Milk production response to varying protein supply is independent of forage digestibility in dairy cows

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ABSTRACT

The aim of this experiment was to examine whether the positive response in milk production to increased crude protein (CP) supply in dairy cows was dependent on the digestibility of the forage. Forty-eight lactating Danish Holstein cows were used in a 4 × 4 Latin square design experiment with 4 rations: (1) high digestibility and high CP concentration (HdHp), (2) high digestibility and low CP concentration (HdLp), (3) low digestibility and high CP concentration (LdHp), and (4) low digestibility and low CP concentration (LdLp). All rations contained 30% corn silage, 25% grass-clover silage, and 45% concentrate on a dry matter (DM) basis. Different digestibilities were obtained by replacing a high-digestible grass-clover silage combined with a high-digestible corn silage with a low-digestible grass-clover silage combined with a low-digestible corn silage. Organic matter digestibilities were 79.8 and 74.7% in the high- and low-digestibility rations, respectively. Dietary CP concentration in the ration was increased by substituting barley and sugar beet pulp with rapeseed meal and soybean meal, whereby CP increased from 13.9 to 14.0% (Lp) to 15.7 to 16.0% (Hp). All cows were offered 3 kg of the same concentrate per day in the automatic milking system in addition to the mixed ration. Every feeding period lasted 3 wk, and DM intake and milk yield were measured in the last week in each period, and milk samples for determining milk composition, including fatty acid content, and blood samples were taken during the last 3 d of each period. Dry matter intake increased by 2.2 kg/d on Hd compared with Ld and by 0.7 kg/d on Hp compared with Lp. The positive effect on DM intake was reflected in the energy-corrected milk (ECM) yield, as a higher ration digestibility increased the ECM yield by 1.7 kg/d and a higher CP concentration increased it by 1.2 kg/d. We detected no interaction between forage digestibility

and CP concentration on milk production. Reduced digestibility was accompanied by an increase in the plasma level of glucose, suggesting that other nutrients were limiting to milk production. In conclusion, milk production responses to dietary CP supply appeared independent of forage digestibility.

Key words: corn and grass clover silage, mammary nutrient supply and extraction, milk fatty acids

INTRODUCTION

Protein requirements of lactating dairy cows are high due to their need for AA for milk protein synthesis. In an environmental context, improving the efficiency of the conversion of dietary CP to milk protein is desirable, and this is mainly achieved by reducing the dietary CP supply (Nielsen et al., 2003) and consequently the N output in urine (Børsting et al., 2003). However, a considerable decrease in voluntary DMI by dairy cows is expected when dietary protein supply is limited (Fisher, 2002), because a protein-deficient diet can limit microbial activity in the rumen and impair digestion (Oldham, 1984). This will affect the DMI negatively and this negative effect has been found to increase progressively as protein supply decreases (Weisbjerg et al., 2010). A higher dietary protein concentration increases DMI, although responses vary according to the level of protein supplementation (Allen, 2000; M'hamed et al., 2001). This positive effect on DMI can be explained by a range of effects on digestibility in the rumen (Oldham, 1984). Nutrient intake and associated energy balance in the cow will affect nutrient composition in the blood and hence also the patterns of mammary nutrient supply (Safayi and Nielsen, 2013) and milk FA composition (Gross et al., 2011). Generally, older cows have a higher milk yield response to increased nutrient supply than do first-lactation cows (Smith et al., 1978; Bossen and Weisbjerg, 2009).

Increasing the concentration of protein in the ration to improve the productivity of cows results in lower utilization of nitrogen when N is consumed in excess of nutritional requirements (Walker et al., 2005). The

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process of transforming the excess protein into urea is energy-demanding.

Weisbjerg et al. (2010) compared recent studies where highly digestible forages were used with earlier experiments where lower digestible forages were used; they concluded that cows eating rations containing high-digestible forages were less sensitive to low protein concentration than cows eating rations containing low-digestible forages. The hypothesis of this study was therefore that feed intake and milk production would be less negatively affected by a reduction in CP supply when forage digestibility is high than when it is low.

The objective of this experiment was to study the interactive effect of CP supply and forage digestibility on dairy cow DMI, mammary nutrient uptake, ECM yield, and milk composition. Mammary nutrient uptake was assessed based on analyses of blood parameters, and milk composition was assessed based on analyses of total fat, protein, and lactose, as well as FA composition.

MATERIALS AND METHODS

Cows, Experimental Design, Facilities, and Feeds

The experiment was carried out at the Danish Cattle Research Centre (Foulum, Denmark) and complied with the guidelines set out by the Danish Ministry of Justice with respect to animal experimentation and the care of animals used for scientific purposes.

Forty-eight lactating Danish Holstein cows that had reached peak yield and would remain lactating throughout the experiment were used, and included 20 first-, 12 second-, 12 third-, and 4 fourth-lactation cows. At the beginning of the experiment, cows averaged ($\pm$ SD) 643 $\pm$ 63 kg of BW, 33.9 $\pm$ 7.6 kg of daily ECM yield, 21.3 $\pm$ 4.4 kg of daily DMI, and 115 $\pm$ 45 DIM (range: 33 to 214 DIM).

Cows were blocked according to parity (first lactation and later) and DIM and randomly assigned to 1 of 4 dietary treatments in a replicated 4 $\times$ 4 Latin square design with a 2 $\times$ 2 factorial arrangement of treatments. Each experimental period lasted 21 d.

The treatments were (1) high OM digestibility and high CP concentration (**HdHp**), (2) high OM digestibility and low CP concentration (**HdLp**), (3) low OM digestibility and high CP concentration (**LdHp**), and (4) low OM digestibility and low CP concentration (**LdLp**). All rations consisted of 30% corn silage, 25% grass-clover silage, and 45% concentrate on DM basis. Differences in ration digestibilities were obtained by use of Ld or Hd silages (Table 1 and Table 2). Organic matter digestibilities were 74.7 and 79.8% in Ld and Hd, respectively. Dietary CP was increased by substituting barley and sugar beet pulp with rapeseed meal and soybean meal to reach protein concentrations of 13.9 to 14.0% CP for Lp and 15.7 to 16.0% CP for Hp (Table 3). Rations were adjusted using urea to reach similar CP concentrations (per kg of DM) for the 2 different forage qualities.

Cows were kept in a loose-housing system with slatted floor and cubicles with mattresses. A cow-traffic system for free access to the automatic milking system (**AMS**; DeLaval AB, Tumba, Sweden) was used, and the AMS was equipped with a device for automatic measurement of milk yield and milk sampling. Cows in first lactation had a milking allowance if the interval from the last milking exceeded 6 or 10 h for cows <21 DIM or $\geq$ 21 DIM, respectively, or if predicted milk yield per milking exceeded 9 kg. Older cows had a milking allowance if the interval from the last milking exceeded 6 or 10 h for cows <21 DIM or $\geq$ 21 DIM, respectively, or if predicted milk yield exceeded 10 kg. The AMS also measured the amount of concentrate dispensed and weighed concentrate refusals at the end of each cow visit. A platform scale (Danvaegt, Hinnerup,

Table 1. Chemical composition (g/kg of DM unless otherwise noted) of feedstuffs including high-digestible (Hd) and low-digestible (Ld) silages

Feedstuff	DM, g/kg of fresh matter	OM	CP	Crude fat	NDF	Starch	Energy, MJ/kg of DM	OMD, ¹ %
Hd Grass-clover silage	414	913	162	32.1	358	—	7.4	81.0
Ld Grass-clover silage	464	912	148	28.7	500	—	5.3	66.2
Hd Corn silage	340	971	81	27.4	373	283	7.2	77.8
Ld Corn silage	235	967	101	17.3	509	134	6.2	72.0
Barley	854	981	115	29.0	174	527	8.8	84.5
Beet pulp dried	891	931	92	9.3	389	—	7.9	87.7
Rapeseed meal	880	922	405	49.1	260	2.7	8.9	78.7
Soybean meal	873	930	524	29.6	120	8.2	11.0	90.6
Urea, ² 80%	980	—	2,300	—	—	—	—	—
Concentrate, AMS ³	869	931	214	37.0	259	—	8.2	81.4

¹OMD = estimated in vivo OM digestibility; calculated from measured in vitro digestibility according to Åkerlind et al. (2011).

²Comprising 80% urea, 11% NaSO₄, 9% CaCO₃.

³AMS = automatic milking system.

Table 2. Quality of fermentation of high-digestible (Hd) and low-digestible (Ld) silages

Feedstuff	pH	Ammonia N, % of N	Lactic acid, % of DM	Acetic acid, % of DM	Propionic acid, % of DM	Butyric acid, % of DM	Ethanol, % of DM
Hd Grass-clover silage	4.4	6.2	4.6	1.0	0	0	0.02
Ld Grass-clover silage	4.5	6.1	2.2	0.6	0.005	0	0.02
Hd Corn silage	3.8	11.2	5.8	1.8	0.052	0.04	0.09
Ld Corn silage	3.7	8.7	8.5	2.1	0	0.01	0.31

Denmark) was installed below each AMS for automatic recording of cow live weight. For automatic recording of intake of mixed ration, the Insentec RIC system was used (Insentec, Marknesse, the Netherlands). Cows had free access to drinking water. For further details, see Bossen et al. (2009).

Experimental Procedures

Feed Composition. Fresh silage samples were taken every week for DM determination. Samples of concentrates were also taken weekly. The weekly samples were

pooled for 6-wk periods for chemical analysis, giving 2 pooled samples per feed for the whole experiment.

After drying (60°C for 48 h), the feed samples were milled using a hammer mill through a 1-mm screen before chemical analysis. Pooled samples were analyzed for DM by drying at 60°C for 48 h and for ash by combustion at 525°C for 6 h (AOAC International, 2000). Crude protein was calculated as $6.25 \times$ total N measured by the Dumas principle, as described by Hansen (1989), using a Vario MAX CN (Elementar Analysensysteme GmbH, Hanau, Germany). Crude fat was measured by Soxhlet extraction with petroleum

Table 3. Ration ingredients and chemical composition of treatments

Item	Treatment ¹			
	HdHp	HdLp	LdHp	LdLp
Ingredient, g/kg of DM unless noted				
Hd Grass-clover silage	240	240	0	0
Ld Grass-clover silage	0	0	241	234
Hd Corn silage	303	303	0	0
Ld Corn silage	0	0	304	322
Barley	95	123	95	120
Beet pulp dried	113	140	113	137
Rapeseed meal	87	52	87	51
Soybean meal	19	0	19	0
Urea, 80%	2.6	2.6	0	0
Vitamin and mineral mix ²	15	16	15	15
Concentrate (automatic milking system)	126	124	126	121
Composition				
DM, g/kg of fresh matter	604	604	583	574
OM	927	927	928	929
CP	157	139	160	140
Crude fat	29.3	28.0	25.5	24.0
FA				
C16	3.80	3.80	3.58	3.56
Total C18	16.15	14.94	13.51	12.16
NDF	317	320	393	399
Starch	136	151	91	107
AAT ₂₀ , ³ g/kg of DM	86.1	78.2	81.1	74.8
PBV ₂₀ , ⁴ g/kg of DM	23	13	15	2
NE _L , ⁵ MJ/kg of DM	7.7	7.6	6.9	6.8
OMD, ⁶ %	79.8	79.8	74.7	74.7

¹High digestibility and high protein concentration (HdHp), high digestibility and low protein concentration (HdLp), low digestibility and high protein concentration (LdHp), and low digestibility and low protein concentration (LdLp).

²Including monocalcium phosphate and sodium chloride.

³Predicted AA absorbed in the small intestine at 20 kg of DMI (Volden, 2011).

⁴Protein balance in the rumen at 20 kg of DMI (Volden, 2011).

⁵NE_L is calculated according to Weisbjerg and Hvelplund (1993).

⁶OMD = estimated in vivo OM digestibility; calculated from measured in vitro digestibility according to Åkerlind et al. (2011).

ether (Soxtec 2050, Foss Analytical, Hillerød, Denmark) after hydrolysis with HCl (Stoldt, 1952). Ash-free NDF was measured using a Fibertec M6 System (Foss Analytical) using heat-stable α -amylase and sodium sulfite as described by Mertens (2002). Total sugar in grass silage was analyzed by the Luff-Schoorl method (71/250/EEC; Schoorl, 1929) and starch was measured by an enzymatic colorimetric technique (Knudsen et al., 1987).

The concentration of VFA in forage samples was analyzed according to the method described by Canibe et al. (2007) using a Hewlett Packard GC (model 6890) equipped with a flame-ionization detector and a 30-m SGE BP1 column (Scientific Instrument Services, Ringoes, NJ). The concentration of ethanol was analyzed as described by Beutler (1984). For determination of NH_3 , the feed was alkalized with KOH, and NH_3 was determined by titration after distillation using a Kjeltac 2400 (Foss Analytical). The FA content of individual feed items was analyzed as described by Larsen et al. (2013).

In vitro digestibility of OM in forages was determined by anaerobic incubation with diluted rumen fluid for 48 h, followed by incubation of undissolved material with pepsin HCl-solution for 48 h (Tilley and Terry, 1963). In vitro digestibility of OM of concentrates was determined by treating samples with pepsin-HCl solution followed by incubation with cellulytic enzymes (Weisbjerg and Hvelplund, 1993).

Feed Intake. Individual intakes of mixed ration and concentrate fed in the AMS were calculated daily. The total daily concentrate allowance (maximum 3 kg) allocated at each visit in the AMS was proportional to the length of time since the last milking for the individual cow. Concentrate was offered at a rate of 400 g/min, and cows were allowed up to 50% of the daily concentrate ration per visit. It was possible for the cows to carry over 50% of daily concentrate allowance to the next day. The individual concentrate intake was calculated as the amount of concentrate offered corrected for recorded individual refusals.

Milk Yield and Quality. Milk yield was measured at every visit in the AMS using the DeLaval Free Flow meter MM25 (DeLaval AB) based on optical milk flow measurement. Every week, representative milk samples were taken at each milking during a 48-h period. Samples were analyzed for protein, fat, and lactose contents on a Milkoscan 4000 infrared analyzer at Eurofins Steins (Holstebro, Denmark). The analyzed milk samples and the corresponding milk yield recordings were used to calculate average concentrations of protein, fat, and lactose. Lactose was expressed as lactose monohydrate, with the energy factor as given by Sjaunja et al. (1991).

Milk urea was analyzed using flow injection analysis (Nielsen et al., 2005) using a FIAstar 5000 Ana-

lyzer (Foss Tecator AB, Höganäs, Sweden). Urease (EC 3.5.1.5, Toyobo Enzymes Ltd., Osaka, Japan) was added to the diluted milk sample. After hydrolysis of urea, a strong alkali solution was added and the developed ammonia was dialyzed through a membrane. pH changes in the passing aqueous phase were followed via a pH indicator by spectrophotometer. User instructions given by the manufacturer were followed (Foss Tecator AB).

For analysis of milk FA, proportionate amounts of the milk samples taken during a 48-h period (see above) in the last week of each experimental period were pooled to obtain 1 milk sample per cow. Composite samples were frozen and stored at -18°C until analysis. The FA composition of the milk fat was analyzed as described by Larsen et al. (2013), except that heptane was used as solvent instead of pentane.

Blood Samples. Blood samples were collected on the last day of each feeding period. Six animals on each ration were randomly selected and samplings on these animals were repeated every feeding period. Blood samples were taken by vein puncture from the jugular vein (JV) and milk vein (MV) between 1030 and 1430 h and were collected in EDTA tubes, after which plasma was separated by centrifugation at $2,000 \times g$ for 15 min at 4°C and stored at -20°C until analyzed. Blood samples were analyzed for cholesterol, triglyceride, urea, serum γ -glutamyltransferase (GGT), butyrate, NEFA, lactate, and glucose as described by Khanal et al. (2014). The concentration in the JV was used as a measure of systemic arterial concentrations because differences in plasma concentrations for the metabolites studied in JV and arterial vessels are insignificant, and the arterio-venous difference was estimated as the difference between the JV and MV concentrations (Nielsen et al., 2001).

BW and BCS. Cows were automatically weighed at each visit in the AMS, and data were treated according to Bossen et al. (2009). Furthermore, all cows were scored for BCS once in each period, based on the visual method described by Ferguson et al. (1994). This method operates with 0.25-point intervals on the scale from 1 to 5.

Calculations and Statistical Analysis

Net energy content of feedstuffs was measured as Scandinavian Feed Units (SFU) and converted to NE_L (MJ/kg of DM) using the fixed conversion coefficient 7.89 MJ of NE_L /SFU (Weisbjerg and Hvelplund, 1993; Bossen et al., 2009).

Energy-corrected milk (3.140 MJ/kg) was calculated as described by Sjaunja et al. (1991) using the following equation: ECM yield (kg) = milk yield (kg) $\times$ [(38.3 $\times$

fat (g/kg) + 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7)/3,140]. Energy efficiency was calculated as ECM (kg/d)/NE_L (MJ/d), and N efficiency (N_{milk}/N_{intake}) as [(milk protein (kg/d)/6.38)/N intake (kg/d)].

Experimental data were analyzed with the MIXED procedure of SAS software (version 9.2, 2010; SAS Institute Inc., Cary, NC), where model parameters included fixed effect of period (1–4), fixed effect of lactation number (1; 1–4), fixed effect of digestibility (d; Hd, Ld), fixed effect of CP concentration (p; Hp, Lp), interaction between digestibility and CP concentration, interaction between lactation number and digestibility, and interaction between lactation number and CP concentration; cow (1–48) was treated as a random variable. Residuals were assumed normally distributed with mean value 0 and constant variance $\varepsilon \sim N(0, \sigma^2)$. For analysis on milk yield and composition, AMS visit frequency, energy and protein efficiency, and concentrations in jugular vein and jugular–milk vein (JMV) difference across the mammary gland, the following parameters were excluded: interaction between lactation number and digestibility and interaction between lactation number and CP concentration, due to lack of significance ($P > 0.10$). For milk yield and composition, AMS visit frequency, and energy and protein efficiency significance levels were $P > 0.12$ for $d \times l$ and $P > 0.11$ for $p \times l$, and for concentrations in the jugular vein and JMV difference across the mammary gland, P -values were > 0.17 for $d \times l$ and $P > 0.38$ for $p \times l$.

To test if a given cow's milk production level affected its sensitivity to the protein supply and digestibility, the mean milk yield per cow was calculated. The difference in ECM between Hp and Lp was calculated for each cow: $(\text{ECM}_{\text{HdHp}} + \text{ECM}_{\text{LdHp}} / 2) - (\text{ECM}_{\text{HdLp}} + \text{ECM}_{\text{LdLp}} / 2)$; and the difference in ECM between Hd and Ld was calculated for each cow: $(\text{ECM}_{\text{HdHp}} + \text{ECM}_{\text{HdLp}} / 2) - (\text{ECM}_{\text{LdHp}} + \text{ECM}_{\text{LdLp}} / 2)$. The difference values for protein and digestibility were analyzed in PROC GLM as a simple regression on mean milk yield of the cow.

Results reported in tables are, if not otherwise stated, treatment least squares means (LSM) and standard errors of mean (SEM). Multiple comparisons of treatment means were performed using PDIF in SAS, if $P \leq 0.05$ for the interaction between digestibility and CP concentration; P -values ≤ 0.05 were regarded as significant, $P \leq 0.10$ as tendencies.

RESULTS

Feed Intake

The chemical composition of feedstuffs is shown in Table 1 and silage quality in Table 2. Ration ingredients and chemical compositions are shown in Table 3.

Total feed intake (DM and NE_L) was affected by treatment, with an interaction between digestibility and CP ($P < 0.05$), where the effect of dietary protein concentration was smaller at low digestibility than at high digestibility (Table 4). When digestibility decreased, total DMI also decreased, from 24.4 to 22.1 kg of DM/d ($P < 0.001$, SEM 0.37). Likewise, when the CP concentration decreased, total DMI decreased, from 23.6 to 22.8 kg DM/d ($P < 0.001$, SEM 0.37). The combination of low digestibility and low CP concentration thus resulted in the lowest total DMI of 21.9 kg/d (SEM 0.38). Low forage digestibility increased concentrate intake in the AMS from 2.2 to 2.4 kg of DM/d ($P < 0.005$, SEM 0.05; Table 4). Dietary CP content did not affect concentrate intake. The response in DMI to increased digestibility in the ration was greater for older cows than for first-lactation cows ($P = 0.003$), as the increase in DMI from Ld to Hd was 1.5 kg (SEM 0.48), 2.8 kg (SEM 0.62), 2.5 kg (SEM 0.62), and 2.5 kg (SEM 1.1) of DMI/d for first-, second-, third-, and fourth-lactation cows, respectively (data not shown).

Milk Yield and Quality, BW, and BCS

Data on milk yield and composition are given in Table 5. Daily ECM yield increased from 32.4 kg/d on Ld to 34.1 kg/d on the Hd ration ($P < 0.001$, SEM 0.87) and from 32.7 kg/d on Lp to 33.8 kg/d on the Hp ration ($P = 0.003$, SEM 0.87). Increased lactation number increased milk yield ($P = 0.002$), with ECM yields of 27.8 (SEM 1.12), 32.3 (SEM 1.45), 35.6 (SEM 1.45), and 37.4 (SEM 2.51) kg/d for first-, second-, third-, and fourth-lactation cows, respectively (data not shown). The milk fat percentage decreased with increased forage digestibility from 4.26 to 4.12% ($P < 0.001$, SEM 0.08), whereas the milk protein percentage increased with increased forage digestibility from 3.56 to 3.62% ($P < 0.001$, SEM 0.04). The urea content in milk was higher when the high CP concentration rations were fed (3.32 vs. 2.54 mM urea; $P < 0.001$, SEM 0.04). Concentrations of individual FA in milk were affected by forage digestibility, where increased digestibility resulted in higher concentrations of medium-chain (C10–C14) FA (23.1 vs. 22.4 g/100 g of FA; $P < 0.001$, SEM 0.19), odd-chain (C11–C17) FA (2.9 vs. 2.7 g/100 g of FA; $P < 0.001$, SEM 0.04), and C18 FA (27.3 vs. 26.3 g/100 g of FA; $P = 0.005$, SEM 0.33). Additionally, Hd resulted in lower concentrations of short-chain (C4–C8) FA (9.9 vs. 10.3 g/100 g of FA; $P = 0.002$, SEM 0.11) and C16 FA (35.4 vs. 36.9 g/100 g of FA; $P < 0.001$, SEM 0.35) compared with Ld. A higher dietary CP concentration decreased C16 FA concentrations from 36.9 to 35.4 g/100 g of FA ($P <$

Table 4. Feed intake of mixed ration, concentrate, and total ration

Item	Treatment ¹					P-value ²					
	HdHp	HdLp	LdHp	LdLp	SEM	d	p	l	d × p	d × l	p × l
Mixed ration DM, kg/d	22.7	21.6	20.1	19.5	0.38	<0.001	<0.001	<0.001	0.06	0.009	0.43
Concentrate DM, kg/d	2.2	2.2	2.3	2.4	0.06	0.005	0.10	0.88	0.51	0.30	0.02
Total DM, kg/d	24.9 ^a	23.8 ^b	22.4 ^c	21.9 ^d	0.38	<0.001	<0.001	<0.001	0.04	0.003	0.37
Total NE _L , MJ/kg of DM	191.7 ^a	181.5 ^b	155.5 ^c	150.5 ^d	2.74	<0.001	<0.001	<0.001	0.02	<0.001	0.32
Total CP, kg/d	4.0 ^a	3.4 ^b	3.5 ^c	3.1 ^d	0.06	<0.001	<0.001	<0.001	0.004	0.005	0.14
Total AAT ₂₀ , ³ kg/d	2.4 ^a	2.2 ^b	2.0 ^c	1.9 ^d	0.03	<0.001	<0.001	<0.001	0.009	0.003	0.31

^{a-d}Means within a row with different superscripts are significantly different ($P < 0.05$).

¹High digestibility and high CP concentration (HdHp), high digestibility and low CP concentration (HdLp), low digestibility and high CP concentration (LdHp), and low digestibility and low CP concentration (LdLp).

²Lactation number (l), digestibility (d), and crude protein (p).

³Predicted AA absorbed in the small intestine at 20 kg of DMI (Volden, 2011).

0.001, SEM 0.35) and increased C18 FA from 26.1 to 27.4 g/100 g of FA ($P < 0.001$, SEM 0.33).

Lower forage digestibility resulted in a greater frequency of visits to the AMS (2.33 vs. 2.25 visits/d; $P = 0.05$, SEM 0.08). Energy efficiency was higher for cows fed forage with a low digestibility (0.22 kg of ECM/MJ of NE_L) compared with cows fed forage with a high digestibility (0.18 kg of ECM/MJ of NE_L; $P < 0.001$, SEM 0.005) and energy efficiency was not affected by the CP concentration. Nitrogen efficiency decreased with higher CP concentrations (0.31 vs. 0.35 kg of N_{milk}/N_{intake}; $P < 0.001$, SEM 0.008).

Average BW changed from 643 to 667 kg throughout the experiment (84 d), resulting in an average daily gain of 278 g; at the same time the BCS changed from 2.9 to 3.0.

Blood Parameters

Data on concentrations in the JV and the JMV difference across the mammary gland are given in Table 6. Increased forage digestibility was associated with increased plasma levels of cholesterol from 5.24 to 5.46 mmol/L ($P = 0.005$, SEM 0.23) and a decrease from

Table 5. Milk yield and composition, automatic milking system (AMS) visit frequency, and energy and protein efficiency¹

Item	Treatment ²					P-value ³			
	HdHp	HdLp	LdHp	LdLp	SEM	d	p	l	d × p
Milk, kg/d	34.1	32.8	32.0	30.9	1.01	<0.001	<0.001	0.002	0.69
ECM, kg/d	34.8	33.5	32.9	31.9	0.90	<0.001	0.003	0.002	0.78
Milk fat, %	4.10	4.13	4.24	4.29	0.08	<0.001	0.10	0.81	0.66
Milk protein, %	3.63	3.62	3.57	3.55	0.05	<0.001	0.09	0.04	0.75
Lactose, ⁴ %	4.85	4.86	4.81	4.80	0.02	<0.001	0.91	0.001	0.11
Urea, mM	3.22	2.45	3.42	2.63	0.05	<0.001	<0.001	0.27	0.76
Protein:fat	0.89	0.88	0.85	0.83	0.01	<0.001	0.01	0.29	0.67
Fatty acids, g/100 g of FA									
Short chain: C4 + C6 + C8	9.8	10.0	10.2	10.4	0.13	0.002	0.06	0.11	0.96
Medium chain: C10 + C12 + C14 + C14:1	23.3	23.0	22.5	22.3	0.21	<0.001	0.06	0.66	0.78
Odd chain: C11 + C13 + C15 + C17 + C17:1	2.9	2.9	2.8	2.7	0.04	<0.001	0.76	0.03	0.07
C16 FA: C16 + C16:1	34.9	36.0	36.1	37.7	0.38	<0.001	<0.001	0.65	0.23
C18 FA: C18 + C18:1 + C18:2 + C18:3	27.8	26.7	27.1	25.6	0.38	0.005	<0.001	0.43	0.47
Visits to AMS (d ⁻¹)	2.27	2.23	2.32	2.34	0.08	0.047	0.85	0.98	0.47
ECM, kg/kg of DMI	1.40	1.42	1.52	1.47	0.04	<0.001	0.41	0.49	0.15
Energy efficiency, kg of ECM/MJ of NE _L	0.18	0.18	0.22	0.21	0.005	<0.001	0.97	0.45	0.18
N efficiency ⁵	0.30	0.35	0.32	0.35	0.01	0.004	<0.001	0.83	0.07

¹Interaction between lactation number and digestibility and between lactation number and CP concentration were excluded from the model due to lack of significance ($P > 0.11$). One exception was C18 FA, where $P = 0.04$ for $l \times d$.

²High digestibility and high CP concentration (HdHp), high digestibility and low CP concentration (HdLp), low digestibility and high CP concentration (LdHp), and low digestibility and low CP concentration (LdLp).

³Lactation number (l), digestibility (d), and crude protein (p).

⁴Lactose is expressed as lactose monohydrate, with the energy factor as given by Sjaunja et al. (1991).

⁵Calculated as $N_{milk}/N_{intake} = [\text{milk protein (kg/d)}/6.38]/[\text{N intake (kg/d)}]$.

3.69 to 3.12 mmol/L ($P = 0.005$, SEM 0.18) in plasma levels of urea in the JV. An increased dietary CP concentration in the ration decreased JV plasma levels of cholesterol from 5.45 to 5.25 mmol/L ($P = 0.009$, SEM 0.22) and increased JV plasma levels of urea from 2.91 to 3.91 mmol/L ($P < 0.001$, SEM 0.18), respectively. The higher CP concentration also decreased plasma levels of triglyceride from 0.22 to 0.20 mmol/L ($P = 0.04$, SEM 0.006) and GGT from 40.5 to 37.8 mmol/L ($P = 0.04$, SEM 2.74), and tended to increase NEFA from 0.31 to 0.42 mmol/L ($P = 0.08$, SEM 0.05).

We observed no effects of forage digestibility or CP concentration in the ration on JMV differences for the different metabolites (see Table 6), except for glucose ($P = 0.05$, SEM 0.08), where JMV differences were lower on rations with high digestibility (0.56 mmol/L) compared with rations with low digestibility (0.73 mmol/L). The JMV differences for triglyceride tended to be lower ($P = 0.09$, SEM 0.005) when digestibility was increased (0.036 vs. 0.046 mmol/L).

Lactation number affected the concentration in the jugular vein only for triglyceride ($P = 0.05$), with higher concentrations in first-lactation cows (0.24 mmol/L, SEM 0.01) compared with second- (0.20 mmol/L, SEM 0.006) and third- (0.19 mmol/L, SEM 0.007) lactation cows. The JMV differences for glucose were higher ($P = 0.005$) for first-lactation cows (0.98 mmol/L, SEM 0.18) than for second- (0.40 mmol/L, SEM 0.07) and third- (0.56 mmol/L, SEM 0.08) lactation cows, and this was also the case for triglyceride ($P = 0.02$), with

JMV differences in first-lactation cows of 0.068 mmol/L (SEM 0.01) compared with 0.023 mmol/L (SEM 0.005) in second-lactation cows and 0.031 mmol/L (SEM 0.005) in third-lactation cows.

DISCUSSION

Rations and Intake

We expected that low CP concentration as well as low forage digestibility would reduce the DMI. Protein requirement is determined by the cow's requirement for AA for protein synthesis in different tissues and the requirement for RDP to sustain microbial protein synthesis in the rumen. The DMI for lactating cows is often positively related to the protein concentration in the ration (Oldham, 1984; Broderick, 2003; Habib et al., 2006; Weisbjerg et al., 2010). In the present study, DMI increased by 0.8 kg ($P < 0.001$, SEM 0.37) when the CP concentration was increased from approximately 14.0 to 15.9%, due to an increase in intake of mixed ration.

It has been suggested that RDP fed under the level needed to sustain ruminal microorganisms may negatively affect intake due to depression of ruminal digestion (Olmos Colmenero and Broderick, 2006; Lee et al., 2012). Increased dietary CP content increases fiber digestibility and thereby DM digestibility of the feed, partly due to increased RDP effects on the digestibility of feedstuffs (Oldham, 1984; Allen, 2000),

Table 6. Concentrations in jugular vein and jugular–milk venous (JMV) difference across the mammary gland¹

Item	Treatment ²					P-value ³			
	HdHp	HdLp	LdHp	LdLp	SEM	d	p	l	d × p
Concentrations in jugular vein									
Glucose, mmol/L	3.79	3.76	3.77	3.68	0.09	0.47	0.36	0.12	0.71
Triglyceride, mmol/L	0.21	0.21	0.20	0.22	0.01	0.36	0.04	0.05	0.32
NEFA, mmol/L	0.35	0.30	0.48	0.33	0.07	0.20	0.08	0.21	0.38
Butyrate, mmol/L	0.63	0.68	0.65	0.72	0.05	0.43	0.12	0.64	0.74
Lactate, mmol/L	0.50	0.48	0.44	0.54	0.11	0.94	0.64	0.63	0.49
Cholesterol, mmol/L	5.35	5.56	5.14	5.34	0.23	0.005	0.009	0.75	0.96
Blood urea-N, mmol/L	3.71	2.54	4.11	3.27	0.21	0.005	<0.001	0.75	0.29
γ-Glutamyl transferase, U/L	38.42	41.75	37.25	39.33	2.89	0.17	0.04	0.30	0.63
JMV difference across the mammary gland									
Glucose, mmol/L	0.63	0.48	0.70	0.76	0.10	0.05	0.61	0.02	0.21
Triglyceride, mmol/L	0.03	0.04	0.04	0.05	0.01	0.09	0.14	0.02	0.89
NEFA, mmol/L	0.09	0.08	0.11	0.09	0.07	0.78	0.76	0.14	0.89
Butyrate, mmol/L	0.14	0.14	0.18	0.18	0.03	0.14	0.87	0.63	0.90
Lactate, mmol/L	0.23	0.12	0.12	0.22	0.10	0.96	0.99	0.83	0.25
Cholesterol, mmol/L	0.18	0.11	0.13	0.13	0.06	0.76	0.49	0.37	0.40
Blood urea-N, mmol/L	0.001	−0.056	−0.056	0.223	0.15	0.38	0.39	0.70	0.19

¹Interaction between lactation number and digestibility and between lactation number and CP concentration were excluded from the model, due to lack of significance ($P > 0.17$). One exception was JMV difference of cholesterol where $P = 0.04$ for $p \times l$.

²High digestibility and high CP concentration (HdHp), high digestibility and low CP concentration (HdLp), low digestibility and high CP concentration (LdHp), and low digestibility and low CP concentration (LdLp).

³Lactation number (l), digestibility (d), and crude protein (p).

although the marginal response diminishes if the CP concentration increases and is expected to be minimal when the CP concentration exceeds 15% of DM. The marginal increase may still be positive when the CP content exceeds 20% (Oldham, 1984), probably due to the replacement of starch with CP, as starch may have a negative effect on the rumen. Increased ruminal digestibility of the feed is assumed to be responsible for increased DMI up to approximately 15% dietary CP. Roffler et al. (1986) evaluated different studies to predict responses in DMI to increased content of dietary CP. They found an exponential decline in the increase of DMI per percentage unit increase of CP. At 18% CP they predicted that the increase in DMI would more or less disappear. A possible reason for the increase in DMI at high dietary CP concentration could be a reduction in rumen propionate production as protein is substituted for starch in the ration (Allen, 2000). When protein is oversupplied it may represent a loss of urea energy to the urine, plus the transformation of excess protein into urea is energy-demanding.

Hristov et al. (2002) found that DMI was strongly related to intake of total digestible nutrients or intake of NE_L (metabolizable energy). In the present study, DMI was reduced by 0.7 kg for Ld compared with Hd rations ($P < 0.001$, SEM 0.37). This could also be due to a higher fill value, defined by NDF, in the Ld ration (396 g of NDF/kg of DM) compared with Hd (319 g of NDF/kg of DM). Negative effects on DMI of low grass digestibility (mature grass) have been reported in previous studies (Kuoppala et al., 2008, 2010).

Weisbjerg et al. (2010) compared recent studies where forages with high digestibility were used with earlier studies where forages with lower digestibility were used, and concluded that cows eating rations containing forages with high digestibility were less sensitive to low protein concentration than cows eating rations containing forages with low digestibility. This gave rise to the hypothesis that the moderate effect of protein supply on DMI was caused by a progression in forage digestibility and quality. The more pronounced effect of protein supplementation on high- than on low-digestibility rations on DMI is in contrast to the proposed hypothesis. In the present study, low dietary CP content had a less negative effect on DMI when forage digestibility was low (-0.5 kg of DM/d) than when forage digestibility was high (-1.1 kg of DM/d), which was the reverse of the hypothesis. It seems that energy supply from the lower digestible forage was more limiting for DMI than for protein (N). This was probably because the high microbial synthesis of the high-digestible silage increased the requirement for RDP. Furthermore, the RDP supply on LdLp, although low, was probably not limiting for rumen digestion. Protein balance in the rumen at 20

kg of DMI (PBV_{20}) ranged from 13 to 23 g/kg of DM for Hd and 2 to 15 g/kg of DM for Ld. The differences in PBV_{20} values between Hd and Ld were mainly due to supplementation of urea to the Hd rations.

Milk Yield and Quality

The frequency of visits to the AMS was higher when the cows were fed rations with low forage digestibility (2.33 visits/d) compared with high forage digestibility (2.25 visits/d; $P = 0.05$, SEM 0.08), probably because a less-digestible mixed ration makes the concentrate in the AMS more attractive. However, the difference was numerically small and probably had only a minor effect on milk yield and composition.

Olmos Colmenero and Broderick (2006) found that increased dietary protein concentration can stimulate milk yield without affecting DMI, probably due to an increased digestibility, because they found a quadratic effect ($P < 0.01$) of the CP concentration on OMD. Weisbjerg et al. (2012) observed a more pronounced increase in milk production than in DMI when increasing the protein concentration in the diet. They found that the response in milk yield was higher for high-yielding cows than for lower-yielding cows. In the present study, sensitivity to CP supply was not affected significantly by the cows' level of milk production, as the difference in milk production between Lp and Hp increased by only 0.017 kg of ECM per 1-kg increase in cow milk production level ($P = 0.8$). The difference between Ld and Hd increased with 0.25 kg of ECM per 1-kg increase in cow milk production level ($P = 0.001$). The increased sensitivity to digestibility should probably be seen in relation to a higher DMI on Hd than Ld. We detected no interaction between CP concentration and lactation number or digestibility and lactation number. Normally, the response to increased nutrient supply is higher in older cows (Smith et al., 1978; Bossen and Weisbjerg, 2009).

Composition of milk was affected by both forage digestibility and CP concentration in the ration, such that the milk fat percentage was higher on the low-digestibility ration (4.26%) compared with the high-digestibility ration (4.12%; $P < 0.001$, SEM 0.08). Veerkamp et al. (1994) also found this difference in milk fat percentage between low (4.44%) and high energy rations (4.11%), which can be explained by a dilution in volume when yield is increased. The dietary protein level has little effect on milk fat concentration within the normal range of supplementation (Veerkamp et al., 1994), and in our study there was only a tendency for low dietary CP concentration to increase milk fat, from 4.17 to 4.21% ($P < 0.1$, SEM 0.08). Moreover, changes in protein supply within the normal range seldom affect

milk protein concentration appreciably (Sutton, 1989), which agrees with the current study where Hp tended to have higher milk protein concentration (3.60%) than Lp (3.58%; $P = 0.09$, SEM 0.04). By supplementation of AA to the ration, it is possible to increase the protein concentration in milk, as shown by Robinson (2010), however, depending on the CP level in the control ration. Milk protein concentration was lower on the Ld rations (3.56%) than on the Hd rations (3.62%; $P < 0.001$, SEM 0.04), which is in agreement with Rinne et al. (1999), who reported that milk protein concentrations increase when high-digestibility silages are fed and that this is coupled to an overall improved energy status and increased intestinal supply of AA to the cow. The urea concentration increased as expected with an increased concentration of CP in the rations (from 2.54 to 3.32 mM; $P < 0.001$, SEM 0.04), as also shown by Groff and Wu (2005).

The observed effects of treatments on milk FA composition were generally minor (relative changes of 3–5%) compared with other feeding experiments, especially those where different fat supplements have been investigated (Larsen et al., 2013). The C18 FA content of the Hd rations was higher than on Ld rations (15.5 vs. 12.8 g/kg of DM) and this resulted in higher content of C18 FA (27.3 vs. 26.3 g/100 g of FA; $P = 0.005$, SEM 0.33) and lower content of C16 FA (35.4 vs. 36.9 g/100 g of FA; $P < 0.001$, SEM 0.35) in milk from cows on Hd rations. The differences in short- and medium-chain FA in milk from cows on Hd and Ld rations showed that a higher proportion of these were short-chain FA when the concentration of C18 FA in milk fat was higher. The differences in FA composition in milk from cows on Hp and Lp rations were limited to a higher content of C18 FA in milk from cows on Hp compared with cows on Lp (27.4 vs. 26.1 g/100 g of FA; $P < 0.001$, SEM 0.33) and a lower content of C16 FA in milk from cows on Hp compared with cows on Lp (35.5 vs. 36.9 g/100 g of FA; $P < 0.001$, SEM 0.35), which was a reflection of the difference in C18 FA content of the Hp and Lp rations (14.83 vs. 13.55 g/kg of DM).

Based on mean values, the balance between FA intake from feed and FA secretion in milk was calculated and the intake of C16 corresponded to 14 to 17% of the output of C16 FA in milk; thus, at least 83% of the C16 in milk was derived from de novo synthesis. For C18 FA, the intake from feed relative to the output in milk corresponded to 109 and 104% for the HdHp and HdLp treatments, respectively, whereas it was 87 and 86% for the LdHp and LdLp treatments. These results show that the fat supply from feed was low and the de novo synthesis was high, and for the Ld treatments part of the C18 FA in milk did not derive directly from the feed. The other main source of C18 FA is from the body

fat turnover and as cows did not lose weight or score lower for body condition, a net body synthesis of C18 FA had occurred.

Nutrient Provision to and Extraction by the Mammary Gland

The plasma level of urea increased for cows fed the Ld (3.69 mmol/L) compared with the Hd (3.12 mmol/L) ration ($P = 0.005$, SEM 0.18), and this was also reflected in milk urea levels. Plasma urea levels are generally believed to reflect hepatic urea synthesis, which, in turn, is coupled to hepatic AA oxidation and absorption of ammonia from forestomach fermentation. It appears unlikely that microbial protein or ammonia synthesis should have been increased due to decreased ration digestibility, where both fermentable OM and dietary urea supply were limited compared with the high-digestibility rations. Therefore, one explanation for the higher plasma levels of urea on Ld rations could be that digestibility was more depressed than supply of N, and therefore less N would be captured in the rumen, leading to a possible increase in plasma urea. Another explanation could be increased hepatic AA oxidation, although it is puzzling that this appears to have occurred despite a decreased surplus of AA [estimated as intake of AA absorbed in the small intestine at 20 kg of DMI (AAT_{20}) minus milk protein output] on Ld compared with Hd rations.

We cannot explain the dietary effects on plasma cholesterol. The higher plasma cholesterol on the Lp rations (5.45 mmol/L) compared with the Hp rations (5.25 mmol/L; $P = 0.009$, SEM 0.22) was associated with higher plasma levels of GGT (40.5 vs. 37.8 mmol/L; $P = 0.04$, SEM 2.74) and triglyceride (0.22 vs. 0.20 mmol/L; $P = 0.04$, SEM 0.006), which could not be a result of increased fat absorption because the dietary fat content was lower on the Lp ration (13.6 vs. 14.8 g of C18/kg of DM). Nor is it likely to be explained by increased hepatic lipoprotein synthesis because GGT (a marker of hepatic function) was increased rather than decreased. The depression in cholesterol on Hp rations is interesting, as cholesterol plays an important role in the synthesis of membranes. It has been suggested that uptake of cholesterol in the mammary gland may be a limiting factor for the formation of cellular membranes and hence for overall milk secretion (Nielsen and Jakobsen, 1994).

Plasma concentrations in arterial blood of the major organic metabolites utilized for milk synthesis in the mammary gland closely resembled plasma concentrations found in the JV, as the quantitative utilization of these nutrients in the brain is negligible (Nielsen et al., 2001). Thus, JMV concentration differences for

the metabolites shown in Table 6 will be equivalent to arterio-venous differences across the mammary gland. The only effect of dietary changes on the uptake of nutrients from plasma in the mammary gland was found for glucose, where both JMV difference and extraction were increased ($P = 0.05$), and JMV also tended to be increased for triglyceride ($P = 0.09$) when forage digestibility was decreased. However, because milk production was lowest on the Ld rations, the quantitative mammary glucose uptake must also have been lower on the Ld diets. According to Madsen et al. (2005), increased efficiency in mammary glucose and triglyceride uptake on the Ld rations could be explained by a compensatory response to a depression of mammary blood flow caused by a decreased mammary AA supply when on a ration with a lower protein supply and N content. This is, however, unlikely because JMV concentration differences for glucose were independent of changes in dietary CP concentration, and other studies have shown that mammary glucose uptake is quite independent of arterial glucose supply during the mid-to-late stages of lactation in ruminants (Nielsen et al., 2001). Acetate is the major nutrient generating the physiological energy (ATP) to sustain milk synthetic processes in the mammary gland, and increased supply of acetate to the mammary gland could sustain milk protein synthesis and compensate for insufficient dietary provision of energy and essential AA (Safayi and Nielsen, 2013). Higher mammary glucose extraction on the Ld rations may be linked to a lowered mammary supply and uptake of acetate, because the microbial fermentation in the rumen would be expected to be lower on Ld rations.

CONCLUSIONS

Lower concentrations of dietary CP and forage digestibility reduced both DMI and milk production. We detected no interactive effect on milk production of CP concentration and forage digestibility, indicating that the reduction in milk production with a lower CP concentration could not be compensated by increased forage digestibility.

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