# Experimentally induced *Escherichia coli* mastitis in lactating primiparous cows

Frédéric Vangroenweghe

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Promoter: Prof. Dr. C. Burvenich
Co-promoter: Dr. P. Rainard

Faculty of Veterinary Medicine
Department of Physiology-Biochemistry-Biometrics

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To *my* Ellen

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# LIST OF FREQUENTLY USED ABBREVIATIONS

| | |
|---|---|
| AOAH | acyloxyacyl hydrolase |
| AUC | area under the curve |
| BHBA | beta-hydroxybutyrate |
| C5a | activated complement fragment 5 |
| CD14 | cluster of differentiation 14 |
| CFU | colony-forming units |
| CL | chemiluminescence |
| $Cl^-$ | chlorine |
| CNF | cytotoxic necrotizing factor |
| CONT-SPL | machinal milk sampling technique |
| COX | cyclo-oxygenase |
| DNA | deoxyribonucleic acid |
| eae | attaching and effacing capacity |
| ELISA | enzyme-linked immunosorbent assay |
| FS | forward scatter |
| HR | heart rate |
| HRP | horse-radish peroxidase |
| IDF | International Dairy Federation |
| IL-8 | interleukin-8 |
| $K^+$ | potassium |
| LBP | LPS-binding protein |
| LPS | lipopolysaccharide |
| LT | heat-labile toxin |
| MAN-SPL | manual milk sampling technique |
| mCD14 | membrane-associated CD14 |
| NAP | neutrophil alkaline phosphatase |
| $Na^+$ | sodium |
| NSAID | non-steroidal anti-inflammatory drug |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PCR | polymerase chain reaction |
| PCV | packed cell volume |
| $PGE_2$ | prostaglandin $E_2$ |
| PI | propidium iodide |
| PIH | post-infusion hour |
| PMA | phorbol 12-myristate 13-acetate |
| PMN | polymorphonuclear leukocytes |
| rbosCD14 | recombinant bovine sCD14 |
| ROI | region of interest |
| RR | respiration rate |
| RT | rectal temperature |
| SCC | somatic cell count |
| sCD14 | soluble CD14 |
| SEM | standard error of the mean |
| SSC | side scatter |
| ST | heat-stabile toxin |
| STER-SPL | sterile milk sampling technique |
| TLR | Toll-like receptor |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNF | tumour-necrosis factor |
| $TXB_2$ | thromboxane $B_2$ |
| *vs.* | versus |
| WBC | white blood cell |

# PATHOGENESIS, SEVERITY PREDICTION AND TREATMENT OF *ESCHERICHIA COLI* MASTITIS: A REVIEW

# CONTENTS

## 1. ETIOLOGY OF COLIFORM MASTITIS

### 1.1. *Escherichia coli*, a specific environmental pathogen

*Escherichia coli* is a Gram-negative, non-spore-forming rod, which belongs to the family *Enterobacteriaceae*. Gram-negative bacteria have a cell wall that typically consists of three layers, the cytoplasmic membrane, the peptidoglycan layer and the outer membrane (Fig. 1). The outer cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). Lipopolysaccharide comprises lipid-A, the lipopolysaccharide core and repeated polysaccharide units, called O-antigens (Fig. 2) (Cullor, 1996). Lipid-A is the lipophilic, inner part of LPS, which causes the toxic effects of LPS, also known as endotoxin (Cullor, 1996; Hogan and Smith, 2003). On the outer surface, bacteria may have fimbriae, which protrude from the cell wall. The surface may also be covered with a thick polysaccharide layer, called a capsule. Based on the different structures of O-antigens, K-antigens (capsular) and H-antigens (flagellar), *E. coli* can be divided into O:H:K serotypes (Cullor, 1996).

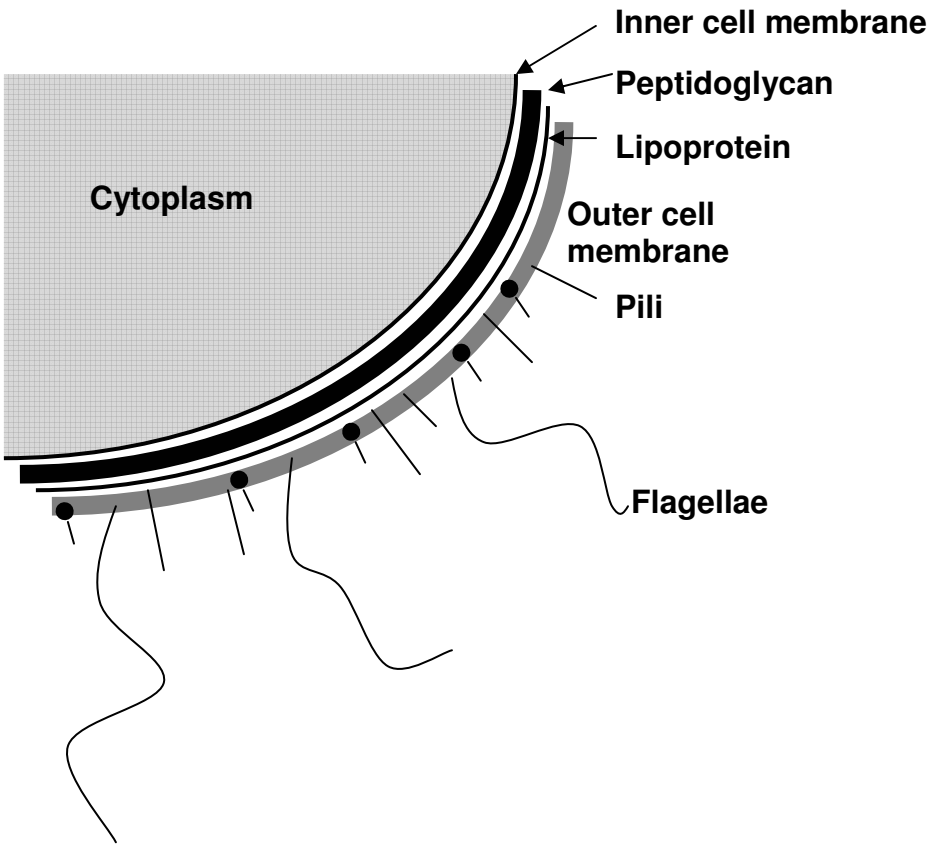



**Figure 1.** Schematic illustration of the cell wall components of Gram-negative bacteria.

*Escherichia coli* is part of the normal intestinal flora of humans and animals, and is the most common facultative anaerobic bacterial species in the gut. The bacteria are constantly excreted in the faeces to the environment. Pathogenic *E. coli* bacteria can cause intestinal and extra-intestinal infections in mammalian and avian hosts (Cullor, 1996; Nagy and Fekete, 1999). Infections of the gastrointestinal tract may lead to various kinds of diarrhoeic diseases, which, in case of Shiga toxin, may even progress to systemic haemolytic uremic syndrome in humans and oedema disease in pigs (Cullor, 1996). *Escherichia coli* is the predominant cause of urinary tract infection in humans, and also causes invasive diseases, such as bacteraemia and meningitis, in humans and animals (Cullor, 1996).

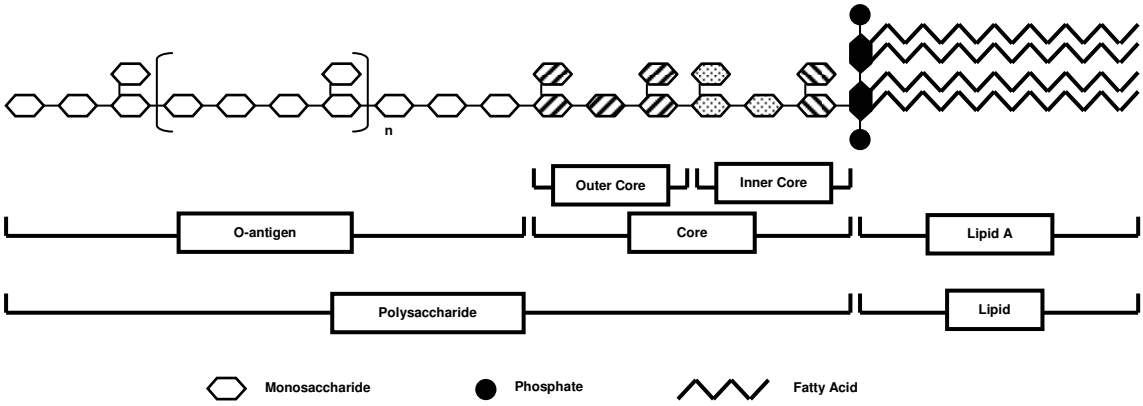



**Figure 2.** Schematic diagram of the structure of LPS. Various monosaccharides are present. The number (n) of repeating subunits in the O-antigen is quite variable and may be > 20 (based on Tobias et al., 1999).

*Escherichia coli* strains involved in acute clinical mastitis have, however, no specific virulence factors (Lehtolainen, 2004). Over the last decades, several potential virulence factors have been studied in bovine mastitis isolates from clinical cases of *E. coli* mastitis. No specific O-serotypes could be associated with bovine mastitis, and only serum resistance could consistently be identified as a possible virulence factor of importance, with a prevalence of 64 to 100% depending on the study. Other potential virulence factors, such as adhesins (F17-fimbriae, S and P fimbriae), toxins (heat-stable toxin (ST), heat-labile toxin (LT), Shiga-like toxins (slt), cytotoxic necrotising factors (CNF) 1 and 2, Vero-toxin), hemagglutination, colicin V, K-antigen, invasiveness, presence of capsule, aerobactin, TraT and attaching and effacing capacity (eae) have extensively been studied, but could not be identified in a consistent number of strains.

Colonies on agar have a smooth or rough appearance. Smooth colonies are characterised by a shiny surface and an entire edge. They have developed polysaccharide side chains as part of their LPS outer membrane. In contrast, rough forms appear as dry, wrinkled colonies, which have lost their polysaccharide side chains by mutation.

## 1.2. Incidence and severity of *E. coli* mastitis in high-yielding dairy cows

Mastitis incidence is the frequency of newly occurring events in a population over a given time period (Smith, 1999). Throughout the lactation cycle, two distinct periods of increased mastitis incidence occur, namely the periparturient period, from 2 weeks before calving until peak lactation (8 weeks), and around drying-off (Natzke, 1981; Burvenich et al., 2000) (Fig. 3).

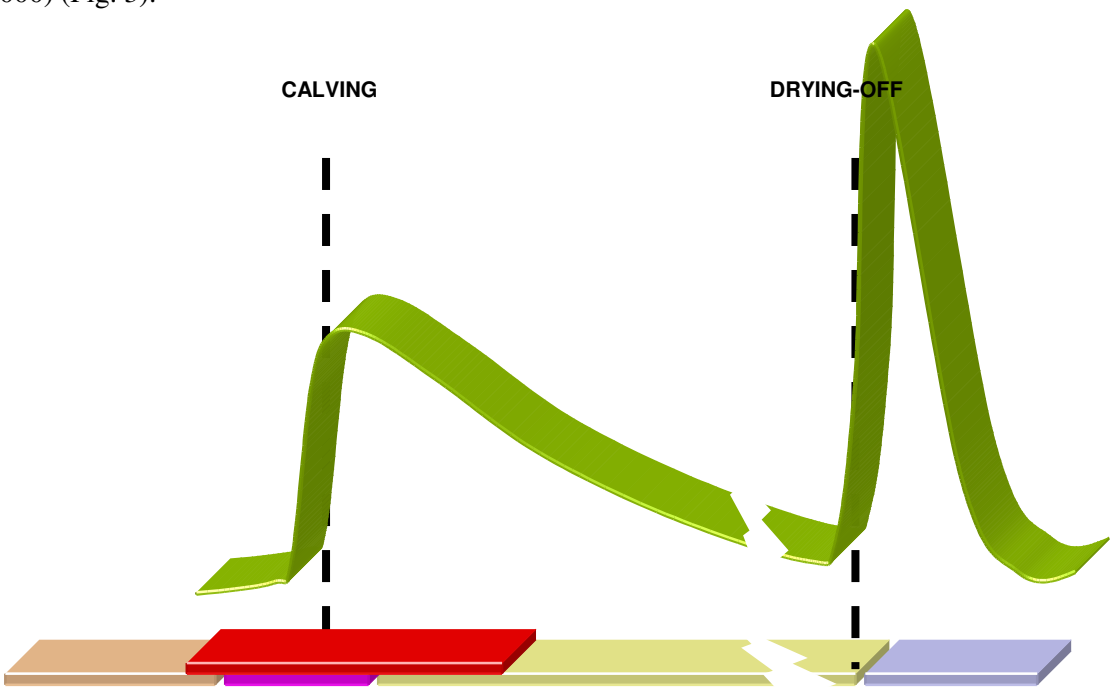



**Figure 3.** Incidence of clinical mastitis throughout the lactation cycle. Two distinct peak moments occur, namely the periparturient period and late lactation – drying-off period (Burvenich et al., 2000, based on results of Natzke, 1981). Time is represented in the X-axis, incidence of clinical mastitis in the Y-axis. The different segments are respectively mammogenesis ( ■ ), periparturient period ( ■ ), lactation ( ■ ) and involution ( ■ ). The transition period ( ■ ) goes from mammogenesis over colostrogenesis to milk secretion during early lactation.

Acute mastitis is an udder inflammation characterised by its sudden onset with visible signs of abnormal milk (Smith, 1999). When all cases of acute coliform mastitis during the entire lactation are considered, a significant percentage occurs before peak lactation (Erskine et al., 1988). Twenty-five % of the cases of clinical coliform mastitis occur in the first two weeks of lactation. However, when the first month of lactation is considered, this percentage increases to 45% and to 60% in the period before peak lactation at 8 weeks post-partum (Erskine et al., 1988; Burvenich et al., 2000) (Fig. 4).

The severity of the disease is, however, quite variable throughout the lactation cycle (Burvenich et al., 2000). During the transition period and in early lactation (until 8 weeks post-partum), a variable degree of self-curing can be observed with moderate to severe clinical responses, whereas during mid- and late lactation, a moderate reaction with a high degree of self-curing is apparent. Dry cows are very resistant to clinical coliform mastitis (Hill, 1981; Todhunter et al., 1991a; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Burvenich et al., 2000) (Fig. 5).

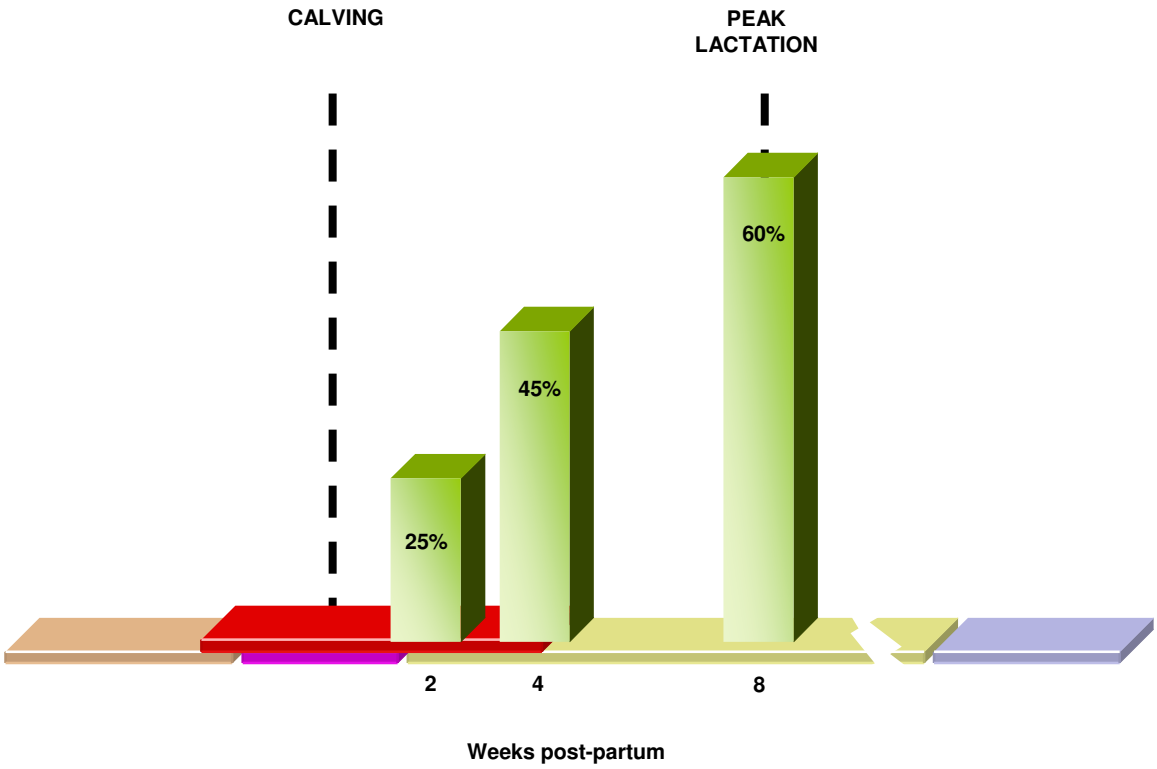



**Figure 4.** Incidence of clinical coliform mastitis during the periparturient period until peak lactation (Burvenich et al., 2000, based on results of Erskine et al., 1988). Twenty-five % of the cases of clinical coliform mastitis already occur in the first 2 weeks of lactation. This increases to 45% after 4 weeks and 60% at peak lactation (8 wks). Time is represented in the X-axis, cumulative percentage of cases of clinical coliform mastitis in the Y-axis. The different segments are respectively mammogenesis (■), periparturient period (■), lactation (■) and involution (■). The transition period (■) goes from mammogenesis over colostrogenesis to milk secretion during early lactation.

Multiple reports on incidence of *E. coli* mastitis in low SCC herds are available (Schukken et al., 1989; Barkema et al., 1998; Surayasathaporn et al., 2000; de Haas et al., 2002). Most research on increased risk for clinical mastitis in low bulk milk SCC has been performed in The Netherlands. Except for Surayasathaporn et al. (2000), who observed an incidence for *E. coli* mastitis of 42.8% in a low bulk milk SCC herd, in most other studies the incidence of clinical mastitis due to *E. coli* was around 20% (Schukken et al., 1989; Barkema et al., 1998; de

Haas et al., 2002). The incidence of *E. coli* mastitis was little higher in multiparous cows (21.5%) as compared to primiparous cows (16.7%) (de Haas et al., 2002). The distribution of cases of *E. coli* mastitis throughout lactation was similar for primiparous and multiparous cows, with 50% of all cases occurring within 56 and 61 days of lactation, and 75% of all cases due to *E. coli* within 118 and 123 days of lactation, respectively (de Haas et al., 2002).

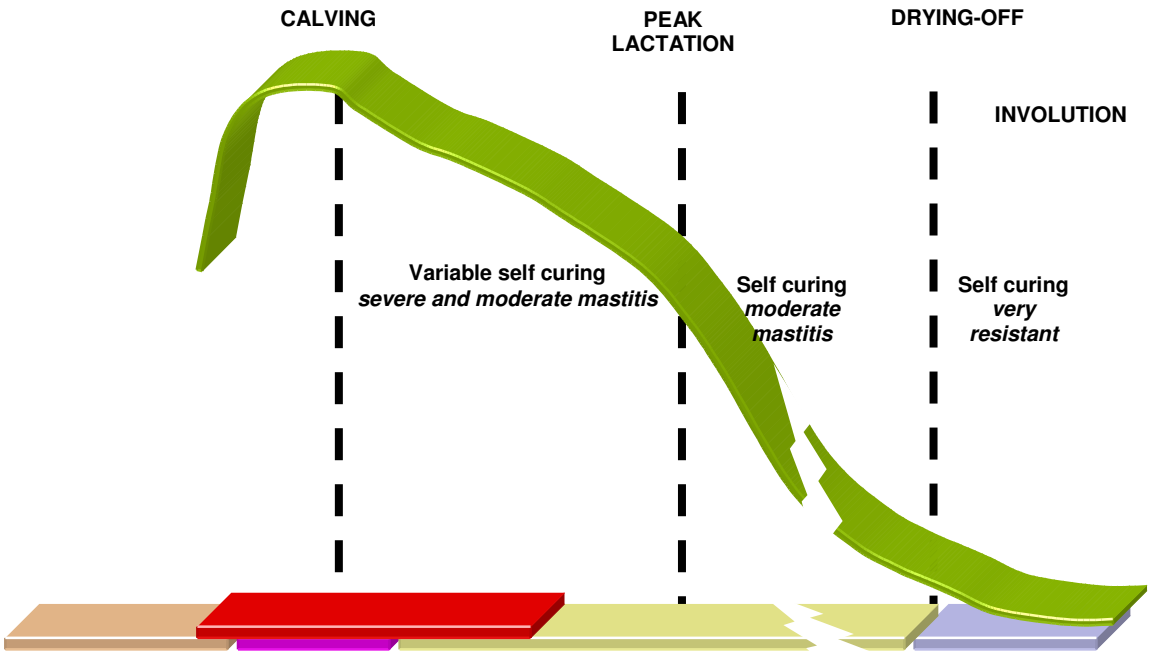



**Figure 5.** Severity of clinical coliform mastitis throughout the lactation cycle (Burvenich et al., 2000). Time is represented in the X-axis, disease severity in the Y-axis. The different segments are respectively mammogenesis (■), periparturient period (■), lactation (■) and involution (■). The transition period (■) goes from mammogenesis over colostrogenesis to milk secretion during early lactation.

## 1.3. Economical impact of coliform mastitis

Accurate estimation of the actual costs and the economic impact of mastitis in general, and *E. coli* mastitis in particular, is difficult (Esslemont and Kossaibati, 1997). Mastitis can occur in many different forms; however, a distinct difference in clinical course can be defined based on the stage of lactation and the cow's parity. The greatest loss caused by clinical mastitis is due to lost milk production, which originates from two different sources, namely the reduced milk yield and milk withdrawal because of drug use. As for *E. coli* mastitis, which occurs frequently during early lactation, these losses can be serious. The prevalence of mild, severe and fatal mastitis in dairy practice was assumed to be 70, 29 and 1%, respectively (Blowey, 1986). In mild cases of clinical mastitis, reduced milk yield seems to be the most important financial loss, whereas in severe cases veterinary costs become almost as high as the cost due to the reduction

in milk yield due to the prolonged negative effects of mastitis on the secretory epithelium (Esslemont and Kossaibati, 1997). In fatal cases of clinical mastitis, major costs are attributed to replacement costs for dairy heifers (Table 1).

**Table 1.** Costs of clinical mastitis in dairy cows (adapted from Esslemont and Kossaibati, 1997).

| | *Mild (70%)* | | *Severe (29%)* | | *Fatal (1%)* | |
|---|---|---|---|---|---|---|
| | Unit | Cost (€) | Unit | Cost (€) | Unit | Cost (€) |
| Drugs | | 15.8 | | 39.8 | | 54.8 |
| Herdsman's time (min) | 15 | 1.9 | | | | |
| Discarded milk (l) | 80 | 28.8 | 120 | 43.2 | | |
| Reduced milk yield (l) | 247 | 74.1 | 450 | 135.0 | | |
| Veterinarian's time (min) | | | 50 | 94.0 | 135 | 244.9 |
| Increased risk of culling (%) | | | 20 | 231.0 | | |
| Cost of fatality | | | | | | 3021.9 |
| **DIRECT COSTS** | | **120.6** | | **543.0** | | **3321.6** |

The determining parameter for the total cost of mastitis in a dairy herd is, however, the number of animals affected by the disease per 100 animals present in the herd. In a British survey of 90 dairy herds, the average number of cases per 100 cows per year was 37.4, although a very wide range existed between the best herds in the study (2.8 cases per 100 cows) and the worst herds (215.4 cases per 100 cows). Taking this into account, mastitis is considered the most important production disease in terms of reduced profitability with an average yearly cost of € 35.9 per cow on an average herd (Esslemont and Kossaibati, 1997).

## 2. PATHOGENESIS

### 2.1. Virulence factors of *E. coli*

*Escherichia coli*, involved in bovine coliform mastitis, is part of the normal intestinal flora of dairy cows. The strains isolated from bovine mastitis are essentially not different from strains isolated from bovine faeces (Nemeth et al., 1994). This supports the hypothesis that mastitic *E. coli* are simply opportunistic pathogens. Nevertheless, several studies have been performed to identify potential virulence factors of *E. coli* associated with bovine coliform mastitis (Linton and Robinson, 1984; Sanchez-Carlo et al., 1984; Barrow and Hill, 1989; Hogan et al., 1990; Nemeth et al., 1991; 1994; Thomas et al., 1992; Fang et al., 1993; Pohl et al., 1993; Lipman et al., 1995; Cray et al., 1996; Kaipainen et al., 2002). However, only LPS, the endotoxin originating from the bacterial outer cell membrane, has been shown to be a consistent virulence factor in all *E. coli* strains isolated from bovine coliform mastitis. Lipopolysaccharide

is a potent inducer of inflammatory cytokines (Shuster et al., 1993) and is released by bacteria during growth and killing (Burvenich, 1983). The second most important virulence characteristic of bovine mastitis isolates is their serum resistance (Carroll and Jasper, 1977; Sanchez-Carlo et al., 1984; Valente et al., 1988; Barrow and Hill, 1989; Nemeth et al., 1991; 1994; Fang and Pyörälä, 1996), and 64 to 100% of the strains were reported to be resistant. Two specific structures on the outer cell membrane, TraT – a cell surface-exposed lipoprotein – and K1 – a capsular antigen –, have been assumed to act in concert to inhibit the correct assembly or membrane insertion of the membrane attack complex of the complement system (Sukupolvi and O'Connor, 1990). No relation between both TraT and K1, and serum resistance could, however, be established (Nemeth et al., 1991; Kaipainen et al., 2002).

In order to colonise the mammary gland and induce mastitis, invading bacteria should be able to proliferate in normal and abnormal mastitis milk (Fang et al., 1993), which means that it may be necessary for the bacterial strains to adapt to changing growth conditions. *Escherichia coli* has improved growth capacity in abnormal mastitis milk, which could be explained through the presence of growth-enhancing nutrients, although elevated antibacterial activities during mastitis would rather inhibit bacterial growth (Fang et al., 1993). *In vivo* adhesion of *E. coli* to the epithelial surface of the mammary gland is thought to be unimportant during the initial phase of infection (Bramley et al., 1979), because in healthy udders collagen or fibronectin are not exposed. Recent *in vitro* experiments, using epithelial cell cultures (Döpfer et al., 2000) or tissue explant cultures (Thomas et al., 1992), have found indications for *in vitro* adhesion to mammary epithelial cells. However, strains in one study were isolated from recurrent cases of coliform mastitis (Döpfer et al., 2000), and a continuous epithelial cell line of MAC-T-cells was used. Thomas et al. (1992) only observed *in vitro* adhesion of *E. coli* when epithelial surface was damaged and underlying tissues, containing fibronectin and collagen, were displayed.

Although over 100 serotypes of *E. coli* have been recognised, no specific O-serotypes have been conclusively related to bovine *E. coli* mastitis (Linton and Robinson, 1984). Nevertheless, intramammary challenge with *E. coli* 487 caused more severe clinical signs of mastitis than did *E. coli* 727 (Todhunter et al., 1991b; Hogan et al., 1992; 1995; 1999). In accordance with *E. coli* strains causing extra-intestinal diseases in humans, hemagglutination and hemolysis of erythrocytes have been considered as virulence factors in bovine mastitis (Hogan et al., 1990), because they enable the bacteria to increase iron availability. However, neither of both characteristics was related to duration or severity of bovine intramammary infection from which the bacteria were isolated (Hogan et al., 1990).

Further research related to the presence of F17 fimbriae, genes encoding for enterotoxins (LT and ST1), verotoxins and cytotoxic necrotising factors (CNF1 and CNF2), attaching and

effacing capacity (eae), Shiga-like toxin (slt) I and II, and heat-stable enterotoxin did not add substantial evidence on the presence of multiple virulence factors in *E. coli* involved in bovine coliform mastitis (Pohl et al., 1993; Lipman et al., 1995; Cray et al., 1996).

## 2.2. Cow factors that influence the outcome of the disease

### 2.2.1. Polymorphonuclear leukocytes and their role in mammary defence

Once environmental bacteria, such as *E. coli*, have passed the first functional barrier against invasion, formed by the teat canal, they are able to proliferate in the milk at the level of the teat and udder cistern. However, milk is not the ideal growth medium, mainly due to the presence of several inhibitory factors, such as lysozyme, lactoferrin, and phagocytic cells, originating from the blood. The polymorphonuclear leukocyte (PMN) and macrophage are the functional phagocytic cells of the body. The resident cells in the normal healthy mammary gland are predominantly macrophages, followed by lymphocytes, whereas only a small portion of PMN (10-15%) is present (Dulin et al., 1982; Östensson et al., 1988; Leitner et al., 2000; Pillai et al., 2001).

In the healthy mammary gland, PMN migrate from the blood circulation through the endothelial gaps in the mammary epithelium to the milk compartment. Directed migration of PMN into the mammary gland is stimulated by nursing or milking (Paape et al., 1992), supplying the normal sterile mammary gland with a constant source of fresh PMN (Paape and Wergin, 1977). Once in the mammary gland, PMN start to phagocytose fat globules and casein, which induces an activated status of the cells, finally leading to a progressive exhaustion of cellular functionality, as can be observed with milk PMN chemiluminescence (CL) (Mehrzad et al., 2001b).

Polymorphonuclear leukocytes have several receptors on their cell membrane, which serve in the process of directed migration from the blood into the milk compartment, called diapedesis. Rolling and attachment of PMN to the endothelium is the first step in the recruitment process and is accomplished by interaction between L-selectin on PMN and its ligand on the endothelial cells (Kishimoto and Rothlein, 1994). Subsequently, the $\beta_2$-integrins are responsible for a strong and sustained attachment, followed by transendothelial PMN migration into the extracellular matrix and through the mammary gland epithelium. The respective role of the two subunits, namely CD11b and CD18, in the migration process has recently been studied using an *in vitro* diapedesis model (Smits et al., 2000). Migration across the endothelium is almost completely dependent on CD18 and to a lesser extent on CD11b, whereas the diapedesis across

the mammary epithelial barrier is more dependent on CD11b. Migration across the collagen of the extracellular matrix was partly dependent on CD18, but completely independent of CD11b (Smits et al., 2000).

Following diapedesis through the blood-milk barrier, functionality of milk PMN has been shown to be decreased (Mehrzad et al., 2001b). This was not only attributed to the ingestion of fat globules and casein from the milk environment (Paape et al., 2003). *In vitro* diapedesis showed a reduction in phagocytosis and oxidative burst activity following migration across a mammary epithelial cell layer (Smits et al., 1999). The migration process also influenced the appearance of programmed cell death or apoptosis in PMN following migration. *In vitro* migration through a collagen-coated membrane induced an apoptotic response, which was downregulated by the addition of a monolayer of endothelial cells, but negated by a mammary epithelial cell monolayer (Van Oostveldt et al., 2002a). It was suggested that L-selectin might play an important role in the PMN apoptosis-inducing effect after *in vitro* migration through the collagen-coated membrane inserts (Van Oostveldt et al., 2002a), whereas CD11b/CD18 could induce an attenuation of the rate of apoptosis after migration through the blood-milk barrier (Smits et al., 2000).

During the periparturient period, a temporary change in several blood PMN characteristics and functions has been described: oxidative burst activity (Moreira da Silva et al., 1998), L-selectin (Monfardini et al., 2002), acyloxyacyl hydrolase (AOAH) (Dosogne et al., 1998a); whereas $\beta_2$-integrins (CD11a, CD11b, CD11c and CD18) did not change over time (Diez-Fraile et al., 2003a).

Acyloxyacyl hydrolase is thought to be one of the mechanisms responsible for the local intramammary detoxification of released LPS (Dosogne et al., 1998a), besides the recognition and uptake of LPS by the LPS-binding protein (LBP) / cluster of differentiation (CD) 14 system (Thomas et al., 2002). Following intramammary endotoxin challenge with high inoculum doses, clinical signs were less pronounced as compared to intravenous endotoxin challenge (Lohuis et al., 1988b). This suggests that endotoxemia as such is not the major cause of systemic clinical signs following LPS or *E. coli* mastitis. Following *E. coli* challenge, few peaks of LPS have been detected in circulation (Dosogne et al., 2002). In contrast, significant differences in circulating concentrations of TNF-$\alpha$ have been observed between moderate and severe responding animals (Hoeben et al., 2000a). This suggests that animals with *E. coli* mastitis rather suffer from mediator shock than from endotoxemia (Hoeben et al., 2000a; Dosogne et al., 2002). However, under practical circumstances bacteraemie has been described in a substantial number of cows with acute clinical mastitis (Wenz et al., 2001). A significant difference in potential bacteraemie

existed between mild-moderate and severe responders, with as much as 42% of the severe responders having positive bacterial cultures.

Subsequently, endothelial cells of the blood vessel walls in the mammary gland are activated, leading to an increased margination, attachment and migration into the mammary gland tissue under the chemotactic guidance of locally produced chemoattractants, such as activated complement fragment 5 (C5a) and interleukin-8 (IL-8) (Shuster et al., 1997; Rainard, 2003). Meanwhile, an increase in mammary blood flow can also be observed (Dhondt et al., 1977), providing the mammary gland with a larger amount of fresh reactive blood PMN. Once the inflammation actively eliminates the invading pathogens from the mammary gland, several regulatory mechanisms to limit the inflammation and the associated local epithelial damage are enhanced. During *E. coli* mastitis, an increase in programmed cell death has been observed (Van Oostveldt et al., 2002c), whereas the margination decreases through a downregulation of L-selectin (Monfardini et al., 1999). Moreover, *in vitro* induction of PMN apoptosis through addition of LPS or TNF-α resulted in a decreased phagocytic and oxidative burst capacity, which could also play a role in the resolution of inflammation (Van Oostveldt et al., 2002b). Nevertheless, functional activity of the viable blood PMN is enhanced during experimental *E. coli* mastitis, as the number of circulating PMN with unstimulated respiratory burst activity is higher (Van Oostveldt et al., 1999). Within the mammary gland, PMN oxidative burst, as quantified through CL, is higher in severely diseased animals (Mehrzad, 2002), which could explain the larger, long-lasting decrease in milk production in the affected quarters of these animals.

At the mammary gland level, activated PMN recognise, phagocytose and kill bacteria through their oxygen-dependent and oxygen-independent bactericidal mechanisms (Burvenich et al., 2003). Finally, they become apoptotic and are taken up by macrophages, without release of their toxic compounds into the surrounding environment (Paape et al., 2003).

### 2.2.2. Hormonal and metabolic profile during the periparturient period

During the periparturient period, hormonal and metabolic profile of the high-yielding dairy cow undergoes some tremendous changes which are mainly related to the process of calving, with its associated hormonal regulation, and the initiation of milk production, the lactogenesis, which changes metabolic demands quite abruptly. Besides the sudden decrease in progesterone, there is a rise in oestrogen, and cortisol peaks on the day of calving. Freshly calved cows meanwhile undergo a decrease in energy balance, characterised by increased blood concentrations of β-hydroxybutyrate (BHBA) and non-esterified fatty acids (Hoeben et al.,

2000b), in conjunction with a slight and short-lasting dip in glucose (Moreira da Silva et al., 1998; Hoeben et al., 2000b).

High concentrations of progesterone and to a lesser extent oestrogen have been shown to decrease blood PMN oxidative burst activity (Moreira da Silva, 1996). β-hydroxybutyric acid has been shown to have a direct negative effect on circulating blood PMN function (Hoeben et al., 1997c), but an indirect inhibiting effect on bone marrow progenitor cloning *in vitro* has also been reported (Hoeben et al., 1999). Taking these findings into account, the previously described depression in PMN functions can easily be explained.

However, the described hormonal and metabolic changes all occur within the same period, which makes a causal interpretation difficult. Using mammectomy, Kimura et al. (1999) showed two major factors affecting the changes in PMN functionality, namely the process of calving and its related hormonal changes on the one hand, and the onset of lactation, the lactogenesis, on the other hand. Eliminating the second factor, decreased PMN functionality could still be demonstrated as an effect of parturition alone (Kimura et al., 1999).

### 2.2.3. Severity of experimentally induced *E. coli* mastitis

A clear distinction must be made between risk factors for severe clinical mastitis and severity determining factors. Risk factors are parameters or characteristics which have a causative relation with the occurrence of severe clinical mastitis. There are, however, no possibilities to manipulate these factors. In contrast, severity determining factors are mechanisms or parameters which can actively be changed or manipulated, resulting in a different outcome of the course of clinical *E. coli* mastitis.

Several studies have identified the number of PMN in blood immediately prior to infection as an important risk factor in the pathogenesis of mastitis (Hill, 1981; Kremer et al., 1993c; Dosogne et al., 1997). It has been shown that a decreased number and function of blood PMN predisposes cows to a severe clinical response to intramammary *E. coli* challenge (Burvenich et al., 1994). A large pool of circulating PMN is apparently required for an effective resistance against intramammary infections (Heyneman et al., 1990; Sordillo and Peel, 1992; Kremer et al., 1993c; van Werven et al., 1999), and considerable difference can be observed in the number of blood PMN or white blood cells (WBC) before infection between moderate and severe responding animals following *E. coli* challenge (Heyneman et al., 1990; Sordillo and Peel, 1992; Kremer et al., 1993c; van Werven et al., 1997) (Table 2).

Many of these functions are associated with the maturity of the circulating PMN (Moreira da Silva et al., 1998; Van Merris et al., 2002), although the dramatic hormonal and

metabolic changes that occur around parturition and at onset of lactation can also influence these functions (Dosogne et al., 1999; Hoeben et al., 1999). The circulating number of PMN represents a dynamic balance between cells that disappear from the circulation through margination and diapedesis on the one hand, and the rate at which cells are introduced from the bone marrow or by demargination. Within an individual animal, the number of blood PMN seems to be relatively constant. High coefficients of correlation ($r$ = 0.68 to 0.83) could be observed between blood PMN number during 5 consecutive days before experimental challenge (van Werven et al., 1997). Moreover, older cows (> 4 parities), which have been shown to be more susceptible to coliform mastitis, had significantly lower numbers of circulating PMN on the day of challenge as compared to young animals ($2^{nd}$ parity). This discrepancy was confirmed by the course of the bacterial counts in the infected quarters, where younger animals had a much lower peak bacterial count ($\approx 10^5$ CFU/ml) in comparison with the older animals ($\approx 10^8$ CFU/ml) (van Werven et al., 1997). However, no data are available on the inflammatory response in primiparous cows.

**Table 2.** Number of WBC or PMN in blood (x $10^6$/ml) of severe and moderate responding cows immediately before infection.

| Reference | Cell Type | Severe | Moderate |
|---|---|---|---|
| Heyneman et al., 1990 | PMN | 2.3 | 3.2 |
| Kremer et al., 1993c | WBC | 5.7 | 7.8 |
| Sordillo and Peel, 1992 | WBC | 6.1 | 7.8 |
| van Werven et al., 1997 | WBC | 10.5 | 12.5 |

Modulation of the number of circulating PMN available for intramammary defence against invading pathogens has been performed (Jain et al., 1971; Paape et al., 1986). Administration of an equine anti-bovine leukocyte serum resulted in neutropenia in all treated cows. Following intramammary *Aerobacter aerogenes* challenge, neutropenic cows only developed slight udder swelling and little leukocytosis in milk, resulting in a massive intramammary proliferation of *A. aerogenes* within 30 h post-infusion. Subsequently, a large amount of endotoxin was generated, which resulted in an extreme inflammatory reaction, leading to necrosis and irreversible tissue damage (Jain et al., 1971). In contrast, the insertion of an intramammary device induced a permanent moderate recruitment of PMN into the mammary gland, resulting in significant higher milk SCC. The continuous PMN activation in the mammary gland resulted in a lower number of acute clinical mastitis cases as compared to the control animals (Paape et al., 1986).

In contrast to the number of circulating PMN, the pre-infection adhesion molecule expression is not a good predictor for the ability to recruit leukocytes to an intramammary infection during the periparturient period (Burvenich et al., 1994). Nevertheless, cows with a

higher CD11c/CD18 and a lower CD11b/CD18 expression on their blood PMN before infection typically showed less severe disease symptoms (van Werven et al., 1997).

During intramammary infection, it is of importance that a high number of circulating PMN can reach the site of infection within reasonable time, and therefore, an optimal chemotaxis and diapedesis of these cells is the determining step for the final outcome of coliform mastitis during early lactation (Burvenich et al., 1994). Pre-infection *in vitro* PMN chemotaxis was higher in moderately diseased animals than in severely diseased cows (Kremer et al., 1993a; van Werven et al., 1997). The same difference was observed by Shuster et al. (1996) when comparing the inflammatory response of early and mid-lactating dairy cows. *In vivo*, rapid influx of PMN into the infected quarters occurred in moderate responders, resulting in efficient suppression of bacterial growth, whereas in severe responders excessive bacterial growth appeared to be related to a delayed diapedesis of PMN into the glands (Vandeputte-Van Messom et al., 1993). Therefore, it can be concluded that one of the most important risk factors for a severe clinical response is a slow migration of PMN from the blood into the infected mammary gland (Table 3).

**Table 3.** Chemotactic differential (ratio of chemotactic *versus* (*vs.*) random migration) of blood PMN of severe and moderate responding cows immediately before infection.

| Reference | Lactation Stage | Severe | Moderate |
|---|---|---|---|
| Kremer et al., 1993a | early | 3.5 | 6.4 |
| Shuster et al., 1996 | early / mid | 0.5 | 0.7 |
| van Werven et al., 1997 | early | 2.9 | 3.2 |

In contrast to the previously described functions and characteristics of blood PMN, phagocytosis does not seem to be critically reduced during early lactation, and therefore, no significant correlation with severity of *E. coli* mastitis could be observed (Dosogne et al., 1997). However, a significant correlation between the number of PMN (number of circulating PMN * % phagocytic PMN) and the severity of clinical mastitis existed in that study, which confirms the importance of a high number of immunologically active cells in the circulation for an effective resistance against intramammary infections by *E. coli* during the periparturient period (Burvenich et al., 1994).

The oxidative burst activity of blood PMN following phagocytosis is, however, an important predictive parameter for the clinical outcome of experimentally induced *E. coli* mastitis (Heyneman et al., 1990). The competence of PMN to generate reactive oxygen species following stimulation with opsonised particles prior to infection was negatively correlated with severity of subsequently induced *E. coli* mastitis.

Binding and detoxification of LPS is strictly controlled upon entrance in the body: after binding to the serum-derived LBP, the complex is recognised by CD14 on macrophages and PMN, and subsequently internalised for further metabolisation by enzymes, such as phosphatases and hydrolases. In milk, the number of PMN expressing CD14 and their receptor density is significantly higher as compared to blood PMN (Paape et al., 1996), and the expression of CD14 molecules on PMN was not significantly reduced during the periparturient period (Dosogne et al., 1998a). The average AOAH activity, however, was reduced during this period (Dosogne et al., 1998a). No relation could be observed between pre-infection blood PMN AOAH activity and severity following experimental *E. coli* challenge (Dosogne et al., 2000). It can therefore be concluded that AOAH activity is not a good marker to predict the final outcome of clinical coliform mastitis.

Neutrophil alkaline phosphatase (NAP) activity increased during experimentally induced *E. coli* mastitis, which may suggest this enzyme plays a role in the pathogenesis of the disease. During mastitis, NAP activity was much higher in severe responders (van Werven et al., 1998), and was associated with an increased percentage of immature PMN, suggesting a higher production in these cells (Heyneman and Burvenich, 1992; van Werven et al., 1998). Despite the association of NAP activity and severity during infection, no relation between pre-infection NAP activity and outcome of clinical *E. coli* mastitis could be observed (van Werven et al., 1998).

Besides its role as a parameter for milk quality and udder hygiene, milk SCC can also be considered as a potential risk factor for mastitis, as a high SCC in milk of healthy cows during early lactation protected against severe clinical symptoms during subsequent experimental *E. coli* challenge (van Werven, 1999). In a comparative study between early and mid-lactating cows, Shuster et al. (1996) also observed lower SCC in the cows during early lactation. Moreover, within early lactating cows, a significant lower pre-infection SCC has been reported in severe responders (Hirvonen et al., 1999; Vandeputte-Van Messom et al., 1993) (Table 4). Nevertheless, a high degree of variation can be observed in pre-infection milk SCC between different studies.

**Table 4.** Somatic cell count (cells/ml) of severe and moderate responding cows immediately before infection.

| Reference | Lactation Stage | Severe | Moderate |
|---|---|---|---|
| Hirvonen et al., 1999 | early | 10,000 | 35,000 |
| Shuster et al., 1996 | early / mid | 20,000 | 63,000 |
| Vandeputte-Van Messom et al., 1993 | early | 99,630 | 132,720 |

In conclusion, several parameters associated with number and function of blood PMN have shown predictive capacity in relation to the outcome (moderate/severe) of experimentally induced *E. coli* mastitis. Besides the absolute number of circulating PMN, their chemotactic migratory capacity, diapedesis and oxidative burst activity have a significant impact on subsequent outcome of intramammary infection.

## 2.3. Role of complement, LPS-binding protein and soluble CD14

### 2.3.1. Complement system and its role in the innate defense

The complement system plays an important role in the innate immunity against microorganisms through its various functions. Proteins of the complement system are not only able to lyse micro-organisms and erythrocytes, as initially thought; they also play a role in recognition and ingestion of micro-organisms by phagocytes. Complement can contribute at three pivotal steps of phagocytosis, which is an essential defence mechanism against mastitis (Craven and Williams, 1985; Burvenich et al., 1994), namely the opsonisation of bacteria through deposition of complement fragments at the bacterial surface, which are recognised by phagocyte receptors; chemotactic attraction of phagocytes to the site of inflammation; and priming or activation of ingestion and/or intracellular killing of pathogens (Rainard, 2003). Moreover, the elements of the complement system are important operators in the initiation and control of inflammation (Frank and Fries, 1991). The concentrations of complement components in milk in different physiological situations have amply been studied. Complement component C1q is known to be relatively deficient, whereas C3 is present in relative abundance. Until now, the origin of the complement components found in bovine milk is essentially a matter of speculation (Rainard, 2003). Although transudation of complement proteins is likely to contribute to the total amount of complement present in normal healthy milk, this route of supply is probably limited due to the relative impermeability of the mammary epithelium. This may be one of the main reasons for the lack of C1q, the largest component (900 kDa) in normal milk (Rainard and Poutrel, 1995). During mastitis, the selective blood-milk barrier is damaged, which enables complement components to temporarily exudate to the affected mammary glands together with other plasma proteins, such as serum albumin and fibrinogen. The concentration of C5 in milk can be highly variable between cows during inflammation (Rainard and Poutrel, 2000), although plasma C5 concentrations in these animals were quite comparable. In milk from uninflamed, uninfected quarters, generated C5a concentrations range from 0.6 ng/ml to 20 ng/ml (Rainard and Poutrel, 2000), whereas in milk from quarters affected by clinical *E. coli* mastitis

C5a concentrations can exceed 50 ng/ml (Shuster et al., 1997). In normal healthy milk, little complement-dependent bactericidal activity is present. The low level of the bactericidal activity, possibly due to low concentrations of immunoglobulins for most mastitis pathogens in this milk, can be increased by systemic immunisation of cows against a defined pathogen (Korhonen et al., 2000). In contrast to normal milk, milk derived from mastitis affected quarters exhibits pronounced bactericidal and haemolytic complement-mediated activity (Rainard, 2003), which is highly correlated with the magnitude of inflammation. Following injury, damage or infection, complement activation results in the production of pro-inflammatory mediators C4a, C3a and C5a, of which C5a is the most biologically relevant peptide in relation to PMN (Rainard, 2003). Complement fragment C5a has various biological functions: increased vascular permeability, potent chemoattractant for PMN, basophils, macrophages and lymphocyte subpopulations, modulation of phagocyte receptor for opsonins, increased oxidative metabolism, release of eicosanoids and degradative enzymes, and stimulation of cytokine synthesis (Damereau, 1987; Frank and Fries, 1991; Tomlinson, 1993).

### 2.3.2. LPS-binding protein and sCD14 recognise and neutralise LPS

The early innate immune response is considered to be the first line of defence against infectious diseases. The principal challenge of the host is to detect the pathogen within a reasonable time; and rapidly mount a defensive response to limit the pathogen growth in order to obtain a total elimination of the pathogen, if possible. Within the first line of defence, the leukocytes, such as macrophages and PMN, are the predominant cell types. They can phagocytose and kill the pathogens and concurrently co-ordinate additional host responses through the synthesis of a wide range of inflammatory mediators and cytokines (Aderem and Underhill, 1999). An important aspect of the innate defence system is the ability to recognise a large number of potential pathogens with a limited number of available receptors. An additional problem is the tendency of pathogens to mutate. However, the host has tried to overcome this by the development of receptors, recognising conserved motifs on pathogens. These motifs have essential roles in the biology of the invading agents, and are therefore not subjected to high mutation rates (Aderem and Ulevitch, 2000). Janeway and Medzhitov (1998) have defined these patterns as pathogen-associated molecular patterns (PAMP's), and their respective binding sites on the phagocytes as pattern-recognition receptors.

In Gram-negative bacteria, LPS is the most important PAMP, and the most essential structural feature governing interactions with the innate immune system is known as lipid A. Lipid A is composed of a diglucosamine backbone, containing ester-linked and amide-linked

long-chain fatty acids. Lipopolysaccharide is released from the outer membrane of the Gram-negative cell membrane by actively growing, damaged and dead bacteria (Petsch and Anspach, 2000). The pattern-recognition receptors on the phagocytes for this PAMP are LBP and CD14, which enhance the inflammatory response mediated by Toll-like receptors (TLR); and TLR-4 and TLR-2, which recognise LPS and initiate the inflammatory response (Aderem and Ulevitch, 2000).

Lipopolysaccharide, a constituent of the outer membrane of Gram-negative bacteria, is apparently one of the major toxins responsible for initiating the pathophysiological cascade resulting in sepsis or a systemic inflammatory response (Rietschel et al., 1996). LPS-binding protein recognises bacterial LPS and transfers it to CD14, thereby enhancing internalisation of LPS or host cell stimulation. Therefore, LBP can be considered as an opsonin, whereas CD14 is an opsonic receptor for complexes of LBP-LPS or LPS-containing particles, such as Gram-negative bacteria. Lipopolysaccharide-mediated stimulation of CD14-positive cells, such as monocytes, macrophages and PMN, is enhanced 100 to 1,000-fold when LBP is added to a serum-free system. Although CD14 alone can efficiently interact with LPS, the presence of LBP has been demonstrated to increase not only the association rate, but also the association constant of LPS with CD14 by three orders of magnitude (Thomas et al., 2002). In addition, LBP can catalytically transfer monomeric LPS from LPS aggregates onto membrane-associated CD14 (mCD14) or soluble CD14 (sCD14) molecules (Tobias et al., 1993; Hailman et al., 1994), which has been suggested a major advantage compared to binding proteins, such as bactericidal/permeability-increasing protein (Tobias et al., 1997).

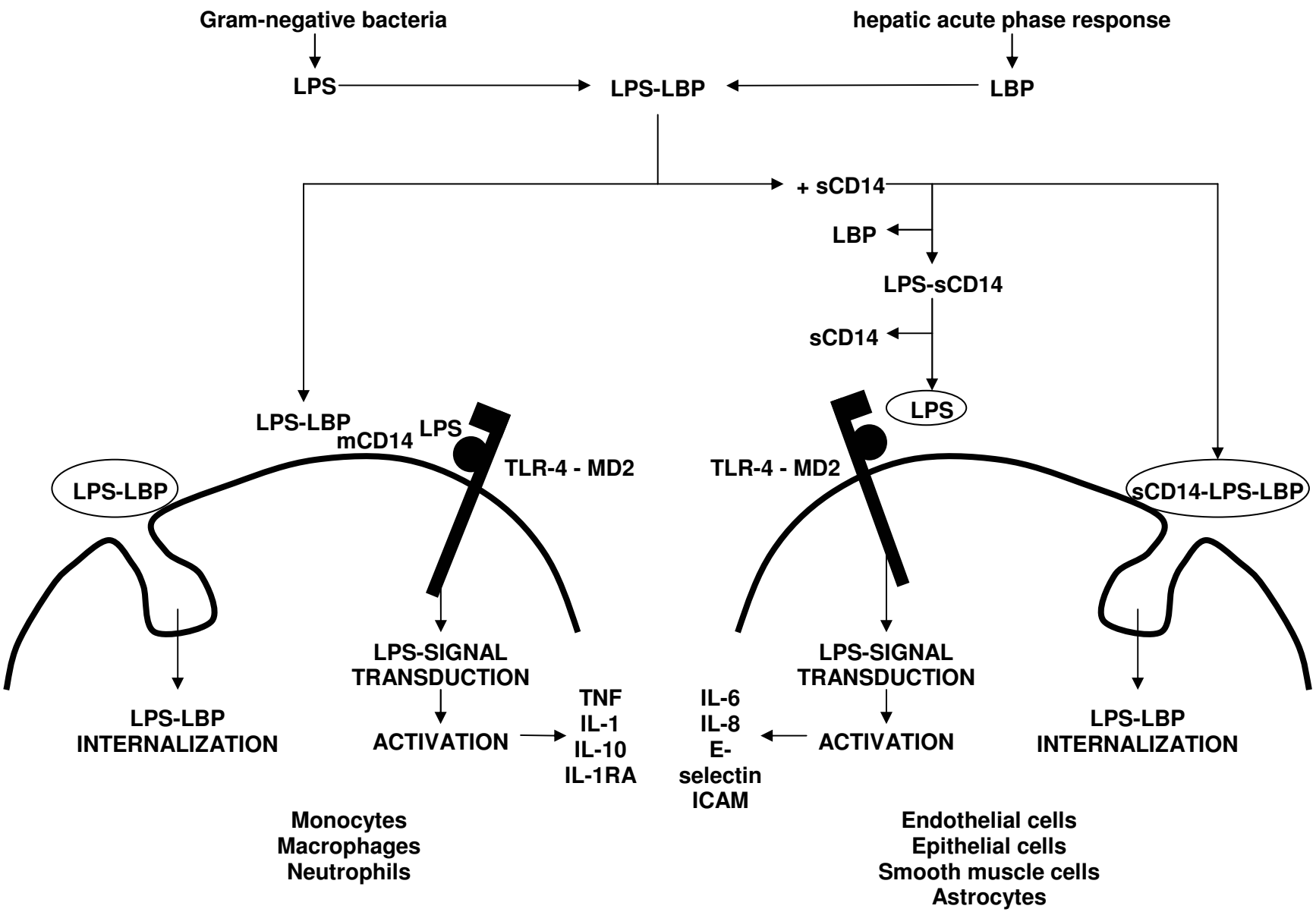
Therefore, LBP participates in the pathogenesis of sepsis (Schumann et al., 1990; Mathison et al., 1992), although it is unclear why the host would release large quantities of LBP during acute inflammation, thereby further enhancing LPS-induced cytokine secretion. However, it has been demonstrated that the acute phase LBP has a protective effect against LPS and bacterial infection; and may represent a physiologic defence mechanism against Gram-negative infection (Lamping et al., 1998). LPS-binding protein not only mediates binding of LPS to CD14, but also the binding of whole Gram-negative bacteria to CD14 leading to phagocytosis and subsequent clearance of bacteria (Lengacher et al., 1996). This LBP-mediated CD14-dependent phagocytic uptake of bacteria could be a mechanism for improved survival following bacterial infection.

*In vitro*, high concentrations of LBP have been shown to block the LPS-induced stimulation of a murine macrophage cell line, both in the absence and in the presence of murine serum (Lamping et al., 1998). The LBP dependency in this cell culture system was of bipolar nature. Addition of murine LBP at concentrations corresponding to the constitutive murine LBP

levels resulted in an increased secretion of TNF-α *in vitro*, whereas high LBP concentrations, simulating the acute phase rise of LBP, resulted in a pronounced decrease in TNF-α response (Lamping et al., 1998). A somehow bipolar nature of biological activity has been described for CD14, another protein involved in LPS recognition. *In vitro*, intermediate concentrations of sCD14 increased the LBP dependent stimulation of PMN, whereas high concentrations of sCD14 inhibited the PMN activation (Hailman et al., 1996), and protected against the toxic properties of LPS (Haziot et al., 1995). It can be concluded that LBP and sCD14 *in vivo* both seem to protect from LPS toxicity and bacteraemie if elevated, although *in vitro* both proteins seem to contribute to LPS recognition and LPS-mediated cell stimulation (Lamping et al., 1998). This bipolar physiologic defence mechanism could have potential applications in therapeutic intervention strategies during sepsis or Gram-negative infections, using a natural host defence mechanism against overstimulation by bacterial products (Lamping et al., 1998) (Fig. 6).

In the bovine, only a few studies have been performed in order to elucidate the kinetics and the role of sCD14 and LBP during LPS-induced or Gram-negative mastitis (Bannerman et al., 2003; Lee et al., 2003a). Lee et al. (2003a) followed sCD14 concentrations throughout lactation and observed the highest concentrations (11.4 ± 0.17 μg/ml) of milk sCD14 during the transitional period. Intermediate concentrations (± 5.5 μg/ml) were present during early (5-100 d post-partum) and late lactation (> 200 d post-partum), and lowest concentrations (4.6 ± 0.27 μg/ml) were found in mid-lactating animals. Increased sCD14 concentration was associated with higher SCC, although sCD14 concentration in subclinically infected quarters did not differ from sCD14 concentration in healthy mammary glands. Upon induction of acute LPS mastitis, milk sCD14 rapidly increased to reach maximal levels between 12 and 24 h post-challenge (Lee et al., 2003a). Possible sources of sCD14 in milk were postulated to be blood serum leakage through the damaged blood-milk barrier or cells present in the mammary gland. Milk sCD14 appeared much later than the initial breakdown of the blood-milk barrier, as indicated by the increase in serum albumin concentrations, ruling out the first possibility. Bovine PMN have been demonstrated capable of releasing sCD14 into milk upon contact with LPS. Moreover, the increase in sCD14 paralleled the increase in SCC following LPS challenge. It is therefore most likely that the PMN influx into the inflamed mammary gland induces the increase in milk sCD14 (Lee et al., 2003a).

**Figure 6.** An overview of the association of LPS with cells mediated by (LBP) and CD14 (based on Tobias et al., 1999).


Gram-negative bacteria
hepatic acute phase response
LPS
LPS-LBP
LBP
+ sCD14
LBP
LPS-sCD14
sCD14
LPS
LPS-LBP
mCD14
LPS
TLR-4 - MD2
LPS-LBP
TLR-4 - MD2
sCD14-LPS-LBP
LPS-SIGNAL TRANSDUCTION
LPS-SIGNAL TRANSDUCTION
LPS-LBP INTERNALIZATION
ACTIVATION
TNF
IL-1
IL-10
IL-1RA
IL-6
IL-8
E-selectin
ICAM
ACTIVATION
LPS-LBP INTERNALIZATION
Monocytes
Macrophages
Neutrophils
Endothelial cells
Epithelial cells
Smooth muscle cells
Astrocytes

Following intramammary LPS challenge, peak fever, a marker of the acute phase response, preceded LBP increase both in blood and milk (Bannerman et al., 2003). The increase in milk LBP paralleled increments of sCD14 levels. From a host perspective, the simultaneous increase of both molecules should be considered advantageous, because they act in concert to facilitate activation of the host defence mechanisms by presenting LPS to the transmembrane LPS receptor, TLR-4 (Bannerman and Goldblum, 2003). However, maximal levels of IL-8 were already observed before increases in either LBP or sCD14, which suggests that initial host cell activation can take place in the presence of only basal levels of sCD14 and LBP (Bannerman et al., 2003). In contrast to sCD14, LBP increases in the affected glands occur coincidently with the disruption of the blood-milk barrier, as indicated by the rise in serum albumin concentration (Bannerman et al., 2003), which is in accordance with the fact that LBP is of hepatic origin and is already present in plasma at high concentrations before the onset of increases in milk.

During the initial phase of inflammation, sCD14 mediates the activation of non-CD14-bearing cells, including epithelial cells (Frey et al., 1992). Low concentrations of LPS-recombinant bovine sCD14 (rbosCD14) have been demonstrated to induce IL-8 transcription *in vitro* in mammary ductal epithelial cell cultures (Wang et al., 2002). Interleukin-8 is an important early chemotactic factor, inducing a massive influx of leukocytes, predominantly PMN into the infected mammary gland (Shuster et al., 1997). A delay in leukocyte recruitment after intramammary coliform infections resulted in 10 times more bacteria (Erskine et al., 1989), indicating the importance of a rapid and early inflammatory reaction in protecting the host from overwhelming bacterial infections. Indeed, delayed leukocyte recruitment was not only associated with overwhelming bacterial growth, but could also be related with increased severity of the inflammatory reaction (Vandeputte-Van Messom et al., 1993). Therefore, the signalling role of sCD14 during the early stage of infection, inducing activation of mammary epithelial cells with the production of IL-8, is an essential step in innate host defence (Wang et al., 2002). Recently, biological functionality of rbosCD14 has been demonstrated in a mouse mastitis model using experimental *E. coli* challenge (Lee et al., 2003b). Intramammary administration of rbosCD14 reduced TNF-$\alpha$ production by phagocytes in milk, possibly by competition with mCD14 for LPS. Moreover, rbosCD14 administration reduced the number of *E. coli*, most likely through an early infiltration of PMN, induced by activation of epithelial cells with the rapid production of IL-8 upon infection (Lee et al., 2003b).

## 2.4. Recurrent intramammary *E. coli* infections in the bovine

Intramammary infections caused by *E. coli* are commonly considered to be limited in duration. Sometimes, the micro-organisms may even be eliminated before or shortly after the onset of clinical symptoms (Hogan et al., 1989). Therefore, the host defence system appears to eliminate *E. coli* efficiently (Hill et al., 1978), especially when the intramammary infection occurs late in lactation (Hill and Shears, 1979). Recurrent clinical mastitis caused by *E. coli* in a cow that experiences persistent intramammary infection is known to exist, but is considered to be exceptional (Hogan et al., 1989; Lam et al., 1996). It has been observed that quarters with recurrent clinical episodes of mastitis caused by *E. coli* were infected by the same *E. coli* strain, as confirmed by serotyping and deoxyribonucleic acid (DNA) polymorphism patterns with REP (= repetitive extragenic palindromic) and ERIC (= enterobacterial repetitive intergenic consensus) primers (Lipman et al., 1994). The estimated occurrence of episodes of clinical mastitis during persistent intramammary infection caused by *E. coli* within the same quarter ranged from 4.77% (Döpfer et al., 1999), over 7.5% (Hogan et al., 1989) to 9.1% (Lam et al., 1996), depending on the study design and confirmation techniques used. The *E. coli* bacteria causing persistent intramammary infection may survive intracellularly after invading the udder epithelial cells (Döpfer et al., 1999), which is, however, in contrast with the general perception that strains of *E. coli* from mastitis cases are non-invasive for the udder epithelial cells (Linton et al., 1979; Sanchez-Carlo et al., 1984; Valente et al., 1988) and mainly opportunistic environmental pathogens (Nemeth et al., 1994). A limited number of *E. coli* strains from recurrent mastitis has been studied with respect to adhesion and invasion mechanisms towards mammary epithelial cells (Döpfer et al., 2000; 2001). Besides occurrence of persistent intramammary infection in the same quarter, multiple cases of recurrent mastitis in different quarters of the same cow with the same genotype of *E. coli* occurred (Döpfer et al., 1999). Possible explanations could be that 1) cows were re-infected with the same strain from the environment, 2) quarters were infected simultaneously but showed clinical symptoms of mastitis at different times, or 3) transmission of *E. coli* from one quarter to another occurred (Döpfer et al., 1999).

Recently, it was observed that quarters infected with an enterobacterial organism during the last third of the dry period were more likely to develop mastitis due to the same pathogen than were uninfected quarters in the subsequent lactation cycle (Bradley and Green, 2000). Previously, the ability of infections acquired during the dry period to remain quiescent within the udder until calving, subsequently causing clinical mastitis in early lactation, has been illustrated (McDonald and Anderson, 1981). The dry period has been implicated as a crucial period for

acquisition of new coliform intramammary infections (Todhunter et al., 1991a), with more than 60% of all new intramammary infections occurring at that time. Although, it has been suggested that the dry gland is highly resistant to enterobacterial infection as a result of the high levels of lactoferrin; more recent research, applying polymerase chain reaction (PCR) DNA fingerprinting techniques, has shown that the mammary gland is not resistant to the acquisition of new enterobacterial intramammary infections (Bradley and Green, 2000). Multiple aspects should be taken into account concerning this new phenomenon, such as potential pathogen adaptation and changed host susceptibility (Bradley and Green, 2001a). Few intramammary enterobacterial infections appeared to persist throughout the entire dry period (Bradley and Green, 2001b).

## 3. EXPERIMENTAL INFECTION MODELS

Several experimental infection models for the induction of intramammary *E. coli* infection have been explored throughout the years in an attempt to mimic naturally occurring *E. coli* mastitis. During natural intramammary infection, bacteria have to overcome the physical barrier formed by the teat canal and its keratinisation before they can enter the teat sinus and start to proliferate in the milk. External contamination of the teat ends with a bacterial suspension of *E. coli* did, however, not induce acute clinical mastitis (Vandeputte-Van Messom et al., 1992). In order to obtain an intramammary infection following bacterial exposure, researchers passed the physical barrier of the teat canal by direct infusion of the bacterial suspension or the bacterial cell wall compounds, such as endotoxin, into the teat and gland sinus.

### 3.1. Lipopolysaccharide model

Lipopolysaccharide (endotoxin) is a component of the Gram-negative cell wall, and is composed of three basic subunits: 1) the O-polysaccharide, providing serospecificity for Gram-negative bacteria, 2) the lipid moiety, generally called lipid A, which is considered to be the toxic component of the cell wall, and 3) the R core, consisting of hexoses, hexamines, and heptose, which acts as a bridge between the O-polysaccharide and the lipid (Westphal, 1975). The extraction of LPS from Gram-negative bacteria can be performed through different methods (Burvenich, 1983): 1) trichloracetic acid (Boivin-extraction), 2) diethylene glycol, 3) phenol / water (Westphal-extraction), 4) water / ether, 5) phenol / chloroform / petroleumether and butanol. Most commercially available endotoxin preparations are prepared by the Boivin- or Westphal-extraction (Burvenich, 1983). Boivin-extracted LPS contains some protein and

possesses therefore much stronger antigenic properties. In contrast, Westphal-extracted LPS contains some peptides, nucleic acids and sugar residues.

The toxicity of LPS is determined by two of its subunits. Lipid A, the lipid moiety of LPS, is identical for LPS originating from different Gram-negative bacterial species. Primary toxicity is due to the lipid A fraction of the LPS, whereas secondary toxicity is related to antigen-antibody reactions, mainly directed against the sugar residues on the O-polysaccharide, which are weakly antigenic. Host hypersensitivity determines the impact of secondary LPS toxicity. A longer O-polysaccharide chain with more sugar residues has higher immunogenic properties and can therefore result in more pronounced secondary toxicity (Burvenich, 1983).

Intramammary LPS challenge is frequently used to study inflammation in the bovine mammary gland, mainly due to its resemblance to *E. coli* challenge (Lohuis et al., 1988b; 1988c). Nevertheless, some fundamental differences exist between both challenge models. In the LPS model, peak fever is reached within 6 h post-challenge, depending on the infused dose of LPS, and is accompanied by acute local and systemic clinical symptoms (Lohuis et al., 1988b), although no depression in reticulorumen motility could be observed following intramammary LPS challenge. Duration of clinical symptoms and general illness in the LPS model is limited, and therefore, quarter milk production is only temporarily depressed and rapidly returns to pre-infection values (Hoeben et al., 2000a; Mehrzad et al., 2001a). In contrast to earlier assumptions, general clinical symptoms following intramammary LPS challenge are not due to LPS resorption into circulation, but mediated through locally produced and systemically active inflammatory cytokines (Dosogne et al., 2002).

Throughout the years, a wide range of LPS inoculum doses, varying from 0.0001 (van der Vliet et al., 1989) to 20,000 µg (Frost et al., 1984), has been applied, although currently used doses appear in a more narrow range of 100 to 1,000 µg per quarter (Table 5). The tendency to use a specific LPS inoculum dose or range of doses also seems to be research group-related. In contrast to the wide variety of applied inoculum doses, only a small selection of strains was used for the extraction and preparation of LPS (Table 6).

A general trend in LPS-induced mastitis is the use of multiparous cows (78.3 %) during the mid-lactation stage (Table 7-8). Although in many, predominantly older studies, neither parity group nor stage of lactation are defined, the most recent research reports on endotoxin-induced mastitis provide more detailed information on the experimental animals and conditions applied in the studies. Taking into account that cow factors may determine the outcome of mastitis (Burvenich et al., 2003), the trend towards a more standardised choice of experimental animals is positive and should be encouraged at all levels.

**Table 5.** LPS inoculum dose for intramammary endotoxin challenge.

| LPS inoculum dose | References |
|---|---|
| 100 pg | van der Vliet et al., 1989 |
| 1 ng | Persson et al., 1993 |
| 10 ng | Persson et al., 1993; Schultze, 1981; Verheijden et al., 1982 |
| 100 ng | Mattila et al., 1985; Persson et al., 1993; Schultze, 1981 |
| 1 μg | Needs and Anderson, 1984; Persson et al., 1993; Persson-Waller, 1997; Schultze et al., 1978; Schultze, 1981; Schultze and Bright, 1983; Verheijden et al., 1982 |
| 2 μg | Hopster et al., 1998; Salih and Anderson, 1979 |
| 5 μg | Giri et al., 1984; Persson, 1990; Persson et al., 1992a; Sladek and Rysanek, 2001 |
| 10 μg | Anderson et al., 1986a; 1986b; 1986c; Anderson and Hunt, 1989; Barrett et al., 1997; Brownlie, 1979; Brownlie et al., 1979; Giri et al., 1984; Guidry et al., 1983; Ishikawa and Shimizu, 1983; Lohuis et al., 1989; Mattila et al., 1987; Mattila and Frost, 1989; Morkoc et al., 1993; Moussaoui et al., 2002; Needs and Anderson, 1984; Paape et al., 1996; Prin-Mathieu et al., 2002; Raulo et al., 2002; Shuster and Harmon, 1992; Shuster et al., 1991a; 1991b; 1993; Verheijden et al., 1982 |
| 12.5 μg | Gorewit, 1993 |
| 15 μg | Bouchard et al., 1999 |
| 20 μg | Kaartinen et al., 1990; Persson and Hallén-Sandgren, 1992; Persson et al., 1992a; Persson-Waller, 1997 |
| 25 μg | Giri et al., 1984; Gorewit, 1993 |
| 50 μg | Carroll et al., 1974; Jain and Lasmanis, 1978; Jain et al., 1978; Mueller et al., 1983; Oliver, 1991; Östensson, 1993; Persson-Waller et al., 2003; Saad and Östensson, 1990 |
| 100 μg | Bannerman et al., 2003; Brooker et al., 1981; Chaiyotwittayakun et al., 2002; Frost et al., 1984; Giri et al., 1984; Guidry et al., 1983; Jackson et al., 1990; Kaartinen et al., 1988; Lappalainen et al., 1988; Lohuis et al., 1988a; 1990b; 1991; Mattila et al., 1985; Schmitz et al., 2004; Shuster and Harmon, 1991; Verheijden et al., 1982; Ziv et al., 1998 |
| 200 μg | Carroll et al., 1965; Jain et al., 1972 |
| 500 μg | Blum et al., 2000; Carroll et al., 1974; Diez-Fraile et al., 2003b; Dosogne et al., 2002; Hoeben et al., 2000a; Lefcourt et al., 1993; Mehrzad et al., 2001a; Paape et al., 1974; Van Oostveldt et al., 2002c; Yagi et al., 2002 |
| 750 μg | Jain et al., 1972 |
| 900 μg | Brooker et al., 1981 |
| 1 mg | Brooker et al., 1981; DeGraves and Anderson, 1993; Dhondt et al., 1977; Frost et al., 1984; Kassa et al., 1986; Tyler et al., 1992; 1993; 1994a; 1994b; Verheijden et al., 1982; Welles et al., 1993; Ziv et al., 1983 |
| 3 mg | Dhondt et al., 1977 |
| 5 mg | Brooker et al., 1981; Carroll et al., 1974; Frost et al., 1984; Jain et al., 1969 |
| 10 mg | Verheijden et al., 1982; Ziv et al., 1976 |
| 15 mg | Ziv and Jochle, 1981 |
| 20 mg | Carroll et al., 1965 |

**Table 6.** Strain diversity used to prepare endotoxin for intramammary inoculation.

| Strain | References |
|---|---|
| *Aerobacter aerogenes* | Carroll et al., 1965 |
| *E. coli* undefined | Fox et al., 1981; Jain et al., 1969; 1972; Oliver and Smith, 1982; Oliver, 1991; van der Vliet et al., 1989 |
| *E. coli* B117 :O8 | Brooker et al., 1981; Frost et al., 1984 |
| *E. coli* O111 :B4 | Bannerman et al., 2003; Blum et al., 2000; Chaiyotwittayakun et al., 2002; Diez-Fraile et al., 2003b; Hoeben et al., 2000a; Kaartinen et al., 1988; Lappalainen et al., 1988; Lohuis et al., 1988a; 1989; 1990a; 1991; 1992; Mattila et al., 1985 ; Mehrzad et al., 2001a; Tyler et al., 1992; 1993; 1994a; 1994b; Van Oostveldt et al., 2002c; Verheijden et al., 1982; Welles et al., 1993; Yagi et al., 2002 |
| *E. coli* O128 :B12 | Lefcourt et al., 1993; Mattila et al., 1985; Schultze, 1981; Schultze and Bright, 1983; Sladek and Rysanek, 2001 |
| *E. coli* O26 :B6 | Anderson et al., 1986a; 1986b; 1986c; Anderson and Hunt, 1989; Barrett et al., 1997; Carroll et al., 1974; DeGraves and Anderson, 1993; Dhondt et al., 1977; Jain and Lasmanis, 1978; Jain et al., 1978; Morkoc et al., 1993; Moussaoui et al., 2002; Mueller et al., 1983; Paape et al., 1974; 1996; Prin-Mathieu et al., 2002; Schmitz et al., 2004; Schultze et al., 1978; Schultze, 1981; Ziv and Jochle, 1981; Ziv et al., 1976; 1983; 1998 |
| *E. coli* O55 :B5 | Bouchard et al., 1999; Brooker et al., 1981; Brownlie, 1979; Brownlie et al., 1979; Frost et al., 1984; Giri et al., 1984; Gorewit, 1993; Guidry et al., 1983; Hopster et al., 1998; Ishikawa and Shimizu, 1983; Jackson et al., 1990; Kaartinen et al., 1990; Mattila et al., 1987; 1989; Needs and Anderson, 1984; Persson-Waller et al., 2003; Raulo et al., 2002; Salih and Anderson, 1979; Schultze, 1981; Shuster and Harmon, 1991; 1992; Shuster and Kehrli, 1995; Shuster et al., 1991a; 1991b; 1993 |
| *E. coli* O157 | Lohuis et al., 1990a |
| *S. typhimurium* SH4809 | Kassa et al., 1986; Östensson, 1993; Persson, 1990; Persson and Hallén-Sandgren, 1992; Persson et al., 1992a; 1992b; 1993; Persson-Waller, 1997; Saad and Östensson, 1990 |

**Table 7.** Frequency and percentage of LPS mastitis studies in different cow parity groups.

| Parity group | Frequency | Percentage |
|---|---|---|
| undefined | 6 | 7.0 |
| primiparous | 10 | 11.5 |
| ***multiparous*** | ***62*** | ***71.3*** |
| primiparous-***multiparous*** | ***7*** | ***8.0*** |
| variable | 2 | 2.2 |
| TOTAL | 87 | 100 |

**Table 8.** Frequency and percentage of LPS mastitis studies in different stages of lactation.

| Parity group | Frequency | Percentage |
|---|---|---|
| undefined | 19 | 22.0 |
| early | 13 | 15.0 |
| ***mid*** | ***23*** | ***26.4*** |
| late | 4 | 4.6 |
| dry | 7 | 8.0 |
| variable | 7 | 8.0 |
| early-***mid*** | ***4*** | ***4.6*** |
| ***mid***-late | ***8*** | ***9.2*** |
| late-dry | 1 | 1.1 |
| early-mid-late | 1 | 1.1 |
| TOTAL | 87 | 100 |

Most frequently, 1 or 2 quarters are challenged with LPS (86.6 %) (Fig. 7).

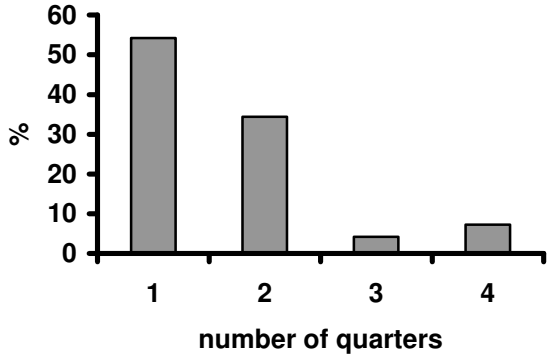



**Figure 7.** Percentage of LPS mastitis studies inoculating 1, 2, 3 or 4 quarters of each dairy cow.

In conclusion, experimental endotoxin mastitis is mostly induced through a single dose administration. This is in contrast with the more dynamic release of LPS in the *E. coli* mastitis model, where LPS is released during growth and subsequent killing of the inoculated bacteria over a much longer time interval (Petsch and Anspach, 2000). Therefore, the model is suitable in the study of inflammatory kinetics, without major health risk for the experimental animals with respect to persistent clinical disease, as is occasionally observed following *E. coli* challenge.

### 3.2. *Escherichia coli* model

The *E. coli* mastitis model is the more realistic approach for an experimental intramammary infection, as it can be observed in field cases. The most important difference with the previously described LPS mastitis model is the occurrence of a continuous release of LPS during bacterial growth and killing (Burvenich, 1983; Petsch and Anspach, 2000), although an initial delay in this release can occur. Intramammary inoculation is performed in one or more quarters, leaving the other quarters as negative controls during inflammation (Fig. 8). The distribution of the number of inoculated quarters with live *E. coli* bacteria is comparable to the previously discussed LPS model.

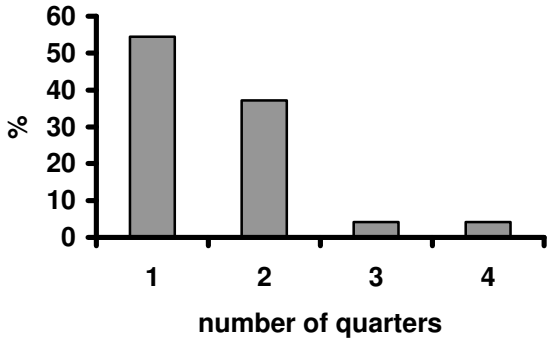



**Figure 8.** Percentage of *E. coli* mastitis studies inoculating 1, 2, 3 or 4 quarters of each dairy cow.

Inflammatory characteristics and kinetics related to experimentally induced *E. coli* mastitis are mainly influenced by the initial inoculum dose. When low numbers of bacteria are inoculated, several hours of bacterial growth are needed before initiation of an inflammatory response (Shuster et al., 1996; Riollet et al., 2000), whereas inoculation of high numbers of bacteria rapidly induce local and systemic clinical symptoms (Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000a; Dosogne et al., 2002). Therefore, it is not easy to clearly define the exact time point when peak fever and maximal systemic and local clinical symptoms are reached. A major clinical difference between the LPS and the *E. coli* mastitis model is the pronounced suppression of reticulorumen motility, which can only be observed in the *E. coli* challenge model (Verheijden et al., 1982; Lohuis et al., 1988b).

For induction of intramammary challenge, a great variety of strains has been used, however, only a few have regularly been applied (Table 9).

**Table 9.** Strain diversity of coliform bacteria used for intramammary challenge.

| Strain | References |
|---|---|
| *Aerobacter aerogenes* | Jain et al., 1969 |
| *A. aerogenes* 2414-1 | Carroll et al., 1973; Zia et al., 1987 |
| *A. aerogenes* 2413-2 | Carroll et al., 1973 |
| *Klebsiella* K644 | Carroll et al., 1973 |
| *Klebsiella* K-6 | Carroll et al., 1973 |
| *K. pneumoniae* | Bramley and Neave, 1975 |
| *E. coli* 12795 | Carroll et al., 1973 |
| *E. coli* | Harmon et al., 1976 |
| *E. coli* S-16 | Carroll et al., 1973 |
| *E. coli* Lilly | Carroll et al., 1973 |
| *E. coli* 1128 | Schultze et al., 1978 |
| *E. coli* McDonald 487 | Hogan et al., 1994b; Shuster et al., 1996; 1997 |
| *E. coli* 727 | Barrett et al., 1997; Hogan et al., 1994a; 1995; Scaletti et al., 2003; Smith et al., 1999; Tomita et al., 2000; Weiss et al., 2004 |
| *E. coli* B117:O8 | Frost et al., 1980; 1982 |
| *E. coli* FT238 | Pyörälä et al., 1994 |
| *E. coli* O157 | Dosogne et al., 1997; Kremer et al., 1993a; 1993b; 1993c; Lohuis et al., 1990b; Roets et al., 1999; van Werven et al., 1997 |
| *E. coli* O5 | Griel et al., 1975 |
| *E. coli* P4:O32 | Anderson et al., 1985; Blum et al., 2000; Dosogne et al., 2002; Heyneman et al., 1990; Heyneman and Burvenich, 1992; Hill et al., 1978; Hoeben et al., 2000a; Monfardini et al., 1999; Riollet et al., 2000; Shpigel et al., 1997; Van Oostveldt et al., 2002c; Vandeputte-Van Messom and Burvenich, 1993; Vandeputte-Van Messom et al., 1993 |
| *E. coli* Saskatchewan | Sordillo and Babiuk, 1991; Sordillo and Peel, 1992 |

Throughout the years, different inoculum doses have been used from 5 CFU (Schultze et al., 1988) up to 1.8 x $10^{10}$ CFU (Frost et al., 1980), although currently used inoculum doses were 2 x $10^{2}$ to 1 x $10^{4}$ CFU (Carroll et al., 1973; Fox et al., 1981; Frost et al., 1982; Heyneman et al., 1992; Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000a) (Table 10). Inoculum doses above 1 x $10^{4}$ CFU per quarter have mainly been used in older studies. Another remarkable observation is that American mastitis research groups predominantly inoculate low numbers (< 100 CFU) of *E. coli* into the mammary gland, whereas European investigators prefer to use higher numbers ( > 100 CFU). Therefore, care should be taken when study results in terms of inflammatory kinetics are compared between both types of experimental design for reasons discussed earlier.

**Table 10.** Inoculum doses used for experimental intramammary inoculation with coliform bacteria.

| Inoculum dose (CFU) | References |
|---|---|
| < 20 | Schultze et al., 1978 |
| 21-30 | Hogan et al., 1994b; Scaletti et al., 2003; Schultze et al., 1978; Shuster et al., 1996; 1997 |
| 31-50 | Barrett et al., 1997; Frost et al., 1982; Hill et al., 1978; Riollet et al., 2000; Sordillo and Babiuk, 1991; Sordillo and Peel, 1992 |
| 51-100 | Carroll et al., 1973; Hogan et al., 1994a; 1995; Smith et al., 1999; Tomita et al., 2000; Weiss et al., 2004 |
| 101-500 | Carroll et al., 1973; Frost et al., 1982; Griel et al., 1975; Harmon et al., 1976; Shpigel et al., 1997; Van Oostveldt et al., 2002c |
| 501-1,000 | Anderson et al., 1985; Carroll et al., 1973; Dosogne et al., 1997; Kremer et al., 1993a; 1993b; 1993c; Roets et al., 1999; Shpigel et al., 1997; van Werven et al., 1997 |
| 1,001-10,000 | Anderson et al., 1985; Blum et al., 2000; Bramley and Neave, 1975; Carroll et al., 1973; Dosogne et al., 2002; Griel et al., 1975; Heyneman et al., 1990; Heyneman and Burvenich, 1992; Hill et al., 1978; Hoeben et al., 2000a; Lohuis et al., 1990b; Monfardini et al., 1999; Pyörälä et al., 1994; Vandeputte-Van Messom and Burvenich, 1993; Vandeputte-Van Messom et al., 1993; Zia et al., 1987 |
| 10,001-100,000 | Bramley and Neave, 1975; Carroll et al., 1973 |
| 100,001-1,000,000 | Bramley and Neave, 1975; Carroll et al., 1973; Hill et al., 1978 |
| > 1,000,001 | Carroll et al., 1973; Jain et al., 1969; Frost et al., 1980 |

Experimentally induced *E. coli* mastitis has mainly been studied in multiparous animals (84.5 %), whereas the immune response in primiparous cows (6.7 %) has hardly been explored (Table 11). In contrast to LPS mastitis which was predominantly studied in mid-lactation, *E. coli* challenge has mainly been performed in early lactation animals (66.7 %) (Table 12).

**Table 11.** Frequency and percentage of *E. coli* mastitis studies in different cow parity groups.

| Parity group | Frequency | Percentage |
|---|---|---|
| undefined | 2 | 4.4 |
| primiparous | 3 | 6.7 |
| ***multiparous*** | ***30*** | ***66.7*** |
| primiparous-***multiparous*** | ***8*** | ***17.8*** |
| variable | 2 | 4.4 |
| TOTAL | 45 | 100 |

**Table 12.** Frequency and percentage of *E. coli* mastitis studies in different stages of lactation.

| Parity group | Frequency | Percentage |
|---|---|---|
| undefined | 4 | 8.9 |
| ***early*** | ***28*** | ***62.3*** |
| mid | 3 | 6.7 |
| late | 4 | 8.9 |
| dry | 0 | 0 |
| variable | 2 | 4.4 |
| ***early***-mid | ***1*** | ***2.2*** |
| mid-late | 2 | 4.4 |
| late-dry | 0 | 0 |
| ***early***-mid-late | ***1*** | ***2.2*** |
| TOTAL | 45 | 100 |

## 4. DIAGNOSIS AND TREATMENT OF *E. COLI* MASTITIS

### 4.1. Diagnosis

Coliform mastitis affects the quality of milk, may induce partial or total loss of milk production capacity in the affected quarters or can even result in a life-threatening situation for the diseased animal. Therefore, rapid and correct diagnosis of the etiological agent of clinical mastitis should be performed. Knowledge on risk factors and severity determining factors may assist in the recognition of a clinical case as being due to *E. coli*.

#### 4.1.1. Clinical and bacteriological diagnosis

Clinical diagnosis, consisting of inspection and visual examination of the diseased animal, the affected quarter and the changes in milk composition, does not systematically result in a correct etiological diagnosis. Appearance of mastitis with acute or peracute clinical signs and watery mammary secretions with flakes is indicative of an intramammary *E. coli* infection, although bacteriological sampling of clinical mastitis cases only resulted in 16.2% *E. coli* cultures (Schukken et al., 1989). If the absence of bacteria in culture would also be considered as *E. coli*, a total of 39.1% of the clinical cases would be due to *E. coli* (Schukken et al., 1989). Therefore, etiological diagnosis solely based on clinical parameters is assumed to be difficult to take the appropriate therapeutic measures to combat acute clinical mastitis. It is clear that further steps should be taken to identify the etiological agent through bacteriological examination or molecular identification methods.

An alternative to clinical diagnosis may be provided by cow-side tests, such as ColiMast™ or LiMast™, which help to identify the etiological agent responsible for the clinical case. In the ColiMast™ cow-side test, coliforms are identified by means of a visual colour change. Reliable and specific results were obtained within 12 h (Rogerson et al., 2000). A disadvantage of the ColiMast™ test is that negative results are difficult to interpret, as presence of coliforms below the detection limit also leads to absence of colour change. The LiMast™ test can be used in cows with moderate to severe clinical symptoms, but detection is also limited to Gram-negative organisms (Yazdankhah et al., 2001). An advantage of the LiMast™ cow-side test is its speed (results within 15 min), although the same disadvantage remains present as pointed out for the ColiMast™ test.

From a therapeutic point of view, the differential diagnosis of clinical mastitis is essential, since the optimal treatment differs between Gram-positive and Gram-negative bacterial infection in the mammary gland (Yazdankhah et al., 2001). Therefore, a rapid procedure (less than 5 min) for differentiation between Gram-positive and Gram-negative mastitis was developed. Although the method has a high detection limit (1 x $10^6$ CFU/ml), application in the diagnosis of moderate or severe clinical mastitis is possible (Yazdankhah et al., 2001). As mild to moderate mastitis caused by coliforms should not be treated with antibiotics, this procedure provides valuable information to limit the excessive use of antibiotics in these clinical cases with a high degree of self-curing.

Bacteriological examination is considered to be the 'golden standard' for routine detection and identification of major mastitis pathogens (IDF, 1971). From a practical point of view, however, bacteriological diagnosis of clinical mastitis is labour-intensive and time-consuming (Riffon et al., 2001), although the final result can be considered reliable and informative, especially for future treatment decision on the same dairy farm. Nevertheless, it has been demonstrated that early detection procedures enhance cure rates and reduce the time required to return to normal milk production when coupled with an appropriate antimicrobial therapy (Milner et al., 1997).

### 4.1.2. Milk SCC and compositional changes

According to International Dairy Federation (IDF) recommendations, mastitis diagnosis should be based on the SCC (Hillerton, 1999). Normal healthy milk SCC mainly consists of macrophages, in addition to PMN, epithelial and mononuclear cells (Dulin et al., 1982; Östensson et al., 1988). The proportion of PMN increases from ± 10% in healthy milk to more than 90% during intramammary infection, and therefore, contribution of PMN to the total milk

SCC has been proposed as an indicator of mastitis (Kelly et al., 2000). This approach would provide more information on the health status of the mammary quarters (Östensson et al., 1988), but further applications did not occur mainly due to practical limitations.

Milk SCC threshold for the diagnosis of mastitis has been reduced from 500,000 to 200,000 cells/ml, and recently a threshold of 100,000 cells/ml was even suggested (Hillerton, 1999) for quarters to be considered as clinically healthy. This seems to be in agreement with SCC results recently obtained through meta-analysis (Djabri et al., 2002), suggesting an average SCC of 68,000 cells/ml in bacteriologically negative quarters. Minor pathogens only result in a slight increase of quarter SCC (110,000 to 150,000 cells/ml), whereas major pathogens induced more pronounced increases (350,000 cells/ml) (Djabri et al., 2002). Recently, SCC patterns have been associated with pathogen-specific cases of clinical mastitis. In that study, clinical *E. coli* mastitis was significantly associated with the presence of a short peak in SCC (de Haas et al., 2004).

Besides SCC, several compositional changes occur in milk during clinical mastitis (Kitchen, 1981; Östensson et al., 1988; Hamann and Krömker, 1997), such as decreased quarter milk yield, lactose, fat, long-chained fatty acids, total casein, α-lactalbumin, β-lactoglobulin, $Ca^{2+}$, $Mg^{2+}$, $P^{2+}$, $Zn^{2+}$ and potassium ($K^{+}$), and increased whey proteins, serum albumin, immunoglobulins, proteose peptone, free fatty acids, short-chained fatty acids, sodium ($Na^{+}$), chlorine ($Cl^{-}$), lactate, lipase, lysozyme, N-acetyl-β-D-glucosaminidase, β-glucuronidase and plasmin activity (Pyörälä, 2003).

### 4.1.3. Molecular identification methods

Recently, a PCR technique was developed for simultaneous detection of several major mastitis pathogens (Riffon et al., 2001). The advantage of PCR, compared to bacteriology, lays in the possibility to use only nanograms of nucleic acid samples, allowing the elimination of bacterial culture, combined with rapidity (within 24 h results) and easy analysis. The detection limit of the assay is set at 5 x $10^3$ CFU/ml of milk in the absence of a pre-PCR enzymatic step, which should be sensitive enough to be used as a diagnostic tool in bovine mastitis.

## 4.2. Treatment

Various preventive measures and management practices have been shown effective to decrease the incidence of coliform mastitis over the years. Nevertheless, a great need exists for therapeutic measures following the occurrence of acute or peracute coliform mastitis. Currently,

these measures include the use of antimicrobials, anti-inflammatory agents (steroidal and non-steroidal) and additional treatments (frequent milk-out, oxytocin administration and fluid therapy) (Ziv, 1992).

Until now, differential therapies, depending on the risk factors and severity determining factors associated with the specific case of acute mastitis, have not been established. The importance of severity estimation should be stressed in relation to subsequent outcome of the clinical disease. Early lactation and ketonemia should be considered important severity determining factors, in combination with higher parity number (Gilbert et al., 1993; Kremer et al., 1993c; Hoeben et al., 1997c; 2000b; van Werven et al., 1997).

### 4.2.1. Antimicrobial treatment of *E. coli* mastitis

Local and systemic antibiotics are frequently used in the treatment of clinical *E. coli* mastitis in the field. Mastitis in general is currently the most frequent reason for antibiotic use in lactating dairy cattle (Gardner et al., 1990; Meek et al., 1986; Guterbock, 1995). Even though antibiotic treatment of mastitis has been performed for decades, our current knowledge about their actual efficacy is still very scarce. Conclusive data on mastitis treatment efficacy through antibiotics should not only be based on clinical field trials, but also consist of *in vitro* studies and experimentally induced mastitis studies (Shpigel, 2001). Antimicrobial drugs are assumed to exert their beneficial therapeutic effect via bactericidal or bacteriostatic action.

*In vitro* studies on the potential effects of local and systemic antimicrobials on bovine blood and milk PMN functionality have demonstrated severe suppressive effects when PMN were exposed to relatively high concentrations of several antimicrobials (Hoeben et al., 1997a; 1997b; 1998a; Dosogne et al., 1998b). In practice, these high doses could only be reached by prolonged multiple dose treatment schedules, mainly through a local administration route (Hoeben et al., 1998a). In these studies a wide range of antimicrobials (Table 13) was tested at different subtherapeutic, therapeutic and supratherapeutic concentrations. Several products had the spectrum required for treatment of infections with Gram-negative bacteria, such as *E. coli* mastitis (Hoeben et al., 1997a) (Fig. 9). All antibiotics, except sulphadiazin and enrofloxacin decreased CL at the highest concentration. The stimulatory effect of enrofloxacin might be due to a stimulation of the production of $H_2O_2$ (Hoeben et al., 1997a) (Fig. 10).

Intravenous administration of enrofloxacin following experimentally induced *E. coli* mastitis in early lactating dairy cows did not result in an improved clinical condition (Hoeben et al., 2000a), as 2 animals in the treated group responded severely following intramammary challenge. Nevertheless, a significantly decreased number of bacteria (1:100) in the infected

quarters could be observed following systemic antimicrobial treatment at 10 h post-challenge (Monfardini et al., 1999). It is known that the administration of a bactericidal antibiotic during intramammary bacterial infection is a double-edged sword, on the one hand decreasing the number of bacteria in the affected quarters, on the other hand inducing a major release of LPS from the destroyed bacteria into the mammary gland compartment (Hoeben et al., 2000a).

In a clinical field trial evaluating the efficacy of parenteral administration of procaine penicillin G, spiramycin and enrofloxacin, bacteriological cure rate in cows with mastitis caused by *E. coli* was 74% for those treated with penicillin G and 71% for the non-treated controls (Pyörälä and Pyörälä, 1998). Following experimentally induced *E. coli* mastitis, treatment with trimethoprim-sulphadiazin or colistin sulphate were apparently no more beneficial than no treatment (Pyörälä et al., 1994). Another study reported beneficial effects of cefquinome treatment on bacteriological cure rate following experimentally induced *E. coli* mastitis, whereas no beneficial effect on bacteriological cure rate was observed for ampicillin or cloxacillin administration (Shpigel et al., 1997).

**Table 13.** Antimicrobial products tested *in vitro* for their influence on blood or milk PMN functionality, more specifically phagocytosis, oxidative burst activity or bactericidal activity (Hoeben et al., 1997a; 1997b; 1998a; Dosogne et al., 1998b).

| Antimicrobial | Antibiotic group | Local or systemic treatment |
|---|---|---|
| ampicillin | aminopenicillins | S |
| cephapirin | 1st generation cephalosporins | L / S |
| chloramphenicol | - | S |
| cloxacillin | penicillinase-resistant penicillins | L |
| danofloxacin | fluoroquinolones | S |
| dihydrostreptomycin | macrolides | S |
| doxycyclin | tetracyclins | S |
| enrofloxacin | fluoroquinolones | S |
| erythromycin | macrolides | S |
| lincomycin | lincosamides | S |
| mecillinam | β-lactam antibiotics | L |
| $Na^+$-ceftiofur | 3rd generation cephalosporins | L / S |
| neomycin | macrolides | S |
| oleandomycin | macrolides | S |
| oxytetracyclin | tetracyclins | S |
| penicillin | natural penicillins | L / S |
| spiramycin | macrolides | S |
| sulphadiazin | sulphonamides | S |

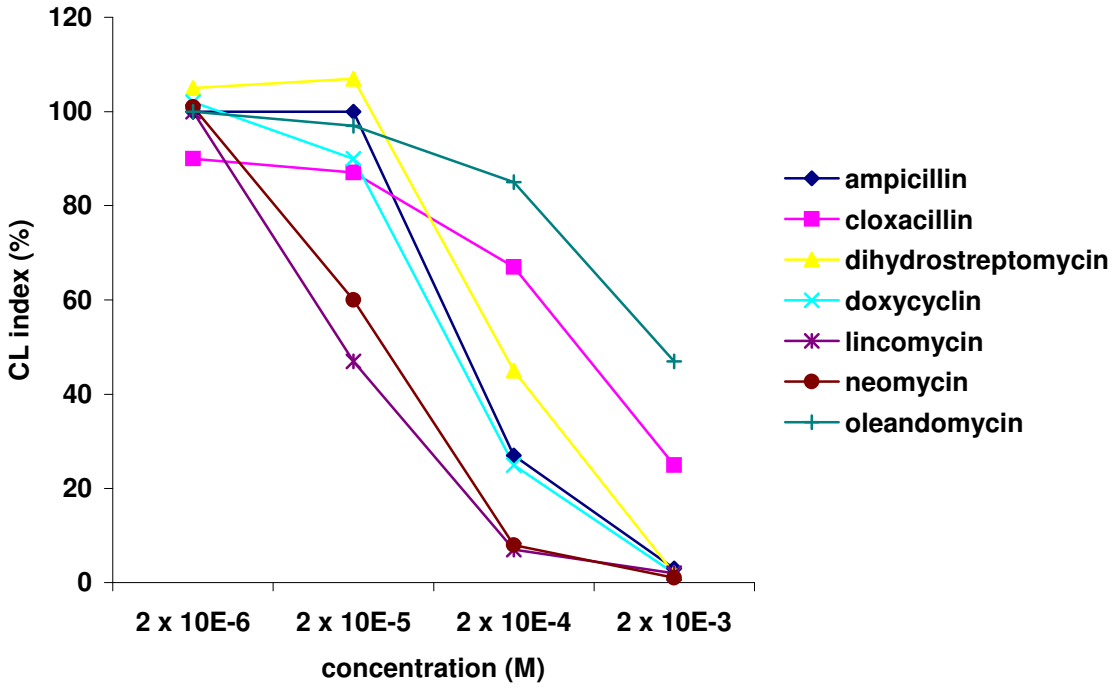



**Figure 9.** Chemiluminescence index (%) of isolated blood PMN incubated with some currently used antibiotics for the treatment of mastitis (Hoeben et al., 1998a).

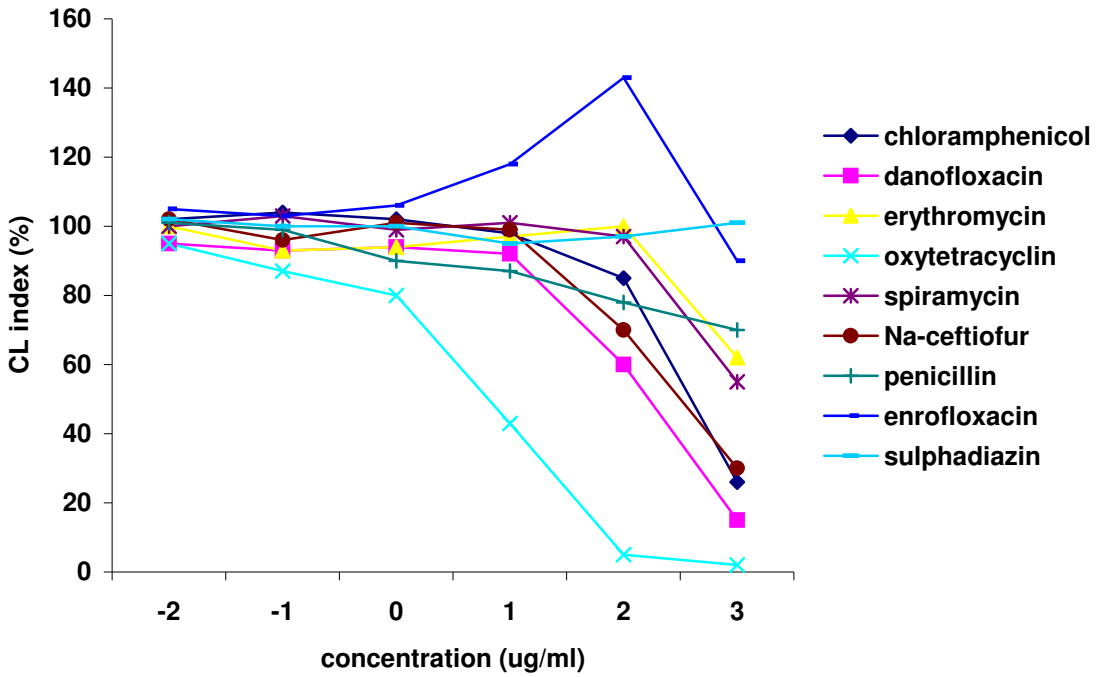



**Figure 10.** Chemiluminescence index (%) of isolated blood PMN incubated with some currently used antibiotics for the treatment of mastitis (Hoeben et al., 1997a; 1997b).

### 4.2.2. Anti-inflammatory treatment of *E. coli* mastitis

Glucocorticosteroids are very well known and widely used anti-inflammatory agents in the treatment of a variety of inflammatory processes, such as acute *E. coli* mastitis in the periparturient cow. Local and systemic administration of glucocorticosteroids is possible. Local administration reduces swelling and oedema in the affected tissue, whereas systemic administration can combat the symptoms of endotoxin shock (Phillips et al., 1987). However, both endogenous and exogenous glucocorticosteroids in high concentrations have been shown to suppress functions of circulating PMN (Hoeben et al., 1998b), although no adverse effects on *in vitro* PMN CL could be shown at therapeutic concentrations. Attention has to be paid to possible immunodepressive effects of glucocorticosteroids after repeated and local administration. It is therefore advisable to avoid the regular use of these drugs during inflammation *in vivo* and to limit potential administration under controlled circumstances (Hoeben et al., 1998b). Furthermore, inhibitory effects of glucocorticosteroids on bone marrow progenitor cells were recently reported (Hoeben et al., 1999). The inhibitory effect on *in vitro* cultured bovine bone marrow progenitors not only appeared at supratherapeutic, but already at physiological concentrations.

Non-steroidal anti-inflammatory drugs (NSAID's) are known for many years for their anti-inflammatory, antipyretic and analgesic effects. They generally prevent the formation of inflammatory mediators by cyclo-oxygenase (COX) inhibition and reduction of prostaglandin $E_2$ ($PGE_2$) production in the brain. Under experimental conditions, NSAID's have been shown to have some beneficial effects (reduced rectal temperature, decrease in quarter inflammation,…), combined with a reduced concentration of inflammatory mediators in milk and plasma (Anderson et al., 1986a; 1986b; Lohuis et al., 1989; Ziv, 1992). A major disadvantage of most experimental studies evaluating NSAID efficacy is the use of an LPS model, in which animals rapidly return to normal milk production, therefore lacking to show any beneficial effects on milk production, rate of return to production or survival (Shpigel, 2001).

Recently, NSAID efficacy was evaluated in the field (Shpigel et al., 1994; 1996). Ketoprofen significantly improved recovery in clinical mastitis, increasing the odds ratio for recovery by as much as 7 (Shpigel et al., 1994). In contrast, phenylbutazone and dipyrone only increased odds ratio to 2.42 and 1.71, respectively (Shpigel et al., 1996). Therefore, ketoprofen can be recommended as an adjunctive therapy in the treatment of Gram-negative clinical mastitis in dairy cows (Shpigel et al., 1994).

### 4.2.3. Additional treatments

Several non-antibiotic methods have been suggested for the treatment of clinical mastitis, such as frequent milk-out with or without oxytocin administration, fluid therapy, hydrotherapy, intramammary infusions of saline, antihistamines, diuretics, hot and cold packing, hypertonic saline infusion and ultrasonic therapy (Roberson et al., 2004). Only two of the most relevant and practical supportive treatments will be discussed here.

Frequent milk-out was encouraged as a possible treatment for mastitis as early as 1869 by Sloan (1869), who claimed that the bad milk should be drawn three to four times a day, for by remaining in the bag, it tends to increase the inflammation. Since then, the practice of frequent milk-out, often facilitated by the administration of oxytocin, has been encouraged, although no scientific reports on potential beneficial effects are available (Leininger et al., 2003). Theoretically, frequent milk-out should have beneficial effects on clinical and bacteriological cure of *E. coli* mastitis, based on the fact that released and accumulated endotoxin in the affected mammary gland would more frequently be removed (Morin et al., 1998; Roberson et al., 2004). Therefore, from a practical point of view, this strategy was frequently recommended in the past (Eberhart et al., 1987). Recently, two studies compared non-treated controls to cows treated by frequent milk-out with oxytocin administration in field cases (Roberson et al., 2004) or experimentally induced *E. coli* challenge (Leininger et al., 2003). Neither of both studies could show beneficial effects of frequent milk-out on the clinical or bacteriological cure rate of the affected animals. This is, however, not surprising as frequent milk-out is rather difficult during acute *E. coli* mastitis due to severe swelling of the affected quarter.

Fluid therapy is widely recommended for the treatment of severe clinical *E. coli* mastitis, although very little published information assessing its efficacy is available (Green, 1998). In practice, several problems can arise when deciding upon a fluid therapy regimen: 1) oral fluid administration alone is probably of little benefit as gastro-intestinal mobility and function are depressed during severe clinical mastitis, resulting in a poor absorption of the administered fluids, 2) no field data are available which indicate improved survival rates following a fluid therapy regimen. Even though no direct clinical benefit would be proven, fluid therapy may nevertheless support the general clinical condition of the treated animal (Green, 1998).

Essentially, two types of intravenous fluid therapy with practical application are available: isotonic and hypertonic fluid therapy. Isotonic solutions contain 0.9% NaCl and are administered in large quantities (50 to 100 ml/kg body weight per 24 h), with a fast rate of administration in the first hours of therapy (50 ml/kg). In contrast, hypertonic solutions contain much higher concentrations of NaCl (7.2%) and are administered concomitantly with an *ad*

*libitum* provision of water. The principal effect of hypertonic saline is thought to be an increase in preventricular load, and hence an increased plasma volume and blood pressure, leading to a general improvement in tissue perfusion (Green, 1998).

## 5. PREDICTION OF THE SEVERITY OF EXPERIMENTALLY INDUCED *E. COLI* MASTITIS

The variability in the severity of clinical signs of acute coliform mastitis is due to the physiological quality of the PMN. The quality seems to be determined by factors acting at the level of the bone marrow, the blood and the mammary gland. It is therefore reasonable and logical to assume that it could be possible to predict the severity of acute mastitis during early lactation through measurement of circulating PMN activity (Burvenich et al., 1999).

The predictability of the number of circulating PMN as a marker for mastitis susceptibility was limited to the moment immediately before infection (Kremer et al., 1993b) (Table 14), although the correlation coefficient between the number of circulating leukocytes immediately before infection (d0) was +0.81 with d-1, +0.83 with d-2, and +0.74 with d-5 (van Werven et al., 1997).

**Table 14.** Correlation between pre-infection parameters determined immediately prior to inoculation (d0), and at d+1, 2, 5, and 6 before inoculation and severity of *E. coli* mastitis characterised by bacterial growth in infected quarters (AUC) (Kremer et al., 1993b).

| Pre-infection parameter | days before infection | r | *P* |
|---|---|---|---|
| number of PMN in blood | 0 | -0.71 | * |
| | 1 | -0.61 | |
| | 2 | -0.61 | |
| | 5 | -0.22 | |
| | 6 | . | |
| chemotactic differential of WBC | 0 | -0.83 | *** |
| | 1 | -0.90 | *** |
| | 2 | -0.88 | *** |
| | 5 | -0.55 | |
| | 6 | -0.16 | |
| chemotactic differential of PMN | 0 | -0.60 | |
| | 1 | -0.72 | * |
| | 2 | -0.74 | ** |
| | 5 | -0.92 | *** |
| | 6 | 0.13 | |

Besides a high number of circulating PMN, a fast migration of these cells into the affected mammary gland upon bacterial infection is critical for the outcome of coliform mastitis

during early lactation (Hill, 1981). Chemotaxis of PMN isolated from blood was negatively correlated with bacterial growth in the milk of the infected quarters (Lohuis et al., 1990b). Moreover, PMN isolated from blood of cows with severe clinical signs of mastitis had a significantly lower pre-infection chemotactic activity than cows with moderate symptoms of mastitis (Kremer et al., 1993a). During 2 consecutive days before infection, the chemotactic activity in severely diseased animals was only about 50% of the normal chemotactic activity, as measured in moderate responders. This resulted in a significant predictive capacity of the chemotactic differential towards severe clinical mastitis (Table 14).

In another study (van Werven et al., 1997), the predictive value of PMN chemotaxis, phagocytosis, oxidative burst and CD11/CD18 adhesion molecules for severity of acute clinical mastitis was evaluated (Table 15). Of all the investigated markers in the circulation, PMN chemotaxis predicted best the outcome of experimentally induced *E. coli* mastitis.

**Table 15.** Correlation between assessments immediately prior to inoculation (d0), and at d+1, 2, 3, 4 5, and 6 before inoculation for values of chemotaxis, phagocytosis, oxidative burst, and CD11/CD18 molecule expression on PMN isolated from blood (van Werven et al., 1997).

| | | **Pre-infection PMN functionality and characteristics** | | | | |
|---|---|---|---|---|---|---|
| *day* | *chemotaxis* | *phagocytosis* | *oxidative burst* | *CD11a expression* | *CD11b expression* | *CD11c expression* |
| -6 | 0.53 | . | 0.30 | 0.73 | 0.69 | 0.24 |
| -5 | 0.75 | 0.50 | . | . | . | . |
| -4 | 0.66 | 0.86 | 0.70 | 0.78 | 0.89 | 0.33 |
| -3 | 0.59 | . | . | . | . | . |
| -2 | 0.35 | 0.84 | 0.85 | 0.47 | 0.80 | 0.68 |
| -1 | 0.05 | 0.77 | 0.84 | 0.46 | 0.31 | 0.91 |

Besides these risk factors for a severe clinical mastitis following intramammary *E. coli* challenge, predictive capacity could also be attributed to several severity determining factors. It is obvious from previously presented data that the incidence of severe clinical mastitis is much higher during early lactation, compared to the other stages of lactation and the dry period (Erskine et al., 1988; Burvenich et al., 2000). Moreover, severe reactions have mainly been observed in older animals in negative energy balance (Kremer et al., 1993c; Vandeputte-Van Messom et al., 1993; van Werven et al., 1997), whereas little is known on severity of the clinical response in primiparous cows.

## 6. CONCLUSIONS

In conclusion, the knowledge on the pathogenesis of *E. coli* mastitis has expanded greatly during the last 20 years. In contrast to infectious mastitis, the incidence and severity of *E. coli* mastitis is mainly due to cow factors, whereas bacterial virulence factors only play a minor role in the disease. Moreover, the circumstances under which moderate and severe clinical responses appear have extensively been studied in experimental *E. coli* mastitis models. In practice, however, the etiological diagnosis of *E. coli* mastitis is still problematic, and therefore, the availability of a practical severity scoring should help the clinician to estimate the cow's clinical response and the related treatment prognosis. An attitude change is needed in order to successfully establish this innovative approach towards treatment of acute coliform mastitis.

However, to be able to make a reliable prognosis, several severity determining factors remain unexplored, such as the parity or age of the affected animals. Until now, older cows are supposed to be more susceptible to severe clinical coliform mastitis, but data on primiparous cows remain scarce. Taking into account the rapid turnover of dairy cows in our dairy herds, knowledge on resistance against or severity during intramammary *E. coli* infection of a large proportion of animals in the dairy herd, the primiparous cows, remains insufficient.

If an age-related resistance would exist, further questions towards the efficacy of therapeutic or prophylactic strategies remain unanswered. It would therefore be of interest to evaluate some of these strategies in an experimental *E. coli* model.

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# HYPOTHESIS AND OBJECTIVES

Review of literature clearly indicates the important role of PMN in the pathogenesis of *E. coli* mastitis, particularly in the elimination of *E. coli* itself and the released LPS. Therefore, it is obvious that small changes in PMN functionality, e.g. diapedesis and oxidative burst, could have a tremendous impact on the pathogenesis of subsequent *E. coli* mastitis (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). From this perspective, the experimental investigation of these factors is of interest to quantify the inflammatory reaction. However, in the past, PMN functionality during different stages of lactation and experimentally induced challenge has mainly been studied in the blood, although the site of infection is situated at the level of the mammary gland. Epidemiological studies have shown an association between low bulk milk SCC and an increased incidence of coliform mastitis (Schukken et al., 1989a; 1989b; Barkema et al., 1999). The role of the resident cells in the healthy bovine mammary gland with a low SCC and the effect of external bacterial contamination during milk sample collection on the PMN functions remain unclear.

Until now, practical treatment of *E. coli* mastitis remains problematic. Standard therapy includes antibiotics, although efficacy and treatment success have never been fully established (Shpigel, 2001). Of major importance is the fact that intramammary infections with Gram-negative bacteria only occur as clinical mastitis, whereas Gram-positive bacteria can have a clinical, subclinical or chronic course of infection. Therefore, there is an urgent need for improved prognosis of the course of clinical disease, mainly to discriminate between moderate cases, which have a high degree of self-curing and do not require treatment, and severe responders, which require rapid and efficient intervention with general supportive therapy and anti-inflammatory drugs. Risk factors for the development of severe clinical *E. coli* mastitis are limited to the stage of lactation (Burvenich et al., 1994; 2003), whereas parity or age of the cow have hardly been studied (Gilbert et al., 1993; van Werven et al., 1997). Nevertheless, some indications for differences in susceptibility between primiparous and multiparous cows have recently been found (Mehrzad et al., 2002). In the context of modern farm management, related with a relatively high turn-over of the dairy cow population, no information is available on resistance against clinical *E. coli* mastitis for a significant proportion of the farm population, namely the primiparous cows.

Using an experimental intramammary *E. coli* challenge model to obtain information on inflammatory reaction kinetics in primiparous cows, it could also be of interest to evaluate possible modulatory effects of inoculum dose, NSAID treatment and prophylactic vaccination against the endotoxin. General perception would link an increased inoculum dose to more pronounced disease symptoms, whereas practically used therapeutic or prophylactic measures are usually thought to accelerate resolution or to protect from severe clinical symptoms.

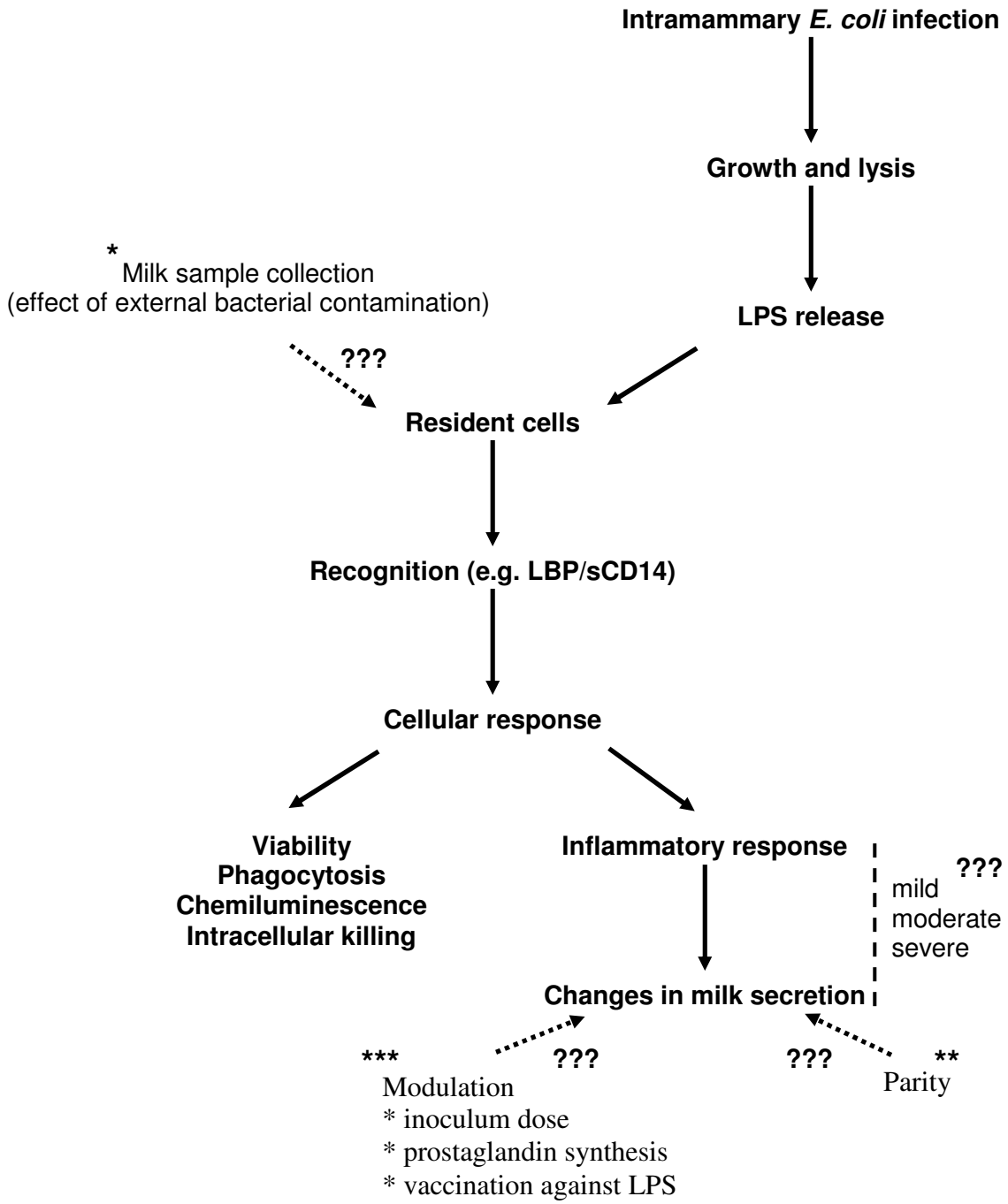


**Figure 1.** Schematic drawing of the hypothesis and objectives of this thesis. Left pathway: study of the effect of external bacterial contamination on cellular response of resident cells in milk (*) (p. 69). Right pathway: following intramammary *E. coli* infection, bacterial growth and lysis leads to the release of LPS, which interacts with the resident cells in milk. Binding of LPS by LBP/sCD14 induces a cellular response, which changes resident milk cell characteristics and elicits an inflammatory response resulting in changes in milk secretion. (**) Effect of parity is studied through intramammary challenge of primiparous cows (p. 125), (***) the modulatory effects of a variation in inoculum dose (p. 145), inhibition of prostaglandin synthesis (p. 167) and vaccination against the endotoxin (p. 189) on the inflammatory reaction (mild-moderate-severe) in primiparous cows are subsequently studied.

In this thesis, the effect of external bacterial contamination through milk sampling on several pre-infection milk related parameters was studied. Moreover, the inflammatory reaction kinetics of primiparous cows were evaluated in an intramammary *E. coli* challenge model and possible modulatory effects were studied. Specific objectives were:

1. to evaluate the effect of milk sampling technique on cellular response of resident cells in low SCC milk of high-yielding dairy cows.
2. to determine the influence of lactation number on the inflammatory reaction following intramammary *E. coli* challenge.
3. to study modulation of a moderate inflammatory reaction through a variation in inoculum dose, inhibition of prostaglandin synthesis and vaccination against the endotoxin in primiparous cows following intramammary *E. coli* challenge.

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# VALIDATION OF MILK SAMPLE COLLECTION UNDER ASEPTICAL CONDITIONS

*Based on:*

Vangroenweghe F., H. Dosogne, J. Mehrzad, and C. Burvenich. 2001. Effect of milk sampling techniques on milk composition, bacterial contamination, viability and functions of resident cells in milk. Vet. Res. 32:565-579.

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# INTRODUCTION

Milk SCC is generally considered to be an important parameter for mastitis detection because inflammation of the mammary gland results in an influx of somatic cells, predominantly PMN, from the blood into the mammary gland (Burvenich et al., 1994). It is of importance that evaluation of animals, based on pre-infection milk parameters, can be performed under standardised conditions. Cows with low milk SCC have been suggested to be more susceptible to intramammary infection (Kehrli and Shuster, 1994). Although the important role of blood PMN in the pathogenesis of mastitis is well described, the contribution of resident milk cells to the intramammary defence mechanism is not well characterised. In healthy cows, the number, differential count and function of leukocytes within the mammary gland could contribute to the defence against invading pathogens. Both artificially induced (Vandeputte-Van Messom et al., 1993; Shuster et al., 1996) as well as naturally occurring (Nickerson et al., 1990) increased SCC have been shown to exert a protective effect against severe inflammatory response to intramammary infections.

Bacterial contamination during sampling could influence various cell functions and may interfere with the interpretation of physiological effects on cell function. Bacteria or yeast have been used in assays of phagocytosis, intracellular killing or oxidative burst of stimulated cells (Paape and Pearson, 1979; Saad and Hageltorn, 1985; Saad, 1987; Hallén-Sandgren et al., 1991; Stevens et al., 1991; Smits et al., 1997). Paape and Pearson (1979) studied the influence of the isolation procedure of PMN in the milk on the variation of phagocytosis. They reported that the variation in milk PMN isolation technique accounted for only 1.5% of the total variation. The variability between duplicate determinations was 4.4% of the total variation.

Not only milk cell function, but also the distribution of the different cell populations in milk has recently been considered a major point of interest. Although several flow cytometric differential leukocyte count methods for bovine milk have been described (Hageltorn and Saad, 1986; Östensson et al., 1988; Redelman et al., 1988; Saad and Östensson, 1990), these methods have predominantly been developed for high SCC milk samples. In addition, little attention has been paid to the standardisation of sample preparation procedures. Milk sample processing varies from the use of centrifuged whole milk samples (Redelman et al., 1988) to dilution with a hypotonic buffer (Hageltorn and Saad, 1986; Östensson et al., 1988); and the temperature of milk sample collection, storage and processing have never been described in detail. Flow cytometric identification of bovine milk cells based on forward scatter (FS) and side scatter (SSC) is difficult because phagocytosis of milk components may alter both size and intracellular

granularity and cellular debris may interfere with the scatter pattern of normal cells. Therefore, most flow cytometric differential milk leukocyte count techniques are based on fluorescent labelling with monoclonal antibodies against CD molecules or DNA labelling (Hageltorn and Saad, 1986; Kelly et al., 2000; Leitner et al., 2000; Pillai et al., 2001). The cells in milk are end-stage cells which have been activated by diapedesis and phagocytosis of milk fat and protein. Therefore, they are particularly sensitive to permeabilising agents, causing drastic alterations of the FS and SSC pattern.

The objectives of the present study are the validation of milk sample collection under aseptical conditions and the evaluation of the effect of bacterial contamination on pre-infection PMN functionality *in vitro*. Moreover, a differential leukocyte count method with minimal effects on milk cell morphology was developed in order to approach the original cellular composition of milk as close as possible. Therefore, alteration of cellular morphology was avoided by using a live cell permeable DNA labelling dye and optimising the collection, processing, storage and labelling conditions.

# MATERIALS AND METHODS

**Experimental Animals**

All cows were free of intramammary infection. Mean milk SCC from each quarter was determined to be < 2 x $10^5$ cells/ml. In part 1 of the study, 10 Holstein-Friesian cows were selected from the Ghent University dairy herd (Biocentrum Agri-Vet, Melle, Belgium). The parity of the cows ranged between 1 and 5. The cows were in early lactation (9 ± 3 days in lactation). In part 2 of the study, 13 healthy Holstein-Friesian cows were selected from the same dairy farm. For method development, cows (n = 6) in mid-lactation (150-210 days in lactation) were used. For method application, cows (n = 7) were in early lactation (22 ± 4 days in lactation). The parity of the cows ranged between 1 and 6.

**Milk Sampling**

In part 1 of the study, 3 different milk sampling techniques were compared: 1) a sterile teat cannula infusion apparatus (STER-SPL), 2) manual hand milking (MAN-SPL) and 3) quarter machine milking (CONT-SPL). Because of practical restrictions, the experiment was divided into 2 separate trials. In trial 1, STER-SPL was compared with MAN-SPL (n = 5). In trial 2, STER-SPL was compared with CONT-SPL (n = 5). In all three sampling techniques, the teat ends were thoroughly disinfected with 70% ethanol containing 0.5% chlorhexidin before sample collection. The same quarter was used to perform the different sampling techniques. During both trials, milk was first collected through STER-SPL, followed by one of both other techniques (MAN-SPL or CONT-SPL). For MAN-SPL, a volume of 1,000 ml was milked into a sterile pyrex container (volume 1,500 ml, diameter opening 50 mm) by manual hand milking. For STER-SPL, a sterile and pyrogen-free cannula (L 7 cm, Ø 2 mm; Me.Ve.Mat, Deinze, Belgium) was inserted into the teat canal, which was then connected to the free end of an infusion set attached to a 2,000 ml sterile collection bag (Uripac®; Vycon, Brussels, Belgium) (Fig. 1). For CONT-SPL, the same fraction of milk was collected using a quarter milking machine. All different milk samples were collected during morning milking at 0800 a.m.

**Transport of Milk Samples**

Milk samples were stored in a coolbox with active cooling immediately after sampling. The time interval between sampling and the arrival at the laboratory was kept as short as possible (max. 1 h).

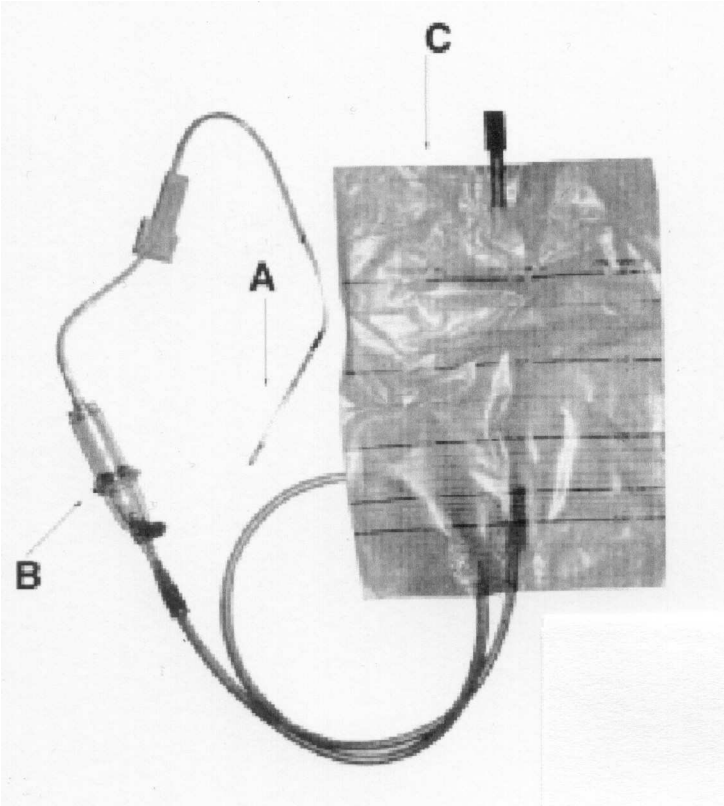


**Figure 1.** The sterile sampling technique devices, namely: (A) sterile and pyrogen-free cannula (Me.Ve.Mat, Deinze, Belgium), (B) sterile infusion set and (C) sterile collection bag (Uripac®; Vycon).

**Bacterial Contamination**

In part 1 of the study, bacteriological examination was carried out through plating of 10 µl onto a Columbia agar with 5% sheep blood (Biokar Diagnostics, Beauvais, France) with a Transferpette® (Brand GmbH, Wertheim, Germany). Subsequently, the milk was smeared out over the plate with a sterile plastic loop. Each sample was plated out in duplicate and incubated for 24 h at 37°C. Colony-forming units were counted using the plate counting method. Detection limit of the bacteriological method was 1 CFU per 20 µl or 50 CFU/ml milk.

**Somatic Cell Count and Milk Composition**

Somatic cell count was determined with a fluoro-opto electronic method (Fossomatic 5000 cell counter; Foss Electrics, Hillerϕd, Denmark). Milk fat, protein and lactose concentrations (mg/ml) were determined by mid-infrared photospectrometry (MilkoScan 4000; Foss Electrics). Glucose concentration (mg/l) was determined using an UV-method (D-Glucose; Roche Diagnostics, Brussels, Belgium) according to manufacturer's instructions.

**Isolation of Resident Milk Cells for Analysis of Milk Cell Characteristics and Functionality**

The isolation of milk cells was performed as described by Paape et al. (1976) with some modifications. Phosphate buffered saline (PBS) solution (25 l) was prepared as follows: 5 l of

stock solution (5.5842 g $KH_2PO_4$, 37.2438 g $Na_2HPO_4$, 40.9 g NaCl, distilled $H_2O$; pH adjusted to 7.5) was diluted to 25 l with distilled $H_2O$ and 180 g NaCl was added. The PBS solution was sterilised through filtration with a cellulose acetate filter (pore size 0.2 µm; Whatman, Maidstone, UK). Milk, diluted 2/1 with sterile PBS, was centrifuged (1,000 x *g*, 15 min, 4°C) and the cream was removed after the first centrifugation. The cell pellet was washed twice in PBS and the cells were finally resuspended in Hanks balanced salt solution (HBSS; Gibco Life Technologies, Paisley, UK) containing $Ca^{2+}$ and $Mg^{2+}$ supplemented with 0.1% bovine serum albumin (BSA, low endotoxin; Sigma Chemicals, St. Louis, MO, USA) and 25 mM HEPES buffer (Sigma).

During isolation, the cells were maintained on melting ice (1°C). Isolated cells were counted using an electronic particle counter (Coulter Counter Z2; Coulter Electronics Ltd., Luton, England) and viability was determined. The milk cells were resuspended to a concentration of 5 x $10^6$ viable milk cells/ml with HBSS containing $Ca^{2+}$ and $Mg^{2+}$ supplemented with 0.1% BSA and 25 mM HEPES buffer.

**Viability, Apoptosis and Differential Counts of Isolated Milk Cells**

Milk PMN viability was determined by propidium iodide (PI) exclusion. Viable PMN having an intact cell membrane, are impermeable to PI (PI-negative); whereas necrotic PMN having a deficient cell membrane, are permeable to PI (PI-positive). Ten µl of PI (50 µg/ml PBS) was added to the cell suspension (400 µl) and analysed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, San José, USA). The PMN were gated, based on their FS and SSC, in accordance with the settings found through flow cytometric analysis of pure PMN populations (Mehrzad et al., 2001) (Fig. 2).

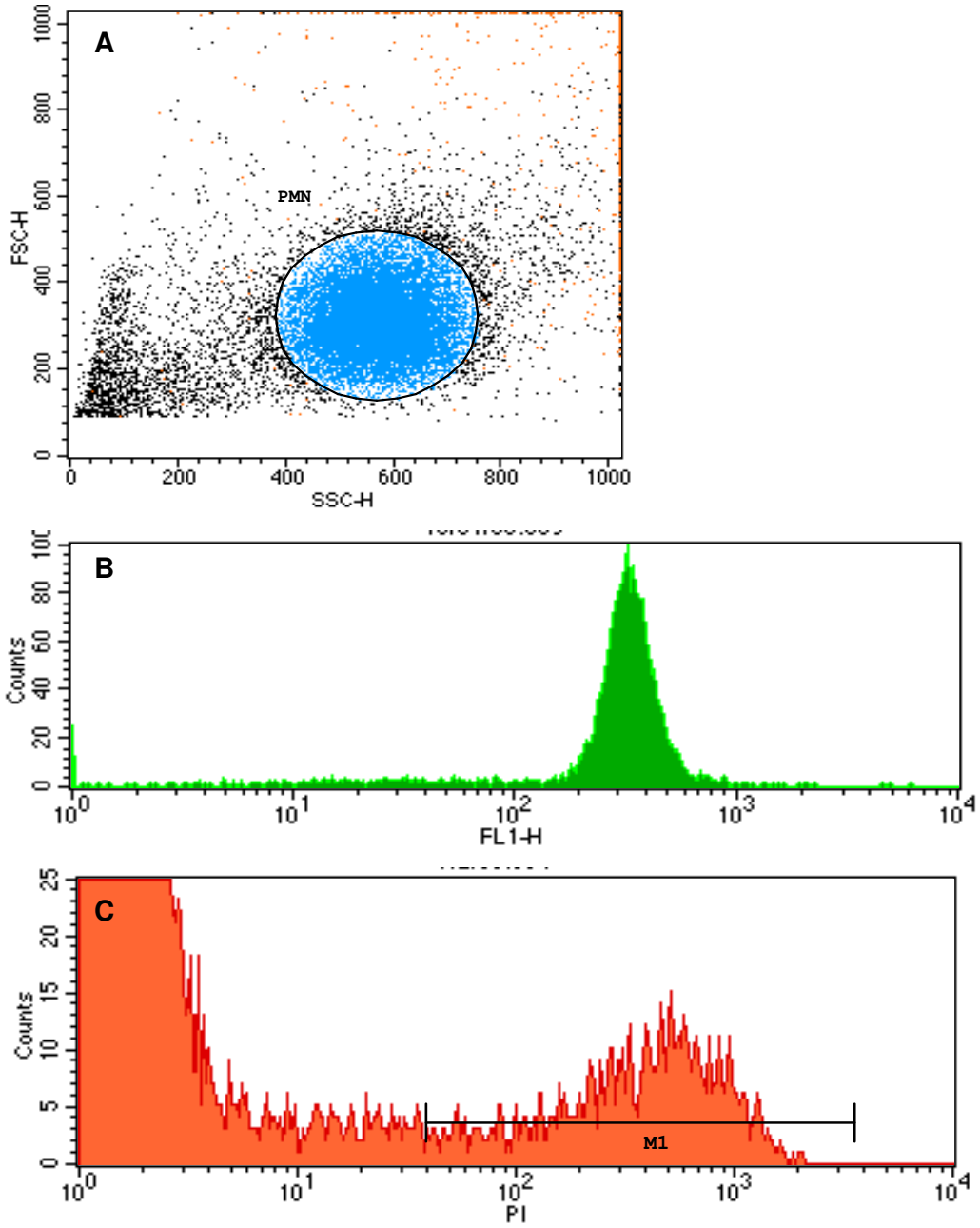


**Figure 2.** Flow cytometric analysis of isolated bovine milk PMN gated in the FS-SSC dot plot (A). Green fluorescence (FL-1) of PMN labelled with a monoclonal antibody specific against bovine granulocytes and with a secondary FITC-labelled antibody (B). Red fluorescence of PI-incubated PMN selectively gated in the FS-SSC dot plot (C). Gate M1 is applied to determine the percentage of dead PMN (for the quantification of the viability).

Milk PMN apoptosis was assessed by addition of annexin-V-FITC and PI according to Van Oostveldt et al. (1999) with slight modifications. Briefly, 50 µl of annexin-V-FITC/PI was added to the cell suspension and incubated for 10 min. After incubation, 300 µl of RPMI 1640 (Gibco Life Technologies) were added and apoptosis was quantified through flow cytometry

(FACScan). Polymorphonuclear leukocytes were again gated, based on their FS/SSC. Apoptotic PMN (programmed cell death) are also PI-negative (viable), but can be distinguished within the viable PMN population by their expression of phospholipid phosphatidyl-serine, binding with annexin-V-FITC, on their outer cell membrane (Fig. 3).

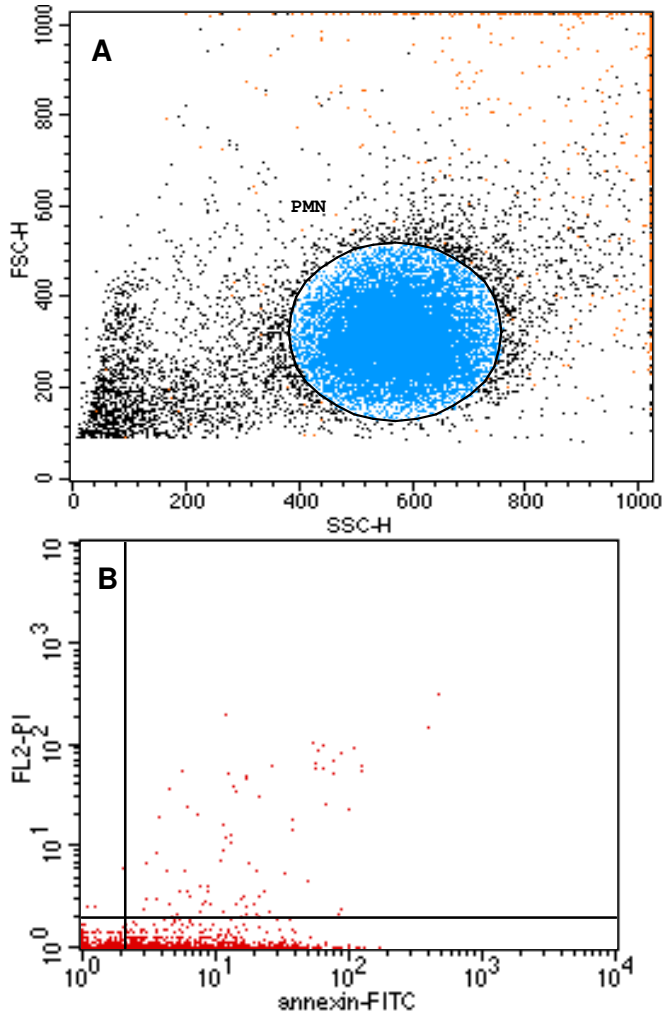


**Figure 3.** Flow cytometric analysis of isolated bovine milk PMN gated in the FS-SSC dot plot (A). Green fluorescence (FL-1) of annexin-V-FITC in the X-axis and red fluorescence (FL-2) from PI in the Y-axis of the flow cytometric analysis program (B). Cells in the lower left quadrant are negative for both fluorescent markers and are classified as living PMN. Cells in the lower right quadrant are positive for annexin-V-FITC (apoptosis marker), though negative for PI and are classified as apoptotic. Cells in the upper right quadrant are positive for both annexin-V-FITC and PI and are classified as dead (necrotic).

Differential counts of isolated milk cells were carried out as described by Dulin et al. (1988). Two hundred cells were counted on each of the smears and the percentages of lymphocytes-monocytes (LM), macrophages (MΦ) and PMN were determined. These data were used to correct intracellular killing and CL for the percentage of PMN in the isolated milk cells.

**Neutrophil Function Tests**

Phagocytosis and intracellular killing of *Staphylococcus aureus* Newbould 305 was evaluated in a bacteriological assay according to Barta (1993) and Barrio et al. (2000) with few modifications for milk. 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: 500 µl isolated cells (5 x $10^6$ viable cells/ml), 100 µl *S. aureus* Newbould 305 suspension (1 x $10^8$ CFU/ml) and 400 µl pooled bovine serum diluted to a final concentration (v/v) of 5% (complement-inactivated, 56°C, 30 min). Control samples contained bacteria, HBSS and serum without PMN. The bacteria to PMN ratio was between 4:1 and 5:1.

The results from the bacteriological assay were expressed as the percentages of killed (% killing) and phagocytosed (% phagocytosis) bacteria and were corrected for the percentage of PMN and MΦ in the samples of isolated milk cells as described by Dosogne et al. (2001).

Luminol-enhanced PMA (phorbol 12-myristate 13-acetate) –stimulated cellular CL of milk PMN was quantified on isolated milk cells as described by Mehrzad et al. (1999). Non-stimulated CL was compared with PMA-stimulated CL. The area under the curve (AUC) was calculated for the registrated impulse rate over a 30-min period. The CL response was corrected for 1,000 viable PMN.

**SYTO®13 Labelling**

A stock of SYTO®13 (5 mM, Molecular Probes, Eugene, USA), a live cell permeable DNA labelling dye, in DMSO was stored at −18°C and diluted 1:40 with RPMI 1640 (Gibco Life Technologies) immediately before labelling, according to the manufacturer's prescriptions. This corresponds with a final concentration of 200 nM in the samples. Two other dilutions (1:200 and 1:400; 40 and 20 nM final concentrations, respectively) were also evaluated with low SCC milk samples from 5 cows, but 1:40 was used for the standard procedure. Four hundred and ninety µl RPMI was added to the cell pellets (1 x $10^5$ – 4 x $10^6$ cells/ml), followed by 10 µl diluted SYTO®13 solution. This mixture was incubated during 0, 5, 10, 20 and 30 min. A 10-min incubation period was necessary for optimal labelling of all cells (Table 5) and was further used for the standard differential leukocyte count procedure.

**Flow Cytometry and Cell Sorting**

A flow cytometer (FACScan) equipped with an argon ion laser was used for measurement of differential leukocyte count. The excitation wavelength of the laser was 488 nm. The FS signal was amplified with a factor 1.98, whereas SSC and fluorescence signals were not amplified. The voltage applied on the SSC detector was 381 V. Forward scatter and SSC were

measured on a linear scale. Four hundred and thirty V was applied on the photomultipliers for both green and red fluorescence. Green fluorescence was measured using a 530-nm band-pass filter and registered on a log scale, whereas red fluorescence emission was measured using a 650 nm long-pass filter and also registered on a log scale. The data were analysed using the CellQuest® (Becton Dickinson) software. Flow cytometric differential leukocyte count of milk after SYTO®13 staining was quantified using the SSC - green fluorescence (FL-1) dot plot. Flow cytometric cell sorting of SYTO®13-labeled milk cells was performed using a FACSVantage cell sorter (Becton Dickinson).

**Confocal Laser Scanning Microscopy**

A lambda (wavelength) scan was performed on cytospin smears of SYTO®13-labeled milk cells using an inverted confocal laser scanning microscope (DMIRBE HCB fluoro microscope with a TCS SP2 confocal system; Leica Microsystems, Wetzlar, Germany). Four different regions of interest (ROI) were defined around 4 different cell types based on size and shape. The fluorescence emission intensity at wavelengths between 498 and 644 nm was registered for these 4 ROI's.

**Milk Sample Preparation for Differential Leukocyte Count**

All milk samples were gently mixed before dilution and further processing. For the flow cytometric cell sorting procedure, cells were isolated from milk according to Hoeben et al. (1997) and finally resuspended to a concentration of 5 x $10^6$/ml after counting with an electronic particle counter (Coulter Counter Z2). For the differential leukocyte count procedure developed in this study, 1.5 ml milk samples were layered on 3.5 ml PBS into 12 x 75 mm polystyrene flow cytometry tubes (Vel, Leuven, Belgium) and centrifuged during 10 min at 180 x *g*. Fat was removed, the supernatant was discarded and the tubes were kept upside down until they were carefully cleaned with a cotton swab to remove all fat adhering to the test tube wall. The pellet was then resuspended with 490 µl RPMI for flow cytometric differential leukocyte count or with 1 ml tryptic soy broth (TSB; Sigma Chemicals) for preparation of microscope slides.

Different dilution buffers were tested with low SCC milk samples from 6 cows: PBS; RPMI 1640 supplemented with 1% bovine serum albumin (Sigma) and 0.2% sodium azide (Sigma); and PBS supplemented with 0.1% paraformaldehyde (Sigma).

The following dilutions of milk samples were evaluated: 0.5, 1.0, 1.5 and 2.0 ml milk + 4.5, 4.0, 3.5 and 3 ml PBS, respectively, using low SCC milk samples from 6 cows. In order to evaluate the effect of temperature on the differential leukocyte count of milk, low SCC milk samples from 6 cows were maintained at 4, 20 and 37°C from immediately after collection, over

transportation and processing until flow cytometric analysis. The optimal dilution (1.5 ml milk with 3.5 ml PBS) and transportation and processing temperature (20°C) were further used for validation of the standard differential leukocyte count procedure.

**Variability of Differential Leukocyte Count Method**

The variability of the flow cytometric measurement of differential leukocyte count was tested by performing 6 repeated measurements on one sample. Variability of the complete differential cell count method was evaluated by performing 5 repetitions of the identification procedure for low SCC milk samples from 6 different cows. Significant differences in identification between the milk samples were evaluated for 2, 3, 4 and 5 repeated differential cell counts. The variability of the method was also evaluated at 500; 1,000; 2,000 and 3,000 analysed cells, with 6 repeated measurements for each number of cells analysed in a single sample. In the standard procedure, 3,000 cells per sample were analysed (Table 5).

**Preparation of Microscope Slides and Comparison Between Flow Cytometric and Microscopic Differential Leukocyte Count**

A described procedure for preparing microscope slides was used (Dulin et al., 1982). Slides were prepared in a cytocentrifuge (Shandon, Astmoor, UK) using 200 µl cell suspension with a concentration between 1 and 5 x $10^5$ cells/ml. The suspensions were centrifuged during 10 min at 113 x *g*. The smears were stained with May-Grünwald's eosin-methylene blue (Merck Diagnostica, Darmstadt, Germany). At least 200 cells were identified per sample, at a magnification of 1,000. Polymorphonuclear leukocytes were identified based on their multi-lobed dark purple stained nucleus and lightly blue stained cytoplasm. Polymorphonuclear leukocytes were characterised for apoptosis based on the morphologic features of pycnotic nuclei. Mature MΦ were large, with a light purple stained round nucleus and a vacuolated cytoplasm. Milk LM had the same appearance as their counterparts in the blood. Epithelial cells were large, mostly without nucleus, irregularly shaped and homogeneously light blue stained. For comparison of the flow cytometric differential leukocyte count method with microscopy, low SCC milk samples from 7 cows in early lactation were prepared in duplicate using the sample preparation method described above. One sample was used for flow cytometry and from the other one, slides were prepared in duplicate. Correlation between the % LM, PMN and mature MΦ obtained with the 2 methods was calculated.

**Statistical Analysis**

In part 1, differences between sampling techniques for SCC, milk composition, bacterial contamination, viability, phagocytosis, intracellular killing, PMA-stimulated and non-stimulated CL were evaluated by least squares ANOVA with Bonferroni's correction (Statistix®, Analytical Software, Tallahassee, FL, USA). Significant differences were determined at $P < 0.05$*, $P < 0.01$**, and $P < 0.001$***.

In part 2, the effects of sample dilution, temperature and storage time at 4°C were evaluated using ANOVA. For evaluation of the method variability, the results were subjected to a Wilk Shapiro rankit plot to examine the normality of the distribution of repeated differential cell count analyses.

# RESULTS

### Somatic Cell Count and Milk Composition

No significant difference in SCC, fat, protein, lactose and glucose content was found between STER-SPL and MAN-SPL, nor between STER-SPL and CONT-SPL milk sampling (Table 1).

**Table 1.** SCC and milk composition of STER-SPL *vs.* MAN-SPL and STER-SPL *vs.* CONT-SPL milk samples during early lactation. Data are means (± SEM).

| | **Milk Sampling Technique** | | |
|---|---|---|---|
| | *STER-SPL* | *MAN-SPL* | *CONT-SPL* |
| SCC ($log_{10}$/ml) | 4.4 ± 4.1 | 4.5 ± 3.9 | |
| | 4.4 ± 4.0 | | 4.9 ± 4.7 |
| FAT (mg/ml) | 24.2 ± 5.2 | 22.3 ± 4.6 | |
| | 25.5 ± 5.8 | | 42.8 ± 7.0 |
| PROTEIN (mg/ml) | 39.5 ± 2.6 | 40.1 ± 2.8 | |
| | 39.0 ± 3.7 | | 37.3 ± 3.6 |
| LACTOSE (mg/ml) | 49.3 ± 1.5 | 50.1 ± 1.6 | |
| | 49.3 ± 2.1 | | 46.2 ± 2.7 |
| GLUCOSE (mg/l) | 93.6 ± 11.2 | 102.0 ± 0.8 | |
| | 106.9 ± 12.9 | | 94.0 ± 9.7 |

### Bacterial Contamination

Bacterial contamination was not significantly different between STER-SPL and MAN-SPL milk sampling, whereas it was significantly ($P < 0.001$) higher in CONT-SPL compared to STER-SPL milk sampling (Table 2).

**Table 2.** Bacterial contamination (CFU/ml) from STER-SPL *vs.* MAN-SPL and STER-SPL *vs.* CONT-SPL milk samples during early lactation. Data are means (± SEM). Significant difference: [a] STER-SPL *vs*. CONT-SPL ($P < 0.001$).

| | **Milk Sampling Technique** | | |
|---|---|---|---|
| | *STER-SPL* | *MAN-SPL* | *CONT-SPL* |
| bacterial count (CFU/ml) | 0 | 660 ± 413 | |
| | 0 [a] | | 6,620 ± 2,339 [a] |

**Resident Milk Cell Viability**

No significant difference in viability was found between STER-SPL and MAN-SPL, nor between STER-SPL and CONT-SPL milk sampling (Fig. 4). During early lactation, milk PMN viability was on average 38.6 ± 6.83 %.

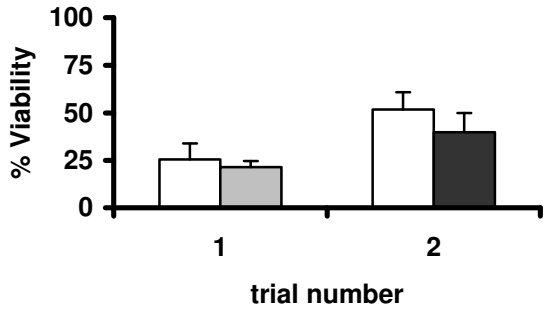


**Figure 4.** Viability of resident PMN: (a) trial 1: STER-SPL □ *vs.* MAN-SPL ▒ milk samples, and (b) trial 2: STER-SPL □ *vs.* CONT-SPL ■ milk samples during early lactation. Data (expressed as %) are means (± SEM).

**Phagocytosis and Intracellular Killing of *Staphylococcus aureus***

Neither of both PMN functionality tests were significantly influenced by the different milk sampling techniques (Fig. 5). Phagocytosis and intracellular killing during early lactation were on average 83.4 ± 2.63 % and 14.8 ± 7.33 %, respectively.

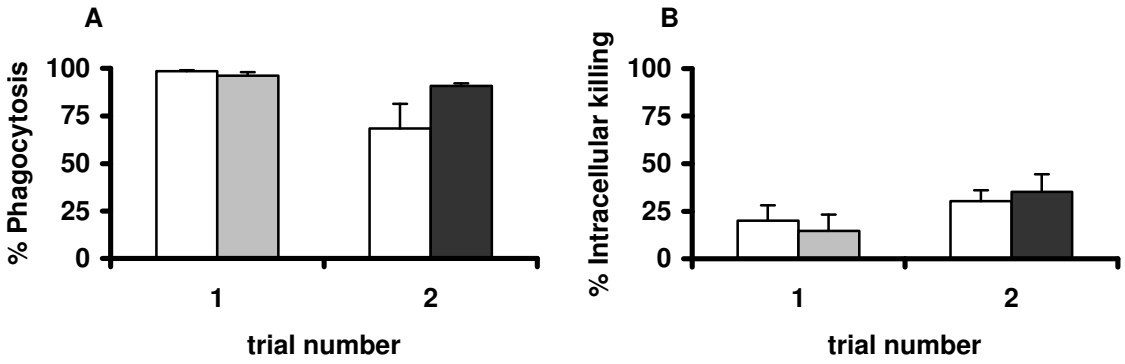


**Figure 5.** Phagocytosis (A) and intracellular killing (B) of *S. aureus*: (a) trial 1: STER-SPL □ *vs.* MAN-SPL ▒ milk samples, and (b) trial 2: STER-SPL □ *vs.* CONT-SPL ■ milk samples during early lactation. Data (expressed as %) are means (± SEM).

**Non-Stimulated and PMA-Stimulated Chemiluminescence**

Non-stimulated and PMA-stimulated CL were not significantly different between STER-SPL and MAN-SPL, nor between STER-SPL and CONT-SPL milk sampling (Fig. 6). During early lactation, non-stimulated and PMA-stimulated CL were 1,150 ± 220 AUC and 2,087 ± 351 AUC, respectively.

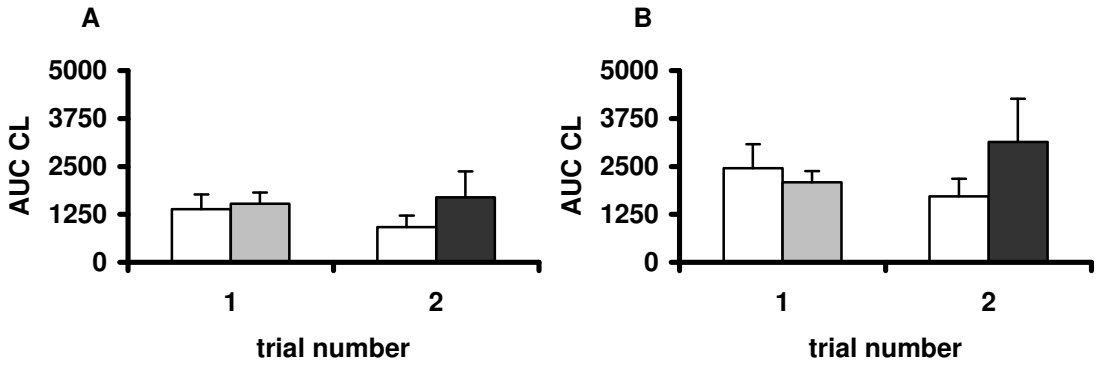


**Figure 6.** Non-stimulated chemiluminescence (A) and PMA-stimulated chemiluminescence (B): (a) trial 1: STER-SPL □ *vs.* MAN-SPL ▒ milk samples, and (b) trial 2: STER-SPL □ *vs.* CONT-SPL ■ milk samples during early lactation. Data (expressed as AUC) are means (± SEM).

**Flow Cytometry and Cell Sorting was Performed Based on Green Fluorescence (FL-1) and Side Scatter (SSC)**

Both red (FL-3) and green (FL-1) fluorescence emission of bovine milk cells was observed following SYTO®13 labelling. Because the best resolution between the populations was obtained with FL-1 and SSC, these parameters were used for the differential leukocyte count procedure. Red fluorescence was reduced at lower SYTO®13 concentrations, but this also resulted in decreased resolution in the green fluorescence (Table 3). In the FL-1 – SSC dot plot of isolated cells, 5 different leukocyte populations were identified (Fig. 7A). Following flow cytometric cell sorting of SYTO®13-labeled milk cells using the FL-1 – SSC dot plot (Fig. 7A), the different cell populations were identified as: 1) $SSC_{low}$ – $FL\text{-}1^{high}$: high FL-1 LM (R1); 2) $SSC_{low}$ – $FL\text{-}1_{intermediate}$: intermediate FL-1 LM (R1); 3) $SSC_{low}$ – $FL\text{-}1_{low}$: cells with apoptotic features (R2); 4) $SSC^{high}$ – $FL\text{-}1^{high}$: mature MΦ (R4) and 5) $SSC^{high}$ – $FL\text{-}1_{intermediate}$: PMN (R5) (Fig. 7B). Uptake of SYTO®13 did not occur immediately and differed between the cell types (Table 4). Saturation of the fluorescence intensity was obtained in most cell types after a 10-min incubation and remained nearly constant during 20 min. The highest green fluorescence intensity was observed in mature MΦ and viable PMN. Lower fluorescence intensity values were found in cells with apoptotic features, $FL\text{-}1_{low}$ LM and debris. For the standard procedure, a 10-min incubation time of cells with SYTO®13 was used.

**Table 3.** Green (FL-1) and red (FL-3) fluorescence emission intensity (mean fluorescence intensity) of different cell types at different SYTO®13 concentrations, measured by flow cytometry. Values are means (± SEM) of 5 cows.

| | **SYTO®13 concentration (nM)** | | | | | |
|---|---|---|---|---|---|---|
| | *20* | | *40* | | *200* | |
| | FL-1 | FL-3 | FL-1 | FL-3 | FL-1 | FL-3 |
| Lymphocytes | 20 ± 4 | 2 ± 0 | 53 ± 21 | 4 ± 1 | 176 ± 41 | 16 ± 3 |
| Macrophages | 286 ± 34 | 46 ± 24 | 1,816 ± 125 | 148 ± 34 | 2,825 ± 156 | 243 ± 28 |
| PMN | 37 ± 4 | 11 ± 8 | 378 ± 21 | 30 ± 3 | 479 ± 31 | 57 ± 5 |

**Table 4.** Effect of incubation time of bovine milk cells with SYTO®13 on green fluorescence emission intensity (mean fluorescence intensity). Values are the average of 3 repeated identification assays.

| | **Incubation time (min)** | | | | |
|---|---|---|---|---|---|
| | *0* | *5* | *10* | *20* | *30* |
| Lymphocytes-monocytes | 10 | 556 | 1,199 | 1,829 | 2,140 |
| Macrophages | 177 | 3,279 | 3,365 | 3,626 | 3,802 |
| PMN | 162 | 3,116 | 3,302 | 3,628 | 3,668 |
| Cells with apoptotic features | 4 | 158 | 410 | 831 | 1,056 |
| Debris | 24 | 132 | 140 | 147 | 155 |

**Confocal Laser Scanning Microscopy Could Confirm and Identify the Different Cell Types Sorted by Flow Cytometry**

Using a lambda scan of SYTO®13-labeled milk cells with confocal laser scanning microscopy, a different morphology could be associated with different fluorescence emission characteristics (Fig. 8A-B). The cells in ROI1 had an intermediate size, were round-shaped with a round nucleus and had a maximal fluorescence emission at 514 nm. This is consistent with the characteristics of LM. The cells in ROI2 were large, irregularly shaped and also had a maximal fluorescence emission at 514 nm but their fluorescence intensity was much higher than any of the other cell types. These cells conform to the characteristics of mature MΦ. The cells in ROI3 had a multi-lobed nucleus and a maximal but low fluorescence intensity at 498 nm, which are typical features of PMN. Finally, the cells in ROI4 had similar fluorescence characteristics as the cells in ROI3 but the fluorescence intensity was higher, especially in the higher emission wavelength region. These cells contain all characteristics of high FL-1 LM.

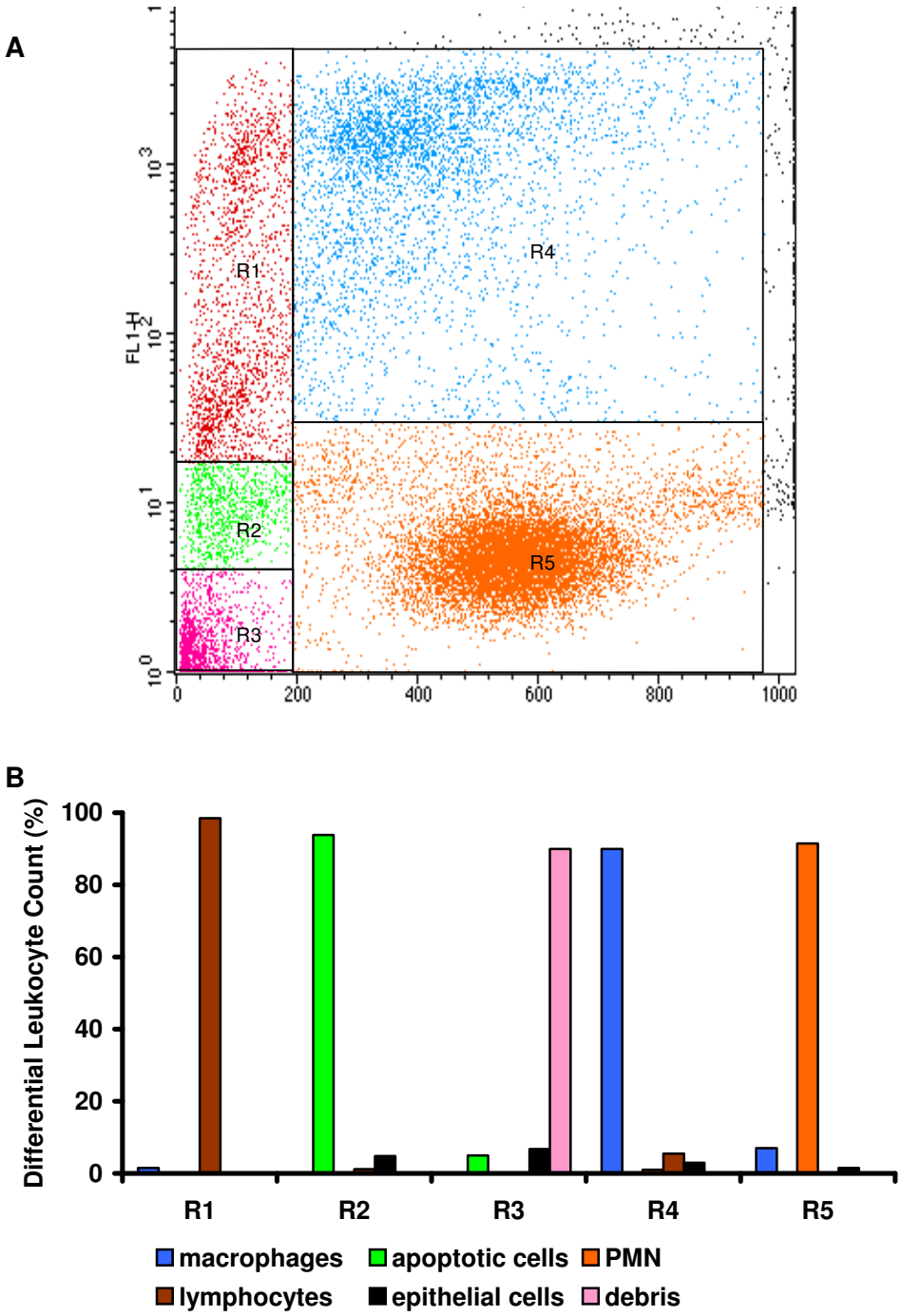


**Figure 7.** Flow cytometric analysis (A) of SYTO®13-labeled bovine milk cells: green fluorescence (FL-1) – side scatter (SSC) dot plot of isolated milk cells used for flow cytometric cell sorting and subsequent microscopic identification (B). R1: LM; R2: apoptotic cells; R3: cellular debris; R4: MΦ; and R5: PMN.

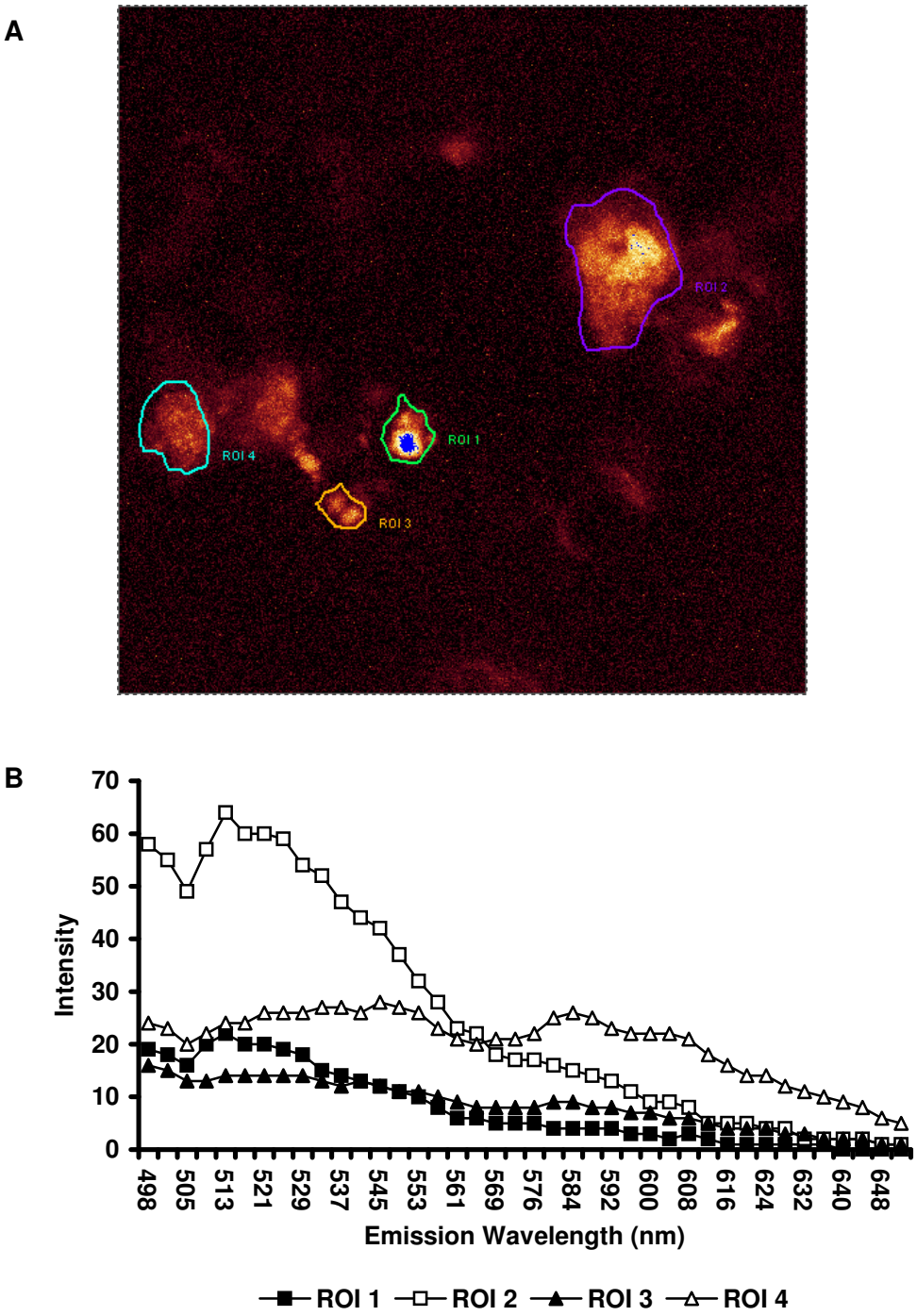


**Figure 8.** Confocal laser scanning microscopic analysis of fluorescence emission of different SYTO®13-labeled bovine milk cells using a wavelength scan. A) identification of different regions of interest (ROI); B) fluorescence emission characteristics of the different ROI's identified in A). ROI1: LM (high FL-1); ROI2: MΦ; ROI3: PMN; and ROI4: LM (low FL-1).

**Milk Sample Preparation and Effect of Storage**

Optimal conditions for milk sample preparation were the following: 1) milk sample dilution with PBS or RPMI-BSA; 2) 30% (v/v) dilution (i.e. 1.5 ml milk + 3.5 ml PBS) (Fig. 9); 3) a sample collection, transportation and processing temperature of 20°C (Fig. 10). Both the SCC and the differential leukocyte count remained constant throughout a 48-h storage period at 7°C (Fig. 11).

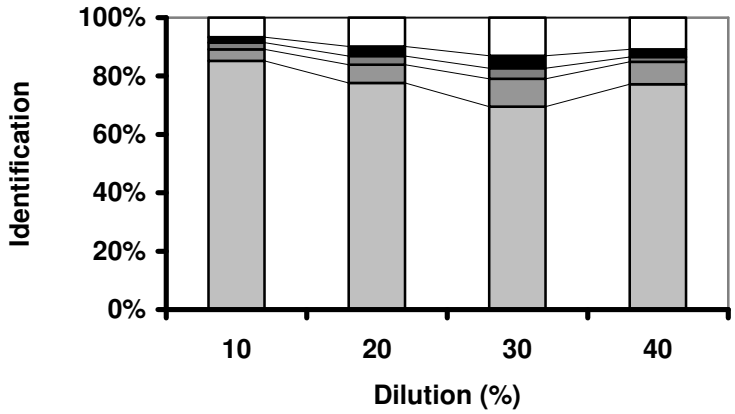


**Figure 9.** Effect of sample dilution on the differential leukocyte count of SYTO®13-labeled bovine milk cells in low SCC (SCC < 200,000 cells/ml) milk samples. The following dilutions were performed: 0.5, 1.0, 1.5, and 2.0 ml milk with 4.5, 4.0, 3.5 and 3.0 ml PBS, respectively. Average values for 6 cows are presented. Different cell types analysed from bottom to top are: debris, cells with apoptotic features, PMN, mature MΦ and LM.

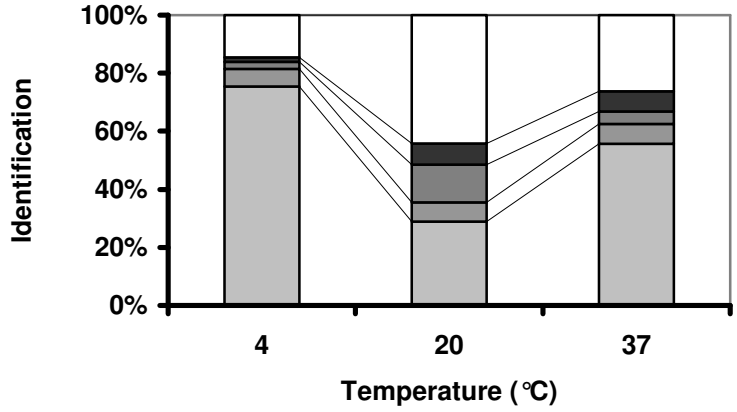


**Figure 10.** Effect of sample collection, transportation, processing and labelling temperature on the differential leukocyte count and on % debris. Average values of 6 cows are presented. Different cell types analysed from bottom to top are: debris, cells with apoptotic features, PMN, mature MΦ and LM.

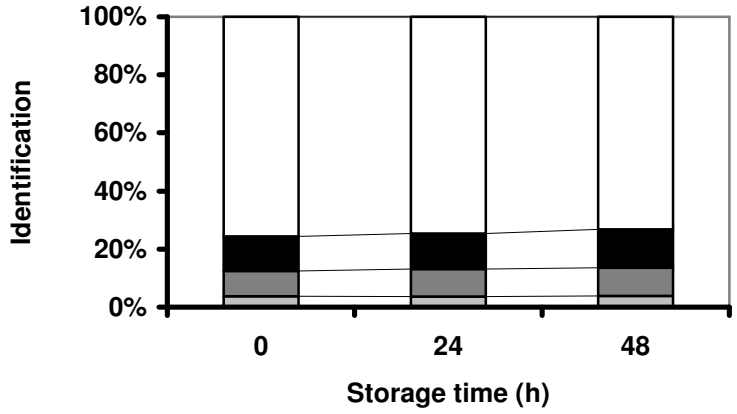


**Figure 11.** Effect of sample storage at 4°C during 24 and 48 h on the differential leukocyte count of bovine low SCC (SCC < 200,000 cells/ml) milk samples. Average values for 6 cows are presented. Different cell types analysed from bottom to top are: debris, PMN, mature MΦ and LM.

**Variability of the Differential Leukocyte Count Method**

The coefficient of variation for repeated measurements in identical cell suspensions was 1.5% for LM, 1.4% for mature MΦ, 0.7% for PMN and 0.8% for cells with apoptotic features. Average coefficients of variation for the complete differential leukocyte count method including sample dilution, resuspension and SYTO®13 labelling of milk samples from 6 cows were 2.5% for LM, 15.8% for mature MΦ, 11.8% for PMN and 10.7 % for cells with apoptotic features (Fig. 12). According to the Wilk-Shapiro rankit plot against the ordered data, which resulted in a straight line, the samples conformed to a normal distribution. Significant differences between the samples were obtained at $P < 0.05$ for duplicate differential leukocyte count assays and at $P < 0.01$ for 3 or more repeated assays. The highest variability between repeated measurements was observed with 500 analysed cells. The variability further decreased from 1,000 over 2,000 to 3,000 cells (Table 5). The average % cells in the different regions was not dependent on the number of analysed cells.

**Table 5.** Variability of the differential leukocyte count method at different numbers of analysed cells. Values are the average percentage of each cell type (± SEM) for 6 repeated measurements.

| | **Number of analysed cells** | | | |
|---|---|---|---|---|
| | *500* | *1,000* | *2,000* | *3,000* |
| Lymphocytes | 69 ± 14 | 68 ± 4 | 67 ± 3 | 67 ± 1 |
| Macrophages | 11 ± 3 | 11 ± 1 | 12 ± 1 | 12 ± 1 |
| PMN | 9 ± 3 | 9 ± 1 | 9 ± 1 | 9 ± 1 |
| Cells with apoptotic features | 11 ± 1 | 12 ± 1 | 12 ± 1 | 12 ± 1 |

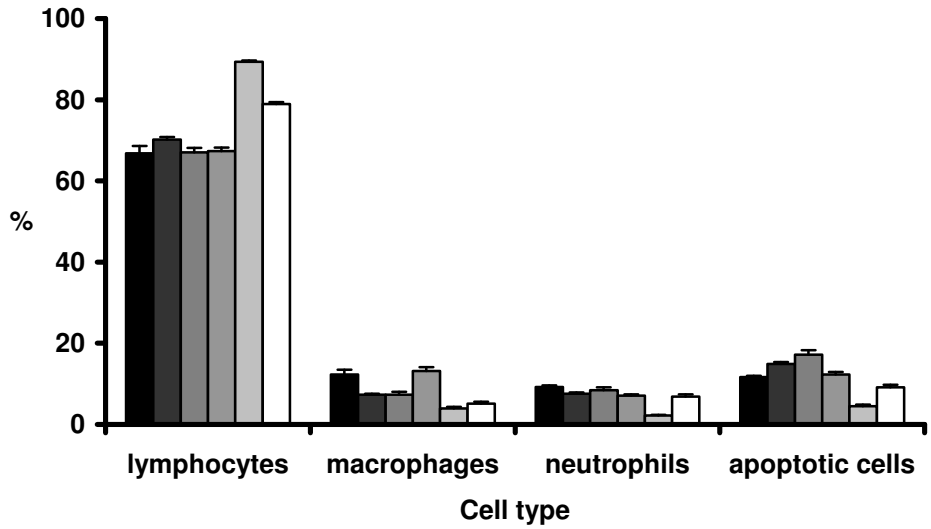


**Figure 12.** Triplicate differential leukocyte count of milk samples from 6 mid-lactating cows. The average coefficients of variation for the complete differential leukocyte count method including sample dilution, resuspension and SYTO®13 labelling of milk samples from these cows were 2.5 % for LM, 15.8 % for mature MΦ, 11.8 % for PMN and 10.7 % for cells with apoptotic features. Data (expressed as %) are means (± SEM).

**Correlation between Flow Cytometric and Microscopic Differential Leukocyte Count Methods**

For the LM population, a coefficient of correlation of 0.81 ($P < 0.05$) and for the PMN population, a coefficient of correlation of 0.90 ($P < 0.01$) was obtained between flow cytometric and microscopic differential leukocyte count. The coefficient of correlation for the mature MΦ count was 0.71 (not significant) between the 2 methods. For LM, flow cytometric results were systematically higher and for PMN and mature MΦ, flow cytometric results were systematically lower than microscopic results. During early lactation, milk SCC was composed of 76.7 ± 2.45% LM, 5.2 ± 1.31% MΦ, 13.1 ± 1.62% PMN and 4.9 ± 1.09% apoptotic cells.

# DISCUSSION

In the present study, milk sample collection was validated and the effect of external bacterial contamination on viability and functions of milk cells was studied in healthy mammary glands with low SCC. Therefore, normal milk was obtained without stimulation of the mammary gland and all milk samples had low SCC (< 2 x $10^5$ cells/ml). In previous studies, PMN recruitment into the mammary gland was stimulated by either the insertion of an intramammary device (Paape et al., 1976; Saad and Hageltorn, 1985), or by intramammary infusion of *E. coli* endotoxin (Dulin et al., 1988).

A new sterile milk sampling technique (STER-SPL) was introduced as reference technique, which allowed milk sample collection under aseptical conditions without external bacterial contamination. Although manual milk sampling (MAN-SPL) could increase the risk of external contamination, bacterial counts observed with this sampling technique were unexpectedly low. In this milk, no change in viability and different cell functions of isolated milk cells was observed. In contrast, machine milking (CONT-SPL) had a significantly higher contamination level as compared to STER-SPL. Despite this bacterial contamination, no significant differences in viability and different cell functions of isolated milk cells were found. It seems that the bacterial contamination present in CONT-SPL milk samples could not significantly activate the isolated resident milk cells. However, it is important to mention that the whole study was performed on milk, which was slowly cooled down to 1°C. Under these conditions, cell functions are inhibited and cells do not react when challenged by these contaminating bacteria. Nevertheless, milk cells did not loose their reaction potential, since they performed CL, intracellular killing and phagocytosis when collected and maintained under the specified conditions. It can be concluded that all three milk sampling techniques are equally appropriate for the study of cellular and non-cellular qualities of bovine milk and resident cells. The reference technique (sterile cannula) is the best for eliminating bacterial contamination, although for practical routine milk sample collection manual milk sampling (MAN-SPL) can be used.

In the present study, a simple, accurate and reproducible flow cytometric differential leukocyte count method for low SCC milk from healthy cows was developed. The method is complementary to flow cytometric differential leukocyte count methods for high SCC milk (Redelman et al., 1988; Pillai et al., 2001). The low amount of cells in milk from healthy cows (< $10^5$ cells/ml; Laevens et al., 1997), makes the identification of leukocytes more difficult than in high SCC milk ($10^9$ cells/ml during coliform mastitis; Dosogne et al., 1997). A maximal amount

of identifiable cells in low SCC milk was obtained with 1) a 30 % (v/v) dilution of milk samples (which corresponds with large-scale isolations; Hoeben et al., 1997); 2) centrifugation and removal of the fat layer; 3) resuspension of the pellet with RPMI 1640; 4) a sample collection, storage and processing temperature of 20 °C. Although the initial SCC of the milk samples was low, a sufficient amount of cells was obtained from 1.5 ml samples. Thus, our method is applicable for small volumes of low SCC milk samples. Another advantage of the method described here is that milk samples can be stored for 2 days at 7°C without any effect on the differential leukocyte count. The method was also shown to be reproducible, even at a very low number of cells analysed: variability was not significantly different between 1,000; 2,000 or 3,000 analysed cells and the average values for differential milk leukocyte count were not dependent on the number of analysed cells.

Four populations with different fluorescence emission characteristics were obtained upon SYTO®13 staining of milk leukocytes. The high fluorescence of mature MΦ is consistent with previous results obtained with acridine orange labelling (Hageltorn and Saad, 1986) and could be explained by a higher degree of mitochondrial RNA staining. Indeed, green fluorescence obtained with the SYTO® dyes is not only governed by stoichiometric DNA binding, but also by cytoplasmic mitochondrial labelling (Poot et al., 1997). The emission wavelength maximum of SYTO®13-labeled RNA (514 nm) is similar to DNA (509 nm) (Molecular probes, SYTO® green fluorescent nucleic acid stains product information sheet). Mitochondrial labelling could also explain the high green fluorescence of some LM and is indicative of a high metabolic rate. Polymorphonuclear leukocytes and LM had similar fluorescence characteristics but could be distinguished on the basis of cytoplasmic granularity, which was typically higher for PMN than for LM.

# CONCLUSION

Following validation of milk sample collection under aseptical conditions, no effect of limited bacterial contamination on PMN viability and functions could be observed. Manual milk sample collection will be preferred for further use during experimental challenge, because the technique results in a small bacterial contamination and is easy to perform during repeated sample collection. During early lactation, pre-infection milk SCC is mainly composed of lymphocytes, whereas only small percentages of monocytes, PMN and apoptotic cells are present.

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# MATERIALS AND METHODS EXPERIMENTAL INFECTIONS

## EXPERIMENTAL ANIMALS AND STUDY FACILITIES

The Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, Merelbeke, Belgium) approved all experimental infections. All primiparous cows (n = 74) were between 24 and 30 months of age at calving. Depending on the study design, cows arrived at the commercial dairy farm (Oudenaarde, Belgium) in their 7th month of pregnancy (n = 33) or within 7 days after calving (n = 41). The animals were on a system of zero-grazing from arrival until the end of the inoculation trial and were fed twice daily at 0700 a.m. and 1700 p.m. The ration consisted of corn silage, good quality hay and water *ad libitum*. Concentrates (Sandilac™; Dumoulin Voeders Sanders, Moorslede, Belgium) were distributed according to milk production.

For inclusion into the intramammary inoculation trial, treatment of clinical diseases (retentio secundinarum, mastitis, metritis, …) was not allowed within 10 d before the intramammary inoculation. Therefore, only healthy animals, free of major mastitis pathogens through 3 consecutive bacteriologically negative examinations were included for the intramammary *E. coli* challenge (IDF, 1971; Hillerton, 1999). Primiparous cows accepted for the intramammary challenge were inoculated between 12 and 28 d post-parturition and had a quarter milk SCC of 57,145 ± 16,478 cells/ml before infection.

Milking was performed daily at 0800 a.m. and 1800 p.m. using a quarter milking device (Packo & Fullwood, Zedelgem, Belgium). Daily quarter milk production, the yield of the evening and subsequent morning milking, expressed as l/d, was measured at d-7, d-4, d-1, d0 (post-infusion hour (PIH) 0-24), d+1 (PIH 24-48), d+2 (PIH 48-72), d+3 (PIH 72-96), d+6 (PIH 144-168), d+9 (PIH 216-240) and d+13 (PIH 312-336) (Fig. 1). Before infection, cows (n = 74) had an average milk production of 15.9 ± 0.38 l/d.

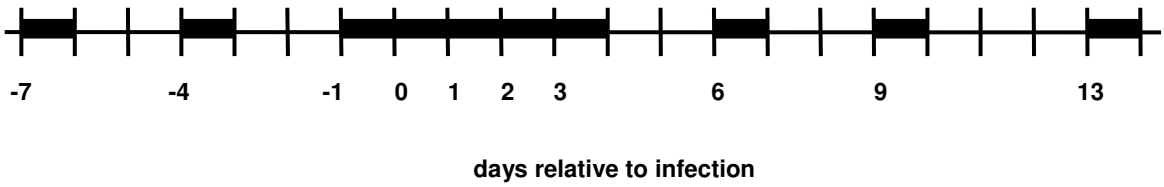


**Figure 1.** Time schedule of registration points for daily quarter milk production before, during and after experimentally induced *E. coli* mastitis in primiparous cows.

# INOCULATION DOSE

The generation time of *E. coli* in mammary secretions can be as short as 20 min (Petsch and Anspach, 2000; Burvenich et al., 2003). Because we were interested in bacterial elimination rather than bacterial growth in the affected mammary glands, a high inoculum dose was used in all the experiments. Practically, two different high inoculum doses were applied, depending on the experiment: 1 x $10^4$ and 1 x $10^6$ CFU.

During *in vitro* bacterial growth in a batch culture system, in which nothing is added to or removed from the environment once the medium is inoculated with the living cells, several distinct phases can be identified (Fig. 2). Due to the closed nature of a batch culture system, cell multiplication can only be supported for a limited time and during bacterial growth, progressive changes in the original growth medium occur.

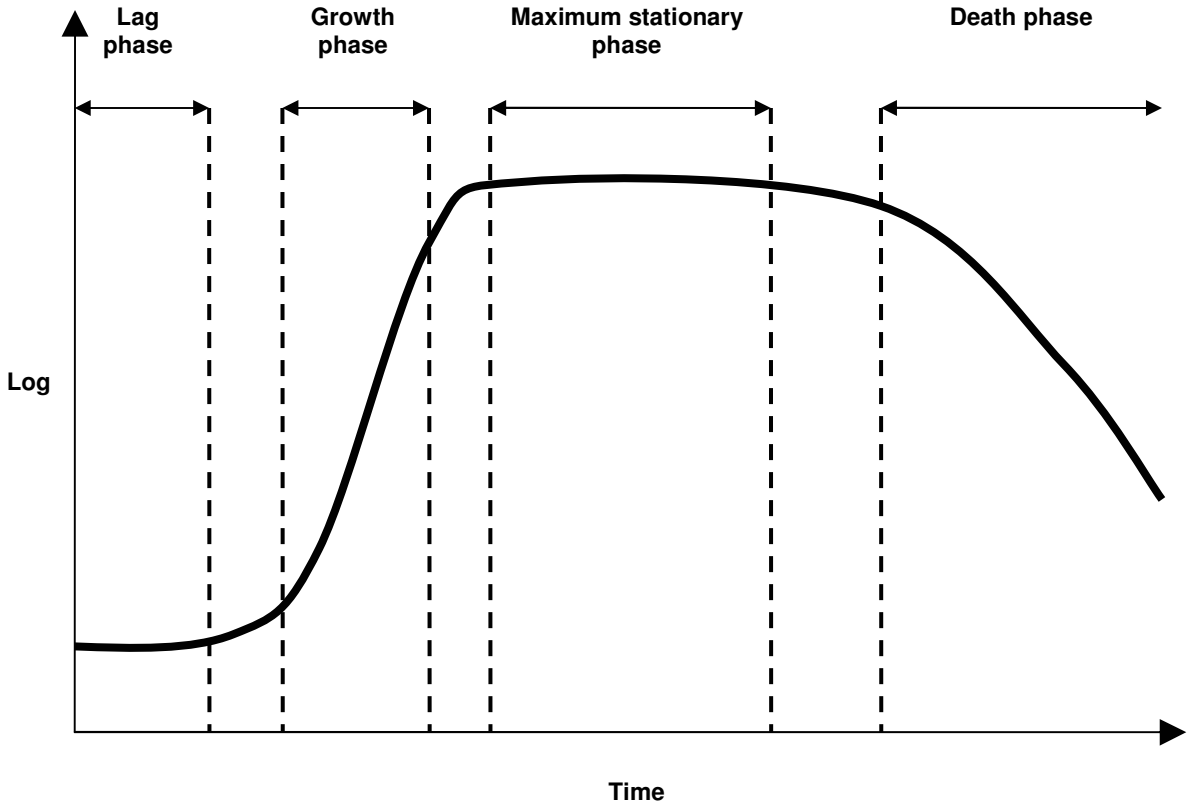


**Figure 2.** Idealised normal growth cycle for a bacterial population (expressed as $\log_{10}$ of the number of viable bacteria) in a batch culture system. Time is expressed in relative units.

Growth proceeds through a lag phase, during which cell numbers do not increase. This is followed by a growth phase, which is usually characterised by an exponential increase in

bacterial numbers. Ultimately, changes in the chemical and/or physical environment of the medium result in a phase of no net increase in bacterial numbers, the maximum stationary phase. Cells in stationary phase still require an energy source for viability maintenance. By definition, the availability of an energy source is limited in a batch culture, and hence a death phase follows, often characterised by an exponential decrease in the number of living cells.

Following overnight culture, bacteria harvested for intramammary challenge were in maximum stationary phase at the time of inoculation. Although a change in growth medium (i.e. healthy mammary gland) should enhance their growth, it is clear from several previous experiments that intramammary bacterial kinetics are variable depending on the initial inoculum dose. When a low number of bacteria is infused, an exponential growth may occur (Shuster et al., 1996), which is followed by subsequent clearance of the bacteria after peak numbers are reached (Fig. 3a). In contrast, stationary phase is immediately reached when a high number of bacteria is inoculated (Hoeben et al., 2000a), which is followed by clearance of bacteria (Fig. 3b).

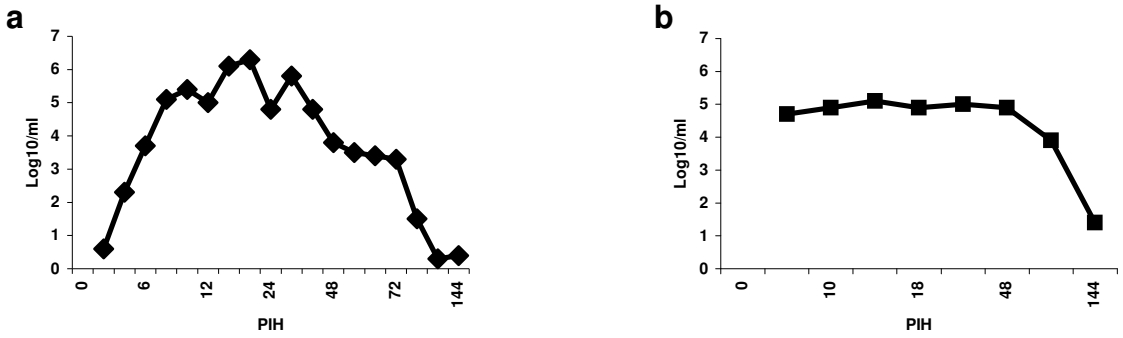


**Figure 3.** Intramammary growth of inoculated *E. coli* bacteria starting from a) a low inoculum dose (~ 30 CFU; Shuster et al., 1996) or b) a high inoculum dose (~ 10,000 CFU; Hoeben et al., 2000a).

## INTRAMAMMARY INOCULATION PROCEDURE

Inoculation was performed as described before (Hoeben et al., 2000a; 2000b). Briefly, *E. coli* P4:O32 (H37, β-glucuronidase +, haemolysin -), maintained as a stock in lyophilisation medium at –20°C, was subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, USA) at 37°C during 3 consecutive days and subsequently washed 3 times with pyrogen-free PBS, and resuspended in PBS. Just before inoculation, the suspension was diluted in pyrogen-free PBS to a final concentration of 1 x $10^4$ CFU/ml or 1 x $10^6$ CFU/ml, depending on the inoculum dose required in each specific study. On d0, 30 min after morning milking (1.5 h after feeding), the

cows were inoculated in the left front and rear quarters with a total volume of 10 ml, containing 1 ml of inoculum and 9 ml of pyrogen-free saline solution (NaCl 0.9%; Baxter N.V., Lessines, Belgium) per quarter. All bacterial suspensions were infused into the teat cistern using a sterile, pyrogen-free teat cannula (L 7 cm, Ø 2 mm; Me.Ve.Mat, Deinze, Belgium). Before challenge, the teat ends were disinfected with 70% ethanol containing 0.5% chlorhexidin. After infusion, the bacterial suspension was thoroughly distributed into the udder cistern through a 30 s massage (Fig. 4).

Following inoculation, a control sample of the inoculum was diluted, plated out on Columbia agar with 5% sheep blood (Biokar Diagnostics, Beauvois, France) and incubated for 24 h at 37°C to check for correct inoculum preparation and adequate inoculum dose administration at intramammary *E. coli* challenge.

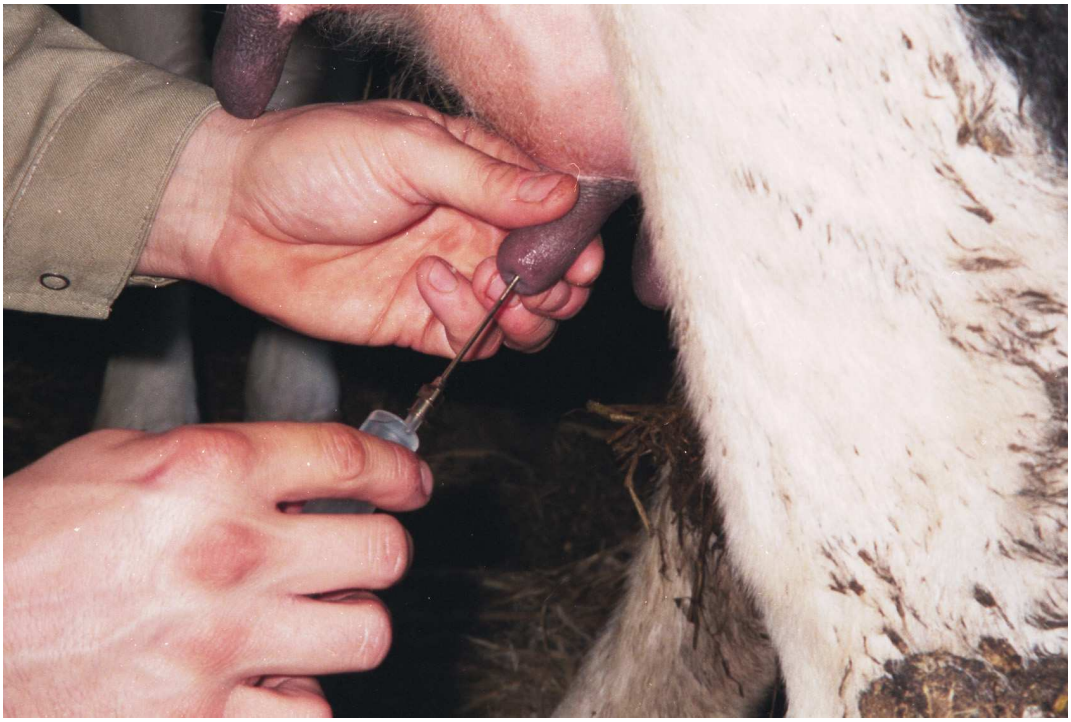

**Figure 4.** Intramammary inoculation in the left rear quarter (picture of MMRC, Merelbeke, Belgium).

## EXPERIMENTAL DESIGN

To study the influence of lactation number on severity of inflammation and kinetic response of the innate immunity, two different high inoculum doses were used, namely 1 x $10^4$ and 1 x $10^6$ CFU per quarter.

For modulation of moderate inflammatory reaction through variation of the inoculum dose, both previously used doses were randomly assigned to animals during the experimental trial. Modulation of moderate inflammatory reaction through inhibition of prostaglandin synthesis was performed following inoculation of 1 x $10^4$ CFU in the two left quarters of all animals. In the study on effect of vaccination against the endotoxin, 6 animals were inoculated with 1 x $10^4$ CFU, the others (n = 17) received 1 x $10^6$ CFU in both left quarters.

## TREATMENT PROTOCOLS

The modulation of moderate inflammatory reaction in primiparous cows has been performed through variation of the inoculum dose, inhibition of prostaglandin synthesis and vaccination against the endotoxin.

In the first study, no specific treatments were performed following the random assignment of the two inoculum doses to the experimental animals. In this study, clinical response in animals receiving the usual inoculum dose of 1 x $10^4$ CFU was compared to animals inoculated with a higher dose of 1 x $10^6$ CFU (study p. 145).

In the second study, the inducible COX-2 enzyme was inhibited using a NSAID, carprofen, administered at PIH 9, when acute clinical symptoms were already present. Carprofen (1.4 mg/kg, Rimadyl™; Pfizer Animal Health, Sandwich, UK) was administered according to body weight (2.9 ml/100 kg). Control animals received saline solution (0.9% NaCl; Baxter N.V., Lessines, Belgium) according to body weight (2.9 ml/100 kg) (study p. 167)

In the third study, vaccination against the endotoxin was performed following a tight vaccination schedule. Animals received three 2 ml doses and each animal received the same treatment (vaccine or saline) for all three injections. The first dose was administered at 56 (±7) d prior to expected calving, followed by a second dose at 28 d after the first vaccination. The third dose was administered at 7 (±7) d post-parturition. The active ingredients of the J5 vaccine (Enviracor™; Pfizer Animal Health, Sandwich, UK) were the *E. coli* J5 mutant, mixed with a proprietary adjuvant. Controls received saline solution (0.9% NaCl; Tomlinson Ltd., Corby, UK). The vaccine and saline were injected subcutaneously behind the shoulder and one hand's width away from it. The first injection for all animals was made on the left side, the second injection on the right side and the third again on the left side (study p. 189).

## SAMPLING PROCEDURE

Blood and milk samples were collected at d-7, d-4, d-1, d0, d+1, d+2, d+3, d+6, d+9, and d+13 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21 (Fig. 5).

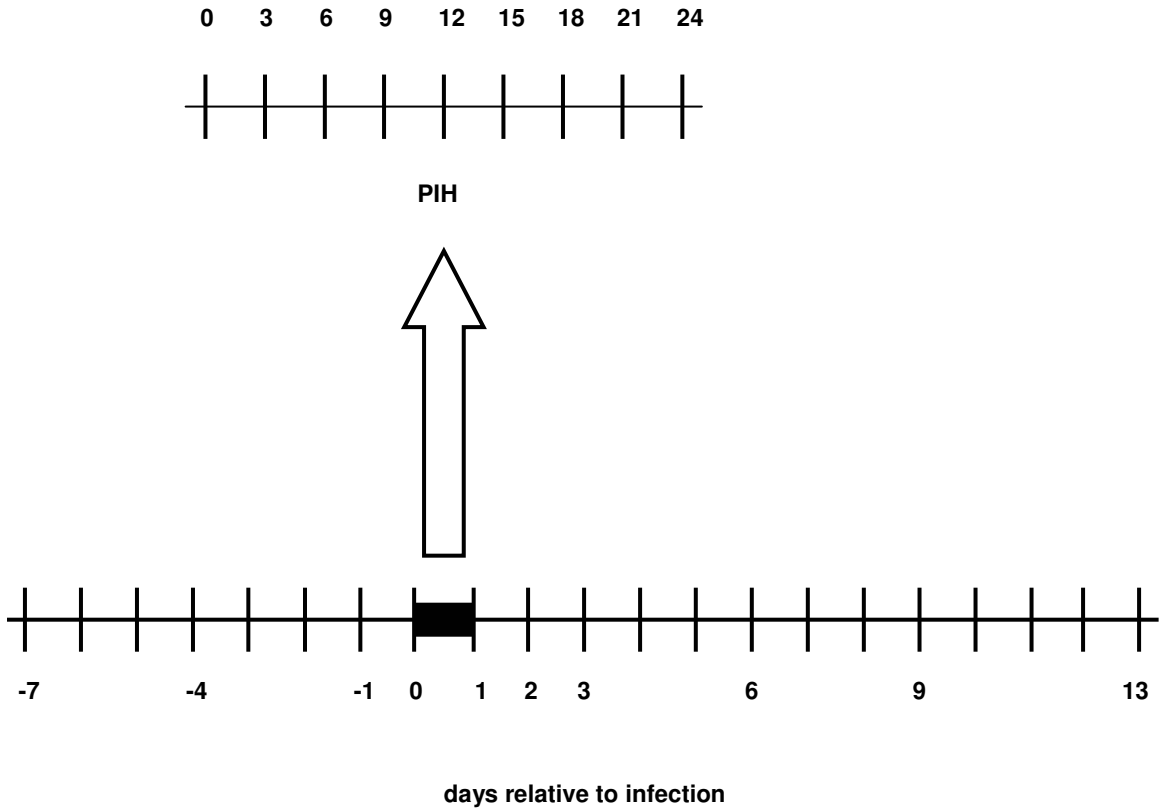


**Figure 5.** Schedule for blood and milk sampling before, during and after experimentally induced *E. coli* mastitis in primiparous cows.

Blood samples were drawn aseptically from the external jugular vein of each cow by venipuncture in evacuated tubes (Table 1).

Foremilk (5 ml) was aseptically collected for diagnostic bacteriology on major pathogens before inoculation and for quantification of the population of *E. coli* (CFU) from PIH 3 onward. Furthermore, milk samples (100 ml) were collected manually, using a procedure which was validated previously, for determination of SCC, milk composition and the preparation of milk whey through centrifugation. All samples were kept on melting ice (1°C) during transport and at the laboratory until analysis was performed or aliquots were prepared for freezing (–20°C).

**Table 1.** Evacuated tubes for venous blood collection and subsequent analytical possibilities.

| Type | Evacuated tube | Analysis |
|---|---|---|
| Unclotted blood - plasma | BD vacutainer LH 143 I.U | Blood leukocyte count<br>Differentiation blood smears<br>Packed cell volume<br>$PGE_2$<br>$TXB_2$ |
| Clotted blood - serum | BD vacutainer SST | LBP<br>BHBA |
| Unclotted blood - NaF | BD vacutainer FX 12.5 mg 10 mg | Glucose |

# CLINICAL EXAMINATION

Classical clinical parameters were examined. Rectal temperature (RT), heart rate (HR), respiration rate (RR), reticulorumen motility, skin turgor, fecal appearance, appetite, general attitude, body condition score (Edmondson et al., 1989) and aspects of the mammary gland (abnormal milk, swelling, temperature, pain, teat relaxation and milk leakage (Massart-Leën et al., 1988)) were recorded at each blood and milk sampling. Normal values of different clinical parameters before infection were calculated (Table 2).

**Table 2.** Normal values of different clinical parameters obtained during clinical examination before intramammary *E. coli* inoculation (n = 74).

| Clinical parameter | Mean ± SEM |
|---|---|
| Rectal temperature (°C) | 38.5 ± 0.04 |
| Heart rate (beats/min) | 71 ± 0.9 |
| Respiration rate (/min) | 22 ± 0.6 |
| Body condition score (0-5) | 2.8 ± 0.03 |

## SEVERITY DETERMINATION AND CLINICAL SEVERITY SCORE

Throughout the years, severity prediction following experimentally induced *E. coli* mastitis has been performed using different clinical and laboratory parameters, such as number of *E. coli* in the infected quarters (Kremer et al., 1993; van Werven et al., 1997), capacity of reactive oxygen species production (Heyneman et al., 1990) and quarter milk production (Burvenich, 1983; Burvenich and Peeters, 1983; Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997).

Burvenich (1983) first quantified severity of an intramammary E. coli inflammation through the reduction in quarter milk production of the uninfected quarters. This severity classification was subsequently used in many experimental *E. coli* mastitis studies (Heyneman et al., 1990; Vandeputte-Van Messom and Burvenich, 1993; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997). Two mechanisms might be involved in production losses during mastitis. The first is a direct effect on milk synthesis by local alteration of the mammary epithelial activity, which is most pronounced in the infected glands. The second mechanism causing a decrease in milk production is rather indirect. During mastitis, milk secretion may be suppressed as a result of general illness in the animals, thereby reducing the availability of milk precursors. This mechanism is responsible for losses in production in the uninfected quarters, since no indication of severe alteration of the blood-milk barrier could be demonstrated in these quarters (Vandeputte-Van Messom et al., 1993). A striking difference in degree and onset of different phenomena of inflammatory parameters could be observed between moderate and severe responders. Besides the pronounced reduction in quarter milk production in the uninfected quarters, blood PMN count was severely reduced and a drastic shift in circulating cell types was established in the days following experimental *E. coli* challenge (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). Moreover, a significant correlation existed between reactive oxygen species production on d-1 and the reduction in quarter milk production (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). Significant differences in several blood and milk constituents already existed before intramammary challenge (Vandeputte-Van Messom et al., 1993).

The severity of *E. coli* mastitis was determined based on quarter milk production in the uninfected quarters at d+2 post-infusion. Animals with a quarter milk production in the uninfected quarters at d+2 higher than 50% of the quarter milk production at d-1 in the same quarters were scored as moderate responders, whereas animals with a quarter milk production at

d+2 lower than 50% were scored as severe responders (Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; 1999) (Fig. 6).

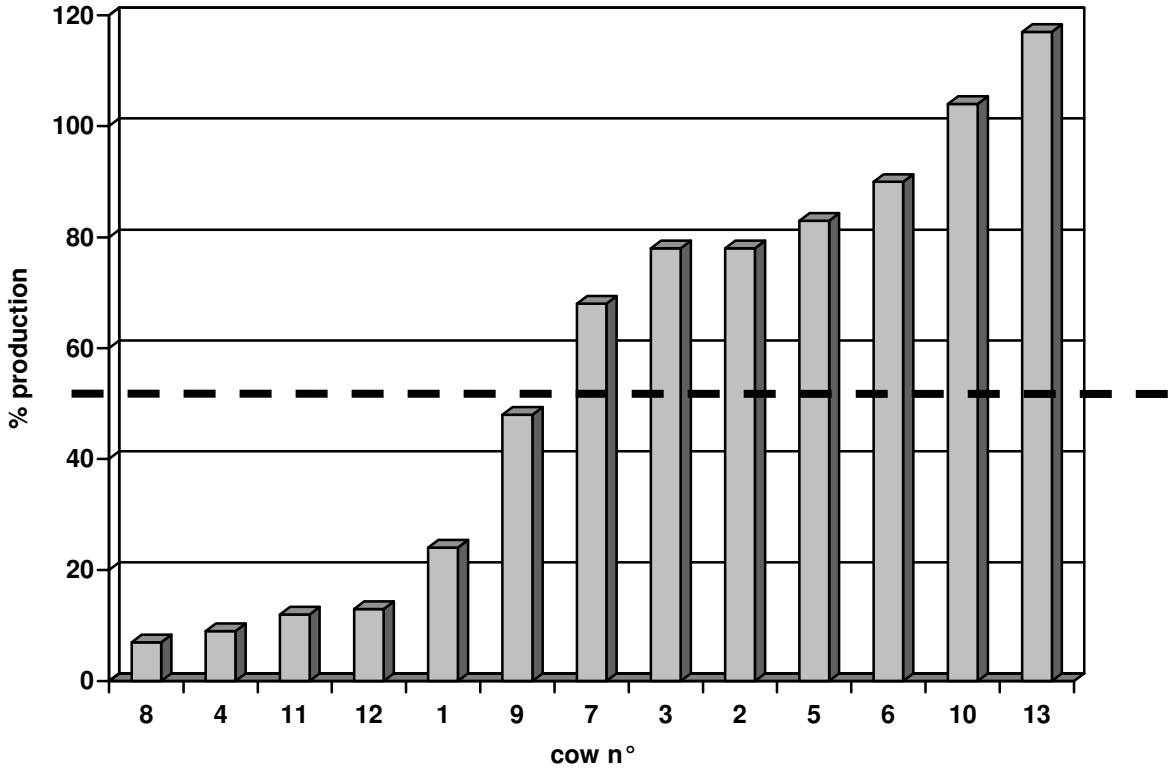


**Figure 6.** Classification of newly calved cows into moderate and severe responders following intramammary *E. coli* challenge, based on quarter milk production in the uninfected quarters at d+2 post-infection. The dashed line indicates the border between severe (under line, < 50 %) and moderate (above line, > 50 %) responders (Dosogne et al., 1997; 1999).

The assessment of disease severity using systemic disease signs in dairy cows with acute coliform mastitis was introduced by Wenz et al. (2001). We developed a classification scheme based on readily observable systemic disease signs, which used rectal temperature, hydration status, rumen contraction rate and attitude as major parameters. In contrast to Wenz et al. (2001), hydration status (assessed through degree of enophtalmus) and attitude (assessment of signs of depression) were scored using another scale (Vangroenweghe et al., 2004). Therefore, severity in our studies was scored based on Wenz et al. (2001) with some slight modifications. Clinical data (RT, skin turgor, reticulorumen motility and general attitude) obtained from PIH 9 to 48 were scored as described in Table 3 and based on their total score, primiparous cows were classified into mild, moderate and severe responders.

**Table 3.** Severity scoring system, based on systemic disease signs, for the classification of primiparous cows, following an experimental intramammary inoculation with *E. coli* P4:O32 (according to Wenz et al., 2001; with slight modifications). Briefly, the 4 parameters are scored, total score is calculated and compared to respective ranges for classification into mild, moderate or severe disease.

| Variable | Criteria | Score |
|---|---|---|
| Rectal temperature (°C) | 37.80 - 39.25 | 0 |
| | 39.30 – 39.80 | 1 |
| | > 39.80 or < 37.80 | 2 |
| Skin turgor | regains normal shape in < 5 sec | 0 |
| | > 5 sec to regain normal shape | 1 |
| Rumen motility rate (contractions/min) | 3 x / 2 min | 0 |
| | 1 – 2 x / 2 min | 1 |
| | 0 x / 2 min | 2 |
| General attitude (signs of depression) | Alert | 0 |
| | Lethargic | 1 |
| | depressed – unable to stand | 2 |
| | extremely sick – recumbent | 3 |
| *Total score* | *Mild disease* | *0 - 2* |
| | *Moderate disease* | *3 - 5* |
| | *Severe disease* | *6 - 8* |

## SOMATIC CELL COUNT AND MILK COMPOSITION

The terminology 'somatic cell count' was first introduced by Paape et al. (1963), because of the presence of epithelial cells in milk, and has become the internationally accepted IDF terminology. Somatic cell count was determined using a fluoro-opto electronic method (Fossomatic 5000 cell counter; Foss Electrics, Hillerϕd, Denmark) by the Milk Control Centre (MCC, Lier, Belgium) under accreditation circumstances (ISO 13366-2; Ubben et al., 1997). Briefly, whole milk samples are heated to 42 ± 2°C, thoroughly homogenised by gentle mixing and analysed within 25 min. Nuclear DNA of the somatic cells present in the milk is stained with a fluorescent probe (ethidiumbromide) and followed by flow cytometric analysis and quantification (cells/ml). Somatic cell count values range from 10,000 to 9,999,000 cells/ml. Control of SCC analysis is performed at three subsequent levels. The first level control includes blank sample analysis every 400 determinations and standard control sample analysis every 35 determinations. Second level control is performed by governmental officials, whereas third level

control includes participation in organised national (Belgian) and international (IDF collaborative test) ring test analyses.

Fat, protein and lactose concentration (mg/ml) were determined using mid-infrared-photospectrometry (MilkoScan 4000; Foss Electrics) (Fig. 7) by the Milk Control Centre (MCC) under accreditation circumstances (IDF standard 141; Trossat and Leray, 1996).

**Figure 7.** MilkoScan 4000 mid-infrared-photospectrometer (Foss Electrics) for the quantitative analysis of fat, protein and lactose in whole milk samples under accreditation circumstances (picture of MCC, Lier, Belgium).

Briefly, whole milk samples are prepared as previously described for SCC determination. Fat, protein and lactose each contain specific chemical structures with different wavelength absorption spectra in the mid-infrared spectrum. The electromagnetic absorption measured at these wavelengths enables quantitative determination (mg/ml) of the different components. Whole milk determination for fat, protein and lactose ranges from 20-70 mg/ml, 25-50 mg/l and 20-70 mg/ml, respectively. Control of milk composition analysis is also performed at three subsequent levels as described for SCC analysis.

Milk samples for the determination of serum albumin (mg/dl), sodium ($Na^+$), chlorine ($Cl^-$) and potassium ($K^+$) concentration (mmol/l) were centrifuged at 1,000 x *g* (30 min, 4°C). Fat was removed and samples of skim milk were taken and immediately frozen at –80°C until analysis. After thawing, serum albumin was quantified using a radial immunodiffusion kit (bovine low level albumin, Bethyl VET-RID; Bethyl Laboratories, Montgomery, TX, USA). Briefly, 10 µl of milk whey was added to each well and incubated at 20°C for 24 h before the diameter of the radial immunodiffusion reaction was assessed. Squared diameters ($d^2$) were compared with a constructed standard curve of known amounts of serum albumin. Ion concentration was analysed using an ion-selective electrode analyser (Ilyte®; Instrumentation Laboratories, Milan, Italy). Briefly, milk samples are brought into contact with specific electrodes consisting of a particular glass composition, which are sensitive to one of the analysed ions, namely $Na^+$, $Cl^-$ or $K^+$. This leads to potential differences, which can be measured and

transformed, into ion concentration values. Average concentrations of different milk components before intramammary challenge are given in Table 4.

**Table 4.** Normal values of different parameters for milk composition obtained before intramammary *E. coli* inoculation.

| Milk component | Mean ± SEM | N |
|---|---|---|
| Fat (mg/ml) | 18.7 ± 1.27 | 130 |
| Protein (mg/ml) | 30.7 ± 0.27 | 114 |
| Lactose (mg/ml) | 49.8 ± 0.22 | 130 |
| Serum albumin (mg/dl) | 17.0 ± 1.37 | 104 |
| $Na^+$ (mmol/l) | 28.5 ± 1.11 | 111 |
| $K^+$ (mmol/l) | 43.5 ± 0.91 | 111 |
| $Cl^-$ (mmol/l) | 33.2 ± 1.14 | 111 |

## PACKED CELL VOLUME, BLOOD LEUKOCYTE COUNT AND DIFFERENTIATION OF BLOOD SMEARS

Blood packed cell volume (PCV) (%) was determined in hematocrit-capillaries (60 µl/75 mm; Hirschmann Laborgeräte, Eberstadt, Germany) using a micro-hematocrit centrifuge (Hawksley, London, UK). Normal values before inoculation were 29 ± 0.4% (n = 66). Blood leukocyte count ($log_{10}$/ml) was determined using an electronic particle counter (Coulter Counter Z2; Coulter Electronics Ltd., Luton, UK). Briefly, 25 µl of whole heparinised blood was added to 20 ml of isotonic solution (Coulter® ISOTON®II Dilution; Beckman Coulter Diagnostics GmbH; Krefeld, Germany). Following addition of 3 drops of zapoglobin (ZAP-OGLOBIN®II Lytic Reagent; Beckman Coulter Diagnostics GmbH), the sample was gently mixed and duplicate measurements were performed. Settings for the electronic particle counter were at gain 256, current 0.707 and lower threshold 30 fl. Following assessment, concentration per ml was automatically calculated (x 40) and displayed as such. For statistical analysis, results were $log_{10}$-transformed. Blood leukocyte count was 8.5 ± 0.24 x $10^6$/ml (6.8 ± 0.04 $log_{10}$/ml) before infection (n = 74).

Differential counts were performed on blood smears. Briefly, 10 µl of homogenised whole blood was added onto a microscope slide and very thin smears were prepared, following drying and staining as described previously (Mehrzad et al., 2001). Differential counts of the blood smears were carried out according to Wilde (1964). Two hundred cells were counted on each smear and percentages of PMN (myelocytes-metamyelocytes, band cells and mature PMN), monocytes, lymphocytes and eosinophils were determined (Fig. 8). Myelocytes-metamyelocytes

were characterised by their round-oval to elongated-arciform nuclei with yellowish cytoplasm. Band cells had longer, circular formed nuclei with more condensed chromatin, situated near the periphery of the cell. Mature PMN had clearly defined nuclei with 3 to 6 lobes and typical neutral granules. Monocytes were round to irregular in shape containing greyish-blue cytoplasm, with foamy appearance in which fine red granules could be found. The nuclear substance had a fine reticulate structure with evenly distributed chromatin. Lymphocytes had compact nuclei, surrounded by cytoplasm varying from a narrow band to a wider, more irregular mass. Eosinophils had their specific eosinophilic granules in the cytoplasm and nuclei with 2 to 3 lobes. Normal values of different cell types before infection were calculated (Table 5).

**Table 5.** Normal values (expressed as %) of different cell types before intramammary *E. coli* inoculation (n = 33).

| **Cell type** | **Mean ± SEM** |
|---|---|
| Myelocytes-metamyelocytes | 9.6 ± 0.78 |
| Band cells | 2.1 ± 0.26 |
| Mature PMN | 26.5 ± 2.17 |
| Macrophages | 8.8 ± 0.88 |
| Lymphocytes | 51.2 ± 2.36 |
| Eosinophils | 1.9 ± 0.28 |

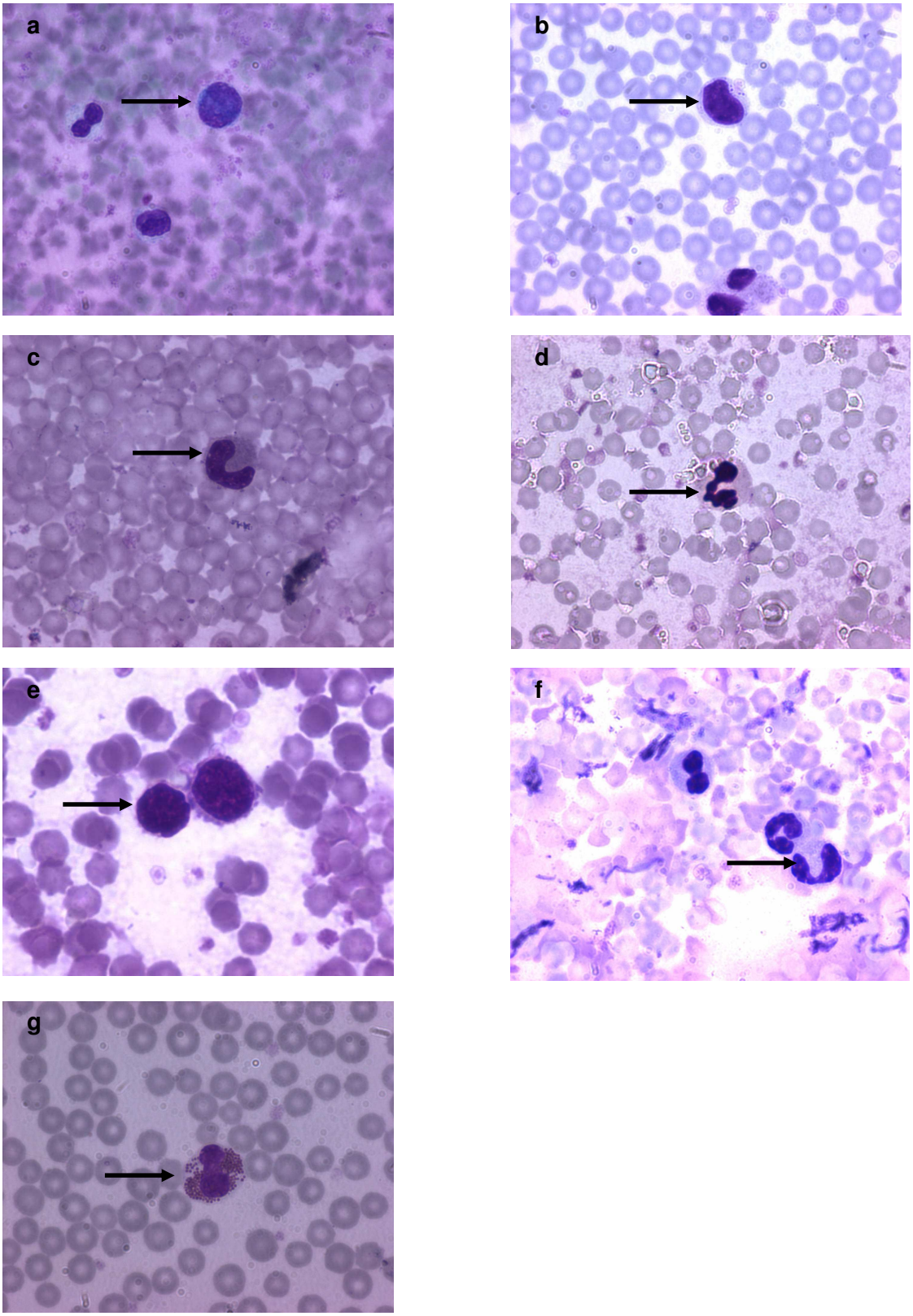


**Figure 8.** Differential leukocyte count of blood smears: a) myelocyte, b) metamyelocyte, c) band cell, d) mature PMN, e) lymphocyte, f) monocyte, and g) eosinophil (pictures of MMRC, Merelbeke, Belgium). ⟶ indicates the specified cell type.

## BACTERIOLOGY ON MAJOR PATHOGENS

Diagnostic bacteriology on major pathogens (i.e. *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, *Corynebacterium bovis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*) was carried out at the laboratory of Dierengezondheidszorg-Vlaanderen (Drongen, Belgium) under accreditation circumstances. Briefly, quarter foremilk samples were plated out onto Columbia agar with 5% sheep blood (Biokar Diagnostics) and incubated at 37°C for 24 h. Suspect colonies were tentatively identified based on colony morphology and growth characteristics, and subsequently typed using specific laboratory identification standards. Results of diagnostic laboratory analysis were reported as negative or positive for major pathogens with specifications of the isolated bacterial species.

## COLONY-FORMING UNITS IN THE INOCULATED QUARTERS

The number of *E. coli* (CFU/ml) after experimental inoculation was determined by appropriate 10-fold dilutions of each milk sample in PBS. Ten µl of these dilutions was plated out on Columbia agar with 5% sheep blood (Biokar Diagnostics). All dilutions were performed in duplicate. Colonies were counted after a 24 h incubation at 37°C. The colony count was converted to CFU/ml based on the factor of dilution and finally expressed as $\log_{10}$/ml for statistical analysis.

Typical colony morphology of inoculated *E. coli* was rough with a predominantly grey colour (Fig. 9a). Occasionally, mutation to smooth colonies could be observed during intramammary passage (Fig. 9b).

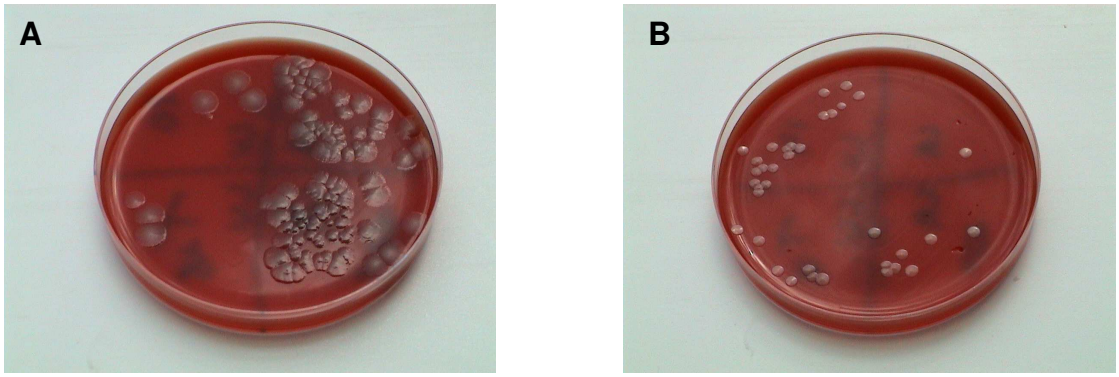


**Figure 9.** Colony morphology of inoculated *E. coli* following a 24 h incubation at 37°C on Columbia agar with 5% sheep blood. A. Rough mutant, B. Smooth mutant (pictures of MMRC).

## β-HYDROXYBUTYRATE

On arrival at the laboratory, clotted blood was incubated for 2 h at 37°C to neutralise the cryoglobulins present in bovine serum. After centrifugation (1,000 x *g*, 20 min, 20°C), serum was aliquoted and stored at –80°C until analysis of BHBA.

β-hydroxybutyrate (mmol/l) was determined twice in the week before challenge to check the status of the cow's energy balance. For analysis, the method with acetone oxidation, described by Williamson and Mellanby (1970), was used. Briefly, 1 ml of $HClO_4$ (0.9 M) was mixed with 1 ml of plasma and subsequently centrifuged (1,000 x *g*, 10 min). Four ml KOH was added to 1 ml supernatant fluid (pH 7.0) and mixed. Subsequently, 2 ml of the mixture was transferred into photospectrometry cups and 1 ml of Tris-hydrazin-EDTA buffer (pH 8.5 in NaOH) and 50 µl NAD-solution were added. After gently mixing, OD was determined at 340 nm. After the first measurement ($OD_1$), 5 µl of β-hydroxybutyrate dehydrogenase was added, followed by incubation at 4°C for 90 min before the second measurement ($OD_2$). β-hydroxybutyrate concentration in the sample was calculated as following:

$$\textbf{β-hydroxybutyrate} = (OD_2 - OD_1) \times 2.89$$

## GLUCOSE

NaF-collected blood was centrifuged (1,000 x *g*, 20 min, 20°C) and plasma was collected and stored at –20°C until analysis of glucose. Glucose concentration (mmol D-glucose/l) was determined using an UV-method (D-Glucose; Roche Diagnostics, Brussels, Belgium). Briefly, 1 ml $HClO_3$ was added to 100 µl plasma and centrifuged at 1,000 x *g* for 10 min to purify the samples. Subsequently, D-glucose was enzymatically transformed to D-glucose-6-phosphate by addition of hexokinase and ATP. Then, D-glucose-6-phosphate was oxidised by glucose-6-phosphate-dehydrogenase into D-gluconate-6-phosphate, with a simultaneous reduction of $NADP^+$ into NADPH. The produced amount of NADPH is a measure for the quantity of D-glucose in the initial plasma sample and is determined by spectrophotometry (Beckman DU® 640D; Beckman Coulter Diagnostics GmbH) at 340 nm wavelength. D-glucose concentration is calculated according to manufacturer's instructions, taking into account dilution effects, molecular weight, and extinction coefficient of the analysed solution. Normal glucose concentration before challenge was 3.3 ± 0.20 mmol/l (n = 23).

## DETERMINATION OF IL-8

Milk IL-8 concentration was determined from undiluted whey samples assayed with a commercially available human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, USA) (Fig. 10). The range of the IL-8 kit was 31.2 to 2,000 pg/ml. The antibody pairs used in this kit have previously been shown to cross-react with bovine IL-8 (Shuster et al., 1996; 1997).

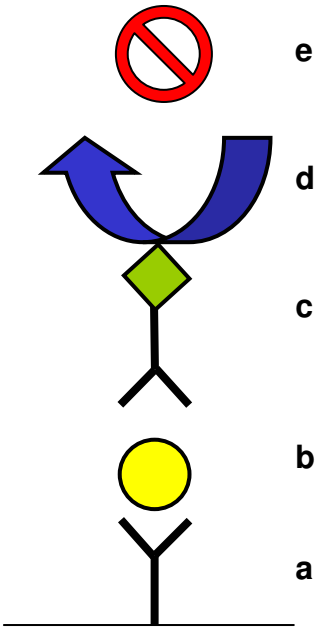


**Figure 10.** Schematic principle of direct sandwich ELISA for the determination of IL-8 in bovine milk whey samples. (a) mouse monoclonal IL-8 antibody, (b) milk whey sample or standard, (c) polyclonal HRP-conjugated anti-IL-8 antibody, (d) TMB substrate solution resulting in a blue product colour, and (e) $H_2SO_4$ stop solution.

Briefly, 100 µl of milk whey sample was added to flat-bottomed microtiter plates, pre-coated with mouse monoclonal anti-IL-8 antibodies, and incubated for 1 h at 20°C. Following washing, polyclonal anti-IL-8 antibodies conjugated to horse-radish peroxidase (HRP) were added and incubated (1 h, 20°C). The substrate solution (3,3',5,5'-tetramethylbenzidine (TMB)) was freshly prepared and added to each well following washing of the wells. After a 30 min incubation at room temperature, the reaction was stopped with 2 N $H_2SO_4$. The optical density at 450 nm and a correction wavelength of 550 nm were measured on an automated microplate reader (Bio-Kinetics Reader; Bio-Tek Instruments, Winooski, USA). Values expressed in picograms per millilitre were extrapolated using linear regression from a standard curve of known amounts of human IL-8. Normal concentration of IL-8 before challenge was 1.9 ± 0.21 pg/ml (n = 82).

## DETERMINATION OF C5a

Milk C5a was quantified by ELISA as previously described (Rainard et al., 1998) (Fig. 11). The range of the C5a ELISA test was 20 to 5,000 pg/ml.

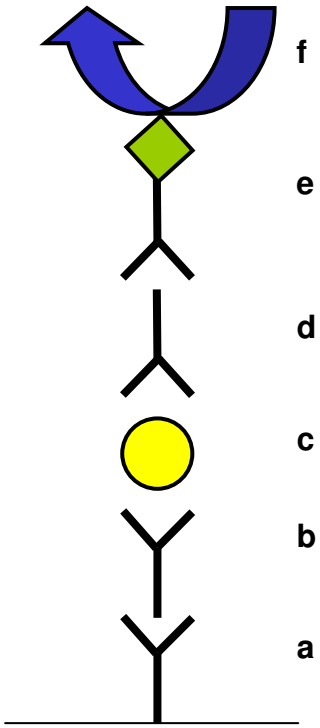


**Figure 11.** Schematic principle of indirect sandwich ELISA for the determination of C5a in bovine milk whey samples. (a) goat anti-mouse IgG, (b) monoclonal anti-C5a antibody, (c) milk whey sample or standard, (d) rabbit anti-bovine C5a/C5 antibody, (e) goat anti-rabbit HRP-conjugated antibody, and (f) ABTS substrate solution resulting in a green product colour.

Briefly, flat-bottomed microtiter plates (Immulon 1; Dynatech, Chantilly, USA) were coated with 100 µl of goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, USA) diluted to 2 µg/ml in 0.1 M carbonate bicarbonate buffer pH 9.6 for 1.5 h at 39°C. After each incubation, the plates were washed five times with PBS supplemented with 0.1% (v/v) Tween 20 (PBST). Unsaturated binding sites were blocked with a solution of 0.5% (w/v) gelatin. The sequence of incubation steps with 100 µl reagents diluted with PBST plus 0.1% gelatin (PBSTG) was as follows: (i) a 1/10,000 dilution of the anti-C5a monoclonal antibody (mAb) 6G4 for 1 h; (ii) twofold dilutions of purified $C5a^{desArg}$ for the calibration curve, or appropriate dilutions of the milk whey sample under test, diluted in PBSTG containing 1 mM EDTA for 1.5 h; (iii) a 1/5,000 dilution of rabbit anti-bovine C5a/C5 for 30 min; (iv) a 1/10,000 dilution of HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 30 min; (v) 52 mM 2,2'-azido-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) in 0.1 M citrate buffer pH 4.2 with 7.5 mM hydrogen peroxide (Sigma). The absorbance at 415 nm was read after about 30 min with an automated microplate reader (Bio-Kinetics Reader; Bio-Tek

Instruments). Values expressed in picograms per millilitre were extrapolated using linear regression from a standard curve of known amounts of $C5a^{desArg}$ (Rainard et al., 1998). Concentration of $C5a^{desArg}$ before intramammary *E. coli* inoculation was 210.1 ± 22.08 pg/ml (n = 82).

## DETERMINATION OF sCD14

A sandwich ELISA was used to quantify sCD14 levels in milk whey as described by Bannerman et al. (2003) (Fig. 12). The range of the sCD14 ELISA test was 780 to 50,000 ng/ml.

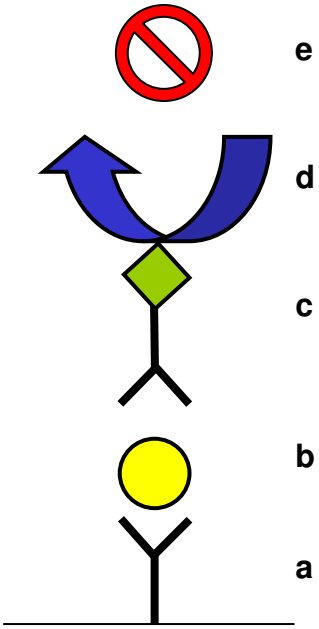


**Figure 12.** Schematic principle of direct sandwich ELISA for the determination of sCD14 in bovine milk whey samples. (a) monoclonal mouse anti-bovine CD14 antibody, (b) milk whey sample or standard, (c) polyclonal HRP-conjugated mouse anti-bovine CD14 antibody, (d) TMB substrate solution resulting in a blue product colour, and (e) $H_2SO_4$ stop solution.

Briefly, flat-bottom 96-well plates were coated overnight with 5 µg/ml of mouse anti-bovine CD14 monoclonal antibody (CAM36A; VMRD, Inc., Pullman, USA) diluted in 0.05 M sodium carbonate-bicarbonate (Sigma), pH 9.6 at 4°C. The plates were washed x4 with 0.05% Tween 20 diluted in 50 mM Trizma-buffered saline (TBS; Sigma), pH 8.0, and subsequently blocked with 2% fish skin gelatin (Sigma) for 1 h at room temperature. Plates were washed and 100 µl of diluted whey samples (1:10) were added to each well in duplicate. Plates were incubated for 1 h at room temperature and subsequently washed as above. Mouse anti-bovine CD14 antibody (MM61A clone, VMRD, Inc.) was conjugated to HRP using a commercially available kit (EZ-Link™ Plus Activated Peroxidase Kit; Pierce Chemical Co., Rockford, USA) and used as the detection antibody. This HRP-conjugated anti-bovine CD14 antibody was diluted 1:1,000 in TBS wash buffer containing 2% gelatin, and 100 µl of the resulting solution

was added to each well. Plates were incubated for 1 h at room temperature, washed as above, and 100 µl of 3,3',5,5'-tetramethylbenzidine substrate solution (TMB; Pierce Chemical Co.) was added to each well. The reaction was stopped by the addition of 100 µl of 2 M $H_2SO_4$ and the absorbance read at 450 nm on a microplate reader (Multiskan PLUS; Labsystems, Helsinki, Finland). A background correction reading of 550 nm was subtracted from the 450-nm absorbance readings. Values expressed in micrograms per millilitre were extrapolated using linear regression from a standard curve of known amounts of rbosCD14 (Wang et al., 2002). Soluble CD14 concentration in healthy mammary glands before *E. coli* challenge was 7.0 ± 0.84 µg/ml (n = 82).

## DETERMINATION OF LBP

Milk whey and plasma LBP levels were determined with a commercially available LBP ELISA kit that cross-reacts with bovine LBP (Cell Sciences, Inc., Norwood, USA) (Fig. 13). The range of the LBP kit was 1.6 to 100 ng/ml. Milk and plasma samples were diluted 1:400 and 1:1,500, respectively, and assayed according to the manufacturer's instructions.

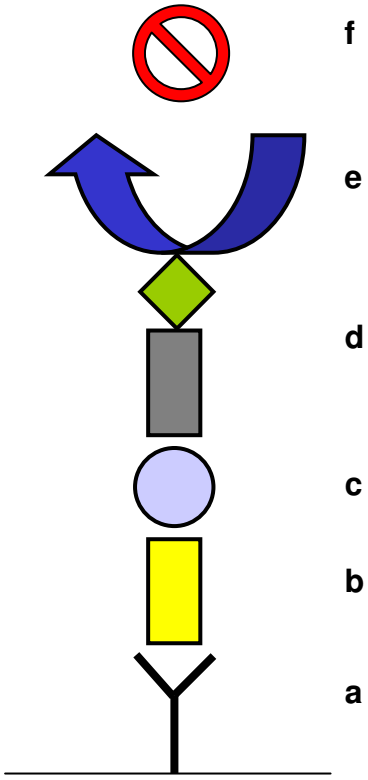


**Figure 13.** Schematic principle of indirect sandwich ELISA for the determination of LBP in bovine plasma and milk whey samples. (a) anti-human LBP antibody, (b) plasma/milk whey sample or standard, (c) biotinylated LPS tracer, (d) streptavidin HRP-conjugated mouse LBP, (e) TMB substrate solution resulting in a blue product colour, and (f) citric acid stop solution.

Briefly, diluted milk whey or plasma samples were added to flat-bottomed microtiter plates, pre-coated with solid bound antibodies recognising LBP of a wide variety of species, and incubated at room temperature for 1 h. The plates were washed 3 times and biotinylated LPS-tracer molecules were added. Following incubation (1 h, 20°C) and three washings, streptavidin-peroxidase conjugated LBP was added (1 h, 20°C). After washing the non-bound conjugated LBP, freshly prepared substrate solution (TMB) was added and the plates were incubated for 30 min (20°C). The enzymatic reaction was stopped by addition of citric acid. The optical density at 450 nm and a correction wavelength of 550 nm were measured on a microplate reader (Multiskan PLUS; Labsystems). The concentration of LBP (expressed in micrograms per millilitre) was calculated by extrapolation using linear regression from a standard curve of known amounts of human LBP. LPS-binding protein concentration in milk whey and plasma before intramammary *E. coli* mastitis were 17.2 ± 2.04 µg/ml (n = 82) and 65.4 ± 7.24 µg/ml (n = 41), respectively.

## DETERMINATION OF $PGE_2$

Milk whey and plasma $PGE_2$ concentrations were determined with a commercially available $PGE_2$ competitive ELISA kit (Neogen, Lexington, USA) according to manufacturer's instructions (Fig. 14). The range of the $PGE_2$ kit was 100 to 4,000 pg/ml.

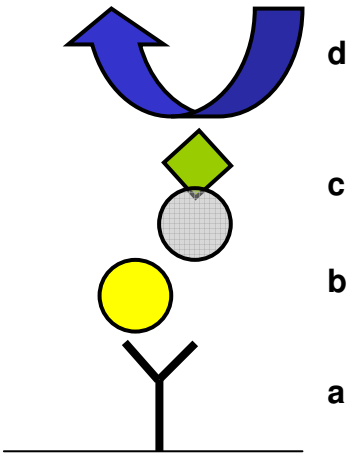


**Figure 14.** Schematic principle of competitive ELISA for the determination of $PGE_2$ in bovine plasma and milk whey samples. (a) monoclonal mouse anti-$PGE_2$ antibody, (b) plasma/milk whey sample or standard, (c) HRP-conjugated $PGE_2$, and (d) TMB substrate solution resulting in a blue product colour.

Briefly, quarter milk samples from acute *E. coli* mastitis were filtered with a 70 µm cell strainer (Becton Dickinson; Erembodegem, Belgium) in order to discard cell clusters. One ml of milk or plasma sample was diluted with 1 ml of distilled water, and 1 ml of the mixture was loaded on a 100 mg C18 column (Varian, St.-Katelijne-Waver, Belgium) after conditioning with 2 ml of distilled water followed by 2 ml of methanol. The column was subsequently washed with

1 ml of each of the following substances: distilled water, methanol:distilled water (30:70), and hexane. The column was centrifuged at 3,200 x *g* for 3 min to withdraw any trace of hexane. Finally, eicosanoids were eluted from the C18 column with 1 ml of methanol. The collected eluate was evaporated to dryness under a stream of nitrogen. Dried samples were reconstituted in an appropriate volume of assay buffer.

Fifty µl of the sample was added to the flat-bottomed microtiter plates, pre-coated with mouse monoclonal anti-$PGE_2$ antibodies. Subsequently, 50 µl of diluted enzyme conjugate was added and the mixture was incubated at 20°C for 1 h. After washing the plates, 150 µl of substrate solution (TMB) was added and incubated (30 min, 20°C). The optical density at 630 nm and a correction wavelength at 490 nm were measured on a microplate reader (Multiskan PLUS; Labsystems). The concentration of $PGE_2$ (expressed in picograms per millilitre) was calculated by extrapolation using linear regression from a standard curve of known amounts of $PGE_2$. Concentrations of $PGE_2$ in milk and plasma before intramammary *E. coli* mastitis were 334.8 ± 21.05 pg/ml (n = 50) and 429.2 ± 25.19 pg/ml (n = 25), respectively.

## DETERMINATION OF $TXB_2$

Milk and plasma $TXB_2$ concentrations were determined with a commercially available $TXB_2$ competitive ELISA kit (Neogen, Lexington, USA) according to manufacturer's instructions (Fig. 15). The range of the $TXB_2$ kit was 4 to 400 pg/ml. Samples were prepared for analysis as described above for $PGE_2$.

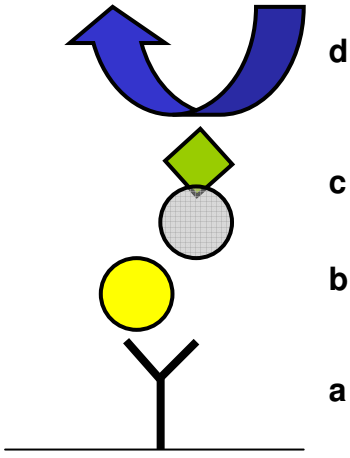


**Figure 15.** Schematic principle of competitive ELISA for the determination of $TXB_2$ in bovine plasma and milk whey samples. (a) monoclonal mouse anti-$TXB_2$ antibody, (b) plasma/milk whey sample or standard, (c) HRP-conjugated $TXB_2$, and (d) TMB substrate solution resulting in a blue product colour.

Briefly, 50 µl of the sample was added to the flat-bottomed microtiter plates, pre-coated with rabbit monoclonal anti-$TXB_2$ antibodies. After addition of 50 µl of diluted enzyme

conjugate, the mixture was incubated at 20°C for 1 h. Following washing, 150 µl of substrate (TMB) was added and incubated (30 min, 20°C). The optical density at 630 nm and a correction wavelength at 490 nm were measured on a microplate reader (Multiskan PLUS; Labsystems). The concentration of $TXB_2$ (expressed in picograms per millilitre) was calculated by extrapolation using linear regression from a standard curve of known amounts of $TXB_2$. Concentrations of $TXB_2$ in milk and plasma before intramammary *E. coli* mastitis were 84.3 ± 5.07 pg/ml (n = 50) and 180.4 ± 26.50 pg/ml (n = 25), respectively.

## STATISTICAL ANALYSIS

The general linear model is a commonly used statistical tool in agricultural research to study the relationship between a normally distributed dependent variable and one or more independent variables. The independent variables can be either continuous or discrete. If an independent variable is discrete, it only takes a restricted number of values and the effect of each level of the discrete variable generates a separate parameter in the model. If an independent variable is, on the other hand, continuous, an underlying functional relationship has to be assumed between the independent variable and the response variable. The number of model parameters corresponds to the number of parameters in the function describing the underlying relationship (Duchateau et al., 1998). The model parameters arising from either a continuous or a discrete independent variable can be fixed or random effects. The mixed model methodology was specifically developed to describe and analyse experiments, which contain both random and fixed effects factors (Duchateau et al., 1998). A major advantage of a mixed model framework is that complex data structures can be described and analysed in a natural way (Duchateau, 1997).

The mixed model was used to analyse the data of the experimental infection randomised trials because animals were measured repeatedly over time during the infection and these repeated measures are not independent. By introducing cow as a random effect in the model, a compound symmetry structure was imposed on the repeated measures, thus accounting for the correlation between the repeated measures.

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# INFLUENCE OF PARITY ON SEVERITY OF INFLAMMATION

*Adapted from:*

Vangroenweghe F., L. Duchateau, and C. Burvenich. 2004. Moderate inflammatory reaction during experimental *E. coli* mastitis in primiparous cows. J. Dairy Sci. 87:886-895.

# INTRODUCTION

Several factors have been shown to play a role in the clinical outcome of *E. coli* mastitis, namely farm management (Jackson and Bramley, 1983; Oz et al., 1985; Smith et al., 1985; Jones, 1986; Pankey et al., 1987; Schukken et al., 1989a; 1989b; Oliver et al., 1990; Lam et al., 1995; Barkema et al., 1999), bacterial factors (Frost et al., 1980; Hill, 1981; Linton and Robinson, 1984; Sanchez-Carlo et al., 1984a; 1984b; Todhunter et al., 1991; Hogan et al., 1992; 1995; 1999; Cross et al., 1993; Nemeth et al., 1994; Nagy and Fekete, 1999) and physiological factors (Heyneman et al., 1990; Gilbert et al., 1993; Kremer et al., 1993a; 1993b; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; 2001; van Werven et al., 1997; Mehrzad et al., 2001; 2002; Vangroenweghe et al., 2001; Burton and Erskine, 2003).

Preventive measures, that are known to be efficient against contagious mastitis, such as post-milking teat disinfection (Schukken et al., 1989a; 1989b; Barkema et al., 1999) have been shown to be inefficient in the control of *E. coli* mastitis (Smith et al., 1985; Oliver et al., 1990; Lam et al., 1995). In contrast, a few studies (Pankey et al., 1987; Oliver et al., 1993) showed that pre-milking teat disinfection may prevent infections with environmental pathogens. Several epidemiological studies have demonstrated a negative correlation between SCC and the incidence of *E. coli* mastitis. Therefore, SCC is thought to be a farm related risk factor for the susceptibility to *E. coli* mastitis (Schukken et al., 1989a; 1989b; Barkema et al., 1999). Significant management factors that are related to a higher incidence of clinical coliform mastitis are: infection pressure from the environment (Jackson and Bramley, 1983), the use of saw dust as bedding material (Oz et al., 1985) and permanent indoor housing during winter (Jackson and Bramley, 1983). Prevention of cows from laying down after milking is often advised to omit contact with bedding material (Jones, 1986).

Various bacterial virulence factors have been studied during *E. coli* mastitis (Sanchez-Carlo et al., 1984a; 1984b), however, only a few have been found to substantially influence the eventual outcome of the disease. *Escherichia coli* is of environmental origin (Nemeth et al., 1994). Although over 100 serotypes have yet been recognised, no specific O-serotypes have been related to bovine *E. coli* mastitis (Linton and Robinson, 1984). Nevertheless, intramammary challenge with *E. coli* 487 caused more severe clinical signs of mastitis than did *E. coli* 727 (Todhunter et al., 1991; Hogan et al., 1992; 1995; 1999). Moreover, a capsulated *E. coli* B117 strain appeared to cause more severe clinical symptoms of mastitis, because it was more difficult to opsonise than a non-capsulated *E. coli* P4 strain (Hill, 1981). Clinical expression after challenge with low doses of *E. coli* is comparable with those after inoculation of

high doses (Frost et al., 1980). *In vivo* adhesion of *E. coli* to the epithelial surface of the mammary gland is thought to be unimportant during the initial phase of infection (Bramley et al., 1979), because in healthy udders collagen or fibronectin are not exposed. Recently, Döpfer et al. (2000) found some indications for *in vitro* adhesion to mammary epithelial cells (MAC-T cells). However, these strains were isolated from recurrent cases of coliform mastitis, a rare disease (Döpfer et al., 2000). The adhesion factors present in enterotoxigenic *E. coli*, which are essential during the initial steps of adhesion in the pathogenesis, do not play a role in the pathogenesis of *E. coli* mastitis (Nagy and Fekete, 1999). A bacterial factor, that plays an important role in the pathogenesis of *E. coli* mastitis, is endotoxin or LPS. Lipopolysaccharide is a potent inducer of inflammatory cytokines (Shuster et al., 1993) during growth and killing (Burvenich, 1983; Petsch and Anspach, 2000). Clinical signs following experimentally induced *E. coli* mastitis are rather due to mediator shock than to endotoxin shock, because endotoxin mainly plays a local role (Hoeben et al., 2000; Dosogne et al., 2002). The capability of some *E. coli* strains to resist the bactericidal activity of serum has also been demonstrated as a virulence factor (Barrow and Hill, 1989).

Physiological factors have a big impact on the clinical outcome of *E. coli* mastitis (Burvenich et al., 2003), far more than farm management and bacterial characteristics that were cited above. Several markers to predict the clinical outcome of *E. coli* mastitis have been studied, such as: the number of circulating leukocytes, production of reactive oxygen species by neutrophils (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993) and chemotactic activity of PMN (Kremer et al., 1993a; 1993b; Dosogne et al., 1997; van Werven et al., 1997). However, predictability of clinical outcome is only significant during 1 to 2 days pre-challenge and does not explain any causal relationship. These markers were mainly studied in multiparous cows, varying between $2^{nd}$ lactation (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997) to $2^{nd}$ – $6^{th}$ lactation (Kremer et al., 1993a; 1993b; van Werven et al., 1997). Because BHBA alters both PMN chemotactic activity and reactive oxygen species production (Heyneman et al., 1990; Kremer et al., 1993a; 1993b; Vandeputte-Van Messom et al., 1993), many *E. coli* challenges that were done at Ghent University and the University of Utrecht were performed on cows with a serum BHBA concentration < 1.4 mmol/l (Kremer et al., 1993b).

Early lactating cows, infected with *E. coli*, are much more severely affected than cows after peak lactation (Hill, 1981). This is mainly due to the impairment of early lactation leukocyte function, as observed by many research groups (Burton and Erskine, 2003). During this period a decrease in cell function of the PMN, resident in the healthy mammary gland (Dosogne et al., 2001; Mehrzad et al., 2001; Vangroenweghe et al., 2001) has been observed. The decrease in cell function was mainly related to the decrease in viability, oxidative burst and

intracellular killing by PMN (Dosogne et al., 2001; Mehrzad et al., 2001; Vangroenweghe et al., 2001).

Besides energy balance and stage of lactation, cow parity was also found to be an important physiological factor that influences severity of clinical mastitis (Gilbert et al., 1993; van Werven et al., 1997; Mehrzad et al., 2002). Blood PMN function was higher in younger animals than in cows after their $4^{th}$ parturition (Gilbert et al., 1993; van Werven et al., 1997). Moreover, viability and oxidative burst have been found to be significantly different between primiparous cows and multiparous cows during the periparturient period (Mehrzad et al., 2002).

In conclusion, many studies indicate that physiological factors determine the clinical outcome of *E. coli* mastitis. In this study, the clinical outcome of *E. coli* mastitis was studied in primiparous cows using the same criteria for severity as in other studies using multiparous cows (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; van Werven et al., 1997; Hoeben et al., 2000).

The purpose of the present study was to evaluate the outcome of intramammary *E. coli* inoculation in primiparous cows under identical conditions as described before with multiparous cows (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; Hoeben et al., 2000; Dosogne et al., 2002). Moreover, the relation between pre-infection parameters and the outcome of infection in term of severity was assessed. Finally, two high inoculum doses, with a 100-fold difference ($1 \times 10^4$ and $1 \times 10^6$ colony-forming units (CFU)), were used because the amount of LPS produced is related to the number of *E. coli* bacteria (Burvenich, 1983; Cross et al., 1993; Monfardini et al., 1999). The primiparous cows in this study were inoculated with the same strain as described before (Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000; Dosogne et al., 2002).

# MATERIALS AND METHODS

### Experimental Animals and Study Facilities

All primiparous cows (n = 19) were in their 7th month of pregnancy on arrival at the commercial dairy farm. At calving, cows were between 24 and 30 months of age.

### Experimental Design

Animals were challenged with *E. coli* P4:O32 on 4 different trial days. The 1 x $10^4$ CFU inoculum group (group A) was challenged in a group of 4 and 5 animals, the 1 x $10^6$ CFU inoculum group (group B) in two groups of 5 animals.

### Sampling Procedure

Blood and milk samples were collected at d-4, d-1, d0, d+1, d+2 and d+3 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21.

### SCC and Milk Composition

In this study, SCC and milk composition were determined. The daily production of fat, protein and lactose (g/24 h per quarter) was calculated based on daily quarter milk production and concentrations of these parameters.

### Statistical Analysis

The two inoculum groups were not formally compared because inoculum dose was not randomly assigned to animals. Pre-infection values of blood and milk constituents of the inoculum groups were compared just before intramammary *E. coli* inoculation using a paired two-sided t-test, assuming unequal variances (Statistix; Analytical Software, Tallahassee, USA) (Table 1). The relationship between pre-infection concentrations of blood and milk constituents and the milk production reduction in the uninfected quarters at d+2 (PIH 48-72) was assessed by linear regression with an adjustment for inoculum size.

**For Further Details** see chapter 'Materials and Methods Experimental Infections', **p. 97**.

# RESULTS

**Relation Between Pre-infection Blood and Milk Constituents and Percentage Reduction in Milk Production at d+2 Post-Infection**

No significant differences were observed in pre-infection levels of blood and milk constituents (Table 1).

**Table 1.** Pre-infection levels of blood and milk constituents (expressed as means ± SEM) in primiparous cows infused with 1 x $10^4$ (group A; n = 9) and 1 x $10^6$ (group B; n = 10) CFU *E. coli* P4:O32.

| | *(Values are means ± SEM)* | |
|---|---|---|
| | **Group A** (n = 9) | **Group B** (n = 10) |
| **Blood constituents** | | |
| β-hydroxybutyric acid (mmol/l) | 0.89 ± 0.20 | 0.66 ± 0.05 |
| Glucose (mmol/l) | 3.8 ± 0.3 | 3.2 ± 0.2 |
| Blood leukocyte count ($log_{10}$/ml) | 6.942 ± 0.024 | 6.903 ± 0.027 |
| **Milk constituents** | | |
| SCC ($log_{10}$/ml) | 4.46 ± 0.10 | 4.18 ± 0.07 |
| Albumin (mg/dl) | 23.4 ± 3.3 | 27.8 ± 3.1 |
| Milk production (l/24 h per quarter) | 3.4 ± 0.4 | 3.8 ± 0.2 |
| Fat (g/24 h per quarter) | 155 ± 22 | 164 ± 29 |
| Protein (g/24 h per quarter) | 101 ± 5 | 110 ± 8 |
| Lactose (g/24 h per quarter) | 169 ± 18 | 186 ± 12 |

No significant relationships between pre-infection blood and milk constituents and the reduction in milk production at d+2 post-infection could be demonstrated (Table 2).

**Clinical and Laboratory Results of Primiparous Cows in Group A Inoculated with 1 x $10^4$ CFU**

All primiparous cows in group A had a moderate clinical outcome of *E. coli* mastitis, based on their quarter milk production of the uninfected quarters on d+2 post-infection. Milk production in the uninfected quarters returned to pre-infection level on d+2 post-infection (Fig. 1).

Rectal temperature increased from PIH 9 onward, with peak fever reached at PIH 12 (Fig. 2a). Heart rate and RR peaked at PIH 12 (Fig. 2b-c). Rumen motility was depressed from PIH 9 onward, resulting in a decrease in appetite. Primiparous cows returned to normal appetite and reticulorumen motility around PIH 21. Udder parameters, such as swelling and elevated

quarter temperature, appeared at PIH 9 in the infected quarters. Teat relaxation, milk leakage and diarrhoea, considered as indicators for severe clinical illness, only appeared in a small number of animals. Changes to abnormal milk with clots, flakes and a watery appearance were maximally present in all infected animals at PIH 18 (results not shown). Body condition score slightly decreased from 3.5 to 2.5 between calving and the end of the experimental challenge period.

**Table 2.** Relationship between pre-infection blood and milk constituents and the reduction in quarter milk production at d+2 (PIH 48-72) in the uninfected quarters of primiparous cows from both groups, assessed by linear regression with correction for inoculum dose. The slope value indicates the change in milk production reduction for one unit change in the blood and milk constituents.

| | **Slope** | **Standard error** | ***P*-value** |
|---|---|---|---|
| **Blood constituents** | | | |
| β-hydroxybutyric acid (mmol/l) | -24 | 14 | *0.110* |
| Glucose (mmol/l) | -11 | 6 | *0.089* |
| Blood leukocyte count ($log_{10}$/ml) | -44 | 64 | *0.497* |
| | | | |
| **Milk constituents** | | | |
| SCC ($log_{10}$/ml) | +12 | 20 | *0.529* |
| Albumin (mg/ml) | -0.8 | 0.5 | *0.126* |
| Milk production (l/24 h per quarter) | +0.013 | 0.014 | *0.120* |
| Fat (g/24 h per quarter) | +0.14 | 0.07 | *0.060* |
| Protein (g/24h per quarter) | +0.37 | 0.20 | *0.089* |
| Lactose (g/24 h per quarter) | +0.23 | 0.12 | *0.067* |

Based on the severity scoring adapted from Wenz et al. (2001), primiparous cows in group A predominantly reacted with a mild response (n = 7) at PIH 9. At PIH 12, the clinical symptoms progressed mostly to a moderate response (n = 7). Thereafter, only one primiparous cow with a moderate response was observed at PIH 15. No severe responses were observed at any time point between PIH 9 and 48 in group A, which received the 1 x $10^4$ CFU *E. coli* inoculum dose.

Milk production in the infected quarters maximally decreased (± 55%) on the day of challenge. At d+1 post-infection, milk production gradually increased and almost totally recovered by d+3 post-infusion (Fig. 2d). The number of *E. coli* in the infected quarters peaked at PIH 6, followed by a rapid elimination until PIH 15. At PIH 72, only 5 animals still had low numbers of *E. coli* in the infected quarters (Fig. 2e). Somatic cell count rapidly increased from PIH 6, reached a maximum at PIH 12, and remained high until the end of the experiment at PIH 72 (Fig. 2f).

Blood leukocyte number decreased to nadir at PIH 12 and recovered to pre-infection level at PIH 48 (Fig. 2j). The presence of early immature PMN (myelocytes-metamyelocytes) in

circulation increased at PIH 6, reaching peak levels at PIH 21 (Fig. 2g). Similarly, maximal percentages of late immature PMN (band cells) were reached at PIH 21 (Fig. 2h). Concomitantly, circulating mature PMN decreased at PIH 6 with nadir at PIH 12 (Fig. 2i).

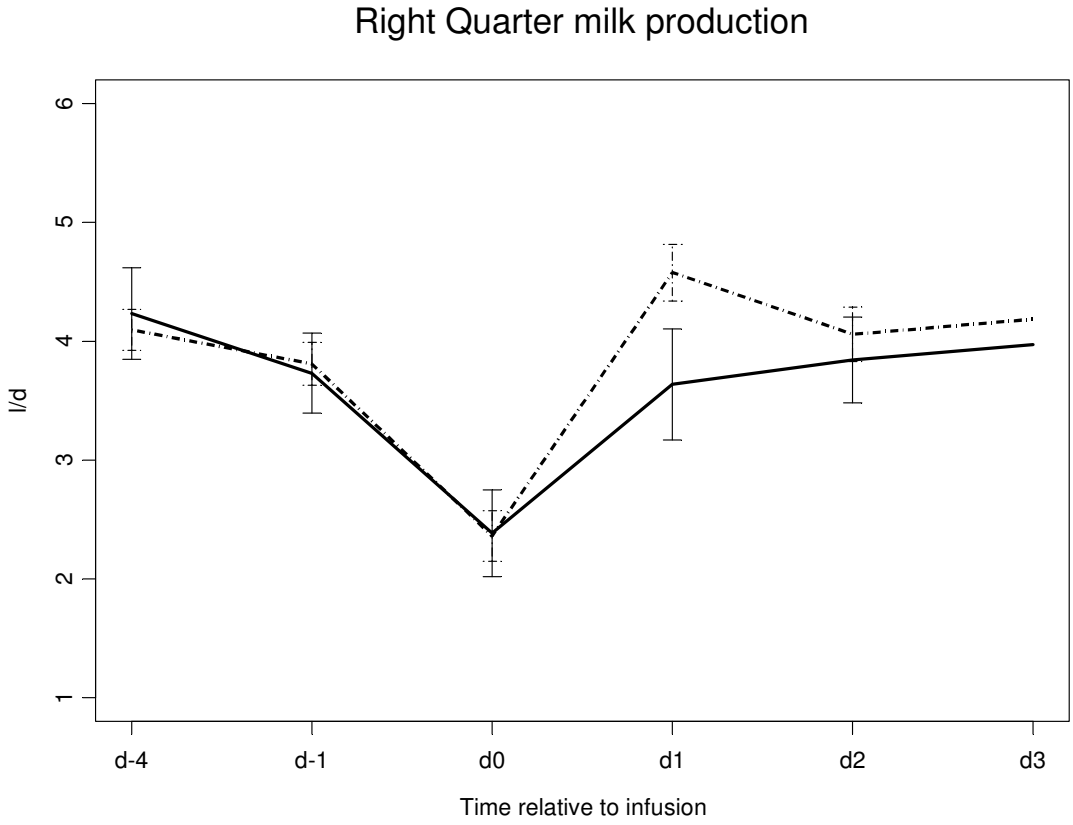


**Figure 1.** Milk production (l/d) of the uninfected right quarters from d-4 until d+3 relative to infusion from primiparous cows infused with 1 x $10^4$ (——; group A; n = 9) and 1 x $10^6$ (------; group B; n = 10) CFU *E. coli* P4:O32. Data are means (± SEM).

The concentration of lactose in the infected quarters decreased from PIH 9 with a minimum at PIH 21-24 (Fig. 2k). Serum albumin in the infected quarters increased from PIH 9, and peaked at PIH 15 (Fig. 2l). Sodium and chlorine concentrations peaked at PIH 12 (Fig. 2m-n), whereas potassium concentration decreased from PIH 9, and reached its greatest reduction at PIH 12 (Fig. 2o). All indicators for the presence of mastitis gradually recovered to pre-infection levels at PIH 72, after reaching their peak values (Fig. 2k-o).

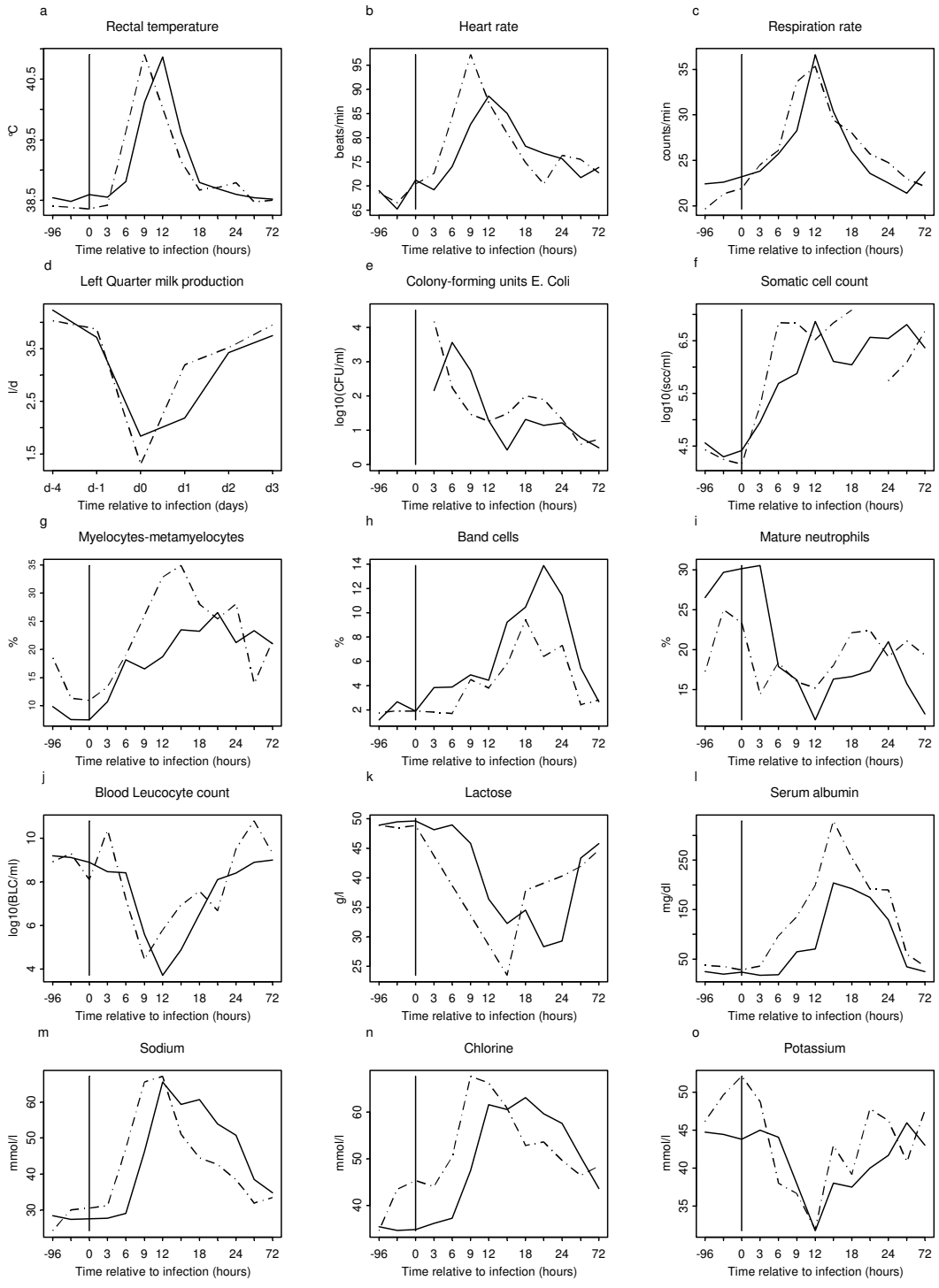


**Figure 2.** Rectal temperature (a), heart rate (b), respiration rate (c), left quarter milk production (d), number of CFU *E. coli* P4:O32 (e), SCC (f), % myelocytes-metamyelocytes (g), % band cells (h), % mature neutrophils (i), blood leukocyte count (j), lactose (k), serum albumin (l), milk sodium (m), milk chlorine (n) and milk potassium (o) from PIH –96 until PIH 72 in infected quarters or blood, respectively, from primiparous cows infused with 1 x $10^4$ (——; group A; n = 9) and 1 x $10^6$ (------; group B; n = 10) CFU *E. coli* P4:O32. Data are means.

**Clinical and Laboratory Results of Primiparous Cows in Group B Inoculated with 1 x $10^6$ CFU**

Clinical reaction and changes in laboratory parameters were similar to the above described results for group A. However, the changes during inflammation generally appeared more rapidly (approximately 3 h) in primiparous cows from group B, infused with the 1 x $10^6$ CFU *E. coli* inoculum dose, compared to group A, infused with the 1 x $10^4$ CFU *E. coli* inoculum dose.

All primiparous cows in this group reacted with a moderate clinical response to intramammary *E. coli* infusion. Milk production in the uninfected quarters was only decreased until d+1 post-infection (Fig. 1). Rectal temperature increased from PIH 6, peaked at PIH 9 and gradually decreased to pre-infection values at PIH 18 (Fig. 2a). Peak tachycardia was reached at PIH 9 (Fig. 2b), whereas maximal RR was reached at PIH 12 (Fig. 2c). Reticulorumen motility and all other clinical parameters seemed to change some 3 h earlier in group B. The same trend in BCS could be observed as in group A.

Clinical scores, adapted from Wenz et al. (2001), showed an earlier reaction (approx. 3 h) in group B compared with group A. At PIH 9, most of the primiparous cows reacted moderately (n = 8), gradually decreasing at PIH 12 (n = 5). Only one animal in group B had a prolonged moderate response until PIH 24. No severe responses were present in this group at any time point between PIH 9 and 48.

Milk production reduction in the infected quarters was maximal (± 65%) on the day of infusion and recovered rapidly to pre-infection production at d+3 post-infection (Fig. 2d). The number of *E. coli* in the infected quarters peaked at PIH 3 (Fig. 2e). A rapid influx of cells into the infected quarters was observed from PIH 3, with a peak at PIH 9 (Fig. 5f).

The number of circulating blood leukocytes decreased from PIH 6, peaking at PIH 9. A pronounced rebound effect was observed between PIH 15 and 48 (Fig. 2j). The increase in circulating early immature cells was pronounced and peaked at PIH 15 (Fig. 2g). Band cells increased at PIH 9, and peaked at PIH 18, thereafter gradually decreasing to reach pre-challenge levels at PIH 72 (Fig. 2h). The greatest reduction in circulating mature PMN was reached at PIH 3, gradually increasing to pre-infection values at PIH 21 (Fig. 2i).

The concentrations of lactose, serum albumin, sodium, chlorine and potassium followed similar kinetics as in group A, although the initial changes appeared approximately 3 h earlier (Fig. 5k-o).

# DISCUSSION

In this study, the same strain and range of high inoculum doses of *E. coli* was used to induce *E. coli* mastitis as previously described (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000; Dosogne et al., 2002). In contrast to previous studies, primiparous cows were used. Similar clinical symptoms, such as quarter inflammation, fever, depression of reticulorumen motility, loss of appetite and general discomfort were observed as expected. In previous studies, however, the clinical responses showed large variations from mild-moderate to severe; whereas in the present study, the variation in clinical response was quite narrow. Quarter inflammation was associated with a temporary loss of milk production, combined with the secretion of abnormal milk from the infected glands. Maximal decrease in milk production in the infected and uninfected quarters occurred on the day of challenge, in contrast to previous observations where maximal decrease in milk production was observed later (Heyneman et al., 1990; Hoeben et al., 2000). In the present study, none of the animals reacted severely following intramammary *E. coli* challenge (Fig. 3). In both infected and uninfected quarters, milk production seemed to recover more rapidly on d+1 post-infection in primiparous cows from group B, which received the 1 x $10^6$ CFU *E. coli* inoculum dose. Animals were scored as previously described (Vandeputte-Van Messom et al., 1993) and compared with the clinical score used by Wenz et al. (2001). This resulted in a similar classification of mild and moderate responses. The only difference that could be observed between group A and B was the time of latency that was shorter when higher inoculum doses were used.

Body condition score and the concentration of BHBA and glucose indicated that the primiparous cows in this study were not ketotic at the time of challenge (Kremer et al., 1993b), although their quarter milk production was comparable to the multiparous cows previously challenged (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993).

The number of circulating PMN, a marker to predict the clinical outcome of the disease, was similar to the levels previously observed in moderate responders (Vandeputte-Van Messom et al., 1993). Primiparous cows in this study could therefore be expected to react with a moderate clinical response. Although no PMN functionality was determined in this study, it is expected that PMN reactive oxygen species production was high in these primiparous cows. Mehrzad et al. (2002) recently found that in the period from 5 weeks before until 5 weeks post-parturition the CL activity of PMN was higher in primiparous cows than in multiparous cows. Polymorphonuclear leukocyte reactive oxygen species production and chemotactic activity were inversely correlated to the clinical outcome of *E. coli* mastitis (Heyneman et al., 1990; Kremer et

al., 1993a; 1993b). No significant relationship with the reduction in quarter milk production on d+2 post-infection was observed, possibly due to the limited number of animals in this study.

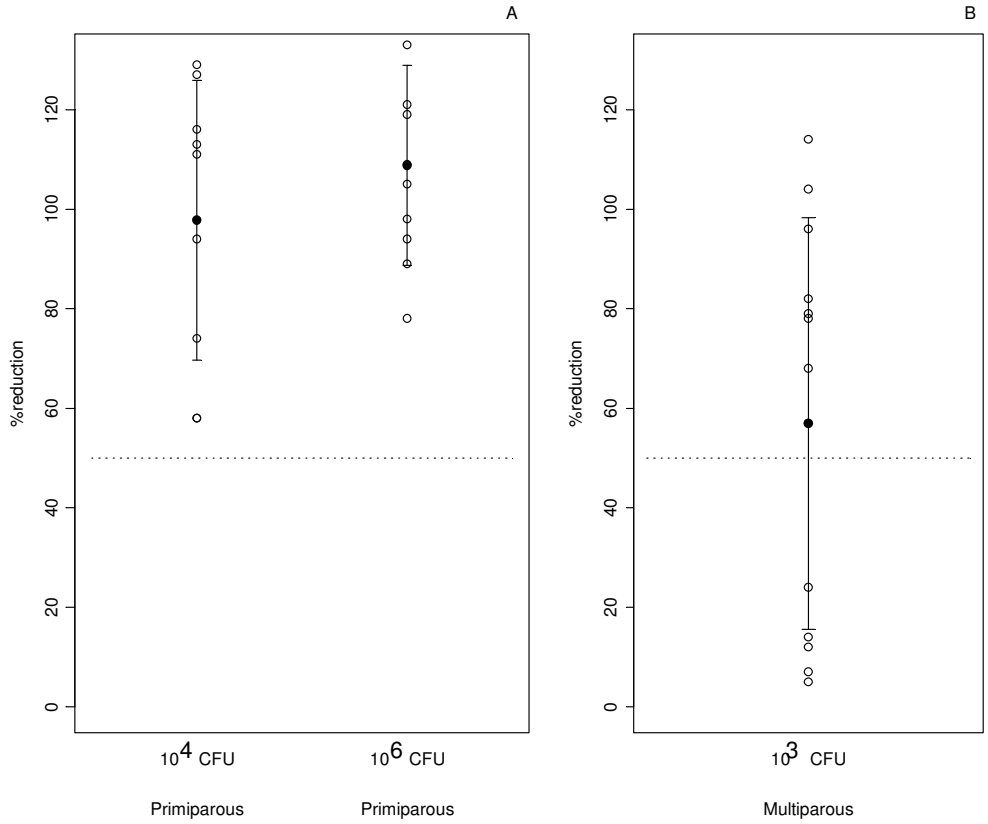


**Fig. 3.** Percentage of the initial quarter milk production in the uninfected quarters on d+2 (PIH 48-72) relative to infection. A. Primiparous cows infused with 1 x $10^4$ (group A; n = 9) or 1 x $10^6$ (group B; n = 10) CFU *E. coli* P4:O32 in both left quarters (present study). B. Multiparous cows infused with 1 x $10^3$ CFU *E. coli* O157 in both left quarters and scored into moderate (n = 7) and severe (n = 5) clinical response (Dosogne et al., 1997; historical control). Means (•) and standard deviation (I) of percentages of the initial milk production in the uninfected quarters were given for each group. Line (……) at 50% of the initial milk production arbitrarily indicates the difference between moderate and severe responders (Vandeputte-Van Messom et al., 1993).

The onset of local clinical signs of mastitis, characterised by quarter swelling, coincided with the influx of PMN to the infected quarters. Somatic cell count increased rapidly in both groups, which is in accordance with earlier observations, where moderate responders had a rapidly occurring leukocytosis in the infected glands (Vandeputte-Van Messom et al., 1993). The extraction of mature PMN from the blood to the infected glands is known to result in early and late immature PMN recruitment from the bone marrow to restore the number of circulating PMN. In this study the recruitment of immature PMN was of short duration, compared to Heyneman et al. (1990), who observed immature forms in circulation for at least 3 d in moderate responders and for almost 10 d in severe responders.

The rapid influx of PMN into the infected glands was associated with fast elimination of bacteria from the quarters. In this study, high inoculum doses were used for experimental induction of *E. coli* mastitis, because we were mainly interested in bacterial elimination rather than bacterial growth in the mammary gland. In contrast to an earlier study with the same inoculum dose in multiparous animals (Vandeputte-Van Messom et al., 1993), peak bacterial numbers were already reached around PIH 3 to 6. This peak number of bacteria was followed by a rapid elimination from the affected glands. Contrary to the induction of *E. coli* mastitis with low inoculum doses, where elimination is preceded by excessive bacterial growth, in this study, peak numbers were reached within 6 h post-infection and followed by a subsequent bacterial elimination. Therefore, PMN influx is thought to be fast and strong enough to rapidly clear the bacteria from the affected glands. It can be presumed that the bactericidal capacity of the PMN migrated to the infected quarters is high, because more efficient PMN functionality was reported in primiparous cows recently (Mehrzad et al., 2002).

Several indicators (lactose, serum albumin, sodium, chlorine and potassium) for the presence of mastitis were determined in this study. Generally, the changes could be observed approximately 3 h earlier in group B, although peak levels were almost identical for both groups, which coincides with all other data, indicating that the animals reacted with a mild to moderate response and little variation in the clinical response was present in this study.

From this study, it appears that the inflammatory response in primiparous cows from group B has an earlier onset compared to group A. One possible explanation for this observation could be the 100-fold difference in the number of *E. coli* infused into the mammary glands, because the amount of LPS produced is related to the number of *E. coli* bacteria (Burvenich, 1983; Cross et al., 1993; Monfardini et al., 1999). A direct effect of LPS present in the inoculum can be excluded, because the bacterial cultures were washed three times in pyrogen-free PBS before further dilutions were made. Lipopolysaccharide, known as a potent inducer of inflammatory cytokines (Shuster et al., 1993), can be produced quite rapidly during bacterial growth following intramammary *E. coli* infusion. Therefore, a sufficient amount of inflammatory cytokines should be produced early during inflammation in both groups, resulting in the rapid attraction of PMN from the blood into the mammary gland, with a subsequent pronounced increase of SCC in the infected glands.

Following *E. coli* mastitis, treatment with a bactericidal antibiotic at PIH 10 has been shown inefficient to alter local and systemic symptoms already present, although the number of bacteria in the infected quarters decreased 100-fold (Monfardini et al., 1999). Therefore, it can be suggested that early inflammatory events (first 3 h) could play an important role in the further regulation of the inflammatory response to combat the invading organisms. Experimental design

in this study, particularly in relation to the number and time of samplings, was however not suitable to unravel these elements of early acute phase response.

Nevertheless, the moderate inflammation model with primiparous cows in the present study clearly indicates that a mild to moderate response following intramammary challenge with *E. coli* is sufficient to combat the induced mastitis with a maximal resolution of mammary gland functionality.

# CONCLUSION

In conclusion, despite the use of relative high inoculum doses, primiparous cows react with a moderate inflammatory response following intramammary infusion. This moderate response was evident from the pre-infection number of circulating leukocytes, the concentration of BHBA and glucose, the prompt clinical response, the rapid influx of PMN into the infected quarters, the efficient bacterial elimination of the affected glands and the fast recovery of milk production in both infected and uninfected glands. To follow-up the bacterial elimination from the affected glands, the use of a high inoculum dose was of particular interest. In contrast to previous studies, pre-infection parameters were not significantly related to the clinical outcome of the disease in terms of severity. The absence of a significant relation between these parameters could be due to the narrow variation in clinical response in the present study, especially compared to the large variation, ranging from mild-moderate to severe, observed in previous studies. The difference in time of latency between both inoculum doses could also be considered as an interesting observation. However, to further elucidate the effect of inoculum dose, a completely randomised study should be designed for this purpose. The moderate inflammation model with primiparous cows presented here, clearly indicates that a mild to moderate response following intramammary challenge with *E. coli* is sufficient to combat the induced mastitis with a maximal resolution of mammary gland functionality.

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# MODULATION OF THE INFLAMMATORY REACTION

1. **Variation of the inoculum dose**

2. **Inhibition of prostaglandin synthesis**

3. **Vaccination against the endotoxin**

# 1.

# VARIATION OF THE INOCULUM DOSE

*Adapted from:*

Vangroenweghe F., P. Rainard, M.J. Paape, L. Duchateau, and C. Burvenich. 2004. Increase of *Escherichia coli* inoculum doses induce faster innate immune response in primiparous cows. J. Dairy Sci. 87:4132-4144.

# INTRODUCTION

The importance of innate immunity in recognising microbial pathogens and mounting a response against them is now widely recognised. The immediate, innate immune response is mediated largely by white blood cells such as PMN and macrophages, cells that phagocytose and kill the pathogens, and that concurrently co-ordinate additional host responses by synthesising a wide range of inflammatory mediators and cytokines (Aderem and Ulevitch, 2000). Several species of Gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae*, and various species of *Enterobacter*, are common mastitis pathogens and are all characterised by the presence of endotoxin or LPS in their outer membrane. Lipopolysaccharide is a pro-inflammatory molecule that is shed from the bacterial surface during bacterial replication or death (Burvenich, 1983; Petsch and Anspach, 2000; Burvenich et al., 2003). Clinical signs following experimentally induced *E. coli* mastitis contributed to mediator shock rather than to endotoxin shock, because endotoxin mainly plays a local role (Hoeben et al., 2000; Dosogne et al., 2002).

Several of the pro-inflammatory cytokines that mediate the localised and systemic response to Gram-negative mastitis, including IL-1β, IL-6, IL-8, and TNF-α are upregulated by LPS (Shuster et al., 1993; Guha and Mackman, 2001). The upregulation of these cytokines is mediated by the interaction of LPS with the accessory proteins, LBP and CD14 (Guha and Mackman, 2001). LPS-binding protein, an acute phase protein binding with circulating LPS, facilitates the transfer of LPS to mCD14, found on PMN and cells of the monocytic lineage (Wright et al., 1990). Membrane-associated CD14 is a glycosyl phosphatidylinositol-anchored protein that lacks an intracellular cytoplasmic domain, rendering it incapable of signal transduction across the cell membrane. In cells lacking mCD14, such as epithelial and endothelial cells, activation is dependent on cellular recognition of LPS-LBP complexes bound to circulating sCD14, which is derived from the shedding of mCD14 (Tapping and Tobias, 1997) from CD14-bearing cells. Toll-like receptor-4 has been identified in both cells of the monocytic lineage and non-mCD14-bearing cells as a LPS transmembrane receptor capable of activating cells (Chow et al., 1999; Faure et al., 2000). Recently, it was shown that LBP in addition to transferring LPS to CD14 also forms an integral part of a trimolecular LPS-LBP-sCD14 complex. Monocytes can therefore detect the presence of LPS at concentrations as low as 10 pg/ml (Thomas et al., 2002). Transmembrane signalling and cell activation in cells lacking mCD14 is thought to be associated with TLR-4, through a cell surface assembly of a multi-protein recognition complex consisting of CD14, MD-2, and TLR-4 (Akashi et al., 2000).

Activation and transmembrane signal transduction through the TLR-4 complex activates several NF-κB controlled genes such as IL-8 in endothelial cells (Aderem and Ulevitch, 2000).

A role for sCD14 and LBP in mediating bovine host responses to intramammary LPS or *E. coli* challenge have recently been demonstrated (Wang et al., 2002; Bannerman et al., 2003; Lee et al., 2003a; 2003b). Following intramammary LPS infusion, sCD14 increases in milk (Bannerman et al., 2003; Lee et al., 2003a) paralleled by an increase in LBP (Bannerman et al., 2003). Moreover, sCD14 has been shown to sensitise the mammary gland to LPS (Wang et al., 2002) and to reduce the severity of experimental *E. coli* mastitis in mice (Lee et al., 2003c) and cows (Lee et al., 2003b). Interestingly, maximal levels of the chemoattractant IL-8 were observed before increases in either milk LBP or sCD14. This suggests that initial host cell activation can occur in the presence of basal levels of sCD14 and LBP (Bannerman et al., 2003). Furthermore, PMN influx as determined by SCC, was similarly elevated before increases in sCD14 and LBP, indicating that heightened levels of these molecules were not required for immediate host innate immune responses (Bannerman et al., 2003).

Several physiological factors impact on the clinical outcome of *E. coli* mastitis (Burvenich et al., 2003). Early lactating cows, infected with *E. coli*, are more severely affected than cows after peak lactation (Hill, 1981). This is mainly due to the impairment of early lactation leukocyte function which begins a few weeks before parturition and only recovers several weeks post-partum (Kehrli et al., 1989; Sordillo and Babiuk, 1991a; 1991b; Sordillo and Peel, 1992; Detilleux et al., 1995; Dosogne et al., 1999; Mehrzad et al., 2002). This pronounced immunosuppresion is not only related to parturition itself (Kimura et al., 1999), but is influenced by several periparturient diseases (Kehrli and Goff, 1989) and has a consequence on many other diseases, such as retained placenta (Kimura et al., 2002). In addition to stage of lactation, parity was also reported to be an important physiological factor that influences severity of clinical mastitis (Gilbert et al., 1993; van Werven et al., 1997; Mehrzad et al., 2002; Vangroenweghe et al., 2004). Blood PMN function was higher in younger animals than in cows after their 4$^{th}$ parturition (Gilbert et al., 1993; van Werven et al., 1997). Moreover, PMN viability and oxidative burst activity have been found to be significantly different between primiparous cows and multiparous cows during the periparturient period (Mehrzad et al., 2002). In a non-randomised intramammary challenge study using large numbers of *E. coli*, primiparous cows reacted as moderate responders based on their quarter milk production in the uninfected quarters on d+2 post-infection. Based on clinical severity, all of the primiparous animals were scored as mild to moderate in their clinical response throughout the entire experimental challenge period (Vangroenweghe et al., 2004).

The purpose of the present study was to quantify several inflammatory mediators and cytokines and to evaluate the outcome of intramammary *E. coli* inoculation in primiparous cows under identical conditions as described previously (Vangroenweghe et al., 2004) with a fully randomised study design, using two high inoculum doses, with a 100-fold difference ($1 \times 10^4$ and $1 \times 10^6$) in CFU. This difference in inoculum dose was based on the amount of LPS produced related to the number of *E. coli* bacteria injected (Burvenich, 1983; Monfardini et al., 1999). The hypothesis of the present study is that the application of 2 different inoculum doses elicits differences of the innate immune response. An effect of 2 different inoculum doses in highly resistant primiparous cows has never been studied before, and is important to have a better insight into the mechanism of innate immune response in these animals.

# MATERIALS AND METHODS

**Experimental animals and study facilities**

All primiparous cows (n = 16) calved within 3 days before arrival at the commercial dairy farm.

**Experimental Design**

Animals were randomly assigned to one of both treatment groups (1 x $10^4$ CFU (group A) or 1 x $10^6$ CFU (group B)) and challenged with *E. coli* P4:O32 on 2 different trial days (8 cows per challenge day, 4 cows per treatment group).

**Sampling Procedure**

Blood and milk samples were collected at d-4, d-1, d0, d+1, d+2 and d+3 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21.

**Determination of cytokines in milk and plasma**

The following cytokines were determined in milk from the affected quarters: IL-8, C5a, sCD14 and LBP. In plasma, only LBP was quantified.

**Statistical Analysis**

In order to compare the two inoculum groups with respect to the various parameters analysed in blood and milk, a mixed model was used with cow as random effect and time, inoculum and their interaction as categorical fixed effects. Furthermore, for the SCC, CFU, RT, IL-8, C5a, sCD14 and LBP, the two inoculum doses were compared at PIH 6, 9 and 12, using Bonferroni's multiple comparisons procedure with an overall type I error equal to 5%. The effect of the inoculum size on the clinical score was tested by the Fisher Exact test.

**For Further Details** see chapter 'Materials and Methods Experimental Infections', **p. 97**.

# RESULTS

**Local and Systemic Inflammatory Response**

Rectal temperature rapidly increased after the intramammary *E. coli* inoculation in both infusion groups and was significantly higher ($P < 0.002$) at PIH 6 and 9 in group B compared to group A. The fever peak appeared 3 h earlier and was highest in group B, which received the 1 x $10^6$ CFU inoculum (Fig. 1a). As indicated by the significant interaction between time and inoculum ($P < 0.0001$), the kinetics of the RT curve significantly differed between the infusion groups. Heart rate and RR followed identical kinetics as RT (results not shown).

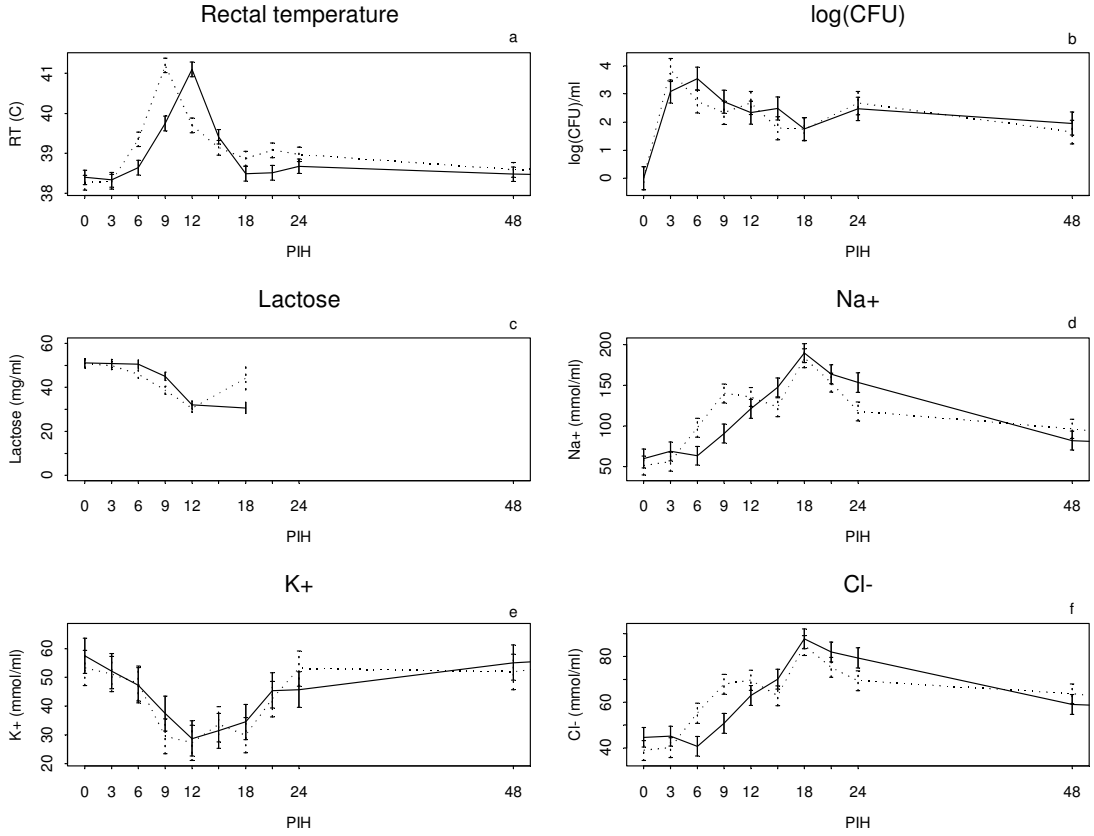


**Figure 1.** Rectal temperature (a), number of CFU *E. coli* P4:O32 (b), lactose (c), sodium (d), potassium (e) and chlorine (f) from PIH 0 until PIH 48 in the infected quarters, respectively, from primiparous cows infused with 1 x $10^4$ (——; group A; n = 8) and 1 x $10^6$ (-----; group B; n = 8) CFU *E. coli* P4:O32. Data are means (± SEM).

Rumen motility was reduced in both infusion groups from PIH 9 onwards. However, the depression of reticulorumen motility was less pronounced in group A with only 3 animals showing a slight decrease in motility (1-2 contractions/2 min) and 1 animal with a complete

absence of reticulorumen activity at PIH 9. In group B, the depression of rumen motility was also maximal at PIH 9: 3 animals with absence of motility and 4 animals showing a slight decrease in rumen motility. Reticulorumen returned to normal motility by PIH 24 in both infusion groups (results not shown).

Local clinical signs at the level of the infected mammary glands appeared early during inflammation. In group B, the first changes in milk appearance (colour, flakes, …) occurred at PIH 9, with a maximum around PIH 15. Swelling of the infected quarters occurred at PIH 6, with a maximum on PIH 9, at the time when PMN influx in the glands became apparent through increases in SCC. Pronounced swelling disappeared gradually, and was already at a low level (6 quarters with moderate swelling) at PIH 72. In the group receiving the low inoculum dose, changes in milk appearance and quarter swelling were equal to group B, but the onset was about 3 h later. Teat relaxation, milk leakage and diarrhoea only appeared in a small number of animals in both infusion groups. Body condition score decreased slightly from 3.5 to 2.5 from arrival at the dairy facility until the end of the intramammary *E. coli* challenge.

**Clinical Severity Scoring**

Based on the clinical severity scoring by Vangroenweghe et al. (2004), clinical changes appeared earlier in group B compared to group A. At PIH 9, 6 animals in group B (n = 8) had a moderate clinical score, whereas in group A (n = 8), only 1 animal reacted moderately, while the others still exhibited a mild response ($P = 0.041$). By PIH 12, only 4 animals in group B (n = 8) reacted moderately, whereas in group A (n = 8) 6 animals showed a moderate response ($P = 0.61$) (Table 1). The clinical score normalised from PIH 15 onward, with only one animal in each group reacting moderately at PIH 15, and none at PIH 18. No severe responses were observed in either of the infusion groups at any time point between PIH 9 and 48.

**Table 1.** Classification of primiparous cows into mild-moderate-severe responders based on the severity scoring system at PIH 9 and 12. The numbers mentioned in the body of the table represent the number of animals having the specified score (sum of clinical parameters; see Materials and Methods Experimental Infections, p. 97) at the respective PIH. In the present study, the total score did not exceed 5, meaning that no severe responses were observed throughout the entire experimental study period.

| PIH | Group | *Mild response* | | | *Moderate response* | | | *Severe response* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| 9 | A | 2 | 2 | 2 | **2** | *0* | *0* | *0* | *0* | *0* |
| | B | *0* | 0 | 1 | **5** | **2** | 1 | *0* | *0* | *0* |
| 12 | A | *0* | 1 | 1 | **5** | **1** | *0* | *0* | *0* | *0* |
| | B | *0* | 2 | 2 | **3** | **1** | *0* | *0* | *0* | *0* |

**Quarter Milk Production**

Milk production in the infected quarters decreased ($P < 0.0001$) on d0, the day of intramammary inoculation. The maximal decrease of 60-70% appeared already on the day of infection (Fig. 2a). Milk production decrease (68%) was slightly more pronounced in group B, with a slightly more rapid recovery on d+1, but no significant differences between the two doses were observed. On d+3, the recovery in the infected quarters was still incomplete and averaged 86 and 76% of the pre-infection milk production in group A and B, respectively.

The milk production decrease in the contralateral uninfected quarters is considered to be an indicator of general systemic illness due to intramammary infection in the left quarters. A moderate and short-termed decrease in the milk production of the contralateral quarters was present in both infusion groups (Fig. 2b). Based on quarter milk production at d+2, no severe responders were observed in the two groups. At d+3, relative to the day of infusion, milk production of these quarters was at 97 and 99% of the pre-infection milk production in group A and B, respectively.

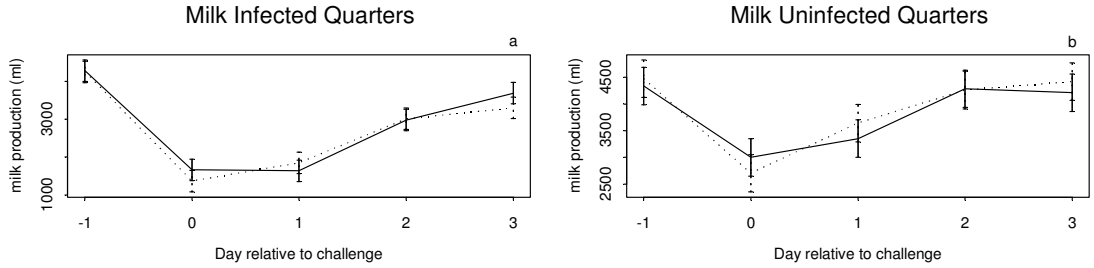


**Figure 2.** Milk production of the infected left (a) and uninfected right quarters (b) from d-1 until d+3 from primiparous cows infused with 1 x $10^4$ (——; group A; n = 8) and 1 x $10^6$ (-----; group B; n = 8) CFU *E. coli* P4:O32. Data are means (± SEM).

**Colony-Forming Units, Somatic Cell Count and Blood Leukocyte Count**

The number of *E. coli* increased to peak values (3.96 and 3.20 $\log_{10}$(CFU)/ml) at PIH 3 and 6 in the high and low dose infusion group, respectively. On average, the number of *E. coli* did not significantly differ between both infusion groups. Furthermore, no significant interaction between time and inoculum dose was found, indicating that the number of *E. coli* changed over time in a similar fashion for the two inoculum doses (Fig. 1b).

Somatic cell count rapidly increased following intramammary *E. coli* infection and was significantly ($P < 0.0001$) higher at PIH 6 in the high dose infusion group compared to the group receiving the low dose. At PIH 9, both infusion groups reached a plateau level of $10^{6.57}$ cells/ml (Fig. 3f). Peak SCC levels were reached at PIH 15 and 18 in group B and A, respectively. Somatic cell count kinetics significantly differed ($P = 0.0005$) between the inoculum groups,

especially due to the earlier cellular influx (PIH 6) in the infected quarters in group B. At PIH 72, SCC did not yet attain pre-infection SCC levels.

Blood leukocyte count decreased after intramammary *E. coli* infection, but kinetics of the blood leukocyte count decrease did not differ significantly between the inoculum groups. Nadir blood leukocyte count was reached at PIH 12 in both infusion groups and normal levels of circulating blood leukocytes appeared at PIH 48 (Fig. 3g).

**Differentiation of Blood Smears**

Following intramammary challenge, marked changes in leukocyte differentiation appeared, resulting in an increased number of early (myelocytes-metamyelocytes) and late (band cells) immature PMN in the blood circulation. Band cells increased from PIH 3 to peak at PIH 12 (group B; 29.1 ± 6.4%) and PIH 15 (group A; 27.2 ± 2.4%). A second peak was observed at PIH 21 (32.3 ± 2.8 and 31.6 ± 4.0% in group A and B, respectively), after which percentage of band cells decreased to normal levels by PIH 216 (Fig. 3i). Early immature cells (myelocytes-metamyelocytes) appeared more frequently in the circulation from PIH 12 (group B) and PIH 15 (group A) onward, with peak values at PIH 18 (group A; 24.1 ± 2.5%) and PIH 21 (group B; 18.8 ± 1.1%). Myelocytes-metamyelocytes gradually decreased in the following 96 h to reach normal levels at PIH 144 to 216 (Fig. 3h).

Mature PMN migrated from the circulation into the infected quarters, which resulted in a decreased percentage from PIH 9 onward in both groups, with the nadir at PIH 15 (1.0 ± 0.6 and 3.5 ± 2.0% in group A and B, respectively). The percentage of circulating mature PMN gradually recovered and reached pre-infection values by PIH 144 (Fig. 3j).

**Milk Composition**

Lactose, sodium, potassium and chlorine are good indicators to assess the presence of intramammary infection. Lactose decreases significantly in group B from PIH 6 onward, to nadir at PIH 12 in both groups. In group A, the disruption of the blood-milk barrier appears 3 h later with a decrease in lactose content at PIH 9 (Fig. 1c). Similarly, $Na^+$ and $Cl^-$ concentrations in group B increased from PIH 6 to peak a first time at PIH 9. Following a slight decrease at PIH 15, a second peak was reached at PIH 18. In group A, infused with 1 x $10^4$ CFU per quarter, the onset of increased concentrations of $Na^+$ and $Cl^-$ appeared at PIH 9, with maximal concentrations at PIH 18 (Fig. 1d and f). In contrast with the observed changes in $Na^+$ and $Cl^-$ concentrations, $K^+$ kinetics did not significantly differ between both infusion groups. Nadir was reached at PIH 12, followed by a slow restoration of initial $K^+$ levels by PIH 48 (Fig. 1e).

**Milk IL-8 and C5a**

Before challenge, IL-8 was very low (2.19 ± 0.93 and 1.89 ± 0.84 pg/ml in group A and B, respectively) in the quarters that were to be infused with *E. coli*. Interleukin-8 kinetics of the two infusion groups differed significantly from each other. In group B, a significant increase in IL-8 appeared as early as PIH 6 and reached a peak of 305 ± 36 pg/ml at PIH 12 (Fig. 3a). At PIH 6 and 9, the concentration of IL-8 in the inflamed quarters was significantly ($P < 0.05$) higher in group B, receiving the 1 x $10^6$ CFU inoculum dose. Following infusion of 1 x $10^4$ CFU (group A), significant increases in IL-8 occurred at PIH 9 and reached peak levels of 240 ± 40 pg/ml at PIH 12. Elevated levels of IL-8 persisted until PIH 24 in both groups, after which the levels returned to baseline.

The complement component C5a had similar kinetics in both groups. The initial increase in C5a following intramammary challenge appeared at PIH 6 (± 1.5 ng/ml), and rapidly reached maximal values of 37.1 ± 16.0 ng/ml and 30.6 ± 12.4 ng/ml at PIH 12 for group A and B, respectively. Elevated levels of C5a persisted until PIH 24 in both groups, after which the levels returned to baseline (Fig. 3b).

**Milk sCD14**

To determine whether *E. coli* could alter mammary gland levels of sCD14, a sandwich ELISA was used to quantitate milk sCD14 (Fig. 3c). Before challenge, sCD14 in mammary quarters (7.7 ± 2.7 μg/ml and 10.4 ± 3.1 μg/ml for group A and B, respectively) was in the range previously described (Lee et al., 2003a) for early lactating uninfected glands (5.46 to 6.90 μg/ml). At PIH 6, a significant increase in milk sCD14 was observed in group B, whereas in group A, the increase in milk sCD14 only appeared at PIH 9. Concentrations of sCD14 were significantly ($P < 0.0001$) different between the two groups at PIH 12. Milk sCD14 peaked at PIH 15 in both groups, although the level of sCD14 was significantly ($P = 0.0323$) higher (205 ± 44 μg/ml) in group B compared to group A (151 ± 54 μg/ml) (Fig. 3c).

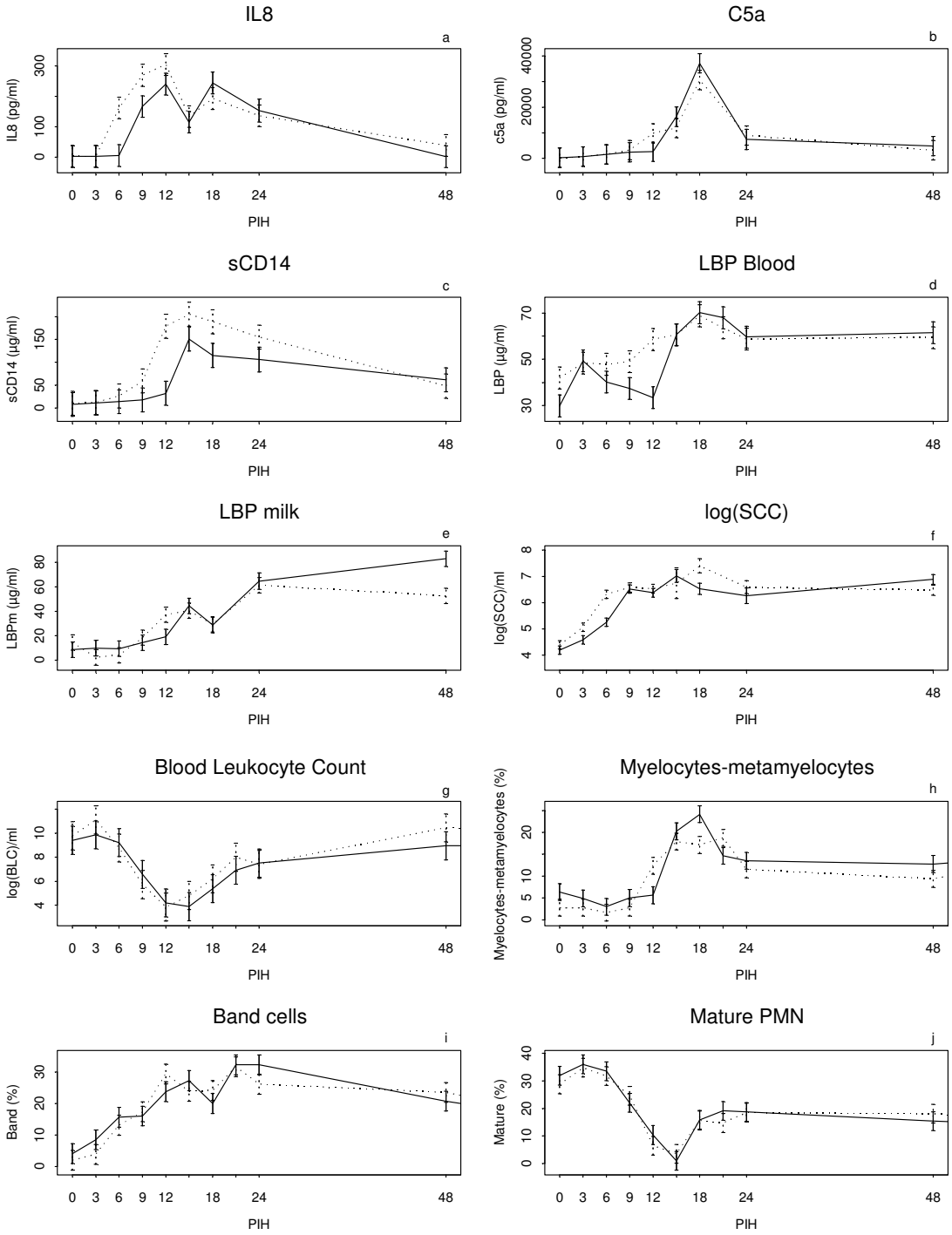


**Figure 3.** Interleukin-8 (a), C5a (b), sCD14 (c), plasma LBP (d), milk LBP (e), SCC (f), blood leukocyte count (g), % myelocytes-metamyelocytes (h), % band cells (i) and % mature PMN (j) from PIH 0 until PIH 48 in the infected quarters or blood, respectively, from primiparous cows infused with 1 x $10^4$ (——; group A; n = 8) and 1 x $10^6$ (-----; group B; n = 8) CFU *E. coli* P4:O32. Data are means (± SEM).

**Plasma and Milk LBP**

It has been shown previously (Bannerman et al., 2003) that elevated levels of sCD14 are associated with similar increases in milk LBP, thereby providing an environment for optimal host recognition of LPS, originating from infused *E. coli* bacteria. Plasma LBP was also assayed as it is known that hepatic synthesis of this protein increases during the acute phase response, mainly due to hepatic cell stimulation by IL-1β and IL-6 (Tobias et al., 1999). Under basal conditions, LBP was detected in bovine blood at concentrations of 29.8 ± 7.5 μg/ml and 41.9 ± 5.4 μg/ml for group A and B, respectively; whereas the concentration of LBP in milk was lower with a 8.5 ± 5.1 μg/ml and 14.5 ± 11.5 μg/ml for group A and B, respectively. At PIH 9, plasma LBP in group B increased and this increase persisted throughout the entire study period (Fig. 3d). Plasma LBP levels reached maximal levels of 70.1 ± 1.5 and 68.8 ± 1.1 μg/ml at PIH 18, after which they slightly declined to plateau until the end of the study. In quarters inoculated with *E. coli*, significant elevation of milk LBP was observed as early as PIH 9, resulting in a first peak at PIH 15 (44.4 ± 10.6 and 40.4 ± 8.7 μg/ml in group A and B, respectively). Milk LPB was significantly ($P = 0.0431$) higher in group B at PIH 12. A second peak (61.6 ± 12.3 and 61.2 ± 12.0 μg/ml in group A and B, respectively) was reached at PIH 24, after which milk LBP levels remained high until the end of the study (Fig. 3e). Peak levels of milk LBP were observed 6 h later than the maximal elevation of plasma LBP levels.

# DISCUSSION

In this study, the same strain and range of high inoculum doses of *E. coli* was used to induce *E. coli* mastitis as previously described (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000; Dosogne et al., 2002; Vangroenweghe et al., 2004). The outcome of experimental *E. coli* inoculation was expected based on the results obtained in a previous study with primiparous cows under identical conditions (Vangroenweghe et al., 2004). In the present study, none of the animals reacted severely following intramammary *E. coli* challenge. Animals were scored as described by Vangroenweghe et al. (2004), which resulted in a similar classification of mild and moderate responses. Interestingly, the time of latency to become moderately ill was approximately 3 h shorter in group B compared to group A.

The number of circulating PMN, a marker to predict the clinical outcome of the disease, was similar to the levels previously observed in moderate responders (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; van Werven et al., 1997; Vangroenweghe et al., 2004). Primiparous cows in this study could therefore be expected to react with a moderate clinical response. Following intramammary *E. coli* challenge, a rapid decrease of circulating PMN was observed, which coincided with the influx of PMN into the infected quarters and started around PIH 6-9. No significant difference in blood leukocyte count could be observed between both infusion groups, whereas for SCC a significantly earlier influx was observed in the high dose inoculum group.

The onset of local clinical signs of mastitis, characterised by quarter swelling, coincided with the influx of PMN to the infected quarters. Somatic cell count increased rapidly in both groups, which is in accordance with earlier observations, where moderate responders had a rapidly occurring leukocytosis in the infected glands (Vandeputte-Van Messom et al., 1993). However, in the present study, distinct differences in the onset of PMN influx into the infected glands could be observed. The extraction of mature PMN from the blood to the infected glands is known to result in early and late immature PMN recruitment from the bone marrow to restore the number of circulating PMN. Recently, IL-8 has been shown to be responsible for rapid granulocytosis with the release of PMN from the bone marrow (Terashima et al., 1998). In the present study, IL-8 was released through activation of the mammary epithelial cells from PIH 6-9, which coincided with the appearance of significantly increased numbers of band cells in the blood circulation. However, the recruitment of PMN was of short duration, compared to Heyneman et al. (1990), who observed immature forms in circulation for at least 3 d in moderate responders and for almost 10 d in severe responders.

The rapid influx of PMN into the infected glands was associated with rapid elimination of bacteria from the quarters. In the present study, high inoculum doses were used for experimental induction of *E. coli* mastitis, because we were mainly interested in bacterial elimination rather than bacterial growth in the mammary gland. In contrast to an earlier study with the same inoculum dose in multiparous animals (Vandeputte-Van Messom et al., 1993), peak bacterial numbers were already reached around PIH 3 to 6, which was in accordance with a previous study in primiparous cows (Vangroenweghe et al., 2004). This peak number of bacteria was followed by a rapid elimination from the infected glands. Contrary to the induction of *E. coli* mastitis with low inoculum doses, where elimination is preceded by excessive bacterial growth (Shuster et al., 1996; 1997; Riollet et al., 2000; Scaletti et al., 2003), in this study, peak numbers were reached within 6 h post-infection and followed by a subsequent bacterial elimination. Therefore, PMN influx rapidly cleared the bacteria from the affected glands. It can be presumed that the bactericidal capacity of the PMN that migrated to the infected quarters was high, because more efficient PMN functionality has recently been reported in primiparous cows (Mehrzad et al., 2002).

Several indicators (lactose, sodium, chlorine and potassium) for the presence of mastitis were determined in this study. The changes appeared significantly earlier (approximately 3 h) in group B, receiving the 1 x $10^6$ CFU inoculum dose, although peak levels were almost identical for both groups, which coincides with all other data, indicating that the animals reacted with a mild to moderate response and little variation in clinical response was present in this study.

Complement component C5a and its derivative $C5a^{desArg}$ play a potential role in the inflammatory response accompanying mastitis (Rainard et al., 1998). The level of C5a/$C5a^{desArg}$ on the day of challenge was within the range previously described by Rainard et al. (1998), taking into account that the average SCC immediately before intramammary infusion was a little higher (approximately 90,000 cells/ml *vs.* 25,000 cells/ml). In the present study, increased levels of C5a/$C5a^{desArg}$ could be observed from PIH 3 onwards, which is in accordance with an earlier study (Rainard et al., 1998), although in this study the induction of inflammatory symptoms was performed with *E. coli* endotoxin and not with live bacteria. The assessment of $C5a^{desArg}$ concentrations in milk following infusion of *E. coli* endotoxin or live bacteria showed that biologically significant amounts of C5a/$C5a^{desArg}$ are present in mastitis milk. The bulk of complement-derived components in mastitis milk are likely to have their origin in blood plasma, exudating through the damaged blood-milk barrier following inflammation of the mammary gland (Rainard et al., 1998). However, milk concentrations of C5a/$C5a^{desArg}$ are probably not a reliable indicator of exudation, as C5a/$C5a^{desArg}$ is rapidly taken up by several cell types present in inflamed milk, in particular PMN (Rainard et al., 1998). Major elimination mechanism of this

inflammatory mediator is believed to rely on the binding of C5a/C5a$^{desArg}$ to cell surface receptors. As milk from inflamed quarters contained high concentrations of cells, mainly PMN, it can be put forward that the concentrations of C5a/C5a$^{desArg}$ measured in milk were underestimates of the total amounts of C5a/C5a$^{desArg}$ which had originally been generated. Complement component C5a generated in milk could well contribute to the activation of the recruited phagocytic cells, with the consequence of improving their bactericidal activity (Rainard, 2003).

Interleukin-8 is considered to play an important role in PMN recruitment to the inflamed quarters (Baggniolini and Clark-Lewis, 1992; Barber and Yang, 1998). In contrast to Shuster et al. (1997), who suggested the importance of C5a to be greater than IL-8 during the early inflammatory response mainly due to earlier peak maxima, in the present study, peak levels of IL-8 appeared 6 h earlier than C5a. The increase of IL-8 chemotactic activity appears coincident with the increment in SCC at the level of the infected quarters. The increase in C5a also appeared as early as PIH 3-6, but its peak maximum was only reached at PIH 18. The induction of IL-8 production and release is known to be independent of the presence of IL-1β and TNF-α (Persson-Waller et al., 2003). Induction of IL-8 is rather associated with the formation of the tripartite LPS-LBP-sCD14 complex, that subsequently activates the mammary gland epithelial cells through a cell surface assembly of a multi-protein recognition complex consisting of CD14, TLR-4 and MD-2 (Akashi et al., 2000), resulting in the activation of the NF-κB controlled IL-8 gene (Aderem and Ulevitch, 2000).

Detectable increases in milk LBP were observed after initial increases in blood LBP, and maximal levels of LBP in milk were observed at PIH 24, some 6 h after the peak levels of plasma LBP. In accordance to Bannerman et al. (2003), the increases in milk LBP paralleled increments in sCD14 levels. From a host perspective, the simultaneous increase in both LBP and sCD14 levels would be expected to be advantageous as both molecules act in concert to facilitate activation of host defence mechanisms by presenting LPS, released during bacterial growth and death, to the transmembrane LPS receptor, TLR-4 (Bannerman and Goldblum, 2003). Interestingly, the increase in IL-8 already started at PIH 6 in group B, receiving the 1 x $10^6$ CFU inoculum dose, whereas significant increases of sCD14 and LBP only appeared from PIH 9, suggesting that initial host cell activation can take place in the presence of basal levels of sCD14 and LBP. Although the increase in sCD14 was earlier and significantly higher in group B, PMN influx as determined by SCC in the infected quarters was similarly elevated before pronounced increment of SCD14. It was proposed that heightened levels of these molecules are not required for immediate host innate immune responses (Bannerman et al., 2003). However, peak levels of SCC were not observed until PIH 15-18, at a time when levels of both sCD14 and LBP in milk

were elevated. Whether increments in sCD14 and LBP are necessary for maximal recruitment of PMN to the inflamed mammary glands remains unknown.

From this study, it appears that the inflammatory response in primiparous cows from group B has an earlier onset compared to group A. One possible explanation for this observation could be the 100-fold difference in the number of *E. coli* infused into the mammary glands, because the amount of LPS produced is related to the number of *E. coli* bacteria (Burvenich, 1983; Monfardini et al., 1999). Because the bacterial cultures were washed three times in pyrogen-free PBS before further dilutions were made, a direct effect of LPS present in the inoculum can be excluded. Lipopolysaccharide, known as a potent inducer of inflammatory cytokines (Shuster et al., 1993), can be produced quite rapidly during bacterial growth following intramammary infusion. In the present study, the increase in sCD14 and LBP, which are known to bind to LPS, facilitated the release of IL-8 from mammary endothelial cells in the infected quarters. Moreover, the infused bacteria rapidly activated the complement system through the alternative pathway, resulting in an early abundant production of C5a. The combined effects of these events resulted in a rapid attraction of PMN from the blood into the mammary gland, with a subsequent pronounced increase of SCC in the infected glands.

The novelty of the present study is the fact that primiparous cows, recently shown to react as mild to moderate responders (Vangroenweghe et al., 2004), are responding faster following an 100-fold increase in the inoculum dose. This quicker response is related to an earlier activation of innate host immunity.

Following *E. coli* mastitis, treatment with antibiotics at PIH 10 did not alter local and systemic symptoms despite a 100-fold decrease in CFU (Monfardini et al., 1999). From the present study, it is evident that at the set time point, antibiotic treatment is unable to alter the production or release of various inflammatory mediators. Therefore, it can be suggested that early inflammatory events (first 3 h) could play an major role in the further regulation of the inflammatory response to combat the invading pathogens.

# CONCLUSIONS

Despite the use of relatively high inoculum doses, primiparous cows react with a moderate inflammatory response following intramammary *E. coli* infusion. This moderate response was evident from the pre-infection number of circulating leukocytes, the prompt clinical response, the rapid influx of PMN into the infected quarters, the efficient bacterial clearance of the affected glands and the fast recovery of milk production in both infected and

uninfected glands. In the present study, the difference in time of latency between both inoculum doses could be confirmed and documented with kinetics of various inflammatory mediators such as sCD14, LBP, IL-8 and C5a. The early increase in IL-8 following activation of the mammary gland epithelium appeared before increases in sCD14 or LBP, indicating that innate host cell activation can occur in the presence of basal levels of sCD14 and LBP. Although C5a increased during early innate host immune response, maximal levels were reached after IL-8 had peaked. In conclusion, primiparous cows were able to efficiently clear an intramammary *E. coli* infection and the increase in inoculum dose induced a more rapid clinical response, mainly due to the earlier activation of the innate host immune response. To further elucidate the regulation of early events, mammary gland biopsies and milk sample collection during the early phase of inflammation should be performed.

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# 2.

# INHIBITION OF PROSTAGLANDIN SYNTHESIS

# INTRODUCTION

Production costs attributed to mastitis are the largest economic loss to the dairy industry. Mastitis caused by coliform bacteria is responsible for a major portion of these losses, predominantly as a result of acute disease. Coliform mastitis in cattle may be associated with systemic clinical disease, which occurs predominantly in the period immediately after calving (Hill, 1981; Wilesmith et al., 1986; Burvenich et al., 2003). Systemic signs include general depression, fever, tachycardia, inhibition of reticulorumen motility, and many non-specific responses, such as neutropenia, followed by leukocytosis (Verheijden et al., 1983). Considerable losses in milk production may occur in cows suffering from *E. coli* mastitis (Hill, 1981; Lohuis et al., 1989; Dosogne et al., 1997; Hoeben et al., 2000). Inhibition of reticulorumen motility is a frequent complication of *E. coli* mastitis (Verheijden et al., 1983) and as a result the microbial degradation of ingested feed is disturbed and the milk yield is reduced. Moreover, marked decreases in plasma tyrosine levels occur (Vandeputte-Van Messom et al., 1987), which are not directly related to peak fever.

The pathophysiology of *E. coli* mastitis is characterised by the presence of endotoxin or LPS in the outer membrane of the etiological bacteria. Lipopolysaccharide is a pro-inflammatory molecule that is shed from the bacterial surface during bacterial replication or death (Burvenich et al., 2003). Clinical signs following experimentally induced *E. coli* mastitis are attributed to mediator shock rather than to endotoxin shock, because endotoxin mainly plays a local role (Hoeben et al., 2000; Dosogne et al., 2002). Several of the pro-inflammatory cytokines that mediate the localised and systemic response to Gram-negative mastitis, including IL-1β, IL-6, IL-8, and TNF-α, are upregulated by LPS (Shuster et al., 1993; Guha and Mackman, 2001). Binding of LPS to cell membranes also activates the membrane-bound enzyme, phospholipase A2, liberating arachidonic acid which can be metabolised by two major enzyme systems, COX and lipoxygenase. The prostaglandins, prostacyclin and thromboxanes are the products of the cyclo-oxygenase pathway, whereas the leukotrienes are synthesised following lipoxygenase enzyme activity. The eicosanoids are important mediators and modulators of inflammation, and play a role in the mediation of acute inflammation (Anderson, 1989; Rose et al., 1989). Intracerebroventricular injection of 50 to 200 μg $PGE_2$ in lactating goats elicited fever in normal lactating goats, the increment and decrement stage being accompanied respectively by a decrease and an increase of mammary blood flow (Burvenich et al., 1982). Pre-treatment with flurbiprofen, administered through intracerebroventricular cannulation, inhibited fever and changes in HR following experimentally induced LPS mastitis, but had little effect on the $Cl^-$

concentration in the affected mammary gland. Nevertheless, the first peak in the mammary blood flow curve occurred with some delay, whereas the second peak was clearly decreased (Burvenich, 1985). Intravenous pre-treatment with flurbiprofen totally abolished fever and delayed the changes in milk ion concentration (Burvenich et al., 1989). It is also known that the release of plasma cortisol after intravenous LPS administration is partially controlled by prostaglandins (Massart-Leën et al., 1992).

Cyclo-oxygenase has two isoforms, the COX-1, which is constitutively expressed in most tissues and is responsible for maintenance of various physiological processes, such as normal body temperature, and the inducible COX-2, a pro-inflammatory enzyme. Induction of COX-2 synthesis is stimulated by several cytokines (IL-1, TNF-α), growth factors and LPS (Lees et al., 2000). Nearly all currently used veterinary NSAID's are non-selective COX-1 and COX-2 inhibitors. It was recently demonstrated in a rat model of inflammation that the acute early peak expression of COX-2 which was pro-inflammatory, was followed by a second, larger increase of COX-2 expression, being anti-inflammatory. Cyclo-oxygenase-2 inhibitors, therefore, inhibited inflammation at the early stage, but significantly exacerbated inflammation later on at 48 h (Gilroy et al., 1999). However, there is considerable interest in the development of specific COX-2 inhibitors for therapeutic use. The COX antagonists currently used in veterinary medicine are all regarded as non-specific inhibitors of COX-1 and 2, with similar potencies against both enzymes, or even some selectivity for COX-1 inhibition (Lees et al., 2000). Exceptions are nimesulide, carprofen and meloxicam, which have a partial selectivity for COX-2 inhibition and may be described as preferential COX-2 inhibitors (Lees et al., 2000).

A role for sCD14 and LBP in mediating bovine host responses to intramammary LPS or *E. coli* challenge has recently been demonstrated (Wang et al., 2002; Bannerman et al., 2003; Lee et al., 2003a; 2003b). Following intramammary LPS infusion, sCD14 increases in milk (Bannerman et al., 2003; Lee et al., 2003a) are paralleled by an increase in LBP (Bannerman et al., 2003). Moreover, sCD14 has been shown to sensitise the mammary gland to LPS (Wang et al., 2002) and to reduce the severity of experimental *E. coli* mastitis in mice (Lee et al., 2003c) and cows (Lee et al., 2003b). Interestingly, maximal levels of the chemoattractant IL-8 and C5a were observed before increases in either milk LBP or sCD14. This suggests that initial host cell activation can occur in the presence of basal levels of sCD14 and LBP (Bannerman et al., 2003; Vangroenweghe et al., 2004b). Furthermore, PMN influx as determined by SCC, was similarly elevated before increases in sCD14 and LBP, indicating that heightened levels of these molecules were not required for immediate host innate immune responses (Bannerman et al., 2003; Vangroenweghe et al., 2004b).

Non-steroidal anti-inflammatory drugs have been reported to alter the clinical course of *E. coli* or endotoxin-induced mastitis in the bovine. A distinct difference has to be made between their potential antipyretic and antiphlogistic activity. Various NSAID's have different physico-chemical characteristics, which result in unequal tissue distribution throughout the body and cause variations in pharmacokinetics, efficacy and toxicity among species (Brune, 1990). In addition, the mechanism of action of NSAID's may vary by selective inhibition of the synthesis of a particular class of prostaglandins and endoperoxides and by inhibition of biochemical reactions, in addition to COX inhibition (Kopcha and Ahl, 1989; Kopcha et al., 1992). The use of NSAID's has been suggested as an adjunctive or alternative therapy to systemic or intramammary antibiotics (Shpigel et al., 1994).

The effect of NSAID's has been studied in experimental coliform and endotoxin-induced mastitis models. These NSAID's have included flunixin meglumine (Anderson et al., 1986; Anderson, 1989; Lohuis et al., 1989), carprofen (Lohuis et al., 1991), flurbiprofen (Burvenich and Peeters, 1982; Vandeputte-Van Messom et al., 1987; Burvenich et al., 1989; Lohuis et al., 1989), ketoprofen (Ziv and Longo, 1991), ibuprofen (DeGraves and Anderson, 1993), indomethacine (Burvenich and Peeters, 1982), suprofen (Burvenich and Peeters, 1982) and meloxicam (Banting et al., 2000). In these studies, NSAID's positively affected various clinical, hematological, biochemical and pathophysiological parameters. Recently, ketoprofen, phenylbutazone and dipyrone have been shown beneficial in the treatment of field cases of clinical mastitis (Shpigel et al., 1994; 1996). All NSAID's in this study were used as an adjunctive therapy in combination with the administration of a systemic antibiotic, trimethoprim-sulphonamide.

Carprofen ((±)-6-chloro-α-methylcarbazole-2-acetic acid) is an NSAID that is well tolerated in the bovine (Ludwig et al., 1989). In healthy cows, carprofen is pharmacokinetically characterised by a small distribution volume (0.09 l/kg), a relatively low systemic clearance (2.4 ml/h/kg) and a long elimination half-life (30.7 h) (Lohuis et al., 1991). During an endotoxin-induced mastitic episode, systemic clearance decreased, whereas elimination half-life significantly increased (43.0 h). Following carprofen treatment at 2 h post-challenge, a significant reduction in severity of clinical parameters was observed.

Until now, few reports have been published on the treatment of experimentally induced *E. coli* mastitis with NSAID's and the effects of these drugs have been mainly assessed for clinical symptoms and much less for laboratory parameters and eicosanoids. The objective of the present study was to examine the modulatory effect of carprofen treatment on different clinical, blood and milk parameters following moderate inflammation in primiparous cows.

# MATERIALS AND METHODS

### Experimental Animals and Study Facilities

All primiparous cows (n = 25) calved within 7 days before arrival at the commercial dairy farm.

### Inoculation Dose

Primiparous cows were inoculated with 1 x $10^4$ CFU *E. coli* P4:O32 in both left quarters.

### Sampling Procedure

Blood and milk samples were collected at d-4, d-1, d0, d+1, d+2, d+3 and d+6 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21.

### Determination of Inflammatory Cytokines and Eicosanoids

Two specific eicosanoids, $PGE_2$ and $TXB_4$, were quantified besides IL-8, C5a, sCD14 and LBP in order to assess for the effect of NSAID treatment following intramammary *E. coli* challenge.

### Statistical Analysis

In order to compare the two treatment groups with respect to the various parameters analysed in blood and milk, a mixed model was used with cow as random effect and treatment, time and their interaction as categorical fixed effects. Furthermore, the two treatment groups were compared at PIH 12 for all analysed parameters and some determined parameters were tested at post-treatment intervals PIH 12-18 (RT, HR, blood leukocyte number and PCV), PIH 12-48 (IL-8, C5a, sCD14, LBP, $PGE_2$ and $TXB_2$) and PIH 12-72 (SCC, CFU, lactose, $Na^+$, $K^+$, $Cl^-$ and serum albumin), respectively, for significant differences between both treatment groups. Bonferroni's multiple comparisons procedure with an overall type I error equal to 5% was used to adjust for multiple comparison. Quarter milk production in both infected and uninfected quarters were tested for significant differences between treatments at d+1 and the interval d+1 - d+3. The effect of treatment on the local aspects of the mammary gland was tested by the Wilcoxon rank sum test.

**For Further Details** see chapter 'Materials and Methods Experimental Infections', **p. 97**.

# RESULTS

**Local and Systemic Inflammatory Response**

Following intramammary *E. coli* inoculation, RT rapidly increased from PIH 9 onward, to reach its maximum at PIH 12 in the saline treated group. Carprofen administration immediately reduced RT significantly ($P < 0.0001$) at 3 and 6 h post-treatment, and RT normalised at PIH 15 (6 h post-treatment). No relapses of RT increase were observed in the carprofen treated group, whereas in the saline treated group, RT increased above 39°C again at PIH 24 (Fig. 1a).

Heart rate followed almost identical kinetics as described for RT. In the carprofen treated group, HR was significantly ($P < 0.05$) lower at PIH 12, 15 and 18. From 9 h post-treatment onward, HR in the carprofen treated animals remained lower, although not significant, until the end of the observation period (Fig. 1b).

Carprofen treatment following intramammary *E. coli* inoculation had a beneficial effect on the duration of reticulorumen motility depression. Although reticulorumen motility was equally depressed in both treatment groups at PIH 9 (time of treatment), the motility increased following carprofen treatment ($P < 0.01$), whereas in the saline treated group, depression of reticulorumen motility reached its maximum at PIH 12 (Fig 1c).

Local clinical symptoms at the level of the mammary gland were also assessed during clinical examination. Local swelling appeared at PIH 6 and reached its maximum at PIH 12 in both treatment groups. In the carprofen treated animals, quarter swelling decreased from PIH 18 and normal quarter consistency was present at PIH 144. Quarter swelling disappeared more slowly in control animals, although normal quarter consistency at palpation was also reached by PIH 144. Maximal quarter swelling score was significantly lower ($P < 0.05$) in the carprofen treated animals. Milk appearance, quarter milk leakage scores and maximum milk leakage scores were significantly ($P < 0.05$) higher in the carprofen treated group at PIH 12. Quarter pain and temperature did not differ between both treatment groups (results not shown).

**Clinical Severity Scoring**

Based on the clinical severity scoring by Vangroenweghe et al. (2004a), clinical scores increased to a maximum at PIH 9 and 12 in the carprofen and saline treated animals, respectively. The clinical severity score in the carprofen treated group decreased more rapidly from 3 h post-treatment onwards. At PIH 12 ($P < 0.0001$) and 15 ($P < 0.01$), clinical severity score was significantly lower in the carprofen treated group compared to the saline treated group.

At PIH 9, the time of treatment, approximately equal numbers of animals in both treatment groups displayed a mild and moderate response. Following carprofen administration, all animals (n = 12) responded mildly (score 0-2), whereas in the saline treated group, 6 animals reacted with a moderate response (score 3-5) and only 7 animals had a mild response. By 6 h post-treatment, 10 animals in the carprofen treated group had a score 0, whereas in the other group, only 3 animals had score 0 and the others respectively scored 1 (n = 4), 2 (n = 5) or 3 (n = 1). During the following observations, the clinical severity score in both groups seemed to normalise with only a slight flare-up of 3 animals (score 2) in the saline treated group at PIH 24.

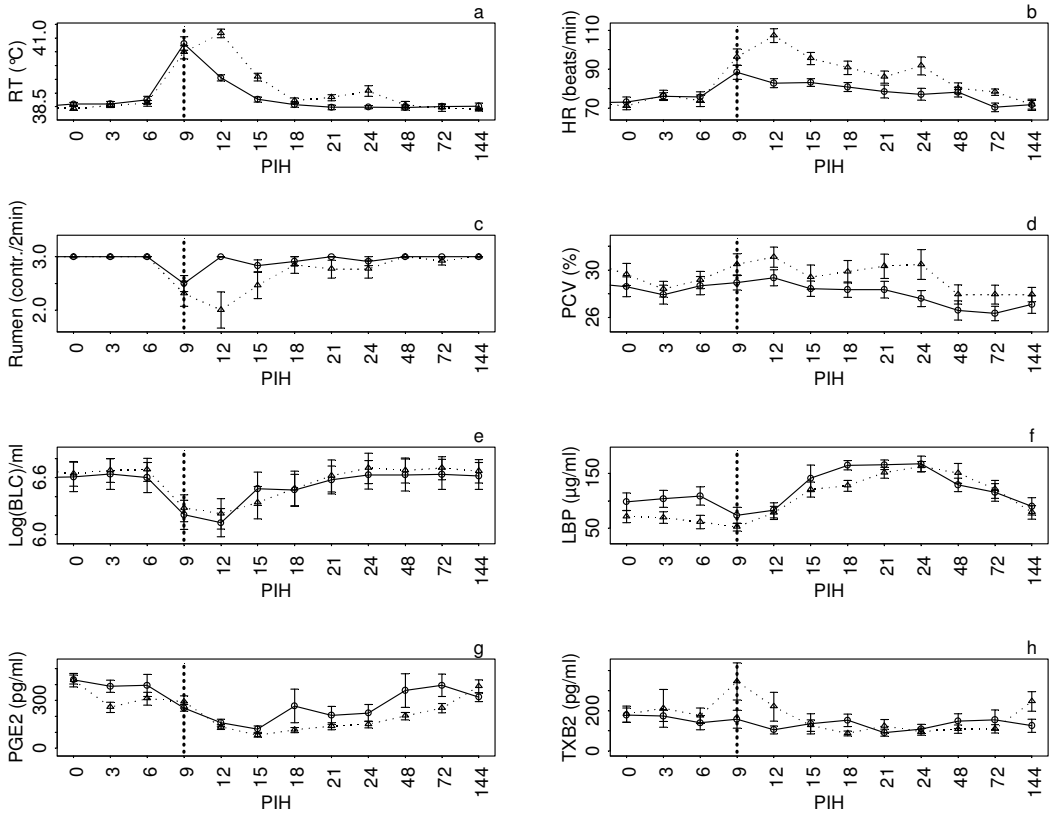


**Figure 1.** Rectal temperature (a), heart rate (b), reticulorumen motility (c), packed cell volume (d), blood leukocyte count (e), plasma LPB (f), plasma $PGE_2$ (g) and plasma $TXB_2$ (h) from PIH 0 until PIH 144 from primiparous cows infused with 1 x $10^4$ CFU *E. coli* P4:O32 and treated at PIH 9 (dashed vertical line) with carprofen (——; n = 12) or saline (-----; n = 13). Data are means (± SEM).

**Quarter Milk Production**

Milk production in the infected quarters decreased equally on d0, the day of intramammary *E. coli* challenge, in both treatment groups. No significant effect of carprofen treatment on the recovery of milk production in the infected quarters could be observed (Fig. 2a).

In the uninfected control quarters, no significant differences in milk production could be observed throughout the entire study period (Fig. 2b). As expected, none of the animals in both

treatment groups reacted as severe responder, based on the quarter milk production of the uninfected quarters at d+2 compared to the quarter milk production in these quarters at d-1 (Vandeputte-Van Messom et al., 1993). This is in agreement with recent findings that primiparous cows react moderately following intramammary *E. coli* challenge (Vangroenweghe et al., 2004a; 2004b).

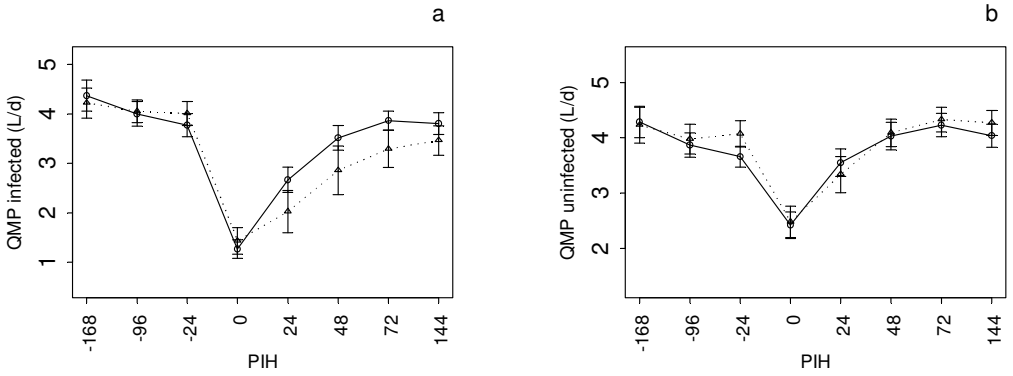


**Figure 2.** Quarter milk production in the infected (a) and uninfected (b) quarters from PIH –168 until PIH 144 from primiparous cows infused with 1 x $10^4$ CFU *E. coli* P4:O32 and treated at PIH 9 with carprofen (——; n = 12) or saline (-----; n = 13). Data are means (± SEM).

**Intramammary Growth of the Inoculated *E. coli*, Somatic Cell Count, Packed Cell Volume and Blood Leukocyte Count**

The number of *E. coli* increased to plateau (4.07 and 3.89 $\log_{10}$(CFU)/ml) at PIH 6. This plateau was followed by a slow, but steady decrease in both treatment groups. No significant differences in number of *E. coli* (PIH 12-48) could be observed throughout the entire experimental period (Fig. 3a). Somatic cell count increased during the early phase of inflammation, although SCC only exceeded 1 x $10^6$ cells/ml at PIH 9 in both treatment groups. Following treatment at PIH 9, no significant differences in SCC (PIH 12-48) could be observed throughout the entire experimental period (Fig. 3b)

A decrease in blood leukocyte number occurred from PIH 9 onward, and nadir blood leukocyte number was reached at PIH 12. This was followed by a steady increase to pre-infection levels at PIH 24 (Fig. 1e). Blood leukocyte count did not differ significantly between the two treatment groups during the period immediately following intravenous carprofen treatment (PIH 12-18).

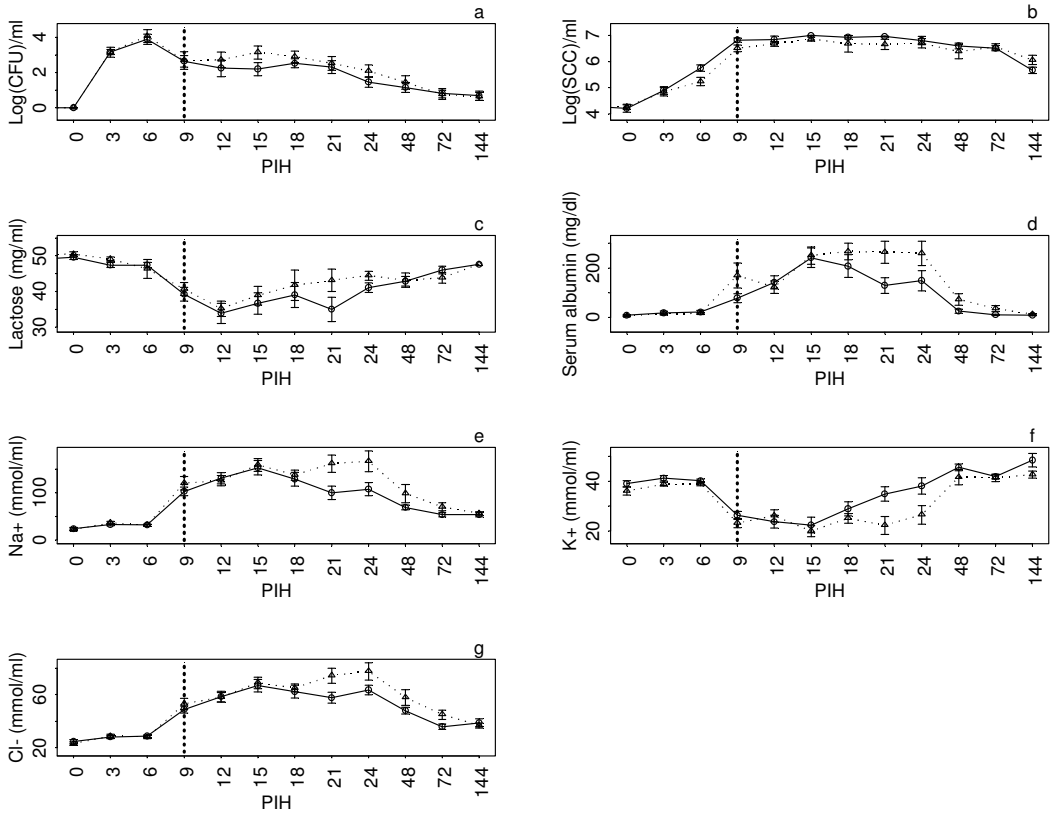


**Figure 3.** Colony-forming units of *E. coli* (a), SCC (b), lactose (c), serum albumin (d), sodium (e), potassium (f) and chlorine (g) in the infected quarters from PIH 0 until PIH 144 from primiparous cows infused with 1 x $10^4$ CFU *E. coli* P4:O32 and treated at PIH 9 (dashed vertical line) with carprofen (——; n = 12) or saline (-----; n = 13). Data are means (± SEM).

**Milk Composition**

Lactose, serum albumin, sodium, potassium and chlorine are indicators of changes in milk composition following mastitis (Burvenich, 1983). Following *E. coli* challenge, the lactose concentration started to decrease at PIH 9, reaching the nadir at PIH 12 (3 h post-treatment) in both treatment groups. No significant differences (PIH 12-48) in lactose concentration were observed following carprofen treatment (Fig. 3c). Maximal concentrations of serum albumin in milk were reached at PIH 15. In the interval PIH 12-48, serum albumin concentration showed a significant interaction between time and treatment ($P$ = 0.013), meaning that serum albumin kinetics were different between both groups. At PIH 21, carprofen treated animals had significant ($P$ = 0.0099) lower concentrations of serum albumin in the milk of the affected quarters (Fig. 3d).

The $Na^+$ concentration was significantly lower in the carprofen treated animals at PIH 21 ($P$ = 0.005) and PIH 24 ($P$ = 0.007), whereas the $K^+$ concentration was significantly higher in the carprofen treated animals at PIH 21 ($P$ = 0.002) and PIH 24 ($P$ = 0.005) (Fig. 3e and f). The $Cl^-$

concentration just failed to be significantly different at PIH 21 ($P$ = 0.013 compared to Bonferroni adjusted comparisonwise significance level of 0.05/6 = 0.0083) (Fig. 3g).

**Milk IL-8, C5a and sCD14**

Before challenge, IL-8 was very low (1.66 ± 0.23 and 2.11 ± 0.35 pg/ml in the saline and carprofen treated groups, respectively) in the quarters that were to be infused with *E. coli*. Until PIH 9, the time of treatment, IL-8 kinetics did not differ between both treatment groups. Peak concentrations (471 ± 50 pg/ml and 389 ± 61 pg/ml for the saline and carprofen treated group, respectively) were reached in the period PIH 12-15. No significant differences between both treatments could be observed throughout the study period (Fig. 4a).

The complement component C5a had similar kinetics in both treatment groups until PIH 18, 9 h post-treatment. From PIH 21, C5a concentration decreased in the carprofen treated animals, whereas animals in the saline treated groups reached peak C5a levels at PIH 24, followed by a decrease at PIH 48, but no significant differences between the two groups were observed. At PIH 72, C5a levels in both groups reached similar values (Fig. 4b).

To determine whether carprofen treatment following intramammary *E. coli* challenge could alter mammary gland levels of sCD14, an ELISA was used to quantitate milk sCD14. Before challenge, sCD14 in mammary quarters (7.33 ± 1.72 μg/ml and 3.77 ± 0.70 μg/ml in saline and carprofen treated animals, respectively) was in the range previously described (Lee et al., 2003a) for early lactating uninfected glands (5.46 to 6.90 μg/ml). In both treatment groups, a first peak was observed at PIH 12 (11 ± 2 μg/ml and 13 ± 2 μg/ml, respectively) followed by a second higher peak at PIH 24 (27 ± 4 μg/ml and 31 ± 6 μg/ml, respectively). Following peak concentrations, sCD14 decreased from PIH 48 onwards to reach normal pre-infection values by PIH 144. No significant differences in sCD14 concentrations were observed throughout the entire experimental period (Fig. 4c).

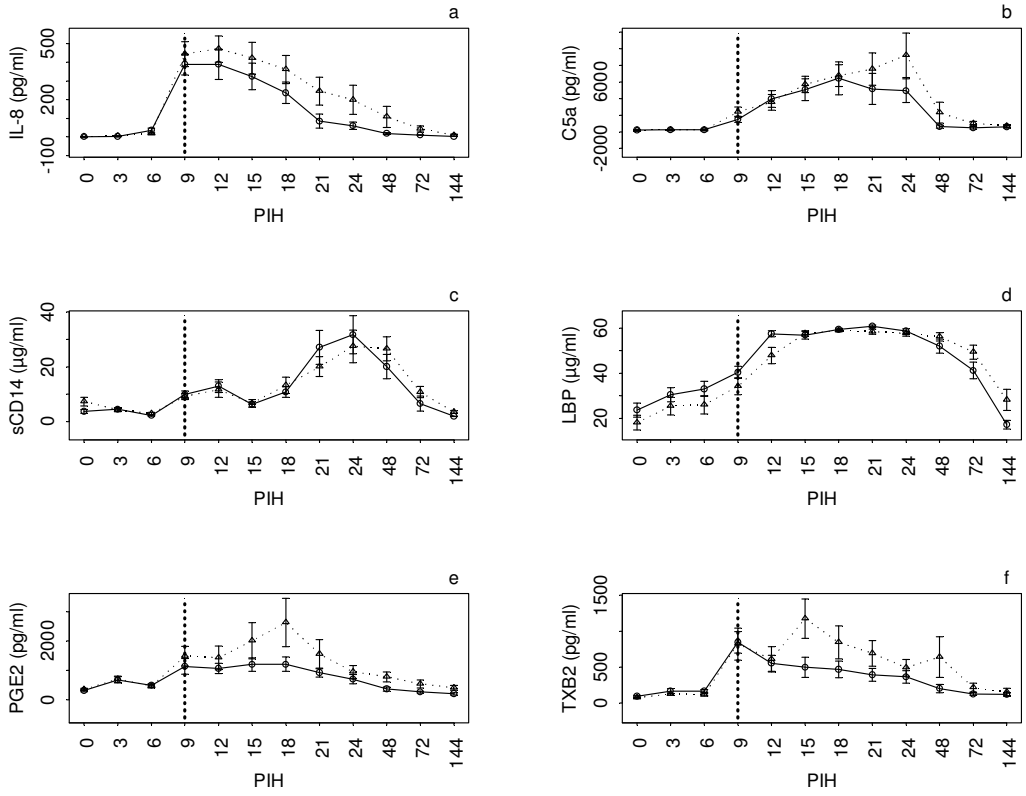


**Figure 4.** IL-8 (a), C5a (b), sCD14 (c), LBP (d), $PGE_2$ (e) and $TXB_2$ (f) in the infected quarters from PIH 0 until PIH 144 from primiparous cows infused with 1 x $10^4$ CFU *E. coli* P4:O32 and treated at PIH 9 (dashed vertical line) with carprofen (——; n = 12) or saline (-----; n = 13). Data are means (± SEM).

**Plasma and Milk LBP**

It has been shown previously (Bannerman et al., 2003) that elevated levels of sCD14 were associated with similar increases in milk LBP, thereby providing an environment for optimal host recognition of LPS, originating from inoculated *E. coli* bacteria. Besides the quantification of milk LBP to assess possible effects of carprofen treatment on the levels of LBP in the infected quarters, plasma LBP was assayed, as it is known that hepatic synthesis of this protein increases during the acute phase response, mainly due to hepatic cell stimulation by IL-1β and IL-6 (Tobias et al., 1999), which could be influenced by NSAID treatment through the intermediate eicosanoid products, $PGE_2$ and $TXB_2$. Under basal conditions, LBP was detected in bovine blood at concentrations of 71.3 ± 10.9 µg/ml and 98.3 ± 16.2 µg/ml for saline and carprofen treated animals, respectively; whereas the concentration of LBP in milk was lower with 18.1 ± 2.3 µg/ml and 23.7 ± 2.2 µg/ml for the treatment groups, respectively. Plasma LBP increased from PIH 15 onward to reach maximal values at PIH 24 (162 ± 10 µg/ml and 167 ± 14 µg/ml for saline and carprofen treated animals, respectively). Thereafter, concentrations of

plasma LBP declined to reach pre-infection levels at PIH 144 (Fig. 1f). No significant difference in plasma LBP was present between the treatment groups throughout the experimental period. In quarters inoculated with *E. coli*, significant elevation of milk LBP appeared from PIH 9 onward, with maxima (58 ± 1 μg/ml and 61 ± 1 μg/ml in saline and carprofen treated animals, respectively) at PIH 21. From PIH 48 on, milk LBP concentrations declined (Fig. 4d). A significant difference ($P = 0.0001$) in milk LBP was observed between the carprofen and saline treated animals at PIH 12.

**Plasma and Milk Prostaglandin $E_2$ and Thromboxane $B_2$**

Plasma $PGE_2$ concentration was 429.2 ± 25.2 pg/ml at the time of inoculation in both treatment groups. Prostaglandin $E_2$ started to decrease in plasma as early as PIH 9 and reached nadir at PIH 15 in both treatment groups. Plasma $PGE_2$ concentrations did not differ between carprofen and saline treated animals (Fig. 1g). Thromboxane $B_2$ concentration in plasma was as high as 180.4 ± 26.5 pg/ml at the time of infection. Throughout the inflammatory episode, no changes over time and no differences between treatment groups were observed (Fig. 1h).

In milk, $PGE_2$ concentration was lower than in plasma and was 334.8 ± 21.1 pg/ml at the time of intramammary inoculation. Prostaglandin $E_2$ subsequently increased from PIH 9 onward in both treatment groups. No significant difference in milk $PGE_2$ concentration was observed between the carprofen and the saline treated animals (Fig. 4e). Initial milk $TXB_2$ concentrations (84.3 ± 5.1 pg/ml) were also markedly lower than concentrations observed in plasma. Thromboxane $B_2$ increases occurred at PIH 9, which was also the maximal concentration in the carprofen treated animals. In saline treated animals, $TXB_2$ further increased and reached its maximum at PIH 15. The milk $TXB_2$ concentrations continued to be higher in the saline treated groups as compared to the carprofen treated animals until PIH 72.

# DISCUSSION

The aim of the present study was to evaluate the potential modulatory effects of treatment with carprofen, a PG synthetase inhibitor through COX-2 inhibition, on a moderate inflammatory reaction following *E. coli* challenge. Therefore, the study design included carprofen administration following the appearance of initial clinical symptoms, which was mainly based on practical field data (Shpigel et al., 1994) and inflammatory dynamics of the moderate inflammatory model used (Vangroenweghe et al., 2004a; 2004b). It is known that pre-treatment far more effectively inhibits the inducible COX-2 enzyme (Burvenich, 1985; Burvenich et al., 1989), however, from a practical point of view, the earliest occasion at which field cases of clinical *E. coli* mastitis can be diagnosed and subsequently treated, occurs at the milking following the infection of the mammary quarter (Shpigel et al., 1994; 1996). The first clinical signs in the present experimental model, using a 1 x $10^4$ CFU inoculum dose, have been shown to appear around PIH 9. Therefore, the choice for a carprofen administration at PIH 9 was considered most suitable, as clinical signs would be present at that time, although maximal RT was not yet reached (Vangroenweghe et al., 2004a; 2004b).

In the present study, the same strain and high inoculum dose of *E. coli* was used to induce a moderate inflammatory reaction in primiparous cows as described before (Vangroenweghe et al., 2004a; 2004b). The clinical course and changes in different laboratory parameters following intramammary *E. coli* challenge in the control animals were similar to animals receiving the same dose (1 x $10^4$ CFU) in previous experiments (Vangroenweghe et al., 2004a; 2004b). Carprofen treated animals elicited an immediate and significant decrease in RT at 3 h post-treatment, whereas pyrexia continued in the control animals with a peak fever at PIH 12. Single dose carprofen administration in the present study resulted in a more pronounced and prolonged antipyretic effect compared to meloxicam treatment in an *E. coli* endotoxin model (Banting et al., 2000), where peak fever was reached 2 h post-treatment in both groups. Reticulorumen motility was equally depressed in both treatment groups at the time of carprofen administration. However, 3 h post-treatment, reticulorumen motility in the carprofen treated animals restored to normal activity, whereas a maximal depression was reached in the control group at PIH 12. In contrast, in an LPS model, no reticulorumen motility depression occurs, and therefore beneficial effects of NSAID treatment on the reticulorumen motility depression can not be quantified.

Improvement of local clinical signs at the level of the affected mammary quarters by carprofen treatment was limited to swelling. There are fewer beneficial effects than observed by

Anderson et al. (1986), who reported significant improvement of quarter temperature, oedema, pain and size following flunixin meglumine treatment of cows suffering from endotoxin-induced mastitis. However, the administration of flunixin meglumine in that study was performed much earlier (PIH 2) as compared to our study, where carprofen was only administered at appearance of the first clinical symptoms.

Clinical scores, combining several clinical parameters, have been described (Wenz et al., 2001; Friton et al., 2002; Vangroenweghe et al., 2004a). Using the clinical severity score described by Vangroenweghe et al. (2004a), carprofen treated animals had a significant lower clinical score at PIH 12 and 15 as compared to the saline treated group. Although, as expected from previous trials, all animals in both groups responded mild to moderate following *E. coli* challenge, the carprofen treated animals generally showed a lower clinical severity score upon NSAID administration. This was mainly due to the rapid restoration of reticulorumen motility and the immediate antipyretic activity following the NSAID administration.

Quarter inflammation was associated with a temporary loss of MP, combined with secretion of abnormal milk from the infected glands. Maximal depression in MP in the infected and uninfected quarters occurred on the day of challenge (d0), and was followed by a rapid recovery during subsequent days. Carprofen treatment did not exert any beneficial effect on milk yield in the infected quarters following intramammary *E. coli* challenge. The absence of any effect of carprofen on the milk production in the uninfected right quarters can mainly be attributed to the moderate inflammatory signs of the mastitis model (Vangroenweghe et al., 2004a).

In the present study, no significant effect of carprofen treatment on bacterial elimination occurred, which could be expected as carprofen treatment was administered alone and not as an adjunctive therapy to intramammary or systemic antibiotics. Enrofloxacin is known to enhance bacterial clearance during experimentally induced *E. coli* mastitis (Monfardini et al., 1999). Rantala et al. (2002) reported a more rapid decline in bacterial number in the infected quarters following combined flunixin meglumine and enrofloxacin treatment, which could probably be attributed to the enrofloxacin treatment and not the adjunctive NSAID therapy.

The leukocyte influx into the infected glands following *E. coli* challenge was not significantly affected by carprofen treatment. This observation is in accordance with previous results obtained using flurbiprofen in experimentally induced LPS mastitis in goats (Burvenich, 1985; Burvenich et al., 1989). Comparable results were obtained following ibuprofen treatment at 2 h post-endotoxin infusion (DeGraves and Anderson, 1993). Although the dose of carprofen (1.4 mg/kg BW) administered intravenously in the present study was twice as high as the dose (0.7 mg/kg BW) used by Lohuis et al. (1991), oedema and leukocyte infiltration in the affected

glands could not be prevented. Nevertheless, the applied treatment was potent enough to reduce eicosanoid generation in the affected mammary glands in the carprofen treated animals. In the blood, similar kinetics could be observed in blood leukocyte number between both treatment group. Nadir blood leukocyte number was reached at PIH 12, 3 h post-treatment in both treatment groups, and was followed by progressive recovery of blood leukocyte number to pre-infection levels by PIH 24.

Dehydration is often assessed during acute coliform mastitis as an easy tool to judge clinical severity. The assessment of dehydration can be performed by skin turgor estimation. In the present study, no difference in skin turgor assessment could be observed throughout the entire study period. This could have been expected based on the mild to moderate responses usually obtained using the experimental infection model. Packed cell volume did not differ among treatments throughout the study either.

Lactose, serum albumin, sodium, potassium and chlorine concentrations in milk of the infected quarters are often determined to assess the severity and duration of the intramammary inflammation. Serum albumin and all ions ($Na^+$, $K^+$ and $Cl^-$) showed similar kinetics, although a significant interaction between time and treatment was only present for serum albumin from 12 h post-treatment (PIH 21) onward in the carprofen treated animals. At PIH 21 and 24, both $Na^+$ and $K^+$ concentration significantly improved in the carprofen treated animals. These results indicate that carprofen induces a more rapid recovery of normal milk composition in animals intramammarily challenged with *E. coli*. Previous studies using ibuprofen in an endotoxin model were unable to demonstrate any effect on milk composition (DeGraves and Anderson, 1993). The elimination half-life of carprofen in healthy cows has been calculated 30.7 h, which is shorter than the half-life of phenylbutazone (31.2 – 82.1 h), but considerably longer than flunixin meglumine (8.1 h) (Lohuis et al., 1991). Therefore, it is not surprising that a single dose of carprofen had prolonged beneficial effects on clinical parameters compared to the control group.

Following intramammary *E. coli* challenge, significant increases in $PGE_2$ (Peter et al., 1990; Zia et al., 1987) and $TXB_2$ (Anderson et al., 1985; 1986; Zia et al., 1987) have been observed in milk. Moreover, a significant effect of flunixin meglumine administration on $TXB_2$ concentrations was reported (Anderson et al., 1986). Even in the uninfected quarter a slight increase in $PGF_{2\alpha}$ and $TXB_2$ concentration was reported (Anderson et al., 1985), although this increase disappeared more rapidly than in the infected quarters. Following flunixin meglumine treatment, $TXB_2$ concentrations remained at baseline levels, whereas in the saline treated animals, a pronounced peak value was observed at PIH 8 in the endotoxin challenged animals (Anderson et al., 1986).

Although the effect of NSAID's on eicosanoids production following inflammation is well documented in literature (Anderson et al., 1986), no reports are available on the possible effect on other immunological inflammatory parameters, such as the chemotactic agents IL-8 and C5a or the early innate immune molecules sCD14 and LBP. Interleukin-8 kinetics in the present study were similar with previous reports using the same inoculum dose (Vangroenweghe et al., 2004b). Local IL-8 production started at PIH 6, although major increases only occurred from PIH 9 onward. Because IL-8 is a well-known chemotactic agent, it is obvious from these results that the initial leukocyte influx into the infected quarters, which occurred around PIH 9, was not affected by the decline of IL-8 occurring much later during inflammation. The appearance and evolution of C5a concentrations in the affected quarters did not differ between both groups until PIH 21, 12 h post-treatment. In the control group, C5a increased until maximal values at PIH 24, which is 6 h later than previously observed (Vangroenweghe et al., 2004b). The marked difference in the onset of carprofen effects on chemotactic agents IL-8 and C5a could be explained by the location of mediator production. Interleukin-8 is directly produced by the epithelial cells in the mammary gland (Baggniolini and Clark-Lewis, 1992; Barber and Yang, 1998), whereas C5a precursors are obtained from the blood through leakage of the disintegrated blood-milk barrier (Rainard et al., 1998; Rainard, 2003).

In accordance with previous reports (Bannerman et al., 2003; Vangroenweghe et al., 2004b), the increases in milk LBP paralleled increments in sCD14 levels. From a host perspective, the simultaneous increase in both LBP and sCD14 levels would be expected to be advantageous as both molecules act in concert to facilitate activation of host defence mechanisms by presenting LPS, released during bacterial growth and death, to the transmembrane LPS receptor, TLR-4 (Bannerman and Goldblum, 2003). Carprofen treatment did not affect either parameter.

The present study showed that carprofen treatment following experimental *E. coli* challenge in a moderate inflammation model with primiparous cows has only slight modulatory effects, which are mainly limited to RT and reticulorumen motility. Administration of carprofen at PIH 9, when first clinical signs occurred, showed beneficial effects on general clinical condition, recovery of milk composition, and reduced production of eicosanoids. Mediators of early innate immune response, IL-8, C5a, sC14 and LBP, were not affected by NSAID treatment.

# CONCLUSIONS

Although carprofen treatment was administered late during the acute phase reaction, when first clinical signs appeared, the NSAID had some modulatory effects on clinical, production and immunological parameters in a moderate inflammation model with primiparous cows. The main modulatory potential occurred at the level of improved clinical condition, mainly due to the antipyretic effects and the ability of carprofen to improve reticulorumen motility. Milk production could not efficiently be modulated through the administration of carprofen at PIH 9. Milk composition (serum albumin, $Na^+$ and $K^+$) was significantly affected by carprofen treatment at PIH 21 and 24, but no further effects on milk composition occurred. Carprofen treatment did not result in a decrease of chemotactic inflammatory mediators, IL-8 and C5a, and no effect was observed for early innate immune molecules, such as sCD14 and LBP. Carprofen treatment did not affect $PGE_2$ and $TXB_2$ in plasma or milk, although there were trends for decreased concentrations of both eicosanoids in milk of the affected quarters. In conclusion, the inflammatory model using primiparous cows during the periparturient period is a minimal and moderate state of inflammation, necessary to eliminate the invading pathogens from the affected mammary quarters. Major modulatory effects from NSAID administration were therefore not observed in this model.

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# 3.

# VACCINATION AGAINST THE ENDOTOXIN

# INTRODUCTION

It is estimated that 20% of all cases of clinical mastitis in some dairy herds are caused by coliform bacteria, particularly in herds where contagious pathogens are well controlled (Schukken et al., 1989a; 1989b; Barkema et al., 1999). The most common of these organisms are *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *K. oxytoca* (Eberhart et al., 1977; Jain, 1979; Smith et al., 1985), which are all known as coliforms. Since most of these organisms are either normal gut inhabitants (Eberhart et al., 1977; Smith et al., 1985) or are found in bedding materials (Oz et al., 1985), there is an almost constant exposure of the mammary gland to these environmental bacteria (Jackson and Bramley, 1983).

The clinical manifestation of an intramammary *E. coli* infection is acute, sometimes peracute and associated with high fever and toxaemia (Hill, 1981). Chronic infections characterised by quiescent periods and periodic acute flare-ups may also occur (Bradley and Green, 2001). A factor, that plays an important role in the pathogenesis of *E. coli* mastitis, is endotoxin or LPS. Lipopolysaccharide may cause release of pre-formed inflammatory compounds, resulting in a mediator shock (Hoeben et al., 2000; Dosogne et al., 2002), which may sometimes progress to fatal disease. The effects of these mediators reflect their normal biological activities and include smooth muscle contraction, increased vascular permeability resulting in oedema, and an early increase in vascular resistance, which may be followed by vascular collapse if sufficiently high concentrations of these mediators are present. Uncorrected, the clinical signs of mediator shock are followed by metabolic acidosis, cyanosis, changed vascular resistance and cardiac output, coma and eventually death (Hill, 1981).

Treatment of acute and peracute clinical coliform mastitis can be difficult and is quite costly to the dairy producer. Moreover, antibiotic treatment immediately following clinical diagnosis has been shown to have little effect on clinical signs present, although the number of *E. coli* in the challenged quarters decreased 100-fold (Monfardini et al., 1999). Therefore, prevention of the disease is considered to be the best option for control. Methods of prevention include decreasing the exposure of the teat end to coliforms from the environment (Smith et al., 1985; Oliver et al., 1990) and increasing the animal's resistance to infection. Maintaining a good sanitation program and practising good milking procedures should reduce the amount of exposure to coliform organisms (Schukken et al., 1989a; 1989b). Taking these control measures into account, however, dairy cattle exposed to the organisms may not possess the appropriate immune status to resist the wide variety of coliform organisms present in the environment, especially as it is known that no specific O-serotypes have been related to bovine *E. coli* mastitis

(Linton and Robinson, 1984). Pre-parturient cows and cows in high production and under nutritional stress during early lactation are particularly susceptible to coliform mastitis, due to a decrease in the effectiveness of their non-specific resistance mechanisms at the mammary gland resident milk cell level (Dosogne et al., 2001; Mehrzad et al., 2001; Vangroenweghe et al., 2001).

The severity of the inflammation, following an intramammary infection with *E. coli*, may be reduced by the use of an effective immunogen (Gonzales et al., 1989). Immunisation of dairy cattle with J5 *E. coli* bacterins has been shown to reduce the occurrence of clinical coliform mastitis under field conditions (Gonzales et al., 1989). The vaccine was developed with the concept of the exposure of the core antigen common to Gram-negative organisms in the mutant J5 strain. Vaccination should result in the development of cross-reactive antibodies, able to enhance cow's immunity to Gram-negative pathogens that cause coliform mastitis (Tomita et al., 2000).

Recently, primiparous dairy cows have been shown to be more resistant to severe clinical *E. coli* mastitis, resulting in a moderate inflammatory reaction with complete resolution (Vangroenweghe et al., 2004a; 2004b). The purpose of the present study was to evaluate if a bacterin formulation made with a J5 mutant strain of *E. coli* used to immunise the animals against the endotoxin (Enviracor™; Pfizer Animal Health, Sandwich, UK) could modulate the moderate inflammation in primiparous dairy cows challenged intramammarily with an *E. coli*.

# MATERIALS AND METHODS

### Experimental Animals and Study Facilities

All primiparous cows (n = 23) were in their 7th month of pregnancy on arrival at a commercial dairy farm.

### Inoculation Dose

Primiparous cows were inoculated with 1 x $10^4$ (n = 6) or 1 x $10^6$ CFU (n = 17) *E. coli* P4:O32 in both left quarters. Control animals were predominantly inoculated with 1 x $10^6$ CFU (10 out of 11 animals), whereas inoculum doses were equally distributed in animals vaccinated against the endotoxin (n = 5 for 1 x $10^4$ CFU; n = 7 for 1 x $10^6$ CFU).

### Sampling Procedure

Blood and milk samples were collected at d-7, d-4, d-1, d0, d+1, d+2, d+3, d+6, d+9 and d+13 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18 and 21.

### Glucose Serum

Glucose was determined in NaF-plasma.

### Statistical Analysis

In order to compare the two treatment groups with respect to the various parameters analysed in blood and milk, a mixed model was used with cow as random effect and treatment, time and their interaction as categorical fixed effects. Pairwise comparisons were adjusted using Bonferroni's multiple comparisons procedure with an overall type I error equal to 5%. Quarter milk production in both infected and uninfected quarters was tested for significant differences between treatments at d+1 and the interval d+1 − d+3. The effect of treatment on the local aspects of the mammary gland was tested by the Wilcoxon rank sum test.

**For Further Details** see chapter 'Materials and Methods Experimental Infections', **p. 97**.

# RESULTS

### Local and Systemic Clinical Signs

Following intramammary challenge, RT (Fig. 1a) and HR (Fig. 1b) increased from PIH 6 onward and reached a peak at PIH 9 in both treatment groups. Rectal temperature and HR rapidly decreased following peak values and normal values were reached from PIH 18 on in both treatment groups. Although on average RT did not differ between the two treatment groups from 0 to 48 hours ($P$ = 0.43), RT evolved differently over time in the two treatment groups ($P$ = 0.01). Rectal temperature was significantly higher at PIH 6 ($P$ = 0.007), but significantly lower at PIH 12 ($P$ = 0.009) in the vaccinated group. No significant differences were found for HR or RR (Fig. 1c). Maximal RR appeared between PIH 9 and 12 in both treatment groups.

Following intramammary *E. coli* inoculation, typical local clinical symptoms occurred at the level of the affected quarters with swelling and abnormal milk (flakes and clots). During the acute phase of inflammation (PIH 0-48), no significant differences in local swelling and milk appearance were observed.

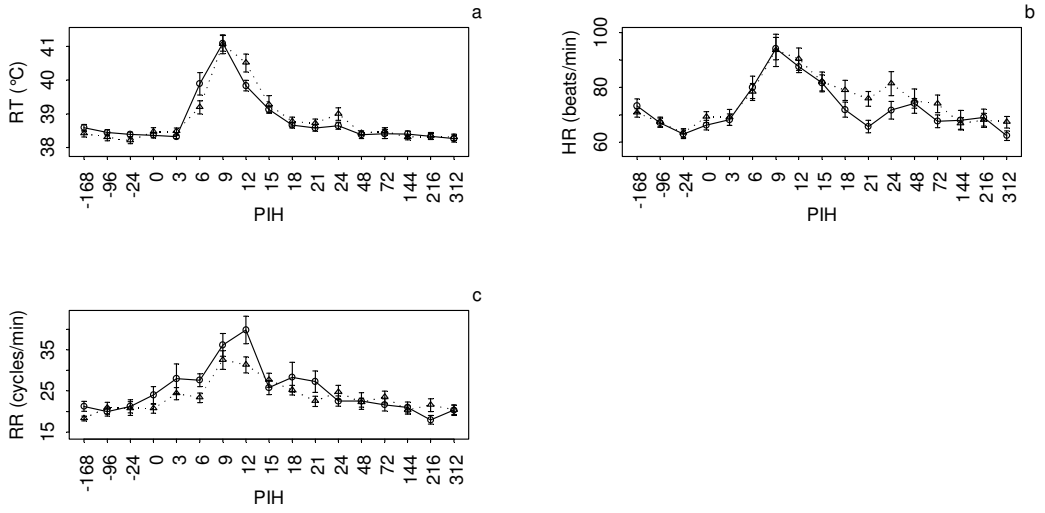


**Figure 1.** Rectal temperature (a), heart rate (b), and respiration rate (c) from PIH -168 until PIH 312 from primiparous cows vaccinated against the endotoxin (——; n = 12) or the placebo (-----; n = 11) and intramammarily challenged with *E. coli* P4:O32. Data are means (± SEM).

### Quarter Milk Production

Following *E. coli* challenge, milk production decreased in the infected and uninfected quarters (Fig. 2a-b). During the days following experimental challenge, milk production rapidly recovered and almost reached pre-infection values in the uninfected quarters (Fig. 2b). In the infected quarters, production at d+13 (PIH 312) was still slightly lower than pre-infection in both

treatment groups (Fig. 2a). No significant difference in quarter milk production at d+2 and the interval d+1 – d+3 could be observed between the treatment groups in infected and uninfected quarters.

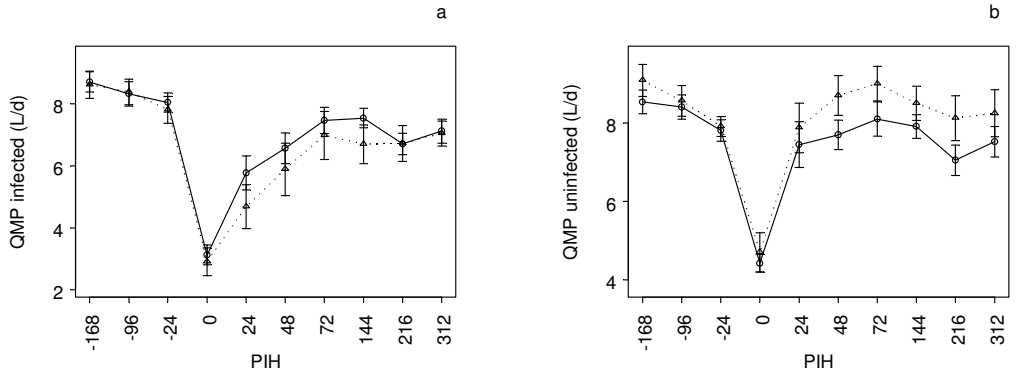


**Figure 2.** Quarter milk production in the infected (a) and the uninfected (b) quarters from PIH -168 until PIH 312 from primiparous cows vaccinated against the endotoxin (——; n = 12) or the placebo (-----; n = 11) and intramammarily challenged with *E. coli* P4:O32. Data are means (± SEM).

**Bacterial Growth and Milk Composition**

Bacterial growth rapidly reached maximal levels in both treatment groups (Fig. 3a). The vaccinated animals eliminated bacteria from the challenged quarters slightly faster during the first hours post-infusion, resulting in a significantly different evolution of CFU over time in the two treatments group ($P = 0.0034$). Bacterial population (CFU/ml) was significantly lower in the vaccinated group at PIH 9 ($P = 0.006$).

Intramammary *E. coli* challenge resulted in a rapid influx of PMN from the blood into the mammary gland, inducing a major increase in SCC in the infected quarters (Fig. 3b), but no significant differences between the two groups were observed. The highest SCC values were observed at PIH 6, staying at that level for an extended time. Following the acute phase of inflammation, SCC slowly decreased, although at d+13, it was still higher than pre-infection.

Changes of milk composition, as measured by the ions ($Na^+$, $K^+$ and $Cl^-$), serum albumin and lactose are indicative for the presence of mastitis in the mammary quarters. The evolution of $Na^+$ over time differed significantly between the two treatment groups ($P = 0.023$), with a faster increase in the vaccinated animals until 12 PIH, after which time the control group had higher $Na^+$ concentrations than the vaccinated group (Fig. 3d). A similar but non-significant relationship was observed for $Cl^-$ (Fig. 3f). Changes in $K^+$ followed the same kinetics with nadir around PIH 9-12 and subsequent recovery to normal pre-infection levels by d+6 to d+13 (Fig. 3e). Serum albumin rapidly increased following intramammary challenge and reached a maximal

concentration at PIH 15 in both treatment groups (Fig. 3g), subsequently followed by normalisation from PIH 48 onward. No significant difference was observed between the two treatment groups.

Lactose concentration decreased in both groups from PIH 9 onward to reach nadir at PIH 15 in vaccinated animals and at PIH 21 in placebo animals (Fig. 3c). The evolution of lactose over time differed significantly in the two groups ($P$ = 0.0004) with the vaccinated animals having a more pronounced decrease of lactose during the acute phase of intramammary *E. coli* challenge.

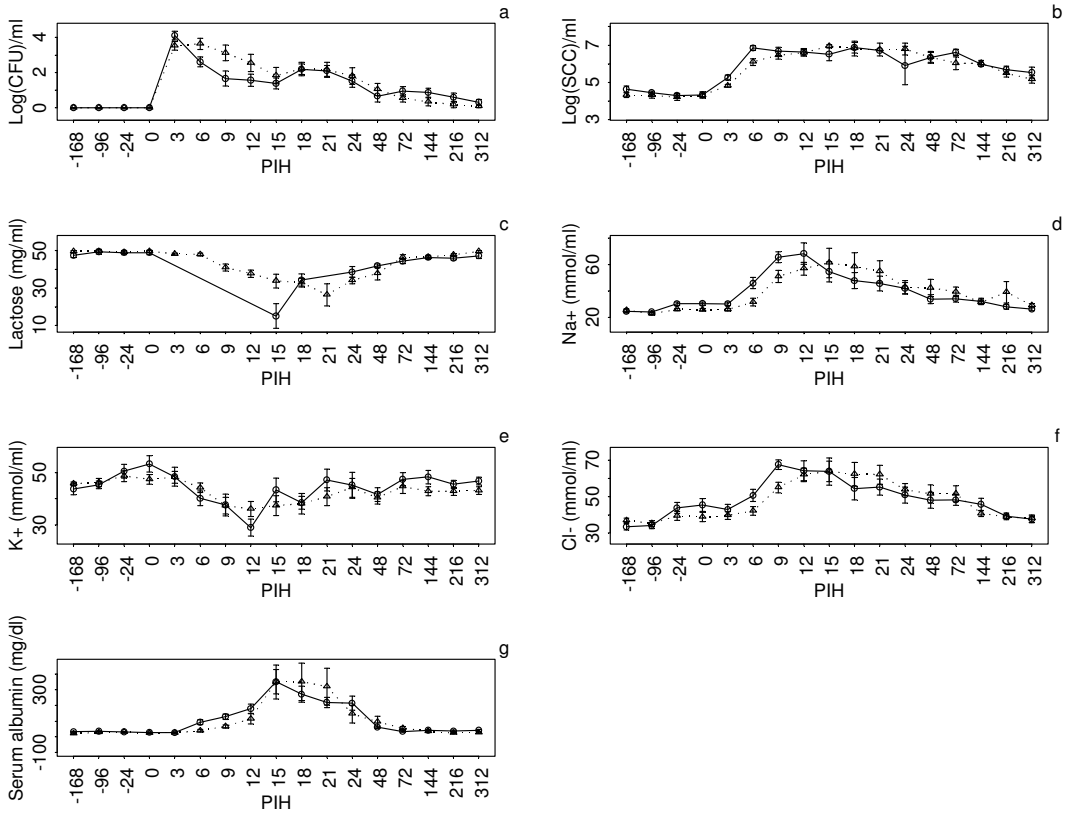


**Figure 3.** Colony-forming units of *E. coli* (a), SCC (b), lactose (c), sodium (d), potassium (e), chlorine (f), serum albumin (g) in the affected quarters from PIH -168 until PIH 312 from primiparous cows vaccinated against the endotoxin (——; n = 12) or the placebo (-----; n = 11) and intramammarily challenged with *E. coli* P4:O32. Data are means (± SEM).

**Hematology**

Packed cell volume did not significantly differ between both treatment groups throughout the experimental challenge (Fig. 4a). Following *E. coli* challenge, blood leukocyte number rapidly decreased and reached nadir at PIH 9 and 12 in the vaccinated and placebo animals, respectively (Fig. 4b). Blood leukocyte count was on average (marginally) significantly

higher in the vaccinated group ($P$ = 0.054). In the placebo group, blood leukocyte number remained lower than in vaccinated animals until PIH 144.

Leukocyte differentiation did not significantly differ between both treatment groups in terms of mature PMN (Fig. 4c), early (myelocytes-metamyelocytes) immature PMN (Fig. 4e), lymphocytes (Fig. 4f), and monocytes (Fig. 4g). Overall, a significantly lower percentage of late (band cells) immature cells ($P$ = 0.052) was, however, observed in the vaccinated group (Fig. 4d) as compared to the placebo group.

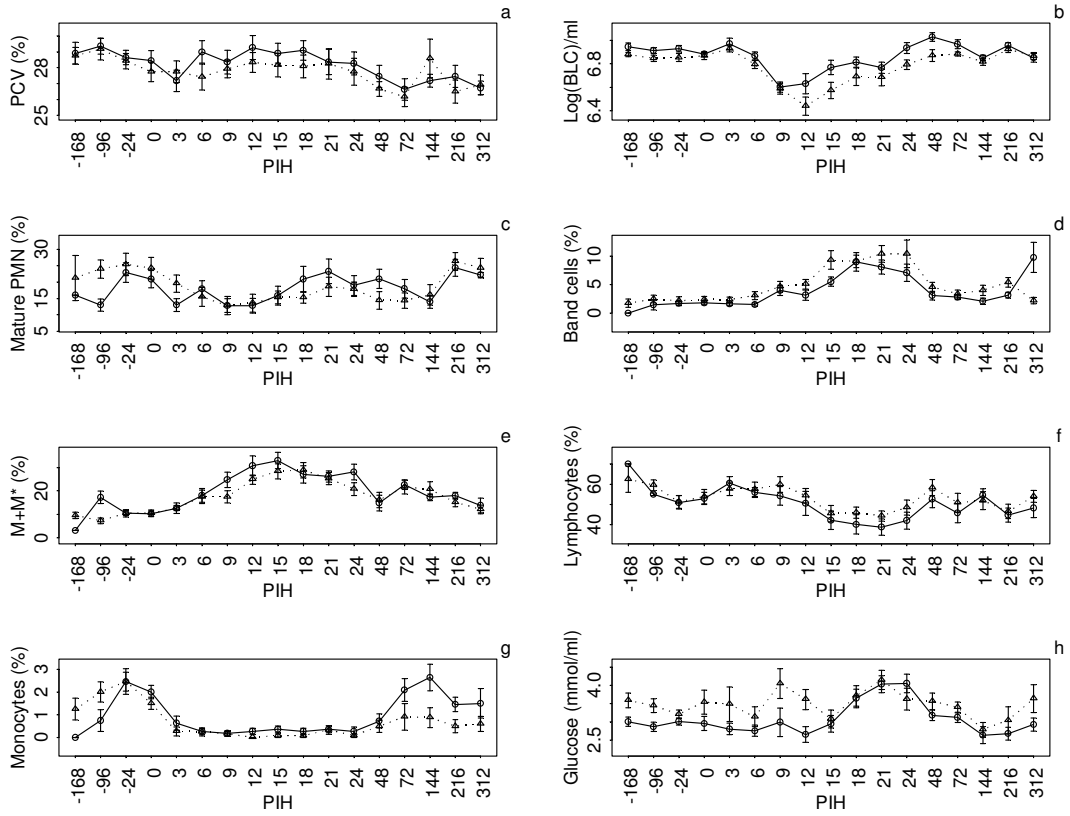


**Figure 4.** Packed cell volume (a), blood leukocyte count (b), percentage of mature PMN (c), band cell (d), myelocytes-metamyelocytes (e), lymphocytes (f), monocytes (g), and glucose (h) in the blood from PIH -168 until PIH 312 from primiparous cows vaccinated against the endotoxin (——; n = 12) or the placebo (-----; n = 11) and intramammarily challenged with *E. coli* P4:O32. Data are means (± SEM).

**Glucose**

The response of blood glucose to intramammary *E. coli* challenge was mainly characterised by two peak concentrations and a dip during the first 24 h post-challenge. In the vaccinated animals, the glucose concentration evolved differently ($P$ = 0.045) with a significant lower glucose concentration during the acute phase of inflammation (Fig. 4h).

# DISCUSSION

Peak *E. coli* concentrations in the infected quarters have been reported to be indicative for the severity of experimental *E. coli* mastitis (Vandeputte-Van Messom et al., 1993), and have been shown to be both higher and prolonged in severe cases (1 x $10^{10}$ CFU/ml) than in moderate (1 x $10^{7}$ CFU/ml) or mild (1 x $10^{4-5}$ CFU/ml) cases (Shuster et al., 1996; van Werven et al., 1997; Hirvonen et al., 1999; Vangroenweghe et al., 2004a; 2004b). Moreover, mild cases of clinical coliform mastitis showed a more rapid elimination of the bacteria from the infected quarters compared to moderate and severe clinical cases. The $AUC_{0\text{-}126}$ of CFU/ml has repeatedly been shown to be a significant indicator of severity of clinical *E. coli* mastitis (Lohuis et al., 1990; Kremer et al., 1993; van Werven et al., 1997). These data, obtained during experimentally induced coliform mastitis, are consistent with field observations by Wenz et al. (2001), who reported higher proportions of cases with > 1 x $10^{5}$ CFU/ml *E. coli* concentrations in severe (77%) than in moderate (51%) and in mild (28%) cases.

Hogan et al. (1992) conducted an experimental challenge comparing cows vaccinated with the *E. coli* J5 strain, using formulations different from Enviracor™, to a placebo group and showed favourable results for the J5 vaccinates when the infection model resulted in a rather high CFU/ml in milk. The mean $\log_{10}$ CFU/ml in the control group at the time point with the highest value was about 4 $\log_{10}$ CFU/ml. Hence, the CFU challenge obtained in the present study should be considered as mild. With the exception of Hogan et al. (1992), most other published challenge studies resulted in peak CFU/ml that were lower than those cited above. In such studies, few modulatory effects on the course of inflammation were observed following vaccination against the endotoxin (Hogan et al., 1995; Smith et al., 1999). This is also the case in the present study.

In cows with *E. coli* mastitis, the milk production can be greatly affected in severe responders (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Dosogne et al., 1997; van Werven et al., 1997) in which it does not normalise rapidly (Hirvonen et al., 1999). Although milk yield has been measured in intramammary challenge studies, evaluating the effect of J5 vaccination, none of these studies (Hogan et al., 1992; 1995; 1999; Smith et al., 1999) showed a significant improvement in milk yield loss of the J5 vaccinates over placebo animals. Milk yield loss in infected and uninfected quarters has been found to be highly correlated with other indicators of severity, in particular AUC of *E. coli* CFU/ml (Lohuis et al., 1990; Kremer et al., 1993). As expected, milk yield recovered fast in the presently used moderate inflammation model (Vangroenweghe et al., 2004a; 2004b) and vaccination against the

endotoxin could not accelerate this phenomenon.

Increases in RT and HR have been found to be higher in animals with a severe than with a moderate clinical response (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Ohtsuka et al., 2001). In moderately reacting primiparous cows, RT was normalised around PIH 18 (Vangroenweghe et al., 2004a; 2004b). Rectal temperature and HR have been shown to correlate significantly positive to both *E. coli* CFU/ml (Kremer et al., 1993) and milk production in the infected and uninfected quarters (Lohuis et al., 1990). Hence, it is not surprising that, based on the results of quarter milk production and CFU, no significant differences in HR were observed between the vaccinated group and the placebo animals. However, significant differences in RT could be observed during the acute phase of inflammation. The significant difference between both groups in RT at PIH 6 and 12 could not be explained by the unequal distribution of the inoculum dose in both treatment groups, as placebo animals predominantly (10/11) received the high inoculum dose (1 x $10^6$ CFU per quarter), which should induce an earlier and more rapid fever response (Vangroenweghe et al., 2004a; 2004b).

Rapid and efficient increase in SCC appears to be the SCC characteristic associated with a moderate inflammatory reaction (Vangroenweghe et al., 2004a; 2004b). None of the previously reported experimental challenges comparing cows vaccinated with the *E. coli* J5 strain to a placebo group showed a significant difference in SCC between both treatment groups (Hogan et al., 1992; 1995; 1999; Smith et al., 1999). A significant lower SCC in the J5 vaccinates was only reported in one experimental challenge study, however, this difference in SCC was at a single time point and at 7 d post-infusion (Hogan et al., 1995). In the present study with moderately reacting animals, the same observations concerning the changes in SCC during the acute phase of inflammation were present.

The milk composition variables determined in the present study are all indicators of the presence of mastitis in the affected glands. In a moderate inflammation model, changes in the concentrations of lactose, potassium, sodium, chlorine and serum albumin were similar to previous studies (Vangroenweghe et al., 2004a; 2004b). Serum albumin has been found to be significantly less increased in J5 vaccinates (Hogan et al., 1995). In the present study, no treatment effect was observed for chlorine, potassium and serum albumin, whereas an earlier change was present for sodium and lactose in the vaccinated animals.

Only one study (Vandeputte-Van Messom et al., 1993) showed a significant lower PCV in severe than in moderate responders. In the present study, being a moderate inflammatory model, no significant difference in PCV was observed between both treatment groups. Following *E. coli* mastitis challenge, blood leukocyte number will first decrease and subsequently increase,

when compared to baseline values (Heyneman and Burvenich, 1992; van Werven et al., 1997; Ohtsuka et al., 2001). Both the initial decrease and the subsequent increase are larger in severe than in moderate responders (Heyneman et al., 1990; Heyneman and Burvenich, 1992; Sordillo and Peel, 1992; Ohtsuka et al., 2001). This typical pattern of blood leukocyte changes has been shown to also occur with endotoxin challenge (Tennant et al., 1975; Morris et al., 1986). From the present study, it is clear that although no significant difference in SCC was present between both treatment groups during the first 72 h post-challenge, significant differences in the blood leukocyte patterns were present. Blood leukocyte number was significantly higher throughout the experiment in the vaccinated animals as compared to the placebo animals. However, significant mobilisation of late immature PMN (band cells) from the bone marrow could only be observed in the placebo group.

The presently used *E. coli* mastitis challenge model consistently results in clinical mastitis and has, therefore, frequently been used to study risk factors influencing the severity of the response to challenge, rather than the occurrence of clinical coliform mastitis (Heyneman et al., 1990; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; van Werven et al., 1997; Hoeben et al., 2000; Dosogne et al., 2002). However, using this model in J5 vaccination studies, it has often been difficult to produce a severe response. A first possible explanation for this observation is that, due to the need for vaccination from dry-off onward, these vaccination studies are often much longer than the classical experimental *E. coli* mastitis challenges. This means that these cattle have remained in the same familiar environment for a longer period prior to challenge. In a classical challenge study, the observation period to challenge can be much shorter, mostly 1 to 2 weeks (Dosogne et al., 1997; Blum et al., 2000 ; Hoeben et al., 2000), meaning that these animals had much less time to adjust to the study settings (experimental facilities and personnel) as well as to the extra interventions (blood and milk sampling, clinical examination), all of which cause extra stress. In this regard, it has been shown that even 1 h of isolation of a cow from her herd mates caused enough stress to result in an increase of leakiness of the tight-junctions between the endothelial cells in the mammary gland, thereby allowing an influx of serum components (serum albumin, chlorine, sodium) into the milk and a loss of milk components (lactose) to the serum (Stelwagen et al., 2000). Another major limitation to obtain a severe clinical response is the fact that in most of the cited J5 vaccine studies the challenge did not take place within days after calving, the period when animals are most likely to develop severe coliform mastitis. A third aspect is that primiparous cows are less susceptible to severe coliform mastitis than older animals (van Werven et al., 1997; Vangroenweghe et al., 2004a; 2004b).

# CONCLUSION

In the present study, pre-calving vaccination against the endotoxin through a J5 vaccine administration could slightly alter the clinical course of a moderate inflammatory reaction. Although vaccinated animals had favourable RT and blood leukocyte kinetics, no major clinical improvement could be observed following a moderate inflammatory reaction during *E. coli* challenge. Therefore, primiparous cows seem to have an optimal inflammatory reaction following intramammary *E. coli* inoculation, characterised by a rapid and efficient influx of PMN into the affected mammary quarters, resulting in a fast and short-lasting inflammation, finally leading to resolution of mammary gland functionality.

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# GENERAL DISCUSSION

*Escherichia coli*, involved in bovine coliform mastitis, is part of the normal intestinal flora of animals. The strains isolated from bovine mastitis are essentially not different from strains isolated from bovine faeces. The etiological bacteria do not possess specific virulence factors that could be attributed to disease severity (Lehtolainen, 2004). In contrast, cow factors such as stage of lactation, PMN functionality and metabolic status are associated with disease severity (Burvenich et al., 2003; 2004). Concerning *E. coli*, numerous epidemiological data are available (Schukken et al., 1989a; 1989b; Barkema et al., 1999), in addition to the results obtained from experimental infection models, which can be used to generate practical conclusions. However, the specific experimental model used, i.e. *E. coli vs.* endotoxin, has a tremendous effect on the results obtained from pathogenesis studies. The inflammatory reaction in multiparous dairy cows has extensively been studied throughout the last decade (Heyneman and Burvenich, 1992; Vandeputte-Van Messom et al., 1993; Kremer et al., 1993a; 1993b; 1993c; van Werven et al., 1997; Dosogne et al., 1997; Hoeben et al., 2000). However, taking into account the present turn-over of high-yielding dairy cows (approximately 25 to 30% per year) in modern dairy herds, a relatively large population of primiparous cows is present, from which no data on resistance or susceptibility towards *E. coli* mastitis are available.

Although the influence of stage of lactation has extensively been studied, until now, little information on the effect of parity is available. Younger cows ($2^{nd}$ parity) have been postulated to be more resistant to intramammary *E. coli* infection, mainly based on the lower bacterial count and the higher number of peripheral circulating leukocytes during experimentally induced *E. coli* mastitis (van Werven et al., 1997). Evaluation of impairment of periparturient neutrophil function revealed a significantly lower superoxide anion production in cows in their $4^{th}$ or greater lactation than in cows in the first three lactations (Gilbert et al., 1993). Superoxide formation plays an important role in the intracellular killing of *E. coli* inside the PMN. A longitudinal study of PMN viability and functions in primiparous and multiparous dairy cows around calving showed significant differences between both groups (Mehrzad et al., 2002). Although a periparturient decrease in PMN viability occurred in both groups, overall PMN viability was much higher in primiparous cows (Mehrzad et al., 2002). It seems logic that animals in their first lactation have markedly different pre-inflammatory parameters, which may result in differences in inflammatory kinetics following intramammary *E. coli* challenge (Gilbert et al., 1993; van Werven et al., 1997; Mehrzad et al., 2002).

Normal milk contains a heterogeneous cell population, with its composition dependent on the stage of lactation. The normal proportion of PMN in the clinically healthy mammary gland is low (< 15%). During inflammation, due to for instance mastitis, the proportion of PMN increases to more than 90%. Therefore, inflammation should be considered a PMN related

condition with a well-controlled cell functionality. In this thesis, milk sample collection under pre-infection conditions was validated in relation to composition of the cell population and PMN functionality in milk, collected under different aseptical and non-aseptical conditions, because external bacterial contamination was thought to activate the PMN functionality *in vitro*. In low SCC milk, the number of PMN was only 13% and the average cell viability was decreased (40%). This observation was in accordance to Mehrzad et al. (2001), which also reported decreased PMN viability and functionality during early lactation. Validation of aseptical milk sample collection revealed no significant effect of bacterial contamination on milk PMN functionality under basal pre-infection conditions, and therefore, manual milk sampling was preferred to perform milk sample collection in the subsequent experimental *E. coli* challenges.

Number and composition of the somatic cell population could be considered as an important determining factor in the immunological status of the mammary gland. Numerous studies have been performed in order to identify the various cell populations present in the mammary gland, however, most of these studies have focused on bovine high SCC milk (Hageltorn and Saad, 1986; Östensson et al., 1988; Redelman et al., 1988; Saad and Östensson, 1990). Moreover, most techniques were complicated and labour-intensive, and therefore without practical applicability. In epidemiological studies, low SCC has been demonstrated as an important risk factor for the occurrence of clinical coliform mastitis (Schukken et al., 1989a; 1989b; Barkema et al., 1999). In this thesis, a rapid, simple and accurate bovine low SCC milk differentiation technique was developed using flow-cytometry (Dosogne et al., 2003). With this technique, PMN, macrophages, lymphocytes and apoptotic cells could easily be distinguished. Questions can arise to what extent the isolation method could influence the proportional composition of SCC, but this problem occurs in all published differentiation techniques. The present method could limit preparative manipulations to a minimum, but obligatory centrifugation for the removal of fat, interfering with flow cytometric analysis, could in fact bias the proportional distribution of cell types obtained in differentiation. However, compared to previous differentiation procedures, milk sample collection and preparation, cell staining and flow cytometric analysis were validated.

As proposed in the study objectives, inflammatory kinetics and severity of the experimentally induced intramammary *E. coli* challenge were described using an inoculum dose of 1 x $10^4$ and 1 x $10^6$ CFU of *E. coli* P4:O32 per quarter (Vangroenweghe et al., 2004a). Primiparous cows generally appeared to react as moderate responders, based on their quarter milk production in the right uninfected quarters on d+2 post-infection. Moderate responders had a higher number of circulating PMN (Heyneman et al., 1990; Sordillo and Peel, 1992; Kremer et al., 1993c; van Werven et al., 1997), and the functionality of circulating blood PMN pre-

infection, such as chemotactic differential (Kremer et al., 1993a; van Werven et al., 1997) and oxidative burst activity (Heyneman et al., 1990), was clearly increased. Some of these aspects, such as circulating number of PMN, were also observed in this thesis (Vangroenweghe et al., 2004a). Therefore, an effect of parity or age can be concluded from the present experiment.

Comparison of historical data of quarter milk production in the uninfected quarters at d+2 with data obtained in this thesis showed that primiparous cows had a remarkably higher average milk production in their uninfected quarters following intramammary challenge as compared to multiparous cows. Several animals already exceeded their pre-infection milk production at d+2 post-infection (Vangroenweghe et al., 2004a). Following *E. coli* challenge, primiparous cows exhibited a moderate inflammatory reaction, which could not be altered through an increase ($1 \times 10^4 \rightarrow 1 \times 10^6$ CFU per quarter) in intramammary inoculum dose. The inflammatory reaction was characterised by a rapid onset of clinical symptoms, such as rectal temperature increase, udder swelling, and changes in milk composition, which also disappeared rather fast, resulting in a complete resolution of the functionality of the affected mammary quarters. To better diversify between different clinical responses, a severity scoring system, used to score cases of clinical mastitis in practice (Wenz et al., 2001), was adapted for use in the experimental infection model. Evaluation of clinical responses through this system neither revealed any severe responses in the challenged primiparous cows (Vangroenweghe et al., 2004a). The severity scoring system could therefore be used as an additional scoring tool, besides the established measurement of quarter milk production in the uninfected quarters at d+2 post-infection.

Literature data revealed a wide diversity of inoculum doses and strains used to provoke experimental *E. coli* mastitis in the bovine. However, strain diversity could not be held responsible for variations in clinical outcome of *E. coli* mastitis, because several researchers were unable to characterise specific virulence factors, which might be associated with severity or virulence following intramammary *E. coli* challenge (Lehtolainen, 2004). Differences in inoculum dose have been shown to result in variable inflammatory kinetics. Following inoculation of low doses (~ 30 CFU), bacterial growth preceded subsequent elimination (Shuster et al., 1996). Inoculation of high doses (~ 10,000 CFU) resulted in rapid bacterial elimination (Hoeben et al., 2000). However, studies in which inoculum doses were randomised have rarely been performed (Jain et al., 1969; Carroll et al., 1973; Bramley and Neave, 1975; Frost et al., 1980). The variation in inoculum dose in primiparous cows, performed in this thesis, could modulate the inflammatory response, which was characterised by an earlier onset (approximately 3 h) of clinical signs and chemotactic activity in the animals inoculated with the highest inoculum dose ($1 \times 10^6$ CFU *E. coli* P4:O32) (Vangroenweghe et al., 2004b). These results seem

to confirm that a minimal number of bacteria should be present in the mammary gland to trigger the innate immune response in its activation of several defence mechanisms, such as activation of the mammary gland epithelium to produce chemotactic factors (IL-8) or macrophage activation with inflammatory cytokine production (Bannerman et al., 2003).

Therapeutic and prophylactic strategies are claimed to modulate the course of an inflammatory reaction. Administration of an inhibitor of prostaglandin synthesis before or during experimentally induced LPS mastitis has been shown beneficial in reducing the clinical episode, characterised by the absence of fever or a shorter or lower fever peak (Burvenich and Peeters, 1982; Anderson et al., 1986; Lohuis et al., 1989; 1991; Banting et al., 2000), mainly through the interaction with fever inducing mechanisms, such as central $PGE_2$ production in the thermoregulatory centre (Burvenich and Peeters, 1982). In primiparous cows, the modulatory effect of carprofen treatment following experimentally induced *E. coli* mastitis was limited to a reduced peak fever and a shorter episode of reticulorumen motility depression. Moreover, eicosanoid ($PGE_2$ and $TXB_2$) production in the affected quarters decreased following carprofen treatment. The inflammatory reaction mounted in these animals, therefore, seems to be on the edge of clinically detectable inflammation, especially as increased rectal temperature is only present for 12 h (from PIH 6 until PIH 18). Furthermore, mammary gland functionality, as quantified by quarter milk production, is rapidly resolved.

From a prophylactic point of view, vaccination against the endotoxin, using a J5 vaccine, has not been able to reduce the incidence of clinical coliform mastitis (Tyler et al., 1993), although a shift in clinical response from severe to moderate has been observed (Gonzales et al., 1989; Tyler et al., 1993). To show efficacy in an experimental model, it is known that a severe inflammatory reaction is needed (Hogan et al., 1992). The present study with primiparous cows resulted in a homogenous population of moderate responders (Vangroenweghe et al., 2004a; 2004b), and it is therefore not surprising that little modulatory effects of vaccination against the endotoxin were observed.

Prognostic evaluation of clinical severity before treatment, combined with rapid etiological diagnosis, is difficult to achieve under practical conditions. However, based on experimentally induced intramammary *E. coli* challenge, several severity determining and risk factors have been identified, which could be associated with the potential occurrence of a severe inflammatory response (Burvenich et al., 2003). One of these factors could be age or parity of the affected animal (Gilbert et al., 1993; van Werven et al., 1997). The inflammatory reaction in primiparous cows following intramammary *E. coli* challenge has been shown mild to moderate based on clinical severity scoring (Vangroenweghe et al., 2004a). Therefore, primiparous cows with acute clinical mastitis during the early post-partum period should be considered low risk

candidates for a severe clinical response due to *E. coli*. Moreover, prophylactic and therapeutic interventions have been shown unable to modulate the already moderate inflammatory reaction, resulting in a maximal resolution of original functionality of the affected quarters. In case of clinical mastitis in primiparous cows, antibiotic treatment should be focused on combating Gram-positive infections, because infection with Gram-negative bacteria does not seem to cause a potential risk for severe inflammatory reactions. In contrast, high-yielding multiparous cows in negative energy balance during the early post-partum period are striking potential candidates for a severe clinical response (Kremer et al., 1993c; Hoeben et al., 1997; van Werven et al., 1997), and treatment should therefore be focused on these animals, providing them antimicrobial and anti-inflammatory therapy, eventually in combination with other additional treatments.

# CONCLUSION

In conclusion, validation of milk sample collection under aseptical conditions to establish basal pre-infection values has revealed no effect of minor bacterial contamination on the milk PMN functionality *in vitro*. Therefore, manual milk sample collection was performed in the subsequent experimental *E. coli* challenges. Moreover, flow cytometric differentiation of the milk cell population present in bovine low SCC milk was validated. Following intramammary *E. coli* challenge during the periparturient period, primiparous cows reacted as moderate responders with a rapid and efficient inflammatory reaction and short-lasting depression in milk production. Variation of the inoculum dose could modulate the inflammatory reaction and a higher inoculum dose ($1 \times 10^6$ CFU per quarter) resulted in a faster innate immune response. Inhibition of the prostaglandin synthesis through NSAID administration at PIH 9, when clinical symptoms were present, improved general clinical condition by rapid defeverescence and restoration of depressed reticulorumen motility. In addition, eicosanoid production ($PGE_2$ and $TXB_2$) decreased following NSAID treatment. Pre-calving prophylactic vaccination against the endotoxin could slightly alter the clinical course of the moderate inflammatory reaction in primiparous cows. Although vaccinated animals had favourable RT and blood leukocyte kinetics, no major clinical improvement could be observed following intramammary *E. coli* challenge. Therefore, primiparous cows can be considered low risk candidates for severe clinical mastitis following experimental intramammary *E. coli* challenge. Care should be taken with respect to generalisation of the obtained results of experimentally induced mastitis to the field situation, especially due to some major differences, such as the inoculum dose, which exist between experimental models used in research and field cases.

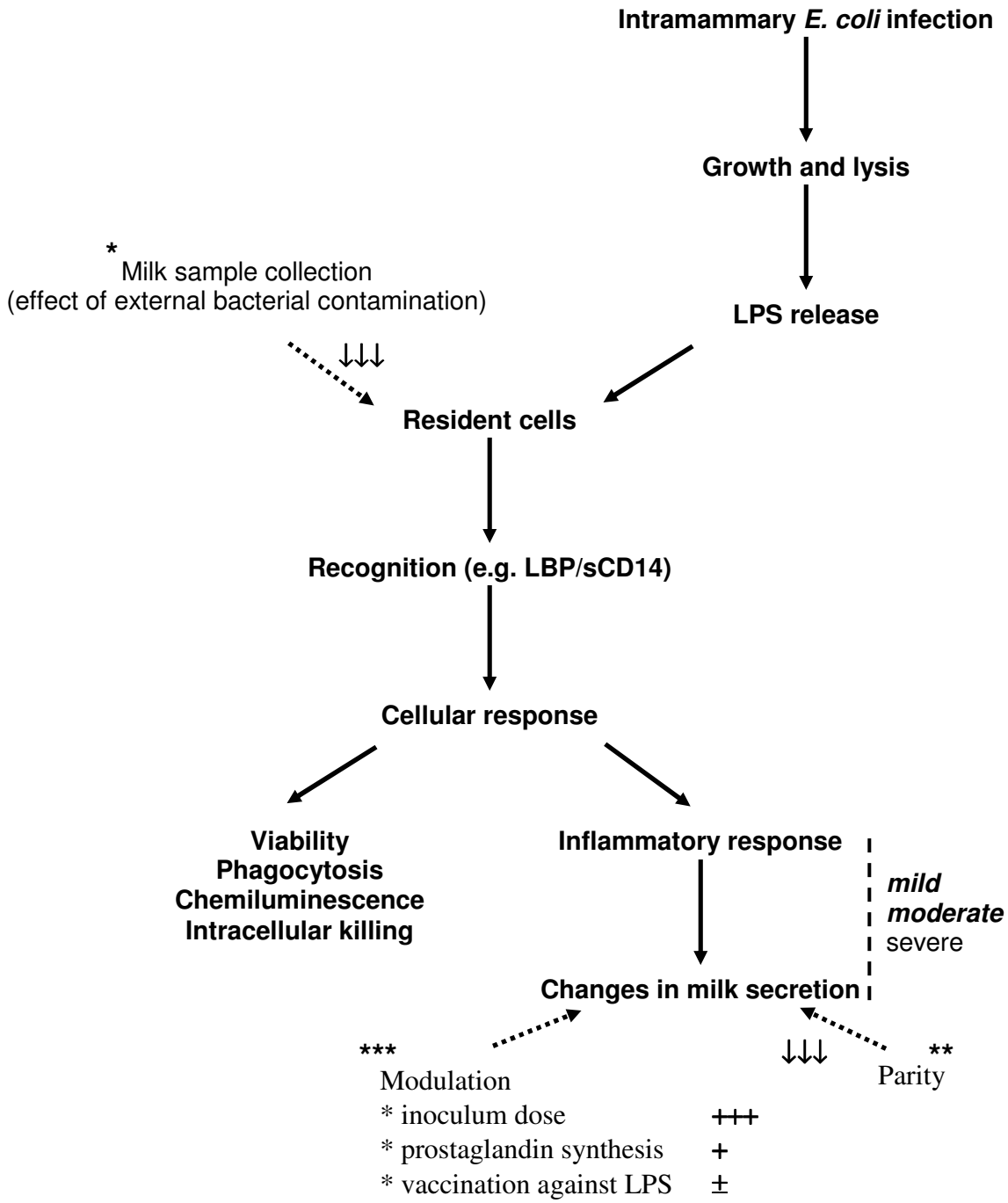


**Figure 1.** Schematic drawing of the hypothesis and objectives of this thesis. Left pathway: external bacterial contamination has no effect on cellular response of resident cells in milk (*) (p. 69). Right pathway: following intramammary *E. coli* infection, bacterial growth and lysis leads to the release of LPS, which interacts with the resident cells in milk. Binding of LPS by LBP/sCD14 induces a cellular response, which changes resident milk cell characteristics and elicits an inflammatory response resulting in changes in milk secretion. (**) Primiparous cows mainly react as moderate responders following intramammary *E. coli* challenge (p. 125), (***) inoculum dose can modulate the kinetics of the moderate inflammatory reaction in primiparous cows (p. 145), whereas inhibition of prostaglandin synthesis only affects general clinical condition and eicosanoid production in milk (p. 167), vaccination against the endotoxin could slightly alter the clinical course of the moderate inflammation in primiparous cows (p. 189).

# PRACTICAL IMPLICATIONS AND FUTURE RESEARCH

1. Until now, milk quality and immune status of the mammary gland are assessed through quantification of the cells in milk (SCC measurement). However, in the future, functional examination of the resident cells in milk will become an additional tool in the study of mammary gland immunity and milk quality. In this thesis, manual milk collection has been shown sufficient for these purposes.
2. Questions will arise concerning the future approach of *E. coli* mastitis, especially in relation to treatment or prevention/prophylaxis. Focusing on prevention, one or more stable immune parameters should be available that can be measured throughout the transition period, in order to obtain quantitative feedback about the currently performed farm management practices. Therefore, the immune status of the dairy cows should be measured through the assessment of specific markers in the population. Besides the preventive approach, treatment of clinical mastitis will still be performed. The present work contributes, together with other data of our laboratory and world-wide mastitis research groups, to the knowledge of the physiological limits and the scientific basis of disease severity during clinical *E. coli* mastitis. This knowledge helps to answer the question: "which animals should not be treated ?" or rather: "should we treat with broad spectrum antibiotics or antibiotics with a Gram-positive spectrum ?". Due to the high degree of self-curing, it is clear that all animals in mid-lactation and the primiparous cows during early lactation are low risk candidates for a severe clinical response due to *E. coli* mastitis and should therefore not be treated. Moreover, EU directives will also determine use of antimicrobials in the near future. However, until now, rapid etiological diagnosis is still difficult. Therefore, mild to moderate clinical mastitis occurring in primiparous cows during early lactation, which could be caused by Gram-positive bacteria, should be treated with antimicrobials with a unique Gram-positive spectrum. In contrast, primiparous cows exhibiting a severe clinical response following naturally occurring mastitis can be treated with an antibiotic with broad spectrum.
3. Under practical circumstances, the use of a severity scoring system will help to obtain a good prognostic evaluation in animals suffering from clinical mastitis. A mild or moderate response in primiparous cows during early lactation may be due to *E. coli* or coagulase-negative staphylococci, which do both not require antimicrobial treatment. In practice, severe cases of clinical mastitis can occur in primiparous cows during the periparturient period. Under these circumstances, bacteriological diagnosis is an

essential tool in etiological diagnosis to differentiate between *E. coli*, which is predominantly self-curing, and *S. aureus*, which should efficiently be treated to omit chronic pathogen survival into the mammary gland. Moreover, differences in the clinical picture can occur between experimentally induced *E. coli* mastitis and naturally occurring cases of *E. coli*, which are observed under field circumstances.

4. Clinical and subclinical mastitis should clearly be distinguished. Primiparous cows are susceptible to coagulase-negative staphylococci, whereas they react mild to moderate following intramammary *E. coli* challenge. However, clinical *E. coli* mastitis is mainly determined by the host (Burvenich et al., 2003), whereas subclinical mastitis by coagulase-negative staphylococci is rather a problem of the bacteria (De Vliegher, 2004). Veterinary clinicians should therefore be forced to perform an etiological diagnosis of mastitis, especially because the term 'mastitis' is rather broad and more specifications should be made in terms of 'environmental mastitis' or 'contagious mastitis' to diversify between the two major groups of etiological bacteria. The more correct description of mastitis problematics does not discharge the veterinary clinician from his duty to perform an etiological diagnosis, because inventarisation of various bacteria, causing mastitis, present at the farm is advisable in good dairy farm management.

5. Primiparous cows suffering from mild to moderate clinical mastitis due to *E. coli* should not be treated with antimicrobials or anti-inflammatory drugs, but care should be taken towards the potential risk to develop a chronic *E. coli* mastitis problem, although this is a rare disease. The presence of multiple cases of clinical *E. coli* mastitis in primiparous cows may be indicative for more fundamental problems in relation to a decreased immunological status of the cows during the early lactation period. This can eventually spread to the population of multiparous cows on the farm, which are far more susceptible to severe clinical coliform mastitis.

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# SUMMARY

*Escherichia coli*, involved in bovine coliform mastitis, is part of the normal intestinal flora of animals. The strains isolated from bovine mastitis are essentially not different from strains isolated from bovine faeces. The bacteria do not possess specific virulence factors contributing in its pathogenesis. Several risk factors and severity determining factors, such as PMN number and functionality, and stage of lactation, have already been studied in relation to severe clinical mastitis. Many information is available on the inflammatory response following intramammary *E. coli* challenge in multiparous cows, although little is known on the susceptibility and reactivity towards *E. coli* mastitis of primiparous cows, which are an important population at dairy herd level.

The objective of this study was to characterise the inflammatory reaction following experimentally induced *E. coli* mastitis in primiparous cows during the periparturient period. Therefore, milk sample collection under aseptical conditions was validated in order to establish the effect of external bacterial contamination on pre-infection PMN functions assessed *in vitro*. Primiparous cows were intramammarily challenged with *E. coli* and the inflammatory reaction was studied. Furthermore, modulatory effects of variation of the inoculum dose, inhibition of prostaglandin synthesis and vaccination against the endotoxin were studied in the same experimental infection model.

In the chapter 'Validation of Milk Sample Collection under Aseptical Conditions' three different milk sampling techniques were compared with respect to the degree of external bacterial contamination obtained during sampling and its effect on pre-infection PMN functions *in vitro*. Limited external bacterial contamination during sampling did not interfere with PMN functionality assays and therefore, manual milk sample collection was preferred for further use in the subsequent experimental *E. coli* infections.

The chapter 'Materials and Methods Experimental Infections' contains all details on materials and methods used in the different experimentally induced *E. coli* challenges using primiparous cows. This chapter also contains pre-infection values of all measured parameters with the respective variation (expressed as SEM).

In the chapter 'Influence of Parity on Severity of Inflammation' primiparous cows are intramammarily challenged with high inoculum doses of *E. coli* and the inflammatory reaction is studied. Primiparous cows react as moderate responders, based on their quarter milk production in the uninfected quarters on d+2 post-infection. Decreased quarter milk production in primiparous cows is very short-lasting and resolution of local inflammation appears rapidly. A clinical severity scoring, using rectal temperature, reticulorumen motility, skin turgor and general attitude, is established to score the inflammatory reaction during clinical examination under practical conditions.

'Modulation of the inflammatory reaction' contains three chapters respectively studying variation of the inoculum dose, inhibition of prostaglandin synthesis and vaccination against the endotoxin.

In the chapter 'Variation of the inoculum dose' primiparous cows are randomly inoculated with 2 different inoculum doses (1 x $10^4$ and 1 x $10^6$ CFU *E. coli* per quarter) and inflammatory reaction is studied by quantification of cytokines and markers of the innate immune response (IL-8, C5a, sCD14 and LBP). All primiparous cows react with a moderate inflammatory reaction. However, intramammary inoculation of the highest dose (1 x $10^6$ CFU) results in an earlier activation of the innate immune response, although recovery of milk production does not differ between both inoculum doses.

In the chapter 'Inhibition of prostaglandin synthesis' an inhibitor of inducible enzymes of the cyclo-oxygenase pathway (COX-2) is administered to primiparous cows, intramammarily challenged with *E. coli*, when first clinical symptoms appear (9 h post-infection). Carprofen, a COX-2 inhibitor, treatment results in an improvement of general clinical condition, by rapid defeverescence and restoration of the reticulorumen motility, which is depressed during *E. coli* mastitis. The COX-2 inhibitor decreases eicosanoid production ($PGE_2$ and $TXB_2$) in the affected mammary quarters, but no effect on the inflammatory mediators of the innate immune response (IL-8, C5a, sCD14 and LBP) is observed.

Chapter 'Vaccination against the endotoxin' studies the effect of pre-calving vaccination with a J5 vaccine on subsequent experimentally induced *E. coli* mastitis in primiparous cows. No improvement in clinical condition could be observed in vaccinated animals, although favourable blood leukocyte kinetics were present in these animals. Vaccination against the endotoxin could barely modulate the inflammatory response following moderate inflammation in primiparous cows.

Primiparous cows during the periparturient period can be considered low risk candidates for severe clinical mastitis due to *E. coli*. The inflammatory reaction following experimentally induced *E. coli* mastitis is short-lasting, limited and self-curing. Only limited modulation is possible through the variation of the inoculum dose or inhibition of prostaglandin synthesis, whereas prophylactic vaccination against the endotoxin barely showed any effects on the moderate inflammatory reaction.

# SAMENVATTING

Acute mastitis, tengevolge van *Escherichia coli*, is van praktisch en economisch belang voor de melkveesector en de zuivelindustrie. De kiem, die geen specifieke virulentiefactoren met een rol in de pathogenese van de aandoening bezit, is een reguliere darmbewoner bij dieren en wordt dan ook in grote aantallen met de feces in de omgeving uitgescheiden. In de voorbije jaren zijn reeds verschillende risicofactoren en determinerende factoren in verband met de ernst van een intramammaire *E. coli* infectie, zoals het aantal en de functionaliteit van de neutrofielen en het lactatiestadium, bestudeerd. De ontstekingsreactie na intramammaire *E. coli* inoculatie is bij multipare koeien al uitgebreid onderzocht, doch de gevoeligheid en reactiviteit tegen *E. coli* mastitis van primipare koeien, een belangrijk deel van de populatie op een melkveebedrijf, is tot op heden nauwelijks bestudeerd.

De doelstelling van dit proefschrift was de karakterisatie van de ontstekingsreactie bij experimenteel geïnduceerde *E. coli* mastitis bij primipare koeien tijdens de peripartum periode te bestuderen. Hiervoor diende eerst de melkstaalname onder aseptische omstandigheden gevalideerd te worden met betrekking tot het effect van externe bacteriële contaminatie op de *in vitro* gemeten pre-infectie neutrofiel functionaliteit. Vervolgens werden primipare koeien intramammair besmet met *E. coli* om de ontstekingsreactie, en de vroege gastheer immuunrespons in het bijzonder, nader te bestuderen. Verder werden modulerende effecten van een variatie van de inoculum dosis, inhibitie van de prostaglandine synthese en vaccinatie tegen het endotoxine in hetzelfde experimentele infectiemodel onderzocht.

In het hoofdstuk 'Validatie van de melkstaalname onder aseptische omstandigheden' werden drie verschillende melkstaalname technieken, met name een aseptische, een manuele en een machinale techniek, vergeleken met betrekking tot de graad van externe bacteriële contaminatie tijdens de bemonstering en het effect van deze externe bacteriële contaminatie op de verschillende klassieke *in vitro* functietesten op boviene neutrofielen. Hieruit bleek dat een beperkte bacteriële contaminatie tijdens de bemonstering geen significante invloed had op deze *in vitro* functietesten. Tevens bleek dat de manuele melkstaalname voldoende garanties zou bieden voor een representatieve staalname tijdens de *E. coli* experimenten.

In het hoofdstuk 'Materiaal en methoden experimentele infecties' zijn alle details omtrent de gebruikte materialen en methodes in de verschillende *E. coli* experimenten bij primipare koeien opgenomen. In dit hoofdstuk zijn eveneens de pre-infectie waarden van alle gemeten parameters met hun respectievelijke variatie (uitgedrukt als SEM) opgenomen.

In het hoofdstuk 'Invloed van pariteit op de ernst van de ontsteking' werden primipare koeien intramammair geïnoculeerd met hoge inoculum doses *E. coli* en werd de ontstekingsreactie onderzocht. De ernst van de intramammaire infectie werd geëvalueerd door het meten van de kwartiermelkproductie in de niet-geïnfecteerde kwartieren op d+2 na infectie.

Daarnaast werd de ernst van de infectie gekwantificeerd door gebruik van een klinisch scoresysteem, rekening houdend met de rectale temperatuur, de voormagenmotiliteit, de huidturgor en de algemene indruk, die tijdens het klinisch onderzoek werden gemeten. Primipare koeien reageerden als 'moderate responders' op basis van beide scoresystemen. Op d+2 na infectie was de kwartiermelkproductie van de niet-geïnfecteerde kwartieren steeds > 50% van de productie voor infectie. Bovendien was de daling van de kwartiermelkproductie bij deze primipare koeien zeer kortstondig en trad vrij snel herstel van de locale intramammaire ontsteking op. De eenduidige 'moderate' klinische respons bij deze dieren stond in sterk contrast met de wijde variatie van het klinisch ziektebeeld dat bij multipare koeien in vorige studies kon worden waargenomen.

Het hoofdstuk 'Modulatie van de ontstekingsreactie' bevat drie onderdelen: de variatie van de inoculum dosis, de inhibitie van de prostaglandine synthese en de vaccinatie tegen het endotoxine.

In het onderdeel 'Variatie van de inoculum dosis' werden primipare koeien *at random* met 2 verschillende hoge inoculum doses ($1 \times 10^4$ en $1 \times 10^6$ kolonievormende eenheden (KVE) per kwartier) *E. coli* geïnoculeerd. De ontstekingsreactie werd vervolgens bestudeerd aan de hand van een kwantificatie van cytokines en merkers van de vroege immuunrespons (IL-8, C5a, sCD14 en LBP). Alle primipare koeien reageerden opnieuw met een 'moderate' ontstekingsreactie. Intramammaire inoculatie met de hoogste dosis ($1 \times 10^6$ KVE) resulteerde niettemin in een vroegere activatie van de immuunrespons. Hierbij trad een duidelijke verschuiving van de activeringskinetiek van IL-8, C5a, sCD14 en LBP op tijdens de vroege fase van de immuunrespons. Niettegenstaande de vroegere activatie van de afweer bij dieren geïnoculeerd met de hoge inoculum dosis, verschilde het herstel van de kwartiermelkproductie niet tussen beide inoculum doses. Uit dit experiment blijkt duidelijk dat een variatie in de inoculum dosis een beperkte modulatie van de ontstekingsreactie kan veroorzaken.

In het onderdeel 'Inhibitie van de prostaglandine synthese' werd een inhibitor van de induceerbare enzymen van de cyclo-oxygenase weg (COX-2) toegediend aan primipare koeien, die intramammair geïnoculeerd waren met *E. coli*. De toediening van het therapeuticum gebeurde op het ogenblik van het verschijnen van de eerste klinische symptomen (9 h na infectie). Carprofen, een COX-2 inhibitor, leidde tot een verbetering van de algemene klinische conditie door het sneller verdwijnen van de koorts en een vlotter herstel van de voormagenmotiliteit. De eicosanoid productie ($PGE_2$ en $TXB_2$) in de geïnfecteerde kwartieren werd door de COX-2 inhibitor onderdrukt. Niettemin had het niet-steroidale anti-inflammatoire product geen positief effect op de ontstekingsmediatoren van de vroege immuunrespons (IL-8, C5a, sCD14 en LBP), daar geen wijzigingen in de kinetiek tussen beide behandelingsgroepen

konden vastgesteld worden. Een inhibitor van de prostaglandine synthese blijkt dus in staat te zijn tot een beperkte modulatie van de ontstekingsreactie bij primipare koeien, vooral door het verbeteren van de algemene klinische conditie van het behandelde dier. Een vroege behandeling, op het ogenblik van het verschijnen van de eerste klinische symptomen, is hierbij van essentieel belang.

In het hoofdstuk 'Vaccinatie tegen het endotoxine' werd het effect van vaccinatie met een J5 vaccin tijdens de dracht op een daaropvolgende experimenteel geïnduceerde *E. coli* mastitis bij primipare koeien bestudeerd. Het vaccin werd tweemaal toegediend tijdens het laatste deel van de dracht (56 en 28 dagen voor de verwachte afkalfdatum) en éénmaal tijdens de eerste week na het afkalven. Vervolgens werden de primipare koeien intramammair met *E. coli* geïnoculeerd en werden een aantal klinische en laboratoriumparameters verzameld. Vaccinatie tegen het endotoxine liet weinig verbetering van de klinische conditie zien, hoewel een gunstige evolutie in de kinetiek van de bloedleukocyten waargenomen werd. De kwartiermelkproductie keerde in beide behandelingsgroepen vrij snel terug tot het pre-infectie niveau. Vaccinatie tegen het endotoxine was vrijwel niet in staat om de ontstekingsreactie bij primipare koeien te moduleren.

Primipare koeien tijdens de peripartum periode kunnen als laag risico kandidaten met betrekking tot ernstige klinische mastitis, te wijten aan *E. coli*, aanzien worden. De ontstekingsreactie bij experimenteel geïnduceerde *E. coli* mastitis is kortdurend, afgelijnd en geneest zonder behandeling. Een beperkte modulatie van de ontstekingsreactie is mogelijk door variatie van de inoculum dosis of inhibitie van de prostaglandine synthese. Vaccinatie tegen het endotoxine had vrijwel geen effect op de 'moderate' ontstekingsreactie.

De extrapolatie van de resultaten van dit experimenteel onderzoek naar de praktijk toe moet met de nodige voorzichtigheid gebeuren, daar onder praktijkomstandigheden het optreden van een ernstige klinische reactie bij *E. coli* mastitis niet steeds kan worden uitgesloten. In dit opzicht is en blijft een bacteriologisch onderzoek voor het stellen van de etiologische diagnose van cruciaal belang om een duidelijk onderscheid te kunnen maken tussen de verschillende mastitisvormen.

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Frédéric

# CURRICULUM VITAE

Frédéric Vangroenweghe was born on January 30th 1975 in Menen (Belgium). He obtained his Degree in Veterinary Medicine (DVM) with the greatest distinction in 1999 and received the Vétoquinol Price for this dissertation on vaccination against *Actinobacillus pleuropneumoniae* in swine and the Price of the Faculty of Veterinary Medicine. From September 1st 1999 onward, he worked as assistant at the Milk Secretion and Mastitis Research Centre (MMRC, Department of Physiology-Biochemistry-Biometrics), where he received training in veterinary physiology and bovine *Escherichia coli* mastitis. He participated in the practical teaching of 2nd and 3rd year students Veterinary Medicine.

Under the guidance of Prof. Dr. Christian Burvenich, he performed scientific research on the role of resident cells in bovine low somatic cell count milk and the inflammatory reaction following *Escherichia coli* mastitis in primiparous cows. He worked abroad from several weeks at the Institut National de Recherche Agronomique (INRA) in Nouzilly - Tours under the supervision of Dr. Pascal Rainard. He actively participated at several national and international congresses with oral communications and poster presentations and is author or co-author of several scientific papers in peer-reviewed national and international journals.

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