10). This effect is mainly explained by an increase in the concentration of *cis*-9–10 18:1 in milk of cows fed DMP_EE. Compared with other diets, DMP_EE had a greater concentration of most fatty acids, especially 16:0 and *cis*-9 18:1 (Table 2), the 2 main fatty acids contained in Ca salts of long-chain fatty acids used in the current study. Long-chain fatty acids derived from the diets inhibit the synthesis of malonyl-CoA and, thus, the de novo synthesis of fatty acids with 6 to 16 carbons by the mammary cells (Beaulieu and Palmquist, 1995; Emery, 1973). As opposed to other de novo fatty acids, milk 16:0 concentration was not significantly different among treatments, most probably due to the double origin (de novo synthesis and preformed) of this specific fatty acid. Several intermediates of the biohydrogenation pathway of unsaturated fatty acids (*trans*-4 18:1, *trans*-5 18:1, *trans*-6–8 18:1, *trans*-9 18:1, and *trans*-10 18:1) were in greater concentration in milk of cows fed DMP_EE in comparison with cows fed all other diets. *Trans*-18:1 fatty acids are derived from incomplete biohydrogenation of dietary UFA in the rumen (Jenkins and McGuire, 2006). The effect of DMP_EE on milk fat concentrations of biohydrogenation intermediates suggests that the Ca salts of long-chain fatty acids, added in the largest proportion in this diet (Table 2), may not be totally inert in the rumen. As demonstrated by Jenkins and Bridges (2007), protected fat sources, such as Ca salts, still release some fatty acids in the

rumen. In addition, because of its potential inhibitory effect on rumen lipolysis and subsequent biohydrogenation (Gerson et al., 1983), the lack of RDP in DMP_EE diet might also have contributed to this observation (Leduc et al., 2017).

CONCLUSIONS

Our results showed that a reduction of MP supply below estimated MP requirements does not reduce milk, milk component, and ECM yields as long as His, Lys, Met, and energy requirements are met. This dietary strategy, however, improved N efficiency and the efficiency of utilization of MP and EAA, while decreasing N excretion by 24%. Such changes would reduce N excretion and thereby decrease the environmental impact of milk production. This study shows that, when formulating diets for dairy cattle, emphasis should be placed on covering EAA rather than MP requirements, as long as RDP requirements are met.

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