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Impact of nutrient restriction at dry-off on performance and metabolism

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ABSTRACT

Thanks to improvements in genetics, nutrition, and management, modern dairy cows can still produce large amounts of milk at the end of lactation, with possible negative effects on health and welfare, particularly when milking is stopped abruptly. To limit yield at dry-off, strategies involving different types of dietary restriction have been used worldwide. Thus, we aimed to investigate the effects of a reduced nutrient density at dry-off on milk production, metabolism, the pattern of rumen fermentation, and milk fatty acid profile around dry-off and in the ensuing periparturient period. During the last week before dry-off, 26 Holstein cows were enrolled in pairs according to the expected calving date and either fed ad libitum ryegrass hay (nutrient restricted, NR; 13 cows) or continued to receive lactation diet (control group, CTR; 13 cows). After dry-off, both groups received only grass hay for 7 d, and free access to water was always provided. Blood, milk, and rumen fluid samples were collected from 7 d before dry-off to 28 DIM. Milk production, DMI (during the periparturient period), and rumination times were recorded daily. At dry-off, NR cows had decreased milk yield (−62%) and milk lactose compared with CTR but had higher fat and protein contents. In the subsequent lactation, no significant differences were observed in milk yield and composition. The BCS did not differ between groups during the transition period, but it decreased in NR after dry-off. Before dry-off, NR had decreased glucose, urea, and insulin, but higher creatinine, BHB, and nonesterified fatty acids (NEFA). The day after dry-off, NEFA were lower in NR, but they were higher 7 d after calving. At dry-off, NR had higher rumen pH, lower lactate, urea, and total volatile fatty acid concentrations. Considering volatile fatty acid molar proportions, NR had increased acetate but decreased propionate and butyrate at dry-off. Rumination time dropped 6 d before dry-off in NR and after dry-off in CTR, but no differences were observed in the periparturient period. Milk fatty acid profile revealed a remarkably lower proportion of short-chain fatty acids in NR at dry-off and a higher proportion of medium- and long-chain ones. These results confirmed that decreasing nutrient density reduce milk yield before dry-off. However, metabolism around dry-off was significantly affected, as suggested by plasma, rumen fluid, and milk analyses. Further research is required to investigate the impact of the metabolic effects on the inflammatory response, liver function, and immune system, particularly concerning the mammary gland.

Key words: mammary involution, stress, milk cessation, transition period

INTRODUCTION

The dry-off process represents a critical phase of the lactation cycle in dairy cattle, with the transition to a nonlactating period. At this time, several changes in the cow's routine, physiology, and diet happen, and cows need to adapt to them. Among those, the cessation of milking promotes the beginning of the mammary involution process (Zhao et al., 2019), which allows the regeneration of the secretory tissue. The diet is changed to more fibrous rations to which the rumen papillae need to adapt (Dieho et al., 2016). Furthermore, cows are often moved to a different pen, requiring the establishment of a new social hierarchy (von Keyserlingk et al., 2008). Together, these factors can develop a potentially stressful situation for the animal (Zobel et al., 2015). The latter is suggested by the decrease in rumination time (Abuelo et al., 2021), the increase in blood concentrations of nonesterified fatty acids (**NEFA**), haptoglobin, oxygen metabolites, and cortisol occurring after dry-off (Putman et al., 2018; Mezzetti et al., 2020; Cattaneo et al., 2021b). These changes can be observed also at the gene expression level (Cattaneo et al., 2021a), with the upregulation

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

of the genes coding for the pro-inflammatory cytokines IL-8 and IL-18 in circulating leukocytes.

In addition, due to progress in management, nutrition, and genetics, modern cows have increased persistency of lactation, often resulting in relatively high milk yield in late gestation (Cattaneo et al., 2023). Cows dried off with production over 15 kg/d can have increased risk of developing IMI during the dry period, potentially affecting the ensuing lactation (Vilar and Rajala-Schultz, 2020). In particular, cows with yields beyond this threshold have a delayed process of involution, with a less effective antibacterial response and a slower plasmin system activation (Silanikove et al., 2013), an exacerbated and prolonged systemic inflammatory response (Mezzetti et al., 2020; Cattaneo et al., 2021a), and increased milk leakage following milking cessation (Dingwell et al., 2004). To avoid these adverse reactions, many strategies have been proposed (Vilar and Rajala-Schultz, 2020), and most of them include a dietary restriction to reduce production and promote the onset of the involution process. Restriction can be quantitative (i.e., limiting the intake allowance, also referred to as feed restriction) or qualitative (i.e., reducing concentrate inclusion in the diet, diluting the diet with fibrous forages, or feeding forages only; also referred to as nutrient restriction).

We hypothesized that reducing the nutrient density of the diet could decrease yield before dry-off without impairing future performance. Indeed, most of the studies available in the literature limited their evaluation to the dry-off phase without focusing on the following early lactation. The aim of the present study was to investigate the effect of nutrient restriction at dry-off on milk production, metabolism, rumen fermentation, and milk fatty acids (**FA**) composition around dry-off and in the subsequent transition period.

MATERIALS AND METHODS

Animal Management and Experimental Design

The research was carried out at the Università Cattolica del Sacro Cuore dairy barn (Cerzoo, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation and ethics (Italian Health Ministry authorization N 139/2021-PR in agreement with D. Lgs. n. 26, 04/03/2014). Clinically healthy Holstein cows were continuously enrolled in the study from March 2021 to March 2022 according to the expected date of calving. Cows were selected based on milk yield and udder health 21 d before dry-off. The inclusion criteria were a milk yield of at least 15 kg/d, composite milk SCC below 400,000 cells/mL, and absence of major pathogens in any quarter, as described in Cattaneo et al. (2021b).

Table 1. Ingredients and chemical composition of diets served as TMR during the study of the effects of nutrient restriction before dry-off

Item	Lactation	Dry period
Ingredient, % of DM		
Corn silage	32.6	11.6
Alfalfa hay	24.9	—
Corn ground	13.8	—
Soybean meal	11.4	4.8
Barley ground	9.2	—
Wheat silage	3.3	47.5
Sunflower meal	2	5.1
Mineral and vitamin	1.9	0.9
Hydrogenated fat	0.9	—
Straw	—	17.7
Ryegrass hay	—	12.4
Chemical composition		
NE_L, Mcal/kg	1.65	1.28
CP, % of DM	16.6	12.7
NSC, % of DM	30	9.7
NDF, % of DM	33.4	57
Ca, % of DM	0.79	0.45
P, % of DM	0.42	0.34

During lactation, cows were housed in a freestall pen with rubber mattresses covered by shavings. They received the lactation diet based on corn silage, alfalfa hay, and concentrates (0800 h; Table 1), and were milked twice daily (0500 and 1700 h). Rations were formulated according to NRC (2001) guidelines and their ingredients and chemical composition are reported in Table 1. Feed intake was measured daily with roughage intake control troughs (Hokofarm Group B.V., Marknesse, the Netherlands), that records the visits to the feed bin (i.e., number and duration of visits and feed intake). Water was provided ad libitum.

Seven days before dry-off, cows were moved to a straw-bedded pen, where they remained for 14 d. Here, they either were fed ryegrass hay (CP = 7.1 ± 1.6% DM; NDF = 59.8 ± 4.3% DM) ad libitum (nutrient restricted, **NR**) or continued to receive the lactation diet (**CTR**) until dry-off. In this pen, feed intake measuring troughs were not present. Initially, 32 cows (parity 1.8 ± 1.6; mean ± SD) were enrolled in pairs to each treatment (i.e., NR or CTR) in an effort to reduce social stress with movement. Cows in each pair were assigned to a feed bin of the same treatment and underwent the drying-off protocol together. Thus, 16 pairs were enrolled at first. Among those, 6 cows did not meet the inclusion criteria (i.e., low production or high SCC) and were not included in the study (i.e., not monitored and sampled as described afterward). Therefore, 26 cows (NR = 13 cows, 8 pairs; CTR = 13 cows, 8 pairs) enrolled in the 16 sessions were included. Groups were balanced for lactation number, previous lactation milk yield, and dry-off month.

Fifty-five days before expected calving, cows were dried off with a single infusion in each quarter of intra-

Table 2. General characteristics of the cows enrolled in the study of the effects of nutrient restriction before dry-off

Item	Mean	SD
Parity at enrollment	1.79	1.14
DIM at enrollment, d	350	71.2
Days carrying calf at enrollment, d	218	3.26
Milk yield at −21 DFD, kg/d	24.0	4.59
SCC at −21 DFD, log_{10} SCC	1.76	0.40
Mature cow equivalent milk yield, kg/d	11,301	1,065
Average milk yield (previous lactation), kg/d	29.4	3.15
Average SCC (previous lactation), log_{10} SCC	2.07	1.05

mammary antibiotic (Mamyzin A; Boehringer Ingelheim Animal Health Italia S.p.A, Milan, Italy) and internal teat sealant (Noroseal; Norbrook Laboratories Limited, Newry, UK). Afterward, both groups were fed ryegrass hay ad libitum for 7 d. Then, cows were moved to the dry pen, which had the same design as the lactation pen and in which measuring troughs were present. Here, they received the dry period diet, based on wheat and corn silages, straw, ryegrass hay, and concentrates (Table 1). A descriptive analysis of animals involved in the trial is shown in Table 2.

Blood Sampling and Analysis

Blood samples were collected by jugular venipuncture into heparinized tubes (BD Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ) before the morning feeding at –7, –3, 0, 1, 4, 7, and 14 d from dry-off (**DFD**) and at –14, –3, 3, 7, and 14 d from calving (**DFC**). Samples were immediately placed into an ice-water bath and processed as described by Calamari et al. (2016) for the determination of the markers of energy and protein metabolism. Analyses were performed at 37°C with a clinical auto-analyzer (ILAB-650; Werfen, Milan, Italy), using commercial kits for glucose (#18250840), urea (#18255440), creatinine (#18255540, Werfen), BHB (RB1007, Randox Laboratories Ltd., Crumlin, UK), and NEFA (#434–91795, Wako Chemicals GmbH, Neuss, Germany). Concentrations of insulin were analyzed using an ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia Inc., Uppsala, Sweden). Intra- and interassay coefficient of variation were 2.3% and 6.7%, respectively.

Milk Yield and Rumination Time

Before dry-off and from 4 to 28 DFC, milk yield was automatically recorded daily in the milking parlor. From 0 to 21 DFD and from –21 to 28 DFC, rumination time was recorded using the Hr-LD tags (SCR by Allflex, Netanya, Israel). Rumination time was measured in 2-h periods and summed to a daily value.

BW, BCS, Rectal Temperature, Health Status

Body weight was recorded after each milking with a single walk-in scale (SAE Afikim, Afikim, Israel). With the same schedule of blood sampling, BCS was assessed by the same person (ADAS, 1986) and rectal temperature was measured with a digital thermometer. Health status was monitored by study personnel and veterinary staff operating in the farm facilities, and any sign of disorder (mastitis, lameness, retained placenta, metritis, ketosis) was noted. Mastitis was diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases.

Milk Samples

At –21, –7, –3, and 0 DFD, and at 7, 14, and 28 DFC, composite milk samples were collected in the morning milking to determine milk composition and milk FA profile. Fat, protein, casein, lactose, and urea were determined with Milkoscan FT120 analyzer (Foss Analytics, Hillerød, Denmark). On –21 DFD samples, SCC was measured by Fossomatic 180 (Foss Analytics) and log_{10}-transformed. Extraction and methylation of milk FA were performed following the method of O'Fallon et al. (2007). Briefly, 1 mL of milk samples were placed into a 16 × 125-mm glass vial (Pyrex Culture Tube, Corning Glass Co., Corning, NY), and 0.7 mL of 10 *N* KOH in water and 5.3 mL of MeOH were added. Tubes were incubated in a water bath (1.5 h at 55°C) and vigorously hand-shaken for 5 s every 20 min. After cooling in a cold tap water bath (10–15°C), 0.58 mL of 24 *N* H_2SO_4 in water was added to each tube. After mixing and precipitation of K_2SO_4, tubes were incubated again (1.5 h at 55°C), hand-shaken for 5 s every 20 min, and, after FA methyl ester synthesis, cooled in a cold-water bath. The FA methyl esters were extracted by addition of 3 mL of hexane followed by mixing and centrifugation (5 min at 3,000 × *g*, 16°C). The hexane layer containing FA methyl esters was put into a GC vial and used for analysis. The assay was performed using equipment and conditions previously reported (Mezzetti et al., 2022). Milk FA yield was calculated as described by Glasser et al. (2007).

Before enrollment (−21 DFD), a milk sample was aseptically collected from each quarter. Briefly, after predipping application with foaming wipes, each teat was cleaned with a cotton swab soaked in ethyl alcohol, and the first 5 streams of milk were discarded. Then, samples were collected into 5-mL sterile tubes, immediately refrigerated, and transferred at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (Piacenza, Italy), where they were analyzed within 24 h from the collection to determine bacterial growth, as described in Cattaneo et al. (2021b).

Rumen Fluid Samples

Rumen fluid samples were collected at –7, 0, and 7 DFD and –14, 7, and 28 DFC before the morning feeding with a ruminal probe specially designed for cattle (Ruminator), as previously described (Wallace et al., 2019). Samples were immediately placed in an ice-water bath. Within 30 min, the pH was measured with a pH meter (GLP 21; Crison Instruments SA). Then, samples were gently mixed, and 5 aliquots of 1 mL each were pipetted into 2-mL tubes and immediately stored at −20°C for VFA measurement. Concentrations of total and individual VFA were determined by GC (model 7820A; Agilent Technologies, Santa Clara, CA) as described by Minuti et al. (2014) and expressed as molar proportions. Ammonia was measured using a urea nitrogen kit (#18255440, Werfen, Milan, Italy) with a clinical autoanalyzer (ILAB-650; Werfen), whereas D- and L-lactic acid were concurrently determined with a D-/L-lactate assay kit (K-DLATE, Megazyme International Ltd., Wicklow, Ireland), following manufacturer's instruction.

Statistical Analysis

Data were analyzed with SAS software (version 9.4; SAS Institute Inc., Cary, NC). Sample size was based on the availability of cows in the herd and supported by a power analysis (POWER procedure of SAS). From data recently collected on the same farm, considering high-producing cows in late lactation, a sample size of 12 subjects/group would be sufficient to detect a significant difference of 6 kg/d of milk during the treatment period (with mean = 24 kg/d and SD = 5 kg/d) with $\alpha = 0.05$ and $\beta = 0.20$. Before analysis, the normality of distributions was checked calculating kurtosis and skewness of data (UNIVARIATE procedure of SAS), and non-normally distributed variables were submitted to the natural logarithm transformation. Data with a single measure were subjected to mixed models (MIXED procedure of SAS). Data with multiple observations were analyzed with repeated measures mixed models (GLIMMIX procedure of SAS) with the compound symmetry covariance structure. The periods around dry-off and calving were analyzed separately. One NR cow left the herd before the end of the trial and was excluded from the analysis of the periparturient period. The models included the fixed effects of treatment (**Trt**; CTR vs. NR), time (**T**), their interaction (**Trt** × **T**), and parity (primiparous vs. multiparous), and the random effect of the enrollment session. For BCS and BW, baseline values were included as covariates. The cow was considered as the observational unit and the enrollment session as the experimental unit. Pair-wise comparisons were performed using the least significant difference test using Tukey adjustment. Moreover, the milk FA profile (expressed as molar proportions) was evaluated by a principal component (**PC**) analysis (PRINCOMP procedure of SAS). Principal component analysis is a model-free technique that summarizes the information from a high number of features per observation in a smaller set of new variables (i.e., PC). Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Milk Production

During the week before dry-off, milk yield dropped in the NR group, but was constant in the CTR group (21.7 ± 1.20 kg/d; $P < 0.01$; Figure 1). In particular, NR cows produced 12.7 ± 1.49 kg/d after 2 d of treatment, and 7.92 ± 1.49 kg/d the day before dry-off (−62% compared with CTR). In the subsequent early lactation, milk yield did not differ between groups (39.9 vs 39.5 ± 1.1 kg/d for CTR and NR, respectively; $P = 0.79$).

The week before dry-off, milk composition was mostly stable in the CTR group (Figure 2), whereas in the NR group milk fat ($P < 0.01$) and fat:protein ratio ($P < 0.01$) increased, protein tended to increase ($P = 0.09$), and lactose decreased ($P < 0.01$). In the first month of lactation, no difference of milk composition was observed between groups (data not shown).

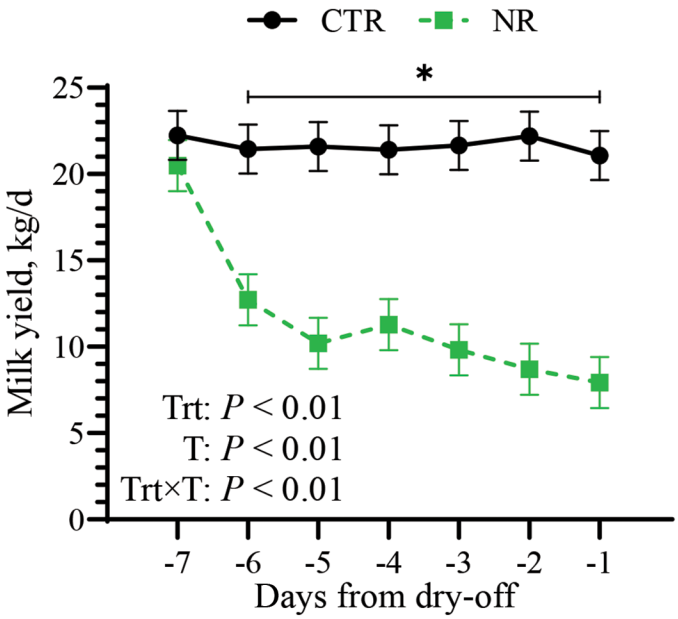

Figure 1. Milk yield in the week preceding dry-off in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported. Error bars represent SE.

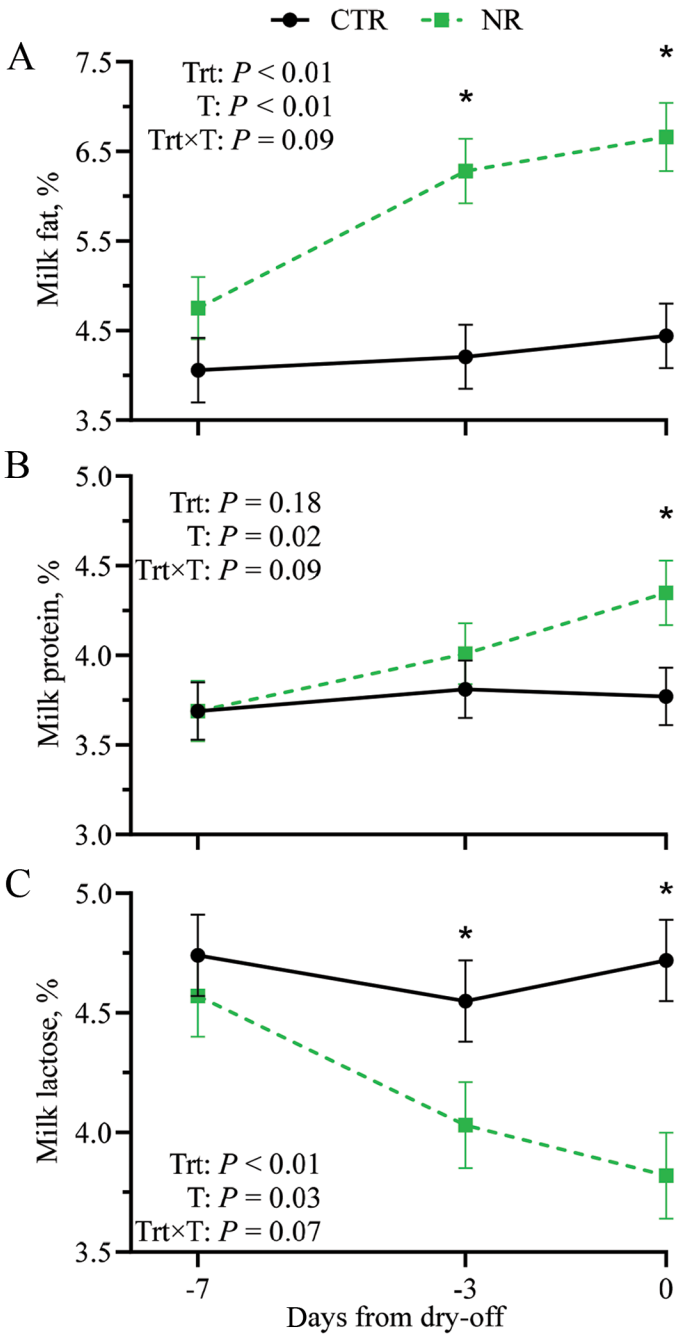

Figure 2. Milk composition (fat, A; protein, B; lactose, C) in the week preceding dry-off in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported. Error bars represent SE.

BW, BCS, and Rectal Temperature

Body weight was lower in NR than in CTR the week before dry-off (Trt; $P < 0.01$). It decreased by around 20 kg in NR during the last week of lactation (669 vs. 651 ± 4.6 kg at –8 and –1 DFD, respectively; $P < 0.01$), but was unchanged in CTR (669 vs. 676 ± 4.5 kg at –8 and –1 DFD, respectively), resulting in a significant interaction Trt × T effect ($P < 0.01$). No differences in BW were observed at calving (600 vs. 612 ± 10.4 kg in CTR and NR, respectively; $P = 0.40$). Around dry-off, the BCS was overall lower in NR that CTR (2.59 vs. 2.61 ± 0.01; $P < 0.01$) as a result of the decrease observed after milking cessation (2.60 vs. 2.54 ± 0.01 at 7 DFD; $P < 0.01$; Figure 3). Nevertheless, BCS was not different between groups at calving. Rectal temperature was overall similar between groups ($P = 0.12$), but at −3 (38.18 vs. 38.61 ± 0.11°C; $P < 0.01$; Figure 3) and 1 DFD (37.94 vs 38.40 ± 0.12°C; $P < 0.01$) was lower in NR compared with CTR. No difference was observed during the transition period.

Feeding Behavior

Dry matter intake was different over time between groups during the transition period ($P < 0.01$; Figure 4). Compared with CTR, NR cows ate slightly more before calving, but less during the first and second weeks of lactation. Particularly, during the second week after calving, DMI was greater in CTR than NR (21.1 vs. 18.7 ± 0.7 kg/d; $P = 0.02$). At drying-off, rumination time had a different trend between groups ($P < 0.01$; Figure 5). It dropped at −6 and −5 DFD in NR (474 and 486 ± 25.9 min/d, respectively in NR, compared with 536 and 549 ± 25.7 min/d in CTR), and then increased again to similar values to CTR until dry-off. Conversely, values were stable in CTR before dry-off, dropped at 1 DFD (465 vs. 597 ± 25.9 min/d in CTR and NR), and rose again afterward. No difference was observed around calving (556 vs. 551 ± 15.5 min/d for CTR and NR, respectively; $P = 0.81$).

Rumen Fermentation

Rumen fluid composition is reported in Table 3. Rumen pH did not differ between groups at −7 and 7 DFD but was higher at 0 DFD in NR than in CTR (6.90 vs. 7.36 ± 0.05 in CTR and NR, respectively; $P < 0.01$; Figure 6). At dry-off, concentrations of D-lactate (48.7 vs. 12.6 ± 3.64 mg/L for CTR and NR at 0 DFD; $P < 0.01$), L-lactate (40.6 vs. 13.1 ± 3.21 mg/L at 0 DFD; $P < 0.01$), and ammonia (6.19 vs. 2.41 ± 0.56 mmol/L at 0 DFD; $P < 0.01$) were greater in CTR, and they decreased in both groups after dry-off (i.e., 7 DFD) compared with −7 DFD. No differences in pH, D- and L-lactate, or ammonia were observed around calving. Total VFA production decreased in both groups after dry-off, but at 0 DFD, it was lower in NR compared with CTR (91.9 vs 58.4 ± 4.02 mmol/L in CTR and NR, respectively; $P < 0.01$; Figure 6); there was no difference at calving. With regard to molar proportions of individual VFA, acetate increased

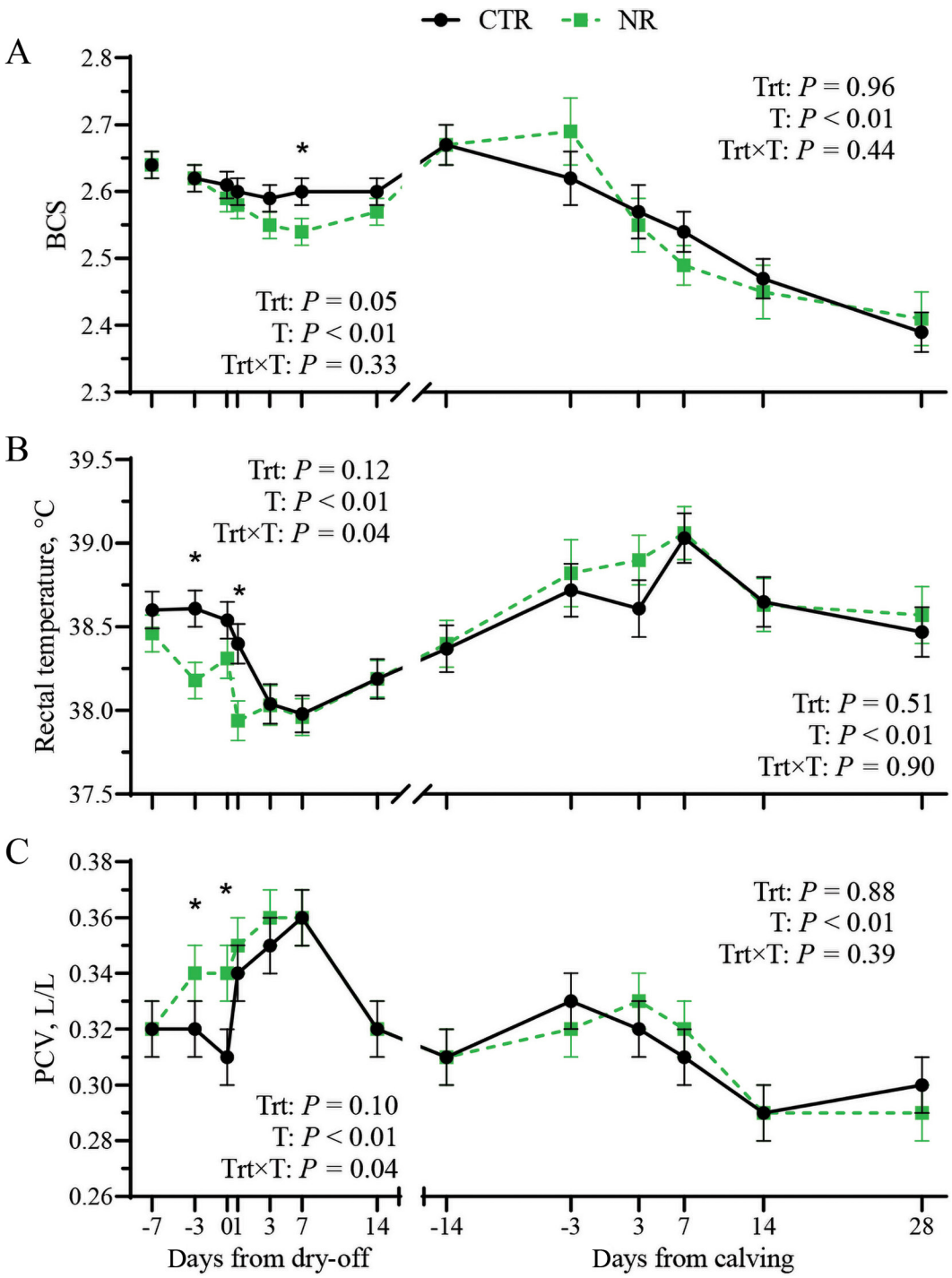

Figure 3. Body condition score (A), rectal temperature (B), and packed cell volume (PCV; C) from −7 d to 14 DFD and from −14 d to 28 DFC in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported. Error bars represent SE.

after dry-off in both groups and at 0 DFD was higher in NR compared with CTR (61.7 vs. 72.0 ± 0.82% in CTR and NR, respectively; $P < 0.01$). Other VFA (propionate, butyrate, valerate, isovalerate, hexanoate, and heptanoate; $P < 0.01$) had the opposite trend, increasing after dry-off and being higher in CTR at 0 DFD. No difference was observed between groups in isobutyrate. At calving, acetate differed between groups (Trt × T; $P = 0.05$), with

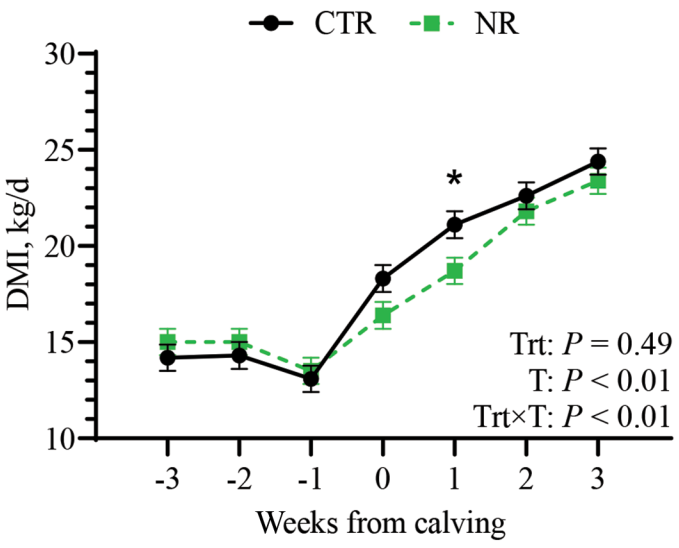

Figure 4. Dry matter intake from −3 to 3 wk from calving in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Significance levels of the main effects of the model are reported. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

a lower proportion in CTR compared with NR at 7 DFC (61.5 vs. 64.1 ± 0.9%; $P = 0.04$). Propionate had the opposite trend (Trt × T; $P = 0.03$), with a greater proportion in CTR at 7 DFC (24.3 vs. 21.4 ± 0.9%; $P = 0.03$).

Plasma Metabolic Markers and Hormones

Packed cell volume increased during restriction in NR (Trt × T, $P = 0.04$; Figure 3) but no differences were noted at calving. Glucose concentrations decreased (on average −10%) at −3 and 0 DFD in NR, whereas it was stable in CTR (4.73 vs. 4.24 ± 0.11 mmol/L at −3 DFD and 4.61 vs. 4.15 ± 0.11 mmol/L at 0 DFD in CTR and NR, respectively; $P < 0.01$; Figure 7). After dry-off and in the transition period, however, no difference was noted. Similarly, urea levels decreased before dry-off in NR but not in CTR (3.38 vs. 5.42 ± 0.34 mmol/L at −3 DFD and 3.44 vs. 5.40 ± 0.34 mmol/L at 0 DFD in CTR and NR, respectively; $P < 0.01$), and at 1 DFD (2.97 vs. 5.46 ± 0.34 mmol/L). Subsequently, urea concentrations were not different between groups. Creatinine was higher overall around dry-off in NR compared with CTR (91.4 vs. 101.8 ± 2.00 µmol/L in CTR and NR, respectively;

Table 3. Rumen fluid composition around dry-off (from −7 to 7 DFD) and calving (from −14 to 28 DFC) in dairy cows fed either an ad libitum ryegrass hay (NR) or a lactation diet (CTR) from −7 to 0 DFD

	Treatment			*P*-value[1]		
Item	CTR	NR	SEM	Trt	T	Trt × T
Around dry-off						
pH	7.05	7.20	0.03	0.01	<0.01	<0.01
L-Lactate, mg/L	31.6	22.3	1.92	0.01	<0.01	<0.01
D-Lactate, mg/L	36.2	23.2	2.00	<0.01	<0.01	<0.01
Ammonia, mmol/L	4.81	3.41	0.45	0.05	<0.01	<0.01
Total VFA, mmol/L	80.3	68.5	2.39	0.01	0.01	<0.01
Acetate, %	66.0	69.8	0.45	<0.01	<0.01	<0.01
Propionate, %	19.6	17.7	0.43	0.01	<0.01	0.02
Butyrate, %	10.33	8.94	0.15	<0.01	<0.01	<0.01
Isobutyrate, %	1.00	0.99	0.06	0.89	<0.01	0.08
Valerate, %	1.09	0.86	0.05	<0.01	<0.01	<0.01
Isovalerate, %	1.46	1.28	0.08	0.16	<0.01	<0.01
Hexanoate, %	0.51	0.38	0.04	0.04	<0.01	<0.01
Heptanoate, %	0.040	0.027	0.005	0.10	<0.01	<0.01
Around calving						
pH	7.0	7.0	0.05	0.95	<0.01	0.11
L-Lactate, mg/L	90	94	12.62	0.81	0.03	0.72
D-Lactate, mg/L	92.4	99.9	15.57	0.74	0.06	0.73
Ammonia, mmol/L	8.0	8.4	0.45	0.51	<0.01	0.13
Total VFA, mmol/L	79.6	79.0	2.40	0.86	<0.01	0.18
Acetate, %	65.1	65.6	0.60	0.54	<0.01	0.05
Propionate, %	21.2	20.6	0.64	0.53	<0.01	0.03
Butyrate, %	9.84	9.63	0.18	0.43	<0.01	0.94
Isobutyrate, %	0.96	1.02	0.05	0.45	0.02	0.05
Valerate, %	1.17	1.19	0.04	0.78	<0.01	0.04
Isovalerate, %	1.44	1.55	0.07	0.25	0.02	0.06
Hexanoate, %	0.41	0.43	0.04	0.63	<0.01	0.48
Heptanoate, %	0.033	0.034	0.003	0.78	<0.01	0.13

[1]Overall effect of the treatment (Trt), time (T), and their interaction (Trt × T).

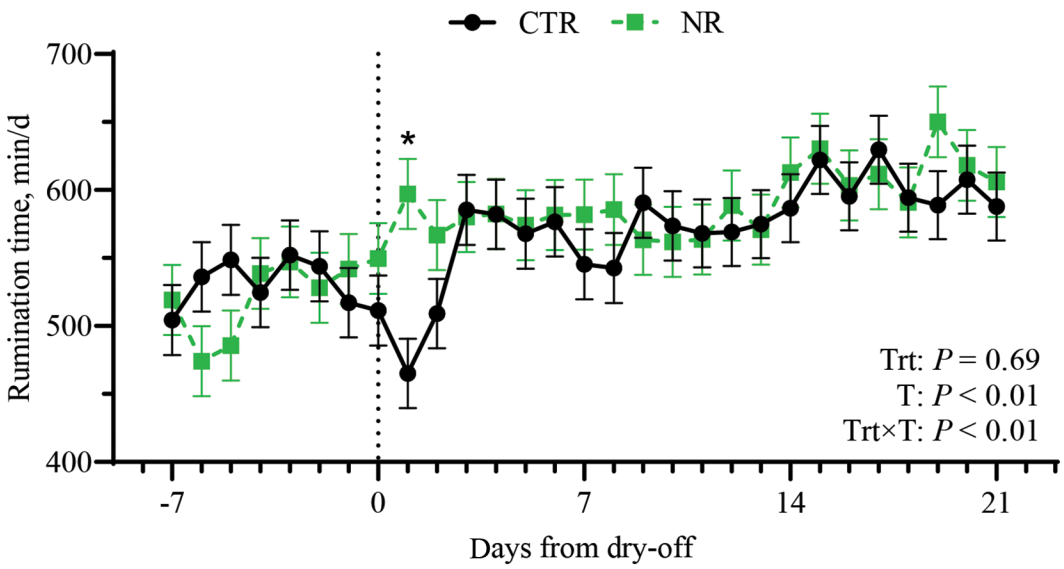

Figure 5. Daily rumination time from 7 d before dry-off to 21 d after in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported. Dotted vertical line indicates day of dry-off. Error bars represent SE.

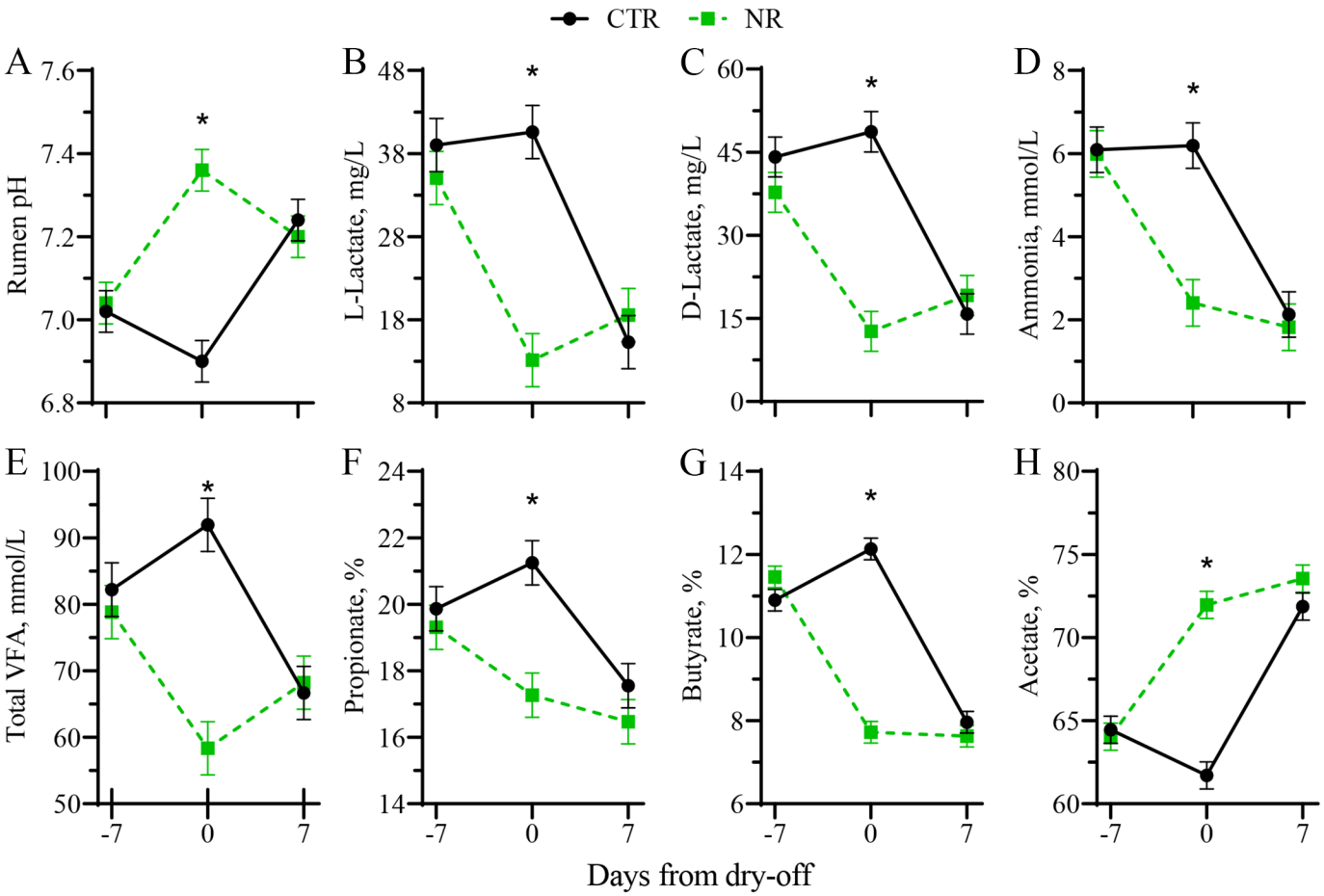

Figure 6. Rumen fluid pH (A), L-lactate (B), D-lactate (C), ammonia-N (D), total VFA concentration (E), and molar proportions of propionate (F), butyrate (G), and acetate (H) from −7 to 7 DFD in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

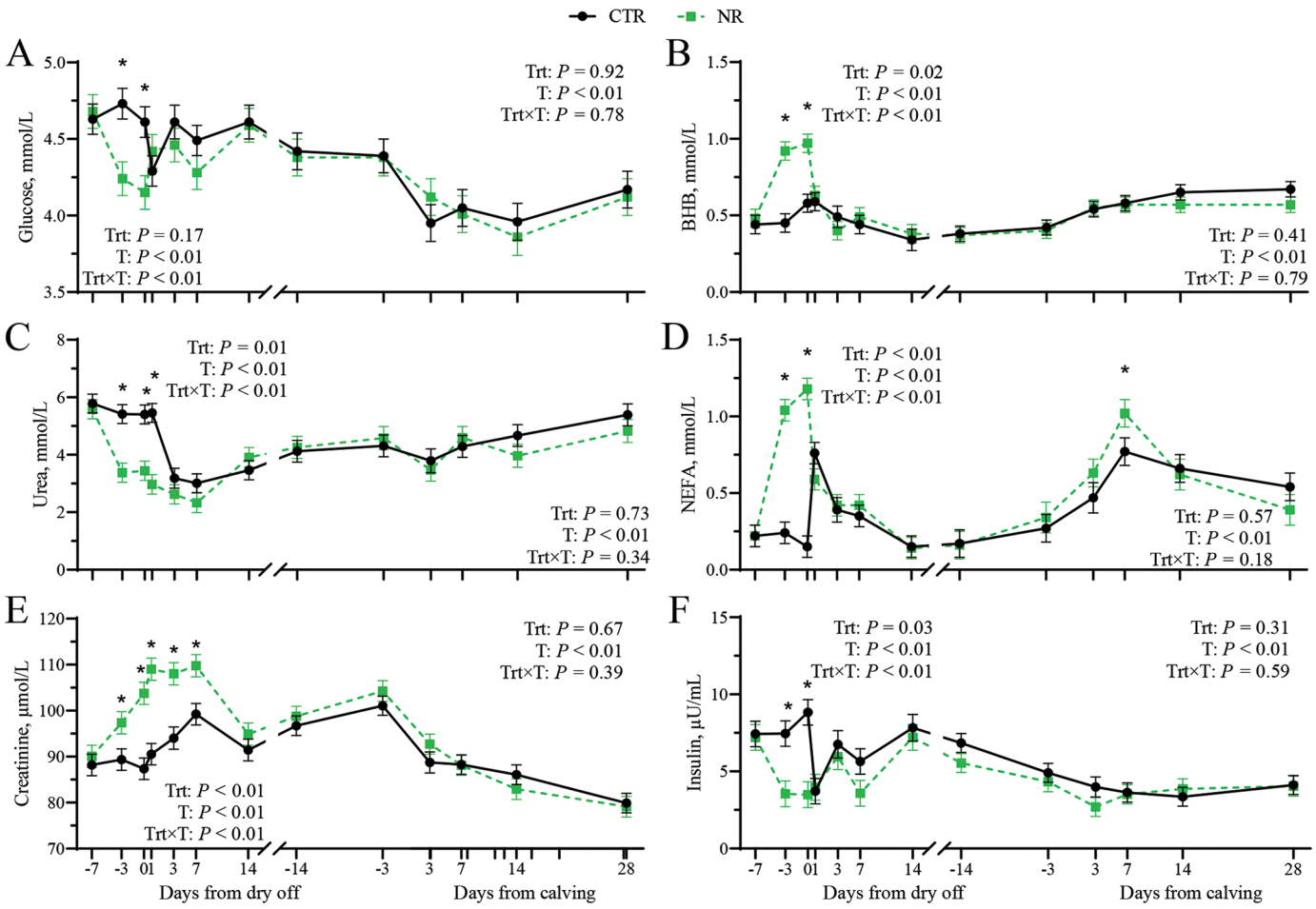

Figure 7. Plasma concentrations of glucose (A), BHB (B), urea(C), NEFA (D), creatinine (E), and insulin (F) from −7 to 14 DFD and from −14 to 28 DFC in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported. Error bars represent SE.

Trt, $P < 0.01$), in particular from −3 to 7 DFD (Trt × T, $P < 0.01$). At calving, creatinine values were not different between groups.

Plasma BHB steadily increased before dry-off in NR but not in CTR cows (0.45 vs. 0.92 ± 0.06 mmol/L at –3 DFD and 0.58 vs 0.97 ± 0.06 mmol/L at 0 DFD in CTR and NR, respectively; $P < 0.01$). Nonesterified fatty acids had a similar trend (0.24 vs. 1.04 ± 0.07 mmol/L at −3 DFD and 0.15 vs. 1.18 ± 0.07 mmol/L at 0 DFD in CTR and NR, respectively; $P < 0.01$), but at 1 DFD, NEFA levels increased in CTR and decreased in NR (0.76 vs. 0.59 ± 0.07 mmol/L; $P = 0.10$). Afterward, no main differences were observed, even though NEFA tended to be higher in NR compared with CTR at 7 DFC (0.77 vs. 1.02 ± 0.09 mmol/L; $P = 0.06$).

Insulin dropped in NR during the restriction, but it remained fairly constant in CTR (Trt × T; $P < 0.01$). After dry-off, insulin concentrations in the CTR group decreased as well, but no difference was noted between groups. At calving, circulating insulin was not affected by treatment.

Milk FA

Overall, FA yield decreased in NR whereas it remained stable in CTR (510 vs. 984 ± 48.6 g/d at dry-off in NR and CTR, respectively; $P < 0.01$). Outcomes of PC analysis carried out on FA molar proportions and the eigenvectors among all the variables considered are shown in Figure 8. The analysis of eigenvectors allows to give a meaning to the relationship between the extracted PC and the original variables. The first 2 PC explained over 78% of the total variance in each time point. No clear separation between groups was observed at −7 DFD. However, the first PC (**PC1**) discriminated very efficiently among the samples at −3 and 0 DFD according to dietary treatment. The PC eigenvectors revealed that medium- and long-chain FA (C18:1n9c, C17:1, C17:0, C16:1, C20:

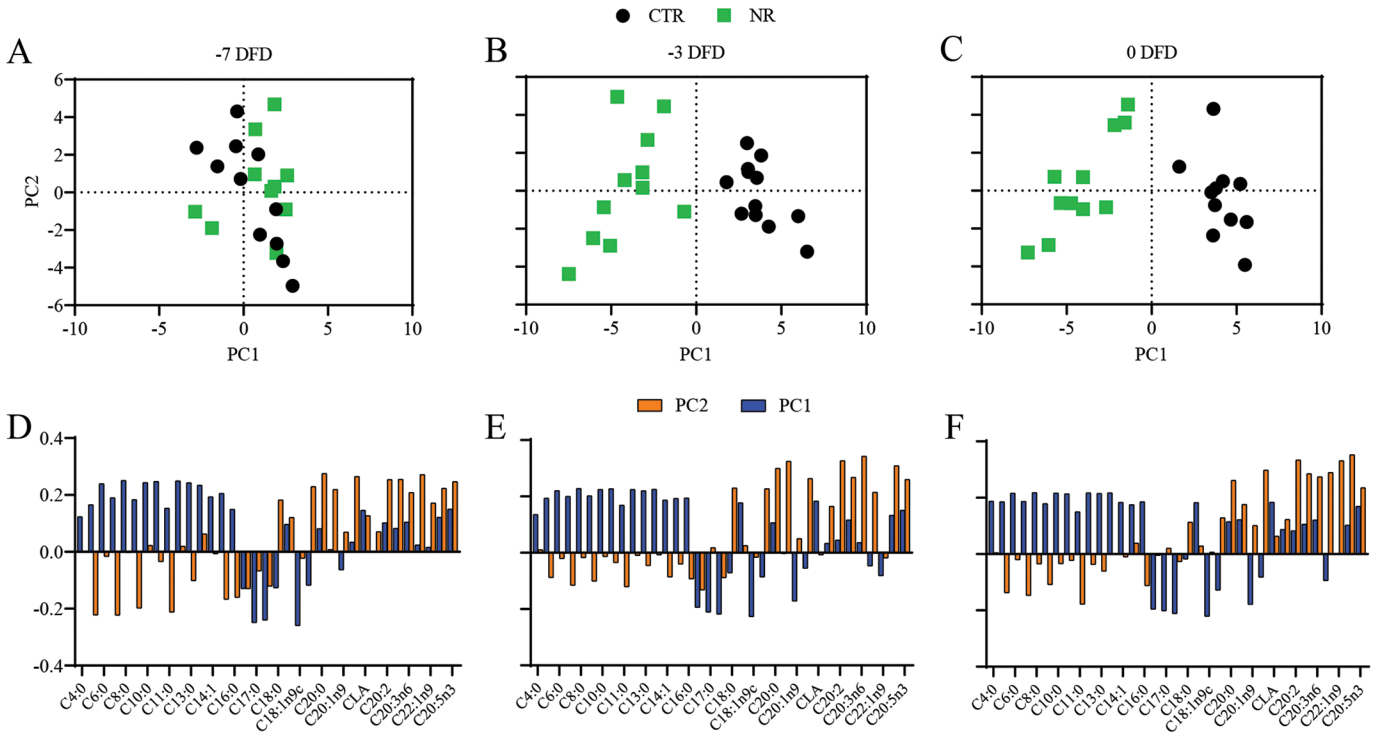

Figure 8. Principal component analysis and eigenvectors on milk fatty acid molar proportions at −7 (A), −3 (B), and 0 DFD (C) in dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from −7 to 0 DFD. PC1 = first principal component; PC2 = second principal component.

1n9, C18:2n6c) were the predominant variables with a negative effect on PC1, and short-chain FA (C8:0, C12:0, C14:0, C10:0, C6:0, C13:0, C10:1) were the predominant variables with a positive effect on PC1.

DISCUSSION

Management of the dry-off phase has been gaining importance in recent years. Protocols including different intensities of restriction (either in terms of limiting feed or energy intakes) are used in commercial farms to reduce excessive milk yield in some cows, but the impact of these practices has not yet been fully explored. The current study investigated the effect of a nutrient restriction strategy at dry-off on milk production and metabolic responses up to the subsequent early lactation phase.

The nutrient restriction (i.e., feeding grass hay to limit the energy intake) adopted in the present study effectively reduced milk yield in the last week of lactation (−62% compared with CTR). On the last day before dry-off, NR cows produced on average ~8 kg/d, far less than the safety threshold of 15 kg/d proposed by Vilar and Rajala-Schultz (2020). The milk reduction achieved is consistent with previous studies adopting a similar protocol (Ollier et al., 2014, 2015), whereas a more moderate nutrient restriction resulted in a lower drop (~30%; Larsen et al., 2021). In our trial, the restriction was approximately equal to 40% of the lactation diet, comparable to the intensities used in other studies (Leduc et al., 2021). Although we did not measure intramammary pressure and milk leakage, these low production levels should ensure the mitigation of the risk of those phenomena (Dingwell et al., 2004). Conversely, the concurrent comparatively higher yield in the control group (~22 kg/d) was previously linked to increased intramammary pressure, glucocorticoid production, risk of milk leakage and entrance of pathogens into the teat canal, and delayed involution (Bertulat et al., 2013; Silanikove et al., 2013).

During restriction, cows lost BW (~20 kg) but not BCS. We could not determine exactly what caused the weight loss. It might be linked to the lower weight of rumen contents and digesta caused by the likely decreased intake. We could not accurately measure feed intake at drying-off, but the greater volume of hay compared with the lactation diet likely limited the intake capacity via increased gut distension (Allen, 1996). Considering the greater hematocrit during restriction, hydration status of the cows might have also been implied. In contrast, BCS was not different between groups before dry-off, though we expected a decrease in NR cows. However, the limited duration of the restriction, paired with the limited sample size, likely prevented the observation of significant differences in this short time frame. Conversely, we observed a small but significant decrease in

BCS after dry-off in NR cows. The hormonal framework in late lactation could partly account for the limited drop. In similar intake deficit conditions, early lactation cows mobilize a significant amount of body reserves to support milk synthesis (Drackley, 1999). However, after calving the mammary gland is metabolically prioritized (Bell and Bauman, 1997), circulating insulin is low and cows are in an insulin-resistant state (De Koster and Opsomer, 2013). This metabolic status sustains milk production, mobilizing body reserves and redirecting nutrients available toward the mammary gland at the expense of peripheral tissues, despite deficient feed intake. In contrast, late-lactation cows have higher insulin (Vicini et al., 1991) and the mammary gland is no longer a priority. Thus, cows responded to nutrient restriction by decreasing milk synthesis, preserving body reserves, and sparing energy for the fetus. In our study, insulin was higher in late lactation compared with the postpartum phase. As expected (Leduc et al., 2021), insulin dropped during the dietary restriction. In similar experimental conditions, Odensten et al. (2005) observed a drop in plasma insulin during nutrient restriction, which was more marked when a lower quality forage (i.e., straw) was fed, compared with silage.

Milk composition reflected the change in yield, with a steady increase in fat and protein concentration and a decrease in lactose content. These variations are consistent with a decrease in mammary epithelial cell function, which is related to nutrient restriction (Dessauge et al., 2011). The increase in the contents of fat and protein could also be due to a concentration effect related to the reduced milk fluid volume. The decrease in milk volume could have been caused by a decrease in lactose synthesis, because lactose is the major milk osmotic component (Kronfeld, 1982; Shamay et al., 2003). The reduced energy availability and the consequent hypoglycemia limited glucose availability for the mammary gland. Moreover, lactose synthetase activity declines during involution (Bauman et al., 1974; Hurley, 1989). Nevertheless, other factors were likely involved. Milk protein usually decreases in response to dietary restriction (Leduc et al., 2021), due to lower dietary and microbial protein availability (which in our study was confirmed by the lower blood urea levels). But protein increases during mammary involution (Hurley, 1989), as a result of the increase in immunoglobulins, serum albumin, and lactoferrin (Smith et al., 1971; Hurley, 1989), despite the decrease in milk-specific proteins such as α-LA, β-LG, and caseins (Hurley and Rejman, 1986; Aslam and Hurley, 1997). Thus, the rise in milk protein content might suggest a loss of mammary epithelial cell integrity, which may be interpreted as a marker of anticipated involution. Along with the higher protein content, the lower lactose might also point out a loss of integrity of the mammary epithelium (Zhao et al., 2019). Being the major milk osmotic component, lactose cannot considerably decrease. Normally, mammary epithelium maintains the ionic gradient between milk and interstitial fluid. However, tight junction permeability increases with involution, allowing the paracellular passage of blood components into milk, and vice versa (Stelwagen and Singh, 2014). In this case, the osmotic role of lactose is taken over by protein, Na, and Cl, which move from interstitial fluid to milk. We observed an increase in milk protein but we did not measure milk minerals to fully confirm this hypothesis. Thus, lactose may be lost as tight junctions become compromised, as it occurs during involution. Milk fat content could have been affected by body fat mobilization. Milk FA synthesis decreased during restriction, but NR cows had a reduced proportion of de novo FA and increased that of long-chain FA. A certain degree of body reserve mobilization occurred, as confirmed by the higher levels of NEFA and BHB in NR cows. This is the typical metabolic framework of dietary restriction (Leduc et al., 2021), which is also paired with low circulating insulin. In the present study, insulin markedly decreased during restriction. The increase in the markers of catabolism was relevant, but of a reduced intensity compared with the first days after calving, never reaching the threshold of subclinical ketosis (Duffield, 2000). Concurrently, a higher amount of mobilized FA was likely available in the bloodstream for mammary gland uptake and incorporation into milk fat. Indeed, differences in NEFA were detected in late lactation as nutrient restriction occurred, and milk FA analysis confirmed greater availability of mobilized FA, as we identified a greater proportion of preformed and long-chain FA in NR cows. These FA classes increase when energy balance is negative and fat reserves are mobilized (Stoop et al., 2009; Gross et al., 2011). Another possible explanation might be related to a decline in the activity of acetyl-CoA carboxylase (Bauman et al., 1974; Hurley, 1989), which limits the synthesis of short- and medium-chain FA.

Daily rumination time dropped similarly in the NR group in the first few days of restriction and in the control group immediately after dry-off. This drop was in agreement with previous observations at dry-off (Abuelo et al., 2021). Therefore, its main driver was the stress arising from diet and group change, rather than the milking cessation itself. The increase after dry-off in the NR group was likely a result of the milking cessation, from a time budget standpoint. Decreasing the time spent in the milking operations made more time available for lying and ruminating (Schirmann et al., 2012; Chapinal et al., 2014), similar to what happens, in the opposite direction, at the onset of lactation (Huzzey et al., 2005; Cattaneo et al., 2020). Rumen pH increased as a result of hay feeding. Long forages require longer chewing time

than concentrates and stimulate saliva secretion, which can alter VFA production and buffer rumen pH (Mertens, 1997). The lower total VFA production, rumen lactate, and urea (consistent with the plasma levels) also highlighted a lower intensity of rumen fermentation. This was supported also by the lower rectal temperature in those cows, because reduced activity of heat-producing rumen microorganisms produces less metabolic heat. Moreover, the lower nitrogen level both in rumen fluid and blood was consistent a lower protein supply, given that they are closely interrelated (Marini and Van Amburgh, 2003). Indeed, nutrient restriction at dry-off decreased protozoa number in the rumen, which are responsible for ammonia production (Odensten et al., 2005). Further, creatinine greatly increased in NR cows around dry-off. Creatinine is a breakdown metabolite of creatine phosphate produced because of muscle creatine catabolism. Thus, blood creatinine concentration can serve as a marker of muscle loss or protein catabolism. However, creatinine increase due to muscle catabolism is usually paired with an increase in plasma urea concentrations (Keogh et al., 2015). Conversely, we had a decrease in plasma urea, that may be explained by a reduced flux from the rumen paired with a reduced utilization of AA as gluconeogenic substrate. Higher creatinine can also indicate a decrease in renal filtration ability or increased liver synthesis, as previously reported in a feed restriction trial performed during the peripartum period (Shahzad et al., 2014).

To evaluate the success of dry-off protocols it is essential to also assess the effect on the subsequent lactation. Milk yield is known to be influenced by previous dry period mammary redevelopment (Capuco et al., 1997; Zhao et al., 2019), which might have been affected by nutrient restriction. Different types of stressors can impair mammary involution. Heat stress, for instance, has a detrimental effect on autophagy in the first stages of dry period (Wohlgemuth et al., 2016), eventually reducing milk yield in the subsequent lactation (Cattaneo et al., 2022). We did not observe any difference in milk production in the subsequent lactation. Similarly, cows feed restricted during early lactation did not show any carryover effect at refeeding on milk production, despite a significantly greater daily mammary epithelial cell exfoliation rate during restriction (Herve et al., 2019). But data about mammary cell activity at the resumption of lactation after dietary restriction at dry-off are lacking; therefore, further studies on this aspect are required. However, a lower DMI was transiently observed after calving in cows that underwent nutrient restriction at dry-off, but this difference in DMI remains unclear and requires further study, focusing particularly on inflammatory and liver conditions of the cows. Accordingly, NEFA were also higher in NR cows at the onset of lactation, even though other markers of metabolism and circulating insulin were unaltered. Thus, the reasons behind this difference require additional investigations. Consistent with the intake data, NR cows had also a slightly higher rumen pH 7 d after calving, pointing to a lower fermentation intensity, as supported by the numerically lower total VFA production.

CONCLUSIONS

Nutrient restriction was confirmed to be an effective solution to quickly reduce milk production before dry-off. Low plasma glucose and urea levels, paired with increased NEFA and BHB, suggest that dietary restriction led to lower energy availability and increased fat mobilization. Hay feeding also altered the pattern of rumen fermentation, with an increase in acetate proportion and a decrease in propionate and butyrate proportions. Concurrently, milk fat and protein content increased, and lactose declined. In addition, rumination time dropped because of the drastic diet changes. However, in the subsequent early lactation, DMI was transiently lower in previously restricted cows. Further research is needed to precisely determine the carryover effect (if present) of dietary restriction at dry-off.

NOTES

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Abbreviations used: CTR = control group fed lactation diet; DFC = days from calving; DFD = days from dry-off; FA = fatty acids; NEFA = nonesterified fatty acids; NR = nutrient-restricted group fed ad libitum ryegrass hay; PC = principal component; PC1 = first PC; T = time; Trt = treatment.

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