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## Inflammatory status and metabolic changes at dry-off in high-yield dairy cows

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### ABSTRACT

Our study investigates metabolic changes occurring at dry-off and the contribution of milk yield (MY) in such alterations. Thirteen Holsteins were dried off at 55 days from expected calving day (assumed as 0 days from dry-off, DFD) and divided in two groups according to their average daily MY in the last week of lactation, assuming a cut-off of 15 kg·d<sup>-1</sup>: low MY (7 cows) and high MY (6 cows). From -7 to 34 DFD dry matter intake (DMI) and rumination time were measured. Blood samples were collected at -7, 2, 7, 27 and 34 DFD to assess an haematological and metabolic profile and at -7, 7 and 34 DFD to test functions of circulating white blood cell (WBC) through *ex vivo* challenges. Data were included in a mixed model for repeated measures assuming MY at dry-off, time and their interaction as fixed effects. After dry-off, DMI was reduced and rumination time was increased in all the animals. High MY cows had greater DMI and rumination time than low MY cows. In blood, WBC counts decreased at 7 DFD and increased the production of pro-inflammatory cytokines at 7 and 34 DFD. Plasmatic concentrations of liver enzymes indicators, positive acute phase proteins (APPs); and nitrogen species increased after dry-off. Conversely, negative APPs and antioxidant species decreased. Those alterations were more marked in high MY animals. This study suggests that dry-off decreased liver function, triggered a systemic inflammation and depleted antioxidant systems, especially in the group of cows with high MY at dry-off.

### HIGHLIGHTS

- Inflammation, liver dysfunctions and altered redox balance has been detected after dry-off in all the animals.
- Cows with highest milk yield before halting of milk removal faced the most severe metabolic challenges.
- Such finding indicates the management of dry-off as a key point for dairy cows health.

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## Introduction

The dry period of dairy cows is defined as the time between cessation of milking and resumption of milk production at a subsequent calving. This phase allows the development of the mammary gland and the turnover of the secretory tissue (Oliver and Sordillo 1988) and its essential to maximise milk yield in the following lactation. At dry-off, milk residuals in the mammary gland induce cellular distension and stimulate the release of autocrine mediators (i.e. feedback inhibitor of lactation protein) that act as inhibitor of milk synthesis (Wilde et al. 1998; Capuco and Akers 1999), while fragments arise from the hydrolysis of caseins

acts as putative mediators of mammary gland involution (Shamay et al. 2003).

At dry-off, the priority in redistribution of energy and protein sources shift from mammary gland to foetus (Dingwell et al. 2001). Adaptation to dry ration decreases the surface area of rumen papillae (Dieho et al. 2016). Furthermore, halting of milk removal affects mammary gland both at gross and cellular levels, increasing the susceptibility to infections (Putman et al. 2018). Finally, the hunger related to feed restriction and the modification of social structure after regrouping could induce psychological stress in weaker animals (von Keyserlingk et al. 2008).

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 Supplemental data for this article can be accessed [here](#).

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Nevertheless, the dry-off did not receive as much attention as other challenging periods, such as transition to calving, and only recent concerns about animal welfare promoted a deeper investigation of dry-off as a cause of metabolic stress (Odensten et al. 2005; Zobel et al. 2015). Consequently, milk yield higher than  $12.5 \text{ kg} \cdot \text{d}^{-1}$  (intended as a sum of the milking bouts at the day of dry-off) has been identified as a negative factor for dairy cow's health (Rajala-Schultz et al. 2005). In fact, increased udder pressure that follows milking interruption could induce severe pain (Silanikove et al. 2013) triggering a raise of stress-related hormones immediately after dry-off (Bertulat et al. 2013). Glucocorticoids and catecholamines interact with specific receptors on immune cells regulating different pathways (i.e. nuclear factor kappa-light-chain-enhancer of activated B-cells and cyclic AMP response element binding protein). Such interactions modify the transcription of genes encoding for a variety of cytokines. Altered cytokines production could dysregulate immune function up to early lactation, with a sufficient magnitude to have health implications (Padgett and Glaser 2003; Trevisi et al., 2015), and suggesting stress occurring at dry-off to be related with severe immune dysfunction happening during the periparturition period of dairy cows (Trevisi et al. 2010). Although a wide background exists regarding the 'safety threshold' to dry-off a cow, none evaluated the contribution of high or low MY prior to dry-off in affecting metabolic changes after milking interruption.

Based on above, our objective is to investigate some aspects of metabolic, oxidative and immune changes occurring after an abrupt dry-off during the whole dry period of cows having an average milk yield higher or lower than  $15 \text{ kg} \cdot \text{d}^{-1}$  in the last week of lactation. Such a threshold has been chosen according to the safety level proposed by Rajala-Schultz et al. (2005) to drying off a cow. Our hypothesis is that abrupt dry-off can negatively affect inflammatory parameters during the dry period, especially in cows with high milk yields at the end of lactation.

## Materials and methods

### Experimental design and animal management

The trial was carried out at Università Cattolica del Sacro Cuore research dairy barn (Experiment Station, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 1047/2015-PR). A group of 13 Italian Holsteins dairy cows (number of lactations:  $1.9 \pm 1.1$ ; milk yield in the last lactation:  $11,547.8 \pm 2576 \text{ kg}$ ; average lactation length:  $353.1 \pm 54$

days [mean  $\pm$  SD]) were raised in individual tied stalls with controlled environmental conditions (room temperature of  $20^\circ \text{C}$ , relative humidity of 65%, 14 hours of light) and dried off at 55 days from expected calving day (assumed as 0 days from dry-off, DFD) with a deep milking and a treatment with a mammary antibiotic (Mamyzin A; Haupt Pharma Latina S.r.l, Borgo San Michele – Latina, Italy). Before dry-off, cows were milked twice a day, at 4:00 am and pm, and milk yield was recorded. All the cows were individually fed with a component diet. Before dry period, animals received 1 kg of concentrate every 3 kg of produced milk. Since  $-7$  days from dry-off the concentrate was gradually reduced till the complete elimination at dry-off (Phase 1). After dry-off, animals received only grass hay till 10 DFD. From 11 DFD till the end of the experimental period, animals received a hay-based ration with soybean meal and corn silage (Phase 2). Same batches of hays and corn silage were used during the trial. Feeds were collected fortnightly and, after dry matter determination, samples were pooled for subsequent analyses. Feeds and diet composition are shown in Table 1.

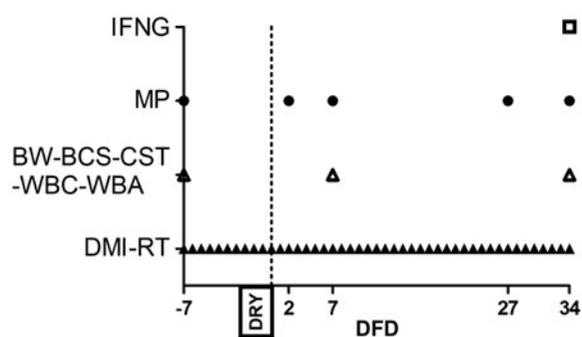
**Table 1.** Composition and characteristics of the experimental diets fed during the two experimental phases.

	Phase 1	Phase 2
DFD	-7; 0	10; 34
Diet, % DM		
Item		
Corn silage	28.50	18.60
Alfalfa hay	16.40	-
Grass hay	23.40	71.40
Concentrate (dry period)	-	10.00
Concentrate (lactation period)	31.70	-
Concentrate composition, %DM		
Corn flour	-	40.00
Barley flour	-	1.40
Soybean meal	90.50	13.10
Sunflower meal	-	4.90
Beet pulp	-	16.60
Wheat bran	-	9.80
Beet molasse slops	-	2.60
Potato protein	-	2.20
Hydrogenated palm oil	-	3.30
Limestone	-	1.39
Dicalcium phosphate	-	1.80
Sodium bicarbonate	-	0.98
Magnesium oxide	2.20	0.64
Sodium chloride	1.40	0.32
Supplement <sup>a</sup>	5.90	1.07
Chemical composition		
NE <sub>L</sub> , Mcal/kg of DM	1.59	1.45
Crude protein, % DM	14.90	13.60
Starch + sugar, % DM	23.70	16.80
Ether extract, % DM	3.80	1.80
NDF, % DM	39.40	49.30
MP <sup>b</sup> , %CP	9.80	9.10
RUP <sup>b</sup> , %CP	4.64	4.48

Between dry-off day ( $-55$  days from expected calving) and 10 days from dry-off (DFD) cows received only grass hay.

<sup>a</sup>Supplements were composited to provide 150,000 U of vitamin A, 10,000 U of vitamin D, 200 mg of vitamin E, 100 mg of vitamin K, 100 mg of vitamin H1, 50 mg of vitamin B1, 0.5 mg of vitamin B12, 500 mg of vitamin PP, 4,000 mg of choline, 350 mg of Mn, 800 mg of Zn, 40 mg of Cu, 20 mg of I, 1 mg of Co, 1 mg Se.

<sup>b</sup>Estimate using NRC 2001.



**Figure 1.** Scheduled time point, expressed as day from dry off (DFD), for dry matter intake and rumination time measurement (DMI-RT), body weight and body condition score determination, white blood cells profiling, whole blood stimulation assay and carrageenan skin test performance (BW-BCS-WBC-WBA-CST), haematic metabolic profile (MP), interferon gamma release assay (IFNG).

From  $-7$  to  $34$  DFD periodical checks were performed and blood samples were collected regularly, according to the time schedule shown in Figure 1 and described in the following sections. In addition, the health status of cows was monitored recording all clinical diseases appeared from  $-7$  DFD till 30 days from subsequent calving. To investigate the effect of milk yield, after dry-off animals were retrospectively divided in two groups according to their average milk yield in the last week of lactation ( $19.9 \pm 8.6 \text{ kg} \cdot \text{d}^{-1}$ ), assuming a cut-off of  $15 \text{ kg} \cdot \text{d}^{-1}$ : low milk yield (LM; 7 cows; average milk yield in the last week of lactation:  $10.6 \pm 3.7$ ; milk yield in the last lactation:  $10,984 \pm 2477 \text{ kg}$ ; average length of the last lactation:  $360.6 \pm 51$  days [mean  $\pm$  SD]) and high milk yield (HM; 6 cows; average milk yield in the last week of lactation:  $16.5 \pm 5.3$ ; milk yield in the last lactation:  $11,503 \pm 2402 \text{ kg}$ ; average length of the last lactation:  $340.0 \pm 50$  days [mean  $\pm$  SD]).

### **Body weight, body condition score, dry matter intake and rumination time**

The body weight was measured with a single walking-in scale, and the body condition score (BCS) was determined from the same operator with a 1–5 scale (Agricultural Development and Advisory Service 1986) at  $-7$ , 7 and 34 DFD. BCS variation ( $\Delta$ BCS) was calculated as the difference between data at  $-7$  and 34 DFD. The daily dry matter intake was measured weighting the amounts of feed administered and residuals for each cow. Rumination time was registered using the Ruminact system (SCR Europe, Podenzano, PC, Italy) and expressed on a daily base (Figure 1).

### **Health status**

The body temperature was measured daily with a rumen bolus (DVM System TempTrack™, HerdStrong, LLC, Greeley, CO). Mastitis were diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases, retained placenta when the foetal membranes were not expelled within 24 h after calving, endometritis and metritis according to Sheldon et al. (2006), milk fever, displacement of abomasum and pneumonia by a veterinary diagnosis. Diarrhoea was diagnosed on visual evaluation of faeces consistency and colour through the faecal score method (Ireland-Perry and Stallings 1993), assuming diarrhoeic faeces those have a faecal score  $\leq 2$ .

### **Blood samples collection**

Blood samples were harvested through jugular venipuncture in evacuated collection tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) before the morning feeding. Samples were used to perform different assays (Figure 1).

### **Metabolic profile assessment**

For metabolic profile assessment (Figure 1), samples were collected at  $-7$ , 2, 7, 27 and 34 DFD into heparinised tubes and processed as described by Calamari et al. (2016). After collection, samples were centrifuged, and packed cells volume was determined. A clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington, MA) was used to determine the concentration of glucose, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, aspartate amino transferase-glutamate oxaloacetate transaminase (AST-GOT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total protein, haptoglobin, ceruloplasmin, albumin, total bilirubin, cholesterol and globulin in accordance with Calamari et al. (2016). Furthermore, reactive oxygen metabolites (ROMt), ferric reducing antioxidant power (FRAP), nitrate ( $\text{NO}_3$ ), nitrite ( $\text{NO}_2$ ) and nitric oxides ( $\text{NO}_x$ ) were determined according to Jacometo et al. (2015), paraoxonase according to Bionaz et al. (2007), thiol groups according to Minuti et al. (2014), myeloperoxidase according to Bradley et al. (1982) and advanced oxidation protein products (AOPP) according to Hanasand et al. (2012). Finally, L- and D-lactic acid were determined with a commercial kit (K-DLATE, Megazyme Co., Wicklow, Ireland). A multi-detection microplate reader (BioTek Synergy 2, Winooski, VT) and commercial kits for ELISA method

were used to determine the concentration of interleukin 1-beta (IL-1B; ESS0029; Thermo Scientific, Frederick, MD) and interleukin 6 (IL-6; ESS0027; Thermo Scientific, Frederick, MD) according to Jahan et al. (2015) and those of serum amyloid alpha (SAA; TP-802, Tridelta D.L., Ireland). Commercial kits for cytokines were validated for cell culture media, and, thus, a validation on plasma has been performed previously (Mezzetti et al. 2019). Furthermore oxygen reactive antioxidant capacity (ORAC) were determined with a fluorometric method according to Jacometo et al. (2015). Retinol, tocopherol and  $\beta$ -carotene were analysed by reverse-phase HPLC (LC-4000, Jasco Europe, Carpi MO, Italy), as described by Jahan et al. (2015). Further details on the analytical procedures adopted in blood analysis are reported in [Supplementary File 1](#).

### **White blood cells profile**

For the white blood cells (WBC) profile (Figure 1), samples were collected with K-EDTA tubes at -7, 7 and 34 DFD and analysed with Cell-DYN 3700 (Abbott Diagnostic Division, Santa Clara, CA). A laser optic assay was used to determine the amounts of total WBC, neutrophils, monocytes and eosinophils. The amount of red blood cells, haematocrit, number of platelets and mean platelet volume were determined via electrical impedance assay. The amount of haemoglobin was determined using spectrophotometry assay.

### **Whole blood stimulation assay**

For whole blood stimulation assay (WBA, Figure 1), blood samples were collected at -7, 7 and 34 DFD with heparinised serum tubes and stimulated with 0 (baseline), 0.01 (low dose; L) and 5  $\mu$ g/mL (high dose; H) of bacterial lipopolysaccharides (LPS, *Escherichia coli* O111:B4; Sigma-Aldrich Company Ltd., Cambridge, UK, Cat. No. L3012), according to the method of Røntved et al. (2005), adapted by Jahan et al. (2015). After WBA, plasma samples were stored at  $-80^{\circ}\text{C}$  for the measurement of glucose, L- and D-lactic acid, IL-1B, IL-6,  $\text{NO}_x$ ,  $\text{NO}_2$  and  $\text{NO}_3$ . Variation of plasma parameters after WBA with L and H doses of LPS were expressed as fold change relative to the baseline.

### **Interferon gamma release assay**

For the interferon gamma (IFNG) release assay, whole blood samples were also collected into heparinised tubes at 34 DFD (Figure 1). After collection, the tubes were stored in vertical position in a warm bath at a temperature of  $38^{\circ}\text{C}$  and transported to the

laboratory within 20 min for the stimulation procedure. Whole blood was used in an IFNG release assay for *Mycobacterium avium* (internal method IZSLER, MP 13/011). Briefly, two 1-mL aliquots of each blood sample were distributed in a 24-well plate. One well was supplemented with 100  $\mu$ l of a 1:10 dilution of *Mycobacterium avium* purified protein derivative (PPD, IZS Umbria e Marche, Perugia, Italy) to PBS, and 1 well with 100  $\mu$ l of sterile PBS as control. The plate was positioned in a heated incubator (Grant Boekel, HIR10 M) set to a temperature of  $38^{\circ}\text{C}$  and with a relative humidity of 95% for 24 h. After incubation, the blood was centrifuged at  $8500 \times g$  for 16 min at  $4^{\circ}\text{C}$  and plasma was stored at  $-20^{\circ}\text{C}$  until use. Plasma was later thawed and analysed in a sandwich ELISA assay for bovine IFNG with a couple of monoclonal antibodies, as previously described (Trevisi et al. 2014). Results were evaluated in terms of optical density difference ( $\Delta$ OD) between avian PPD-stimulated and control wells.

### **Carrageenan skin test**

The carrageenan skin test (CST) was performed according to the method of King (1993), adapted by Jahan et al. (2015), to evaluate peripheral immune responses at -7, 7 and 34 DFD (Figure 1). The skin thickness was measured using a skinfold calliper (cat# 470119-588, VWR, USA) immediately before carrageenan injection (0 days), then 2 and 9 days after the injection. The total response to each challenge was calculated as the area under the curve of the thickness, measured at day 2 and day 9, subtracting the thickness measured at day 0.

### **Statistical analysis**

Data in the tables are presented as mean and standard error. Before analysis, the normality of distributions was verified for each parameter by reckoning skewness and kurtosis according to the Shapiro test of SAS. Non-normally distributed parameters were normalised through natural logarithms (among plasma parameters the IL-1B, IL-6, L-lactic acid, haptoglobin, SAA, NEFA, thiol groups, AOPP and  $\beta$ -carotene, among WBA the fold changes of D-lactic acid, IL-1B, IL-6,  $\text{NO}_2$ ,  $\text{NO}_3$  and  $\text{NO}_x$  and the total response to CST). Original values for log-transformed parameters have been presented in the [Supplementary File 6](#). Incidence of health problems recorded during the study was evaluated by  $\chi^2$  analysis (Freq procedure, SAS Inst. Inc., Cary, NC). Data of body weight, BCS, dry matter intake, rumination

time, metabolic profile, WBC profile, WBA and CST were submitted to ANOVA using a mixed model for repeated measures (Mixed procedure, SAS Inst. Inc., Cary, NC). The statistical model included the fixed effect of milk yield at dry-off (MD; LM and HM), time (t) and their interaction (MD × t)

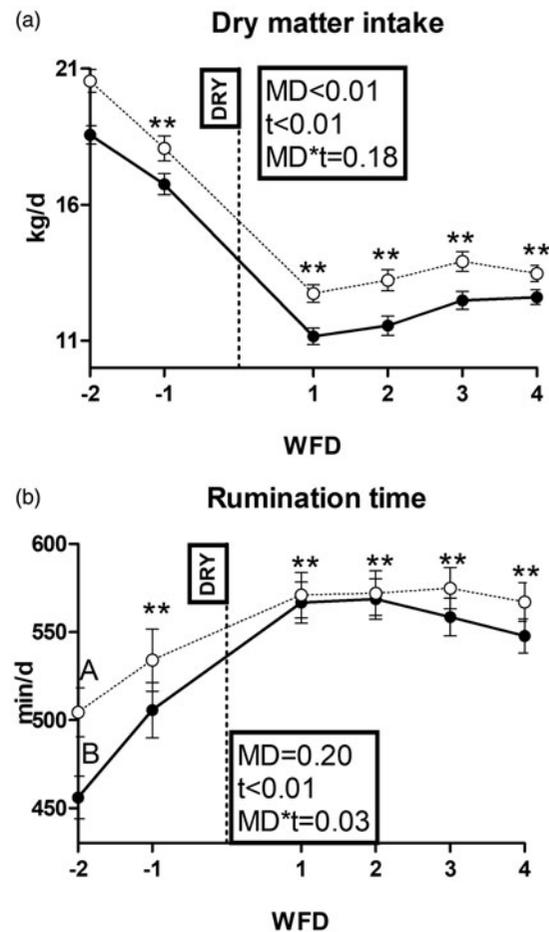
$$Y_{ijk} = \mu + \alpha_i + \delta_{ij} + \tau_k + (\alpha\tau)_{ik} + e_{ijk}$$

where  $y_{ijk}$  is the response at time  $k$  on animal  $j$  in MD group  $i$ ,  $\mu$  is the overall mean,  $\alpha_i$  is a fixed effect of MD group  $i$ ,  $\delta_{ij}$  is a random effect of animal  $j$  in MD group  $i$ ,  $\tau_k$  is a fixed effect of time  $k$ ,  $(\alpha\tau)_{ik}$  is a fixed interaction effect of MD group  $i$  with time  $k$ , and  $e_{ijk}$  is random error at time  $k$  on animal  $j$  in MD group  $i$ . For those parameters that were measured daily (dry matter intake and rumination time) time effect considered the average weekly value, while for other parameters (BW, BCS, metabolic profile, WBC profile, WBA and CST) it considered single DFD. The time was considered as a repeated measure within the cow, and the cow was assumed as a random effect. For WBA, the dose (D; L and H) and the full interaction effect (MD × t × D) also were considered. The analysis was carried out using three covariance structures: autoregressive order, compound symmetry and spatial power with their heterogeneous counterparts. These were ranked according to their Akaike information criterion, with the one having the lowest Akaike information criterion being eventually chosen (Littell et al. 1998). Pairwise comparisons were done using the least significant difference test. For t effect, pairwise comparisons were done between data collected before dry-off (−7 DFD for body weight, BCS, metabolic profile, WBC profile, WBA and CST; −2 weeks from dry-off for dry matter intake and rumination time) and subsequent observations. Data of  $\Delta$ BCS and IFNG release assay were analysed by a one-way ANOVA (GLM procedure, SAS Inst. Inc., Cary, NC), considering only the fixed effect of MD. Post-hoc comparisons were discussed when the  $p$  value for main effect was  $\leq .05$ . Main effects at  $p \leq .10$  are discussed in the context of tendencies.

## Results

### Body weight, body condition score, dry matter intake, rumination time and health status

Body weight was affected by time, while no effect appeared on BCS (Supplementary File 2). Both dry matter intake and rumination time were affected by time (Figure 2(a,b)). Dry matter intake was higher in HM than LM cows ( $p < .01$ ) and rumination time was



**Figure 2.** Pattern of dry matter intake (a) and rumination time (b) in dairy cows with an average milk production lower (LM; solid line) or higher than  $15 \text{ L} \cdot \text{d}^{-1}$  (HM; dotted line) in the week prior to dry off. MD is the effect of milk yield at dry off; t is time effect (\*\* $p < .01$ ); MD × t is the interaction effect (A/B is  $p < .01$ ); WFD is weeks from dry off; DRY is dry-off day (−55 days from expected calving).

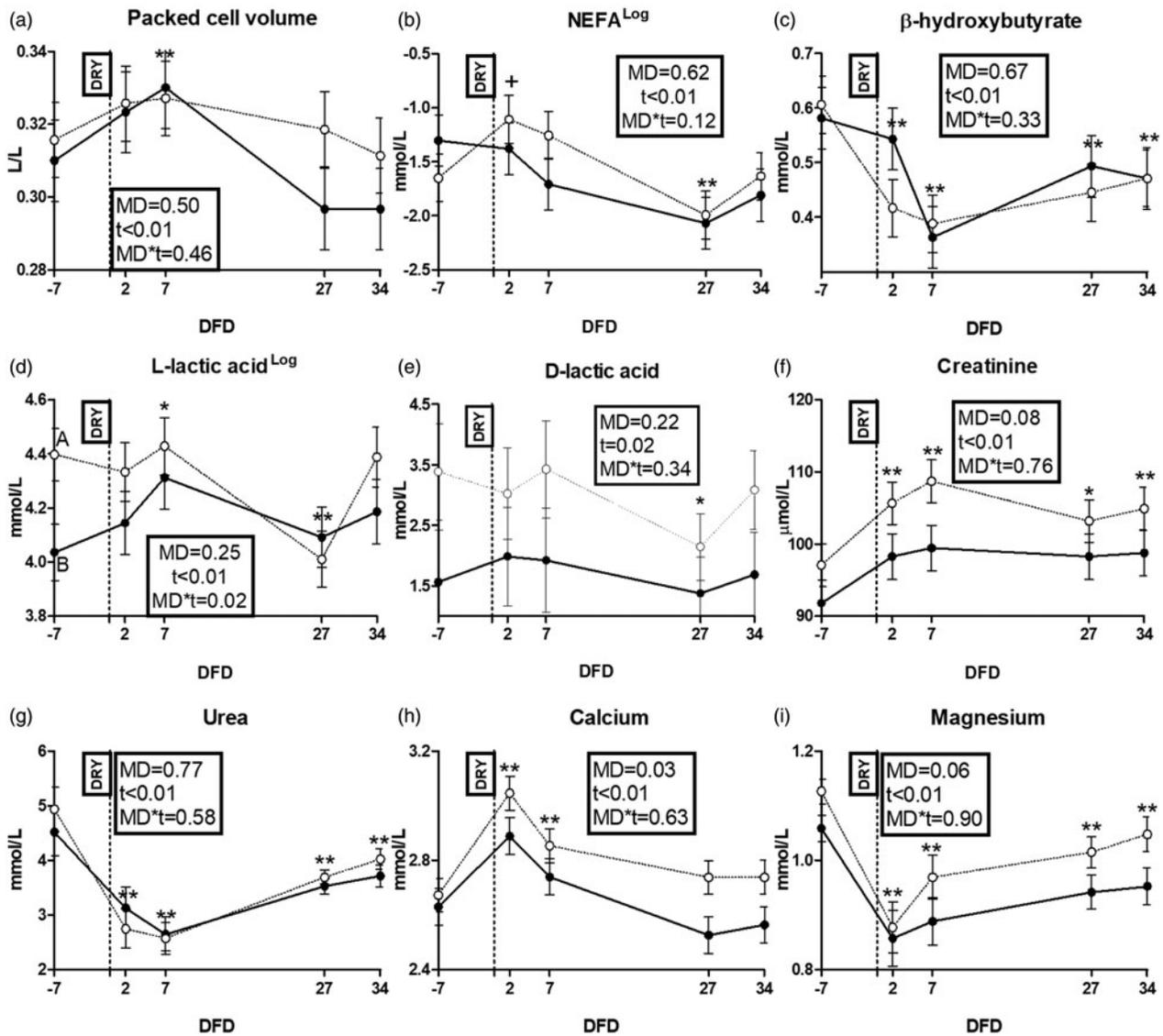
higher in HM than LM cows at −2 weeks from dry-off ( $p < .01$ ).

No clinical diseases were recorded during the whole experimental period, and no MD effect was detected on the incidence of diseases in the first month of the following lactation (Supplementary File 3).

### Metabolic profile

#### Haematocrit, energy, protein and mineral metabolism biomarkers

The packed cell volume was affected by time (Figure 3(a)). Among energy metabolism biomarkers, NEFA, BHB, L- and D-lactic acid were affected by time (Figure 3(b–e)). L-Lactic acid was higher in HM than LM cows at −7 DFD ( $p < .01$ ; Figure 3(d)). Glucose did not show any effect (Supplementary File 4(a)). Among protein



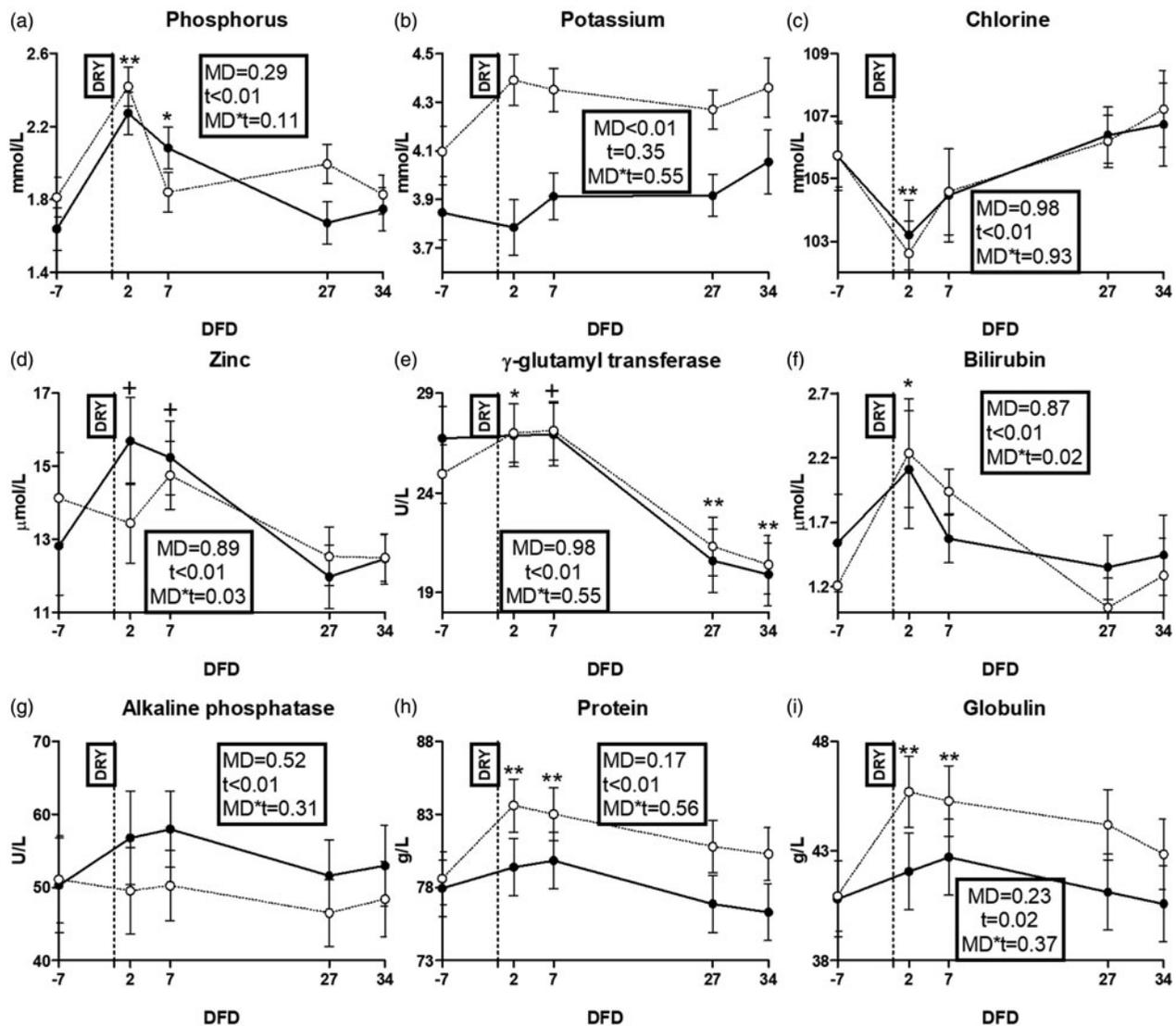
**Figure 3.** Time course of packed cell volume (a) and plasma concentrations of NEFA (b), β-hydroxybutyrate (c), L-lactic acid (d), D-lactic acid (e), creatinine (f), urea (g), calcium (h) and magnesium (i) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 L·d<sup>-1</sup> (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (\*\*p < .01; \*p < .05; †p < .1); MD × t is the interaction effect (A/B is p < .01); DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving); <sup>Log</sup> indicates data expressed as log-transformed.

metabolism biomarkers, creatinine and urea were affected by time (Figure 3(f,g)). Creatinine tended also to be higher in HM than LM cows ( $p = .08$ ). Among mineral metabolism biomarkers, Ca, Mg, P, Cl and Zn were affected by time (Figures 3(h,i) and 4(a-d)). Ca, Mg and K resulted higher in HM than LM cows ( $p = .03$ ;  $= .06$  and  $< .01$ ; Figures 3(h,i) and 4(d), respectively). Na concentration did not show any effect (Supplementary File 4(b)).

#### Liver function and inflammatory status biomarkers

Among liver function biomarkers, GGT, bilirubin and ALP were affected by time (Figure 4(e-g)) while AST-GOT did

not show any effect (Supplementary File 4(c)). Among inflammation biomarkers, total protein and globulin were affected by time (Figure 4(h,i)), while no effect appeared on myeloperoxidase (Supplementary File 4(d)). Among positive acute phase proteins (APP) indicators, ceruloplasmin and SAA were affected by time (Figures 5(b,c)), while haptoglobin resulted higher in HM than LM cows ( $p = .04$ ; Figure 5(a)). Among negative APP indicators, albumin, retinol, paraoxonase and cholesterol were affected by time (Figure 5(d-g)). Among cytokines, IL-1B resulted numerically higher in HM than LM cows from 7 DFD to the end of the experimental period (Figure 5(h)). There was no effect on IL-6 concentration (Supplementary File 4(e)).



**Figure 4.** Time course of plasma concentrations of phosphorus (a); potassium (b), chlorine (c), zinc (d),  $\gamma$ -glutamyl transferase (e), bilirubin (f), alkaline phosphatase (g), protein (h) and globulin (i) in dairy cows with an average milk production lower (LM; solid line) or higher than  $15 \text{ L} \cdot \text{d}^{-1}$  (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (\*\* $p < .01$ ; \* $p < .05$ ; † $p < .1$ ); MD  $\times$  t is the interaction effect; DFD is days from dry-off; DRY is dry-off day (–55 days from expected calving).

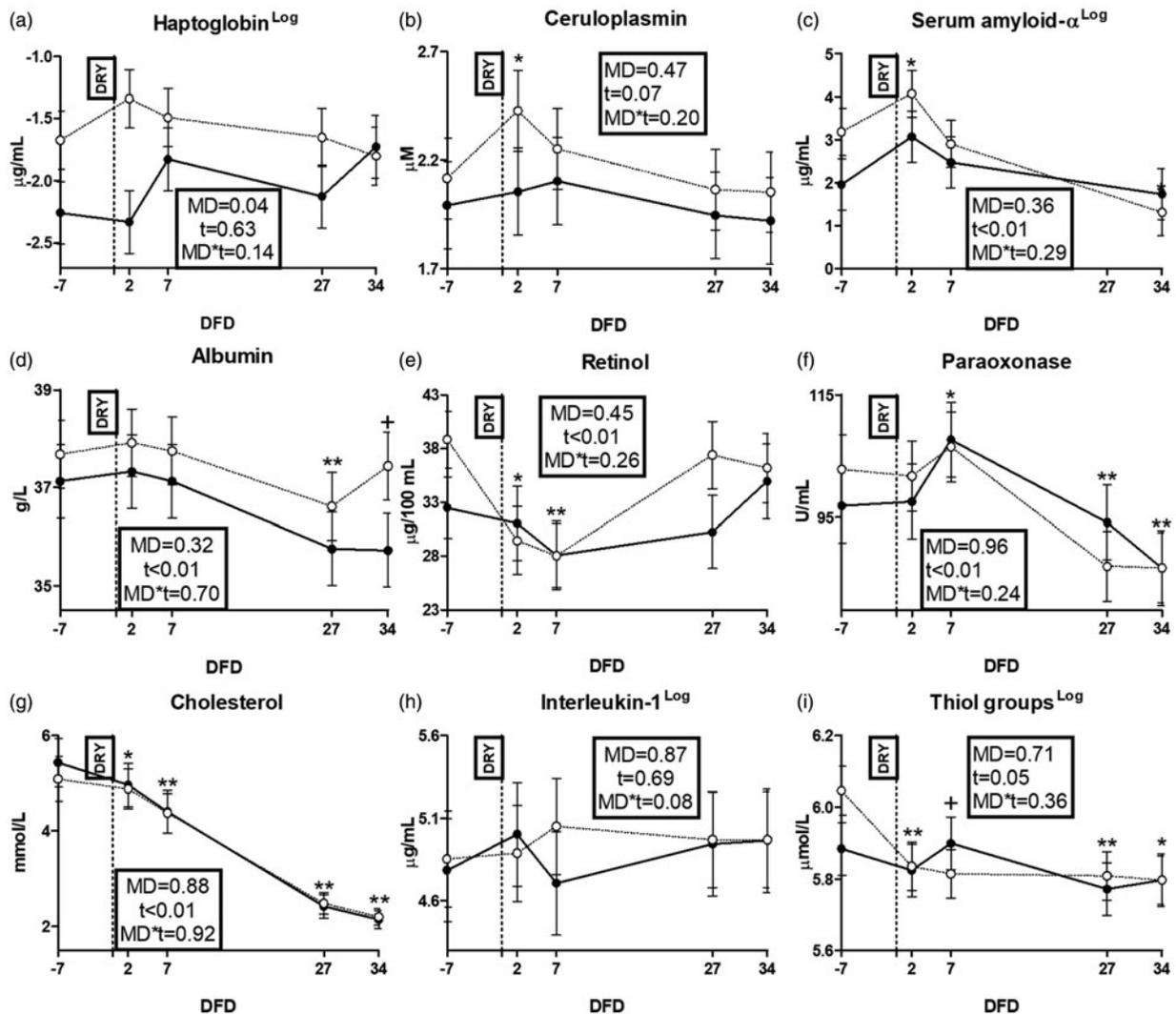
### Oxidant status biomarkers

Among antioxidant systems biomarkers, thiol groups, FRAP, tocopherol and  $\beta$ -carotene were affected by time (Figures 5(i) and 6(a–c)). HM cows had higher concentrations of  $\beta$ -carotene at –7 and 27 DFD ( $p < .1$  and  $< .05$ , respectively; Figure 6(c)) and tendentially higher concentrations of ORAC ( $p = .06$ ; Figure 6(d)) than LM cows. Among oxidative damage biomarkers, a tendency for a time effect was detected for AOPP (Figure 6(e)). Among oxidant species, nitrite, nitrate and nitric oxides were affected by time (Figure 6(f–h)). Nitrite concentrations was higher in HM than LM cows ( $p = .03$ ). No effect appeared on ROMt (Supplementary File 4(f)).

### White blood cells profile, whole blood stimulation assay, interferon gamma release assay and carrageenan skin test

Among WBC profile (Table 2), total WBC, neutrophils, monocytes, eosinophils, red blood cells, haemoglobin and haematocrit were affected by time. Platelets resulted numerically higher in HM than LM cows at 34 DFD. The mean platelet volume was lower in HM than LM cows ( $p = .06$ ).

Fold changes of cytokines after WBA were affected by time and had the greatest increase at the highest LPS dose ( $p < .01$ ; Table 3). Fold change of glucose tended to decrease and those of L-lactic acid increased by increased LPS dose ( $p = .06$  and  $.03$ , respectively).



**Figure 5.** Time course of plasma concentrations of haptoglobin (a); ceruloplasmin (b); serum amyloid- $\alpha$  (c), albumin (d), retinol (e), paraoxonase (f), cholesterol (g), interleukin-1 (h) and thiol groups (i) in dairy cows with an average milk production lower (LM; solid line) or higher than  $15 \text{ L} \cdot \text{d}^{-1}$  (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (\*\* $p < .01$ ; \* $p < .05$ ; † $p < .1$ ); MD  $\times$  t is the interaction effect; DFD is days from dry-off; DRY is dry-off day ( $-55$  days from expected calving); <sup>Log</sup> indicates data expressed as log-transformed.

while those of D-lactic acid was not affected by LPS treatment (Table 4). Fold change of  $\text{NO}_x$  tended to increase by increased LPS dose ( $p = .08$ ) with a tendency for a higher response in LM cows before dry-off ( $p < .1$ ) while those of  $\text{NO}_2$  was affected by time, independently from the LPS dose. Fold change of  $\text{NO}_3$  was not overall affected by WBA (Table 5).

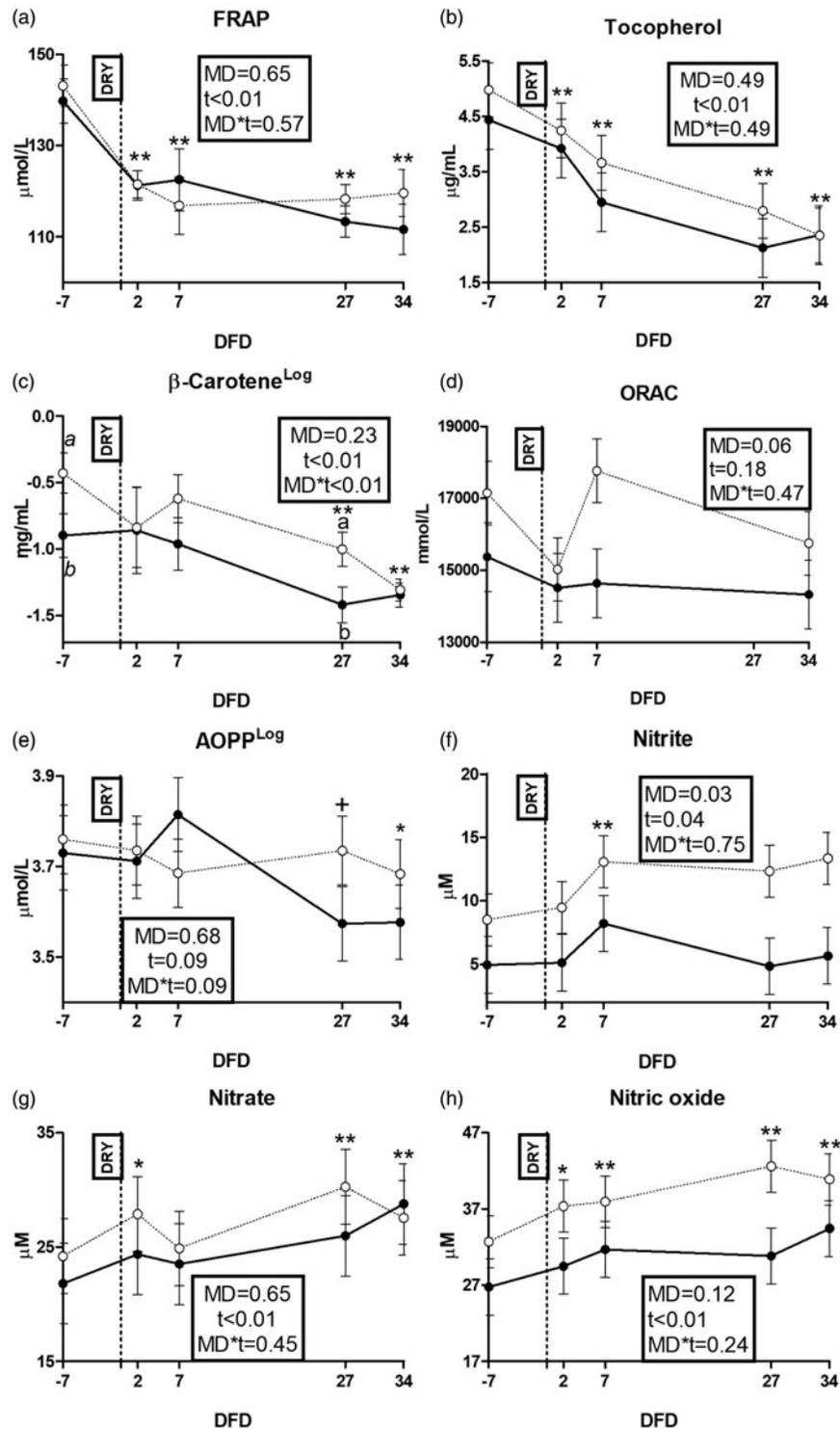
Response to IFNG release assay and CST did not show any effect (Supplementary File 5(a,b)).

## Discussion

### Effects of dry-off on metabolism and immune system of dairy cows

Approaching to dry-off is related to physiological and nutritional changes that deeply affect nutrients

requirements and feeding behaviour in dairy cows (Dingwell et al. 2001). These changes could justify the likelihood to develop a metabolic stress condition, even though the magnitude and duration of variations induced from dry-off in biomarkers related to well-being has been poorly investigated. Our results suggest that adaptation to high fibre content of dry ration had reduce dry matter intake and increased rumination time at dry-off. The further reduction of dry matter intake and the increase of body weight observed in late dry period suggests a reduction of the rumen volume driven by foetal growth, as two-thirds of the development of foetus are completed during this phase (Dingwell et al. 2001). A light mobilisation of lipid sources occurred at two DFD, as suggested by the tendency towards increased NEFA levels



**Figure 6.** Time course of plasma concentrations of ferric reducing antioxidant power (FRAP; a); tocopherol (b),  $\beta$ -carotene (c), oxygen reactive antioxidant power (ORAC; d), advanced oxidation protein product (AOPP; e), nitrite (f), nitrate (g) and nitric oxide (h) in dairy cows with an average milk production lower (LM; solid line) or higher than  $15 \text{ L} \cdot \text{d}^{-1}$  (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off;  $t$  is the time effect (\*\* $p < .01$ ; \* $p < .05$ ; † $p < .1$ ); MD  $\times$   $t$  is the interaction effect (a/b is  $p < .05$ ; a/b is  $p < .1$ ); DFD is days from dry-off; DRY is dry-off day ( $-55$  days from expected calving); <sup>Log</sup> indicates data expressed as log-transformed.

detected, although the lack of any effect on glucose and BHB indicates that glycaemia was not modified, and that liver has been able to fully oxidise the

amount of NEFA received. Previous studies found similar patterns of energy metabolism biomarkers at dry-off paired with the decrease in insulin concentration

**Table 2.** White blood cells profile in dairy cows with an average milk production lower (LM) or higher (HM) than 15 L·d<sup>-1</sup> in the week prior to dry-off.

Item, unit	MD <sup>a</sup>	Days from dry-off			SE <sup>b</sup>	p Value		
		-7	7	34		MD <sup>a</sup>	t <sup>c</sup>	MD × t <sup>d</sup>
White blood cells, K·μL <sup>-1</sup>	LM	6.84	6.52	6.54	0.73	.99	.03	.21
	HM	6.91	6.11	6.92	0.79			
		*						
Neutrophils, K·μL <sup>-1</sup>	LM	3.45	3.00	3.32	0.30	.65	<.01	.79
	HM	3.73	3.05	3.50	0.33			
		**						
Monocytes, K·μL <sup>-1</sup>	LM	0.55	0.41	0.55	0.04	.88	<.01	.78
	HM	0.58	0.43	0.53	0.04			
		**						
Eosinophils, K·μL <sup>-1</sup>	LM	0.19	0.45	0.23	0.11	.32	.04	.82
	HM	0.03	0.31	0.15	0.12			
		*						
Red blood cells, K·μL <sup>-1</sup>	LM	6.59	6.68	6.36	0.18	.34	.02	.65
	HM	6.26	6.59	6.10	0.20			
Haemoglobin, g·dL <sup>-1</sup>	LM	10.70	11.20	10.70	0.38	.39	<.01	.85
	HM	10.30	10.80	10.10	0.41			
		**						
Haematocrit, %	LM	31.30	32.10	31.10	1.23	.57	.02	.38
	HM	30.20	32.20	29.30	1.33			
		*						
Platelets, K·μL <sup>-1</sup>	LM	325.70	318.40	279.10	48.60	.71	.17	.08
	HM	334.00	285.80	348.50	52.50			
MPV <sup>e</sup> , K·μL <sup>-1</sup>	LM	7.16	7.10	7.58	0.41	.06	.44	.29
	HM	5.84	6.69	6.24	0.52			

<sup>a</sup>Effect of milk yield at dry-off.<sup>b</sup>Standard error.<sup>c</sup>Time effect (comparisons are made with respect to -7 days from dry-off value: \**p* < .05; \*\**p* < .01).<sup>d</sup>Milk yield at dry-off × time interaction effect.<sup>e</sup>Mean platelet volume.**Table 3.** Fold changes of cytokines after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 L·d<sup>-1</sup> in the week prior to dry-off.

Item	D <sup>b</sup>	MD <sup>a</sup>	Days from dry-off			SE <sup>c</sup>	Effect	p Value
			-7	7	34			
IL-1B <sup>g</sup>	L	LM	3.74	7.79	8.59	6.54	MD <sup>a</sup>	.61
		HM	4.54	9.20	10.84	7.06	t <sup>d</sup>	<.01
	H	LM	15.66	25.38	25.61	6.54	D <sup>b</sup>	<.01
		HM	15.96	25.67	35.41	7.06	MD × t <sup>e</sup>	.89
	Tot	LM	9.70	16.58	17.10	5.45	MD × t × D <sup>f</sup>	.77
		HM	10.25	17.44	23.13	5.89		
		**	**					
IL-6	L	LM	1.42	1.73	1.36	0.21	MD <sup>a</sup>	.51
		HM	1.46	1.56	1.41	0.23	t <sup>d</sup>	.06
	H	LM	2.21	2.40	2.00	0.21	D <sup>b</sup>	<.01
		HM	1.73	1.93	1.74	0.23	MD × t <sup>e</sup>	.75
	Tot	LM	1.81	2.07	1.68	0.18	MD × t × D <sup>f</sup>	.75
		HM	1.60	1.74	1.58	0.19		
		+						

Values are expressed with respect to baseline (unstimulated sample).

<sup>a</sup>Milk yield at dry-off effect.<sup>b</sup>Dose effect: L is the low dose (0.01 μg LPS/mL whole blood); H is the high dose (5 μg LPS/mL whole blood); Tot is the total effect of LPS stimulation.<sup>c</sup>Standard error.<sup>d</sup>Time effect (comparisons are made with respect to -7 days from dry-off value: + is *P* < .1; \**p* < .05; \*\**p* < .01).<sup>e</sup>Milk yield at dry-off × time interaction effect.<sup>f</sup>Milk yield at dry-off × time × dose interaction effect.<sup>g</sup>Interleukin-1, beta.**Table 4.** Fold changes of glucose and metabolites thereof after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 L·d<sup>-1</sup> in the week prior to dry-off.

Item	D <sup>b</sup>	MD <sup>a</sup>	Days from dry-off			SE <sup>c</sup>	Effect	p Value
			-7	7	34			
Glucose	L	LM	0.950	0.970	0.960	0.022	MD <sup>a</sup>	.170
		HM	0.980	0.950	0.990	0.023	t <sup>d</sup>	.720
	H	LM	0.920	0.930	0.910	0.022	D <sup>b</sup>	.060
		HM	0.940	0.950	0.980	0.023	MD × t <sup>e</sup>	.250
	Tot	LM	0.940	0.950	0.940	0.016	MD × t × D <sup>f</sup>	.850
		HM	0.960	0.950	0.980	0.017		
D-Lactic acid	L	LM	1.000	1.040	1.050	0.032	MD <sup>a</sup>	.570
		HM	1.040	1.020	1.030	0.034	t <sup>d</sup>	.320
	H	LM	1.010	1.020	1.080	0.032	D <sup>b</sup>	.150
		HM	1.070	1.070	1.080	0.034	MD × t <sup>e</sup>	.330
	Tot	LM	1.010	1.030	1.070	0.025	MD × t × D <sup>f</sup>	.790
		HM	1.060	1.040	1.060	0.027		
L-Lactic acid	L	LM	1.030	1.010	1.040	0.015	MD <sup>a</sup>	.620
		HM	1.030	1.030	1.040	0.017	t <sup>d</sup>	.050
	H	LM	1.050	1.020	1.070	0.015	D <sup>b</sup>	.030
		HM	1.060	1.050	1.060	0.017	MD × t <sup>e</sup>	.270
	Tot	LM	1.040	1.020	1.060	0.012	MD × t × D <sup>f</sup>	.970
		HM	1.050	1.040	1.050	0.013		

Values are expressed with respect to baseline (unstimulated sample).

<sup>a</sup>Milk yield at dry-off effect.<sup>b</sup>Dose effect: L is the low dose (0.01 μg LPS/mL whole blood); H is the high dose (5 μg LPS/mL whole blood); Tot is the total effect of LPS stimulation.<sup>c</sup>Standard error.<sup>d</sup>Time effect (comparisons are made with respect to -7 days from dry-off value: + is *P* < .1; \**p* < .05; \*\**p* < .01).<sup>e</sup>Milk yield at dry-off × time interaction effect.<sup>f</sup>Milk yield at dry-off × time × dose interaction effect.

(Odensten et al. 2005; Putman et al. 2018). Fat mobilisation could occur during the last week of lactation consequently to the hormonal response to the withdrawal of concentrates while milk production was still maintained, but adrenaline released at dry-off as a consequence of the milk stasis in the mammary gland could also have a role in the process (Dingwell et al. 2001). In fact, adrenaline is known to provide the primary stimulus for the mobilisation of NEFA from adipose tissue during a stressing event (Buckle 1962). The reduction of NEFA levels observed at 7 DFD suggests a mitigation of stressing conditions and a reduction of energy requirements (also reflected by the decreased dry matter intake) to occur after dry-off.

Changes in diet composition and feeding behaviour could partially account for the increase of lactate and for the reduction of BHB and urea levels found in plasma after dry-off. In fact, fluctuations of rumen pH occurring during adaptation to dry ration inhibit bacterial utilisation of lactate increasing its efflux to blood (Counotte and Prins 1981). On the other hand, a half of haematic BHB is directly related to the ruminal production of butyric acid (Church 1979), while plasmatic urea is paired with its concentration in rumen fluid (Marini and Van Amburgh 2003; Odensten et al. 2005),

**Table 5.** Fold changes of nitrite, nitrate and nitric oxides after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 L·d<sup>-1</sup> in the week prior to dry-off.

Item	D <sup>b</sup>	MD <sup>a</sup>	Days from dry off			SE <sup>c</sup>	Effect	p Value
			-7	7	34			
Nitric oxide	L	LM	1.000	1.000	1.000	0.075	MD <sup>a</sup>	.510
		HM	0.880	1.030	1.020	0.081	t <sup>d</sup>	.420
	H	LM	1.160	1.160	1.040	0.075	D <sup>b</sup>	.080
		HM	0.970	1.010	1.090	0.081	MD × t <sup>e</sup>	.040
	Tot	LM	1.080 <sup>a</sup>	1.080	1.020	0.061	MD × t × D <sup>f</sup>	.510
		HM	0.930 <sup>b</sup>	1.020	1.060	0.066		
Nitrite	L	LM	0.980	0.980	1.030	0.069	MD <sup>a</sup>	.600
		HM	0.960	0.880	1.190	0.074	t <sup>d</sup>	.020
	H	LM	1.000	1.020	1.130	0.069	D <sup>b</sup>	.810
		HM	1.030	0.900	1.010	0.074	MD × t <sup>e</sup>	.340
	Tot	LM	0.990	1.000	1.080	0.053	MD × t × D <sup>f</sup>	.600
		HM	0.990	0.890	1.100	0.057		
Nitrate	L	LM	1.000	1.000	1.000	0.126	MD <sup>a</sup>	.480
		HM	0.870	1.080	0.960	0.136	t <sup>d</sup>	.470
	H	LM	1.230	1.300	1.070	0.126	D <sup>b</sup>	.110
		HM	0.960	1.040	1.120	0.136	MD × t <sup>e</sup>	.110
	Tot	LM	1.110	1.150	1.030	0.098	MD × t × D <sup>f</sup>	.480
		HM	0.910	1.060	1.040	0.105		

Values are expressed with respect to baseline (unstimulated sample).

<sup>a</sup>Milk yield at dry-off effect.

<sup>b</sup>Dose effect: L is the low dose (0.01 µg LPS/mL whole blood); H is the high dose (5 µg LPS/mL whole blood); Tot is the total effect of LPS stimulation.

<sup>c</sup>Standard error.

<sup>d</sup>Time effect (comparisons are made with respect to -7 days from dry-off value: + is  $p < .1$ ; \* $p < .05$ ; \*\* $p < .01$ ).

<sup>e</sup>Milk yield at dry-off × time interaction effect (*a/b* is  $p < .1$ ).

<sup>f</sup>Milk yield at dry-off × time × dose interaction effect.

and both parameters are known to decrease in rumen during dry period consequently to the higher fibre content of the ration and the lower feed intake of the animals in comparison to lactation. Furthermore, blood urea partially arises from amino acids deamination (Broderick and Clayton 1997) and its lower level reflects also a lower utilisation of amino acids in gluconeogenic processes in order to face the energy deficit after dry-off. This interpretation, together with the increased creatinine level after dry-off, suggests the interruption of milk synthesis to have reduced protein requirements, increasing the amount of amino acids addressed to anabolic processes. Indeed, creatinine is the product of the metabolism of one of the main molecules for the storage of energy in the muscle (i.e. phosphocreatine), and it is thus a direct indicator of muscular body mass (Hayden et al. 1992).

Effects of dry-off on mineral metabolism are mainly related to the interrupted milking routine. The presence of higher levels of Ca immediately after dry-off is consistent with previous results (Putman et al. 2018). Such an increase could arise from the sudden interruption of the mammary gland uptake from the haematic circulating pool of calcium. A contribution of the

increased udder pressure arise from the stasis of milk residuals could also be hypothesised, as the tight junctions between epithelial cells are weakened during this phase, increasing paracellular transport of calcium in blood (Aslam and Tucker 1998). Both processes had a transient effect during milk stasis phase only, as the decreased calcium concentration observed from two DFD up to the end of the experimental period suggests the recovery of homeostasis in its haematic pool and the exhaustion of milk residuals in the mammary gland. The role of calcium-regulating hormones on phosphorus homeostasis account for the detection of similar trends for haematic Ca and P concentrations in our experiment, while the lower concentration of Mg found after dry-off was in agreement with its strong direct relationship with milk production (Cavestany et al. 2005).

Early involution of mammary gland that occurs at the beginning of dry period implies an important contribution of leukocytes (Putman et al. 2018). In our study, reduction of WBC, neutrophils and monocytes populations in blood observed after dry-off are consistent with results of Putman et al. (2018) and are related to the migration of those cells to the mammary gland during acute involution (Atabai et al. 2007). The increased concentration of eosinophils in blood after dry-off is also consistent with results of Putman et al. (2018), even though the interpretation is less certain. State that eosinophils are related to allergies and parasites (Gouon-Evans et al. 2000), it has been hypothesised that it may represent a subclinical hypersensitivity to the milk residuals in mammary gland after milking interruption (Putman et al. 2018). Such changes in leukocytes populations in blood are also paired with their augmented sensitivity to biological stressors, as reflected from our results of WBA test with LPS. In fact, increased production of interleukins and NO<sub>2</sub> after dry-off reflects a greater production of metabolites related to inflammation and of oxidant species.

Leukocytes activation during involution phase could be seen as a main cause of the systemic inflammatory status observed after dry-off, and reflected from trends of plasma parameters (Castell et al. 1989). Metabolic inflammation typically affects liver metabolism, implying severe losses in hepatic functions (Bertoni et al. 2008), and this is consistent with the increased GGT and bilirubin concentrations found in our cows during the first week after dry-off. In fact, GGT is an enzyme involved in AA metabolism that increase in blood mostly due to liver damages (Rodriguez-Jimenez et al. 2018), while bilirubin results

from degradation of red blood cells, and its clearance depends on liver enzymes functioning (van den Top et al. 1996). A shift of anabolic priority of the liver named acute phase also occurs during systemic inflammations (Bertoni et al. 2008). In particular, the liver produces more  $\alpha$ -globulins, known as positive APP, i.e. haptoglobin, ceruloplasmin and SAA (Cecilian et al. 2012). This is consistent with the increased globulins and total proteins observed in our cows during the first week after dry-off, that could be driven from the  $\alpha$ -globulins fraction, as they are paired to the increase in ceruloplasmin and SAA (Crisman et al. 2008). Conversely, the liver reduces the synthesis of albumin, retinol binding protein, paraoxonase and lipoproteins during the acute phase (Bertoni et al. 2008; Bertoni and Trevisi 2013) and the drop of retinol found at two DFD in our cows is thus consistent with a systemic inflammation occurred at dry-off. The incapacity to detect any sign of inflammation on blood concentrations of haptoglobin, albumin, paraoxonase and cholesterol (an indicator of lipoproteins synthesis) could depend on the different sensitivity to acute phase of these APPs. In fact, haptoglobin is known to peak instantaneously during acute phase, while negative APPs could require a longer time to reflect inflammatory conditions (Bertoni and Trevisi 2013; Minuti et al. 2015).

An increase in nitrate and nitrite concentrations found in blood after dry-off is consistent with results of Putman et al. (2018), who related it to the altered redox status during early involution of the mammary gland. On the other hand, the increase of nitric oxide could be related to WBC activities at mammary level, as it is mainly produced by activated macrophages as a cytotoxic agent (Coleman 2001). Accumulation of such nitrogen species is known to trigger oxidative damages on macromolecules, as nitric oxide exerts a direct oxidative action, while nitrite is converted in nitrating agent for lipids and proteins by MPO during the neutrophils activation (Dedon and Tannenbaum 2004). Body effort to contain such oxidative damages could account for the depletion of antioxidant systems after dry-off, explaining the decrease in haematic thiol groups, tocopherol and  $\beta$ -carotene levels. In fact, thiol groups (and specifically glutathione) allow the reduction of a wide spectrum of hydroperoxides participating in the glutathione peroxidase enzymatic complex functioning (Sordillo and Aitken 2009), tocopherol is involved in the reduction of the chain propagation and amplification of lipid peroxidation process, while  $\beta$ -carotene indirectly participate in the protection against oxidative stress maintaining other antioxidant

molecules in the reduced form (Ghiselli et al. 1995). Consumption of antioxidant systems in containing oxidative damages, together with their dysregulation during a systemic inflammatory status (Celi 2011), could account for the decrease in the general indicators of body antioxidant capacity (i.e. FRAP) observed after dry-off.

The reduction in AOPP found at 27 and 34 DFD suggests a decreased activity of leukocytes in mammary gland during dry period. In fact, AOPP represent a synthetic marker of protein oxidation exerted by hypochlorous acid, that is produced by MPO during neutrophils activation (Celi and Gabai 2015), and its reduction suggest the interruption of leukocytes contribution in mammary remodelling. The increased blood concentration of retinol and the decreased concentrations of positive APP and bilirubin found at 27 DFD suggest that the interruption of leukocytes activities in mammary gland allowed the organism to face the systemic inflammation occurred at milking interruption, recovering homeostasis in liver metabolism. On the other hand, the altered redox balance did not seem to ameliorate, as the production of nitrogen species and the consumption of antioxidant systems (thiol groups, FRAP, tocopherol and  $\beta$ -carotene) continued up to 34 DFD. At least for  $\beta$ -carotene and tocopherol, a contribution of the increased vitamins requirements in growing foetus could be hypothesised in such a reduction.

### **High milk yield at dry-off is related to greater stress in dry period**

The risk related to dry-off a cow with a milk yield higher than  $25 \text{ kg} \cdot \text{d}^{-1}$  has been deeply investigated previously (Dingwell et al. 2001; Rajala-Schultz et al. 2005). In this respect, the adoption of an average milk yield of  $15 \text{ kg} \cdot \text{d}^{-1}$  in our study demonstrate that 'safety threshold' to dry-off a cow could be much lower, as haematic biomarkers allowed the detection of important alteration in the body homeostasis to occur in animals having the highest milk yield at dry-off. The higher dry matter intake found in HM cattle could be related to the higher production requirements driven by the higher milk production at dry-off, and the greater rumination time is consistent with the greater feed consumption. This is also in agreement with their greater pre dry-off lactate concentration that could be related to sustained high demand for glucose by the mammary gland, resulting in greater Cori cycle activity in other tissues, relative to the LM cows. Furthermore, linkage of Ca and Mg with milk

production (Dingwell et al. 2001) explains the higher concentration of these minerals found in HM cows. In fact, higher mineral requirements related to production in those animals could lead to higher circulating minerals concentrations as soon as cows were dried-off. Those differences demonstrate that HM cows faced deeper changes during the metabolic adaptation to dry-off, reflecting a greater risk to develop metabolic stress in those animals. HM cows showed greater concentrations of haptoglobin and NO<sub>2</sub> after dry-off, indicating that acute phase had a longer duration and that these animals required a longer time to recover homeostasis (Bertoni et al. 2008; Bertoni and Trevisi 2013; Osorio et al. 2014). The greater degree of inflammation is consistent with a longer mammary tissue remodelling phase in HM cows, probably related to the greater amount of parenchymatic tissue (Arslan et al. 2013; Putman et al. 2018). Despite that, higher blood concentrations of β-carotene and ORAC found both before and after dry-off suggested a greater antioxidant power in HM cows. Furthermore, lower fold variation of NO<sub>x</sub> found at WBA before dry-off suggested a less marked oxidant production in their WBC. State the relationship between stress, inflammation, oxidant molecules and metabolic disorders (Biswas and Lopez-Collazo 2009; Van Knegsel et al. 2014) our data suggest high milk yield at dry-off to increase the likelihood of diseases in dry period. Nevertheless, the greater antioxidant availability in blood of our HM cows suggests a greater scavenge capacity against oxidant molecules produced during inflammatory processes, and thus their greater ability to cope with such a potentially harmful condition.

## Conclusions

Dry-off has been revealed as a challenging phase in high-yielding cow's career, related to deep changes in feeding behaviour, metabolism and immune parameters. An inflammation occurred at dry-off in all the animals, probably because of leukocytes contribution in the involution phase of mammary gland, impairing liver function and altering redox balance during the early dry period. Animals with higher milk yield at dry-off showed the worst condition, and this could probably be related to the deeper metabolic changes they faced at dry-off consequently to milking interruption. This study highlights the dry-off as a challenging phase to manage dairy cows' health and could depose for a relationship between dry-off and immune alteration that typically occurs around calving time. In order to demonstrate this, a study where cows are

followed up during the subsequent lactation for all the parameters measured in the current experiment should be performed.

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## Disclosure statement

The authors declare that there is no conflict of interest associated with the paper. The authors alone are responsible for the content and writing of this article.

## Ethical Approval

This study complied with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (authorization of the Italian Ministry of Health n. 1047/2015-PR).

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