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Nitrogen balance in dairy cows fed low-nitrogen diets based on various proportions of fresh grass and maize silage

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A B S T R A C T

To ensure sustainable and efficient production, dairy farms must reduce their environmental impacts and nitrogen losses, which are sources of pollution, while increasing their feed self-sufficiency. Grass-based dairy systems, frequently combine fresh grass with maize silage when grass is scarce or during dietary transitions. However, the effects of combining fresh grass and maize silage on cow performance and N excretion are poorly known. This study aimed to quantify the effects of increasing the proportion of maize silage in a fresh grass diet on cow N flows and metabolism, in the context of grass-based dairy systems. Four proportions of maize silage in a fresh grass diet (objectives of 0, 17, 34 and 51% DM of maize silage) were investigated. The experiment was performed in a 4 × 3 Latin square design using eight lactating cows during three 3-week periods. DM intake (**DMI**), milk yield, faeces and urine outputs, and their N concentrations were measured for each cow. The fresh grass CP concentration was lower than planned (106 ± 13.0 g/kg DM). This resulted in very low dietary CP concentration, which decreased from 108 to 86 g/kg DM when maize silage in the diet increased from 0 to 51% DM, respectively. DM intake and milk yield both decreased linearly by 3.3 kg/day from 0 to 51% DM of maize silage in the diet. Thus, N intake decreased linearly by 100 g/day from 0 to 51% DM of maize silage in the diet. The N concentration of milk was highest for the diet with 0% DM of maize silage. Nitrogen excreted in faeces and urine decreased linearly by 29 and 23 g/day, respectively, from 0 to 51% DM of maize silage in the diet. The low dietary N concentration resulted in low ruminal NH_3-N concentrations (8 mg/L, on average) and urinary urea excretion (down to 8% urea N in urinary N). Increasing the proportion of maize silage in an unusually low-N grass diet, without protein-rich concentrates, induced highly N-deficient diets with minimal N losses in faeces and urine but large and unsustainable decreases in DMI and milk yield.

Implications

In dairy systems, farmers frequently offer maize silage to grazing cows during dietary transitions or when grass is scarce. Grass nitrogen concentration can vary greatly, and combining grass with maize silage may result in nitrogen-deficient diets. This study provides new data on nitrogen balance in lactating cows fed fresh grass and maize silage in situations of high nitrogen deficit but usual energy supply. This study highlights the farmers' difficulty in anticipating periods of poor grass quality, and the importance of finding a trade-off between decreasing nitrogen losses and maintaining dairy performance.

Introduction

To ensure sustainability, dairy farms must increase their feed self-sufficiency while reducing their negative environmental impacts. Ruminant production is frequently highlighted as a contributor to the emission of greenhouse gases and pollutants such as NO_3^- and NH_3 (Lesschen et al., 2011; European Environment Agency, 2019), while using resources which could be consumed as human food. One way to address this challenge is to include more forage in dairy cow diets, like fresh grass, whether grazed or not. Fresh grass is a low-cost feed produced on-farm, with a good nutritive value for lactating cows, especially for nitrogen supply when compared to conserved forages (INRA, 2018; Delaby et al., 2020). However, the availability and composition of fresh grass vary throughout the year. Thus, when grass is scarce and during dietary transitions, farmers frequently supplement grazed grass with other feeds. Grass-based dairy systems, such as organic farms, that use few concentrates, commonly combine fresh grass

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with conserved forages, such as maize silage, in cow diets. Fresh grass is most of the time rich in protein and degradable N in contrast to maize silage (INRA, 2018). Thus, combining fresh grass and maize silage could help balance the nutritional value of diets. French technical guidelines recommend not supplementing fresh grass-based diets with N-rich concentrate when maize silage is less than 50% of the diet's DM (French Livestock Institute, 2010). Effects of this combination of forages on cow N balance and metabolism are not well known compared to those of full-time grazing or a total mixed ration with only conserved forages and concentrates.

The present study aimed to determine to what extent increasing the proportion of maize silage in a fresh grass diet can decrease N excretion without compromising dairy performance in the context of grass-based dairy systems. We hypothesised that increasing the proportion of maize silage in fresh grass diets could decrease N excretion in manure by decreasing dietary N concentration (Castillo et al., 2000; Huhtanen and Hristov, 2009; Spanghero and Kowalski, 2021), with little impact on dairy performance, by maintaining the dietary energy concentration and increasing urea recycling (Reynolds and Kristensen, 2008; Edouard et al., 2016; 2019). To this end, we quantified the effects of increasing the proportion of maize silage in a fresh grass diet with no N-rich supplements on cow N balance and metabolism. Fresh grass N concentration was lower than initially planned. This allowed us to focus on the effect of highly N-deficient diets on cow performance and N use.

Material and methods

Treatments, experimental design and cows

The study was performed at the INRAE PEGASE experimental dairy farm of Méjusseaume (Le Rheu, France, https://doi.org/10.15454/yk9q-pf68) from 12 April to 19 June 2021. Four treatments with an increasing proportion of maize silage in a fresh grass diet were investigated: treatments MS0, MS17, MS34 and MS51 corresponded to objectives of 0, 17, 34 and 51% DM of maize silage, respectively. These proportions were chosen to create regular intervals between treatments until half of the diet's DM was composed of maize silage (French Livestock Institute, 2010). Treatments were tested during three experimental periods according to two 4 × 3 Latin squares (one for primiparous and one for multiparous) balanced for potential carry-over effects. The study was limited to three periods to avoid a shortage of grass availability in late spring. Each period consisted of 13 days for adaptation to the treatment and 6 days for measurements (as in Kristensen et al., 2010; Cantalapiedra-Hijar et al., 2014).

The experiment was performed with eight ruminally cannulated Holstein cows (four primiparous and four multiparous) with two cows per treatment within each period. At the beginning of the experiment, cows were in mid-lactation (166 ± 40 days in milk) and had a mean BW of 601 ± 83.1 kg. During the pre-experimental period from 29 March to 7 April, cows were individually fed the MS34 diet *ad libitum*, and DM intake (**DMI**) and milk yield were 16.0 ± 2.26 and 22.2 ± 4.78 kg/day, respectively. Fresh grass was offered after the morning milking and then at 0930, 1130 and 1600 h, and maize silage was offered only after the afternoon milking, to mimic daytime grazing followed by spending all night indoors.

The grass came from the same paddock, managed to offer grass at the same vegetative stage throughout the experiment, by cutting regularly specific areas 25–35 days prior to be fed to the cows. The grassland was sown with a mixture of grasses (16 kg/ha of *Lolium perenne* L., Trybal cultivar; 8 kg/ha of *L. perenne* L., Ibisal cultivar; and 8 kg/ha of *Festuca arundinacea* Schred., Philona cultivar) in September 2018. The grassland received 30 kg N/ha as ammonium nitrate in March 2021 and after each grass harvest (i.e. ca. every 30–40 days). The botanical composition of the grass was determined on one day during each measurement period from a representative sample of 1 kg of freshly cut grass. On a DM basis, it contained a mean of 96.1 ± 2.8% grasses (mainly *L. perenne*) and 3.9 ± 2.8% other species, none of them legumes.

Housing and feeding management

Cows were housed in tie stalls in two temperature-controlled and mechanically ventilated rooms throughout the entire experiment. Cows were milked twice a day in the rooms. They could see, smell and hear each other during the experiment. Cows were fed *ad libitum* on a 24-h basis in individual troughs and had unlimited access to water and a salt lick. Two feeding management rules were followed throughout the experiment. First, total refusals for the entire day had to exceed but remain close to 10% of the offered diet; thus, at least one forage was offered *ad libitum*. The second rule was to ensure that the diet ingested contained the required proportion of maize silage. This involved adjusting the amounts of forage offered each day depending on the daily DM concentration and individual intake of each forage. For example, if the proportion of maize silage in the diet was too low one day, maize silage was fed *ad libitum* the next day and fresh grass was restricted.

Fresh grass was cut once daily at 0800 h at 6 cm from the ground using a mechanical mower with a cutter bar (Haldrup GmbH, Ilshofen, Germany) and was immediately offered at the trough or conserved in a cool room at 4 °C until the next feeding. The fresh grass offered was spread over four feedings, at 0800, 0930, 1130 and 1600 h, and refusals were removed at 1745 h for all treatments, except for MS0. The first two grass feedings were the most important as they followed the cows' natural feeding behaviour. Cows fed the MS0 diet also received fresh grass for the night at 1800 h (a fifth meal). For MS17, MS34 and MS51, maize silage was fed once per day at 1800 h immediately after removing grass refusals; maize silage refusals were removed at 0745 h the following morning. Thus, for MS17, MS34 and MS51, cows had 10 h per day to eat fresh grass and 14 h per day to eat maize silage. For the MS0, cows had access to grass all day long.

Forage characteristics and intake calculations

Quantities of forages offered and refused were weighed daily and samples were dried in a ventilated oven for 48 h at 60 °C to measure their DM concentrations and calculate the daily DMI of each forage for each cow. To this end, a 1 kg sample of each forage offered and refused per cow was dried daily. The DM concentration of maize silage was corrected by considering the volatilisation of fermentation products in the oven during drying. To this end, NH_3, volatile fatty acids, alcohols and lactic acid in a frozen sample (−20 °C) of maize silage were analysed, and volatilisation was then calculated using the equation of Dulphy et al. (1975). The volatilisation correction factor was 26 g/kg DM. Chemical analyses (organic matter (**OM**), N, NDF, ADF and ADL) were performed per period on pooled lyophilised daily samples of fresh grass and maize silage offered during the six measurement days. Similarly, chemical analyses of grass and maize silage refusals were performed per period on pooled lyophilised daily samples per cow on the same days. Nutrient intake (g/day) was calculated individually from amounts of nutrients offered and refused, thus considering the potential feed preferences of each cow:

$$\text{Nutrient intake} = \sum\nolimits_{n}^{1} [\text{Nutrient offered} - \text{Nutrient refused}]$$

with n the number of forages in the diet (n = 1 for MS0, 2 for the other diets), nutrient offered (g/day) the amount of forage offered (kg DM/day) multiplied by its nutrient concentration (g/kg DM), and nutrient refused (g/day) the amount of forage refused (kg DM/day) multiplied by its nutrient concentration (g/kg DM).

The net energy for lactation (**UFL**, Unité Fourragère Lait, equivalent to the net energy for the lactation, 1 UFL = 7.37 MJ of net energy/kg DM; INRA, 2018) concentration, **PDI** (protein digestible in the small intestine, equivalent to the metabolisable protein, INRA, 2018) concentration and rumen protein balance (**RPB**, CP intake minus the non-ammonia CP flowing from the duodenum (INRA, 2018)) of each forage were calculated from its chemical composition and the INRA 2018 feeding system (PrevAlim® software, https://www.inration-ruminal.fr/en/; INRA, 2018). The UFL and PDI concentrations, UFL and PDI supplies, and RPB of the entire diet were then calculated from the initial forage nutritional values and the DMI of each feed, considering digestive interactions, using the INRA 2018 feeding system (INRAtion® software, https://www.inration-ruminal.fr/en/; INRA, 2018).

Milk yield and composition

Cows were milked twice a day, and individual milk yield was recorded daily. True protein and fat concentrations were measured in fresh milk at each milking from days 15 to 19. For each cow, N concentration was measured in a 50 mL sample of fresh milk taken from pooled morning and afternoon milk once a week (day 17). A subsample of this pool was then ultra-filtered and frozen at −20 °C for later analysis of urea concentration.

Faeces and urine output, sampling and digestibility calculation

Faeces and urine output were determined individually by collecting all faeces and urine during a 5-day period (days 15–19). Faeces were collected in a gutter behind the cow and regularly transferred to a closed bucket. Total amounts were weighed and sampled daily (2% of the faecal output). Half of the sample was dried in a ventilated oven for 72 h at 60 °C to determine the faecal DM concentration, and the other half was frozen and lyophilised for chemical analyses (OM, N, NDF and ADF) of pooled samples per cow and period. The whole-tract digestibility of nutrients was calculated from the amount of each component ingested (intake, kg/day) and excreted in faeces (faecal output, kg/day):

$$\text{Digestibility} = (\text{intake} - \text{faecal output})/\text{intake}$$

The nutrient intake and faecal output used to calculate whole-tract digestibility are given in Supplementary Table S1.

To collect urine separately from faeces, cows were equipped with a harness that held a tube around the vulva to drain urine into a plastic container. Urine was immediately acidified in the container with 500 mL of 20% H_2SO_4 to prevent NH_3 volatilisation. Urine was weighed and sampled daily (1% of the urine output). Samples were pooled per cow and period and frozen before analysing N, urea, allantoin, uric acid and creatinine. Daily samples were successively stored in the same container at −20 °C.

Rumen fermentation and plasma metabolites

Ruminal pH and NH_3 concentration kinetics were determined on day 18 based on 10 sampling times during the day. Basal concentrations were determined at 0745 and 1745 h (before the first morning feeding of 0800 h and the evening feeding of 1800 h, respectively). The other samples were taken 1, 2, 3 and 5 h after these feedings (0900, 1000, 1100 and 1300 h, respectively, for morning grass feeding and 1900, 2000, 2100 and 2300 h, respectively, for evening silage or grass feeding). At each time, 50 mL of rumen fluid was sampled in the ventral sac via the cannula. The pH was immediately measured. Rumen fluid was then filtered through six layers of muslin and frozen at −20 °C (4 mL of rumen fluid in 4 mL of 20% NaCl preservative). The weighted means of ruminal pH and NH_3 concentration for the entire day were calculated based on the sampling times and intervals.

Blood urea was determined from blood sampled before the first morning feeding and the evening feeding (0745 and 1745 h, respectively) and then 3 h later (1100 and 2100 h, respectively) on day 17. Blood was sampled via the caudal vein and centrifuged (2 000g at 4 °C for 15 min), and the plasma was then frozen at −20 °C.

Chemical analyses

The lyophilised offered forages, refused forages and faeces were ground (0.8 mm) to analyse OM, fibre and N. The OM concentration was measured by ashing in a muffle furnace at 550 °C for 8 h (AOAC, 1990). Fibre concentrations (NDF, ADF and ADL) were determined sequentially with a Fibersac extraction unit (Ankom Technology, Fairport, NY, USA) (AOAC, 1990; Van Soest et al., 1991). The pepsin-cellulase digestibility of dried feeds was determined according to Aufrère and Michalet-Doreau (1988). The N concentrations of the offered and refused forages, faeces, urine and milk were analysed using the Dumas method (Leco, Saint Joseph, MI, USA) (AOAC, 1990). Urine, milk and plasma urea concentrations were analysed based on an enzymatic and colourimetric reaction assessed using a multi-parameter analyser (KONE Instruments 200 Corporation, Espoo, Finland). True protein and fat concentrations in the milk were measured by mid-infrared spectrophotometry (Milkoscan, Foss Electric, Hillerød, Denmark). The ruminal NH_3-N concentration was determined using the Berthelot colourimetric reaction method (KONE Instruments 200 Corporation, Espoo, Finland) (Gordon et al., 1978). The urinary concentrations of allantoin, uric acid and creatinine were analysed by high-performance liquid chromatography (HPLC Alliance, Waters Corporation, Milford, MA, USA) (George et al., 2006).

Calculation of unaccounted-for N

Unaccounted-for N was calculated as N intake minus the N exported in milk, N excreted in faeces and urine (g N/day), and retained N (g N/day). The retained N (g N/day) was estimated from the UFL balance (UFL/day, calculated as the dietary UFL supply minus cow UFL needs for lactation, gestation, growth and maintenance), assuming that 6 g N/UFL was retained by protein accretion or mobilised when the UFL balance was positive or negative, respectively (INRA, 2018).

Statistical analyses

One cow was removed from the analysis due to an unexplained deterioration of its health at the end of the first experimental period. Data were averaged per cow and per period (n = 21 statistical units) and analysed using the following mixed model (SAS, 2020; PROC MIXED):

$$Y_{ijk} = \mu + \text{Treatment}_i + \text{Period}_j + \text{Cow}_k + e_{ijk}$$

with Y_{ijk} the analysed variable; μ the overall mean; Treatment_i the fixed effect of the proportion of maize silage in the diet (3 df); Period_j the fixed effect of the experimental period (2 df); Cow_k the random effect of the cow and e_{ijk} the residual error term.

Linear and quadratic responses to the proportion of maize silage in the diet were determined using orthogonal contrasts.

Results

Feed and diet compositions, intake and digestibility

The mean chemical composition of maize silage lay within normal ranges (INRA, 2018) (Table 1). The mean fresh grass CP concentration, PDI concentration and RBP were very low (106, 80 and −28 g/kg DM, respectively), while the mean grass energy value was normal, with low mean NDF and ADF concentrations. The proportion of maize silage in the ingested diet followed a regular interval among diets, as intended (Table 2). As planned, the amount of DM refused was ca. 15% of the DM offered in each diet. Cows were less likely to refuse fresh grass than maize silage. To obtain the expected proportion of maize silage in the diet, the supply of fresh grass had to be restricted to an increasing degree as the proportion of silage in the diet increased. Thus, fresh grass refusals were near zero for the MS51 diet.

Total DMI decreased linearly by 3.3 kg/day from MS0 to MS51 (P < 0.01). The dietary DM and OM concentrations increased linearly as the proportion of maize silage in the diet increased (P < 0.05), while the dietary CP concentration decreased linearly by 22 g/kg DM from MS0 to MS51 (P < 0.01). Dietary NDF and ADF concentrations did not differ significantly among diets. Dietary UFL and PDI concentrations decreased linearly as the proportion of maize silage increased (P < 0.01). Dietary RPB was negative for all diets. Dietary RPB and the dietary PDI:UFL ratio decreased linearly as the proportion of maize silage in the diet increased (P < 0.01). The whole-tract digestibilities of DM, OM, NDF and ADF decreased linearly as the proportion of maize silage in the diet increased (P < 0.05).

Milk yield, milk composition and nitrogen partitioning

Milk yield decreased linearly by 3.3 kg/day from MS0 to MS51 (P < 0.01; Table 3). Milk fat concentration tended to vary quadratically among the diets and was lowest for MS17 and highest for MS51 (P = 0.08). Milk protein concentration of the MS0 diet was higher than those of the other three diets (quadratic effect: P < 0.01).

Nitrogen intake decreased linearly by 100 g/day from MS0 to MS51 (P < 0.01; Table 3). Milk N of the MS0 diet was greater than those of the other three diets (quadratic effect: P < 0.05). Faecal and urinary N decreased linearly by 29 and 23 g/day from MS0 to MS51, respectively (P < 0.01). Diet did not influence urine output. The unaccounted-for N was negative for all diets and tended to decrease linearly as the proportion of maize silage in the diet increased (P = 0.08).

Rumen fermentation, urea and non-urea nitrogen metabolites

The mean ruminal pH increased linearly as the proportion of maize silage in the diet increased (P < 0.01; Table 4). The basal ruminal pH at 0745 h for the MS0 diet was lower than those for the other three diets (linear effect: P < 0.01; Fig. 1a). At 0900 h, just after the first grass feeding, the ruminal pH for the MS34 and MS51 diets was higher than that for the MS0 diet (linear effect: P < 0.01). At 1745 h, just before feeding maize silage, the ruminal pH was highest for the MS51 diet, intermediate for the MS34 and MS0 diets, and lowest for the MS17 diet (quadratic effect: P < 0.01).

The mean ruminal NH_3-N concentration was not influenced by the diet (8.2 ± 3.87 mg/L). The basal ruminal NH_3-N concentration at 0745 h for MS51 tended to be higher than those for the three other diets (linear effect: P = 0.08; Fig. 1b). Ruminal NH_3-N concentrations at 1900 and 2000 h for the MS51 diet were higher than those for the other three diets (quadratic effect: P < 0.05 and P = 0.07 for 1900 and 2000 h, respectively). At 2300 h, the ruminal NH_3-N concentration for the MS34 diet was higher than those for the other three diets (quadratic effect: P = 0.06).

The mean plasma urea concentration decreased linearly by 18.2 mg/L from MS0 to MS51 (P < 0.01; Table 4). Milk and urinary urea concentrations tended to decrease linearly as the proportion of maize silage increased (P = 0.08). Urinary urea N excretion decreased linearly by 2.3 g/day from MS0 to MS51 (P < 0.01). The urinary creatinine and urea N proportion in total urinary N were not influenced by the diet. Urinary allantoin and uric acid decreased linearly as the proportion of maize silage in the diet increased (P < 0.01).

Discussion

Highly nitrogen-deficient diets

All diets had CP concentrations (86–108 g/kg DM) much lower than those usually recommended for dairy cows (140–160 g/kg DM, INRAE, 2018) and reported in the literature (from 100 to more than 250 g/kg DM, Huhtanen and Hristov, 2009), mainly due to the unexpectedly low-CP concentration in the fresh grass. A relatively low mean temperature in spring 2021 (11.9 °C vs 13.4 °C during the same period from 1991 to 2020, AgroClim INRAE, INRAE CLIMATIK platform, 2022) likely decreased N mineralisation in the soil and thus N availability for grass growth (Miller and Geisseler, 2018). Under these conditions, the mineral N fertilisation was certainly insufficient to ensure correct plant nutrition and grass N uptake (Peyraud et al., 1997).

Such conditions may occur in dairy farms and result in low-N diets, while farmers generally do not have the opportunity to mea-

Table 1
Chemical composition and nutritional value of forages offered to dairy cows.

	Maize silage		Fresh grass	
Component	Mean	SD	Mean	SD
DM, g/kg fresh weight	329	4.1	230	37.8
OM, g/kg DM	956	0.6	933	4.5
CP, g/kg DM	64	1.5	106	13.0
NDF, g/kg DM	501	11.1	489	71.7
ADF, g/kg DM	274	7.3	252	41.7
ADL, g/kg DM	21	0.6	17	4.8
Nutritional value				
UFL/kg DM	0.88	0.008	1.00	0.079
PDI, g/kg DM	57	0.4	80	2.9
RPB, g/kg DM	−41	1.2	−28	10.3

Abbreviations: OM = organic matter; PDI = protein digestible in the small intestine; RPB = rumen protein balance, CP intake minus non-ammonia CP flowing from the duodenum; UFL = unité fourragère lait (1 UFL = 7.37 MJ of net energy for lactation).
Means and SD for six samples per forage.

Table 2
Effects of increasing the proportion of maize silage in a fresh grass diet on dairy cow intake, diet composition and digestibility.

Variable	Treatment[1]				RSD	P-value	
	MS0	MS17	MS34	MS51		Linear	Quadratic
Feed intake							
DM intake, kg/day	15.6[a]	13.9[b]	13.4[bc]	12.3[c]	0.99	<0.001	0.550
Fresh grass intake, kg DM/day	15.6[a]	11.4[b]	8.9[c]	6.2[d]	0.93	<0.001	0.126
Maize silage intake, kg DM/day	0[a]	2.5[b]	4.5[c]	6.1[d]	0.53	<0.001	0.118
Fresh grass in the diet, % DM	100[a]	82.2[b]	66.1[c]	51.1[d]	1.12	<0.001	0.021
Maize silage in the diet, % DM	0[a]	17.8[b]	33.9[c]	48.9[d]	1.12	<0.001	0.021
Total DM refused, % DM offered	17.5	14.4	13.5	16.2	3.72	0.526	0.118
Fresh grass refused, % DM grass offered	17.5[a]	14.5[a]	10.7[ab]	4.0[b]	5.00	0.002	0.425
Maize silage refused, % DM silage offered	0[a]	11.8[b]	17.3[bc]	24.7[c]	5.94	<0.001	0.428
Diet chemical composition							
DM, g/kg fresh weight	228[a]	242[ab]	253[ab]	265[b]	22.6	0.021	0.924
OM, g/kg DM	933[a]	937[b]	942[c]	944[c]	2.8	<0.001	0.540
CP, g/kg DM	108[a]	101[b]	93[c]	86[d]	4.7	<0.001	0.998
NDF, g/kg DM	475	485	488	491	23.5	0.303	0.771
ADF, g/kg DM	245	254	256	261	14.1	0.116	0.772
Diet nutritional value							
UFL/kg DM	0.997[a]	0.969[b]	0.937[c]	0.910[d]	0.0157	<0.001	0.922
PDI, g/kg DM	80[a]	76[b]	73[c]	69[d]	1.2	<0.001	0.561
PDI/UFL, g/UFL	81[a]	79[b]	78[bc]	76[c]	1.5	<0.001	0.713
RPB, g/kg DM	−27[a]	−29[ab]	−32[bc]	−34[c]	2.3	<0.001	0.845
Whole-tract digestibility, g/g							
DM	0.733[a]	0.724[ab]	0.703[ab]	0.692[b]	0.0249	0.020	0.897
OM	0.751[a]	0.740[ab]	0.717[b]	0.705[b]	0.0232	0.007	0.950
NDF	0.694[a]	0.672[a]	0.620[b]	0.585[b]	0.0339	<0.001	0.708
ADF	0.710[a]	0.686[a]	0.633[b]	0.598[b]	0.0334	<0.001	0.731

Abbreviations: OM = organic matter; PDI = protein digestible in the small intestine; RPB = rumen protein balance, CP intake minus non-ammonia CP flowing from the duodenum; UFL = unité fourragère lait (1 UFL = 7.37 MJ of net energy for lactation).

[1] Treatments MS0, MS17, MS34 and MS51 correspond to objectives of 0, 17, 34 and 51% DM of maize silage in a fresh grass diet, respectively. In a given row, adjusted means with different superscript letters differ significantly between treatments ($P < 0.05$).

Table 3
Effects of increasing the proportion of maize silage in a fresh grass diet on dairy cow milk yield and composition, and nitrogen partitioning.

Variable	Treatment[1]				RSD	P-value	
	MS0	MS17	MS34	MS51		Linear	Quadratic
Milk yield, kg/day	16.4[a]	14.6[ab]	13.3[b]	13.1[b]	1.27	0.002	0.222
Corrected milk yield[2], kg/day	16.8[a]	14.6[b]	13.6[b]	13.5[b]	1.08	<0.001	0.074
Milk fat concentration, g/kg	42.2[ab]	40.4[a]	42.0[ab]	43.8[b]	1.91	0.135	0.076
Milk true protein concentration, g/kg	32.6[a]	30.6[b]	30.5[b]	31.1[b]	0.70	0.011	0.003
N partitioning, g/day							
Intake	267[a]	223[b]	201[b]	167[c]	17.0	<0.001	0.547
Milk	90[a]	75[b]	69[b]	68[b]	5.9	<0.001	0.037
Faeces	103[a]	89[b]	86[b]	74[c]	7.2	<0.001	0.864
Urine	68[a]	60[ab]	53[b]	45[c]	5.5	<0.001	0.836
Unaccounted-for N[3]	−5	−8	−10	−14	7.0	0.078	0.959
Urine output, kg/day	19.6	20.9	19.5	18.1	4.48	0.527	0.550

Abbreviations: UFL = unité fourragère lait (1 UFL = 7.37 MJ of net energy for lactation).

[1] Treatments MS0, MS17, MS34 and MS51 correspond to objectives of 0, 17, 34 and 51% DM of maize silage in a fresh grass diet, respectively. In a given row, adjusted means with different superscript letters differ significantly between treatments ($P < 0.05$).

[2] Milk yield corrected for standard milk with concentrations of 40 g/kg fat and 31 g/kg protein (INRA, 2018).

[3] Calculated from N intake, in milk, in faeces, in urine and corrected from an estimate of retained or mobilised N. Retained or mobilised N was estimated from the balance of net energy for lactation (UFL balance), assuming 6 g N retained per UFL (INRA, 2018).

sure the grass N concentration regularly. The present study tested an unexplored range of dietary CP concentrations for lactating dairy cows, enabling to better predict the detrimental effects of low-N diets on dairy performance over a short period. This enabled investigating effects of extreme N deficit in relatively high-energy diets, confirmed by the negative RPB (−30 g/kg DM) and the extremely low milk and plasma urea concentrations (27.6 and 37.2 mg/L, respectively). In particular, the mean ruminal NH_3-N concentration (8 mg/L) was lower than typical concentrations for cows fed fresh grass diets (67–275 mg/L for diets with 140–225 g CP/kg DM, Van Vuuren et al., 1993; Delagarde et al., 2008) and even lower than those of low-N fresh grass diets in the literature (11–28 mg/L for diets with 106–113 g CP/kg DM, Peyraud et al., 1997; Delagarde et al., 1999; Rojen et al., 2008). Ruminal pH did not fall below 5.9 at any time, which is uncommon for fresh grass diets, which generally cause it to decrease to at least 5.6 (Kolver et al., 1998; Delagarde et al., 1999; 2008). These ruminal pH and NH_3-N concentrations clearly indicate that microbial activity decreased with such a high dietary N deficit. For most of the measurement hours, the ruminal NH_3-N concentration lay below the critical threshold of 20 mg/L mentioned by Clark et al. (1992) as the minimum for optimal cellulolytic activity in dairy cows. Ruminal NH_3-N concentration varied slightly throughout the day, with a postprandial peak that did not exceed 25 mg/L above the basal level, while it usually reaches up to 80–260 mg/L in dairy cows fed a fresh grass diet with a standard CP concentration (130–

Table 4
Effects of increasing the proportion of maize silage in fresh grass diet on dairy cow ruminal pH and NH_3-N, urea in plasma, milk, urine and non-urea urinary N components.

Variable	Treatment[1]				RSD	*P*-value	
	MS0	MS17	MS34	MS51		Linear	Quadratic
Ruminal pH[2]	6.23[a]	6.28[ab]	6.41[bc]	6.49[c]	0.090	<0.001	0.694
Ruminal NH_3-N concentration[2], mg/L	9.01	7.79	6.53	9.44	3.866	0.997	0.259
Urea concentration, mg/L							
Plasma[3]	45.3[a]	43.4[a]	32.8[ab]	27.1[b]	9.18	0.006	0.657
Milk	29.5[ab]	34.5[a]	27.7[ab]	18.7[b]	9.82	0.072	0.144
Urine	590	562	471	396	165.0	0.079	0.782
Urinary urea N, g/day	5.09[a]	5.56[a]	4.02[ab]	2.84[b]	1.20	0.010	0.213
Urinary urea N, % of urinary N	7.48	9.45	7.76	6.48	2.067	0.300	0.163
Urinary allantoin, g/day	34.1[a]	29.2[ab]	27.6[b]	20.6[c]	3.65	<0.001	0.556
Urinary uric acid, g/day	4.4[a]	3.4[b]	3.4[b]	2.0[c]	0.67	<0.001	0.498
Urinary creatinine, g/day	12.6	12.3	12.7	11.3	1.48	0.261	0.423

[1] Treatments MS0, MS17, MS34 and MS51 correspond to objectives of 0, 17, 34 and 51% DM of maize silage in a fresh grass diet, respectively. In a given row, adjusted means with different superscript letters differ significantly between treatments ($P < 0.05$).
[2] Weighted means of ruminal pH and NH_3-N concentration for the entire day based on the sampling times and intervals between the 10 sampling times (0745, 0900, 1000, 1100, 1300, 1745, 1900, 2000, 2100 and 2300 h).
[3] Mean of four plasma sampling times (0745, 1100, 1745 and 2100 h).

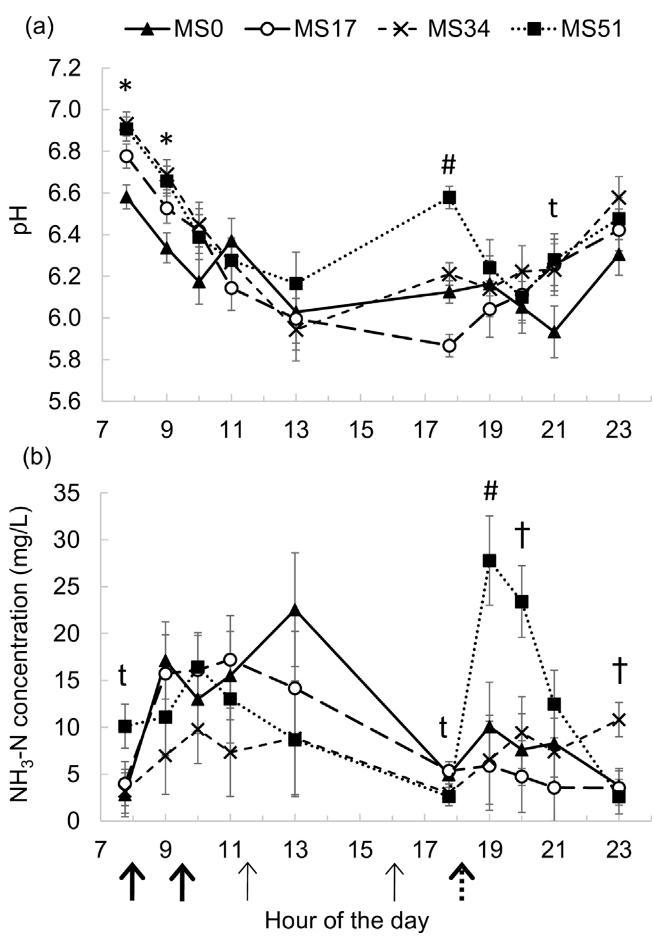

Fig. 1. Effect of increasing the proportion of maize silage in a fresh grass diet on dairy cow (a) ruminal pH and (b) NH_3-N concentration per hour. Treatments MS0, MS17, MS34 and MS51 correspond to objectives of 0, 17, 34 and 51% DM of maize silage in a fresh grass diet, respectively; * = linear effect ($P < 0.05$); t = linear trend ($P < 0.10$); # = quadratic effect ($P < 0.05$); † = quadratic trend ($P < 0.10$); vertical bars = SEM; bold arrows = main fresh grass feedings; fine arrows = supplementary fresh grass feedings; dashed arrow = maize silage feeding (except for MS0: grass feeding).

185 g/kg DM, Van Vuuren et al., 1993; Kolver et al., 1998; Delagarde et al., 2008; Ribeiro Filho et al., 2012). This clearly illustrates the chronic shortage of degradable N in the diet, which was immediately used by micro-organisms in the rumen.

Intake, digestion and milk yield

The large decrease in voluntary DMI as the proportion of maize silage in the diet increased was due to the fact that cows limited their intake of maize silage despite being fed *ad libitum* (strong increase in maize silage refusals). Achieving the planned proportion of maize silage in the diet required strongly restricting the fresh grass supply. The decrease in DMI was unlikely to be due to differences in feeding management between diets, as the cows that ate the least were not those that had the shortest access to the diet. Cows fed the MS51 diet had 14 h each day to eat the maize silage, which is much longer than the time required to eat 6 kg DM of maize silage, given its high intake rate (5–6 kg DM/h; Le Liboux and Peyraud, 1998 and 1999). We assume that the cows' greater preference for fresh grass rather than maize silage was likely enhanced by the low-CP and metabolisable protein concentrations of the diets, which is known to decrease voluntary intake (Faverdin et al., 2003; INRA, 2018). A deficit in degradable N may have decreased microbial activity, fibre ruminal digestion and slowed down passage rate (Köster et al., 1996). A decrease in protein in the intestine may also have directly affected voluntary intake by regulating appetite (Faverdin et al., 2003). The loss of 1.4 kg/day of DMI observed in the present study for every 10 g CP/kg DM decrease in the diet was in complete agreement with Bryant and Donnelly (1974), who observed the same loss of DMI (1.5 kg/day for every 10 g CP/kg DM decrease) for dairy cows fed a combination of fresh grass and maize silage, with dietary CP concentrations similar to those in the present study (94–114 g/kg DM). They even showed that cows stopped eating when their diets consisted entirely of maize silage fed *ad libitum*. The decrease in DMI related to that of dietary CP concentration in our study was greater than that reported in the literature, which ranged from 0 to 0.5 kg/day for dairy cows fed diets ranging from 106 to 173 g CP/kg DM (Peyraud et al., 1997; Kristensen et al., 2010; Yang et al., 2022). Adverse effects on voluntary intake are likely to increase as the CP concentration of the diet decreases, particularly below 100 g CP/kg DM (Rico-Gómez and Faverdin, 2001; Faverdin et al., 2003), which is extremely low for lactating dairy cows.

The decrease in dietary OM and fibre digestibilities as the proportion of maize silage in the diet increased was expected and consistent with the specific fibre digestibility of each forage, as fibre in maize silage is much less digestible (0.51 and 0.45 for NDF and ADF, respectively; INRA, 2018) than that in fresh grass (0.69 and 0.71 for NDF and ADF, respectively, in treatment MS0). The decrease in dietary CP concentration and RPB also likely decreased dietary OM digestibility, but only marginally, as all four diets were deficient in degradable N, with low and similar ruminal NH_3-N concentrations, thus limiting microbial synthesis (INRA, 2018).

The loss of DMI was likely the main cause of the decrease in faecal N as the proportion of maize silage in the diet increased (Castillo et al., 2000). Faecal N output had a range similar to those of other experiments with dairy cows fed low-N diets (Susmel et al., 1995; Peyraud et al., 1997; Rojen et al., 2008). The decrease of 9 g N/day for every 1 kg loss of DMI in the present study is consistent with the decrease in faecal N observed in several studies (Hindrichsen et al., 2006; Yang et al., 2022), which ranged from 6-13 g/day for every 1 kg loss of DMI.

The large decreases in DMI, N intake, diet digestibility and thus energy supply, as the proportion of maize silage in the diet increased were the main factors responsible for the decrease in milk yield and milk protein concentration (Coulon and Rémond, 1991). These decreases in DMI and milk yield as the proportion of maize silage increased in a fresh grass diet agree with the results of Bryant and Donnelly (1974). The decrease in milk yield, milk N concentration and N exported in milk were similar to those observed by Susmel et al. (1995) and Cantalapiedra-Hijar et al. (2014).

In this study, the N use efficiency (N in milk in g/day divided by N intake in g/day) of the MS51 diet was particularly high (41%), and such a high value has rarely been reported in the literature. However, given the severe restrictions on DMI and milk production, such high efficiency is not sustainable, as it requires highly N-deficient diets that strongly disrupt rumen function and can endanger cow health in the medium-to-long term.

Nitrogen metabolism, urea and urinary nitrogen

The urinary N and urinary urea N outputs were low, which is consistent with previous experiments with dairy cows fed low-N diets (Susmel et al., 1995; Peyraud et al., 1997; Cantalapiedra-Hijar et al., 2014). Susmel et al. (1995) observed a urinary urea N (4.7 g/day) similar to that in the present study (4.4 g/day) in dairy cows fed a diet with 94 g CP/kg DM. The low urinary urea concentration resulted from the low plasma urea concentration (37 mg/L), which was lower than those reported in the literature for dairy cows fed a slightly higher dietary CP concentration (76 mg/L with 120 g CP/kg DM) (Cantalapiedra-Hijar et al., 2014). A decrease in the clearance rate of urea (volume of blood cleared per unit time (L/h); urinary urea excretion (g/day) divided by the plasma urea concentration (g/L)) can also explain the low concentrations of urinary urea N, as the latter is known to decrease as dietary CP concentration decreases (Kristensen et al., 2010). In the present study, clearance rate was lower than those in the literature for lactating dairy cows fed higher dietary CP concentrations (11 L/h vs 20–41 L/h with 120–180 g CP/kg DM, according to Kristensen et al. (2010) and Edouard et al. (2016)). The extreme N deficit likely resulted in renal regulation being responsible for the low clearance rate of urea. Eriksson and Valtonen (1982) observed active regulation of urea filtration and reabsorption in the kidneys, which decreased the clearance rate in goats fed extremely low-CP straw-based diets (<20 g/kg DM).

The decrease in urinary N as the proportion of maize silage in the diet increased was primarily due to the decrease in N intake (Spanghero and Kowalski, 2021). However, this decrease was much smaller than those in previous studies which investigated wider ranges of dietary CP concentrations (2 g/day vs 5–9 g/day for every 10 g/day decrease in N intake for Susmel et al. (1995), Peyraud et al. (1997) and Cantalapiedra-Hijar et al. (2014)). The proportion of urea N in urinary N was particularly low in our study (8%). The literature indicates that this proportion is correlated with the dietary protein supply and CP concentration (Dijkstra et al., 2013). It can reach 80% in dairy cows when dietary CP concentration is high (>175 g/kg DM) and decreases to ca. 40% when CP concentration is ca. 120 g/kg DM (Edouard et al., 2016; 2019). It can even decrease to 14% for low-CP diets (<110 g/kg DM) (Susmel et al., 1995; Peyraud et al., 1997). Although a proportion of 8% of urea N in urinary N has never been reported in the literature for lactating dairy cows, it is consistent with the variability previously described and the extremely low dietary CP concentrations observed in the present study. Thus, the main variable component of urinary N – urea N – was small, even for the diet with the highest CP concentration (MS0), which explained the small decrease in urinary N in situations of extreme N deficit (MS51). This suggests that a minimum threshold of urinary N was reached for such low-N intake, likely due to a large and non-compressible part of endogenous N in urinary N (Castillo et al., 2000). According to the literature, calves fed a highly N-deficient diet that was not compatible with production excreted little urinary N, which was composed almost entirely of endogenous N and varied proportionally to their BW (Swanson, 1977). In the present study, mean endogenous urinary N can be estimated as 30 g/day (0.05 $\times$ BW, INRA, 2018), representing up to 50% of the urinary N.

The urinary urea N decreased by only 2.3 g/day from MS0 to MS51, while urinary N decreased 10 times as much (-23 g/day). This suggests a large decrease in non-urea N in urine. Indeed, allantoin and uric acid decreased as the proportion of maize silage increased, in accordance with the decrease in dietary CP concentration (Bristow et al., 1992; Susmel et al., 1995). The decrease in purine derivatives indicates a decrease in microbial protein synthesis in the rumen (Dijkstra et al., 2013). For dairy cows fed fresh grass, Peyraud et al. (1997) showed that despite the large decrease in dietary CP concentration (150 to 106 g/kg DM), the microbial N flow entering the duodenum remained constant (237 g/day), which highlights compensation of the low dietary N by urea recycling in the rumen. However, in the present study, urea recycling was likely restricted by the low plasma urea concentration, as suggested by Peyraud et al. (1997), due to the extreme dietary N deficit.

The present study showed cow metabolic adaptations over a short period that conserve urea and increase its recycling. Although these low-N diets minimise N excretion in manure, such a high N deficit is most likely not sustainable in the medium-to-long term as it does not ensure good rumen function or lactation support and may degrade cow health. These long-term effects of high diet N deficit require further investigation.

Conclusion

Increasing the proportion of maize silage from 0 to 51% DM in a low-N fresh grass diet resulted in very low dietary N concentrations (106 to 86 g CP/kg DM, respectively), with negative RPB (down to -30 g/kg DM) causing a decrease of 3.3 kg/day in DMI and milk yield. In these N-deficit situations with highly digestible diets, most of the ingested N was used, and the N excreted in faeces and urine was minimal, especially for the diet composed of half fresh grass and half maize silage (74 and 45 g/day, respectively). The dietary N deficit resulted in low ruminal NH_3-N concentration (8 mg/L), as well as low plasma and milk urea concentrations (28 and 37 mg/L, respectively), which ended in extremely low urinary urea N excretion (4.4 g/day, i.e. 8% of urinary N). This study illustrated the ability of cow metabolism to adapt to a severe N deficit over a short period. However, it also showed that very low-N diets severely decreased intake and dairy production, and altered rumen function. This study underlines the wide variability of the grass CP concentration and the difficulty for farmers in knowing the grass quality offered to cows. These results highlight a trade-off between limiting N losses and maintaining dairy performance, and clearly show that dietary CP concentrations below 100 g/kg DM are not sustainable in the medium-to-long term for lactating dairy cows.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100976.

Ethics approval

Experiments were performed in accordance with French and European Union legislation on animal experimentation and animal welfare. All procedures related to the care and management of animals were approved by an animal ethics committee of the French Ministry of Agriculture (approval number: APAFiS #23913-2020020315487805_v2).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration interest

None.

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