

## Comparison of blood and milk non-specific immune parameters in heifers after calving in relation to udder health

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**Abstract** – A practical protocol to study udder immune status in field conditions was planned with the aim to assess different non-specific immune parameters in milk samples from dairy heifers during the periparturient period. Five herds located in northern Italy were selected and overall 39 heifers were enrolled in the trial. Milk samples were taken at 7, 14, 21, 28, 45, 60, and 75 days after calving. The parameters assessed were N-acetyl- $\beta$ -glucosaminidase (NAGase), lysozyme, respiratory burst (RB), somatic cell counts (SCC) and serum protein profile. SCC and NAGase were higher in the first sampling after calving, while lysozyme showed large variations during the observation period without a definite trend. The levels of RB observed in the first two weeks after calving, even if lower, were not statistically different from the values observed in samples taken over the following weeks. This study confirmed that the levels of immune components in milk are different from what is observed at blood level in the same cow. A significant decrease in RB in milk polymorphonuclear leukocytes (PMN) post-calving was not observed; milk PMN from healthy cows showed low RB levels, while the values from infected quarters were significantly higher. Significant differences between healthy and infected animals were also observed for milk NAG, lactoglobulin and albumin. These data suggest that udder immune response could be influenced both by the cow immune status and by external factors such as pathogens and management. Therefore, the reduction in immune defences, particularly in heifers, is not unavoidable and methods to boost PMN activity should be explored.

**heifers / periparturient period / blood milk / non-specific immunity**

### 1. INTRODUCTION

The periparturient period is one of the most important and critical periods. It has been demonstrated that around calving some impairment of immune defences can be observed [8, 12]. This impairment increases the frequency of reproductive, production

and udder diseases, as shown experimentally and under field conditions [1, 4, 5, 7, 29].

Most of the information available on cow immune defences are based on blood parameters, while data on milk immune defences are fewer, even if their number is increasing [20, 21, 25, 27].

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In order to evaluate the possibility to develop a practical protocol to study the immune status of dairy cows under field conditions, a study was planned to assess several non-specific immune parameters in blood and milk from dairy heifers during the periparturient period. A detailed description of the results on blood parameters has already been published in this journal [23]. This paper reports the results concerning milk immune parameters, and compares the pattern of the non-specific immune parameters measured contemporarily in blood and milk in relation to the health status of the mammary gland.

## 2. MATERIALS AND METHODS

### 2.1. Animals and samplings

Five herds located in northern Italy, were selected. The herds can be considered as 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 the climate (i.e. a hot and humid climate, heavy rain) on immune parameters. Indeed, in the Italian environment late winter is the most stable period, with a low rain rate and temperature between 0 and 10 °C.

Quarter milk samples (QMS) were collected before milking by an aseptic procedure starting  $7 \pm 1$  days after calving, then at  $14 \pm 1$ ,  $21 \pm 1$ ,  $28 \pm 1$ ,  $45 \pm 1$ ,  $60 \pm 1$ , and  $75 \pm 1$  days after calving. Blood samples were taken at the same intervals as milk samples.

### 2.2. Milk bacteriological analysis

At the laboratory, an aliquot (0.01 mL) of each QMS was spread on blood agar

plate. The colonies were isolated and identified by proper methods according to the National Mastitis Council [17]. Somatic cells were counted on a Bentley Somacount 150 (Bentley, USA). The quarter status was defined following the scheme described by Pyorala [24]. Samples with SCC  $\geq 200\,000$  cells/mL were defined as *positive* for intramammary infection (IMI); a bacteriologically positive sample with SCC  $< 200\,000$  cells/mL as *latent IMI*; a bacteriologically negative sample was defined as *healthy* if SCC were  $< 200\,000$  cells/mL.

### 2.3. Serum, whey and cell isolation

Whey was obtained from skimmed milk by centrifugation at  $60\,000 \times g$  for 30 min at 4 °C, while serum was obtained from centrifugation of the blood at  $1\,500 \times g$  for 15 min at room temperature. Then the whey and serum were aliquoted in 1.500 L tubes and immediately frozen at  $-80$  °C for the enzyme analyses. These analyses were performed in a single session for each enzyme, at the end of the follow-up period.

Polymorphonuclear leukocytes (PMN) were isolated from milk by density gradient separation after centrifugation at  $500 \times g$  for 15 min at 4 °C on Ficoll-Paque Plus (AmeshamBiosciences, Sweden), following the procedure described by Carlson and Kaneko [2] and immediately delivered for the respiratory burst assay. The same procedure was followed for the isolation of blood PMN, applying blood hypotonic lysis instead of centrifugation.

### 2.4. Respiratory burst

Respiratory burst (RB) was assessed by luminol-enhanced chemiluminescence. The assay was performed adding  $0.7 \times 10^6$  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 1 µg/mL, while PMN in the other well were not stimulated (control). After injecting 40 µL of 0.3 mM 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.

### 2.5. Biochemical assays

Lysozyme was assessed in duplicate by the procedure described by Metcalf et al. [16]. The method is based on the lysis of  $\beta$ -1,4 glucosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the mucopeptide cell wall structure of *Micrococcus lysodeicticus*. The lyses of bacteria were measured by changes in optical density at 450 nm after 2 min on a microplate spectrophotometer (Spectramax 340, Molecular Devices, USA). The concentration of unknown samples, in g/mL, was calculated by a standard curve obtained by adding a standard amount of lysozyme in each plate.

N-Acetyl- $\beta$ -glucosaminidase (NAG) was assessed in duplicate by the procedure described by Kitchen et al. [10], and expressed as units defined as pmol of 4-methylumbelliferon released per min at 25 °C catalysed by 1 L of milk, on a microplate fluorimeter at 355 ex and 460 em (Ascent, ThemoLab-system, Finland).

### 2.6. Serum protein electrophoresis

Whey proteins were assessed by agarose gel electrophoresis with Hydragel 15 HR (Sebia, France), a kit that allows a higher resolution of electrophoresis in comparison with the one normally applied to serum proteins. This kit is intended for the separation of serum and whey proteins on an automated multiparametric agarose gel electrophoresis system (Hydrasis, Sebia, France). The gels were analysed by a densitometer and dedicated software (Phoresis, Sebia, France). Protein standards (albumin,  $\alpha$ - and  $\beta$ -globulins, and  $\gamma$ -globulins) were added as a reference for the densitometer analysis. Serum proteins were assessed by the same

procedure and instrument applying the standard kit for blood serum proteins (Hydragel 30, Sebia, France).

Total proteins (TP) were assessed in duplicate by a bicinchoninic acid assay [26] with a commercial kit (BCA Protein Assay Kit, Pierce, USA) on a microplate spectrophotometer at 562 nm (Spectramax 340, Molecular Devices, USA).

### 2.7. Statistical analysis

The data were collected in a database, and the differences among sampling times were analysed by the general linear model for repeated measures procedure on SPSS 11.5 [28]. The between-subject factor was represented by herd (5 levels) and the within-subject factor was represented by sampling time (7 levels) and the model applied was a full factorial, with polynomial contrasts for the within-subject factor. In order to compare the differences in immune parameters between healthy and infected cows, a general linear model procedure on SAS software [26] was applied. The model applied was a full factorial, and included herd (5 levels), health status (2 levels, healthy and positive) and sampling (7 levels), and the appropriate error terms for within- and between-subject factors as suggested by Hatcher and Stepanski [6] were applied. The cows were classified as healthy if all four quarters were healthy and positive if at least one quarter was IMI positive.

## 3. RESULTS

Overall, the frequency of IMI at the quarter level was higher at the first sampling after calving except for herd D (Tab. I). The frequency of negative samples, after the first sampling, was in the range 60–79%, while latent infections had a low frequency, in the range 11–22%. Some differences can be observed among herds. Indeed, herd E showed overall the highest frequency of

**Table I.** Distribution of quarter health status among herds and samplings (%). Quarter status is defined according to [22].

Herd	Status	Samplings							Total
		7	14	21	28	45	60	75	
A	Negative	42.86	85.71	64.29	35.71	50.00	40.00	80.00	56.98
	Latent	0	14.29	14.29	35.71	10.00	30.00	0	15.12
	Positive	57.14	0	21.42	28.57	40.00	30.00	20.00	27.90
B	Negative	26.09	50.00	63.64	68.18	45.00	75.00	65.00	55.70
	Latent	21.74	27.27	18.18	18.18	35.00	5.00	20.00	20.81
	Positive	52.17	22.73	18.18	13.64	20.00	20.00	15.00	23.49
C	Negative	50.00	57.14	64.29	92.86	78.57	78.57	85.71	72.45
	Latent	7.14	14.29	14.29	0	21.43	21.43	14.29	13.27
	Positive	42.86	28.57	21.42	7.14	0	0	0	14.28
D	Negative	87.50	62.50	66.67	50.00	62.50	62.50	75.00	66.67
	Latent	12.50	25.00	16.67	37.50	12.50	12.50	25.00	20.37
	Positive	0	12.50	16.66	12.50	25.00	25.00	0	12.96
E	Negative	43.75	86.67	82.35	88.88	66.67	61.11	88.89	74.16
	Latent	6.25	13.33	11.76	5.56	22.22	22.22	0	11.67
	Positive	50.00	0	5.89	5.56	11.11	16.67	11.11	14.17
Total	Negative	44.00	67.12	68.49	69.73	60.00	65.72	78.57	64.70
	Latent	10.67	19.18	15.07	17.11	22.86	17.14	11.43	16.17
	Positive	45.33	13.70	16.44	13.16	17.14	17.14	10.00	19.13

healthy quarters during the whole follow-up period, while herd A showed the highest frequency of IMI in all samplings. Among the 100 bacteriological positive samples, 45% were coagulase negative Staphylococci, 39% environmental streptococci 14% *Staphylococcus aureus* and 2% other species.

Table II reports the mean values observed for the different milk parameters during the follow-up period. The first sampling after calving was the highest for SCC, NAGase (NAG) and  $\gamma$ -globulins ( $\gamma$ G). Serum albumin (ALB) and lactoferrin (LF) were rather stable; lactoglobulin (LG) and respiratory burst (RB) values were higher after the first sampling, whereas lactoalbumin (LA) showed an increasing trend as the days in milk (DIM) increased. The statistical analysis for repeated measurements (Tab. III) confirmed that the mean values at the first

sampling for NAG, SCC, LG and  $\gamma$ G were statistically different from the values observed in the following samplings. Lactoalbumin showed significantly higher values in samples taken from 28 DIM until the end of the follow-up period, when compared with the samples taken in the first three weeks of lactation.

### 3.1. Factors influencing non-specific immune parameters in milk

The analysis of the influence of the different factors on milk immune parameters (Tab. IV) showed that the interaction of herd and sampling was significant for all immune parameters considered, out of RB. The herd was statistically significant only for ALB and  $\gamma$ G, while sampling was statistically significant for all parameters except for lysozyme, ALB and LF.

**Table II.** Mean values ( $\pm$  SE) for the different milk immune parameters assessed from 7 to 75 days after calving.

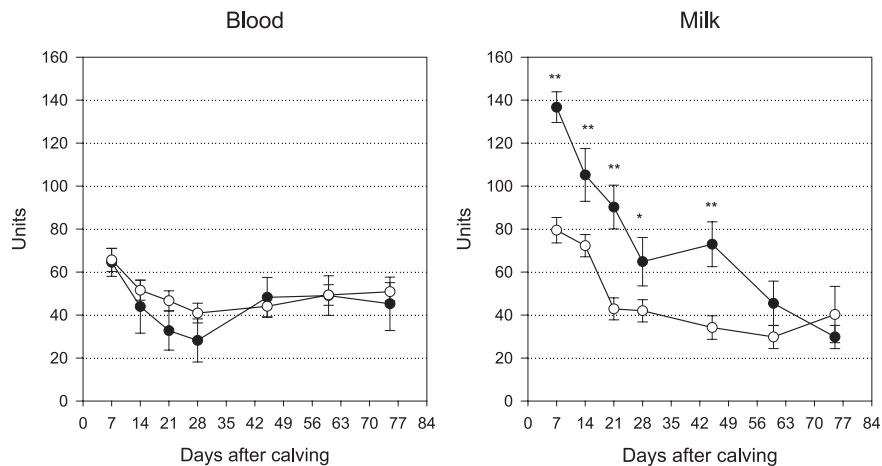
Milk parameter	Days from calving						
	7	14	21	28	45	60	75
Lysozyme ( g/mL)	0.21 $\pm$ 0.11	0.05 $\pm$ 0.05	0.11 $\pm$ 0.06	0.13 $\pm$ 0.08	0.21 $\pm$ 0.15	0.18 $\pm$ 0.18	0.22 $\pm$ 0.16
NAGase (units)	108.0 $\pm$ 7.9	71.9 $\pm$ 4.6	53.3 $\pm$ 3.8	44.1 $\pm$ 2.5	44.2 $\pm$ 3.4	31.2 $\pm$ 3.3	33.4 $\pm$ 3.8
Serum albumin (%)	6.5 $\pm$ 0.2	6.7 $\pm$ 0.2	6.8 $\pm$ 0.3	6.9 $\pm$ 0.3	6.7 $\pm$ 0.3	6.2 $\pm$ 0.2	6.4 $\pm$ 0.3
Lactoglobulin (%)	42.3 $\pm$ 0.9	45.6 $\pm$ 0.6	46.5 $\pm$ 0.6	45.6 $\pm$ 0.5	45.3 $\pm$ 0.6	46.2 $\pm$ 0.5	45.9 $\pm$ 0.7
Lactoalbumin (%)	25.6 $\pm$ 0.5	26.4 $\pm$ 0.4	26.4 $\pm$ 0.4	28.3 $\pm$ 0.5	30.2 $\pm$ 0.5	31.7 $\pm$ 0.4	31.5 $\pm$ 0.9
Lactoferrin (%)	3.21 $\pm$ 0.3	3.14 $\pm$ 0.2	3.19 $\pm$ 0.2	3.25 $\pm$ 0.1	3.21 $\pm$ 0.1	2.89 $\pm$ 0.1	2.63 $\pm$ 0.2
$\gamma$ -globulin (%)	18.77 $\pm$ 0.9	15.12 $\pm$ 0.4	13.9 $\pm$ 0.4	12.96 $\pm$ 0.4	11.55 $\pm$ 0.4	10.14 $\pm$ 0.4	11.04 $\pm$ 1.0
Oxidative burst (mV)	4.01 $\pm$ 1.0	4.95 $\pm$ 1.3	3.94 $\pm$ 1.3	3.43 $\pm$ 1.1	5.66 $\pm$ 2.2	5.37 $\pm$ 1.9	4.43 $\pm$ 2.2
SCC (log <sub>10</sub> cell/mL)	4.90 $\pm$ 0.1	4.14 $\pm$ 0.1	4.28 $\pm$ 0.1	4.07 $\pm$ 0.1	3.93 $\pm$ 0.1	4.03 $\pm$ 0.1	3.96 $\pm$ 0.2

**Table III.** Presence of a significant difference ( $P < 0.05$ ) among samples by the general linear model for repeated measurements of milk parameters.

Days from calving	Days from calving					
	14	21	28	45	60	75
7	NAG <sup>a,b</sup>	NAG	NAG	NAG	NAG	NAG
	LG	LG	LG	LG	LG	LG
			LA	LA	LA	LA
	$\gamma$ G SCC	$\gamma$ G SCC	$\gamma$ G SCC	$\gamma$ G SCC	$\gamma$ G SCC	$\gamma$ G SCC
14		NAG	NAG	NAG	NAG	NAG
		$\gamma$ G	$\gamma$ G	$\gamma$ G	$\gamma$ G	$\gamma$ G
			LA	LA	LA	LA
21					NAG	NAG
				LG	LG	
			LA	LA	LA	LA
			SCC	SCC	$\gamma$ G	$\gamma$ G SCC
28						NAG
					LA	LA
45					$\gamma$ G	$\gamma$ G
					NAG	NAG
60					LA	LA
						RB

<sup>a</sup> The presence of parameter acronym in the cell shows the presence of a significant difference between the samplings indicated by the respective row and column headings.

<sup>b</sup> NAGase: N-acetyl- $\beta$ -glucosaminidase, ALB: serum Albumin, LA: lactoalbumin, LG: lactoglobulin,  $\gamma$ G: gamma-globulin, LF: lactoferrin, SCC: somatic cell counts, RB: respiratory burst.



**Figure 1.** Distribution of the mean  $\pm$  SE of NAGase in blood and milk, by mammary gland health status ( $\bullet$ : IMI;  $\circ$ : healthy) during the follow-up period. Stars on sampling mean a statistical significant difference among negative and positive milk samples (\*  $P < 0.10$ ; \*\*  $P < 0.05$ ).

**Table IV.** Summary of analysis of variance with the general linear model for repeated measurements for the milk immune parameters considered.

Parameter	Within-subject factors		Between-subject factor
	Sampling	Sampling $\times$ Herd	Herd
Lysozyme	n.s. <sup>a</sup>	0.068	n.s.
NAGase	0.000	0.001	n.s.
Albumin	n.s.	0.003	0.047
Lactoglobulin	0.005	0.000	n.s.
Lactoalbumin	0.000	0.000	n.s.
Lactoferrin	n.s.	0.009	n.s.
$\gamma$ -globulin	0.000	0.002	0.057
Respiratory burst	0.071	n.s.	n.s.
SCC	0.000	0.000	n.s.

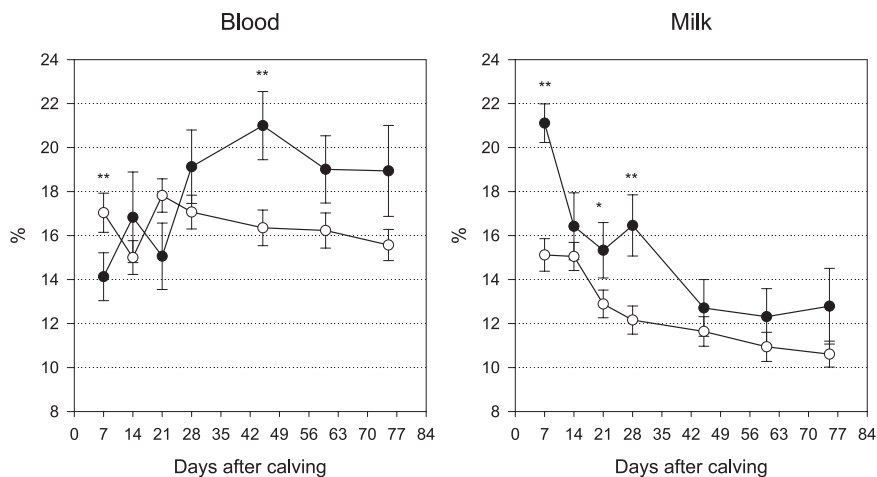
<sup>a</sup> Not significant ( $P > 0.05$ ).

### 3.2. Blood and milk comparison

The most challenging results were observed when blood and milk immune parameters were compared in healthy and IMI animals. Latent infections were not included in the analysis, because of their low frequency.

NAGase in blood showed similar patterns in both samples taken from healthy and unhealthy cows, with a decrease in the

first month of lactation (Fig. 1). A different pattern was observed in milk. Indeed, milk samples showed an overall decline in NAGase activity as lactation advanced. However, mean levels in infected quarters showed values significantly higher than those in negative ones. In the second and third months of lactation, negative quarters showed rather stable values around 40 units, while in IMI the quarters declined progressively, even if the



**Figure 2.** Distribution of the mean  $\pm$  SE of  $\gamma$ -globulins in blood and milk, by mammary gland health status ( $\bullet$ : IMI;  $\circ$ : healthy) during the follow-up period. Stars on sampling mean a statistical significant difference among negative and positive blood and milk samples (\*  $P < 0.10$ ; \*\*  $P < 0.05$ ).

levels were higher than in the negative quarters, until the last sample.

Lysozyme showed a very large variation in blood, when stratified by the mammary gland health status, while milk lysozyme showed very low mean levels independently of sampling and udder health (data not shown).

Gamma-globulins showed a completely different pattern between IMI and negative quarters both in blood and milk (Fig. 2). Blood  $\gamma$ G was significantly lower at first sampling in IMI quarters, increasing significantly at 45 DIM, when compared with negative quarters. In milk,  $\gamma$ G were significantly higher in IMI quarters in comparison to negative quarters, and the values declined in both cases from 7 to 45 DIM.

Milk PMN respiratory burst levels were significantly higher in IMI samples when compared with healthy ones, throughout the follow-up period (Fig. 3). In the first three weeks after calving, RB in blood PMN showed levels lower than in the following samplings, both in healthy and IMI cows.

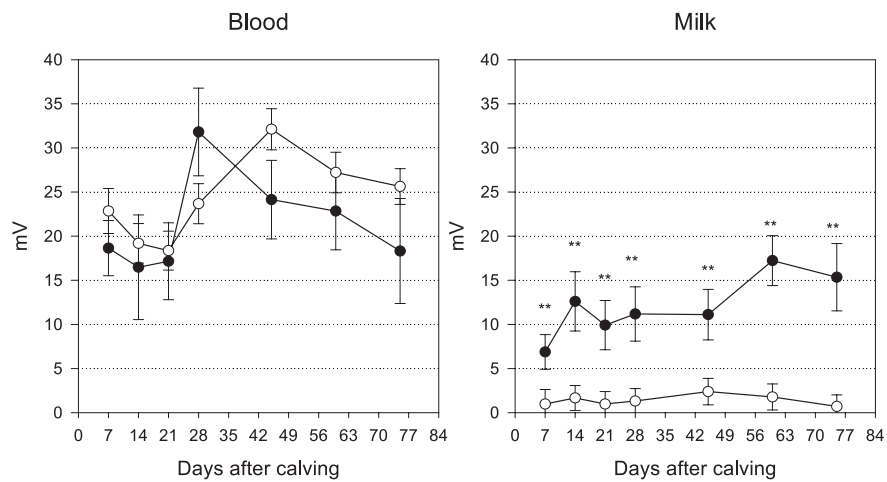
Milk serum albumin (Fig. 4) showed a different pattern from the other parameters

considered. The mean values were similar during the follow-up period in both healthy and IMI quarters, except for samples taken at 21 and 28 DIM when the IMI quarters showed significantly higher levels of ALB in comparison with the healthy quarters. This pattern was the opposite of the one observed in blood, where infected cows showed lower ALB concentration in comparison with healthy cows, with a significant difference at 28 d (Fig. 4).

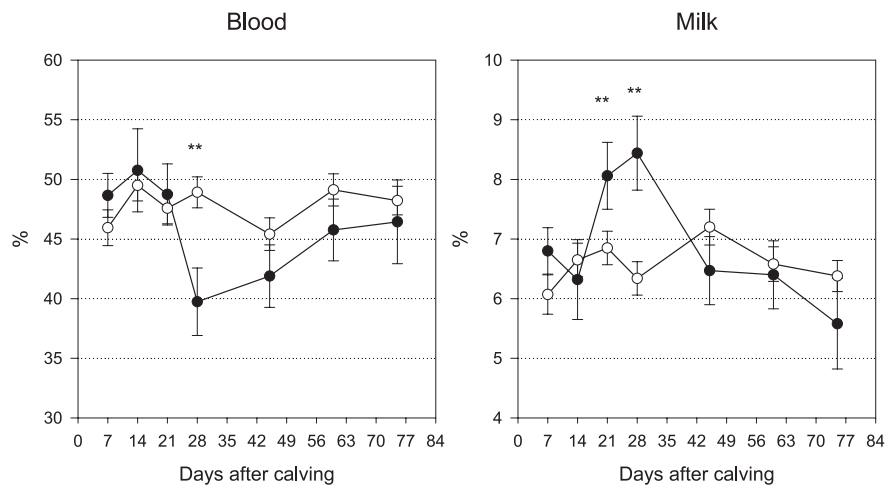
Figure 5 reports the pattern of LG in milk. During the first month of lactation IMI quarters showed significant lower levels in comparison with negative quarters. In blood, globulins showed lower values in the first month of lactation, both in IMI and negative cows, in comparison with the remaining part of the follow-up period.

#### 4. DISCUSSION

The mammary gland is a very peculiar organ; indeed, while its main role is to produce milk, one of the safest foods in nature, it is open to the external environment and



**Figure 3.** Distribution of the mean  $\pm$  SE of respiratory burst in blood and milk, by mammary gland health status (●: IMI; ○: healthy) during the follow-up period. Stars on sampling mean a statistical significant difference among negative and positive milk samples (\*  $P < 0.10$ ; \*\*  $P < 0.05$ ).

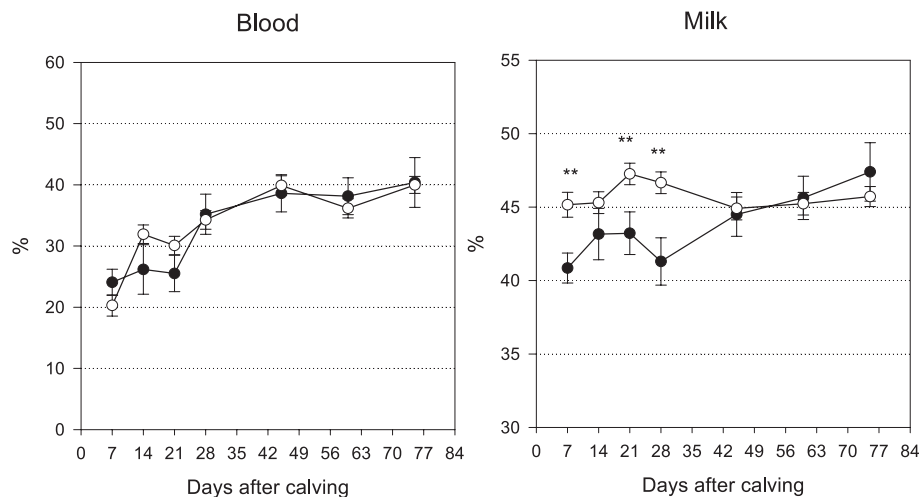


**Figure 4.** Distribution of the mean  $\pm$  SE of albumin in blood and milk, by mammary gland health status (●: IMI; ○: healthy) during the follow-up period. Stars on sampling mean a statistical significant difference among negative and positive blood and milk samples (\*  $P < 0.10$ ; \*\*  $P < 0.05$ ).

therefore exposed to pathogens. This explains the scientific and practical interest in understanding mammary gland immune defences [3, 29].

In this field study, non-specific humoral defences, such as LF and milk  $\gamma$ G, showed higher mean values in the first week after calving, when compared with the following





**Figure 5.** Distribution of the mean  $\pm$  SE of globulins ( $\alpha + \beta$ ) in blood and lactoglobulin in milk, by mammary gland health status ( $\bullet$ : IMI;  $\circ$ : healthy) during the follow-up period. Stars on sampling mean a statistical significant difference among negative and positive milk samples (\*  $P < 0.10$ ; \*\*  $P < 0.05$ ).

samplings. However, the differences were statistically significant only for  $\gamma$ G, as expected, being that this latter component is one of the most important milk constituents for newborn calves.

SCC and consequently milk NAGase, a lysosomal enzyme, were higher in the first sampling after calving, as expected [10, 24]. The levels of RB in milk PMN observed in the first two weeks after calving, even if lower, were not statistically different from the values observed in samples taken over the following weeks. This result was not in agreement with the results observed in blood, where a significant decrease was observed [23], but indirectly confirmed what was observed in experimental studies [15].

Milk lysozyme showed large variations during the observation period, without a definite trend. The influence of different herd IMI prevalence on this result could be hypothesised. Indeed, the role of the pathogens in eliciting different levels of immune response has been demonstrated [14, 22]. However, in our study, the differences observed in

immune parameters were not statistically associated with the different bacteria isolated (data not shown).

Overall, these data confirmed the results of a previous study on blood parameters [23], suggesting that the level of non-specific immune parameters could be influenced, in addition to health status, by management and genetics. These latter aspects need more targeted studies to confirm this hypothesis and to define the role of bacteria.

The comparison of immune parameters in blood and milk confirmed that the immune response at the milk level could be different from what is observed at the blood level, as suggested by [15]. Indeed, NAG activity was higher in milk than in the blood in the first two weeks after calving. Moreover, samples from infected quarters showed significantly higher NAG activity in comparison with healthy ones, suggesting an immune response by milk cells. Gamma-globulin behaviour confirmed these observations; indeed, blood  $\gamma$ G showed a progressive increase, especially in infected

cows, while it was the opposite for milk  $\gamma$ G. In infected animals, we observed an increase of blood  $\gamma$ G at 45 DIM, but not at the milk level. A possible explanation of this result is supplied by the increase of ALB in milk at 21 and 28 DIM, and the parallel decrease in blood. This observation suggests the opening of the milk-blood barrier, due to the release of mediators of inflammation, and the consequent stimulation of  $\gamma$ G production [14].

Lactoglobulin, the major whey protein of ruminants, showed significant differences in IMI quarters in comparison with negative ones. The functions of this protein are still unclear [11], but our data support the hypothesis that LG could be involved in preventing bacterial adhesion as hypothesised at the gut level [11, 18, 19]. If the protein acts in the same way at the udder level, its lower concentration could ease the outcome of bacterial infection.

Lactoglobulin is synthesised at the udder level; therefore the differences observed in milk from healthy and IMI animals, but not in blood globulins was not unexpected. However, the low levels observed in blood globulins during the first month of lactation support the presence of an impairment of immune defences as described [13, 23].

We did not observe a significant decrease in RB in milk PMN post-calving, as happens in blood PMN [23]. However, the RB values from infected quarters at 7 d were lower, even if not significantly, than the values observed during the last part of the follow-up period. Milk PMN from healthy cows showed very low RB levels in all samples, while the values from the infected quarters were significantly higher. This could be explained by the presence of activated PMN as suggested by Mehrzad et al. [15]. The release of mediators of inflammation at the udder level could induce a depletion of activated blood PMN to milk [14, 15] and therefore the decrease of RB in blood PMN from infected animals, after the first month of lactation.

These data suggest that a decrease in non-specific immune defences can be observed during the periparturient period at the milk level, as expected [9, 12, 13, 15], but to a lesser extent in comparison to what is observed at the blood level in the same cow [23]. Cellular enzymes and RB showed different values in comparison to blood ones, but a significant decrease in the first samplings after calving could not be demonstrated. The interaction with herds and with IMI prevalence suggests that the udder immune response could be influenced both by the cow's immune status and by external factors such as pathogens and management. Therefore, the reduction in immune defences, particularly in heifers, is not unavoidable, as already suggested under experimental conditions [14], and the methods to particularly boost PMN activity should be investigated.

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