Original article

The evaluation of non-specific immune status of heifers in field conditions during the periparturient period

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Abstract – During the periparturient period, some impairment of immune defences were observed. Reference values for the different non-specific immune parameters in cows are not generally available, thus limiting the application of these parameters in dairy practice. This paper reports the data on the measurements of different parameters in the blood, and explores the possible influence of the herd on the non-specific immune status of the cow. Five herds located in Northern Italy were selected and overall 39 heifers were enrolled in the trial. Blood samples were taken 14 and 7 days before the expected date of calving, then at 7, 14, 21, 28, 45, 60, and 75 days after calving. The parameters assessed were N-acetyl-β-glucosaminidase (NAGase), lysozyme, nitric oxide, superoxide dismutase, haptoglobin, respiratory burst, and serum protein profile. After calving, a significant decrease of respiratory burst and nitric oxide concentration were observed in comparison with the pre-calving values but not with the post-calving samplings. Total proteins, β- and γ-globulins showed a progressive and significant increase in concentration after calving, in comparison with pre-calving values. The results of the study confirmed that a decrease of immune functions can be observed in commercial dairy herds in the first four weeks after calving. The amplitude of this phenomenon is not common to all animals and all herds, suggesting the possibility to reduce the impairment by improved management and genetic selection.

heifers / periparturient period / blood / non-specific immunity

1. INTRODUCTION

The achievement of an optimal health status in dairy herds is influenced by different factors such as genetics, nutrition, housing, hygiene, and prevention. The current dairy herd management requires maintaining a difficult balance among different man-agement and physiological factors, in order to reduce the frequency of diseases affecting production such as mastitis [11, 34]. Indeed, it has been demonstrated that achieving a proper animal welfare level is essential to reduce the frequency of diseases, reducing the direct and indirect impairment of immunological defences by external

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causes [6]. Therefore, assessing non-specific immune defences represents an essential step in the evaluation of the health status of the animal. Moreover, it allows both to objectively assess the welfare level and to calculate a predictive measure of the risk to develop diseases [15].

Among the different production phases, the periparturient period is one of the most important and critical periods. It has been demonstrated that in this period and, particularly around calving, some impairment of immune defences could be observed [19, 22]. This impairment increases the frequency of reproductive and production diseases as recently reviewed by [17], and of clinical mastitis as shown experimentally [5, 10], and in field conditions [13, 35], as reviewed recently [8]. Indeed, for different reasons evaluating the cow's immune status in the field is difficult. Among these reasons are the costs and the labour required for many analytical procedures, the natural variability of some parameters, the disturbance caused to the routine work at the farm level and, finally, the absence of reference values to define an immune status.

In order to evaluate the possibility to develop a practical protocol to study the immune status of dairy cows in field conditions, a study was planned assessing different non-specific immune parameters in blood samples in dairy heifers during the periparturient period. Indeed, heifers were shown to have functional impairment in oxidative reactions, as compared with pluriparous cows [24]. Besides, sampling heifers at the beginning of their first lactation, allows to measure immune parameters in animals that were unexposed to previous stressors related to lactation (i.e. milking, metabolic diseases, clinical mastitis) that could bias the results.

This paper reports the results of a field study, with the patterns of the selected blood parameters, and explores the possible association between herd and immune status of the cows.

2. MATERIALS AND METHODS

2.1. Animals and samplings

Five herds located in Northern Italy were selected. The herds can be considered as being representative of Italian dairy herds: all of them are free-stall herds, herd size is in the range 80–350 Holstein cows; total mix ratio, dry-cow therapy and post-dip are regularly applied. Overall 39 heifers free from clinical diseases, were enrolled in the trial (7 from herd A, 12 from herd B, 7 from herd C, 4 from herd D, 9 from herd E). All the pregnant heifers with an expected calving date between January and April were enrolled in the study. This period was selected to reduce the influence of climate (i.e. a hot and humid climate, heavy rain) on the immune parameters. Indeed, in the Italian environment late winter is probably the most stable period, with a low rain rate and temperature between 0 and 10 °C.

Blood samples were taken 14 ± 2 and 7 ± 2 days before the expected date of calving, then at 7 ± 1, 14 ± 1, 21 ± 1, 28 ± 1, 45 ± 1, 60 ± 1, and 75 ± 1 days after calving. When calving was delayed, a further sample was taken and the first one was discarded.

2.2. Analysis

Polymorphonuclear leukocytes (PMN) were isolated from the blood by density gradient separation after hypotonic lysis on Ficoll-Paque Plus (AmershamBiosciences, Sweden), following the procedure described by [9] and immediately delivered for the respiratory burst assay.

Serum obtained from centrifugation at $2\,000 \times g$, was aliquoted in $1\,500$ μL tubes and immediately frozen at –80 °C for the enzyme analyses, which were performed in a single session for each enzyme after the end of the follow-up period.

2.3. Respiratory burst

Respiratory burst (RB) was assessed by luminol-enhanced chemiluminescence [25].

The assay was performed adding 0.7×10^6 of viable PMN/mL to two wells of a microplate, in one well PMN were stimulated with phorbol myristate acetate, PMA (Sigma-Aldrich, USA) at a concentration of 6 µg/mL, while the PMN in the other well were not stimulated (control). After injecting 40 µL of luminol (Sigma-Aldrich, USA), the chemiluminescence produced was automatically recorded for 30 min on a microplate luminometer (Luminoskan Ascent, Themolabsystem Finland) and the total chemiluminescence emission, expressed in mV, was calculated.

2.4. Biochemical assays

Lysozyme was assessed in duplicate by the procedure described by [25] based on the lysis of *Micrococcus lysodeycticus* measured by changes of optical density at 450 nm after 2 min on a microplate spectrophotometer (Spectramax 340, Molecular Devices, USA).

N-Acetyl-β-glucosaminidase (NAG) was assessed in duplicate by the procedure described by [20], and expressed as units (pmol of 4-methylubelliferon released per min at 25 °C catalysed by 1 µL of milk) on a microplate fluorimeter at 355 exc and 460 em (Ascent, ThemoLabsystem, Finland).

Superoxide dismutase (SOD) was assessed in duplicate with a commercial kit (SOD Assay Kit-WST, Doijndo, Japan) based on a reaction with a tetrazolium salt on a microplate spectrophotometer at 440 nm [32].

Nitric oxide (NO) was assessed in duplicate with a commercial kit (Nitrite/Nitrate Fluorometric Assay Kit, Cayman, USA), based on the conversion of all the nitrate to nitrite, by nitrite reductase. Then the nitrite fluorescent compound was measured by microplate fluorimeter at 375 exc and 415 em [26, 27].

Haptoglobin (HP) concentration was assessed in duplicate with a commercial kit based on its binding affinity to haemoglobin (Haptoglobin, Tridelta, Ireland) on a microplate spectrophotometer at 630 nm [28].

2.5. Serum protein electrophoresis

Serum proteins were assessed by agarose gel electrophoresis with Hydragel 30 (Sebia, France), a kit intended for the separation of serum proteins on an automated multiparametric agarose gel electrophoresis system (Hydrasis, Sebia, France). The gels obtained were analysed by a densitometer and a dedicated software (Phoresis, Sebia, France). Protein standards (albumin, α- and β-globulins, and γ- globulins) were added as a reference for densitometric analysis.

Total proteins (TP) were assessed in duplicate by a bicinchoninic acid assay [30] with a commercial kit (BCA Protein Assay Kit, Piece, USA) on a microplate spectrophotometer at 562 nm.

2.6. Statistical analysis

Data were collected in a database and analysed by the General linear model for repeated measures on SPSS 11.5 [31]. The between-subjects factor was represented by herds (5 levels) and the within-subjects factor was represented by sampling time (9 levels) and the model applied was a full factorial, with polynomial contrasts for within-subjects factor.

3. RESULTS

3.1. Blood parameters pattern

Only heifers that fulfilled the sampling schedule were included and therefore the study involved 39 heifers from five herds. The heifers considered did not show signs of clinical diseases or acute distressful conditions during the whole follow-up period.

The patterns observed for RB and NAGase means (± SE) are reported in Figure 1, for HP, SOD and NO in Figure 2 and for the different protein fractions in Figure 3.

Table I summarises the results of the statistical analysis on the differences among samplings by the means of a general linear
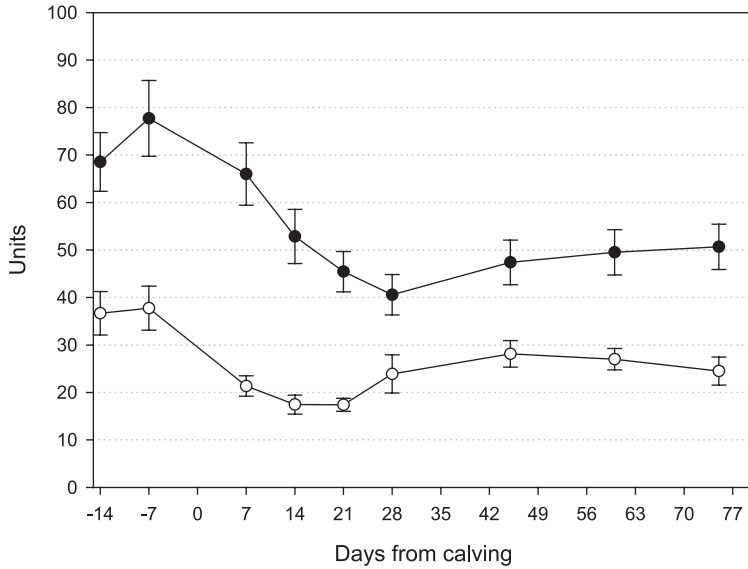
Figure 1. Distribution of the mean ± SE of NAGase (●: units) and respiratory burst (○: mV) during the follow-up period.
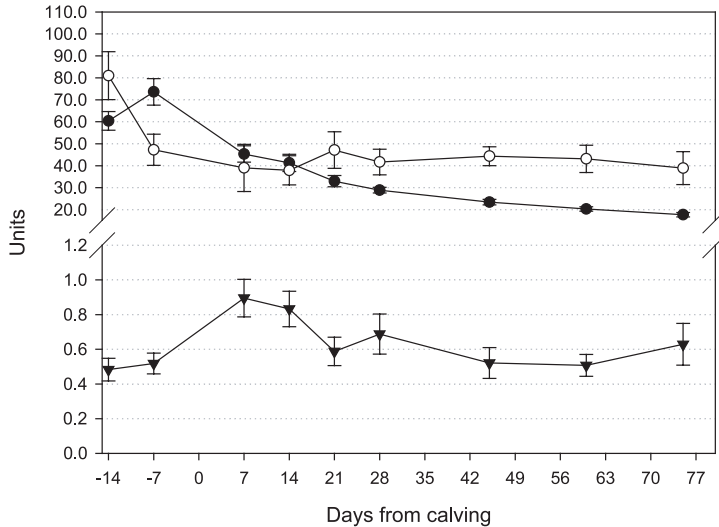

Figure 2. Distribution of the mean ± SE of nitric oxide (●: units), superoxide dismutase (○: μM/mL) and haptoglobin (▼: mg/mL).

model (GLM) for repeated measures. After calving, a significant decrease of respiratory burst was observed in comparison with the pre-calving values until 75 d, while the differences among the post-calving samplings were significant only in the 21 d sampling (lowest level). Significant decreases in NAGase activity and in NO concentration was also observed, in comparison with pre-calving values. Moreover, NO showed
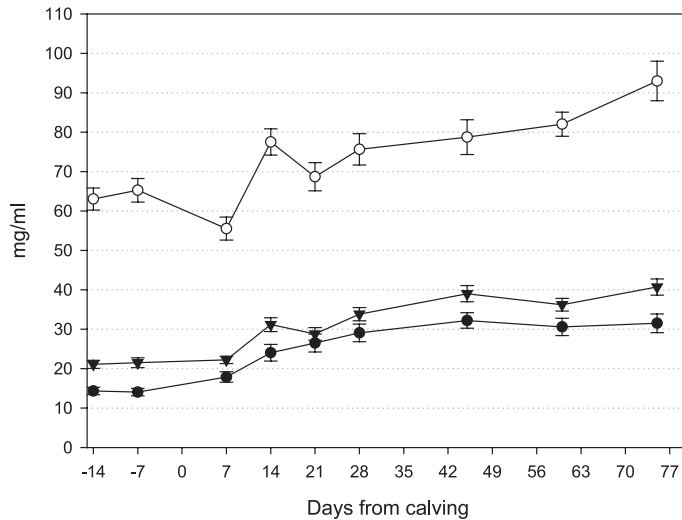
Figure 3. Distribution of the mean ± SE of albumin (○: mg/mL), γ-globulin (●: mg/mL), β-globulin (▼: mg/mL).

Table I. The presence of a significant difference ($P < 0.05$) among samples by a general linear model for repeated measurements of blood parameters (respiratory burst, acute phase response and oxidative stress).

Days from calving	Days from calving							
	−7	7	14	21	28	45	60	75
−14	NAGase[a] SOD NO	RB[b] SOD	RB SOD	RB NO	NAGase NO	NAGase RB NO	NAGase RB SOD NO	NAGase RB SOD NO
−7		RB NO	NAGase RB NO	NAGase RB NO	NAGase NO	NAGase RB NO	NAGase RB NO	NAGase RB NO
7			NAGase SOD	NAGase SOD	NAGase SOD NO	NAGase SOD NO	NAGase NO	NAGase NO
14					RB NO	NO	NO	NO
21					RB NO	RB NO	RB NO	NO
28						NO	NO	NO
45–75								NO[c]

[a] The presence of the parameter acronym in the cell shows the presence of a significant difference between the samplings indicated by the respective row and column headings.
[b] NAGase: N-acetyl- β-glucosaminidase, NO: nitric oxide, RB: respiratory burst, SOD: superoxide dismutase.
[c] Significant differences were observed only with NO values at 75 days.

Table II. The presence of a significant difference ($P < 0.05$) between samples by the general linear model for repeated measurements of the different serum protein fractions.

Days from calving	Days from calving							
	−7	7	14	21	28	45	60	75
−14		Albumin A/G	TP[a,b] Albumin βG, γG A/G	TP Albumin βG, γG A/G	TP Albumin βG, γG A/G	TP Albumin βG, γG A/G	TP Albumin βG, γG A/G	TP Albumin βG, γG A/G
−7		Albumin A/G	TP Albumin βG, γG A/G	TP βG, γG A/G	TP Albumin βG, γG A/G	TP βG, γG A/G	TP Albumin βG, γG A/G	TP Albumin βG, γG A/G
7			TP Albumin βG	TP Albumin βG, γG	TP Albumin βG, γG	TP Albumin βG, γG	TP Albumin βG, γG	TP Albumin βG, γG
14						TP βG, γG A/G		TP
21						βG	βG	TP Albumin βG
28								βG
45–75[c]								

[a] The presence of a parameter acronym in the cell shows the presence of a significant difference between the samplings indicated by the respective row and column headings.
[b] TP: total proteins; βG: β-globulin; γG: γ-globulins; A/G: albumin/globulin ratio.
[c] Significant differences were not observed in this period.

a progressive and significant decrease after calving with differences among the samplings up to 28 d. No other parameter showed any significant difference among the samplings, out of the SOD values in the first two samplings and in the last two samplings after calving in comparison with 14 d precalving samples.

Table II reports the results of the statistical analysis related to the different serum protein fractions. Total proteins, albumin, β-globulins (βG) and γ-globulins (γG) showed a progressive and significant increase in concentration after calving, in comparison with the pre-calving values. Albumin and βG concentrations were significantly lower at 7 d after calving in comparison with the following samplings, while γG concentrations showed significant differences only in comparison with the samples taken at 7 days

after calving. The albumin/γ-globulins (A/G) ratio varied with values > 1 in the pre-calving period and at the first sampling after-calving, then decreased to values < 1 until 75 d of sampling. The values were significantly different among pre-calving samples and all the post-calving ones, but there were not significant differences among the post-calving samples.

The results show that significant differences could not be identified among the values observed in the samples taken from 28 to 75 days post-calving, with very few exceptions.

3.2. Herd influence on non-specific immune status

The influence of the herd, sampling date and their interactions on the variance of the

Table III. The summary of analysis of variance with a general linear model for repeated measurements for the blood immune parameters considered.

Parameter	Within-subjects factors		Between-subjects factor
	Sampling	Sampling × Herd	Herd
NAGase	0.001	n.s.[a]	n.s.
Haptoglobin	n.s.	n.s.	n.s.
Lysozyme	n.s.	n.s.	n.s.
SOD	0.009	0.008	n.s.
Nitric Oxide	0.001	0.001	0.001
Respiratory Burst	0.001	0.039	n.s.
Total proteins	0.001	n.s.	0.032
Albumin	0.001	n.s.	n.s.
Beta-globulins	0.001	n.s.	0.040
Gamma-globulins	0.001	n.s.	n.s.
Albumin/globulin ratio	0.001	0.022	n.s.

[a] Not significant ($P > 0.05$).

different parameters considered are reported in Table III, while the statistical analysis of the values observed for the four parameters with a significant herd × sampling interaction and some objective performance characteristics are reported in Table IV.

The patterns of mean SOD and RB for the five different herds observed during the follow-up period are reported in Figure 4.

The SOD pattern did not show any statistically significant changes in herds C and D, while in herds B and E post-calving values decreased significantly, in comparison with the pre-calving values. In herd A, significant decreases of SOD values were observed only during the first two weeks after calving. Respiratory burst patterns also showed differences among the herds. Indeed, herd D was characterised by overall higher levels of RB and an inconsistent decrease after calving, while herd A showed significant lower RB values during most of the follow-up period. Herd B had lower RB values at 14, 21 and 28 d after calving, then PMN activity rose again, while herds C and E showed a significant decrease only during the first month after calving, in comparison with the second sampling before calving.

Data on NO and A/G ratio are reported in Figure 5; for both parameters the patterns were rather homogeneous between herds, in comparison with SOD and RB. However in herd D, the NO values showed only a few significant changes after calving in comparison with either the first or the second sampling before calving. Herds B, C and E showed significant decreases of NO activity from the third week after calving in comparison with both pre-calving samplings, while in herd A the significance was only towards the first sampling before calving. The A/G ratio showed very low variations in herds D and A, while in herds B and E the ratio decreased significantly during the follow-up period and herd C showed irregular variations of the values.

4. DISCUSSION

Measuring immune parameters in commercial dairy herds could be useful for different aims: as a direct evaluation of health status of the cow, as a measure of cow welfare or as a mean to assess if the efficacy of the treatment could influence the non-specific immune response. To be applied in the field, the assays should be fairly easy to perform in the laboratory, with a relatively high throughput of the samples, and, possibly, cheap. Besides, the measurement should

Table IV. Herd performance characteristics and the presence of a significant difference ($P < 0.05$) for selected markers[a], between post-calving samples and pre-calving samples in the single herds.

Herd	Yield EMC (kg)	Mean daily yield (kg)	Age at 1st calving (months)	Calving interval (days)	Monthly culling rate (%)		7	14	21	28	45	60	75
							Post-calving samples (comparison with pre-calving values)						
A	8 936	22.29	26.52	439	4.94	SOD	A[b]	A					A
						RB	A	A	A		A	A	A
						NO			A	A	A	A	A
						A/G			A		AB		AB
B	11 003	31.79	26.25	416	2.89	SOD	A	A	A	A	A	A	A
						RB		A	A	A			
						NO		B	AB	AB	AB	AB	AB
						A/G	AB		AB	AB	AB	AB	AB
C	11 558	30.91	24.74	432	3.33	SOD							
						RB	B	B	B	B			
						NO			AB	AB	AB	AB	AB
						A/G	AB		AB		AB	AB	AB
D	10 944	30.92	28.89	438	3.47	SOD							
						RB	A		B			B	
						NO		A		B	B		
						A/G				B		B	
E	11 227	29.88	25.96	485	2.86	SOD	A	A		A	A	A	A
						RB	B	B	B	B			
						NO		AB	AB	AB	AB	AB	AB
						A/G	AB	AB	AB	AB	AB	AB	

[a] SOD: superoxide dismutase; NO: nitric oxide; RB: respiratory burst; A/G: albumin/globulin ratio.
[b] A and B mean the presence of a significant difference between respectively the 1st sampling or the 2nd sampling before calving and the sampling defined by column headings.

be both sensitive enough to show changes in the different physiological or pathological conditions, and stable when the physiological conditions are unchanged. Therefore, different parameters that could fulfil these requirements were selected among the ones which could potentially give information about dairy cow non-specific immune defences. Lysozyme and NAGase are two cellular enzymes that could be markers for neutrophil activity of the cells, as components of cytoplasmic secretory granules [2], NAGase is also considered as a marker of inflammation [29]. Respiratory burst represents a measurement of the capacity of PMN to react to the presence of bacteria or exogenous substances [3]. NO is consid-

ered as an important mediator in the inflammatory process [21] and in the immune response [14]. SOD is one of the most important components of the antioxidant enzyme complex related to oxidative stress [4]. HP is one of the most important acute phase proteins in ruminants [18] and finally the serum protein profile was selected because it measures the amount of immunoglobulins and abnormal values are related to the early stages of inflammation [18].

Our work was focused on the periparturient period, because the presence of different physiological and pathological stressors is well-known [19] and on heifers, which show functional changes in oxidative reactions, when compared to pluriparous cows
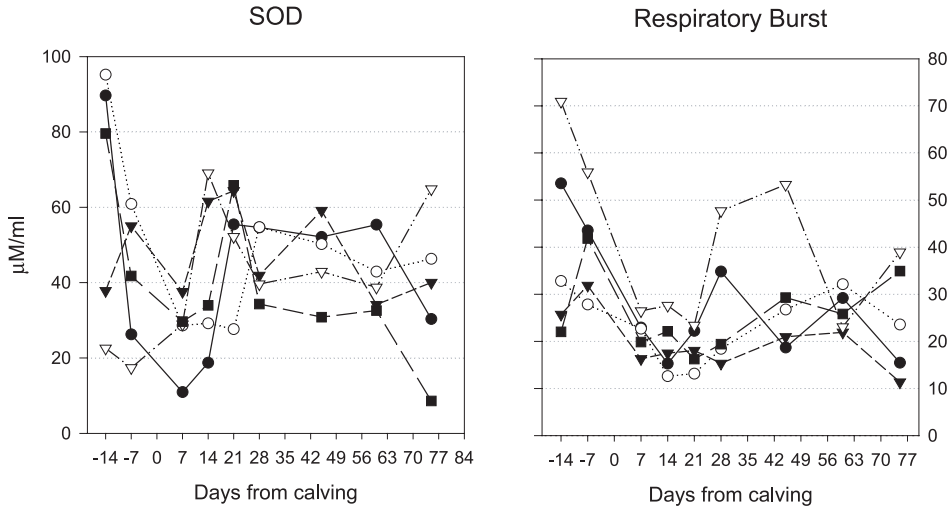
Figure 4. Distribution of mean values observed for SOD (μM/mL) and respiratory burst (mV) in the five herds considered (Herd A: ●; B: ○; C: ▼; D: ▽; E: ■).
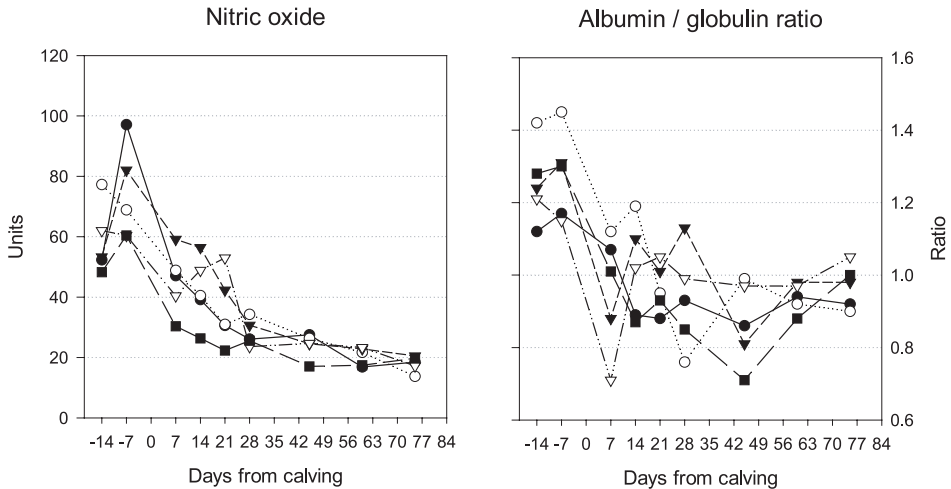

Figure 5. Distribution of the mean values observed for nitric oxide (units) and albumin/globulin ratio (units) in the five herds considered (Herd A: ●; B: ○; C: ▼; D: ▽; E: ■).

[24]. Moreover, heifers at the beginning of lactation have not been previously exposed to stressors related to lactation (i.e. milking, metabolic diseases, clinical mastitis) that could bias the results.

Lysozyme and HP showed non-significant variations in the periparturient period, therefore they cannot be considered useful biological markers to assess variations related to physiological changes. All the other parameters showed significant changes at the different sampling times until the fourth week after calving, then the values for the different parameters were statistically non-different, across sampling times, suggesting that a steady condition state was reached.

The data of this study confirmed that the first four weeks after calving have a significant influence on the non-specific immune defences. Indeed, respiratory burst and NAGase activity reached the lowest level respectively at 14 d and at 28 d after calving. The observed decrease in RB was in agreement with the results reported in recent experimental studies [23, 24]. The impairment of some of the functions related to PMN degranulation is also supported by the decrease in NAGase concentrations. The increase of HP at 7 d post-calving, even if not significant, also supports the presence of an inflammatory response that is harmful to cellular functions as expressed by the low levels of RB. Nitric oxide progressively declined from the pre-calving to the post-calving period, with higher levels during the first two weeks after calving, than in the following weeks. The high NO concentrations around calving could exert an inhibitory effect on different PMN functions such as RB, as suggested both in human [1] and veterinary medicine [7].

The decline in SOD activity during the first two weeks after calving supports the presence of an impairment of oxygen-dependent neutrophil functions, mediated by a high concentration of NO [14].

Serum proteins showed a different pattern: γG increased from calving for the first month of lactation, even if between 25 and 40% of the samples were below the 25th percentile of the parameter distribution (data not shown), supporting the presence of an impairment of the overall non-specific immune conditions. Beta-globulins have been suggested to be activators of PMN functions [33] and their pattern could be related to the RB pattern observed [23].

The preliminary analysis on the effects of the five different herds on the variance of the immune parameters considered shows that SOD, RB, NO and A/G ratio at each sampling date were statistically different across herds. If a decrease of the immune function is common to all the herds, as suggested [12], however, the impairment of

immune functions for both amplitude and length was statistically different across herds. Herd D showed an overall pattern that was different from the other four herds, with the highest levels of RB and of SOD before calving, suggesting a high PMN reactivity without inflammation signs. During the first three weeks after calving RB decreased, while SOD activity and NO values increased. Then, SOD and NO returned to low levels, RB increased, while the A/G ratio remained substantially stable. In herd D, heifers calved with the highest mean age (29 months), unlike herd C, which had similar performances (daily milk yield, calving interval and monthly culling rate), but the lowest mean age at the first calving. In this herd, high levels of NO and low RB and SOD activity were observed before calving. After calving, SOD activity increased, RB declined further and never recovered, while NO was relatively high and A/G ratio changed irregularly, suggesting a general imbalance of the immune system. This imbalance could be related to problems to cope with the physiological and metabolic stressors related to calving. Herd A, which was the poorest performer herd, showed very high levels of NO, SOD before calving and very low levels of RB and NO after calving, while SOD was consistently high after the second week after calving. In this herd, an oxidative stress and metabolic impairment could be hypothesised, justifying the SOD pattern, the low RB response and the overall poor herd performances [16, 17].

This trial shows that assessing different immune parameters is feasible in field conditions. Particularly, NAGase, respiratory burst, nitric oxide, and A/G ratio were shown to be useful markers in assessing the immune status of the cow.

The results of this study confirmed that a decrease of immune functions can be observed in commercial dairy herds during the first four weeks after calving in similar but non-equal management conditions. The amplitude of this phenomenon is not common to all animals and herds, suggesting the

possibility to reduce the impairment by improving management and genetic methods.

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