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Immune-metabolic-inflammatory markers in Holstein cows exposed to a nutritional and environmental stressing challenge

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Abstract

Dairy cows are exposed to multiple stressors during the productive cycle, such as metabolic challenges, overcrowding, grouping change, environmental stress and dietary errors. Thus, it is essential to study reliable markers able to detect stress conditions in dairy farms. This study evaluates dairy cows' immunologic and metabolic markers after the sudden and combined exposition to a high-grain diet (75% concentrates) and the abrupt change of the housing system (from free stall to tie stall). A group of twenty-four Holstein cows were enrolled in a challenge study of 28 days duration. Several immunological and metabolic blood markers were evaluated over the trial. Blood samples were taken at day 0 (normal value) and day 1, 3, 7, 14, 21, and 28 (challenge). Data were submitted to a mixed model for repeated measures, including time as fixed and cows as random effects. The nutritional and environmental challenge had heavy effects on animal welfare and cows responded with a dramatic rumination drop. Our results suggest that the most responsive markers after abiotic stressors in cows were as follows: Serum Amyloid A and ROM in the acute response; Ceruloplasmin and GGT in the mid acute and Albumin, Paroxonase and FRAP in the chronic phase. Serum Amyloid A, Ceruloplasmin, Paraoxonase, GGT and ROM resulted as positive phase proteins, while, Albumin and FRAP resulted as negative phase proteins. Preliminary obtained results could concur to develop strategies able to mitigate stressor effects; moreover, the proposed design can be used as a model to test stress nutritional modulators.

KEYWORDS
dairy cows, immunological markers, metabolic markers, stressors, subacute ruminal acidosis

1 | INTRODUCTION

Dairy cows can experience multiple sources of stress during their life, as recently well-reviewed (Collier et al., 2017). Stressors may have direct (i.e. loss in production, loss in fertility) or indirect (i.e. through glucocorticoids and somatotropin release) effects on

metabolism and immune functions (Baratta et al., 2017; Chebel et al., 2016; Formigoni & Trevisi, 2003; Mammi et al., 2021). Severe or chronic stress in particular can be responsible for a disruption of homeostasis, which alters biological functions and predisposes animals to a number of pathologies (Bertoni & Trevisi, 2013). Common examples of stressors in the modern dairy industry are represented

by metabolic challenges, overcrowding, abrupt group changes, heat stress, mineral and vitamins imbalances, ruminal and intestinal dysbiosis. Digestive disorders associated with high-grain diets (RGD) and lack of physically effective fibre from forages can negatively affect rumen and intestinal function (Khafipour et al., 2009; Trevisi et al., 2018) and result in large economic loss for farmers. Digestive disorders are commonly correlated to subacute ruminal acidosis (SARA) syndrome (Humer et al., 2018). Moreover, the negative effect of RGD was also related to the possible alteration of hindgut permeability, which could increase the peripheral inflammation status (Khafipour et al., 2009; Minuti et al., 2014) predisposing the animals to different pathologies. In addition, cows are often regrouped in dairy farms. Grouping strategies are generally based on age, frame size, stage of lactation and pregnancy status. Commonly, these changes are important to optimize nutrition and management tasks such as health monitoring and breeding (Smid et al., 2019), but they are also responsible for acute stress responses such as decreased feed intake, lying time and milk production (Grant & Albright, 2001).

Considering that stress effects are additive (Bradford et al., 2015), it is important to investigate the evolution pattern of immunological and metabolic markers, in cows exposed to multiple concomitant stressors, here represented by the administration of a rich grain diet and the changing from free to tie stall.

The improvement of this knowledge could concur to develop strategies able to mitigate cows response to stressors that could easily occur in common dairy farms, like abrupt nutritional and environmental changes. Moreover, this experimental design can be used in the future as a model for studies that aimed to investigate the effects of some nutritional modulators of stress.

2 | MATERIALS AND METHODS

2.1 | Experimental design and animals

This study was conducted at the University of Bologna (Italy), in northern Italy, during the months of December 2017 through April 2018.

Twenty-four multiparous high producing Italian Holstein-Friesian cows were used in a 4 weeks environmental-nutritional challenge design study. Summary characteristics of the cows are reported in Table 1. Cows were enrolled in the trial in three consecutively

TABLE 1 Cows' characteristics enrolled for the trial (means ± SD)

Group	24 Italian Holstein cows
Age, year	2.63 ± 0.59
Lactation, n°	1.65 ± 0.65
Days in milk, n°	51.90 ± 29.68
Body weight, kg	631.3 ± 61.2
Rumination time, min/day	522.8 ± 79.6
Milk yield, kg/day	40.27 ± 7.76

replicates, each one with eight cows, in order to study the reproducibility of the trial scheme.

The challenge period was characterized by a simultaneous and sudden change in TMR composition and housing system. TMR change consisted on increasing the grain content of the diet (Control diet vs. Treatment diet). Control (CTR) diet was formulated to mimic the typical Parmigiano Reggiano rations, which are based only on dry forages and approved concentrates. Treatment (TRT) diet was characterized by the same ingredients with different proportions, the concentrates increased and the forages diminished. Rations were balanced using a software-based on CNCPS model (DinaMilk5; Fabermatica) and offered ad libitum intake (approximately $1.10 \times$ expected intake) once a day at 0900 h (Zago Mixer). Detailed characteristics of the diets are reported in Table 2.

The change in the housing system consisted of moving cows from their usual free stall pen to the tie stall area. Free stall pens were characterized by concrete-base, absence of overstocking (Fustini, Galeati, et al., 2017), individual cubicles with abundant straw coverage and automatic ventilation systems (fans and springs). Tie stall was naturally ventilated and characterized by individual feed bunk and water dispenser, mattress and abundant sawdust coverage. Both challenges were provided suddenly to maximize the stress effect on the cows. Detailed characteristics of the experimental model are reported in Table 3.

2.2 | Feedstuffs and diet chemical analysis

Samples of feedstuff and diets were collected twice a week and dried in a forced-air oven at 65°C for DM determination. Crude protein (CP), neutral detergent fibre (aNDFom) and acid detergent fibre (ADF) were analysed according to Mertens et al. (2002) and AOAC 973.18 respectively. Starch was determined according to AOAC official method (AOAC 996.11). Diet samples were used to determine particle size distribution on a DM basis using the RoTap Separator (W.S. Tyler). Physically effective neutral detergent fibre (peNDF) of diets was calculated as:

$$\text{peNDF, \% of DM} = \text{aNDFom} * \text{PEF,}$$

where PEF is the physical effective factor, as described by Heinrichs (2013).

In vitro aNDFom digestibility (24 and 240 h) was determined using in vitro fermentation in buffered media containing ruminal fluid (Palmonari et al., 2016). Digestibility was performed on forages and TMR according to the procedure described by Palmonari et al. (2017). Briefly, in vitro aNDFom digestibility at 24 h and 240 h (IVNDFD24h and IVNDFD240h) was performed using the Tilley and Terry modified technique (Palmonari et al., 2017). The chemical composition of the diets was severely changed with a decrease in fibre fractions (45:55 vs. 25:75 Forage:Concentrate ratio; 36 vs. 28 aNDFom, %DM; 10 vs. 3 uNDF_{240},%DM; 18 vs. 13 peNDF, %DM; in CTR and TRT diet respectively) and increase in starch content (23 vs. 35 starch, %DM in CTR and TRT diet respectively) during the challenge.

TABLE 2 Diets' characteristics and composition

	CTR diet[a]	TRT diet[b]
Ingredient, kg/head/day af		
Grass hay, fine chopped	9.5	6.0
Wheat straw, fine chopped	1.0	1.0
Corn flakes[c]	6.0	13.0
Concentrate[d]	7.5	8.0
Liquid feed[e]	1.0	1.0
F:C ratio[f]	45.4:54.6	24.8:75.2
Chemical composition, %DM		
DM	87.22 ± 3.00	88.11 ± 0.74
Ash	7.50 ± 1.28	8.06 ± 1.58
Ether extract	3.21 ± 0.47	3.27 ± 047
CP[g]	14.79 ± 1.17	14.18 ± 0.84
aNDFom[h]	35.94 ± 4.16	28.38 ± 2.99
ADF	24.55 ± 2.56	16.29 ± 4.75
ADL	5.27 ± 1.09	3.37 ± 1.24
uNDF$_{240h}$[i]	9.93 ± 3.32	3.05 ± 0.18
Starch	22.95 ± 2.62	35.03 ± 2.18
peNDF[j]	17.56 ± 1.35	13.10 ± 0.85
Energy[k], ME/Mcal/ kg of DM	2.37	2.94

[a]CTR: normal diet following Parmigiano Reggiano regulation (Disciplinare di Produzione del Formaggio Parmigiano Reggiano, 2019).

[b]TRT: rich grain diet—challenge diet.

[c]Mycotoxin level lower <0.02 mg/kg.

[d]Lactation mix ingredient: 29.6% wheat bran, 29.4% sorghum grain, 21.6% canola meal, 14.7% flaked fullfat soybean, 2.2% calcium carbonate, 1% sodium chloride, 0.4% magnesium oxide, 0.9% sodium bentonite and 0.3% vitamin and mineral premix (provided 40,000 IU vitamin A, 4000 IU vitamin D3, 30 mg vitamin E 92% α-tocopherol, 5 mg vitamin B1, 3 mg vitamin B2, 1.5 mg vitamin B6, 0.06 mg vitamin B12, 5 mg vitamin K, 5 mg vitamin H1 (para-aminobenzoic acid), 150 mg vitamin PP (niacin), 50 mg choline chloride, 100 mg Fe, 1 mg Co, 5 mg I, 120 mg Mn, 10 mg Cu and 130 mg Zn).

[e]Properly characterize as reported in Palmonari et al. (2020).

[f]Forage and concentrate ratio, % of forages and concentrates on DM basis.

[g]Crude protein

[h]Amylase- and sodium sulfite-treated NDF with ash correction.

[i]Unavailable NDF estimated via 240-h in vitro fermentation.

[j]Physically effective NDF (aNDFom*pef), calculated using the Ro-Tap system (Cavallini, et al., 2018).

[k]Estimated using DinaMilk5; Fabermatica, Ostiano, Italy.

2.3 | Measurements

Before the start of the trial, cows were evaluated by a veterinarian to assess the health status and confirm the absence of subclinical pathologies.

During the trial, animals were milked twice a day (0800 and 1930 h) in a double-5 herringbone-milking parlour (Afimilk System).

Milk samples from two consecutive milkings for each cow were collected on day 0 (baseline), 7, 14, 21 and 28 (challenge period) and analysed by a certified laboratory (Associazione Provinciale Allevatori Bologna, Foss 4000, Foss Technology) for SCC (Somatic Cell Count), expressed on Log 10 base. Individual weight was recorded (Afiweight Scale, Afikim, Israel). Individual dry matter intake (DMI) was determined by recording feed offered and refused using individual feed bunk (Dinamica generale). Drinking water intake (WI) was recorded by individual water meter. To continuously monitor cows' health conditions, the rumination time was selected as farm marker and the Hi-Tag rumination monitoring system was used (SCR Engineers).

2.4 | Blood analysis

Blood was collected on day 0 (control value), 1, 2, 3, 7, 14, 21 and 28 (challenge period), in the morning before new TMR distribution (0830). Samples were obtained from the coccygeal vein into evacuated tubes containing clot-activator (silicate), for serum assay and Li-heparin, for plasma assay (Vacutest, Kima), and EDTA for blood cell count (CBC). Clot-activator and Li-heparin samples were centrifuged at 250 g for 20 min and 500 g for 10 min (15–20°C) in order to obtain serum and plasma respectively (Centrifugette 4203, ALC International Srl) and stored at −80°C until the analysis. EDTA samples kept at 4°C after collection were analysed for CBC within 4 h for white blood cell count (WBC), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), basophils (BAS) and eosinophils (EOS). Serum samples were analysed only at 0, 14 and 28 day for interleukin 6 (IL-6) and interferon gamma (γIFN). Plasma analysed parameters were cholesterol (CHL), beta-hydroxybutyrate (BHB), albumin (ALB), haptoglobin (HPT), serum amyloid alpha (SAA), ceruloplasmin (CER), paraoxonase (PON), gamma-glutamyl transferase (GGT), ferric-reducing antioxidant power (FRAP), reactive oxygen metabolites (ROM), cortisol (CRT) and interleukin 1 beta (IL1β). WBC was obtained using an automated haematology system (ADVIA 2120, Siemens Healthcare Diagnostics) according to Troìa et al. (2018) and Monari et al. (2020). A clinical auto-analyzer (ILAB-650, Instrumentation Laboratory) was used to determine the concentration of BHB, GGT, APT, CER, ALB and CHL in accordance with Calamari et al. (2016). Furthermore, ROM and FRAP were determined according to Jacometo et al. (2015) and PON according to Bionaz et al. (2007). Calibration was performed through commercial standards for ceruloplasmin, albumin, BHB, ROM and FRAP. Calibration for remaining indicators was performed through internal standards. Four different quality controls were used to test the repeatability and precision for each parameter during each assay. A multi-detection microplate reader (BioTek Synergy 2) and commercial kits for ELISA were used to determine the concentration of IL1β (IL-1B; ESS0029; Thermo Scientific) and serum amyloid A (SAA; TP-802, Tridelta D.L.). Bioassays were used to measure IL-6 with a commercial kit (DuoSet ELISA, cat. no. DY8190, R&D Systems) and γIFN with a

TABLE 3 Experimental design

	Normal condition	Challenge period
Duration	—	28 days
Housing system	Free stall	Tie stall
Diet	CTR[a]	TRT[b]
Samples	Day 0	Day 1, 2, 3, 7, 14, 21, 28
Aim	Set base line level	Provide stress-genic challenge

[a]CTR: normal diet following Parmigiano Reggiano regulation (Disciplinare di Produzione del Formaggio Parmigiano Reggiano, 2019).
[b]TRT: rich grain diet—challenge diet.

FIGURE 1 Variation of monocytes (n°/μmc) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)
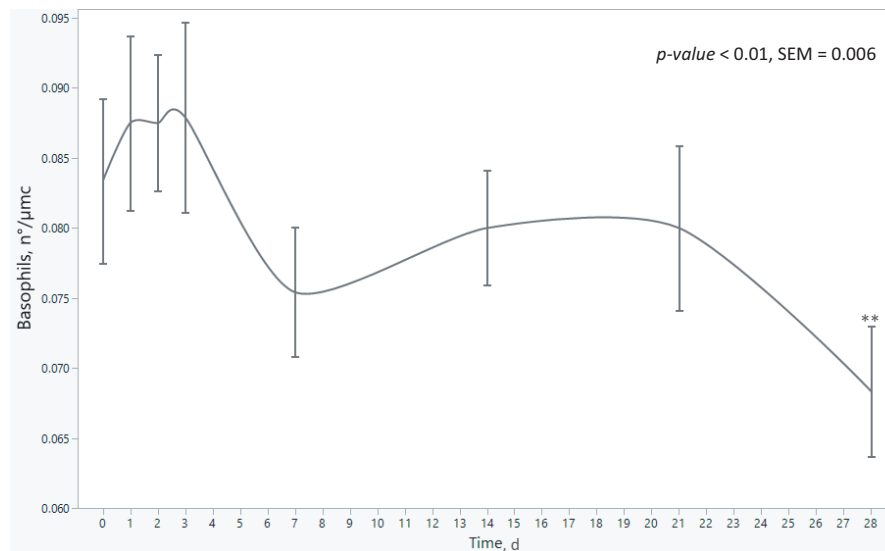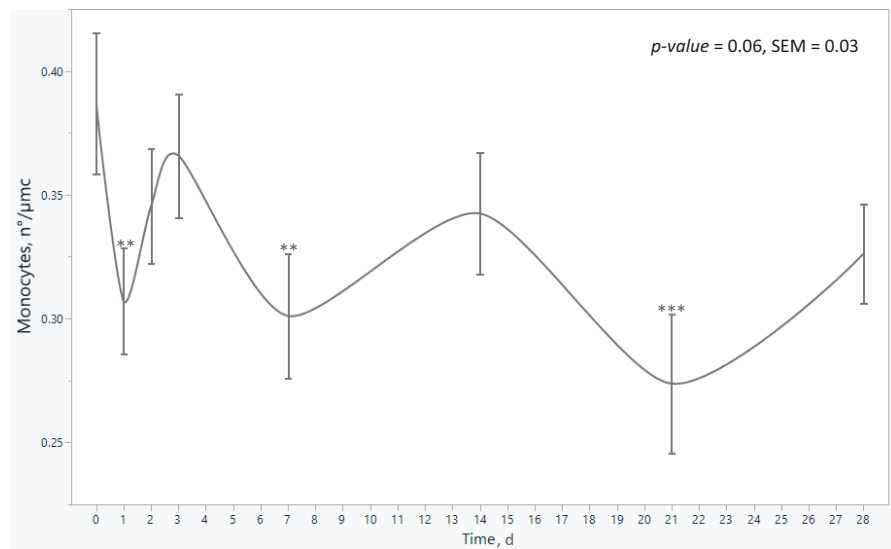
FIGURE 2 Variation of basophils (n°/μmc) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

commercial ELISA kit (Bovigam™ TB Kit, cat. no. 63320, Thermo Scientific Prionics AG), both calibrated through standard solution according to the manufacturer's directions. Blood EDTA samples were analysed at the Clinical Pathology Laboratory University of Bologna Veterinary Hospital; plasma samples were analysed at the Università Cattolica del Sacro Cuore (Piacenza, Italy); serum samples were analysed at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy).

2.5 | Statistics

All data were statistically analysed using the software JMPpro v15 (SAS Institute). Each parameter was tested for normal distribution using the Shapiro test and normalized, when necessary, by different logarithmic or square root transformations according to best final model, determined by the minimum Akaike and Bayesian information criterion (Mammi et al., 2021). Repeated-measures linear mixed-effects models were constructed to assess trends in the parameters measured over the study period. Model fixed effect was the time (t), and a preliminary analysis with the replicates as fixed effect was conducted and resulted in no significance; thus, this parameter was included as nested effect into the random factors. Then, each cow within replicate was considered as experimental unit and used as random variable for all analyses. The specified term for the repeated statement was the time, cow as subject, and the covariance structure used was first-order autoregressive based on the Akaike and Bayesian information criterion. Means were separated on the basis of least square mean. Our purpose was to test variations of each parameter from the normal condition (day 0, control), for this reason, a comparison with control Dunnett's post hoc test was applied. Dunnett's post hoc test p-value results were considered tendency when ≤ 0.10; statistically significant when ≤ 0.05; and highly significant when ≤ 0.01.

3 | RESULTS

3.1 | Overall condition

The abrupt change in diet and housing, as expected, produced a marked decrease in rumination time (−91 min/day, from 494 to 403 min/day in pre- and during challenge period respectively) and milk yield (−1.52 kg/day, from 42.85 to 41.33 kg/day in pre- and during challenge period respectively). During the challenge period, average DMI resulted in 24.66 kg/day. Moreover, during the challenge period SCC LS increased (12.00 vs. 12.81, in T0 and during the challenge respectively) and one cow presented clinical mastitis.

3.2 | Blood and health parameters

On WBC parameters, only Monocytes (n°/μmc) and Basophils (n°/μmc) were affected by the dietetic and environmental challenge (Figures 1 and 2). Monocytes resulted depressed by the challenge at day 1, 7 and 21 ($p = 0.02$, $p = 0.03$ and $p < 0.01$ respectively). Basophils values diminished only at day 28 ($p = 0.03$). Other WBC parameters did not change during the trial (Table 4).

The challenge had heavily affected metabolic, health and immunologic parameters pattern. In fact, SAA, CER, PON, GGT, ROM and ROM/FRAP increased along the challenge period. On the other hand, BHB, Albumin and FRAP values diminished during the stress exposition. BHB resulted reduced from day 3 until the end of the trial ($p \leq 0.05$ since day 3, Figure 3). ALB drop resulted more intense on day 28 ($p = 0.04$, Figure 4). SAA resulted greater from day 2 to the end of the trial ($p \leq 0.10$ since day 2, Figure 5), except on day 3. CER since day 7 increased overall the trial ($p \leq 0.05$, Figure 6). PON raised since day 14, but only a tendency was recorded at day 21 ($p = 0.06$, Figure 7). GGT values reported increased at day 1 ($p = 0.10$) but constantly raised from day 14 to 28 ($p < 0.01$ since day 14, Figure 8). FRAP dropped from day 3; however, only from day 21 became significant ($p \leq 0.05$, Figure 9). ROM curve and ROM/FRAP ratio raised since day 3 ($p = 0.06$) up to the end of the trial ($p < 0.01$, Figures 10 and 11 respectively). Other metabolic and immunologic parameters tested did not varied from the baseline level (day 0) during the trial. In Table 4 are reported mean and range values of all parameters tested at T0 (baseline) and during the challenge.

4 | DISCUSSION

Our objective was to study blood and immunological parameters in lactating dairy cows after a sudden exposition to an environmental and dietary challenge. For this reason, 24 Italian Holstein cows were moved from free stall to tie stall condition and fed a rich grain diet for 4 weeks of trial. Chebel et al. (2016) reviewed this topic and concluded that more efforts are needed to evaluate the effects of stress on dairy cows. In this trial, we combined two stressors to study their effects on early lactation dairy cows. Studying the cumulative effects of different concomitant stressors presents limitations for the interpretation of the results; however, such kind of approach was applied for the first time and obtained results are novel and preliminary.

The nutritional and environmental challenge of this trial had substantial effects on animal wellbeing, and cows responded with a severe drop in chewing time. Rumination time is affected by the health conditions of the animal, and by stressors, whether or not related to sickness (Giaretta et al., 2019; Soriani et al., 2013). The decrease in rumination time related to the stall change highlights the stressfulness of this event, as stated in other experiments (Chebel et al., 2016). During the challenge period, the mean rumination time significantly dropped compared with the previous conditions. This decrease was worsen further by the lower peNDF and uNDF content in the ration, especially in the treatment ration (13.10% and 3.05% of DM), that is lower than those suggested by other authors to ensure an optimum rumen pH (Humer et al., 2018). In previous research projects conducted using dry hay and straw in the diets, as commonly happens in the Parmigiano Reggiano PDO area, it was possible to decrease the amount of peNDF till 11.2% of DM with no rumen health negative effects (Buonaiuto et al., 2021; Cavallini et al., 2021; Fustini et al., 2016; Fustini, Palmonari, et al., 2017). In those experiments, the dietary starch content was lower (23.2, % DM, on avg) and comparable to the CTR diet, offered before the challenge. Simultaneously, the uNDF values should be greater than 9% on DM as recommended by Cotanch et al. (2014). The starch and

TABLE 4 Summary of analysed parameters at day 0 and during the challenge period

Item	Day 0			Challenge period		
	Mean	Lower 95%	Upper 95%	Mean	Lower 95%	Upper 95%
WBC, n°/μmc	7.790	7.298	8.283	7.582	7.347	7.816
LYM, n°/μmc	3.653	3.370	3.936	3.598	3.484	3.712
LYM, %	47.27	44.08	50.46	48.45	47.00	49.91
MON, n°/μmc	0.387	0.328	0.446	0.323	0.305	0.341
MON, %	4.971	4.205	5.737	4.363	4.126	4.600
NEU, n°/μmc	3.411	3.004	3.818	3.311	3.096	3.525
NEU, %	43.36	39.94	46.77	42.57	41.06	44.08
EOS, n°/μmc	0.193	0.139	0.247	0.225	0.200	0.249
EOS, %	2.426	1.823	3.029	2.961	2.652	3.269
BAS, n°/μmc	0.083	0.071	0.096	0.081	0.077	0.085
BAS, %	1.021	0.843	1.199	1.077	1.029	1.124
CHL, μmol/L	7.155	6.754	7.556	7.278	7.092	7.465
BHB, mmol/L	0.636	0.467	0.805	0.478	0.439	0.517
ALB, g/L	36.67	35.64	37.71	36.50	36.12	36.89
HPT, g/L	0.297	0.131	0.463	0.383	0.321	0.446
SAA, μg/ml	65.34	36.94	93.73	137.9	112.4	163.4
CER, μmol/L	1.971	1.768	2.174	2.287	2.196	2.379
PON, U/ml	90.45	80.08	100.82	92.20	88.97	95.44
GGT, U/L	25.06	22.29	27.83	28.17	26.89	29.45
FRAP, μmol/L	170.4	156.7	184.1	160.6	155.2	166.1
ROM, mgH$_2$O$_2$/100 ml	14.12	13.13	15.10	16.11	15.64	16.58
ROM/FRAP	0.086	0.076	0.096	0.106	0.101	0.112
CRT, pg/ml	8439	5992	10,885	10,428	9107	11,749
IL1β, pg/ml	162.5	27.8	297.1	240.0	148.1	332.0
IL6, pg/ml	1809	901	2717	1972	1432	2513
γIFN, pg/ml	192.0	130.7	253.2	224.5	165.9	283.0

Abbreviations: ALB, albumin; BAS, basophils; BHB, beta hydroxyl butyrate; CER, ceruloplasmin; CHL, cholesterol; CRT, cortisol; EOS, eosinophils; FRAP, ferric-reducing antioxidant power; GGT, gamma-glutamyl transferase; HPT, haptoglobin; IL1β, interleukin 1 beta; IL6, interleukin 6; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; PON, paraoxonase; ROM, reactive oxygen metabolites; SAA, serum amyloid alpha; WBC, white blood cell count; γIFN, interferon gamma.

uNDF content of the diet offered in the challenge period were 35% and 3% of DM, respectively, and could be the cause of the reported drop in rumination, which should be recognized as a clinical sign of SARA (DeVries et al., 2009).

In our study, the sudden stall change produced acute stress, with changes in eating, chewing and ruminal patterns as previously reported. In fact, as reported by Cavallini et al. (2018) and Heinrichs et al. (2021) the cows have a great capacity to face management changes, and they can readapt to the new situation within few days. Moreover, Razzuoli et al. (2016) reported that after environmental changes, the serum levels of cytokines in bulls increased between days 0 and 15 since the arrival at the new farm conditions.

The innate immune responses to non-infectious stressors have been highlighted by several authors (Trevisi et al., 2018), which also deal with the metabolic stress of high-yielding dairy cows. Our data agree with the results of previous nutritional challenge works (Trevisi et al., 2018), in which an increase in plasma concentrations of positive acute phase protein (+APP) was observed when rumen pH was below 5.8 for at least 6 h a day, as expected when we fed rich grain diets.

γIFN, IL1β and IL-6 did not varied during the challenge. Interferon and interleukins are pro-inflammatory cytokines and play a pivotal role in mounting and directing the inflammatory response, and moreover, they are activated by infectious and non-infectious stimuli (Bradford et al., 2015).

Interleukin 6 values did not varied and confirmed the finding of previous authors (Danscher et al., 2014), even if our absolute value resulted lower (2500 vs. 5000 pg/ml in our and Danscher et al., 2014 trial respectively). Moreover, in a heifers' starch challenge trial, even lower levels of IL-6 were reported after 1 week of the challenge (900 pg/ml, De Nardi et al., 2014). This fact points to the extreme
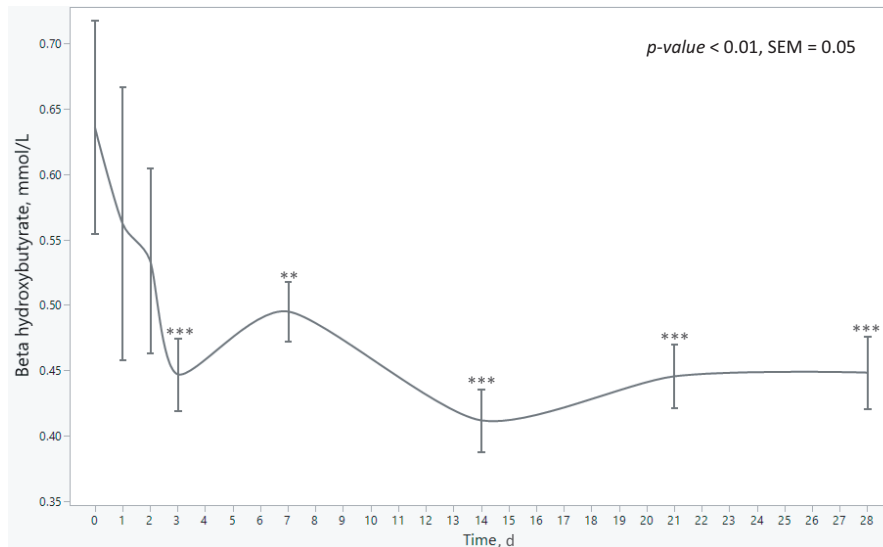
FIGURE 3 Variation of BHOB (mmol/L) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)
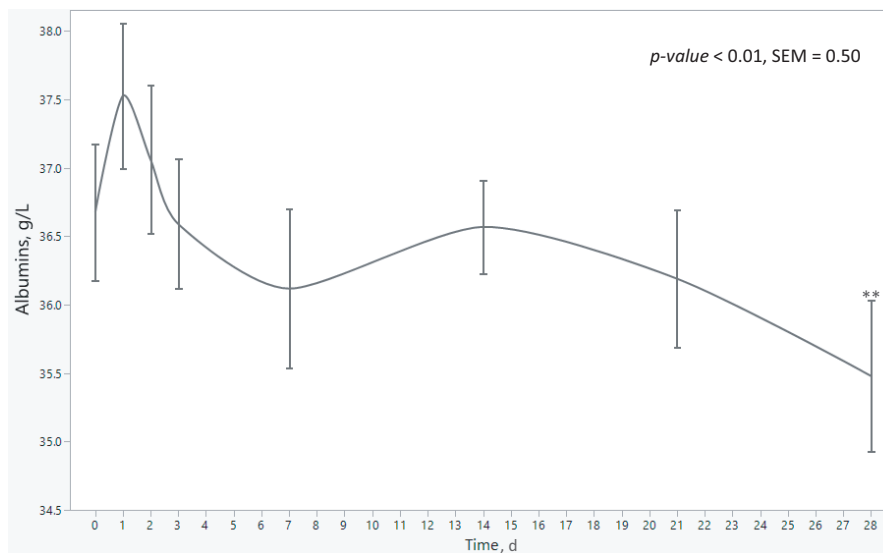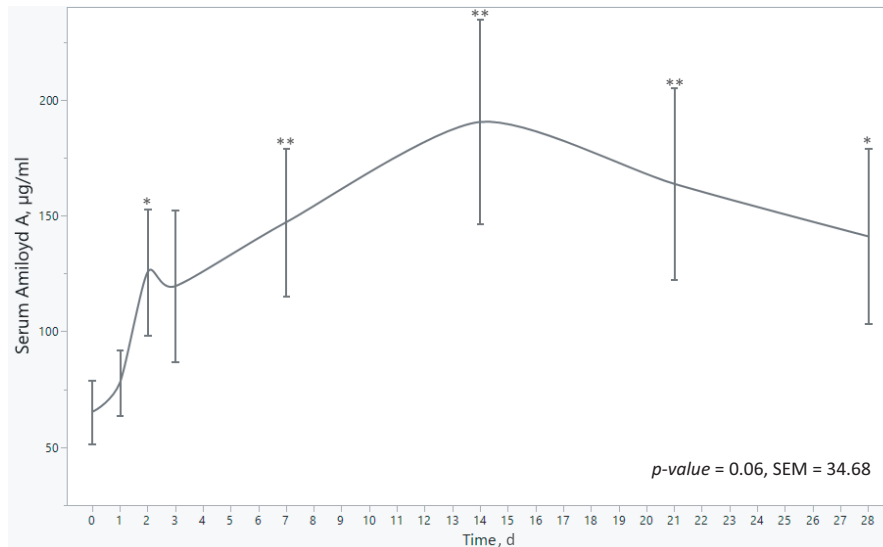

FIGURE 4 Variation of albumin (g/L) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

variability of this compound between trials even when the challenge conditions are comparable.

The arrival of LPS into the systemic circulation is reported to trigger the production of +APP of inflammation, such as SAA and the HPT, which stimulate the macrophages or hepatic Kupffer cells and the release of pro-inflammatory cytokines such as the IL-1β, IL-6 and γIFN (Minuti et al., 2014). Moreover, the increase in circulation of the pro-inflammatory cytokines stimulates a booster release of APP from the liver. At the same time, they reduce blood concentrations of negative APP (−APP), such as ALB and PON (Minuti et al., 2014; Trevisi et al., 2018).

Serum amyloid alpha in our research reported a statistical increase after the first week of the challenge (up to 170 µg/ml, day 14). A similar increase, but more acute, was reported: 116 µg/ml after 3 days challenge

(Danscher et al., 2014) and 446.7 µg/ml at 6 h from the challenge (Khafipour et al., 2009). This compound was measured also in steers exposed to a SARA challenge with 4 weeks of incremental concentrate doses and resulted in 163 µg/ml (Gozho et al., 2007). On the other hand, lower values of SAA were reported in a heifers' starch diet challenge trial after 1 week (37.1 µg/ml, De Nardi et al., 2014). Interestingly, Ceciliani et al. (2012) reported that levels of this compound greater than 115 ± 37 µg/ml are related also to mammary diseases.

Haptoglobin increased in the challenge but not significantly (0.32–0.45 g/L). Other rich grain dietary challenges reported a significant increase of this parameter (0.68 g/L in heifers, De Nardi et al., 2014; 0.48 g/L in dairy cows, Khafipour et al., 2009). Moreover, other authors, in similar conditions, recorded lower values of HPT

FIGURE 5 Variation of SAA (µg/ml) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)
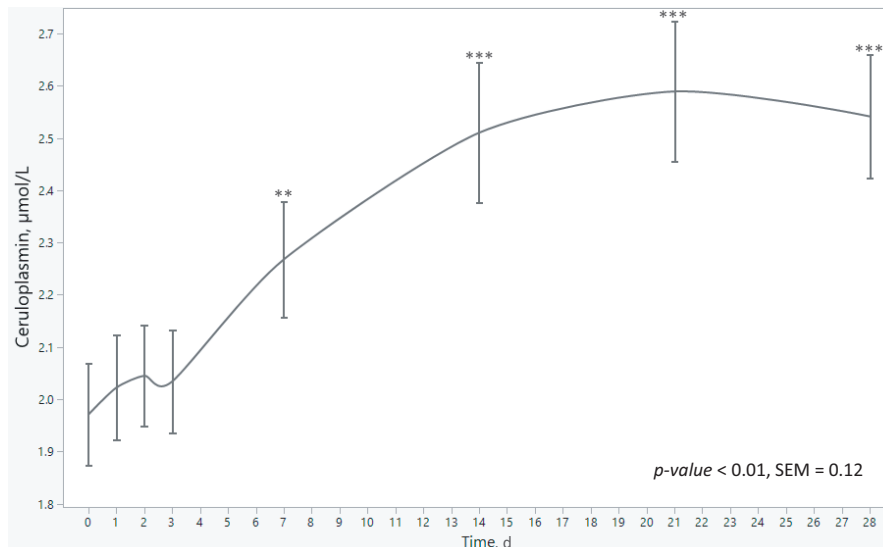
FIGURE 6 Variation of ceruloplasmin (µmol/L) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

than in our trial (0.24 g/L, Xu et al., 2017; 0.22 g/L, Danscher et al., 2014). Finally, in literature, are present extremely higher levels of HPT in dairy cows during metritis (1.62 ± 0.47 g/L, Ceciliani et al., 2012).

Ceruloplasmin increased along with the challenge, with peaks at 2 and 3 weeks (2.7 µmol/L). This increase is comparable with what was found by other authors (Xu et al., 2017), where 2.85 µmol/L was recorded. CER has both anti- and pro-oxidant roles, and means concentrations were within the proposed reference values for lactating Holsteins (2.2–3.15 µmol/L, Bertoni & Trevisi, 2013).

Many +APP, including SAA, CER and HPT, are poorly specific for pathogens and toxins and is reported that an increase of +APP could be related to mammary pathologies (Ceciliani et al., 2012). In

our trial, we observed an increase in SCC and one case of clinical mastitis; therefore, the augment of this +APP could be related to the mammary gland inflammation, more than to the stress challenge provided. However, in our study, their increase is likely due to the translocation of LPS out of the digestive tract into the portal circulation, as widely reported in the literature with similar dietary challenges (Gozho et al., 2007; Khafipour et al., 2009; Minuti et al., 2014). Moreover, an increase in SCC could be secondary to LPS translocation into the systemic circulation and their approach to the mammary gland; thus, SCC raises could be a direct effect of the dietary challenge (Khafipour et al., 2009; Trevisi et al., 2018).

Reported CHL values, even if not significant, resulted higher than what was reported as reference by Bertoni and Trevisi (2013,
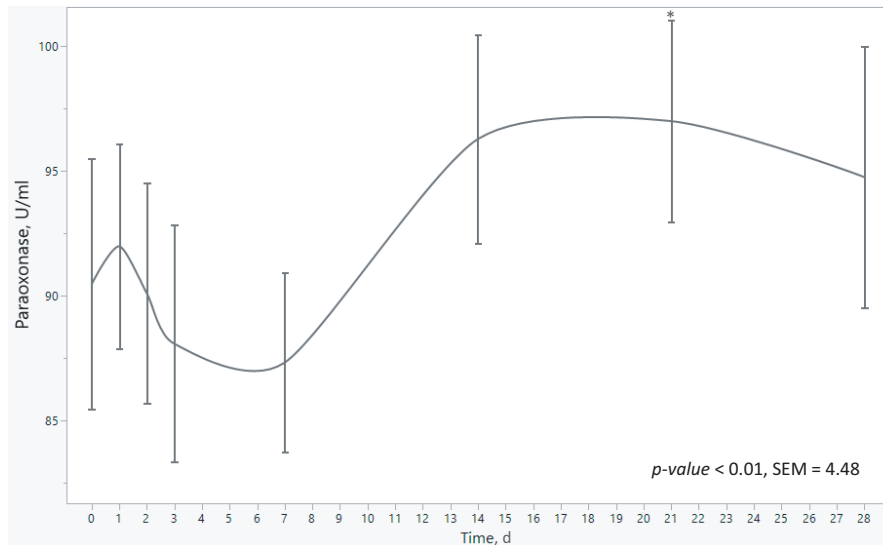
FIGURE 7 Variation of paraoxonase (U/ml) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)
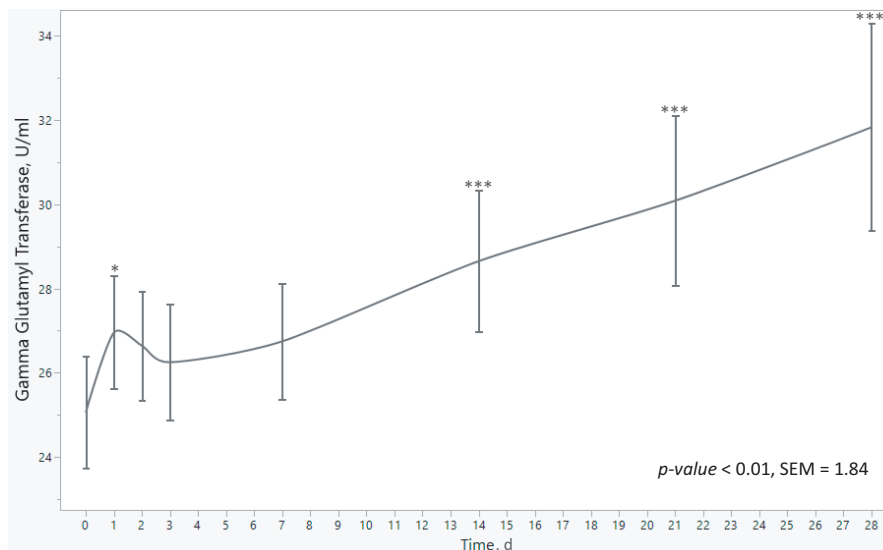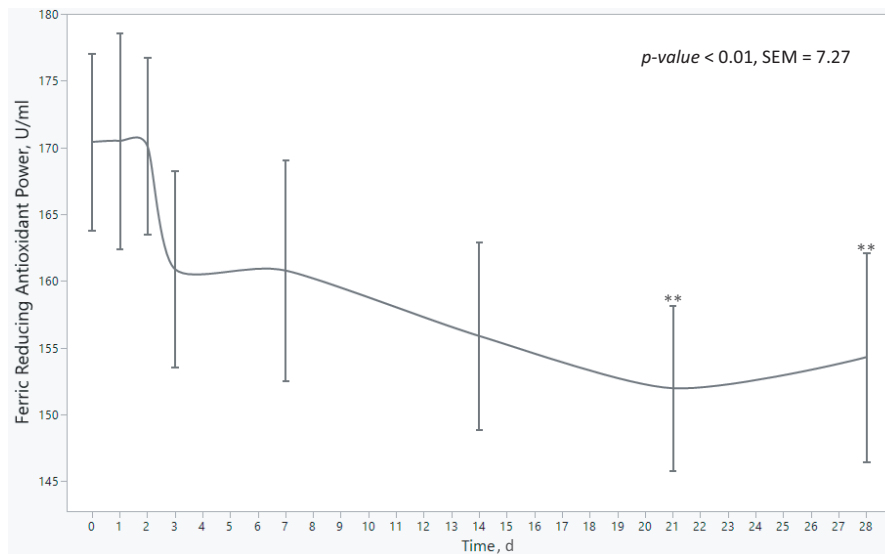

FIGURE 8 Variation of GGT (U/ml) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

2.9–6.8 mmol/L) and Engelking (2015, 2.3–6.6 mmol/L). Moreover, recorded levels were higher than what was reported in Xu et al. (2017), where CHL resulted in 5.53 mmol/L in Holstein cows exposed to a punctual rich grain diet challenge. This variance could be caused by dietary, genetics or environmental differences. In fact, in Xu et al. (2017) the challenge was produced by wheat-barley pellet top dressing addition and in our trial mainly by corn flakes TMR increase, moreover, they sampled the blood after 12 h of feeding the challenge diet. Finally, our CHL values are intermediated between those measured by Piccioli-Cappelli et al. (2014) in early and late lactation fed a high starch diet (8.37 and 6.41 mmol/L respectively).

In our study, BHB resulted within the normal range values (0.2–1, Cornell University, 2020) and decreased from the beginning of the

challenge. This fact reports a better energy balance of the animals, indeed, the energy density of the TRT diet was higher than CTR diet, due to the increase in concentrates. Moreover, BHB levels resulted lower than what reported in Holstein cows during similar rich grain diet challenge trials (1.28 mmol/L, Xu et al., 2017) and comparable to the levels reported in cows fed high starch diets in early and late lactation (0.50 and 0.54 mmol/L, Piccioli-Cappelli et al., 2014). Moreover, in Piccioli-Cappelli et al. (2014) cows fed high starch diet had a reduction in BHB in comparison with the control diet, confirming our findings.

Albumin values significantly dropped only at the end of the challenge period (35.5 g/L, day 28) without reaching pathological levels (<27 g/L, Engelking, 2015, or <33 g/L, Bertoni & Trevisi, 2013;

FIGURE 9 Variation of FRAP (U/ml) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)
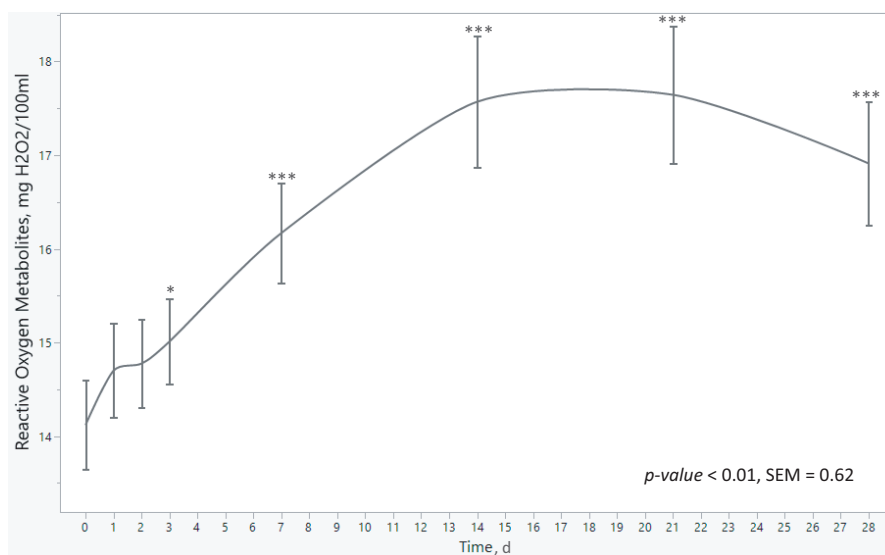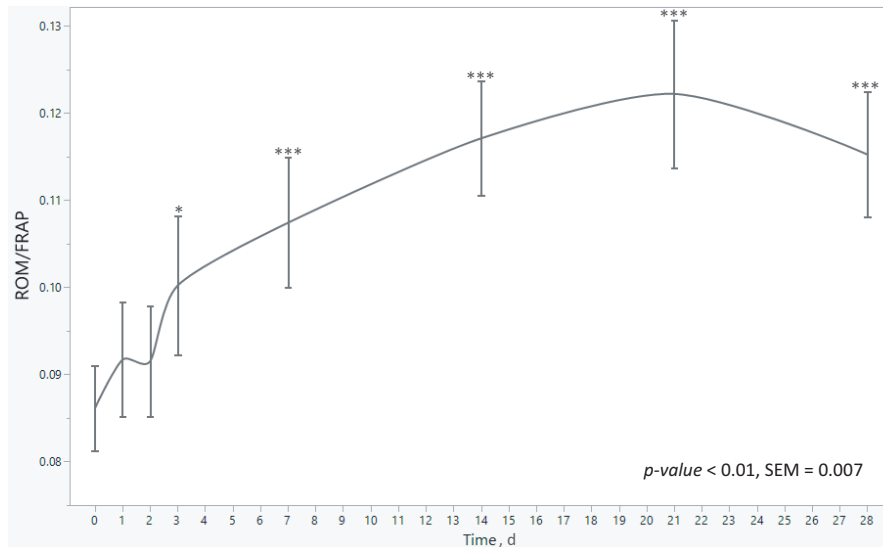

FIGURE 10 Variation of ROM (mg H_2O_2/100 ml) on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

Cornell University, 2020). Our findings confirm that this indicator is a negative acute phase protein and decrease during inflammation due to the shift of liver synthesis on positive acute phase protein (Trevisi et al., 2018). These modifications are characteristics of cows after calving (Giammarco et al., 2018; Mammi et al., 2021). Moreover, albumin have a role in ligand binding and free radical trapping (Trevisi et al., 2018); thus, their diminishment reduced the antioxidant capacity of cows exposed to challenge.

Ferric-reducing antioxidant power values decreased by the abrupt challenge, but this drop resulted significant only in the late inflammation phase. These compounds are known to exert antioxidant activity. FRAP provides a measurement of antioxidant power

via blood concentration of bilirubin, uric acid, proteins and vitamins C and E (Benzie & Strain, 1996). As these compounds with antioxidant capacity directly depend on liver activity (Sordillo & Aitken, 2009), dysregulation on the liver functions reflects on blood concentrations of such biomarkers. Greater depletion of these compounds is already reported around calving (<130 μmol/L, Mezzetti et al., 2020).

At the same time, PON increase during the second phase of the challenge (97 U/ml) and similar levels are reported after 12 h of challenge (108 U/ml, Xu et al., 2017). Normal levels of this compound are set at 83 ± 7 U/ml and below 68 ± 8 U/ml is considered as pathologic (Ceciliani et al., 2012). PON is an antioxidant enzyme synthesized by

FIGURE 11 Variation of ROM/FRAP on Holstein cows exposed to a nutritional and environmental stressing challenge; Error bar indicates SEM. *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$ for differences among specific time point to time day 0 (CTR)

the liver and a negative acute phase protein. In our study, this compound resulted augmented in the late phase. This finding is in contrast with that found by Trevisi et al. (2018), where the reduction in PON was associated with subclinical metabolic disorders. Thus, we can hypnotize that in the chronic stage of the stress exposition the body responds with increased levels of PON levels to face against the oxidative stress generated by other compounds, such as ROM that we found augmented. Moreover, FRAP, as previously stated, resulted increased at the same time. Then, from our results, dairy cows exposed to chronic stress (3 weeks at least) respond with an increase in PON and a reduction in FRAP.

In fact, among oxidant species, ROM had a great growth due to stressors exposition from the early challenge period (up to 17.5 mg H_2O_2/100 ml). Similar values are reported also in extremely acute rich grain challenge (17.6 mg, Xu et al., 2017).

Gamma-glutamyl transferase, an enzyme related to amino acid metabolism in the liver (Rodriguez-Jimenez et al., 2018), increased from 2 weeks (28 U/ml) to 4 weeks (31 U/ml) of challenge, indicating an impaired liver function. However, this parameter is considered pathological when greater than 34.5 U/L (Bertoni & Trevisi, 2013), 48 U/L (Engelking, 2015) or than 54 U/L (Cornell University, 2020) and, in our study, did not reach these levels. Similar values were reported in previous trials (33.7 U/ml, Xu et al., 2017). On contrary, other studies reported greater values in challenge trails with grain or alfalfa pellet (46.9–47.9 U/L, respectively, Rodríguez-Lecompte et al., 2014). However, in Rodríguez-Lecompte et al. (2014) study no differences have been reported with the control group, so this parameter did not increase due to the dietary challenge.

Cortisol is stated as good chronic stress markers (Trevisi & Bertoni, 2009), even if it could be depend on the frequency of the mode of sampling. Its increase in blood is a common consequence of the acute stress; this has generally positive effects, despite not completely understood (Sapolsky et al., 2000). In our trial, it

did not change significantly from the basal levels, even if numerically increased. It is reported that high levels of cortisol seem to be maintained when there is a failure to restore homeostasis or after repeated stress, but some contradictory results emerge from the literature relating to farm animals (Trevisi & Bertoni, 2009). Reported CRT values resulted similar to what was found at the farm level by Bertoni et al. (2005; 6000–9000 pg/ml) and Barrasso et al. (2020, 8800–11,300 pg/ml). Finally, the level of cortisolemia in dairy cows is highly influenced by several and usual manipulations (Bertoni et al., 2005) and, in this trial, all the manipulation and managing actions were the same during the sampling procedure.

Among WBC, only MON and BAS reported a slight depletion but always remaining within the reference values (0–0.9 and 0–0.4 $\times 10^3$/μl, respectively, Cornell University, 2020). Other white blood cells evaluated in this study (LYM, NEU and EOS) resulted in normal values compared with the reference values reported by the Animal Health Diagnostic Center of the Cornell University (2020). Gozho et al. (2007) reported no differences in WBC differentials in challenges performed by grain or alfalfa pellet, confirming our results.

5 | CONCLUSION

These preliminary results showed that an abrupt dietary change, increasing the content of concentrates, and the change in the housing stall system, had a massive effect on animal homeostasis and inflammatory response. The most responsive markers after abiotic stressors in our study were as follows: Serum Amyloid A and ROM in the acute phase; Ceruloplasmin and GGT in the mid acute and Albumin, Paroxonase and FRAP in the chronic phase. Serum Amyloid A, Ceruloplasmin, Paraoxonase, GGT and ROM resulted as positive phase proteins; on the other hand, Albumin and FRAP resulted

diminished. We are conscious that study the cumulative effects of different concomitant stressors presents limitations; however, it permitted us to obtain novel and preliminary results compared to the literature. In fact, the current study contributes additional observations on the ranges of stress biomarkers in dairy cows exposed, for the first time, simultaneously to an environmental and dietary challenge.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and all experimental procedures involving animals were approved by the University of Bologna Animal Care and Use Committee (protocol number 762).

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CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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