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**Phagocytic and bactericidal activity of blood and milk-resident neutrophils against  
*Staphylococcus aureus* in primiparous and multiparous cows during early lactation**

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## Abstract

To examine the effect of parity on polymorphonuclear neutrophils (PMN) function, phagocytic and bactericidal activity of the PMN isolated from blood and milk against *Staphylococcus aureus* was compared between groups of 6 primiparous and 6 multiparous healthy dairy cows during early lactation using bacteriological and PMN-pathogen interaction assays. Latex-stimulated luminol-amplified chemiluminescence (CL) and viability of these PMN were also investigated. The phagocytosis and killing of *S. aureus* by PMN and the CL and viability in blood PMN were markedly higher than in milk PMN. Both in blood and in milk the phagocytosis of *S. aureus* by PMN in primiparous cows was substantially higher than in multiparous cows. The killing activity of blood PMN against *S. aureus* was  $42.3 \pm 3.4$  % and  $23.2 \pm 1.7$  % in primiparous and multiparous, respectively. Milk PMN killed only  $20.7 \pm 2$  % *S. aureus* in primiparous and  $10.2 \pm 1.3$  % in multiparous cows. Blood and milk PMN CL and milk PMN viability were significantly higher in primiparous cows. The pronounced reduction in phagocytic and bactericidal activity in blood and milk-resident PMN from multiparous cows, in part, resulted from pronounced decrease of PMN viability and free radicals production capacity; this suggests that heifers' udders could be more protected against *S. aureus*, which remains to be tested in the field.

**Keywords:** Intracellular killing; Mastitis; Parity; Phagocytosis; *Staphylococcus aureus*

## 1. Introduction

Mastitis is a very common disease that causes major economic losses to the dairy industry worldwide. This inflammatory reaction develops most frequently in response to an intramammary bacterial infection (Smith et al., 1985; Paape et al., 2002; Burvenich et al., 2003). *Staphylococcus aureus* is one of the most virulent organisms with low cure rates involved in bovine mastitis (Miles et al., 1992; Burvenich et al., 2003; Smith et al., 2005). To establish intramammary infection, *S. aureus* has to overcome several host defence barriers (phagocytosis, recruitment of polymorphonuclear neutrophils (PMN), etc.). *Staphylococcus aureus* possesses factors such as extracellular polysaccharide layer (exopolysaccharide or slime) and capsule polysaccharides that boost bacterial evasion by, e.g., impairing complement- and antibody-mediated opsonisation and phagocytosis (Wilkinson et al., 1979; Xu et al., 1992). This evasion mechanism enhances the virulence capacity of *S. aureus* (Quie et al., 1987; Baselga et al., 1994).

Several antimicrobial systems exist in the mammary gland (Paape et al., 2002; Burvenich et al., 2003). It is, however, the massive influx of PMN that will resolve the infection. These cells have enormous potential to eventually kill phagocytosed bacteria (Paape et al., 2002; Burvenich et al., 2003; Mehrzad et al., 2004; 2005). The phagocytic and bactericidal activity of PMN in dairy cows is one of the major protective mechanisms against invading pathogens in the mammary gland (Mehrzad et al., 2001a; Paape et al., 2002; Burvenich et al., 2003). When PMN engulf invading bacteria free radicals are rapidly produced inside the phagosome and phagolysosome. These free radicals react very broadly against Gram negative and positive bacteria, fungi and protozoa (Heyneman et al., 1990; Hampton et al., 1996; Chapman et al., 2002; Reeves et al., 2002; Burvenich et al., 2003). Free radicals production capacity of PMN can be measured following PMN stimulation with particles e.g. zymosan, bacteria, latex beads. The most widely used technique to quantify free

radicals production of PMN is the chemiluminescence (CL) assay (Piccinini et al., 1999, Hoeben et al., 2000; Mehrzad et al 2001a; 2001b; 2005). The CL of PMN reflects intracellular and extracellular events of the PMN-pathogen interactions, which are very critical for the mechanisms of bacterial killing. The increased incidence of severe mastitis during early lactation is correlated with a decreased function of blood and milk PMN (Burvenich et al., 2003; Mehrzad et al., 2004; 2005), further emphasizing the pivotal role of PMN in the defence against intramammary pathogens. Despite their importance, little study has been conducted on the issue of PMN-*S. aureus* interaction in the mammary gland.

It is widely accepted that clinical mastitis in heifers is predominantly local and to a much lesser extent systemic (Burvenich et al., 2003; Vangroenweghe et al., 2004) compared to multiparous dairy cows. It also appears that the transition from pregnancy to parturition is accompanied by a gradual decrease in PMN bactericidal capacity (Hoeben et al., 2000; Mehrzad et al., 2001a, 2002). The severity of clinical mastitis has been reported to be less pronounced in dairy cows with higher pre-infection PMN free radicals production (Heyneman et al., 1990; Mehrzad et al., 2004; 2005). Increased severity of mastitis in dairy cows has also been associated with increased parities (van Werven et al., 1997). All of these changes might be due to the fact that blood and milk PMN in heifers react faster than in multiparous cows. To our best knowledge, the influence of parity on overall killing capacity of blood and milk PMN has never been assessed.

Since *S. aureus* is a common cause of contagious mastitis in dairy cows, blood and milk PMN bactericidal activity against this bacterium was investigated during relatively immune suppression, early lactation (Dosogne et al., 2001; Mehrzad et al., 2001a; 2002; Burvenich et al., 2003). The main objective of this study was, therefore, to compare the phagocytic and bactericidal activity of blood and milk-resident PMN against *S. aureus* in heifers and older cows to get a better understanding of the bactericidal activity of the PMN.

## 2. Materials and methods

### 2.1. *Animals and experimental procedures*

In total, 12 Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected. Primiparous cows (first gestation,  $2.4 \pm 0.5$  yr,  $n=6$ ) and multiparous cows (forth to fifth gestation,  $5.8 \pm 0.7$  yr,  $n=6$ ) were used. After calving (until  $30 \pm 5$  days (mean  $\pm$  SD) after parturition for both groups), 1.5 L mixed cisternal 4-quarter-morning milk samples and 80 ml blood samples were aseptically collected (Mehr-  
 90 et al., 2001a; 2002; 2004; 2005). The cows were clinically healthy and showed no signs of periparturient diseases after calving. To confirm that cows had no mastitis pathogens, 10 ml and 50 ml quarter-foremilk samples were collected after parturition and the day before the  
 95 experiment and examined for bacteriological infection and somatic cell count (SCC), respectively. Only cows with a quarter SCC  $< 2 \times 10^5$  cells/ml and milk samples that cultured negative for major mastitis pathogens were considered as clinically healthy; to do this, the quarter milk samples were aseptically cultured onto Columbia sheep blood agar (Biokar Diagnostics, Beauvois, France) for 18 h at 37°C. Blood samples were aseptically collected  
 100 from the external jugular vein into evacuated tubes (BD-Vacutainer System, Plymouth; UK) containing 125 IU heparin for further processing.

### 2.2. *Blood and milk PMN preparation, enumeration and identification*

105 Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes (Carlson and Kaneko, 1973; Mehrzad et al., 2001a; 2001b; 2002). The isolation

procedure of PMN from blood yielded > 98% of granulocytes (PMN + eosinophils) with  
 predominantly PMN (> 86%) and a viability of > 98% in both groups. After counting the cells  
 using an electronic programmable particle counter (Coulter counter Z2, Coulter Electronics  
 110 Ltd., Luton., UK) and determining the viability as described below and percentage of PMN,  
 the suspension was adjusted to a concentration of  $5 \times 10^6$  viable blood PMN/ml with Hank's  
 Balanced Salt Solution (HBSS) containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Gibco BRL, Life Technologies  
 Inc., MD, Gaithersburg) supplemented with 1 g bovine serum albumin/l (BSA; Sigma  
 Chemicals, St. Louis MO USA) and 25 mM-HEPES buffer (HBSS-BSA; Sigma) for PMN  
 115 functional assays. The milk samples were maintained on smashed ice following sampling and  
 during the isolation procedure. Initial volumes of 1.5 L milk were processed using a high-  
 capacity centrifuge (RC – 3BP, Sorvall, Newtown, CT, USA was carefully) after 60% vol/vol  
 dilution with cold phosphate buffered saline (PBS; 0.01 M phosphate buffer ( $\text{KH}_2\text{PO}_4$ -  
 $\text{Na}_2\text{HPO}_4$ ) – 0.15 M NaCl, pH = 7.2). Fat was carefully removed after the first centrifugation  
 120 ( $600 \times g$ , 15 min,  $4^\circ\text{C}$ ) and the pellet was washed twice at  $300 \times g$  for 10 min and  $200 \times g$  for  
 15 min at  $4^\circ\text{C}$ , in the cold PBS. After counting the isolated milk cells using Coulter counter  
 and determination of the viability, the cells were finally resuspended to a concentration of  $5 \times$   
 $10^6$  viable milk cells/ml with HBSS-BSA for further PMN functional assays. Isolation of cells  
 from milk was performed using three centrifugation steps as previously described (Mehrzaad et  
 125 al., 2001a; 2002), yielding ~60% PMN with different viability throughout the experiment.  
 The isolation procedure time for milk PMN was similar to that of blood PMN ( $55 \pm 4$  min).  
 Milk somatic cell count (SCC) in whole milk was determined with a fluoro-opto electronic  
 method (Fossomatic 400 cell counter, Foss Electric, Hillerød, Denmark). The total number of  
 leukocytes and isolated blood and milk cells were determined using an electronic particle  
 130 counter (Mehrzaad et al., 2001a: b). The total number of circulating PMN was determined  
 using smear preparation of blood sample (Mehrzaad et al., 2001a; b). Similar whole blood



staining procedure was performed to isolated blood and milk cells. The procedure of cell isolation from normal milk will rarely give 100% viable PMN, though the cells in the isolate are predominantly PMN. To precisely assess PMN functions, the isolated cells were always differentiated and the viability of PMN was quantified in the isolated cells. Therefore, the number and percentage of viable PMN per sample were very precisely included, and the calculated functional assay is always for the exact number of PMN.

### 2.3. Phagocytosis and killing of *S. aureus* by blood and milk PMN

Phagocytosis and killing of *S. aureus* Newbould 305 (Naidu and Newbould, 1975) was monitored by a bactericidal assay using sample cultivation according to Barta (1993) and Barrio et al. (2000) with some modifications. The assay was run in duplicate in Eppendorf® tubes (Netheler-Hinz GmbH, Hamburg, Germany) in a final volume of 1 ml with the following composition: 100 µl preopsonised *S. aureus* variant ( $5 \times 10^7$  cfu/ml), 500 µl viable PMN ( $5 \times 10^6$  cells/ml) and 400 µl pooled bovine serum diluted to a final concentration (v/v) of 5% (complement- inactivated serum, 56 °C, 30 min). Control samples ( $C_0$ ,  $C_{60}$ ; expressed as cfu/ml) contained bacteria, HBSS and serum without PMN. The *S. aureus* to PMN ratio was 10/1. The tubes were rotated end-over-end at 37 °C for 60 min. Twenty five µl samples were taken from the mixture assay ( $Ma_0$ ,  $Ma_{60}$ ; expressed as cfu/ml) at 0 and 60 min, diluted into distilled water and kept at 0 °C for 3 h in order to disrupt the PMN. Extracellular (Ec; expressed as cfu/ml) bacteria were separated from the PMN by centrifugation (100×g, 1 min, 4 °C). A 25 µl sample of the supernatant from  $Ma_{60}$  was taken and diluted as for the mixture assay samples. Ten-fold dilutions of  $C_0$ ,  $C_{60}$ , Ec,  $Ma_0$  and  $Ma_{60}$  were performed and the last dilutions were spread onto Columbia sheep blood agar. After overnight incubation at 37 °C, colony counts were related to the original bacterial suspension. The results from the

bactericidal assay were expressed as the percentages of phagocytosed (% phagocytosis) and killed (% killing) bacteria. The percentages were calculated using the following formulas:

$$\% \text{ phagocytosis} = \frac{C_0 - C_{60}}{C_0} \times 100 \quad \text{and} \quad \% \text{ killing} = \frac{Ma_0 - Ma_{60}}{Ma_0} \times 100$$

with Bg = bacterial growth =  $C_{60}/C_0$  where  $C_0$  corresponds to control sample at 0 min and  $C_{60}$  to control sample at 60 min,  $Ma_0$  = mixture assay sample at 0 min,  $Ma_{60}$  = mixture assay sample at 60 min, EC = mixture assay sample supernatant after centrifugation (extracellular). The values for Bg,  $C_0$ ,  $C_{60}$ ,  $Ma_0$ ,  $Ma_{60}$  and EC were expressed as cfu/ml.

#### 2.4. Viability and free radicals production of blood and milk PMN

The viability of isolated PMN was determined in duplicate by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA), using propidium iodide exclusion (Mehrzhad et al., 2001a; 2004). Luminol-enhanced latex-stimulated cellular CL was used to quantify the free radicals production of PMN isolated from blood and milk (Mehrzhad et al., 2001a; 2001b; 2004; 2005). The area under the curve (AUC) was calculated over a period of 30 min. The CL response was corrected for the actual number of viable PMN in each sample. Results from the CL of milk and blood were calculated by performing the corrections according to Mehrzhad et al. (2001a, 2001b; 2005). Compared to blood PMN, we have always had far greater difficulties when working on normal milk PMN functional assay; milk isolates were less homogenous, comprising of macrophages and lymphocytes epithelial cells. It is worth addressing that contribution of especially macrophages to luminol-dependent CL is negligible, as we previously demonstrated (Mehrzhad et al., 2001a). It is, therefore, logical to exclude the macrophages

from the calculation of functional assay. The CL response was expressed per  $10^3$  viable PMN.

## 2.5. Statistical analysis

SAS Version 9.1 was used to analyze the parameters measured. Means and standard errors of the mean (SEM) were calculated, and data were presented as means  $\pm$  SEM. To compare the two groups (primiparous and multiparous) with respect to different response variables in blood and milk, analysis of variance was used. Hypothesis testing was done at the 5% significance level.

## 3. Results

### 3.1. Phagocytosis and killing of *S. aureus* by blood and milk PMN

Both in blood and in milk the phagocytosis of *S. aureus* by PMN was significantly higher in primiparous cows ( $P < 0.001$ ; Figures 1). The phagocytosis and killing of *S. aureus* by PMN in blood were markedly higher than in milk (Figures 1 and 2). The killing activity of blood PMN against *S. aureus* was  $42.3 \pm 3.4$  % and  $23.2 \pm 1.7$  % in primiparous and multiparous cows, respectively ( $P < 0.001$ ; Figure 2). Milk PMN killed only  $20.7 \pm 2$  % *S. aureus* in primiparous and  $10.2 \pm 1.3$  % in multiparous cows ( $P < 0.001$ ; Figure 2).

### 3.2. Viability and free radicals production of blood and milk PMN

No differences were observed for blood PMN viability of primiparous and multiparous cows (Table 1), and blood PMN viability was higher than their milk counterparts. The viability of milk PMN was lower in multiparous cows compared to primiparous cows ( $P < 0.001$ ; Table 1). The overall latex-stimulated CL of blood PMN in multiparous cows was markedly lower than in primiparous cows (Figure 3). Compared to multiparous cows, in blood PMN, the free radicals production was significantly higher in primiparous cows ( $P < 0.001$ ; Figure 3). Additionally, the milk PMN free radicals production in primiparous cows was consistently higher than in multiparous cows ( $P < 0.01$ ; Figure 3).

### 3.3. Enumeration of leukocytes in blood and milk

A significant difference in total white blood cell count ( $P < 0.05$ ) and number of mature neutrophils ( $P < 0.001$ ) between primiparous and multiparous cows was observed, with higher values in primiparous cows (Table 1). The total milk SCC in multiparous cows was significantly higher than in primiparous cows ( $P < 0.01$ ; Table 1). Percentage of isolated milk PMN from primiparous and multiparous cows averaged  $\pm$  SEM of  $56 \pm 3$  and  $54 \pm 2\%$ , respectively. Compared to blood PMN, isolation of PMN from normal milk was far tougher and yielded far less. We were therefore unable to give an exact number of PMN/ $\mu$ l in our original milk samples.

## 4. Discussion

Our results show a pronounced reduction in phagocytic and bactericidal activity in blood and milk-resident PMN against *S. aureus* in multiparous cows, compared with the primiparous ones. This marked suppression may increase cow's susceptibility to many age- or parity-related infections. Although we did not study any cause-effect relationship between

230 parity and PMN bactericidal inefficiency, such an association with increased cow's susceptibility to, for example, mastitis pathogens was nonetheless reported elsewhere (van Werven et al., 1997; Burvenich et al., 2003); this relationship might contribute to the severity of clinical mastitis cases. The severity of clinical mastitis in heifers is less pronounced and largely local (Burvenich et al., 2003; Vangroenweghe et al., 2004) compared to multiparous  
 235 dairy cows; increased severity also closely links to increased parities (van Werven et al., 1997). All of these relations could initially result from changes in the blood and milk PMN phagocytic and bactericidal capacity (Heyneman et al., 1990; Mehrzad et al., 2004; 2005; Hoeben et al., 2000; Mehrzad et al., 2001a, 2002). Our results in bovine innate immunity clearly narrow the bridge between PMN bactericidal capacity and parity, indirectly tightening  
 240 the link between PMN-pathogen interactions and heifer mastitis.

Consistent with other studies (Ferrante et al., 1993; Barrio et al., 2000), the described opsonisation conditions in our *in vitro* bactericidal assay revealed that the differences in phagocytic and bactericidal activity in blood and milk PMN between primiparous and multiparous cows were measured under optimal and constant opsonisation conditions.  
 245 Indeed, sufficient opsonising factors (5% complement-inactivated serum) were used for phagocytosis of the *S. aureus* by blood and milk PMN. The complement-inactivated bovine serum was used to opsonise *S. aureus* Newbould 305, mainly because the presence of complement seemed to be unnecessary for opsonisation and phagocytosis of *S. aureus* by bovine PMN (Rainard et al. 2000). Additionally, we used 1% ZAS containing complement-  
 250 inactivated serum for incubation of *S. aureus* Newbould 305 with blood PMN. The reason for this slightly different incubation condition is that blood PMN are in a quiescent state, whereas milk-resident PMN are in an activated state (Rainard et al. 2000; Mehrzad et al., 2001a; b). The difference from the study of Ferrante et al. (1993) and Barrio et al. (2000), in which less

phagocytosis and killing of *S. aureus* was observed in the presence of complement-inactivated serum, might be explained by the use of a different *S. aureus* strain in our study.

A range of 30 to 53 % of the originally administered *S. aureus* was phagocytosed by blood PMN and 20 to 30 % by milk PMN at bacteria to PMN ratio (10/1) in our study corresponds well to other studies (Saad, 1987; Barrio et al., 2000). Although different opsonisation conditions could explain some of the differences between the results of our study and those of Saad (1987) and Barrio et al. (2000), it appears that PMN phagocytosed less bacteria at a higher bacteria to PMN ratio (Barrio et al., 2000; Dosogne et al., 2001). Indeed, when the ratio of bacteria to PMN was increased from 1/3 to 11/3, the number of phagocytosed bacteria was decreased (Williams et al. 1985). These *in vitro* experiments suggest that, at a given number of bacteria, the presence of a greater number of milk PMN may provide protection for the mammary gland *in vivo* in comparison with a small amount of milk PMN. This hypothesis is supported by our previous findings (Mehrzhad et al., 2004; 2005), demonstrating that a moderately higher SCC before infection had a protective effect against induced *Escherichia coli* mastitis; the protective effect of moderately higher SCC might also be relevant for *S. aureus* mastitis. In addition, recent evidence supports the concept that critically reduced SCC is a risk factor for severe clinical mastitis in dairy herds (Suriyasathaporn et al. 2000). More to the point is that in our earlier study the primiparous cows showed lower SCC, but they did not show severe response to mastitis (Vangroenweghe et al., 2004), strongly confirming the importance of quality or viability of milk PMN, which is mostly higher in primiparous than in multiparous cows. The higher phagocytosis of *S. aureus* by milk PMN from primiparous lactating cows could mainly link to a higher quality or viability of PMN in the mammary gland (Mehrzhad, 2002).

It is indisputable that PMN free radicals are pivotal for bactericidal activity (Burvenich et al., 1994; 2003; Mehrzhad et al., 2005), though how exactly the PMN-*S. aureus* interactions

kill the *S. aureus* is still the subject of debate (Reeves et al., 2002). Central to the debate is  
 280 the issue of infections with different strains of *Staphylococci* in the field. For example,  
 intramammary *S. aureus* infections are typically characterized by high but variable SCC with  
 persistently variable shedding of bacteria and low cure rates (Smith et al., 2005). In contrast,  
 the *S. aureus* Newbould 305 strain is known to be relatively gentle with a moderate increase  
 in SCC and high cure rates (Smith et al., 2005; Piccinini et al., 1999). Because of the atypical  
 285 nature of *S. aureus* Newbould 305 as a representative of *S. aureus* pathogenics for the udder  
 and the small size of our study materials, we can not extrapolate our current results to the  
 field. Nonetheless, the lower free radicals production can, in part, lead to an overall impaired  
 bactericidal capacity of blood and milk PMN in multiparous cows.

We have convincing evidence (Williams et al. 1985; Mehrzad et al., 2001b; Mehrzad,  
 290 2002) to point out the negative correlation between intracellular survival of *S. aureus* and CL  
 activity of bovine blood PMN; this further emphasizes the crucial role of free radicals in the  
 intracellular killing of *S. aureus*. Indeed, both oxygen-dependent and -independent  
 mechanisms are involved in the intracellular killing of *S. aureus* (Hampton et al. 1996;  
 Reeves et al., 2002; Burvenich et al., 2003). Our finding, therefore, suggests that not only  
 295 oxidative bactericidal mechanisms but also non-oxidative mechanisms like PMN elastase and  
 other granule-proteases may inefficiently work in multiparous cows. This idea is in  
 agreement with the general concepts in innate immunity that the oxidative and non-oxidative  
 bactericidal mechanisms are closely interrelated (Reeves et al., 2002; Burvenich et al., 2003).

It is, however, unclear whether other bactericidal mechanisms are restricted to  
 300 multiparous cows. According to this study, the percentage of killed *S. aureus* by blood PMN  
 was significantly higher than that of milk PMN. A reduction of phagocytic and bactericidal  
 activity of milk PMN may result from diapedesis through the blood-milk barrier (Smits et al.  
 1999) and ingestion of fat globules and casein micelles (Paape et al., 1978). Further, milk

PMN could be considered as exhausted cells approaching death. Compared to blood PMN, the phagocytic and bactericidal activity of milk PMN is decreased (Paape et al. 1978; Smits et al. 1999; Mehrzad et al., 2001a; b) and the intracellular glycogen stores are depleted (Naidu and Newbould, 1975). Also, they do have a very short half-life time and show apoptotic features (Van Oostveldt et al. 2002). Nevertheless, several studies suggest that milk-resident PMN may exert a significant antibacterial activity (Burvenich et al., 2003; Mehrzad et al., 2004; 2005). For example, killing of *S. aureus* by milk PMN in the current study was only about 10-20 % lower than blood PMN. The higher growth of *S. aureus* in PMN from multiparous lactating cows was mainly associated with a less efficient intracellular free radicals production (Mehrzad et al., 2002), potentially resulting from protein kinase C, NADPH-oxidase and myeloperoxidase dysfunction (Mehrzad et al., 2001a, 2002).

## 5. Conclusion

For the first time we demonstrated a more pronounced decrease in the phagocytic and bactericidal activities of both blood and milk PMN against *S. aureus* in multiparous cows, as compared to primiparous cows, during early lactation. The higher growth of *S. aureus* in PMN from multiparous lactating cows could mainly result from a lower free radicals production capacity. The pronounced reduction in PMN bactericidal activity in milk of multiparous cows may be involved in the underlying mechanisms that make these animals more susceptible to infectious diseases. In contrast, the prevalence of (sub)clinical mastitis is also high in primiparous cows. However, there might be a difference in the resolution or the persistence of the udder infections between primiparous and multiparous cows which may attribute to differences in the bactericidal activity of the blood



and milk PMN between heifers and old dairy cows. Still, further research in that area is needed to extrapolate conclusions to the field.

330

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### Conflict of interest

None of the authors (J. Mehrzad, L. Duchateau, C. Burvenich) has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled “Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation”.

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### References

345

Barrio, B., Vangroenweghe, F., Dosogne, H., Burvenich, C., 2000. Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. Vet. Res. 31, 603-609.

350

Barta, O. 1993. Phagocytosis and killing of bacteria monitored by sample cultivation. In: Veterinary Clinical Immunology Laboratory. Bar-Lab. Inc., Blacksburg, USA, Chapter B3-IV.

355 Baselga, R., Albizu, I., Amorena, B., 1994. *Staphylococcus aureus* capsule and slime as virulence factors in ruminant mastitis. Vet. Microbiol. 39, 159-204.

Burvenich, C., Paape, M.J., Hill, A.W., Guidry, A.J., Miller, R.H., Heyneman, R., Kremer, W.D.J., Brand, A., 1994. Role of the neutrophil leukocyte in the local and systemic  
360 reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. Vet. Q. 16, 45-49.

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L., 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet. Res. 34, 521-562.

365 Carlson, G.P., Kaneko, J.J., 1973. Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med. 142, 853-856.

Chapman, A.L.P., Hampton, M.B., Senthilmohan, R., Winterbourn, C.C., Kettle, A.J., 2002. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of  
370 *Staphylococcus aureus*. J. Biol. Chem. 227, 9757-9762.

Dosogne, H., Vangroenweghe, F., Barrio, B., Rainard, P., Burvenich, C., 2001. Decreased number and bactericidal activity against *Staphylococcus aureus* of the resident cells in milk of dairy cows during early lactation. J. Dairy Res. 68, 539-549.

375

Ferrante, A., Martin, A.J., Bates, E.J., 1993. Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. J. Immunol. 151, 4821-4828.

380 Hampton, M.B., Kettle, A.J., Winterbourn, C.C., 1996. Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. Infec. Immun. 64, 3512-3517.

Heyneman, R., Burvenich, C., Vercauteren, R., 1990. Interaction between the respiratory  
385 burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. J. Dairy Sci. 73, 985-994.

Hoeben, D., Monfardini, E., Opsomer, G., Dosogne, H., de Kruif, A., Beckers, J.-F.,  
Burvenich, C., 2000. Chemiluminescence of bovine polymorphonuclear leukocytes  
390 during the periparturient period and relation with metabolic parameters and bovine pregnancy-associated glycoprotein. J. Dairy Res. 67, 249-259

Mehrzaad, J., Dosogne, H., Meyer, E., Heyneman, R., Burvenich C., 2001a. Respiratory burst  
activity of blood and milk neutrophils in dairy cows during different stages of lactation.  
395 J. Dairy Res. 68, 399-415.

Mehrzaad, J., Dosogne, H., Vangroenweghe, F., Burvenich, C., 2001b. A comparative study of  
bovine blood and milk neutrophil functions with luminol-dependent  
chemiluminescence. Luminescence. 16, 343-356.

400

Mehrzhad, J., Duchateau, L., Pyörälä, S., Burvenich, C., 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.* 85, 3268-3276.

405 Mehrzhad, J., 2002. Respiratory burst activity and viability of bovine blood and milk neutrophils during different stages of lactation and mastitis, PhD thesis, Ghent University.

Mehrzhad, J., Duchateau, L., Burvenich, C., 2004. Viability of milk neutrophils and severity of  
410 bovine coliform mastitis. *J. Dairy Sci.* 87, 4150-4162.

Mehrzhad, J., Duchateau, L., Burvenich, C., 2005. High milk neutrophil chemiluminescence limits the severity of bovine coliform mastitis. *Vet. Res.* 36, 101-116.

415 Miles, H., Lesser, W., Sears, P., 1992. The economic implications of bioengineered mastitis control. *J. Dairy. Sci.* 75, 596-605.

Naidu, T.G., Newbould, F.H., 1975. Significance of beta-hemolytic staph. aureus as a pathogen to the bovine mammary gland. *J. Vet. Med. B* 22, 308-317.

420 Paape, M.J., Pearson, R.E., Schultze, W.D., 1978. Variation among cows in the ability of milk to support phagocytosis and in the ability of polymorphonuclear leukocytes to phagocytose *Staphylococcus aureus*. *Am. J. Vet. Res.* 39, 1907-1910.

- 425 Paape, M.J., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich C., 2002. Defense of the bovine  
mammary gland by polymorphonuclear neutrophil leukocytes. *J. Mammary Gland  
Biol. Neoplasia*. 7, 109-121.
- Piccinini, R., Bronzo, V., Moroni, P., Luzzago, P., Zecconi, A., 1999. Study on the  
430 relationship between milk immune factors and *Staphylococcus aureus* intramammary  
infections in dairy cows. *J. Dairy Res.* 66, 501-510.
- Quie, P.G., Belani, K.K., 1987. Coagulase-negative staphylococcal adherence and  
persistence, *J. Infect. Dis.* 156, 543-547.
- 435 Rainard, P., Riollot, C., Poutrel, B., Paape, M.J., 2000. Phagocytosis and killing of  
*Staphylococcus aureus* by bovine neutrophils after priming by tumor necrosis factor- $\alpha$   
and the des-arginine derivative of C5a. *Am. J. Vet. Res.* 61, 951-959.
- 440 Reeves, E.P., Lu, H., Jacobs, H.L., Messina, C.G.M., Bolsover, S., Gabella, G., Potma, E.O.,  
Warley, A., Roes, J., Segal, A.W., 2002. Killing activity of neutrophils is mediated  
through activation of proteases by  $K^+$  flux. *Nature*. 416, 291-297.
- Saad, A.M., 1987. Flow cytometric measurement of bovine milk neutrophil phagocytosis.  
445 *Acta Vet. Scand.* 28, 333-342.
- Smith, K.L., D.A. Todhunter, Schoenberger, P.S. 1985. Environmental mastitis: cause,  
prevalence and prevention. *J. Dairy Sci.* 68:1531-1553.

- 450 Smith, E.M., Green, L.E., Medley, G.F., Bird, H.E., Dowson, C.G., 2005. Multilocus  
sequence typing of *Staphylococcus aureus* isolated from high-somatic-cell-count cows  
and the environment of an organic dairy farm in the United Kingdom. J. Clin.  
Microbiol. 43, 4731-4736.
- 455 Smits, E., Burvenich, C., Guidry, A.J., Heyneman, R., Massart-Leën, A., 1999. Diapedesis  
across mammary epithelium reduces phagocytic and oxidative burst of bovine  
neutrophils. Vet. Immunol. Immunopathol. 68, 169-176.
- Suriyasathaporn, W., Schukken, Y.H., Nielen, M., Brand, A., 2000. Low somatic cell count:  
460 a risk factor for subsequent clinical mastitis in a dairy herd. J. Dairy Sci. 83, 1248-1255.
- Van Oostveldt, K., Paape, M.J., Burvenich, C., 2002. Apoptosis of bovine neutrophils  
following diapedesis through a monolayer of endothelial and mammary epithelial cells.  
J. Dairy Sci. 85, 39-147.
- 465 Van Werven, T., Noordhuizen-Stassen, E.N., Daemen, A.J.J.M., Schukken, Y.H., Brand, A.,  
Burvenich, C., 1997. Preinfection in vitro chemotaxis, phagocytosis, oxidative burst,  
and expression of CD11/CD18 receptors and their predictive capacity on the outcome  
of mastitis induced in dairy cows with *Escherichia coli*. J. Dairy Sci. 80, 67-74.
- 470 Vangroenweghe, F., Rainard, P., Paape, M., Duchateau, L., Burvenich, C., 2004. Increase of  
*Escherichia coli* inoculum doses induces faster innate immune response in primiparous  
cows. J. Dairy Sci. 87, 4132-4144.

- 475 Wilkinson, B.J., Sisson, S.P., Kim, Y., Peterson, P.K., 1979. Localization of the third  
component of complement on the cell wall of encapsulated *Staphylococcus aureus* M:  
implications for the mechanism of resistance to phagocytosis. Infect. Immun. 26, 1159-  
1163.
- 480 Williams, M.R., Craven, N., Field, T.R., Bunch, K.J., 1985. The relationship between  
phagocytosis and intracellular killing of *Staphylococcus aureus* by bovine neutrophils.  
Brit. Vet. J. 141, 362-371.
- Xu, S., Arbeit, R.D., Lee, J.C., 1992. Phagocytic killing of encapsulated and  
485 microencapsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes.  
Infect. Immun. 60, 1358-1362.

Table 1

Comparison of number of circulating leukocytes, number of milk somatic cell, number of circulating mature neutrophils and blood and milk PMN viability between primiparous and multiparous cows during the study. Values are means  $\pm$  SEM of 6 primiparous and 6 multiparous cows.

Parameter	Primiparous	Multiparous
Total number of leukocytes / $\mu$ l blood	9634 $\pm$ 409*	8400 $\pm$ 220
Total number of somatic cell / $\mu$ l milk	56 $\pm$ 9**	115 $\pm$ 13
Total number of circulating mature neutrophils / $\mu$ l blood	3298 $\pm$ 221***	1824 $\pm$ 109
Viability of blood PMN (%)	99 $\pm$ 1	98 $\pm$ 1
Viability of milk PMN (%)	71 $\pm$ 4***	49 $\pm$ 3

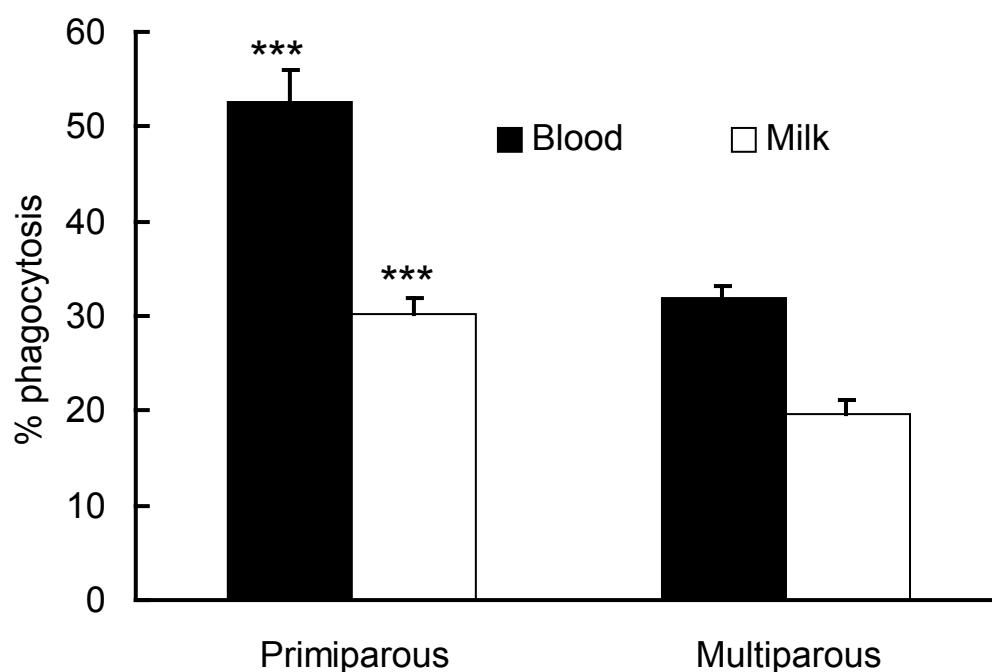
\* =  $P < 0.05$  \*\* =  $P < 0.01$  \*\*\* =  $P < 0.001$



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Figure 1

Phagocytosis (%) of *Staphylococcus aureus* Newbould 305 after incubation with PMN isolated from blood (solid bars) and milk (open bars) of healthy primiparous and multiparous cows during early lactation. The comparison is between primiparous and multiparous cows. Significance of the difference between the two groups is indicated with asterisks (\*\*\*:  $P < 0.001$ ;  $n=6$ ). Bars represent the standard error of the mean.



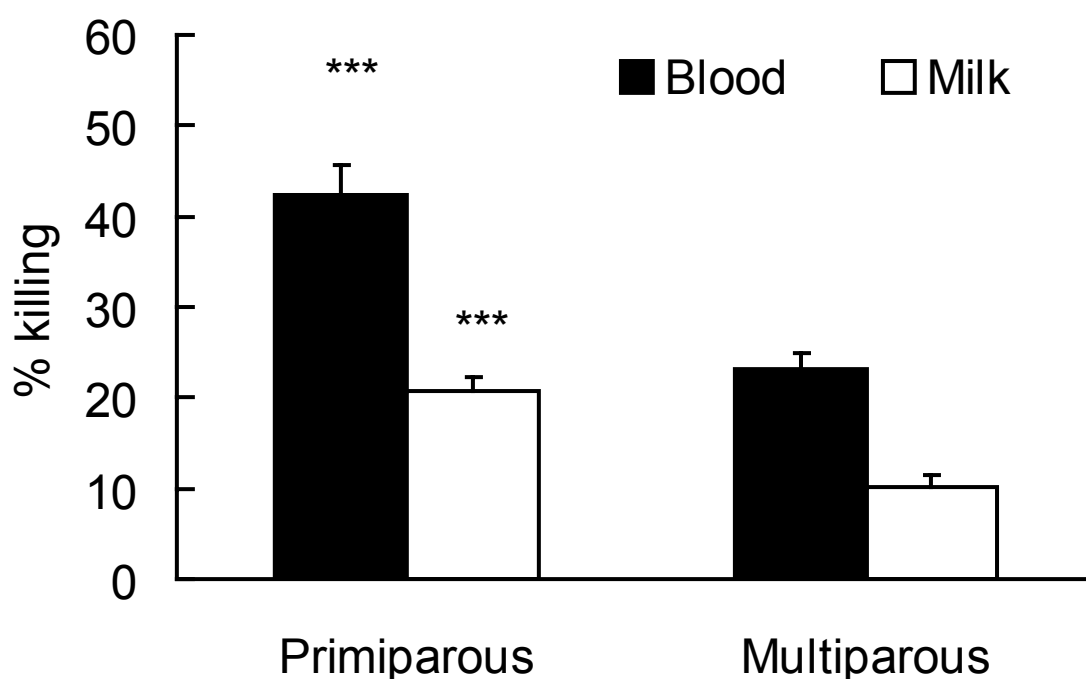
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Figure 2

520 Killing (%) of *Staphylococcus aureus* Newbould 305 after incubation with PMN isolated from blood (solid bars) and milk (open bars) of healthy primiparous and multiparous cows during early lactation. The comparison is between primiparous and multiparous cows. Significance of the difference between primiparous and multiparous cows is indicated with asterisks (\*\*\*:  $P < 0.001$ ;  $n=6$ ). Bars represent the standard error of the mean.

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Figure 3

Free radicals production of latex stimulated of blood (solid bars) and milk (open bars) of healthy primiparous and multiparous cows during early lactation. Data are expressed as the area under the curve (AUC) of continuously free radicals production of  $10^3$  viable PMN for 30 minutes. The comparison is between primiparous and multiparous cows. Significance of the difference between the two groups is indicated with asterisks (\*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ;  $n=6$ ). Bars represent the standard error of the mean.

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