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Impact of severe nutrient restriction before dry-off on inflammatory, hepatic, and immune-related indicators from dry-off through the transition period

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ABSTRACT

Drying-off high-producing dairy cows can induce inflammatory responses associated with mammary involution, particularly when milk yield remains high at milking cessation. Nutrient restriction is often used to reduce milk yield to approximately 15 kg/d before dry-off, a level considered favorable for mammary involution. However, restriction can also alter metabolic status during late lactation and the effects of this strategy on systemic inflammatory status and liver function during the subsequent transition period remain unclear. The objective of this study was to determine whether a severe reduction in dietary nutrient supply before dry-off affects systemic inflammatory status, liver function, and selected immune-related indicators during dry-off and the subsequent transition period. Thirty-two Holstein cows arranged in 16 pairs were initially enrolled and assigned at the pair level to either receive ryegrass hay ad libitum as the sole feed for 7 d before dry-off (NR) or to continue receiving the lactation diet (CTR). After exclusion of cows that did not meet the inclusion criteria, 26 cows producing more than 15 kg/d remained in the final data set (NR, n = 13; CTR, n = 13). Treatments were applied at the pair level, whereas biological measurements were collected at the individual-cow level. After dry-off, carried out 55 d before the expected calving, both groups received only grass hay for 7 d, and free access to water was always provided. Blood samples were collected at –7, –3, 0, 1, 4, 7, 14, and 28 d from dry-off (DFD), and –14, –3, 3, 7, 14, and 28 d from calving (DFC). Milk samples were collected at –7, –3, and 0 DFD and 7, 14, and 28 DFC for SCC determination, bacteriological culture, and flow cytometry analysis. Restriction reduced milk yield by 62% before dry-off. Dry-off significantly altered blood cell count and plasma concentrations of inflammatory biomarkers. Milk SCC increased during restriction but was similar afterward. Overall differences between treatments in blood cell counts were limited. Similarly, no differences in CD3+, CD4+, CD8+, and CD11b+ milk cell populations were detected. Compared with CTR, in NR monocyte count was lower and eosinophils higher at 0 DFD. Lower haptoglobin and ROM concentrations at 4 DFD suggested a different temporal pattern of the systemic response after dry-off, potentially consistent with an earlier onset of involution-related changes. Nevertheless, after calving, NR cows had lower albumin and paraoxonase, and higher globulin, haptoglobin, bilirubin, AST-GOT, and myeloperoxidase at key time points, consistent with a less favorable inflammatory and hepatic biomarker profile. These findings, together with the previously reported reduction in DMI in NR cows, suggest that severe nutrient restriction before dry-off may alter the timing of dry-off-related responses and impair metabolic and inflammatory adaptation during the subsequent lactation. Further research is needed to decipher the mechanism underlying these long-term effects.
Key words: inflammation, acute-phase protein, transition period, liver function

INTRODUCTION

Dry-off is a complex stage in the life of dairy cows. It involves several management, physiological, and behavioral changes and challenges (i.e., milking cessation, diet change, and regrouping) (Cattaneo et al., 2023a). As a result, dry-off triggers an inflammatory response during the days immediately following the event (Gao et al., 2024). Particularly, positive acute-phase proteins (**APP**; i.e., haptoglobin, ceruloplasmin, and serum amyloid-A), which are synthesized by the liver in response to inflammation (Ceciliani et al., 2012), increase in blood and milk

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beginning 2 to 3 d after milk cessation and may remain elevated for up to 2 wk (Putman et al., 2018; Mezzetti et al., 2020; Gao et al., 2024). These variations are even more relevant in cows with a preexisting worse inflammatory condition (Cattaneo et al., 2021a) or in cows with high milk yield (Mezzetti et al., 2020; Cattaneo et al., 2021b). At the same time, concentrations of negative APP (mainly albumin, retinol binding protein, paraoxonase and lipoproteins) decrease during inflammation as a consequence of reduced hepatic synthesis (Bertoni et al., 2008). Consistently, liver biomarkers indicate impaired hepatic function and indicators of oxidant status decrease in blood during early involution (Putman et al., 2018; Mezzetti et al., 2020; Cattaneo et al., 2021b). Interestingly, pre- and post-dry-off inflammation appear to be associated with lower milk production and altered inflammatory and metabolic responses in the subsequent periparturient period (Cattaneo et al., 2021a; Goetz et al., 2022). Therefore, altering cow condition in this phase could have long-lasting effects.

As mentioned above, drying off cows with high milk yield (>15 kg/d; Vilar and Rajala-Schultz, 2020) can impair and delay the involution process (Silanikove et al., 2013), exacerbate the inflammatory response (Mezzetti et al., 2020; Cattaneo et al., 2021b), and increase the risk of new intramammary infections (Dingwell et al., 2004). To reduce milk production in this phase and try to alleviate the typical inflammatory response, which is mainly related to mammary involution and the dry-off process itself (Zobel et al., 2015; Zhao et al., 2019), feed or nutrient restriction strategies have been implemented. Nevertheless, dietary restriction may promote inflammatory signaling through multiple mechanisms. These include lipid mobilization and adipose tissue responses, but also gastrointestinal adaptations related to altered nutrient supply, changes in rumen fermentation, and gut immune activity (Bertoni et al., 2015; Kvidera et al., 2017; Bradford and Swartz, 2020). Moreover, the decreased nutrient availability might jeopardize immunity (Trevisi and Minuti, 2018). However, the impact of restriction in late lactation has yet to be fully elucidated, because most of the available insights stem from early- or mid-lactation studies (Leduc et al., 2021).

Although nutrient restriction is frequently used to reduce milk yield before dry-off, its potential consequences for systemic inflammatory status and liver function during the subsequent transition period remain poorly understood, particularly when restriction is severe. Thus, the objective of this study was to determine whether decreasing dietary nutrient density at dry-off alters inflammatory status, liver function, and some immune-related indicators during dry-off and the subsequent transition period. We hypothesized that reducing milk production before dry-off through a lower dietary nutrient density would modify the inflammatory response after milking cessation. Performance and metabolic responses of the same cows were described in a companion paper (Cattaneo et al., 2024), whereas the present study focuses on inflammatory, hepatic, oxidative, and immune-related responses during dry-off and the subsequent transition period.

MATERIALS AND METHODS

Animal management and experimental design

The research was conducted at Università Cattolica del Sacro Cuore dairy barn (Cerzoo, San Bonico, Piacenza, Italy) in accordance with Italian regulations for animal experimentation and ethics (Italian Health Ministry authorization N 139/2021-PR in agreement with D. Lgs. n. 26, 04/03/2014). Detailed description of experimental design and procedures is reported in the companion paper (Cattaneo et al., 2024). Briefly, during lactation, cows were housed in a freestall pen with rubber mattress covered by shavings, received the lactation diet, and were milked twice daily (0500 and 1700 h). Feed intake was measured daily with Roughage Intake Control (RIC) troughs (Hokofarm Group, AX Emmeloord, The Netherlands). As described in the companion paper (Cattaneo et al., 2024), cows were managed and assigned to treatments in pairs to reduce social stress associated with pen movement. Each pair of cows was assigned to the same feed bin and underwent the dry-off protocol together. Thus, treatment was applied at the pair level, whereas biological measurements were collected at the individual cow level. A total of 32 cows and 16 pairs were initially enrolled. Cows were selected based on milk yield and udder health 21 d before dry-off. The inclusion criteria were milk yield of at least 15 kg/d, composite milk SCC below 400,000 cells/mL, and absence of major pathogens in any quarter. Among the enrolled cows, 6 cows did not meet the inclusion criteria (i.e., low milk yield or high SCC) and were not included in the study (i.e., they were not monitored and sampled as described afterward). Therefore, 26 Holstein parous cows (parity 1.8 ± 1.6; mean ± SD) enrolled in 16 sessions were included in the final data set. The experimental treatment was assigned at the pair level, because paired cows shared the same feed bin and management protocol, whereas measurements were collected at the cow level.

During the 7 d preceding dry-off, the 16 initially enrolled pairs were assigned to one of 2 treatments, with 8 pairs assigned to each treatment. Treatment was applied at the pair level, whereas measurements were collected at the individual-cow level. Treatments were: a severe nutrient restriction treatment in which ryegrass hay was offered ad libitum as the sole feed (**NR**), or a control

treatment in which cows continued to receive the lactation diet (**CTR**), until dry-off. After exclusions, the final data set included 13 cows in NR and 13 cows in CTR, enrolled in 8 sessions/treatment. As detailed in the companion paper, this dietary intervention markedly reduced nutrient intake and milk yield during the week preceding dry-off. Therefore, it should be considered a severe restriction model. Fifty-five days before expected calving, cows were dried off by administering an intramammary antibiotic to each quarter (Mamyzin A; Boehringer Ingelheim Animal Health Italia S.p.A) and internal teat sealant (Noroseal; Norbrook Laboratories Limited). Afterward, both groups were fed ryegrass hay *ad libitum* for 7 d. At the end of drying-off phase, cows were moved to the dry pen, and received the dry period diet. Diets were reported in the companion paper.

Blood sampling and analysis

Blood samples were collected from the jugular vein into heparinized tubes before the morning feeding at –7, –3, 0, 1, 4, 7, 14, and 28 d from dry-off (**DFD**) and at –14, –3, 3, 7, 14, and 28 d from calving (**DFC**). Samples were immediately placed into an ice-water bath and processed as described by Calamari et al. (2016) for determination of the markers of inflammation, liver function, oxidative stress, and mineral status: cholesterol, Ca, P, Mg, Na, K, Zn, total protein, albumin, globulin, ceruloplasmin, haptoglobin, thiol groups, myeloperoxidase, advanced oxidation protein products (**AOPP**), paraoxonase, reactive oxygen metabolites (**ROM**), aspartate aminotransferase-glutamate oxaloacetate transaminase (**AST-GOT**), γ-glutamyl transferase (**GGT**), bilirubin, alkaline phosphatase. Albumin-to-globulin ratio (A:G) was calculated. Moreover, albumin, bilirubin, and cholesterol plasma concentrations at 3 and 28 DFC were used to calculate the Liver Functionality Index (**LFI**; Bertoni and Trevisi, 2013).

Moreover, at –7, 0, and 7 DFD, blood samples were collected into K-EDTA tubes for blood count determination. Blood count was performed with Cell-DYN 3700 (Abbott Diagnostic Division, CA), and white blood cells and their subpopulations (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), red blood cells, hemoglobin, and platelets were determined.

Milk samples

At –21, –7, –3, and 0 DFD, and at 7, 14, 28 DFC, composite milk samples were collected in the morning milking for determination of milk SCC. The SCC was assessed by Fossomatic 180 (Foss Analytics, Hilleroed, Denmark) and log-10 transformed before statistical analysis.

At –21 DFD and at 7 and 28 DFC, a milk sample was aseptically collected from each quarter. Briefly, after pre-dipping application with foaming wipes, each teat was accurately 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 to the "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna" (Piacenza, Italy), where they were cultured within 24 h of collection, as described in Cattaneo et al. (2021), for bacterial presence determination. Quarter-level bacteriology was performed at the enrollment screening and after calving to assess preexisting infection status and postpartum mammary health.

At –7, –3, and 0 DFD, one hour after morning milking, approximately 1 L of milk was sampled through a portable milking system. Before milking, teat ends were accurately cleaned with foaming wipes, and, after milking, iodine solution was applied. Milk was immediately placed in an ice-water bath. Within 2 h, 400 mL of milk were centrifuged at 1000 x g for 20 min at 4°C with slow deceleration. Milk fat and supernatant were discarded, and cell pellet was washed twice using 40 mL of PBS solution (400 x g for 10 min at 4°C). Pellet was then resuspended in 4 mL of freezing medium (50% of RPMI-1640 nutritional medium, 40% of fetal bovine serum, and 10% of DMSO), and frozen slowly (–1°C/min; CoolCell, Corning Inc., NY) at −80°C for subsequent flow cytometric assays. The process of isolation and storage of milk cells for flow cytometry was completed within 4 h after sampling, and all procedures were carried out at 4°C.

Flow cytometry. Frozen milk cell samples were rapidly thawed at 37°C. Then, the cell suspension was diluted with 10 mL of RPMI-1640 at 37°C and centrifuged at 400 x *g* for 5 min at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 3 mL of RPMI-1640 and centrifuged again. Afterward, the supernatant was discarded, and the cell pellet was washed with 3 mL of PBS-EDTA. The cell pellet obtained was diluted to a final concentration of 10^6 cells/mL. Cell solution was incubated with diluted monoclonal primary antibodies (Table 1, Bio-Rad Laboratories, Hercules, CA, USA) for 30 min in the dark at 4°C, washed with PBS, and centrifuged for 3 min at 500 x *g* at 4°C. Cells were subsequently incubated for 30 min at 4°C in the dark with polyclonal secondary antibody conjugated to Alexa Fluor 488 (excitation at 488 nm) [F(ab')2-Goat anti-Mouse IgG, IgM (H^+L) secondary antibody, Alexa Fluor 488, Invitrogen, A10684]. After washing, cells were resuspended in PBS, stained with propidium iodide (PI, Sigma-Aldrich, Merk Life Science, Milan, Italy), and immediately analyzed using flow cytometer (BD Accuri C6 Plus, BD Biosciences, Franklin Lakes, NJ).

A total of 10,000 events were acquired, excluding most of the cellular debris based on forward- and side-scatter properties. Results were extracted with BD Accuri C6 Plus software (BD Biosciences), and corrected for autofluorescence using unlabeled milk cells isolated from the same sample. Because only a limited panel of markers was assessed before dry-off, this analysis was intended as an exploratory evaluation of selected milk-cell subpopulations.

Statistical analysis

Data were analyzed with SAS software (version 9.4; SAS Institute Inc., Cary, NC). Sample size calculation was reported in the companion paper (Cattaneo et al., 2024). Before analysis, normality of distributions was checked, and non-normally distributed variables were log-transformed. Data with a single observation were subjected to ANOVA (GLM procedure of SAS), comparing the effect of treatment (**Trt**; CTR vs. NR). Data with repeated observations were analyzed using mixed models for repeated measures (GLIMMIX procedure of SAS) with the compound symmetry covariance structure. The model included Trt, time (**T**), their interaction (Trt × T), and parity (primiparous vs multiparous) as fixed effects, and enrolled pair as a random effect. The periods around dry-off and calving were analyzed separately. Baseline value was included as a covariate when appropriate. Pairwise comparisons were carried out with the Tukey adjustment. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Blood count

Results of blood count are shown in Table 2. Red blood cells generally increased after dry-off, but in NR slightly decreased at 0 DFD before increasing again (Trt × T; $P < 0.01$). Compared with CTR, NR had greater hemoglobin at 0 DFD, whereas values were similar at −7 and 7 DFD (Trt × T; $P < 0.01$). Mean corpuscular volume tended to decrease more in CTR than in NR after dry-off (Trt × T; $P = 0.07$). No differences were observed in platelets.

White blood cells changed over time in both groups, but the temporal pattern differed between treatments, decreasing progressively in CTR and decreasing at 0 DFD before increasing again at 7 DFD in NR (Trt × T; $P = 0.04$). Neutrophils decreased earlier in NR (0 DFD) than in CTR (7 DFD), resulting in a significant Trt × T interaction ($P < 0.01$). Monocytes had a similar trend and tended to decrease sooner in NR than in CTR (Trt × T; $P = 0.06$). Lymphocyte, eosinophil, and basophil counts did not show any difference between groups. Analysis of leukocyte proportions provided similar results for neutrophils (Trt × T; $P = 0.02$). Lymphocyte proportion showed a peak in NR at 0 DFD, whereas in CTR they were stable in late lactation and slightly increased at 7 DFD (Trt × T; $P = 0.03$). Eosinophil proportion increased from 0 DFD, while in CTR the rise happened at 7 DFD (Trt × T; $P < 0.01$). No treatment differences were noted in monocyte or basophil proportions.

Inflammation and immunity biomarkers

Plasma marker concentrations are reported in Table 3. Among inflammation biomarkers (Figure 1), total plasma protein concentration did not differ between groups around dry-off, whereas albumin was lower in NR compared with CTR at 14 and 28 DFC (36.6 vs 34.4 ± 0.54 g/L in CTR and NR at 14 DFC, respectively, and 37.3 vs 35.7 ± 0.54 g/L at 28 DFC; Trt × T, $P = 0.02$). Moreover, globulin showed numerically greater values in NR during the transition period, particularly at 7 DFC (36.2 vs. 40.7 ± 1.57 g/L in CTR and NR, respectively), although the overall treatment effect only tended to be significant ($P = 0.10$). Haptoglobin was higher in CTR compared with NR at 4 DFD (0.19 vs 0.08 ± 0.03 g/L; $P = 0.02$) but was lower at 14 DFC (0.11 vs 0.35 ± 0.08 g/L; $P = 0.03$). Cholesterol tended to change differently over time between treatments around dry-off (Trt × T, $P = 0.12$), with a greater decrease observed in NR, but it was not different at calving. Paraoxonase was lower before dry-off in NR compared with CTR (99.2 vs 83.3 ± 5.19 U/mL at –3 DFD in CTR and NR, respectively, and 98.1 vs 82.9 ± 5.19 U/mL at 0 DFD; $P < 0.01$). After dry-off, values were similar between groups. In the periparturient period, it was lower in NR at 14 DFC (103.5 vs 85.6 ± 5.71 U/mL; $P = 0.03$). No treatment differences were observed for ceruloplasmin or Zn around either dry-off or calving.

Table 1. Monoclonal antibodies used for differentiation and labeling of bovine milk cells applying flow cytometry analysis

Type	Description	Clone	Isotype	Specificity	Host
CD3	T Lymphocytes	MM1A	IgG1	Bovine	Mouse
CD4	Helper T Lymphocytes	CC8	IgG2a	Bovine	Mouse
CD8	Cytotoxic T Lymphocytes	CC63	IgG2a	Bovine	Mouse
CD11b	Neutrophils, monocytes, and macrophages	CC126	IgG2b	Bovine	Mouse

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Table 2. Blood cell count from –7 to 7 d from dry-off in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d from dry-off to dry-off. Differences between groups ($P \leq 0.05$) at each time point are denoted with an asterisk and tendencies ($0.05 < P \leq 0.10$) with a +

		Days from dry-off				*P*-value		
Item, unit	Treatment	−7	0	7	SEM	Trt	T	Trt × T
White blood cells, K/µL	CTR	8.12	7.82	7.25	0.37	0.74	0.04	0.04
	NR	8.20	7.66	7.99	0.37			
Red blood cells, K/µL	CTR	6.86	6.70	7.65	0.22	0.53	<0.01	<0.01
	NR	6.57	6.89	7.27	0.22			
Hemoglobin, g/dL	CTR	11.2	10.9	12.5	0.22	0.78	<0.01	<0.01
	NR	11.1	11.6*	12.1	0.22			
Platelets, K/µL	CTR	235	283	309	31.82	0.90	0.01	0.15
	NR	250	293	268	31.82			
Leukocyte subpopulations, K/µL								
Neutrophils	CTR	3.31	3.18	2.59	0.22	0.87	<0.01	0.01
	NR	3.41	2.84+	2.99	0.22			
Lymphocytes	CTR	3.39	3.31	3.20	0.22	0.67	0.67	0.44
	NR	3.38	3.46	3.43	0.22			
Monocytes	CTR	0.83	0.81	0.64	0.04	0.85	<0.01	0.06
	NR	0.73	0.64+	0.61	0.04			
Eosinophils	CTR	0.51	0.44	0.78	0.11	0.17	<0.01	0.39
	NR	0.63	0.68*	0.92	0.11			
Basophils	CTR	0.07	0.07	0.06	0.01	0.39	0.82	0.69
	NR	0.06	0.06	0.06	0.01			
Leukocyte proportions, %								
Neutrophils	CTR	40.9	41.1	35.7	1.79	0.77	<0.01	0.02
	NR	41.5	37.0	37.3	1.79			
Lymphocytes	CTR	41.7	42.0	44.2	2.00	0.81	0.11	0.03
	NR	41.4	45.2	43.1	2.00			
Monocytes	CTR	9.73	9.74	8.46	0.35	0.16	<0.01	0.43
	NR	9.51	8.76+	8.09	0.35			
Eosinophils	CTR	6.24	5.75	10.79	0.92	0.19	<0.01	0.01
	NR	7.43	8.75*	11.27	0.92			
Basophils	CTR	0.83	0.87	0.89	0.06	0.19	0.94	0.57
	NR	0.78	0.76	0.75	0.06			

Among liver function biomarkers (Figure 2), bilirubin was greater in NR than in CTR around dry-off (1.44 vs 2.25 ± 0.21 µmol/L in CTR and NR, respectively; Trt, $P < 0.01$), with the largest differences occurring before dry-off (0.92 vs 3.32 ± 0.34 µmol/L at –3 DFD and 0.60 vs 5.19 ± 0.34 µmol/L at 0 DFD; Trt × T, $P < 0.01$). At 1 DFD, it tended to be lower in NR (4.42 vs 2.89 ± 0.34 µmol/L). During transition period, bilirubin was numerically greater in NR and differed significantly at 7 DFC (4.38 vs. 6.37 ± 0.51 µmol/L; $P < 0.01$), whereas the overall treatment effect only tended to be significant ($P = 0.08$). Concentration of AST-GOT tended to decrease in NR after dry-off, whereas the decrease was less evident in CTR (Trt × T, $P = 0.10$). Moreover, AST-GOT increased more after calving in NR (Trt × T, $P = 0.09$), in particular at 7 DFC (109 vs 170 ± 14.2 U/L in CTR and NR; $P < 0.01$). Alkaline phosphatase was overall lower in NR compared with CTR (67.0 vs 55.5 ± 2.14 U/L in CTR and NR, respectively; Trt, $P < 0.01$) and decreased starting from –3 DFD throughout the drying off period (Trt × T, $P < 0.01$). At calving, it was still overall lower in NR ($P = 0.01$), in particular up to 3 DFC (Trt × T, $P = 0.03$). GGT did not differ between treatments either around dry-off or around calving. The LFI tended to be higher in CTR than in NR (1.77 vs 0.47 ± 0.53; $P = 0.09$).

Among oxidative status biomarkers (Figure 3), myeloperoxidase was not different between groups at dry-off but was higher at calving in NR compared with CTR (396 vs 442 ± 15.2 U/L in CTR and NR, respectively; Trt, $P = 0.05$), in particular at 7 DFC ($P = 0.02$). ROM differed between groups at 4 DFD (16.12 vs. 14.35 ± 0.61 U/L in CTR and NR, respectively; $P = 0.04$), whereas the overall interaction was not significant (Trt × T, $P = 0.12$). AOPP had a different trend between groups (Trt × T, $P < 0.01$), with a decrease in NR at 0 and 1 DFD and higher values than CTR at 28 DFD. No treatment differences were observed for thiol groups around dry-off or calving, nor for ROM or AOPP during the transition period.

Among minerals (Figure 4), Ca showed a tendency for a different temporal pattern between treatments (Trt × T, $P = 0.08$), characterized by a peak in NR at 4 DFD (2.82 vs 2.95 ± 0.04 mmol/L in CTR and NR, respectively; $P = 0.01$). During transition period, it was higher in CTR (2.44 vs 2.33 ± 0.03 mmol/L in CTR and NR, respectively; Trt, $P < 0.01$), particularly immediately after calving (2.26 vs 2.04 ± 0.05 mmol/L at 3 DFC and 2.41 vs 2.20 ± 0.04

Table 3. Blood biomarkers around dry-off (from –7 to 14 d) and calving (from –14 to 28 d) in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d from dry-off to dry-off

	Around dry-off						Around calving					
	Treatment			*P*-value			Treatment			*P*-value		
Item, unit	CTR	NR	SEM	Trt	T	Trt × T	CTR	NR	SEM	Trt	T	Trt × T
Creatinine, µmol/L	92.1	101.9	1.78	<0.01	<0.01	<0.01	90.8	91.2	1.56	0.87	<0.01	0.35
Cholesterol, mmol/L	5.07	4.68	0.28	0.34	<0.01	0.12	2.81	2.61	0.14	0.31	<0.01	0.81
Paraoxonase, U/mL	102.2	95.6	4.80	0.34	<0.01	<0.01	97.0	89.5	4.48	0.25	<0.01	0.20
Bilirubin, µmol/L	1.44	2.25	0.21	0.01	<0.01	<0.01	2.69	3.31	0.24	0.08	<0.01	0.21
AST-GOT, U/L	108	111	4.95	0.66	<0.01	0.10	97.5	116.6	7.57	0.16	<0.01	0.07
GGT, U/L	33.3	29.4	1.87	0.15	<0.01	0.35	24.7	22.7	1.51	0.36	<0.01	0.96
Ceruloplasmin, µmol/L	2.66	2.61	0.07	0.64	0.54	0.36	2.79	2.83	0.08	0.72	<0.01	0.90
Haptoglobin, g/L	0.12	0.11	0.01	0.50	0.10	0.35	0.26	0.33	0.04	0.26	<0.01	0.37
Total protein, g/L	79.6	81.0	1.01	0.34	<0.01	0.08	73.7	75.9	1.12	0.17	<0.01	0.73
Albumin, g/L	37.4	38.0	0.41	0.30	<0.01	0.17	36.0	35.3	0.35	0.19	<0.01	0.02
Globulin, g/L	42.2	43.0	1.13	0.64	<0.01	0.07	37.7	40.6	1.19	0.10	<0.01	0.38
A:G	0.89	0.89	0.03	0.96	0.04	0.09	0.98	0.90	0.04	0.12	<0.01	0.08
Thiol groups, µmol/L	380	386	11.3	0.68	<0.01	0.60	329	320	8.33	0.46	0.36	0.31
Myeloperoxidase, U/L	396	400	16.9	0.86	0.01	0.86	396	442	15.1	0.05	<0.01	0.84
AOPP, µmol/L	52.6	50.3	1.82	0.32	<0.01	<0.01	45.2	45.5	2.08	0.91	<0.01	0.58
ROM, mgH_2O_2/100mL	15.3	14.8	0.45	0.49	0.22	0.12	14.0	14.6	0.46	0.32	<0.01	0.92
Alkaline phosphatase, U/L	66.9	55.5	2.15	<0.01	<0.01	<0.01	50.4	40.5	2.57	0.01	<0.01	0.03
Ca, mmol/L	2.72	2.71	0.02	0.65	<0.01	0.08	2.44	2.33	0.03	0.01	<0.01	0.01
P, mmol/L	2.19	2.15	0.06	0.61	<0.01	0.03	1.66	1.61	0.05	0.48	<0.01	0.04
Mg, mmol/L	1.01	1.00	0.02	0.58	<0.01	<0.01	0.94	0.94	0.02	0.84	<0.01	0.06
Na, mmol/L	149	149	0.22	0.61	<0.01	0.07	148	148	0.37	0.80	<0.01	0.30
K, mmol/L	4.26	4.26	0.06	0.95	0.01	0.62	4.07	4.11	0.05	0.56	<0.01	0.98
Cl, mmol/L	106	106	0.38	0.39	<0.01	0.04	107	108	0.43	0.17	<0.01	0.68
Zn, µmol/L	13.1	13.9	0.60	0.36	<0.01	0.29	11.0	10.8	0.73	0.82	0.25	0.15

[1]AST-GOT = aspartate amino transferase-glutamate oxaloacetate transaminase; GGT = γ-glutamyl transferase; A:G = albumin-to-globulin ratio; AOPP = advanced oxidation protein products; ROM = reactive oxygen metabolites.

mmol/L at 7 DFC; $P < 0.01$). P levels had a more marked peak at 1 DFD in CTR compared with NR (2.76 vs 2.31 ± 0.11 mmol/L; $P < 0.01$) and were higher in CTR at 3 DFC (1.59 vs 1.21 ± 0.10 mmol/L; $P < 0.01$). Mg showed a different temporal pattern between groups (Trt × T; $P < 0.01$), because it decreased in NR before dry-off and was greater than CTR at 14 DFD. Similarly, Cl dropped in NR before dry-off (Trt × T, $P = 0.04$), particularly at 0 DFD. No treatment differences were observed in Mg or Cl during the transition period, nor overall in Na or K concentrations.

Milk SCC and mammary gland immunity

Overall, SCC was greater before dry-off in NR than in CTR (Trt; $P < 0.01$; Figure 5), because of the marked increase observed at –3 and 0 DFD (Trt × T; $P < 0.01$). No difference was detected after calving. Results of milk bacteriological cultures are reported in Table 4. Quarter-level bacteriological findings are presented descriptively because of the limited number of infected quarters. No clear treatment-related differences in quarter bacteriological status were detected at enrollment or after calving, and most quarters were uninfected in both groups at 7 and 28 DFC. Results of flow cytometry analysis on milk cells collected during nutrient restriction are shown in Table 5. No treatment differences were detected in the proportions of viable cells or in the selected milk-cell populations evaluated by flow cytometry (CD3+, CD4+, CD8+, CD11b+, and CD4+:CD8+ ratio).

DISCUSSION

Dietary restriction is one of the most commonly used strategies to reduce excessive milk yield at dry-off. In the companion paper (Cattaneo et al., 2024), we showed how severe nutrient restriction at dry-off can effectively decrease yield and may have promoted earlier onset of involution, despite large effects on metabolism. The nutrient restriction adopted in the companion paper was approximately equal to 40% of the lactation diet and reduced milk yield by 62% before dry-off (NR cows produced ~8 kg/d the day before dry-off, compared with approximately 22 kg/d in CTR). Restricted cows showed a marked decline in plasma glycemia (−10% at dry-off), increased NEFA and BHB concentrations (up to 1.18 and 0.97 mmol/L on average in NR at dry-off), and milk compositional changes consistent with reduced mammary synthetic activity. In early lactation, milk yield did not differ between groups, but DMI was transiently

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lower in NR cows during the second week after calving, and NEFA tended to be higher at 7 DFC. These findings provide the metabolic context for interpreting the present inflammatory and hepatic biomarker responses.

Impact during the pre-dry-off restriction and immediate post-dry-off period

In the companion paper, we speculated that mammary involution process may have started before dry-off in the NR cows, based on the increase in milk protein and the decrease in lactose content, that might be interpreted as markers of increased permeability of the mammary epithelium (Hurley, 1989; Zhao et al., 2019). Consistent with our hypothesis, SCC increased over time during the restriction period in NR cows. This increase was not accompanied by clear evidence of a treatment-related difference in intramammary infection status. The increase was likely due in part to the lower milk volume, which would increase somatic cell concentration (Boutinaud and Jammes, 2002; Green et al., 2006). Additionally, during involution, there is a higher rate of cell exfoliation, which increases the number of mammary epithelial cells in milk (Herve et al., 2019), and a migration of immune cells from the bloodstream (Atabai et al., 2007; Putman et al., 2018; Mezzetti et al., 2020), that together contribute to the higher SCC. One of the mechanisms by which dietary restriction is known to reduce milk yield is reducing the number and the activity of mammary epithelial cells in the udder (Dessauge et al., 2011). Moreover, it causes an increase in tight junction leakage and a disruption in mammary epithelium integrity (Herve et al., 2016), similarly to what happens during mammary involution (Zhao et al., 2019). Nevertheless, we could not determine clearly whether these variations represented earlier involution-related changes or simply reflected disruption of the mammary epithelium. Decreased milk citrate can be interpreted as a marker of mammary involution (Nonnecke and Smith, 1984) but in our study its concentration increased in restricted cows. This result agrees with Ollier et al. (2014) and is related with the typical increase in milk citrate occurring with nutrient restriction (Bjerre-Harpøth et al., 2012; Leduc et al., 2021). In this condition, plasma insulin and milk de novo fatty acids are reduced, reducing the need for NADPH (Bell and Bauman, 1997), for which citrate is a precursor (Faulkner and Peaker, 1982). Further studies are needed to determine more precisely the effect of such a severe dietary restriction on mammary involution.

The blood count data were also consistent with a shift in the timing of systemic and mammary-related responses. During involution, leukocyte count greatly increases in the mammary gland (Sordillo and Nickerson, 1988). Thus, the lower leukocyte concentration observed in blood at dry-off in restricted cows may reflect leukocyte redistribution toward peripheral tissues. It is possible to hypothesize a migration of leukocytes toward the mammary gland (Atabai et al., 2007), but leukocyte trafficking to the gastrointestinal tract or other tissues may also have contributed (Janssen et al., 2023). The trends of individual

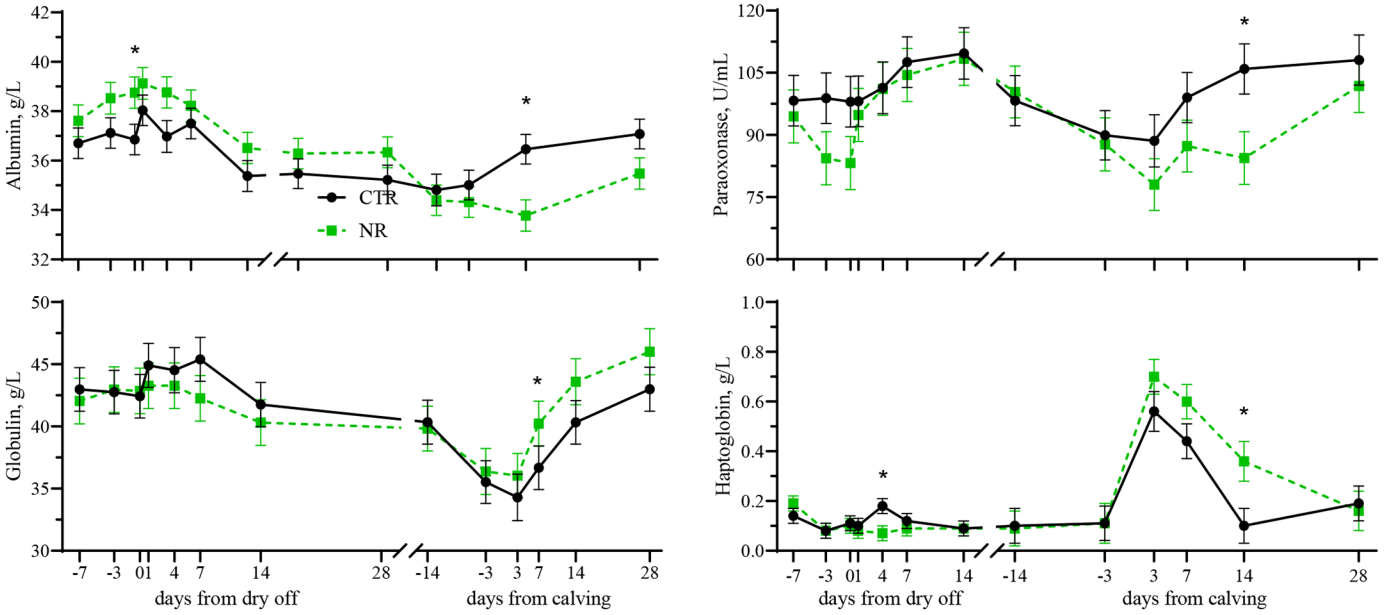


Figure 1. Plasma concentrations of inflammatory and APP-related biomarkers (i.e., albumin, globulin, paraoxonase, and haptoglobin) from –7 to 7 d from dry-off and from –14 to 28 d from calving in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d before dry-off to dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SEM.

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leukocyte populations during the last week of lactation in the restricted cows were comparable to those of control cows during the first week of dry period. In fact, neutrophils and monocytes decreased, whereas eosinophils increased. Similarly, previous studies reported comparable trends after dry-off (Putman et al., 2018; Mezzetti et al., 2020; Gao et al., 2024). During involution, there is an immune system activation, which recruits immune cells, mainly neutrophils and monocytes, to the target tissue (i.e., the mammary gland) (Atabai et al., 2007). The increase in eosinophil proportion, observed at dry-off in NR cows and later in CTR cows, was consistent with the previous reports describing leukocyte dynamics during mammary involution. Eosinophils are commonly associated with allergic and parasitic responses (Gouon-Evans et al., 2000) and their increase after milking cessation has been interpreted as a possible reaction to residual milk components in the mammary gland (Putman et al., 2018). However, the earlier increase observed in NR cows suggests that this response may not depend solely on milk accumulation. Studies in other species indicate that eosinophils may also participate in tissue remodeling processes during mammary gland development and involution (Gouon-Evans et al., 2000) and they are also present in greater amounts in the involuting mammary gland in mice (Stein et al., 2004). Therefore, the temporal pattern observed here may reflect immune-cell recruitment associated with mammary remodeling during involution, although the specific role of eosinophils in this process remains unclear and warrants further investigation. The lack of differences in milk flow cytometry should be interpreted cautiously and only within the limits of the selected markers analyzed. These data do not indicate treatment-related changes in the specific milk-cell subpopulations evaluated, but they do not exclude effects on other mammary immune pathways. The panel

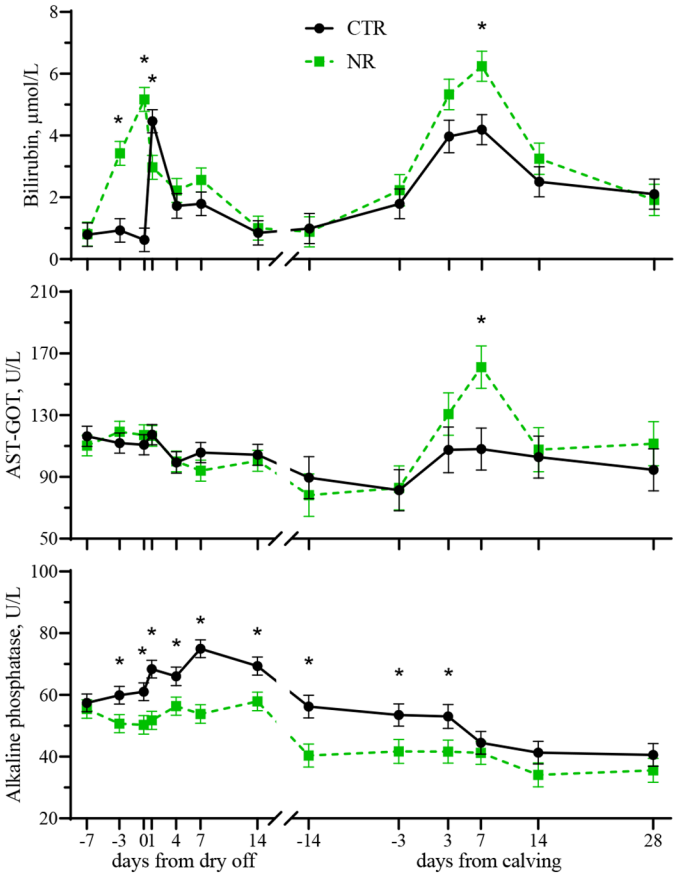


Figure 2. Plasma concentrations of liver function biomarkers (i.e., bilirubin, aspartate amino transferase-glutamate oxaloacetate transaminase [AST-GOT], and alkaline phosphatase) from –7 to 7 d from dry-off and from –14 to 28 d from calving in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d before dry-off to dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SEM.

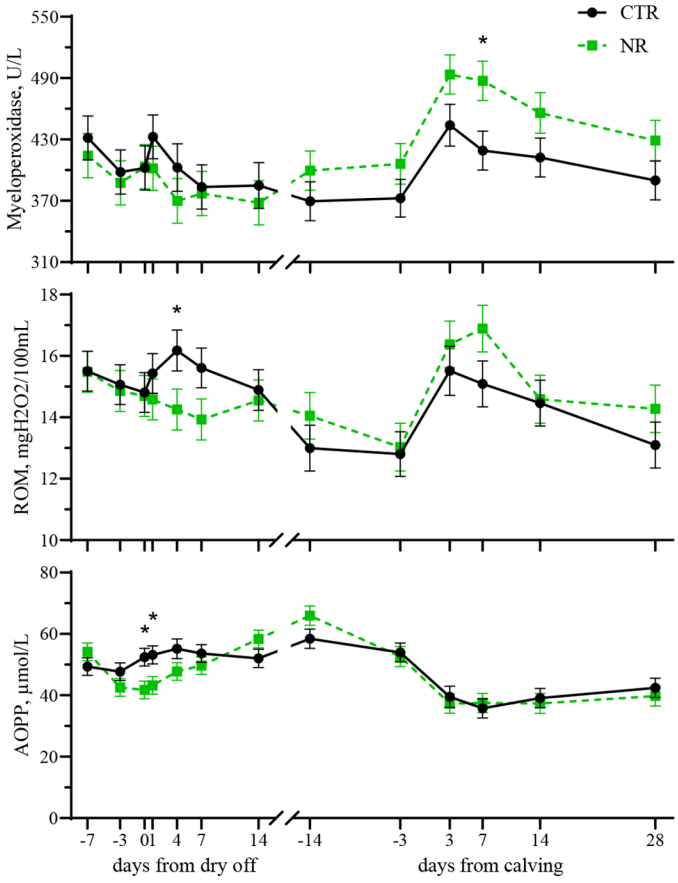


Figure 3. Plasma concentrations of oxidative status biomarkers (i.e., myeloperoxidase, reactive oxygen metabolites [ROM], and advanced oxidation protein products [AOPP]) from –7 to 7 d from dry-off and from –14 to 28 d from calving in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d before dry-off to dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SEM.

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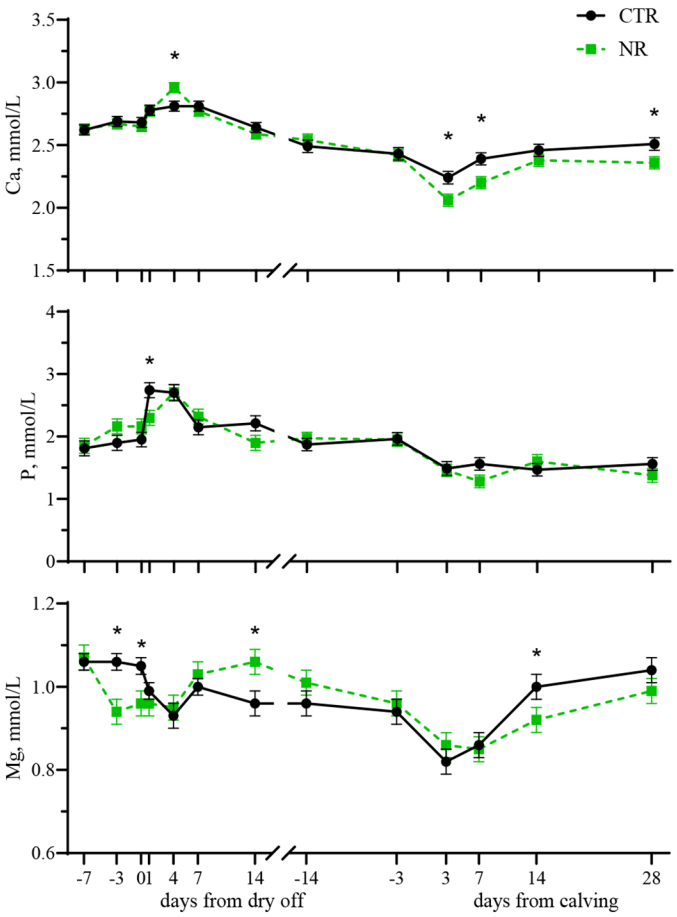


Figure 4. Plasma concentrations of minerals (Ca, P, and Mg) from –7 to 7 d from dry-off and from –14 to 28 d from calving in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d before dry-off to dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SEM.

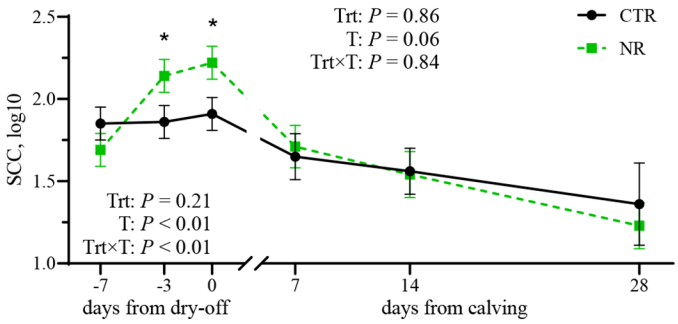


Figure 5. Milk SCC (log10-transformed) in the last week before dry-off and in the first month of lactation in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d before dry-off to dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported in the text box. Error bars represent SEM.

mainly targeted selected lymphocyte-related populations and did not comprehensively assess the innate immune cell populations that are likely to play a major role during involution. In addition, sampling was restricted to the pre-dry-off period, so this component cannot be used to infer immune changes occurring after milk cessation. Also, our sample size could have been too small to detect significant differences in these secondary outcomes.

Nevertheless, consistently with the blood energy status markers reported in (Cattaneo et al., 2024), many changes were noted between groups in the markers of inflammatory condition, liver function, immunity, and minerals. First, despite being mild, the increase in haptoglobin concentration after dry-off in the CTR highlighted that the temporal pattern of response to dry-off differed between groups. Haptoglobin, besides being a positive APP, is also involved in tissue remodeling (Arslan et al., 2013). Its increase was consistent either with a more pronounced inflammatory response or with a greater tissue-remodeling demand, possibly related to the higher milk yield before dry-off and the subsequent milk accumulation and residuals in the udder (Silanikove et al., 2013). The changes observed in other blood metabolites (i.e., ROM and AOPP), although limited, were directionally consistent with this interpretation, since they are, respectively, markers of inflammation processes and products of protein oxidation exerted by hypochlorous acid, that is produced by myeloperoxidase during neutrophils activation (Celi and Gabai, 2015). However, myeloperoxidase similarly increased immediately after dry-off in both groups and neutrophils were not different. Myeloperoxi-

Table 4. Distribution of infected quarters at –21 d from dry-off (DFD), 7 d from calving (DFC), and 28 DFC in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 DFD to dry-off

	–21 DFD		7 DFC		28 DFC	
	CTR (n = 46)	NR (n = 43)	CTR (n = 47)	NR (n = 44)	CTR (n = 46)	NR (n = 43)
Uninfected	76%	74%	89%	93%	98%	91%
Infected with major pathogens[1]	0%	7%	9%	5%	2%	2%
Infected with minor pathogens[2]	24%	19%	2%	2%	0%	7%

[1]*Staphylococcus aureus*, *Streptococcus uberis*, *Enterobacter* spp., *Escherichia coli*.
[2]Coagulase-negative staphylococci and *Corynebacterium bovis*.

Table 5. Flow cytometric analysis of leukocyte populations in milk collected at –7, –3, and 0 d from dry-off in high-producing dairy cows fed either *ad libitum* ryegrass hay (NR) or lactation diet (CTR) from –7 d from dry-off to dry-off

	Treatment			*P*-value		
Item	CTR	NR	SEM	Trt	T	Trt × T
Viable cells, %	29.7	30.5	3.08	0.86	0.14	0.66
$CD3^+$, %	57.0	58.7	2.62	0.65	0.02	0.82
$CD4^+$, %	29.2	31.0	1.67	0.47	<0.01	0.36
$CD8^+$, %	21.9	18.5	1.55	0.13	0.05	0.90
$CD4^+$:$CD8^+$	2.01	2.06	0.33	0.91	0.06	0.82
$CD11b^+$, %	10.5	11.3	1.29	0.66	0.59	0.22

dase is a marker of neutrophil activation, that commonly occurs at milking cessation (Putman et al., 2018; Mezzetti et al., 2020).

Albumin, globulin, and total protein showed minor differences before dry-off, although these differences were not consistently supported by the overall model effects. Their increases may reflect mild hemoconcentration associated with reduced feed intake and water balance changes during the restriction period, as suggested by the slightly higher hematocrit values (Eckersall, 2008). Additionally, the sudden increase in both albumin and globulin in the CTR group the day after dry-off was likely related to the milking cessation (Putman et al., 2018; Mezzetti et al., 2020). Although globulin includes several positive acute-phase proteins that increase during inflammation, previous studies using feed-restriction models have not consistently reported systemic inflammatory responses during the restriction period (Pascottini et al., 2019). In contrast, other indicators suggested some degree of altered hepatic function, as paraoxonase concentrations were lower and bilirubin concentrations were higher in NR cows. Paraoxonase is synthesized by the liver and its activity decreases during inflammation, liver dysfunction, or intense lipid mobilization (Bionaz et al., 2007), whereas bilirubin accumulation reflects reduced hepatic clearance (Bertoni and Trevisi, 2013). The greater plasma alkaline phosphatase concentration in CTR throughout the dry-off period is difficult to interpret biologically, because other markers of liver damage (AST-GOT and GGT) did not indicate overt hepatic impairment. Because total alkaline phosphatase includes isoforms from different tissues, this difference may reflect nonspecific variation in tissue turnover or metabolic adaptation rather than a clear alteration in hepatic function.

Plasma Ca and P showed small and transient differences between treatments around dry-off, the biological relevance of which is uncertain. Plasma P increased briefly in CTR cows immediately after dry-off, consistent with previous observations in high-yielding cows at this stage (Mezzetti et al., 2020). In contrast, Mg dynamics likely reflected differences in dietary intake and rumen conditions. Body reserves of Mg are limited, and plasma concentrations depend largely on dietary supply. In the present study, NR cows likely had lower Mg intake and reduced ruminal fermentation because of the hay-based restriction diet, conditions that may decrease Mg absorption (Fontenot et al., 1989).

Impact on the subsequent transition and early lactation periods

Recently, it has been observed that inflammatory condition at dry-off can influence the adaptation to the subsequent calving (Cattaneo et al., 2021a; Goetz et al., 2022). Moreover, the origin of some diseases takes place way before the onset of the illness (Mezzetti et al., 2019, 2021). Thus, applying drastic protocols during the dry-off phase may affect the ensuing transition period. Moreover, dietary restriction can have long-lasting effects. In the companion study, we observed reduced milk yield and DMI in cows that were nutrient restricted at dry-off, paired with an increase in NEFA post-calving. In the present study, several plasma markers of inflammation, liver function, and oxidative status suggested a poorer systemic condition shortly after calving in NR cows. Both positive (globulin and haptoglobin) and negative APP-related indicators (albumin, paraoxonase, and bilirubin) suggested a slower recovery of homeostasis after calving in NR cows. Moreover, consistently with bilirubin, AST-GOT peaked higher in NR cows, particularly at 7 DFC, which is consistent with greater hepatic challenge (Bertoni et al., 2008). Myeloperoxidase was higher as well, suggesting that also the antioxidant system was altered. The numerically lower LFI was directionally consistent with the other biomarkers and supported the interpretation of less favorable adaptation to lactation in NR cows.

Pascottini et al. (2019) attempted to induce systemic inflammation 2 weeks before calving using a feed-restriction model to mimic the drop in feed intake typical of the period before parturition. Although systemic inflammation was not clearly induced and the level of mobilization

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achieved was lower than in our study, restricted cows showed a numerical increase in haptoglobin immediately after calving, suggesting that nutritional stress before calving may influence the subsequent inflammatory response. In one of our previous studies (Cattaneo et al., 2023b), we pursued the opposite objective by supplementing *Aloe arborescens* at dry-off to mitigate the inflammatory response during this phase. Interestingly, cows receiving Aloe before dry-off exhibited a milder increase in plasma haptoglobin and ceruloplasmin after calving, compared with control cows. Together with previous findings showing that inflammatory status before dry-off is associated with postpartum performance (Cattaneo et al., 2021a), these results support the concept that physiological conditions occurring well before calving may affect the inflammatory response during the subsequent transition period (Mezzetti et al., 2019; Horst et al., 2021). The mechanisms underlying these carryover effects remain unclear but may involve alterations in metabolic status that influence hepatic function and systemic inflammatory regulation.

Overall, these results suggest that severe nutrient restriction before dry-off may modify the short-term inflammatory response associated with milking cessation and may also impair metabolic and inflammatory adaptation during the subsequent transition period. Although reducing milk yield before dry-off is often recommended to facilitate mammary involution, the present results indicate that severe restriction strategies should be carefully evaluated because of potential carryover effects into the next lactation. Further research is needed to clarify the mechanisms linking pre–dry-off nutritional management with systemic inflammatory responses after calving.

CONCLUSIONS

The present study confirms that dry-off is associated with changes in blood cell counts and plasma biomarkers related to inflammation and hepatic status. Severe pre-dry-off nutrient restriction increased milk SCC during the restriction period, but no clear treatment-related differences in postpartum mammary health were detected. Overall, the results suggest that severe nutrient restriction before dry-off altered the temporal pattern of dry-off-related responses and may have attenuated some immediate post-dry-off inflammatory indicators. However, when interpreted together with the companion paper, this strategy was associated with a pronounced metabolic challenge before dry-off and with less favorable inflammatory and hepatic biomarker profiles during the subsequent transition period. These findings raise the possibility that excessive negative energy balance at dry-off may induce longer-term alterations in immune and hepatic function. Therefore, pre-dry-off strategies aimed at reducing milk yield should be evaluated not only for short-term dry-off responses but also for their carryover effects into the next lactation.

NOTES

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Nonstandard abbreviations: A:G = albumin-to-globulin ratio; AOPP = advanced oxidation protein products; APP = acute-phase protein; AST-GOT = aspartate amino transferase-glutamate oxaloacetate transaminase; CTR = control group; DFD = days from dry-off; DFC = days from calving; GGT = γ-glutamyl transferase; LFI = Liver Functionality Index; NR = nutrient restricted group; ROM = reactive oxygen metabolites.

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