Extrusion of lupines with or without addition of reducing sugars: Effects on the formation of Maillard reaction compounds, partition of nitrogen and Nϵ-carboxymethyl-lysine, and performance of dairy cows

E. Manzocchi, A. Ferlay, S. Mendowski, G. Chesneau, Patrick Chapoutot, S. Lemosquet, Gonzalo Cantalapiedra-Hijar, P. Nozière

J. Dairy Sci. 106:7675–7697
https://doi.org/10.3168/jds.2022-22902

Extrusion of lupines with or without addition of reducing sugars: Effects on the formation of Maillard reaction compounds, partition of nitrogen and Nε-carboxymethyl-lysine, and performance of dairy cows

E. Manzocchi,[1,2]* A. Ferlay,[1] S. Mendowski,[2] G. Chesneau,[2] P. Chapoutot,[3] S. Lemosquet,[4] G. Cantalapiedra-Hijar,[1] and P. Nozière[1]†

[1]Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, 63122 Saint-Genès-Champanelle, France
[2]Valorex, La Messayais, 35210 Combourtillé, France
[3]Université Paris Saclay, INRAE, AgroParisTech, UMR Modélisation Systémique Appliquée aux Ruminants, 75005 Paris, France
[4]UMR Physiologie, Environnement et Génétique pour l'Animal et les Systèmes d'Elevage, INRAE, Institut Agro, 35590 Saint Gilles, France

ABSTRACT

The extrusion of leguminous seeds induces the formation of Maillard reaction compounds (MRC) as a product of protein advanced glycation and oxidation, which lowers protein degradability in the rumen. However, the quantitative relationship between the parameters of pretreatment (i.e., addition of reducing sugars) and extrusion, and the formation of MRC has not been established yet. Moreover, the fate of the main stable MRC, Nε-carboxymethyl-lysine (CML), in the excretory routes has never been investigated in ruminants. We aimed to test the effects of the temperature of extrusion of white lupines with or without addition of reducing sugars on the formation of MRC, crude protein (CP) degradability in the rumen, N use efficiency for milk production (milk N/N intake), and performance of dairy cows. Two experiments with a replicated 4 × 4 Latin square design were conducted simultaneously with 16 (3 rumen-cannulated) multiparous Holstein cows to measure indicators of ruminal CP degradability (ruminal NH_3 concentration, branched-chain volatile fatty acids), metabolizable protein supply (plasma essential AA concentration), N use efficiency (N isotopic discrimination), and dairy performance. In parallel, apparent total-tract digestibility of dry matter, organic matter, neutral detergent fibers, N, total Lys and CML, and partition of N and CML were measured with 4 cows in both experiments. The diets consisted on a DM basis of 20% raw or extruded lupines and 80% basal mixed ration of corn silage, silage and hay from permanent grasslands, pelleted concentrate, and a vitaminized mineral mix. Expected output temperatures of lupine extrusion were 115°C, 135°C, and 150°C, without and with the addition of reducing sugars before extrusion. The extrusion numerically reduced the in vitro ruminal CP degradability of the lupines, and consequently increased the predicted supply of CP to the small intestine. Nitrogen balance and urinary N excretion did not differ among dietary treatments in either experiment. Milk yield and N use efficiency for milk production increased with extrusion of lupines at 150°C without addition of reducing sugars compared with raw lupines. Nitrogen isotopic discrimination between dietary and animal proteins (the difference between $\delta^{15}N$ in plasma and $\delta^{15}N$ in the diet) were lower with lupines extruded at 150°C without and with addition of reducing sugars. Regardless of sugar addition, milk true protein yield was not affected, but milk urea concentration and fat:protein ratio were lower with lupines extruded at 150°C than with raw lupines. In the CML partition study, we observed that on average 26% of the apparently digested CML was excreted in urine, and a much lower proportion (0.63% on average) of the apparently digested CML was secreted in milk, with no differences among dietary treatments. In conclusion, we showed that the extrusion of white lupines without or with addition of reducing sugars numerically reduced enzymatic CP degradability, with limited effects on N partition, but increased milk yield and N use efficiency at the highest temperature of extrusion without addition of reducing sugars.

Key words: dairy cattle, white lupine, extrusion, advanced glycation end-product

INTRODUCTION

Alternative indigenous protein feeds such as leguminous seeds are increasingly needed to limit the net protein import into European livestock production systems, thereby promoting environmental protection and meeting societal expectations for more sustainable,

Received October 13, 2022.
Accepted May 12, 2023.
*Current address: Ruminant Nutrition and Emissions, Agroscope, Tioleyre 4, 1725 Posieux, Switzerland.
†Corresponding author: pierre.noziere@inrae.fr

traceable, and safe dairy production systems. In comparison with conventional protein feeds such as soybean meal, CP of raw leguminous seeds is more degradable in the rumen. Thermo-mechanical treatments such as extrusion can reduce their ruminal CP degradability, thereby increasing the supply of MP (Benchaar et al., 1994; Mendowski et al., 2021). Extrusion induces the formation of Maillard reaction compounds (**MRC**) as the result of nonenzymatic reactions between a carbonyl compound and an amino group, leading to protein glycation. However, the quantitative appearance of MRC during the extrusion of leguminous seeds according to their proximate composition is not predictable yet, and thus it is still poorly controllable by the technological parameters. Furthermore, the quantitative relation among the content of MRC in leguminous seeds, the ruminal degradability of CP and intestinal digestibility has not yet been established. To refine the technological parameters and eventually control the ruminal CP degradability of extruded leguminous seeds, it is essential to study the occurrence of MRC during their thermo-mechanical treatments.

Extrusion of different leguminous seeds induces different profiles of MRC, as a result of the interaction among the plant matrix, type of pretreatment, and temperature of extrusion (Mendowski et al., 2019, 2020). Nε-carboxymethyl-lysine (**CML**) is the first stable and relatively inert compound resulting from the glycation of the ε-amino group of Lys and its subsequent oxidation and is considered as a marker of the later steps of the Maillard reaction cascade. Lysine becomes thereby biologically less accessible to bacterial proteinases in the rumen, resulting in lower ruminal CP degradability. Lupines seem to be particularly susceptible to the formation of advanced-stage MRC such as CML during extrusion in comparison to faba beans, which contain compounds resulting from rather early steps of the Maillard reaction such as Nε-2-furoylmethyl-L-lysine (furosine; Mendowski et al., 2019). To date, there is little background information on the digestibility and absorption of MRC in dairy cows. Low molecular weight MRC such as CML may be absorbed in the intestine (Alamir et al., 2013). Mendowski et al. (2019) reported the apparent total-tract digestibility of CML in dairy cows for the first time. However, the fate of apparently digested CML has never been studied in ruminants, and quantitative studies investigating its metabolism (including excretion and secretion) in dairy cows are missing. Chronic exposure to dietary advanced glycation end products is in fact suspected to have immunomodulatory functions with adverse effects on human and animal health, as they interact with specialized receptors forming free radicals, which may induce oxidative stress and inflammation (Teodorowicz et al., 2018; Tessier et al., 2021). Research efforts are therefore increasingly devoted to the fate of dietary advanced glycation end products and their effects on human and animal health, but references regarding dairy cows are, to our knowledge, not available.

The aim of the present experiments was therefore to investigate the effects of extrusion with or without prior addition of reducing sugars on the formation of MRC in white lupines, and the consequences for ruminal fermentation (including kinetics of ruminal parameters on 3 rumen-cannulated cows), N partition and performance of dairy cows. The N isotopic discrimination between animal proteins and the diet ($\mathbf{\Delta^{15}N_{animal\text{-}diet}}$) was also used as biomarker for N use efficiency (Cantalapiedra-Hijar et al., 2018; Correa-Luna et al., 2022). By adding or omitting reducing sugars and applying different temperatures of extrusion to white lupines, we principally aimed at generating different MRC profiles. Indeed, the addition of reducing sugars increases the formation of MRC during extrusion of faba beans (Mendowski et al., 2020). The fate of the ingested CML in the main excretory routes was also investigated. We hypothesized that (1) CP from extruded lupines is less degradable in the rumen than CP from raw lupines, which improves the supply of CP to the small intestine and the predicted supply of MP. We also hypothesized that (2) the extrusion increases N use efficiency estimated as grams of milk N per grams of N intake, and reduces N excretion in urine, and (3) that the addition of reducing sugars before extrusion further increases the formation of MRC and reduces the degradability of CP in the rumen.

MATERIALS AND METHODS

The experiments were conducted at the INRAE Herbipôle research facility in Theix, France (https://doi.org/10.15454/1.5572318050509348E12) from November 2020 to March 2021 and approved by the ethics committee of the Auvergne-Rhône-Alpes region and the French Ministry of Higher Education, Research, and Innovation (approval APAFIS#15401–2017062616304407 v5).

Animals, Experimental Design, and Diets

Sixteen multiparous Holstein cows averaging 3.0 ± 0.24 lactations, 79 ± 17 DIM, 25 ± 1.0 kg of milk/d, and 686 ± 14.3 kg BW, 3 of which were rumen-cannulated, were enrolled in this study. Cows were used in 2 experiments (8 cows per experiment) with a replicated 4 × 4 Latin square design balanced for carryover effects conducted simultaneously. The 4 dietary treatments

tested in the first Latin square experiment (**EXP1**) were white lupines (*Lupinus albus*) extruded at expected output temperatures of 115°C (**EL1**), 135°C (**EL2**), or 150°C (**EL3**), and raw lupines (**RL**). The second Latin square experiment (**EXP2**) compared white lupines pretreated with the addition of 1.25% reducing sugars (i.e., dextrose monohydrate) before their extrusion at expected output temperatures at 115°C (**ELS1**), 135°C (**ELS2**), or 150°C (**ELS3**), and raw lupines with the addition of the same proportion of reducing sugars (**RLS**). Each dietary treatment immediately preceded and followed every other dietary treatment exactly once in each Latin square. The 3 rumen-cannulated cows (3.7 ± 0.7 lactations, 167 ± 76 DIM, 22.1 ± 1.9 kg of milk/d, 748 ± 3.7 kg BW) were all included in EXP1.

Both experiments consisted of 4 periods lasting 4 wk each, including 22 d of dietary adaptation and 6 d of samplings and data collection. All cows were housed in a freestall barn and had access to their own individual assigned feed trough during a pre-experimental period for habituation, as well as during the entire experiment, except during measurements of apparent total-tract digestibility of OM, N, NDF, CML, and total Lys, as well as N, and CML partition (wk 4). At wk 4 of each period, cows (n = 4) of square 1 of each experiment (n = 8; 3.3 ± 0.4 lactations, 99 ± 32 DIM, 24.8 ± 1.9 kg of milk/d, and 729 ± 17.9 kg BW) were tethered in a tiestall barn for the separate and total collection of feces and urine and measurements of apparent total-tract digestibility, N, and CML partition. During the pre-experimental period, all cows were fed the same basal mixed ration with up to 20% raw lupines in the total DM.

All experimental diets consisted (on a DM basis) of 30% corn silage, 24% grass silage (first cut of semi-mountainous grassland with *Dactylis glomerata*, *Arrhenaterum elatius*, and *Lolium perenne* as main botanical species), 15% hay (first cut of semimountainous grassland with *Alopecurus pratensis*, *Holcus lanatus*, *Arrhenaterum elatius*, *Lolium perenne* as main botanical species), 11% pelleted concentrate, and 20% of 1 of the 8 experimental raw (RL, and RLS) or extruded lupines (EL1, ELS1, EL2, ELS2, EL3, and ELS3) supplied as a meal and mixed individually and thoroughly with the basal ration to form a TMR (Table 1). In addition, 200 g of a vitamin-mineral supplement (Galaphos MiDi Duo, CCPA) was provided daily to each cow mixed in the TMR. All diets were designed iso-NE_L (expressed in unité fourragère lait; **UFL**; 1 UFL is equivalent to 7.37 MJ NE_L) and iso-CP, with lupines providing on average 45% of the CP. They were also iso-starch (16% starch in the DM) and iso-NDF (40% NDF in the DM), with forages providing 83% of the NDF. To ensure a nonlimiting Met supply according to INRA (2018) recommendations for intestinal truly digestible Met (**MetDI**) and Lys [**LysDI**; MetDI = 2.1% of protein truly digestible in the intestine (**PDI**), if 6.6 < LysDI < 6.8% of PDI; INRA, 2018], each cow was fed 20 g/d of rumen-protected DL-Met (with 80% bioavailability of Met, PDI = 60% in the DM, and MetDI = 100% PDI; Smartamine M, Adisseo). The mixed ration was offered in 2 equal meals at 0900 and 1600 h. Offered amounts until the third week of each experimental period allowed for at least 10% refusals for ad libitum intake and were adjusted twice per week according to the DM content of the basal mixed ration (determined at 103°C for 24 h) at 95% of the individual ad libitum intake measured during wk 3 of each period, to reduce interday variability during apparent total-tract digestibility measurements, N and CML partition study and thereby reduce experimental error. Dietary transitions between periods were gradual, at a rate of 25% of substitution of the experimental lupines per day, lasting 3 d. During the entire experiment, all cows had free access to water and NaCl blocks. All cows were milked daily at 0730 and 1500 h in a herringbone milking parlor, and during digestibility measurements in the tiestall with a milking pipeline. The individual milk yield was recorded automatically at each milking. Body weight was recorded daily after the morning milking, before the morning meal on an automatic scale, except for the 8 cows used for digestibility measurements from d 22 to 28 of each period. The BCS was graded on a 0-to-5 scale with quarter-unit increments (Bazin, 1984) by 2 trained individuals on d 1 of each period, and at the end of the experiment.

Pretreatment and Extrusion of Lupines

Before the pretreatment, as reported in Table 2, all lupines were ground. The lupines were extruded in a single screw extruder, whereby the generation of the heat to reach the expected output temperatures occurred only by self-heating of the extruded matrix through compression against the screw and the developed shear forces. Indeed, at the same expected output temperatures, extrusion by self-heating provides better protection of the proteins from degradation in the rumen compared with the extrusion of the same matrix with heating, as evidenced in previous in vitro screenings (Chapoutot et al., 2016). The target output temperatures were defined before the experiment based on previous in situ and in vitro measurements to induce the formation of different profiles of MRC in the extruded lupines, without compromising intestinal protein digestibility, which is likely reduced when lupines are extruded at expected output temperatures above 160°C (Mendowski et al., 2019; Chapoutot et al., 2020).

Table 1. Chemical composition and nutritive value according to INRA (2018) of the experimental diets containing lupines (EXP1) or lupines with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]				Lupines + reducing sugars (EXP2)[2]			
Item	RL	EL1	EL2	EL3	RLS	ELS1	ELS2	ELS3
Chemical composition,[3] g/kg DM								
OM	924	924	924	923	924	924	924	923
CP	154	156	156	154	152	155	156	154
NDF	407	405	405	404	406	402	402	401
Ether extract	40	40	40	43	40	40	40	46
CML,[4] μg/g DM	17.49	17.88	18.23	18.66	17.21	18.18	18.37	20.97
Nutritive value[5]								
ME, MJ/kg DM	10.7	10.7	10.7	10.6	10.7	10.7	10.6	10.6
Net energy, UFL/kg DM[6]	0.92	0.92	0.92	0.91	0.92	0.92	0.92	0.92
PDI,[7] g/kg DM	80	91	92	93	79	90	91	93
RPB,[8] g/kg DM	24	16	13	7	20	14	11	9
MetDI,[9] % PDI	2.6	2.4	2.3	2.3	2.6	2.4	2.3	2.3
LysDI,[10] % PDI	6.8	6.6	6.6	6.6	6.8	6.6	6.6	6.6
LysDI:MetDI ratio	2.6	2.8	2.9	2.9	2.6	2.8	2.8	2.9

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugar extruded at 115°C, ELS2 = lupines with reducing sugar extruded at 135°C, ELS3 = lupines with reducing sugar extruded at 150°C.

[3]Ingredients of the experimental diets on a DM basis: 30% corn silage (per kg DM: 86 g CP, 345 g starch, 387 g NDF, 49 g ash), 24% grass silage (per kg DM: 127 g CP, 552 g NDF, 118 g ash), 15% hay (per kg DM: 70 g CP, 586 g NDF, 75 g ash), 11% concentrate (per kg DM: 177 g CP, 355 g starch, 206 g NDF, 79 g ash), and 20% experimental lupines; concentrate consisting on a DM basis of 30% sugar beet pulp, 22.7% wheat, 20% barley, 15% rapeseed meal, 7.9% soybean meal, 1.5% sugar molasses, 1.0% dicalcium phosphate, 1.0% magnesia, 0.9% NaCl, 1,000 IU/kg vitamin A, 1,400 IU/kg vitamin D_3, 15 IU/kg vitamin E, 0.75 mg/kg I, 0.26 mg/kg Co, 15 mg/kg Cu, 60 mg/kg Mn, 90 mg/kg Zn, 0.25 mg/kg Se. In addition, 200 g/d of mineral-vitamin mix (20% Ca, 2.5% P, 4.5% Mg, 3.5% Na, 1% S, 400,000 IU/kg of vitamin A, 120,000 IU/kg of vitamin D_3, 1,600 IU/kg of vitamin E, 1.3 g/kg of Cu, 5 g/kg of Zn, 3.5 g/kg of Mn, 90 mg/kg of I, 36 mg/kg of Co, and 20 mg/kg of Se; Galaphos MiDi Duo, CCPA) were fed.

[4]CML = Nε-carboxymethyl-lysine.

[5]Estimated based on a feeding level of DM corresponding to 3.1% BW/d (DMI = 22.0 kg/d, BW = 700 kg) according to INRA (2018) and actual feed nutrient composition.

[6]UFL = net energy for lactation expressed in unité fourragère lait; 1 UFL is equivalent to 7.37 MJ.

[7]PDI = protein truly digestible in the intestine.

[8]RPB = rumen protein balance.

[9]MetDI = truly digestible Met in the intestine.

[10]LysDI = truly digestible Lys in the intestine.

Measurements and Samplings

Feeds. Intake of DM was measured daily as the difference between the offered mixed ration and refusals during 5 d in wk 1 to 3 and 7 d in wk 4. Samples of forages (corn silage, grass silage, and hay), concentrates (experimental lupines and pelleted concentrate) and basal mixed ration were collected on d 21, 24, and 26 and pooled per period. Feed refusals were removed and weighed daily before the morning feeding. Only when

Table 2. Parameters of pretreatment and extrusion of the experimental lupines without the addition of reducing sugars (EXP1) and with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]				Lupines + reducing sugars (EXP2)[2]			
Item	RL	EL1	EL2	EL3	RLS	ELS1	ELS2	ELS3
Pretreatment								
Duration, min	—	20	29	27	—	24	25	32
Temperature of maturation, °C	—	55	55	55	—	55	55	55
Temperature of extrusion, °C								
Penultimate extruder section N-1	—	90	91	125	—	90	90	127
Ultimate extruder section N	—	115	130	157	—	115	135	161
Intensity, % of extruder potential	—	68.2	66.7	70.6	—	67.3	66.7	73.6
Mechanical energy index, Wh/kg	—	43.2	61.5	59.2	—	50.1	52.8	74.8

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugars extruded at 115°C, ELS2 = lupines with reducing sugars extruded at 135°C, ELS3 = lupines with reducing sugars extruded at 150°C.

the percentage of feed refusals was higher than 5% of the daily offered DM, refusals were sampled and their DM content was determined (105°C, 24 h in air-forced oven). Aliquots of daily refusals were pooled per period, and frozen. One aliquot of each sample of feed and refusal was dried (60°C, 72 h in air-forced oven); another aliquot was lyophilized. Samples of forages and refusals were milled with a centrifugal mill (BJL 8500, Boisson) to pass a 1-mm screen. Experimental lupines and pelleted concentrate samples were ground to pass a 1-mm screen of a cutting mill (Retsch SM 300, VWR International). Lyophilized experimental lupines were pooled over periods for later total Lys and CML analysis.

Apparent Total-Tract Digestibility and N and CML Partition. For 6 d, all feces were collected in a bin located behind the tiestall. Urine was collected separately using urine funnel devices glued around the vulva, which were connected to a 30-L canister secured behind the tiestall. Urine pH was maintained below 4 with the addition of 500 mL of 30% H_2SO_4 to avoid the volatilization of NH_3. Every day at 0800 h total collected feces and urine were removed, weighed, and mixed thoroughly with an electric paint mixer drill. Samples corresponding to 0.5% of the total excreted feces and urine were taken, pooled per period, and frozen at −20°C. Fecal samples were then thawed, thoroughly mixed, oven-dried at 60°C for 72 h, and ground through a 1-mm sieve for NDF and ash analysis, or lyophilized and ground through a 1-mm sieve for N assays and determination of total Lys and CML. Fecal DM content was determined daily (at 103°C for 24 h in air-forced oven) on 0.5% of the collected feces. On d 20 and 26, unacidified spot samples (50 mL) of the first urine excreted after 0800 h were collected and immediately frozen at −20°C until lyophilization before analysis of MRC.

Ruminal Fluid. On d 28 of each experimental period, ruminal fluid samples (500 mL) from all 16 cows involved in the experiments, were collected 5 h after the morning feeding via gastro-esophageal tubing with a manual pump after discarding the first 300 mL drained from the oro-ruminal probe. On d 25 and 27, ruminal fluid (100 mL) was collected in the ventral rumen of the 3 rumen-cannulated cows using a plastic syringe connected to a suction strainer, at 0 (immediately before feeding), 1, 2.5, 5, and 8 h after feeding. Immediately after collection, the pH of all ruminal fluid samples was measured (WTW PH 538, Inlab Expert 51343100 electrode, Mettler-Toledo). After filtration through a 250-μm pore size nylon cloth, ruminal fluid was aliquoted for NH_3 and VFA analysis. For NH_3 analysis, 1 mL of ruminal fluid was preserved with 10% vol/vol H_3PO_4 (5%). Samples for VFA (0.8 mL) were preserved with 0.5 mL of a 0.5 *N* HCl solution with 0.4% (wt/vol) of crotonic acid and 2% (wt/vol) of metaphosphoric acid. All samples were immediately frozen to −20°C. For protozoal counts, 1 mL of filtered ruminal fluid was added to 1 mL of methyl green formalin saline solution (Ogimoto and Imai, 1981), and kept at ambient temperature in the dark until counting.

Blood Plasma. Blood samples were taken on all 16 cows from the tail after the morning milking and before feeding on d 27 of each experimental period with 1 EDTA-containing tube and 1 lithium-heparinized tube (both Greiner, ref. 455036, and 455084, respectively). All samples were centrifuged at 3,500 × *g* for 15 min at 4°C and the plasma obtained was immediately stored at −20°C for later analysis of plasma metabolites, and at −80°C for later analysis of plasma AA and ^{15}N isotope enrichment.

Milk. Milk yield of all 16 cows was recorded at each milking. Milk samples were collected at morning and evening milkings on d 24, 26, and 27 of each period, and preserved with 2-bromo-2-nitropropane-1,3-diol at 4°C until analysis of fat, true protein, lactose, and urea contents by mid-infrared spectrometry (MilkoScan FT6000, Foss) in an accredited commercial laboratory (Agrolab's, Aurillac). Milk samples (200 mL) were collected on 4 consecutive milkings on d 24 and 25 of each experimental period from the 8 cows used for apparent total-tract digestibility measurements pooled proportionately to the milk yield of the respective milking and were frozen at −20°C until analysis of CML.

Laboratory Analyses

Feed, Feces, and Urine Composition. The OM in feed, refusals, and feces was determined by ashing at 550°C for 6 h (ISO 5984:2002; ISO, 2002). The concentration of N in feeds, refusals, lyophilized feces, and urine was determined by the Dumas method (ISO 16634-1:2008 [ISO, 2008], on a Rapid N Cube, Elementar Analysensystem GmbH), and CP in feeds was calculated as N × 6.25. The NDF in feeds and dried feces was determined according to Van Soest et al. (1991) using Gooch crucibles with addition of sodium sulfite and α-amylase. Ether extract was analyzed by ether extraction after acid hydrolysis (ISO 6492:1999 norm; ISO, 1999). Starch was enzymatically determined according to ISO 15914:2004 (ISO, 2004) norms in maize silage and concentrate feed. The enzymatic degradability of CP after 1 h in vitro incubation (**ED1**) of the lupines was determined according to Aufrère et al. (1991). The DM content in urine spot samples was determined by weighing the samples before and after lyophilization. The N stable isotope composition [$\boldsymbol{\delta^{15}N}$; deviation of the ^{15}N-to-^{14}N ratio from the international standard (atmospheric N_2) in parts per 10^3] of the basal mixed

diet and the lupines was analyzed on an isotope-ratio mass spectrometer coupled to an elemental analyzer (both Isoprime, VG Instruments).

Total Lys and CML. The contents of total Lys in feeds, and feces, as well as CML in feeds, feces, urine, and milk were analyzed by LC coupled to ion trap tandem MS (**LC-MS/MS**) after reduction and acid hydrolysis according to Niquet-Léridon and Tessier (2011). The analyzed total Lys includes both free and protein-bound Lys that is not trapped in MRC. All analyses of total Lys and CML were performed in triplicate. The coefficients of variation of the determination (i.e., SD of triplicate determination divided by the mean analyte concentration, averaged over all samples of each matrix) of the concentration of CML in feeds, feces, urine, and milk, were 4.5, 4.5, 4.3, and 5.5%, respectively, whereas that of total Lys in feeds was 4.6%.

Ruminal pH, NH_3, VFA, and Protozoal Counts. Before analysis, the 2 samples of ruminal fluid collected from each cannulated animal at each period and sampling day and sampling time were pooled by sampling time to yield 5 samples (immediately before feeding, and 1, 2.5, 5, and 8 h after feeding) per each cannulated animal per period. The concentration of VFA was analyzed by GC (Clarus 580, fitted with a CP-Wax 58 column, Perkin Elmer) with a flame ionization detector. The concentration of NH_3 in the ruminal fluid was determined with a spectrophotometric method using a urea/ammonia kit (Ref. 10542946035, Roche Diagnostics). Protozoa in ruminal fluid were counted in a Neubauer chamber by microscopy and differentiated into large (>100 μm) and small (<100 μm) *Entodinomorphs*, or *Holotrichs* (*Isotricha* or *Dasytricha*) according to their morphology (Hobson and Stewart, 1997).

Blood Plasma. Plasma concentrations of BHB, glucose, and urea were analyzed with commercial kits (Kit 984325, 981379, and 981818, respectively, all ThermoScientific) on an automatic analyzer (Arena 20XT, Thermo Fisher Scientific). Plasma AA concentrations were analyzed using ultra-high-performance liquid chromatography-mass spectrometry (**UPLC-MS**; Acquity Ultra Performance LC System, Waters, Guyancourt, France) according to the method described by Haque et al. (2012). The N stable isotope composition ($\delta^{15}N$) was analyzed in freeze-dried plasma samples as described for feed samples.

Calculations. The nutritive values of the diets (Table 1) were calculated according to INRA (2018) using the online application INRAtionV5 (www.inration-ruminal.fr) considering a feeding level of 3.1 kg DM/100 kg BW per day, the analyzed composition of the feeds, and measured ED1 values for the lupines (Table 3). For lupines, the effective degradability of N (**ED_{6}_N**) was predicted from the measured ED1, and the intestinal digestibility of rumen undegraded feed protein was assumed to be 89 and 100% for raw and extruded seeds, respectively, based on INRA (2018) reporting data obtained with the mobile bag technique according to Peyraud et al. (1988). Data on DMI was averaged per cow over wk 4 within each period. The ECM yield (kg/d) was calculated with data recorded during wk 4 of each period as [23.8 × protein yield (kg/d) + 38.9 × fat yield (kg/d) + 16.3 × lactose yield (kg/d)]/3.14 (Madsen et al., 2008). Milk total N was calculated by dividing the average daily milk true protein yield during wk 4 of each period by 6.38 and 0.95 according to Spanghero and Kowalski (1997), assuming that 95% of milk N is true protein N. The apparent total-tract digestibility of dietary components was calculated as the difference between intake and fecal excretion of the respective component expressed as a percentage of the intake of that component. Nitrogen and CML daily balances were calculated as intake minus (fecal excretion + urinary excretion + milk secretion), all excretion (feces and urine), and secretion (milk) being measured over 6 consecutive days. The PDI use efficiency was calculated according to INRA (2018), as the ratio of protein synthesis to available PDI (i.e., PDI intake − PDI used for endogenous urinary N losses), whereby protein synthesis accounted for milk protein synthesis, protein endogenous fecal losses, scurf protein synthesis, and protein retention related to net energy (**NE**) balance. Individual NE balance was estimated as the difference between NE supply and the sum of NE requirements for maintenance and production according to INRA (2018) for all cows included in both experiments. Individual ruminal protein balance (**RPB**) was calculated as predicted RPB of each experimental diet reported in Table 1 multiplied by individual DMI. The $\Delta^{15}N_{animal\text{-}diet}$ was calculated for all cows during each experimental period as the difference between the individual $\delta^{15}N$ in plasma and $\delta^{15}N$ in the diet, whereby the latter was calculated as the average of $\delta^{15}N$ of each feed ingredient weighted by the percent of N in the dietary ingredient. The marginal increase of digestibility of CML provided by lupines and the basal mixed ration was determined by fitting a mixed linear model regression defining the apparently digested CML (in mg/d) as a function of the CML provided by the respective dietary component (i.e., lupines or the basal mixed ration; in mg/d) across all observations, with the slope of the regression being the estimated marginal digestibility of CML originating from the respective dietary component. The linear mixed model included period as fixed effect and individual cow as random

effect, whereas the effect of the experiment (i.e., with or without addition of reducing sugars) was excluded from the model, as it was not significant.

Statistical Analysis

All data deriving from both experiments were analyzed separately with linear mixed models with the MIXED procedure of SAS (version 9.4, SAS Institute Inc.). For each experiment, the statistical units were the means of each individual cow in each experimental period. The following model was applied to response variables measured on all cows included in each experiment:

$$Y_{ijkl} = \mu + Trt_i + P_j + S_k + C_l(S)_k + \varepsilon_{ijkl},$$

where Y_{ijkl} is the measured response variable, μ is the overall mean, Trt_i is the fixed effect of the dietary treatment (RL, EL1, EL2, and EL3 in EXP1, and RLS, ELS1, ELS2, and ELS3 in EXP2), P_j ($j = 1$–4) is the fixed effect of experimental period, S_k ($k = 1$–2) is the fixed effect of square, $C_l(S)_k$ ($l = 1$–8) is the random effect of cow nested within square, and ε_{ijkl} is the random residual error term.

All data obtained on apparent total-tract digestibility, as well as N and CML partition measured on cows belonging to 1 square per experiment were analyzed with following model:

$$Y_{ijk} = \mu + Trt_i + P_j + C_k + \varepsilon_{ijk},$$

where Y_{ijk} is the measured response variable, μ is the overall mean, Trt_i is the fixed effect of dietary treatment, P_j is the fixed effect of experimental period, C_k ($k = 1$–4) is the random effect of cow, and ε_{ijk} is the residual error term.

All data related to the kinetics of ruminal parameters (i.e., pH, NH_3, VFA, and protozoal counts) recorded on the 3 rumen-cannulated cows included in EXP1 were analyzed with a linear mixed model with dietary treatment (Trt_i; RL, EL1, EL2, and EL3), experimental period (P_j; period 1 to 4), sampling time (S_l; 0, 1, 2.5, 5, and 8 h), treatment × sampling time interaction as fixed effects, and cow (C_k; $k = 1$ to 3) as random effect according to following equation:

$$Y_{ijkl} = \mu + Trt_i + P_j + C_k + S_l + Trt_i \times S_l + \varepsilon_{ijkl}.$$

Sampling time was specified as a repeated measure with a first-order autoregressive covariance structure [AR(1)], based on best fit according to the Bayesian information criterion. Normality and homoscedasticity of the data and the residues were tested with the Shapiro-Wilk test and by visual inspection of the skewness and kurtosis. Protozoal counts and feed refusals were $\log_{10}$-transformed before statistical analysis to comply with the assumption of normality. All variables in the tables are reported as least squares means and standard error of the means, except protozoal counts and feed refusals, which are reported as arithmetical means of dietary treatments and SE. In all models, degrees of freedom for testing fixed effects were estimated with the Kenward-Roger approximation. Effect of dietary treatment Trt_i was declared significant when $P \leq 0.05$ and as a tendency toward significance when $0.05 < P < 0.10$. Further, least squares means of dietary treatment were separated using the pdiff statement in SAS with the Tukey's honest significant difference adjustment for multiple comparisons.

Welch's 2-sample t-tests with unequal variance were performed with the TTEST procedure in SAS to compare ruminal parameters recorded 5 h after feeding by sampling ruminal fluid through the ruminal cannulas or via stomach tubing. Correlations between individual urea concentrations in plasma and milk as well as between milk urea concentration and individual RPB (in g/d) were estimated with the 'corr' procedure in SAS. Simple linear regressions for absolute excretion of CML in milk and urine (in mg/d) against CML intake (in mg/d), as well as of $\Delta^{15}N_{animal\text{-}diet}$ to N in milk:N intake ratio, N in urine:N intake ratio, and to predicted RPB in g/kg DM were estimate with the 'reg' procedure in SAS. Two regressions between $\Delta^{15}N_{animal\text{-}diet}$ and N in milk:N intake ratio were estimated: a regression included data from the 4 cows per experiment that were involved in the N and CML partition study, a second regression was estimated with data measured on all cows ≥50 DIM. In fact, according to Correa-Luna et al. (2022), $\Delta^{15}N_{animal\text{-}diet}$ has a limited application as biomarker for N use efficiency for milk production in early-lactating cows (DIM <50), because protein mobilization artificially increases both N use efficiency for milk production and $\Delta^{15}N_{animal\text{-}diet,}$ leading to a positive rather than a negative relation.

RESULTS

Extrusion, Characteristics of the Extruded Lupines, and Diets

At identical target temperatures, the addition of reducing sugars numerically decreased the mechanical energy index of the extrusion at 135°C (52.8 vs. 61.8 Wh/kg), whereas it numerically increased it at 115° (50.1 vs. 43.2 Wh/kg) and at 150°C (74.8 vs. 59.2 Wh/kg)

Table 3. Chemical composition, enzymatic CP degradability (ED1), content of Maillard reaction compounds, and nutritive value according to INRA (2018) of the experimental lupines without addition of reducing sugars (EXP1) or with the addition of reducing sugars (EXP2), and Maillard reaction compounds in the basal mixed ration

Item	Basal mixed ration	Lupines (EXP1)[1]				Lupines + reducing sugars (EXP2)[2]			
		RL	EL1	EL2	EL3	RLS	ELS1	ELS2	ELS3
DM, g/kg		866	898	903	922	863	897	901	920
OM, g/kg DM		958	958	957	956	958	958	958	956
CP, g/kg DM		345	354	355	344	336	349	353	344
NDF, g/kg DM		235	228	227	221	235	213	211	206
Ether extract, g/kg DM		94	95	97	109	92	94	95	126
ED1,[3] %	—	74.0	56.4	45.7	33.3	76.2	54.0	47.2	35.8
ED_6_N,[4] %	—	90.0	73.9	70.1	65.6	90.7	73.0	70.6	66.5
Total Lys, g/kg DM	3.90	17.7	18.4	17.8	16.0	16.3	18.0	16.3	16.1
CML,[5] g/kg DM	0.0201	0.0070	0.0089	0.0107	0.0128	0.0056	0.0104	0.0114	0.0244
CML/total Lys, ‰	5.19	0.39	0.48	0.60	0.80	0.34	0.58	0.70	1.51
Nutritive values[6]									
Net energy, UFL/kg DM[7]		1.42	1.42	1.42	1.41	1.44	1.45	1.42	1.42
PDI,[8] g/kg DM		106	157	165	170	101	157	159	171
RPB,[9] g/kg DM		193	157	139	111	171	146	128	118
MetDI,[10] % PDI		1.6	1.3	1.2	1.2	1.6	1.3	1.3	1.2
LysDI,[11] % PDI		6.8	6.3	6.2	6.2	6.8	6.3	6.3	6.2
LysDI:MetDI ratio		4.3	5.0	5.1	5.1	4.2	4.9	5.0	5.1

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugars extruded at 115°C, ELS2 = lupines with reducing sugars extruded at 135°C, ELS3 = lupines with reducing sugars extruded at 150°C.

[3]ED1 = enzymatic CP degradability determined by 1-h in vitro incubation according to Aufrère et al. (1991).

[4]ED_6_N = estimated effective degradability of the N with an outflow from the rumen of 6% per h, predicted from ED1 with equation for raw lupines (ED_6_N = 47.9 + 0.36 × ED1 + 15.4) and for extruded lupines (ED_6_N = 47.9 + 0.36 × ED1 + 5.7) according to INRA (2018).

[5]CML = Nε-carboxymethyl-lysine.

[6]Estimated based on analyzed chemical composition and ED1 according to INRA (2018).

[7]UFL = net energy for lactation expressed in unité fourragère lait; 1 UFL is equivalent to 7.37 MJ.

[8]PDI = protein truly digestible in the intestine.

[9]RPB = rumen protein balance.

[10]MetDI = truly digestible Met in the intestine.

[11]LysDI = truly digestible Lys in the intestine.

compared with the omission of reducing sugars (Table 2). The ED1 of all extruded lupines was numerically lower than that of raw lupines (on average −29 percentage points of the ED1 of RL and −30 percentage points RLS; Table 3). The ED1 did not numerically differ with and without reducing sugars. Except in ELS3 (i.e., extrusion at 150°C with addition of reducing sugars), the CML concentration in lupines extruded at 115°C and 135°C was only marginally higher than in raw lupines, and generally lower than in the basal mixed ration. The proportion of CML provided by the lupines in the diet accounted for 8.0, 10.0, 11.7, and 13.8% of total diet CML with RL, EL1, EL2, EL3, and 6.5, 11.5, 12.4, and 23.3% in RLS, ELS1, ELS2, ELS3, respectively. The ratio of CML to total Lys increased numerically with increasing temperature of extrusion. Addition of reducing sugars resulted in numerically higher CML concentrations compared with extrusion without addition of sugars only at the highest expected temperature of extrusion.

Feed Intake, Milk Yield, Milk Composition, N, and PDI Use Efficiency

In both experiments, DMI ($P = 0.54$ in EXP1, $P = 0.48$ in EXP2), DM refusals during wk 4 ($P = 0.25$ in EXP1, $P = 0.29$ in EXP2), and milk true protein concentration ($P = 0.85$ in EXP1, $P = 0.86$ in EXP2) did not differ among dietary treatments (Table 4). The milk yield was higher with EL3 than with RL (+2.4 kg/d; Tukey-adjusted $P = 0.03$) in EXP1 (i.e., without addition of reducing sugars). In EXP2 (i.e., with addition of reducing sugars) the milk yield tended to be higher with ELS3 compared with RLS (+1.9 kg/d; Tukey-adjusted $P = 0.08$). The ECM yield was unchanged across dietary treatments in either experiment ($P = 0.23$ in EXP1, $P = 0.28$ in EXP2). In EXP1, the milk fat content was lower with EL3 than with RL (−2.8 g/kg, Tukey-adjusted $P = 0.02$), whereas in EXP2, the milk fat content tended to be lower with ELS3 than with RLS (−3.9 g/kg; Tukey-adjusted $P = 0.05$). In either experiment, the milk fat yield did not differ among dietary treatments ($P = 0.61$ in EXP1, $P = 0.51$ in EXP2). The milk true protein yield tended to be higher with EL3 than with RL (+66 g/d; Tukey-adjusted $P = 0.06$) in EXP1. However, in EXP2, milk true protein yield was not affected ($P = 0.14$) by dietary treatments. The fat-to-protein ratio was lower (−0.09 g/g; Tukey-adjusted $P = 0.02$) with EL3 compared with RL and EL1 in EXP1. In parallel, the fat-to-protein ratio was lower with ELS3 than with RLS (−0.11 g/g; Tukey-adjusted $P = 0.02$) in EXP2. The milk urea concentration was lower with EL3 compared with RL (−42 mg/L; Tukey-adjusted $P = 0.02$), and had intermediate values with EL1 and EL2. Similarly, in EXP2, the milk urea concentration was lower with ELS3 than with RLS (−28 mg/L; Tukey-adjusted $P = 0.03$). In EXP1, the milk urea concentration was positively and significantly correlated with the plasma urea concentration [Pearson correlation coefficient (**r**) = 0.44, $P = 0.01$], whereas in EXP2 milk urea was not correlated ($P = 0.29$) with plasma urea concentration. For all observations in both experiments, the individual milk urea concentration was positively correlated with the predicted individual RPB (r = 0.37, $P < 0.01$).

Nitrogen intake did not differ among dietary treatments in both experiments ($P = 0.21$ in EXP1, and $P = 0.48$ in EXP2). Milk N yield tended to be higher (Tukey-adjusted $P = 0.06$) with EL3 than with RL in EXP1. The N in milk:N intake ratio (i.e., N use efficiency for milk production) was higher (Tukey-adjusted $P < 0.01$) with EL3 than with all other treatments in EXP1. In EXP2, milk N yield ($P = 0.14$) and N in milk:N intake ratio ($P = 0.30$) were not affected by dietary treatments. In both experiments, the PDI intake was higher (Tukey-adjusted $P < 0.01$) with extruded than with raw lupines by on average +253 g/d in EXP1, and +280 g/d in EXP2. The PDI use efficiency was lower (Tukey-adjusted $P < 0.01$) with all extruded lupines than with raw lupines in the respective experiment.

Ruminal Parameters in Intact and Rumen-Cannulated Cows

In both experiments, neither the concentration of NH_3 ($P = 0.88$ in EXP1, and $P = 0.57$ in EXP2) nor the concentration of total VFA in the ruminal fluid sampled by stomach tubing 5 h after feeding ($P = 0.57$ in EXP1, and $P = 0.11$ in EXP2) were affected by the dietary treatments (Table 5). The molar proportion of branched-chain VFA (**BCVFA**) in the ruminal fluid was lower (−0.53 mol/100 mol, Tukey-adjusted $P < 0.01$ in EXP1, and −0.51 mol/100 mol, Tukey-adjusted $P < 0.01$ in EXP2, respectively) with lupines extruded at 150°C (EL3 and ELS3) than with raw lupines (RL and RLS, respectively). Indeed, the molar proportions of isobutyrate and isovalerate were lower (−0.18 mol/100 mol, Tukey-adjusted $P = 0.01$; −0.36 mol/100 mol, $P < 0.01$, respectively) with EL3 than with RL in EXP1 (i.e., without the addition of reducing sugars). In EXP2 (i.e., with the addition of reducing sugars), the proportion of isovalerate in total VFA was lower (−0.30 mol/100 mol, Tukey-adjusted $P < 0.01$) with ELS3 than with RL and ELS1. However, the (acetate + butyrate)-to-propionate ratio did not differ among dietary treatments in either experiment ($P = 0.13$ in EXP1, and $P = 0.49$ in EXP2). Ruminal pH was not affected ($P = 0.56$) by dietary

Table 4. Dry matter intake, BW, BCS, milk yield, and N use efficiency for milk production in cows (n = 8 per experiment) fed raw and extruded lupines without the addition of reducing sugars (EXP1) and with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]						Lupines + reducing sugars (EXP2)[2]					
Item	RL	EL1	EL2	EL3	SEM	*P*-value	RLS	ELS1	ELS2	ELS3	SEM	*P*-value
DMI, kg/d	22.7	22.4	22.7	22.2	0.79	0.54	21.1	21.4	21.3	21.0	0.97	0.48
DM refusals,[3] kg/d	1.27	1.14	0.61	0.97	0.23	0.25	0.94	0.75	0.83	1.15	0.167	0.29
BW,[4] kg	727	726	733	738	27.4	0.37	669	673	669	670	18.3	0.65
BCS, 0–5 points	2.7	2.6	2.7	2.8	0.15	0.42	2.6	2.5	2.5	2.4	0.13	0.61
Milk yield, kg/d	28.2^{b}	29.0^{ab}	29.2^{ab}	30.6^{a}	1.53	0.05	27.2	28.6	27.7	29.1	1.76	0.07
ECM yield, kg/d	28.0	28.2	28.1	29.4	1.27	0.23	26.9	28.3	26.5	27.6	1.42	0.38
Milk constituents												
Fat, g/kg	40.1^{a}	39.9^{ab}	38.6^{ab}	37.3^{b}	1.21	0.02	40.5	39.2	38.3	36.6	1.26	0.07
Fat yield, g/d	1,128	1,133	1,106	1,146	50.0	0.61	1,093	1,131	1,058	1,081	56.7	0.51
Protein, g/kg	30.8	30.5	31.0	30.6	0.82	0.85	29.7	29.9	29.7	29.2	1.05	0.86
Protein yield, g/d	868	870	891	934	39.8	0.06	804	866	816	866	38.4	0.14
Fat:protein ratio	1.31^{a}	1.31^{a}	1.25^{ab}	1.22^{b}	0.032	0.02	1.37^{a}	1.31^{ab}	1.29^{ab}	1.26^{b}	0.027	0.03
Urea, mg/L	305^{a}	292^{ab}	292^{ab}	263^{b}	11.9	0.03	303^{a}	298^{ab}	297^{ab}	275^{b}	6.88	0.02
Lactose, g/kg	50.0	50.3	50.5	50.6	0.66	0.83	51.1	50.4	50.3	49.3	1.05	0.53
N intake, g/d	562	559	568	547	20.1	0.21	515	533	526	518	20.8	0.48
N in milk, g/d	143	144	147	154	6.57	0.06	133	143	135	143	6.33	0.14
N in milk, % of N intake	25.6^{b}	25.8^{b}	26.1^{b}	28.4^{a}	1.11	<0.01	25.9	26.8	25.5	28.0	1.20	0.30
NE balance,[5] UFL/d	1.86	1.19	1.59	0.72	0.565	0.06	1.19	1.12	0.58	0.98	0.769	0.87
PDI[6] intake, g/d	1,808^{b}	2,032^{a}	2,087^{a}	2,063^{a}	69.6	<0.01	1,657^{b}	1,939^{a}	1,916^{a}	1,956^{a}	72.0	<0.01
PDI use efficiency, g/g	0.78^{a}	0.68^{b}	0.69^{b}	0.69^{b}	0.016	<0.01	0.79^{a}	0.70^{b}	0.67^{b}	0.70^{b}	0.025	<0.01

[a,b]Least squares means with differing superscript letters significantly differ ($P < 0.05$) according to Tukey's honest significance difference test.

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugars extruded at 115°C, ELS2 = lupines with reducing sugars extruded at 135°C, ELS3 = lupines with reducing sugars extruded at 150°C.

[3]Measured daily on wk 4 of each period.

[4]Measured during wk 4 on n = 4 per treatment.

[5]NE balance estimated as NE supply − NE requirements for maintenance, production, gestation, and gain according to INRA (2018), expressed in unité fourragère lait (UFL); 1 UFL is equivalent to 7.37 MJ.

[6]PDI = truly digestible protein in the intestine.

treatments in EXP1. In EXP2, ruminal pH was lower (Tukey-adjusted $P = 0.02$) with ELS1 than with ELS2, and had intermediate values with RLS and ELS3. Protozoal counts did not differ among dietary treatments in either experiment, except for *Isotricha*, which tended to be less abundant (Tukey-adjusted $P = 0.07$) with ELS3 than with ELS1 in EXP2.

The average total VFA molar concentrations measured in ruminal fluid sampled on rumen-cannulated cows in EXP1 tended to be lower ($P = 0.06$) with EL1 and EL3 than with RL. A lower proportion of propionate (−1.0 mol/100 mol, $P = 0.03$) was observed with EL1 and EL2 compared with RL, and a higher (Tukey-adjusted $P < 0.01$) proportion of butyrate was observed with all extruded lupines than with RL (Supplemental Table S1; https://doi.org/10.57745/U4U77P; Manzocchi et al., 2023). The average NH_3 concentration measured in ruminal fluid of rumen-cannulated cows did not differ among dietary treatments ($P = 0.49$), and the postprandial NH_3 decrease was not affected by dietary treatments either (Treatment × Sampling interaction $P = 0.66$; Supplemental Figure S1, https://doi.org/10.57745/U4U77P; Manzocchi et al., 2023).

The total ruminal VFA concentration 5 h after feeding was higher ($P < 0.01$ for Welch 2-sample *t*-test with unequal variance) in samples collected through the cannula than in those collected via stomach tube (155 ± 2.0 m*M* vs. 123 ± 4.0 m*M*), but the proportion of BCVFA in total VFA did not differ ($P = 0.46$) between sampling methods (2.31 ± 0.140 vs. 2.19 ± 0.058 mol/100 mol, respectively). However, the average ruminal NH_3 concentration 5 h after feeding did not differ ($P = 0.61$) between samples collected through rumen-cannulas (165 ± 18.1 mg/L) or via stomach tubing (155 ± 9.2 mg/L). Eventually, average DMI did not differ ($P = 0.23$) between rumen-cannulated (23.0 ± 0.43 kg/d) and intact cows (22.1 ± 0.61 kg/d) involved in EXP1, but realized feeding level of DM was lower ($P = 0.02$) in the 3 rumen-cannulated (3.0 ± 0.03% BW/d) than in intact cows (3.2 ± 0.08% BW/d).

Apparent Total-Tract Digestibility of Dietary Components, N, and CML Partition

In EXP1 (i.e., with lupines without reducing sugars), the apparent total-tract DM digestibility was lower with EL2 (Tukey-adjusted $P = 0.03$) than with RL (Table 6). However, the apparent total-tract digestibility of the OM was also lower with EL1 than with RL (Tukey-adjusted $P = 0.05$). In EXP2 (i.e., with the addition of reducing sugars), the apparent total-tract digestibility of DM ($P = 0.27$) and OM ($P = 0.13$) did not differ among dietary treatments. In either experiment, the apparent total-tract digestibility of NDF ($P = 0.21$ in EXP1, and $P = 0.15$ in EXP2) and N ($P = 0.13$ in EXP1, and $P = 0.79$ in EXP2) did not differ among dietary treatments. The apparent total-tract digestibility of CML was unchanged across dietary treatments in either experiment ($P = 0.14$ in EXP1, and $P = 0.82$ in EXP2). The apparent total-tract digestibility of total Lys tended to be lower with EL3 than with EL1 (−3.3 percentage points, Tukey-adjusted $P = 0.10$) in EXP1, whereas in EXP2 it was not affected ($P = 0.14$) by dietary treatments.

Nitrogen intake ($P = 0.80$ in EXP1, $P = 0.72$ in EXP2), total excretion of fecal N ($P = 0.42$ in EXP1, $P = 0.66$ in EXP2), urinary N ($P = 0.36$ in EXP1, $P = 0.60$ in EXP2), and N balance ($P = 0.61$ in EXP1, $P = 0.91$ in EXP2) were not affected by dietary treatments in either experiment. However, in EXP1 milk N yield tended to be higher with EL3 then with EL2 (Tukey-adjusted $P = 0.09$), but did not differ among other dietary treatments. The N in milk:N intake ratio was not affected in either experiment ($P = 0.34$ in EXP1 and $P = 0.52$ in EXP2), when it was assessed only on cows belonging to 1 square, which were involved in the N and CML balance study.

Absolute concentrations of CML in fecal DM ($P = 0.40$ in EXP1, $P = 0.95$ in EXP2), urine ($P = 0.25$ in EXP1, $P = 0.19$ in EXP2), and milk ($P = 0.66$ in EXP1, $P = 0.13$ in EXP2) did not differ among dietary treatments in both experiments (Supplemental Tables S2 and S3, https://doi.org/10.57745/U4U77P, Manzocchi et al., 2023). In EXP1, the daily dietary intake of CML ($P = 0.20$), secretion of CML in milk ($P = 0.77$) and total excretion of CML in feces ($P = 0.51$), and urine ($P = 0.24$) did not differ among dietary treatments. Nevertheless, the CML balance was higher with EL3 (+39 mg/d; Tukey-adjusted $P = 0.04$) than with RL in EXP1. In EXP2, the daily CML intake was higher with ELS3 (+71.3 mg/d, Tukey-adjusted $P < 0.01$) than with RLS, ELS1, and ELS2. In EXP2, the CML balance did not differ among treatments ($P = 0.34$). In both experiments, the proportions of fecal CML, urinary CML, and CML secreted in milk to the dietary intake of CML (data not shown), as well as the proportion of apparently retained CML to CML intake was not affected in either experiment ($P = 0.13$ in EXP1, and $P = 0.88$ in EXP2). The excretion of CML in urine as a fraction of apparently digested CML ($P = 0.27$ in EXP1, $P = 0.79$ in EXP2) and secretion of CML in milk as a fraction of apparently digested CML ($P = 0.34$ in EXP1, $P = 0.61$ in EXP2) were not affected by the dietary treatments in either experiment. However, there were positive linear relations between the dietary intake of CML and the amount of appar-

Table 5. Ruminal pH, concentrations of NH_3 and VFA, and protozoal counts 5 h after the morning feeding in cows (n = 8 per experiment) fed raw and extruded lupines without the addition of reducing sugars (EXP1) or with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]						Lupines + reducing sugars (EXP2)[2]					
Item	RL	EL1	EL2	EL3	SEM	*P*-value	RLS	ELS1	ELS2	ELS3	SEM	*P*-value
pH	6.7	6.7	6.6	6.6	0.09	0.56	6.5^{ab}	6.4^{b}	6.7^{a}	6.6^{ab}	0.07	0.03
NH_3, mg/L	158	145	149	166	22.4	0.88	192	163	154	165	21.3	0.57
Total VFA, m*M*	119	124	131	117	7.8	0.57	136	135	125	123	5.3	0.11
VFA, mol/100 mol												
Acetate	65.0	64.0	63.4	65.0	0.73	0.30	64.7	63.9	66.4	65.3	1.18	0.69
Propionate	19.1	20.1	20.3	19.6	0.36	0.16	19.5	20.2	19.0	19.1	0.71	0.53
Butyrate	11.6	11.9	12.5	12.3	0.52	0.46	12.0	12.1	11.1	12.2	0.54	0.79
Isobutyrate	0.98^{a}	0.85^{ab}	0.86^{ab}	0.80^{b}	0.045	0.05	0.86	0.82	0.72	0.76	0.057	0.31
Valerate	1.27	1.21	1.18	1.12	0.057	0.28	1.16	1.22	1.19	1.15	0.047	0.60
Isovalerate	1.48^{a}	1.36^{a}	1.26^{ab}	1.12^{b}	0.064	<0.01	1.32^{a}	1.18^{ab}	1.05^{bc}	0.92^{c}	0.069	<0.01
Caproate	0.61	0.57	0.56	0.54	0.042	0.54	0.55	0.54	0.51	0.61	0.048	0.91
BCVFA,[3] mol/100 mol	2.45^{a}	2.21^{ab}	2.12^{ab}	1.92^{b}	0.102	<0.01	2.18^{a}	2.01^{ab}	1.76^{b}	1.67^{b}	0.105	<0.01
(Acetate + butyrate): Propionate, mol/mol	4.01	3.80	3.75	4.03	0.089	0.13	3.94	3.76	3.79	4.10	0.273	0.49
Total protozoa, × 10^3 cells/mL	1,530	1,517	1,090	1,078	324	0.14	1,060	931	947	980	173	0.93
Entodinomorph												
<100 μm	1,480	1,428	1,015	1,019	327.8	0.13	980	820	860	905	184.1	0.92
>100 μm	32.0	67.1	54.9	43.8	22.3	0.46	68.5	83.4	69.1	62.3	24.46	0.94
Holotricha												
Isotricha	12.5	15.1	11.3	8.9	3.32	0.18	8.5	19.4	12.4	6.9	5.33	0.08
Dasytricha	5.8	7.4	9.5	6.2	2.41	0.18	3.3	7.9	5.9	5.9	2.48	0.20

[a–c]Least squares means with differing superscript letters significantly differ (P <0.05) according to Tukey's honest significance difference test.

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugar extruded at 115°C, ELS2 = lupines with reducing sugar extruded at 135°C, ELS3 = lupines with reducing sugar extruded at 150°C.

[3]BCVFA = branched-chain VFA; includes isobutyrate and isovalerate.

Table 6. Apparent total-tract digestibility, N, and Nε-carboxymethyl-lysine partition in cows (n = 4 per experiment) fed raw and extruded lupines without the addition of reducing sugars (EXP1) and with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]						Lupines + reducing sugars (EXP2)[2]					
Item	RL	EL1	EL2	EL3	SEM	*P*-value	RLS	ELS1	ELS2	ELS3	SEM	*P*-value
Apparent total-tract digestibility, %												
DM	68.5[a]	66.9[ab]	66.8[b]	67.9[ab]	0.73	0.02	67.3	66.6	66.8	65.7	0.83	0.27
OM	70.7[a]	69.1[b]	69.2[ab]	70.1[ab]	0.69	0.03	69.6	69.0	69.1	67.9	0.73	0.13
NDF	58.5	56.3	56.0	58.0	1.36	0.21	55.8	57.3	56.7	54.6	1.09	0.15
N	67.2	67.4	65.8	68.4	0.97	0.13	64.7	64.7	65.1	63.6	1.31	0.79
CML[3]	39.0	42.2	41.4	46.2	3.05	0.14	37.4	38.4	38.2	43.4	5.19	0.82
Total Lys	60.1	61.9	58.6	58.5	0.93	0.09	56.9	59.0	55.3	55.2	1.47	0.14
N partition												
N intake, g/d	600	575	592	584	20.6	0.80	523	542	535	538	30.4	0.72
N in feces, g/d	201	190	206	187	10.6	0.42	184	192	186	195	8.26	0.66
N in milk, g/d	146	152	145	160	8.87	0.08	142	150	139	151	9.92	0.30
N in urine, g/d	226	204	202	192	10.0	0.36	171	166	172	163	9.47	0.60
N balance, g/d	28.4	29.5	39.8	46.1	7.53	0.61	26.2	34.0	38.4	29.6	14.9	0.91
N milk, % of N intake	24.2	26.8	24.4	27.3	1.57	0.34	27.3	27.5	25.9	28.1	1.10	0.52
N urine, % of N intake	37.6	35.2	34.2	32.9	1.67	0.16	32.9	30.9	32.1	30.5	1.63	0.33
CML partition												
CML intake, mg/d	408	417	414	425	19.6	0.20	368[b]	399[b]	393[b]	458[a]	23.1	<0.01
CML in feces, mg/d	252	242	246	231	18.9	0.51	229	243	243	256	19.2	0.81
CML in milk, mg/d	0.94	0.99	1.03	0.98	0.081	0.77	0.90	0.87	0.90	1.01	0.110	0.11
CML in urine, mg/d	39.8	40.3	34.8	38.7	3.06	0.24	37.3	36.9	37.4	46.1	4.68	0.36
CML balance, mg/d	122[b]	133[ab]	138[ab]	161[a]	13.3	0.05	101	119	112	157	25.6	0.34
CML balance, % of CML intake	29.4	32.3	33.1	37.2	3.24	0.13	27.0	29.1	28.8	33.3	5.75	0.88
CML in milk, % of app. dCML[4]	0.73	0.61	0.63	0.50	0.110	0.34	0.74	0.61	0.62	0.52	0.131	0.61
CML in urine, % of app. dCML	30.0	25.4	19.9	19.4	5.21	0.27	33.5	26.2	25.8	23.6	7.26	0.79

[a,b]Least squares means with differing superscript letters significantly differ ($P < 0.05$) according to Tukey's honest significance difference test.

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugars extruded at 115°C, ELS2 = lupines with reducing sugars extruded at 135°C, ELS3 = lupines with reducing sugars extruded at 150°C.

[3]CML = Nε-carboxymethyl-lysine.

[4]App. dCML = apparently digested Nε-carboxymethyl-lysine.

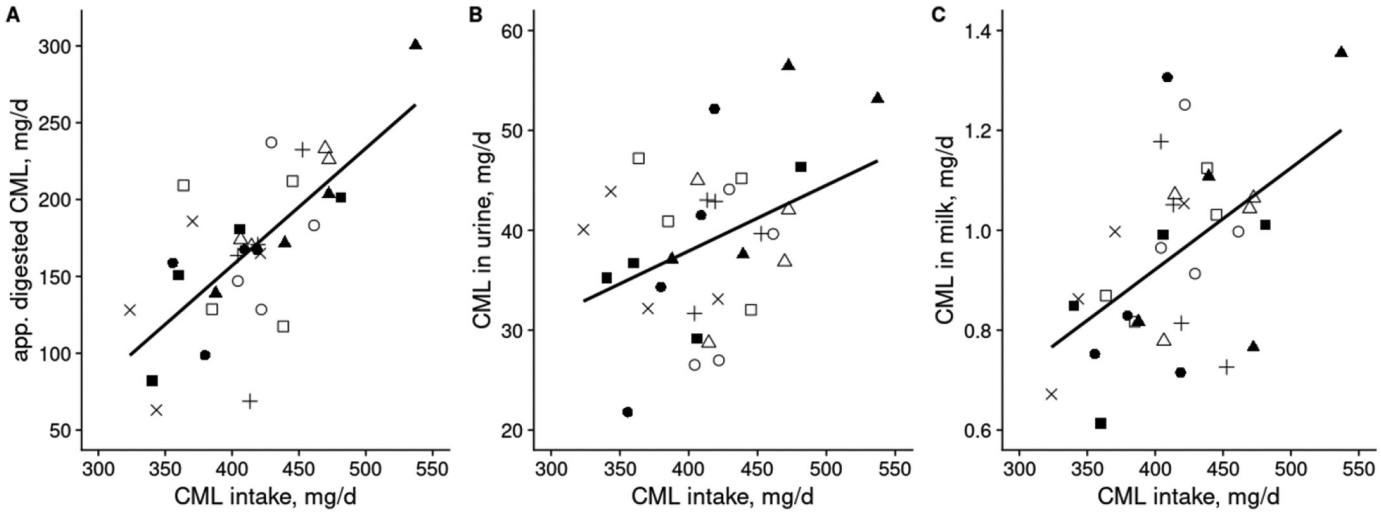

Figure 1. Simple linear regressions between dietary intake of Nε-carboxymethyl-lysine (CML) and apparently (app.) digested CML (A), absolute excretion of CML in urine (B), and secretion in milk (C) based on 32 observations (4 cows × 4 periods × 2 experiments). + RL: raw lupines; □ EL1: lupines extruded at 115°C; ○ EL2: lupines extruded at 135°C; Δ EL3: lupines extruded at 150°C; × RLS: raw lupines with reducing sugars; ■ ELS1: lupines with reducing sugars extruded at 115°C; ● ELS2: lupines with reducing sugars extruded at 135°C; ▲ ELS3: lupines with reducing sugars extruded at 150°C; RMSE: root mean square error. (A) Y = −149 (± 60) + 0.76 (± 0.15) × X (n = 32, adjusted $R^2 = 0.46$, RMSE = 36.6, $P < 0.01$). (B) Y = 11.5 (± 12.2) + 0.066 (± 0.029) × X (n = 32, adjusted $R^2 = 0.12$, RMSE = 7.32, $P = 0.03$). (C) Y = 0.108 (± 0.256) + 0.0020 (± 0.0006) × X (n = 32, adjusted $R^2 = 0.24$, RMSE = 0.155, $P < 0.01$).

ently digested CML ($P < 0.01$), as well as its excretion in urine ($P = 0.03$) and secretion in milk ($P < 0.01$; Figure 1). The estimated marginal increase in digestibility of CML provided by lupines was on average 84 ± 20%, whereas that of CML provided by the basal mixed ration was on average 62 ± 22%.

Blood Plasma Metabolites, AA Concentrations, and $\Delta^{15}N_{animal\text{-}diet}$

The plasma concentrations of BHB ($P = 0.77$ in EXP1, and $P = 0.78$ in EXP2), urea ($P = 0.84$ in EXP1, and $P = 0.57$ in EXP2), and glucose ($P = 0.38$ in EXP1, and $P = 0.56$ in EXP2) did not differ among dietary treatments in either experiment (Table 7). In EXP1 (i.e., with lupines without addition of reducing sugars), the plasma Arg concentration was higher (+18.4 μ*M*; Tukey-adjusted $P = 0.04$) with EL3 than with EL2, and had intermediate values with RL and EL1, whereas in EXP2 (i.e., with the addition of reducing sugars) the plasma Arg concentration did not differ ($P = 0.40$) among dietary treatments. The plasma concentration of His was higher (Tukey-adjusted $P < 0.01$) with EL3 and EL1 than with RL in EXP1, whereas it only tended to be affected ($P = 0.06$) by dietary treatments in EXP2. In EXP1, the plasma concentration of branched-chain AA (**BCAA**) was higher with EL3 than with RL (+100 μ*M;* Tukey-adjusted $P = 0.05$). In particular, the plasma concentration of Leu was higher with EL3 than with RL (+27.1 μ*M*; Tukey-adjusted $P = 0.04$), whereas Val (Tukey-adjusted $P = 0.06$), and Ile (Tukey-adjusted $P = 0.08$) tended to be higher in cows fed EL3 than in those fed RL or EL2. In EXP2, the concentrations of Val ($P = 0.09$), Leu ($P = 0.08$) and His ($P = 0.06$) tended to be affected by dietary treatment. Plasma Met concentration was lower with ELS3 than with RLS (−5.5 μ*M*; Tukey-adjusted $P < 0.01$) in EXP2, whereas it did not differ among dietary treatments in EXP1. The plasma concentration of total nonessential AA did not differ ($P = 0.42$ in EXP1, and $P = 0.14$ in EXP2) among dietary treatments in either experiment. Plasma taurine concentration was lower (Tukey-adjusted $P = 0.02$) in cows fed EL3 than in those fed RL in EXP1. In EXP2, the concentration of taurine was lower (Tukey-adjusted $P < 0.01$) with ELS3 than with all other dietary treatments.

The $\Delta^{15}N_{animal\text{-}diet}$ was lower (Tukey-adjusted $P < 0.01$) with EL3 versus RL and EL1 in EXP1, and also lower (Tukey-adjusted $P < 0.01$) with ELS3 versus RLS, ELS1, and ELS2 in EXP2. Furthermore, the $\Delta^{15}N_{animal\text{-}diet}$ was positively related to urinary N to N intake ($P < 0.01$) and negatively related to the N use efficiency for milk production ($P < 0.01$) in cows involved in the N balance study (Figure 2). The $\Delta^{15}N_{animal\text{-}diet}$ was also negatively related ($P < 0.01$) to the N use efficiency for milk production with all cows ≥ 50 DIM [n = 55, N in milk:N intake (g/g) = 0.343 (±0.025) − 0.038

Table 7. Blood plasma metabolites, AA concentrations, and N isotopic discrimination between animal proteins and diet ($\Delta^{15}N_{animal\text{-}diet}$) measured on d 27 of each experimental period in cows (n = 8 per experiment) fed raw or extruded lupines without the addition of reducing sugars (EXP1) and with the addition of reducing sugars (EXP2)

	Lupines (EXP1)[1]						Lupines + reducing sugars (EXP2)[2]					
Item	RL	EL1	EL2	EL3	SEM	*P*-value	RLS	ELS1	ELS2	ELS3	SEM	*P*-value
Blood plasma metabolites												
BHB, mmol/L	0.477	0.488	0.454	0.478	0.028	0.77	0.551	0.561	0.519	0.575	0.044	0.78
Urea, mg/L	218	223	222	229	12.4	0.84	214	224	230	229	9.44	0.57
Glucose, mmol/L	0.656	0.658	0.666	0.676	0.010	0.38	0.673	0.679	0.664	0.661	0.015	0.56
EAA, μM	674	730	670	817	46.4	0.15	685	687	729	737	56.7	0.37
Arg	64.1^{ab}	66.8^{ab}	62.0^{b}	80.4^{a}	4.79	0.04	66.9	68.3	72.9	70.1	3.61	0.40
His	17.9^{b}	28.6^{a}	22.6^{ab}	28.2^{a}	4.22	<0.01	19.8	20.5	23.1	26.6	4.12	0.06
Ile	87.1	98.6	90.2	113.4	7.66	0.08	95.9	97.5	103.8	111.9	8.86	0.26
Leu	79.6^{b}	86.7^{ab}	82.0^{ab}	106.7^{a}	7.39	0.03	85.4	90.0	98.3	100.8	9.16	0.08
Lys	57.5	64.1	59.0	77.0	6.84	0.15	64.3	62.7	66.9	65.2	5.60	0.87
Met	28.0	27.2	24.1	23.6	2.80	0.37	28.7^{a}	28.4^{ab}	27.6^{ab}	23.2^{b}	1.82	0.03
Phe	34.8	36.3	32.4	37.7	2.44	0.43	37.3	36.3	39.8	35.8	1.91	0.25
Thr	99.0	98.3	85.5	96.5	11.73	0.28	76.6	75.0	72.3	70.5	4.56	0.73
Trp	32.4	32.5	30.0	32.7	2.01	0.73	34.7	33.7	34.2	31.6	1.51	0.35
Val	164^{b}	190^{ab}	173^{b}	210^{a}	12.6	0.06	176	176	190	202	14.9	0.09
BCAA,[3] μM	332^{b}	375^{ab}	346^{ab}	432^{a}	26.7	0.05	357	364	392	414	35.8	0.12
NEAA, μM	1,023	1,079	959	1,072	81.4	0.42	1,183	1,104	1,124	1,026	66.2	0.14
Taurine, μM	30.4^{a}	29.5^{ab}	24.6^{ab}	22.3^{b}	1.10	0.03	26.1^{a}	25.0^{a}	25.5^{a}	20.0^{b}	1.09	<0.01
$\Delta^{15}N_{animal\text{-}diet}$,[4] ‰	2.38^{a}	2.33^{a}	2.26^{ab}	2.11^{b}	0.074	<0.01	2.25^{a}	2.21^{a}	2.19^{a}	2.06^{b}	0.066	<0.01

[a,b]Least squares means with differing superscript letters significantly differ ($P < 0.05$) according to Tukey's honest significance difference test.

[1]RL = raw lupines, EL1 = lupines extruded at 115°C, EL2 = lupines extruded at 135°C, EL3 = lupines extruded at 150°C.

[2]RLS = raw lupines with reducing sugars, ELS1 = lupines with reducing sugars extruded at 115°C, ELS2 = lupines with reducing sugars extruded at 135°C, ELS3 = lupines with reducing sugars extruded at 150°C.

[3]BCAA = branched-chain AA; includes Ile, Leu, and Val.

[4]$\Delta^{15}N_{animal\text{-}diet} = \delta^{15}N_{animal} - \delta^{15}N_{diet}$; N isotopic discrimination between dietary and animal proteins.

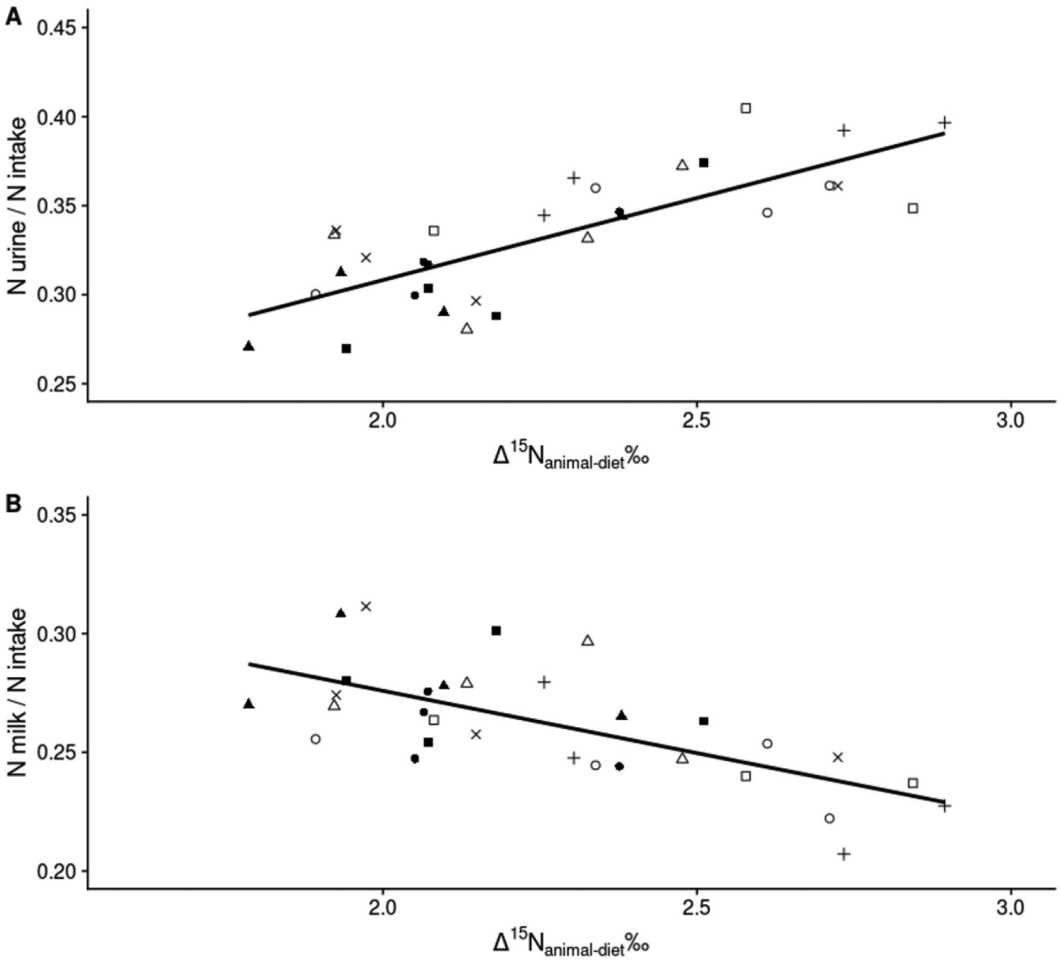

Figure 2. Simple linear regression between N isotopic discrimination between dietary and animal proteins ($\Delta^{15}N_{animal\text{-}diet}$, ‰) and N in urine:N intake ratio (g/g; A) and N use efficiency expressed as N in milk:N intake ratio (g/g; B) based on 31 individual observations performed on dairy cows involved in the digestibility and N partition measurements in both experiments (4 cows × 4 periods × 2 experiments). The data of 1 cow during 1 period with dietary treatment EL1 were outlying and therefore omitted from both relations. + RL: raw lupines; □ EL1: lupines extruded at 115°C; ○ EL2: lupines extruded at 135°C; Δ EL3: lupines extruded at 150°C; × RLS: raw lupines added with reducing sugars; ■ ELS1: lupines added with reducing sugars extruded at 115°C; ● ELS2: lupines added with reducing sugars extruded at 135°C; ▲ ELS3: lupines added with reducing sugars extruded at 150°C; RMSE: root mean square error. (A) Y = 0.124 (± 0.031) + 0.092 (± 0.014) × X (n = 31, adjusted R^2 = 0.60, RMSE = 0.022, $P < 0.01$). (B) Y = 0.381 (± 0.025) + 0.052 (± 0.011) × X (n = 31, adjusted R^2 = 0.43, RMSE = 0.017, $P < 0.01$).

(±0.011) × $\Delta^{15}N_{animal\text{-}diet}$ (‰); adjusted R^2 = 0.16, root mean square error (**RMSE**) = 0.020]. The intra-animal variation of $\Delta^{15}N_{animal\text{-}diet}$ was positively related ($P < 0.01$) to the predicted RPB in g/kg DM of the diets [$\Delta^{15}N_{animal\text{-}diet}$ (‰) = 2.05 (±0.075) + 0.013 (±0.004) × RPB (g/kg DM); adjusted R^2 = 0.61, RMSE = 0.14]. The intra-animal variation of $\Delta^{15}N_{animal\text{-}diet}$ was negatively related to the plasma urea concentration [$\Delta^{15}N_{animal\text{-}diet}$ (‰) = 2.61 (±0.200) − 1.69 (±0.900) × plasma urea (g/L); adjusted R^2 = 0.66, RMSE = 0.15]. However, the $\Delta^{15}N_{animal\text{-}diet}$ was not related ($P = 0.79$) with the milk urea concentration. Eventually, the PDI use efficiency was not related ($P = 0.22$) to the intra-animal variation of $\Delta^{15}N_{animal\text{-}diet}$.

DISCUSSION

In the present study, the main objective of the pretreatment with the addition of reducing sugars in interaction with different expected output temperatures of extrusion (115°C, 135°C, 150°C) was to generate different profiles of MRC with consequently different CML concentrations in the lupines to evaluate their effects on ruminal CP degradability, ruminal fermentation, N use efficiency for milk production, and the productive performance of dairy cows. The effects of the pretreatment of leguminous seeds with the addition of reducing sugars before their extrusion were tested with faba beans in a previous experiment (Mendowski

et al., 2020). Our study was therefore not designed to test the quantitative effect of the addition of reducing sugars to lupines per se on dairy performance and N use efficiency for milk production.

Extrusion of Lupines, Addition of Reducing Sugars, and Dietary Supply of CML

The ED1 of extruded lupines was numerically lower than that of raw lupines, and numerically decreased proportionally to the actual temperature measured in the ultimate section of the extruder, in accordance with our first hypothesis. The predicted ED_6_N values were in accordance with those reported for lupines extruded at 140°C and 160°C by Mendowski et al. (2019). The addition of reducing sugars had the numerical greatest effect on the concentration of CML and the ratio of CML to total Lys at the highest temperature of extrusion (i.e., 150°C). Contrary to our third hypothesis, the addition of reducing sugars did not numerically alter the effect of temperature of extrusion on ED1. Similarly, Mendowski et al. (2020) showed that the addition of reducing sugars before extrusion of faba beans did not reduce the ED1 further than extrusion at the same expected temperature without the addition of reducing sugars, but in faba beans the addition of reducing sugars promoted the initiation of Maillard reactions with the formation of early Maillard compounds (e.g., furosine). Although lupine extrusion numerically increased the ratio of CML to total Lys, the absolute concentration of CML in the basal mixed diet was relatively high compared with extruded lupines, so the basal mixed ration provided most of the dietary CML in both experiments. So far, few publications have reported the content of MRC in forages and common feeds for livestock. Hofmann et al. (2020) quantified CML in commercial feeds with similar concentrations to those found in our experimental feeds. The proteolytic processes occurring during wilting, harvest, and conservation of the forages also induce nonenzymatic glycosylation and consequent formation of MRC (van Soest and Mason, 1991). In addition to the conserved forages (grass and corn silages, hay), the pelleted concentrate (11% of the dietary DM in the present study) including technologically heat-treated feeds and byproducts such as sugar beet pulp, rapeseed meal, soybean meal, and sugar molasses may also supply additional MRC in the basal mixed ration (Hofmann et al., 2020).

The concentration of melanoidins, which are brown-colored compounds derived from the final stages of advanced-stage protein glycation, induced visible differences in color (data not shown) among experimental lupines. Nevertheless, the DMI did not differ among tested lupines in either experiment, which indicates that the palatability of the lupines was not compromised by extrusion even at 150°C. Maillard reaction compounds, and in particular terminal compounds such as CML, may therefore be valuable proxies for the efficiency of technological treatments such as the pretreatment and extrusion.

Effects of Extrusion on Indicators of Ruminal CP Degradability and Protein Intestinal Digestibility

Consistent with the numerically lower ED1 of extruded lupines, the molar proportion of BCVFA to total VFA in the ruminal fluid sampled by stomach tubing 5 h after feeding was lower with lupines extruded at 150°C than with raw lupines in both experiments, suggesting a lower in vivo ruminal CP degradability, as ruminal BCVFA are primarily derived from the bacterial degradation of dietary true proteins, BCAA, and the resulting AA (Miura et al., 1980). In contrast, no effect of dietary treatment on ruminal BCVFA concentrations was observed in rumen-cannulated cows. The lower CP degradability and predicted higher supply of MP in the small intestine with lupines extruded at 150°C are also supported by higher concentrations of BCAA (i.e., Ile, Leu, Val) in the plasma of cows fed EL3 (+100 μM) than in those fed RL and the intermediate values with EL1 and EL2 in EXP1, whereas the difference between RLS and ELS3 (+57 μM) in EXP2 was only numerical. A higher supply of MP, without great variations in the nature of MP and its AA profile, at similar milk true protein yields, results in increased BCAA in plasma (Omphalius et al., 2019). Nevertheless, Omphalius et al. (2019) also observed higher concentrations of total plasma EAA at greater MP supply, which was not the case in either experiment of the present study.

Despite the relatively high proportion of CP provided by lupines in the diet (45% of dietary CP), the expected lower ruminal CP degradability was not supported by a reduction in ruminal NH_3 concentration with extruded lupines. In samples collected by stomach tubing 5 h after feeding, the NH_3 concentration was even numerically lower with RL than with EL3. In rumen-cannulated cows, NH_3 kinetics were also discordant, as the greatest reduction in NH_3 concentration 5 h after feeding was observed with EL1 compared with RL, whereas the ED1 of EL2 and EL3 were numerically lower than that of EL1. Nevertheless, the observed numeric reduction in mean ruminal NH_3 concentration in rumen-cannulated cows fed extruded lupines, was similar (−16.1 mg/L vs. −11.8 mg/L) to that previously reported by Mendowski et al. (2021) in a meta-analysis of the effects of technological treatments of lupines on the ruminal NH_3 concentration. Ruminal NH_3 concentration may not be a completely unbiased indicator of ruminal CP degrad-

ability. In fact, ruminal NH_3 concentration depends on hydrolysis and deamination rates of dietary protein, NH_3 utilization by microorganisms, urea exchange through the rumen wall and urea recycling through saliva, as well as on the digesta passage rate. The high intercow variability of ruminal NH_3 concentration (Huhtanen et al., 2015), the single sampling via stomach tube and its poorly controlled location (Shen et al., 2012), in addition to the stratification of the digesta and their kinetics in the rumen (Smith et al., 1956), may have been sources of variability in ruminal NH_3 and VFA concentrations, leading to results contrasting our hypothesis. In addition, a slightly lower realized feeding level in rumen-cannulated compared with intact cows may have contributed to discrepancies in data obtained from samples collected via stomach tube or ruminal cannula 5 h after feeding in the present experiment.

Effects of Extrusion on N Partition, N Use Efficiency, and Dairy Performance

As expected, the $\Delta^{15}N_{animal\text{-}diet}$ was negatively related to the N use efficiency for milk production, when considering cows ≥50 DIM (Correa-Luna et al., 2022). Furthermore, the $\Delta^{15}N_{animal\text{-}diet}$ was lower ($P < 0.01$) with lupines extruded at 150°C than with all other dietary treatments, except EL2, in the respective experiments. In fact, $\Delta^{15}N_{animal\text{-}diet}$ measured in plasma proteins has been significantly correlated with ruminal NH_3 concentration and ruminal N use efficiency in dairy cows (Cantalapiedra-Hijar et al., 2016). Accordingly, a lower $\Delta^{15}N_{animal\text{-}diet}$ intra-animal (i.e., within the same animal) was related to a lower predicted RPB expressed in g/kg DM, despite a low contribution of RPB to the individual variability of $\Delta^{15}N_{animal\text{-}diet}$ in accordance with Cantalapiedra-Hijar et al. (2016). Assuming similar endogenous CP outflow from the rumen, a lower predicted RPB indicates a higher protection of dietary CP from degradation or higher microbial protein synthesis in the rumen. The observed negative relation between intra-animal variation of $\Delta^{15}N_{animal\text{-}diet}$ and plasma urea concentration may also indicate a higher efficiency of N utilization in the rumen through reduced ruminal CP degradability of extruded lupines. In line with these observations, we found a lower concentration of milk urea (Huhtanen et al., 2015) with lupines extruded at 150°C than with raw lupines, irrespective of sugar addition. Nevertheless, plasma urea concentrations did not differ among dietary treatments in either experiment, probably in relation to the preprandial blood sampling (Cattaneo et al., 2023). In contrast to the highly significant relationship observed by Cantalapiedra-Hijar et al. (2016), the $\Delta^{15}N_{animal\text{-}diet}$ was not related to the milk urea concentration in the present experiments, perhaps as a result of intramammary urea synthesis from Arg, and the consequent weakening of the relationship between $\Delta^{15}N_{animal\text{-}diet}$ and milk urea concentration.

Contrary to our second hypothesis, urinary N excretion and N balance were not affected by dietary treatments in both experiments. However, as expected, the $\Delta^{15}N_{animal\text{-}diet}$ was positively related to the ratio of urinary N to N intake. The mean N balances were 40.0 ± 4.08 g/d in EXP1 and 32.0 ± 6.70 g/d in EXP2, respectively. Experimental determination of N partitioning is often biased by a non-negligible N balance default, which generally overestimates N retention (Spanghero and Kowalski, 2021). Outcomes of both experiments were in line with the average N balance of 38.5 g/d found in the updated meta-analysis of N balance studies by Spanghero and Kowalski (2021), with 307 dietary treatments. Assuming a N retention caused by protein retention (33 g of protein per 1 UFL/d balance; INRA, 2018) for the synthesis of body tissues in relation to the observed positive NE balance (on average +2.05 UFL/d for the set of n = 4 cows in EXP1, and +1.63 UFL/d for the set of n = 4 cows in EXP2) and protein loss with scurf (0.032 g $N/BW^{0.6}$ kg; INRA, 2018), the estimated average N balance default (measured N balance − true N retention) was 27.5 g/d in EXP1 and 21.8 g/d in EXP2. Thus, at least in EXP1, the N balance default may have exceeded the magnitude of the numerical variation in urinary N excretion (−20.3 g/d on average) with extruded compared with raw lupines.

In EXP1, N use efficiency for milk production increased by 10% with EL3 compared with all other lupines, whereas in EXP2 it did not differ among dietary treatments, although it increased numerically by 8.0% from RLS to ELS3. In contrast, irrespective of the addition with reducing sugars, no increase of N use efficiency for milk production was observed with lupines extruded at 115°C and 135°C, as it was previously observed for lupine:linseed (90:10) blends extruded at 140°C and 160°C (Mendowski et al., 2019) or faba beans:linseed (90:10) blends extruded at 140°C (Mendowski et al., 2020). This suggests that the temperature of extrusion may be a key parameter affecting N use efficiency for milk production, which would require testing on a larger number of animals. Pretreatment with reducing sugars may not be as effective and could be further refined.

Milk true protein yield was not affected in either experiment, but observed milk protein yields (on average +30 g/d in EXP1, and +45 g/d in EXP2) were in line with the expected decreased ruminal CP degradability and higher ($P < 0.01$) predicted MP supply with extruded than with raw lupines (+253 g PDI/d in EXP1, and +280 g PDI/d, in EXP2). However, assuming a marginal efficiency of PDI utilization for milk protein synthesis of 19% when cows produce at their potential

and the available PDI covers protein expenditure (Daniel et al., 2016), the observed increased PDI supply leads to a predicted increased milk protein synthesis of +48 g/d in EXP1 and +53 g/d in EXP2. Thus, the observed marginal milk true protein yield obtained with the extrusion of lupines in both experiments was slightly lower than predicted.

In contrast to observations by Cantalapiedra-Hijar et al. (2016), the $\Delta^{15}N_{animal\text{-}diet}$ was not related to the PDI use efficiency estimated in these experiments. However, the lower PDI use efficiency with all extruded lupines than with raw lupines, partly agreed with the observed higher predicted PDI intake with all extruded lupines, as lower efficiency of MP utilization at higher predicted MP supply is classically observed (Metcalf et al., 2008; Moraes et al., 2018). Altogether, these observations suggest that the variations observed in N use efficiency for milk production with the highest temperature of extrusion (150°C) without addition of reducing sugars may be mainly related to ruminal N use efficiency rather than to absorptive or postabsorptive mechanisms.

Still, the extrusion at 150°C may have reduced the bioavailability of Lys in the intestine compared with the lower temperatures of extrusion applied in these experiments, as supported by the tendency ($P = 0.09$) toward an effect on the apparent total-tract digestibility of total Lys in EXP1 in line with the increased CML to total Lys proportion especially with EL3. Lysine is the AA most susceptible to glycation and its lower bioavailability in the intestine may result from overprotection from proteases and peptidases due to CML formation. Nevertheless, this may not have affected the dairy performance, as Lys may not have been limiting compared with Met as LysDI represented 6.6% of predicted PDI, which is close to LysDI requirements (i.e., 7.0% of PDI) according to INRA (2018). Furthermore, dietary Lys accounts for only around one-third of fecal Lys, whereas around two-thirds is of microbial or endogenous origin, which may have masked the effects of extrusion on the bioavailability of Lys in the intestine.

In both experiments, lupines extruded at 150°C induced lower plasma taurine concentrations in comparison with raw lupines and lupines extruded at 115°C. Taurine derives from cysteine following Met catabolism through the transsulfuration pathway (Seymour et al., 1990). It is not used for milk protein synthesis and is therefore only sparingly taken up in the bovine mammary gland. Only in EXP2, the plasma Met concentration was lower in cows fed ELS3 than in cows fed RLS, despite the supplementation with rumen-protected Met to reach 2.3% of MetDI in PDI in diets with lupines extruded at 135°C and 150°C. Overall, this might suggest also a lower intestinal bioavailability of Met, a lower absorption, and consequently a less extensive catabolism of Met in cows fed lupines extruded at 150°C, especially with added reducing sugars.

Eventually, the dietary inclusion of lupines has often been reported to reduce milk fat concentration and yield (Froidmont and Bartiaux-Thill, 2004) as a consequence of their relatively high content of crude fat (9.6% in the DM; INRAE-CIRAD-AFZ, 2022) compared with other leguminous seeds. Thermo-mechanical treatments such as extrusion may further increase the availability of the lipids in the rumen and lead to the observed lower milk fat content in EXP1 or tendency toward lower milk fat content in EXP2 (Mendowski et al., 2021), which was balanced out by higher milk yield in EXP1, and tendency toward a higher milk yield in EXP2.

Apparent Total-Tract Digestibility, Partition, and Excretion of CML

In addition to being potential proxies for ruminal CP degradability in feed production, dietary MRC such as CML are known to have immunomodulatory effects that may also be relevant in livestock (Teodorowicz et al., 2018). Current knowledge on the digestibility and partition of MRC is mainly based on research in humans and rodents, and therefore further investigations on dairy cows were required. In the present experiments, the apparent total-tract digestibility of CML in the total diet was lower (on average 41 vs. 71%) than previously reported by Mendowski et al. (2020), due to the relatively lower CML intake [416 ± 47 mg/d in present experiments vs. 748 ± 120 mg/d in the experiment by Mendowksi et al. (2020)] and similar fecal CML excretion [246 ± 39 mg/d in the present experiments vs. 226 ± 34 mg/d in the study by Mendowski et al. (2020)]. Furthermore, the estimated marginal increase in digestibility of CML with increasing CML intake from lupines (84%) was similar to the estimated digestibility of CML provided by faba beans (88%; Mendowski et al., 2020), whereas the marginal increase in digestibility of CML from the basal mixed ration was relatively lower (62%) and similar to the digestibility of CML provided by all dietary components except for the faba bean:linseed blend (60%) estimated by Mendowski et al. (2020). The absorption of free MRC with low molecular weight such as CML probably occurs by simple diffusion into the intestinal epithelial cells, whereas MRC included in dipeptides may be absorbed through the action of PEPT-1 transporters (Grunwald et al., 2006; Hellwig et al., 2011). Differences between marginal increases in CML digestibility observed for different feeds (lupines vs. basal mixed ration) suggest that CML available after ruminal degradation and enzymatic digestion may differ in chemical form by source. The CML in leguminous seeds might be available in the intestine rather in

free AA form, while CML from the basal mixed ration, in particular from forages, might be in peptides, or undigested proteins, implying more selective absorption into gastrointestinal epithelial cells.

Beyond the dietary supply and fecal excretion of CML measured in the present experiments, we cannot a priori exclude the formation and degradation of CML in the gastrointestinal tract. For example, isolated human gut bacteria have been reported to degrade CML in vitro (Hellwig et al., 2015; Bui et al., 2019). Recently, also a soil bacterium was found to degrade CML (Mehler et al., 2022). However, 5 *Escherichia coli* strains degraded CML only under aerobic conditions in vitro (Hellwig et al., 2019), and other bacteria capable of degrading CML were shown to have highly specialized mechanisms (Hellwig et al., 2015; Mehler et al., 2022), which to our knowledge have not yet been described in the ruminant digestive system.

To our knowledge, this is the first study reporting the partition of apparently digested CML between the urine and milk of dairy cows. We suppose that CML recovered in milk and urine did not derive from proteolysis and glycation occurring after urine and milk harvest, as the samples were frozen to −20°C immediately after collection and lyophilized before CML analysis. Our results showed that independently of the dietary treatment, lactating dairy cows excreted 26 ± 2.1% of the apparently digested CML in urine, whereas the secretion of CML in milk was limited to 0.63 ± 0.05% of the apparently digested CML. Previous literature reports indicate that urinary excretion of apparently digested CML accounts on average for 55% in rats (Alamir et al., 2013) and 26% in young adults (Delgado-Andrade et al., 2012), the kidneys being the main organ responsible for the removal of advanced glycation end products from the bloodstream and their excretion. However, a greater CML urinary excretion in relation to higher dietary CML intake was also observed in human infants, rodents, dogs, and cats (Šebeková et al., 2008; Alamir et al., 2013; Palaseweenun et al., 2021). On the contrary, Delgado-Andrade et al. (2012) found proportionally lower urinary CML excretion at higher CML intake in young adults eating MRC-enriched meals. We did not observe any difference among dietary treatments in either experiment, either in the concentration of CML in urine and milk or in the absolute daily excretion of CML in urine or milk.

Although CML is one of the best studied advanced glycation end products in milk after thermal processing (Milkovska-Stamenova and Hoffmann, 2019; Wölk et al., 2021), to the best of our knowledge the quantitative relation between CML intake and absolute excretion and secretion has never been investigated in any lactating animal. Schwarzenbolz et al. (2016) hypothesized a relation between the dietary intake of MRC and their concentration in bulk milk depending on the dairy production system (e.g., organic, conventional), but this hypothesis has never been tested under controlled experimental conditions. The concentration of CML in raw milk in the present study (33 ± 0.7 μg/kg) was relatively lower than that found in human breast milk (133 ± 83 μg/kg) by Dittrich et al. (2006) and in line with those previously found in bulk milk (on average 21 ± 0.3 μg/kg) from German organic and conventional farms (Schwarzenbolz et al., 2016). Nevertheless, the CML concentration in human breast milk was determined with an immunochemical assay, which may have different specificity for CML, whereas in the survey of Schwarzenbolz et al. (2016) and in our experiments CML was analyzed by LC-MS/MS, which limits to some extent between-species comparisons of the reported absolute CML concentrations in milk.

A substantial proportion of the ingested (i.e., 30 ± 1.8%) and apparently digested CML (i.e., 74 ± 2.2%) was not recovered in the analyzed excretory routes, which is in accordance with observations in other species, including humans (Delgado-Andrade et al., 2012) and rats (Alamir et al., 2013). Interestingly, the estimated amount of CML apparently retained in the body was higher with EL3 than with RL. Despite significantly higher CML supply with ELS3 than with all other treatments and a 55% numerically greater CML balance with ELS3 than RLS in EXP2, we observed no significant effect on the CML balance, hinting toward a low statistical power of EXP2. In mice, CML accumulates primarily in the kidneys but also in other tissues such as in the ileum, colon, and lungs, except in adipose tissue where it seems not to accumulate owing to its hydrophilic properties (Tessier et al., 2016). The endogenous formation of CML through oxidative reactions also at the body temperature (38–39°C) of dairy cows, as reported in other species such as humans (Thorpe and Baynes, 2002) and rodents (Alamir et al., 2013), was not evaluated in the present study but cannot be excluded a priori. Finally, any diurnal pattern in urinary CML concentration is not included in the present measurements and calculations of CML partition and excretion, which may limit to some extent the comparability of our results with those obtained in other species. Further investigations are warranted to determine the metabolism, tissue distribution, excretion kinetics, and possible deposition of CML and other dietary or endogenous advanced glycation end products, and their influence on inflammatory and oxidative status, as well as their long-term effects on the health status of dairy cows.

CONCLUSIONS

Extrusion of lupines numerically decreased enzymatic CP degradability, especially at the highest extrusion temperature (150°C) confirming our first hypothesis. The decreased CP degradability was associated with numerically increased CML concentration and CML to total Lys ratios, irrespective of reducing sugars, partially refuting our third hypothesis. The overall effects of the extrusion on milk production and composition were limited to lower milk urea concentrations and lower fat:protein ratio with lupines extruded at 150°C without greatly affecting N partition, partially refuting our second hypothesis. Milk yield and N use efficiency for milk production increased with extrusion at 150°C only without reducing sugars, in partial agreement with our second but largely refuting the third hypothesis. Regardless of reducing sugars, the $\Delta^{15}N_{animal\text{-}diet}$ was lower with lupines extruded at 150°C than with raw lupines. CML appeared to be partially digested and then excreted mainly in urine and to a much lesser extent in milk.

ACKNOWLEDGMENTS

These experiments were conducted in the framework of the PROLEVAL project supported by Valorex SAS (Combourtillé, France) and financed by bpifrance (Paris, France). We gratefully acknowledge the committed help and technical support provided by F. Fournier, G. De Falvard, L. Mouly, and D. Roux (INRAE, Herbipôle, Saint-Genès-Champanelle, France) during the setting up and performance of the experiments, as well as L. Genestoux, C. Labonne, L. Salis, C. Selebran, I. Constant, and C. Chantelauze (INRAE, UMR Herbivores, Saint-Genès-Champanelle, France) during sampling and laboratory analyses. Many thanks also to C. Léridon (UniLaSalle, Beauvais, France) for the analysis of the Maillard reaction compounds, and to J. Pires (INRAE, UMR Herbivores) for statistical advice. Author contributions were as follows: E. Manzocchi contributed to the investigation, formal analysis, visualization, writing of original draft, and review and editing of text; A. Ferlay contributed to the conceptualization, supervision, investigation, formal analysis, review and editing, and funding acquisition; S. Mendowski contributed to the conceptualization, resources, methodology, review and editing, and project administration; G. Chesneau contributed to the conceptualization, resources, methodology, review and editing, and funding acquisition; P. Chapoutot contributed to the conceptualization, methodology, review and editing, and funding acquisition; S. Lemosquet contributed to the methodology, resources, and review and editing; G. Cantalapiedra-Hijar contributed to the methodology, formal analysis, and review and editing; and P. Nozière contributed to the conceptualization, supervision, investigation, methodology, formal analysis, validation, review and editing, and funding acquisition. Data are available from the corresponding author upon reasonable request. The authors have not stated any conflicts of interest.

REFERENCES

Alamir, I., C. Niquet-Leridon, P. Jacolot, C. Rodriguez, M. Orosco, P. M. Anton, and F. J. Tessier. 2013. Digestibility of extruded proteins and metabolic transit of Nε-carboxymethyllysine in rats. Amino Acids 44:1441–1449. https://doi.org/10.1007/s00726-012-1427-3.

Aufrère, J., D. Graviou, C. Demarquilly, R. Vérité, B. Michalet-Doreau, and P. Chapoutot. 1991. Predicting in situ degradability of feed proteins in the rumen by two laboratory methods (solubility and enzymatic degradation). Anim. Feed Sci. Technol. 33:97–116. https://doi.org/10.1016/0377-8401(91)90049-X.

Bazin, S. 1984. Grille de notation de l'état d'engraissement des vaches Pie Noires. Réseau national d'expérimentation et de démonstration en élevage bovin. Institut technique de l'élevage bovin, Paris, France.

Benchaar, C., R. Moncoulon, C. Bayourthe, and M. Vernay. 1994. Effects of a supply of raw or extruded white lupin seeds on protein digestion and amino acid absorption in dairy cows. J. Anim. Sci. 72:492–501. https://doi.org/10.2527/1994.722492x.

Bui, T. P. N., A. Troise, V. Fogliano, and W. M. de Vos. 2019. Anaerobic degradation of N-ε-carboxymethyllysine, a major glycation end-product, by human intestinal bacteria. J. Agric. Food Chem. 67:6594–6602. https://doi.org/10.1021/acs.jafc.9b02208.

Cantalapiedra-Hijar, G., R. Dewhurst, L. Cheng, A. R. J. Cabrita, A. J. M. Fonseca, P. Nozière, D. Makowski, H. Fouillet, and I. Ortigues-Marty. 2018. Nitrogen isotopic fractionation as a biomarker for nitrogen use efficiency in ruminants: A meta-analysis. Animal 12:1827–1837. https://doi.org/10.1017/S1751731117003391.

Cantalapiedra-Hijar, G., H. Fouillet, J. F. Huneau, A. Fanchone, M. Doreau, P. Nozière, and I. Ortigues-Marty. 2016. Relationship between efficiency of nitrogen utilization and isotopic nitrogen fractionation in dairy cows: Contribution of digestion v. metabolism? Animal 10:221–229. https://doi.org/10.1017/S1751731115002025.

Cattaneo, L., F. Piccioli-Cappelli, A. Minuti, and E. Trevisi. 2023. Metabolic and physiological adaptations to first and second lactation in Holstein dairy cows: Postprandial patterns. J. Dairy Sci. 106:3576–3585. https://doi.org/10.3168/jds.2022-22685.

Chapoutot, P., O. Dhumez, A. Germain, and G. Chesneau. 2016. Influence of extrusion on nitrogen degradability of faba bean or lupine blends including urea. Page 564 in EAAP 67th Annual Meeting, Belfast. Wageningen Academic Publishers.

Chapoutot, P., O. Dhumez, S. Mendowski, G. Chesneau, and P. Nozière. 2020. L'extrusion des protéagineux modifie la dégradation dans le rumen et la digestibilité intestinale de l'azote, de la lysine et des composées de Maillard. Page 384 in 25. Rencontres autour des Recherches sur les Ruminants, Institut de l'Elevage, INRAE.

Correa-Luna, M., M. Johansen, P. Nozière, C. Chantelauze, S. M. Nasrollahi, P. Lund, M. Larsen, A. R. Bayat, L. A. Crompton, C. K. Reynolds, E. Froidmont, N. Edouard, R. Dewhurst, L. Bahloul, C. Martin, and G. Cantalapiedra-Hijar. 2022. Nitrogen isotopic discrimination as a biomarker of between-cow variation in the efficiency of nitrogen utilization for milk production: A meta-analysis. J. Dairy Sci. 105:5004–5023. https://doi.org/10.3168/jds.2021-21498.

Daniel, J. B., N. C. Friggens, P. Chapoutot, H. Van Laar, and D. Sauvant. 2016. Milk yield and milk composition responses to

change in predicted net energy and metabolizable protein: A meta-analysis. Animal 10:1975–1985. https://doi.org/10.1017/S1751731116001245.

Delgado-Andrade, C., F. J. Tessier, C. Niquet-Leridon, I. Seiquer, and M. Pilar Navarro. 2012. Study of the urinary and faecal excretion of Nε-carboxymethyllysine in young human volunteers. Amino Acids 43:595–602. https://doi.org/10.1007/s00726-011-1107-8.

Dittrich, R., I. Hoffmann, P. Stahl, A. Müller, M. W. Beckmann, and M. Pischetsrieder. 2006. Concentrations of N^{ε}-Carboxymethyllysine in human breast milk, infant formulas, and urine of infants. J. Agric. Food Chem. 54:6924–6928. https://doi.org/10.1021/jf060905h.

Froidmont, E., and N. Bartiaux-Thill. 2004. Suitability of lupin and pea seeds as a substitute for soybean meal in high-producing dairy cow feed. Anim. Res. 53:475–487. https://doi.org/10.1051/animres:2004034.

Grunwald, S., R. Krause, M. Bruch, T. Henle, and M. Brandsch. 2006. Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems. Br. J. Nutr. 95:1221–1228. https://doi.org/10.1079/BJN20061793.

Haque, M. N., H. Rulquin, A. Andrade, P. Faverdin, J. L. Peyraud, and S. Lemosquet. 2012. Milk protein synthesis in response to the provision of an "ideal" amino acid profile at two levels of metabolizable protein supply in dairy cows. J. Dairy Sci. 95:5876–5887. https://doi.org/10.3168/jds.2011-5230.

Hellwig, M., C. Auerbach, N. Müller, P. Samuel, S. Kammann, F. Beer, F. Gunzer, and T. Henle. 2019. Metabolization of the advanced glycation end product N-ε-carboxymethyllysine (CML) by different probiotic *E. coli* strains. J. Agric. Food Chem. 67:1963–1972. https://doi.org/10.1021/acs.jafc.8b06748.

Hellwig, M., D. Bunzel, M. Huch, C. M. A. P. Franz, S. E. Kulling, and T. Henle. 2015. Stability of individual Maillard reaction products in the presence of the human colonic microbiota. J. Agric. Food Chem. 63:6723–6730. https://doi.org/10.1021/acs.jafc.5b01391.

Hellwig, M., S. Geissler, R. Matthes, A. Peto, C. Silow, M. Brandsch, and T. Henle. 2011. Transport of free and peptide-bound glycated amino acids: Synthesis, transepithelial flux at caco-2 cell monolayers, and interaction with apical membrane transport proteins. ChemBioChem 12:1270–1279. https://doi.org/10.1002/cbic.201000759.

Hobson, P. N., and C. S. Stewart. 1997. The Rumen Microbial Ecosystem. Springer.

Hofmann, T., A. C. Engling, S. Martens, O. Steinhöfel, and T. Henle. 2020. Quantification of Maillard reaction products in animal feed. Eur. Food Res. Technol. 246:253–256. https://doi.org/10.1007/s00217-019-03406-w.

Huhtanen, P., E. H. Cabezas-Garcia, S. J. Krizsan, and K. J. Shingfield. 2015. Evaluation of between-cow variation in milk urea and rumen ammonia nitrogen concentrations and the association with nitrogen utilization and diet digestibility in lactating cows. J. Dairy Sci. 98:3182–3196. https://doi.org/10.3168/jds.2014-8215.

INRA. 2018. INRA Feeding System for Ruminants. Wageningen Academic Publishers. https://doi.org/10.3920/978-90-8686-292-4

INRAE-CIRAD-AFZ. 2022. Feed tables: Composition and nutritive values of feeds for cattle, sheep, goats, pigs, poultry, rabbits, horses and salmonoids. Accessed Jan. 15, 2022. https://www.feedtables.com/content/lupin-white.

ISO. (International Organization for Standardization). 1999. ISO 6492:1999 Animal feeding stuffs — Determination of fat content. International Organization for Standardization, Geneva, Switzerland.

ISO. (International Organization for Standardization). 2002. ISO 5984:2002 Animal feeding stuffs–Determination of crude ash. International Organization for Standardization, Geneva, Switzerland.

ISO. (International Organization for Standardization). 2004. ISO 15914:2004 Animal feeding stuffs — Enzymatic determination of total starch content. International Organization for Standardization, Geneva, Switzerland.

ISO. (International Organization for Standardization). 2008. ISO 16634-1:2008 Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content — Part 1: Oilseeds and animal feeding stuffs. International Organization for Standardization, Geneva, Switzerland.

Madsen, T. G., M. O. Nielsen, J. B. Andersen, and K. L. Ingvartsen. 2008. Continuous lactation in dairy cows: Effect on milk production and mammary nutrient supply and extraction. J. Dairy Sci. 91:1791–1801. https://doi.org/10.3168/jds.2007-0905.

Manzocchi, E., A. Ferlay, S. Mendowski, G. Chesneau, P. Chapoutot, S. Lemosquet, G. Cantalapiedra-Hijar, P. Nozière. 2023. Supplementary materials for: Manzocchi et al, J Dairy Sci 2022-22902. Recherche Data Gouv, V3. https://doi.org/10.57745/U4U77P.

Mehler, J., K. I. Behringer, R. E. Rollins, F. Pisarz, A. Klingl, T. Henle, R. Heermann, N. S. Becker, M. Hellwig, and J. Lassak. 2022. Identification of *Pseudomnoas asiatica* subsp. *bavariensis* str. JM1 as the first N_{ε}-carboxy(m)ethyllysine-degrading soil bacterium. Environ. Microbiol. 24:3229–3241. https://doi.org/10.1111/1462-2920.16079.

Mendowski, S., P. Chapoutot, G. Chesneau, A. Ferlay, F. Enjalbert, G. Cantalapiedra-Hijar, A. Germain, and P. Nozière. 2019. Effects of replacing soybean meal with raw or extruded blends containing faba bean or lupin seeds on nitrogen metabolism and performance of dairy cows. J. Dairy Sci. 102:5130–5147. https://doi.org/10.3168/jds.2018-15416.

Mendowski, S., P. Chapoutot, G. Chesneau, A. Ferlay, F. Enjalbert, G. Cantalapiedra-Hijar, A. Germain, and P. Nozière. 2020. Effects of pretreatment with reducing sugars or an enzymatic cocktail before extrusion of fava bean on nitrogen metabolism and performance of dairy cows. J. Dairy Sci. 103:396–409. https://doi.org/10.3168/jds.2019-17286.

Mendowski, S., P. Nozière, A. Ferlay, P. Denis, G. Chesneau, and P. Chapoutot. 2021. Raw or technologically treated proteaginous seeds as alternatives to soybean meal for dairy cows: Comparative evaluation by meta-analysis of in situ and in vivo digestive parameters, nitrogen partition and dairy performance. Anim. Feed Sci. Technol. 271:114758. https://doi.org/10.1016/j.anifeedsci.2020.114758.

Metcalf, J. A., R. J. Mansbridge, J. S. Blake, J. D. Oldham, and J. R. Newbold. 2008. The efficiency of conversion of metabolisable protein into milk true protein over a range of metabolisable protein intakes. Animal 2:1193–1202. https://doi.org/10.1017/S1751731108002140.

Milkovska-Stamenova, S., and R. Hoffmann. 2019. Diversity of advanced glycation end products in the bovine milk proteome. Amino Acids 51:891–901. https://doi.org/10.1007/s00726-019-02707-4.

Miura, H., M. Horiguchi, and T. Matsumoto. 1980. Nutritional interdependence among rumen bacteria, *Bacteroides amylophilus*, *Megasphaera elsdenii*, and *Ruminococcus albus*. Appl. Environ. Microbiol. 40:294–300. https://doi.org/10.1128/aem.40.2.294-300.1980.

Moraes, L. E., E. Kebreab, J. L. Firkins, R. R. White, R. Martineau, and H. Lapierre. 2018. Predicting milk protein responses and the requirement of metabolizable protein by lactating dairy cows. J. Dairy Sci. 101:310–327. https://doi.org/10.3168/jds.2016-12507.

Niquet-Léridon, C., and F. J. Tessier. 2011. Quantification of Nε-carboxymethyl-lysine in selected chocolate-flavoured drink mixes using high-performance liquid chromatography–linear ion trap tandem mass spectrometry. Food Chem. 126:655–663. https://doi.org/10.1016/j.foodchem.2010.10.111.

Ogimoto, K., and S. Imai. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press.

Omphalius, C., H. Lapierre, J. Guinard-Flament, P. Lamberton, L. Bahloul, and S. Lemosquet. 2019. Amino acid efficiencies of utilization vary by different mechanisms in response to energy and protein supplies in dairy cows: Study at mammary-gland and whole-body levels. J. Dairy Sci. 102:9883–9901. https://doi.org/10.3168/jds.2019-16433.

Palaseweenun, P., E. A. Hagen-Plantinga, J. T. Schonewille, G. Koop, C. Butre, M. Jonathan, P. A. Wierenga, and W. H. Hendriks. 2021. Urinary excretion of advanced glycation end products in dogs and cats. J. Anim. Physiol. Anim. Nutr. (Berl.) 105:149–156. https://doi.org/10.1111/jpn.13347.

Peyraud, J. L., C. Genest-Rulquin, and R. Vérité. 1988. Mesure de la digestion de l'azote des aliments dans l'intestin des ruminants par la technique des sachets mobiles. 1. Évaluation de la quantité de matières azotées indigestibles en sachet des principaux aliments. Reprod. Nutr. Dev. 28(Suppl. 1):129–130. https://doi.org/10.1051/rnd:19881139.

Schwarzenbolz, U., T. Hofmann, N. Sparmann, and T. Henle. 2016. Free Maillard reaction products in milk reflect nutritional intake of glycated proteins and can be used to distinguish "organic" and "conventionally" produced milk. J. Agric. Food Chem. 64:5071–5078. https://doi.org/10.1021/acs.jafc.6b01375.

Šebeková, K., G. Saavedra, C. Zumpe, V. Somoza, K. Klenovicsová, and I. Birlouez-Aragon. 2008. Plasma concentration and urinary excretion of N^{E}-(carboxymethyl)lysine in breast milk- and formula-fed infants. Ann. N. Y. Acad. Sci. 1126:177–180. https://doi.org/10.1196/annals.1433.049.

Seymour, W. M., C. E. Polan, and J. H. Herbein. 1990. Effects of dietary protein degradability and casein or amino acid infusions on production and plasma amino acids in dairy cows. J. Dairy Sci. 73:735–748. https://doi.org/10.3168/jds.S0022-0302(90)78727-9.

Shen, J. S., Z. Chai, L. J. Song, J. X. Liu, and Y. M. Wu. 2012. Insertion depth of oral stomach tubes may affect the fermentation parameters of ruminal fluid collected in dairy cows. J. Dairy Sci. 95:5978–5984. https://doi.org/10.3168/jds.2012-5499.

Smith, P. H., H. C. Sweeney, J. R. Rooney, K. W. King, and W. E. C. Moore. 1956. Stratifications and kinetic changes in the ingesta of the bovine rumen. J. Dairy Sci. 39:598–609. https://doi.org/10.3168/jds.S0022-0302(56)94791-9.

Spanghero, M., and Z. M. Kowalski. 1997. Critical analysis of N balance experiments with lactating cows. Livest. Prod. Sci. 52:113–122. https://doi.org/10.1016/S0301-6226(97)00138-3.

Spanghero, M., and Z. M. Kowalski. 2021. Updating analysis of nitrogen balance experiments in dairy cows. J. Dairy Sci. 104:7725–7737. https://doi.org/10.3168/jds.2020-19656.

Teodorowicz, M., W. H. Hendriks, H. J. Wichers, and H. F. J. Savelkoul. 2018. Immunomodulation by processed animal feed: The role of Maillard reaction products and advanced glycation end-products (AGEs). Front. Immunol. 9:2088. https://doi.org/10.3389/fimmu.2018.02088.

Tessier, F. J., E. Boulanger, and M. Howsam. 2021. Metabolic transit of dietary advanced glycation end-products—The case of N^{ϵ}-carboxymethyllysine. Glycoconj. J. 38:311–317. https://doi.org/10.1007/s10719-020-09950-y.

Tessier, F. J., C. Niquet-Léridon, P. Jacolot, C. Jouquand, M. Genin, A.-M. Schmidt, N. Grossin, and E. Boulanger. 2016. Quantitative assessment of organ distribution of dietary protein-bound ^{13}C-labeled N^{E}-carboxymethyllysine after a chronic oral exposure in mice. Mol. Nutr. Food Res. 60:2446–2456. https://doi.org/10.1002/mnfr.201600140.

Thorpe, S. R., and J. W. Baynes. 2002. CML: A brief history. Int. Congr. Ser. 1245:91–99. https://doi.org/10.1016/S0531-5131(02)00881-6.

van Soest, P. J., and V. C. Mason. 1991. The influence of the Maillard reaction upon the nutritive value of fibrous feeds. Anim. Feed Sci. Technol. 32:45–53. https://doi.org/10.1016/0377-8401(91)90008-G.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Wölk, M., S. Milkovska-Stamenova, T. Schröter, and R. Hoffmann. 2021. Influence of seasonal variation and processing on protein glycation and oxidation in regular and hay milk. Food Chem. 337:127690. https://doi.org/10.1016/j.foodchem.2020.127690.

ORCIDS

E. Manzocchi https://orcid.org/0000-0002-6397-8300
A. Ferlay https://orcid.org/0000-0002-0651-792X
S. Lemosquet https://orcid.org/0000-0002-1077-1285
G. Cantalapiedra-Hijar https://orcid.org/0000-0001-9486-8238
P. Nozière https://orcid.org/0000-0003-1727-8984