

Impact of Milk Somatic Cell Count on *Escherichia coli* Growth *in Vitro*

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Abstract

Bovine mastitis is of major economic impact on global dairy industries. Overshooting *Escherichia coli* (*E. coli*) growth due to impaired function of polymorphonuclear cells (PMN) increases severity of acute mastitis. During mastitis, PMN account for the majority of somatic cells in milk. Besides PMN, soluble antimicrobial factors are secreted into milk. The objective of the current study was for how long and to what extent immigrating PMN and soluble factors in milk can inhibit *E. coli* growth *in vitro*. In 14 Holstein heifers, with somatic cell counts (SCC) $<5 \times 10^4$ /ml a SCC increase ($>2.5 \times 10^6$ /ml) was induced by infusion of 1 µg Lipopolysaccharide (LPS) per udder quarter. Milk was collected 0, 24, 72 and 240 h after LPS infusion and *E. coli* growth was assessed in whole milk, cell depleted skim milk and Lysogeny Broth (LB). Whole milk containing somatic cells significantly inhibited *E. coli* growth 24 h after LPS infusion ($P < 0.001$). No inhibitory effect could be detected in cell depleted skim milk. There was a significant negative correlation ($R^2 = 0.829$, $P < 0.001$) between *E. coli* growth and SCC levels. After LPS infusion the increased SCC was dominated by PMN ($>90\%$) as assessed by flow cytometry. In conclusion PMN act as the main inhibiting factor for *E. coli* growth in mastitis milk *in vitro*.

Keywords: Mastitis; *E. coli*; LPS; PMN; *in vitro*

Introduction

Due to the current lack of satisfactory treatment or prophylaxis, bovine mastitis has a major economic impact on global dairy industries [1]. Severe clinical mastitis is often caused by an intramammary *E. coli* infection. The main pathogenic component of *E. coli* is lipopolysaccharide (LPS), which can initiate an overshooting immune response [2]. This leads to severe local and systemic signs of disease which may damage the udder tissue permanently or even cause death in affected cows. At this point speed and quantity of PMN influx determine the extent of *E. coli* multiplication in the mammary gland and in consequence the severity of disease [3, 4]. Besides PMN, soluble antimicrobial factors like β defensins and the antibacterial psoriasin are highly abundant in the udder during *E. coli* mastitis [5, 6]. For establishment of intramammary infection, *E. coli* does not depend on specific

virulence factors [2, 7]. It is among bacteria with the highest multiplication rate. Milk plays a key role as an ideal nutrient solution for *E. coli* which is capable of metabolizing lactose [8]. By determining *E. coli* growth in milk attempts were made in the past to mimic establishment of intramammary infection *in vitro*. It was shown that growth varied in milk from different cows by factor 100. Additionally *E. coli* growth was faster in milk serum than in cell reduced skim milk or whole milk. Yet no relation could be detected between *in vitro* growth and *in vivo* growth. Only the initial SCC of experimentally infected cows had an impact on *E. coli* growth *in vivo* [9]. Lohuis et al. found out that growth of *E. coli* in whole and skim milk collected prior to endotoxin infusion was not inhibited whereas inhibition occurred in whole milk samples taken 18 h after infusion of 1 mg endotoxin. In skim mastitic milk growth inhibition varied between cows and between different quarters of the same cow [10]. The objective of the study was to develop an *in vitro* model to examine the antibacterial actions of cellular and soluble factors in milk

towards *E. coli*. By infusing udders of healthy heifers with LPS, defined, sterile mastitis milk should be induced. An increased antibacterial capacity of milk was to be tested in whole milk containing cells, as well as cell depleted skim milk to point out the impact of cellular mechanisms in mastitis milk. Additional milk cell differentiation should underpin the importance of the predominating cell population.

Materials and Methods

Intramammary LPS infusion

The study included 14 healthy Holstein heifers in their 3rd to 6th month of lactation. The animal experiment had been approved by the ethics committee of the regional government of Upper Bavaria (No. 55.2-1-54-2531-108-05). All animals had no history of mastitis, showed a consistently low SCC (<50,000 cells/ml) and had been repeatedly diagnosed free from bacteria in milk of all udder quarters.

LPS of *E. coli* strain 1303 was kindly provided by Sonja von Aulock, University of Konstanz, Germany. It was prepared by butanol extraction procedure and hydrophobic interaction chromatography, as described [11]. This was followed by a purification step with triethylamine and deoxycholate [12]. Finally the LPS was diluted in 0.9% sterile, pyrogen-free saline to a concentration of 0.2 µg/ml and kept stored in 5 ml aliquots at -80°C. After thorough cleaning and disinfection (70% ethanol) of every teat orifice all animals received 1 µg LPS of *E. coli* strain 1303 (in 5 ml 0.9% sterile, pyrogen free saline) intrac-isternally via the teat canal. Over the following 240 h animals were tightly monitored for signs of inflammation. Rectal body temperature, blood leukocyte counts and general condition were assessed at time point 3, 6, 9, and 12 h after trial start and then every 12 h. Milk samples (10 ml) were taken aseptically every 12 h after trial start.

Milk analysis

Milk SCC was determined every 12 h by Fossomatic 5000® in accordance with IDF Standards. To guarantee milk samples were free from bacteria, 15 µl milk were plated on Columbia Sheep Blood Agar (Oxoid, Wesel, Germany). Flow cytometric milk differential cell count was assessed as previously described [13]. Briefly, 10 ml milk in 40 ml PBS were centrifuged (400 x g, 10 min, 4°C) and the fat and skim milk were removed. The cell pellet was diluted in 50 ml PBS and centrifuged (400 x g, 10 min, 4°C); the supernatant was then removed, and the cells were suspended in PBS and kept at a concentration of 10⁵ cells/ml. After addition of propidium iodide (PI, 2 µg/ml final concentration) and acridine orange (AO, 2.5 pg/ml final concentration), the cells were identified and 20,000 events were acquired with a FACScan® cytometer after a live gate was set on green fluorescing particles. The proportions of viable cellular subpopulations were determined after gating for PI-nega-

tive events. PMN were identified on the basis of morphological characteristics by comparison of forward (FSC) and side scatter (SSC) cytometry dotplots. Flow cytometric data was analyzed with the software FCS Express V3.

In vitro growth assay

For *E. coli* growth assays *in vitro*, milk from the animals was collected aseptically after discarding the first 15 ml. Whole milk and cell depleted skim milk were prepared from the same portion of milk. In total 250 ml milk were collected in a sterile flask from the udder quarter with the lowest SCC immediately before LPS infusion (time point 0 h). For the following analysis 250 ml milk were collected 24, 72 and 240 h later from the same udder quarter respectively. The following preparation steps were carried out immediately after collection under a lamina flow to avoid contamination. After transferring 100 ml whole milk to a sterile 0.5 l Erlenmeyer flask on ice, cell depleted skim milk was prepared by centrifugation (4,500 x g, 25 min, 4°C). After carefully removing the fat layer, 100 ml skim milk were transferred to a 0.5 l Erlenmeyer flask. Cell depletion of skim milk was confirmed by flow cytometry. The inoculum dose of bovine mastitis isolate *E. coli* 1303 was prepared as previously described [5]. Bacteria were plated on Columbia Sheep Blood Agar and incubated (37°C) for 24 h. A few colonies were transferred to a tube of Brain-Heart Infusion Broth and incubated for 6 h (37°C); then a 100 µl sample was transferred to a tube containing 9.9 ml Trypticase Soy Broth. Serial dilutions were made after 18 h to prepare the desired concentration of 10⁶ cfu/ml 0.9% sterile, pyrogen free saline. In total 10 µl were transferred to either 100 ml whole milk, cell depleted skim milk or Lysogeny Broth (LB) respectively to obtain an initial concentration of 100 cfu/ml. Subsequently the flasks were placed on a shaker and were incubated over 24 h (37°C). Bacterial counts were determined after 2, 4, 6, 8 and 24 h by spiral plating (Eddy Jet, IUL Instruments, Königswinter, Germany) on Violet Red Bile Agar (Oxoid). Agar plates were incubated over night (37°C) and cfu/ml were determined by computer assisted colony counting (CounterMat Flash, IUL Instruments).

Statistical analysis

Statistical analysis was performed with Microsoft Excel 2007 and SPSS (Version 17). *E. coli* growth was compared after calculating the area under the curve (AUC) of log transformed cfu for each group for the growth phase 0-24 h. Differences in bacterial growth were assessed by non-parametric Kruskal-Wallis-test with post-hoc Mann-Whitney U-test using Bonferroni-correction for multiple testing. Correlation coefficient for AUC and SCC was determined by linear regression.

Results

Intramammary LPS infusion increases milk SCC and PMN

After intramammary LPS infusion animals responded with a significant SCC increase (Table 1). SCC reached its maximum 12 h after LPS infusion (data not shown) and was still significantly elevated after 24 h and 72 h before almost reaching baseline values after 240 h (Table 1). The amount of PMN among the somatic cells in milk increased >90% 24 h after intramammary LPS infusion (Table 1). Visible alterations in foremilk were found in 50% of the animals after LPS infusion but gradually disappeared during the following 72 h. Beside a short lived elevation of body temperature (39.6 ± 0.2 °C), animals showed no systemic signs of clinical mastitis (leukopenia, depressed general condition, data not shown).

Table 1. SCC and PMN in milk after intramammary LPS Infusion.

time after LPS infusion (h)	SCC ¹ (*10 ³ /ml)	PMN ² (%)	
n	mean	SD	Median
0	14 26.6 ±	49.0	34.2
24	14 2639.9 ±	1218.7	91.2
72	14 564.9 ±	230.2	79.6
240	10 41.8 ±	29.8	46.9

significant differences to 0 h bold ($P < 0.001$)

¹somatic cell count ²polymorphonuclear neutrophilic granulocytes

Intramammary LPS infusion inhibits *E. coli* growth in whole milk but not in cell depleted skim milk

The strain *E. coli* 1303 showed a reproducible growth pattern *in vitro* throughout the study (Figure 1, bottom). Every growth assay in milk preparations was accompanied by a simultaneous growth assay in LB medium (Table 2) to guarantee the inoculated strain followed its usual growth pattern. The logarithmic area under the curve (ln AUC) for *E. coli* growth was significantly greater in LB compared to milk preparations (Table 2). When comparing growth in whole milk to cell depleted skim milk and LB medium we could only detect growth inhibition in cell rich milk from cows 24 h after LPS infusion (Table 2, Figure 1 top). There was no statistical significant difference between the time points 0 h, 72 h and 240 h (Figure 1). *E. coli* growth showed to be negatively correlated with SCC levels in whole milk (Figure 2). In whole milk samples collected 24h after LPS infusion we could only detect growth inhibition over the first 8 h of incubation (Figure 1). After 24 h incubation time the number of *E. coli* converged in all milk preparations.

Table 2. Effect of intramammary LPS infusion on *E. coli* growth in whole milk and cell depleted skim milk.

time after LPS inoculation (h)	whole milk (ln AUC ¹)	cell depleted skim milk (ln AUC ¹)	LB ² (ln AUC ¹)			
n	mean	SD	mean	SD	mean	SD
0	14 32.4 ±	2.9	32.4 ±	3.1	37.7 ±	2.1
24	14 19.8 ±	7.4	31.8 ±	3.9	35.5 ±	6.1
72	14 32.2 ±	2.9	33.4 ±	2.7	37.4 ±	3.0
240	10 32.7 ±	1.5	32.9 ±	2.4	38.2 ±	2.5

significant differences to 0 h bold ($P < 0.001$)

¹log transformed area under curve

²Lysogeny Broth

Discussion

After intramammary LPS infusion milk showed a higher SCC. Milk analysis showed that the amount of PMN among the somatic cells in milk increased >90% after 24 h (Table 1). This observation is in line with previous studies, reporting that a SCC increase is mainly determined by immigrating PMN [13].

The intended low dose of 1 µg LPS per udder quarter induced a mild local subclinical inflammatory response which resulted in SCC increase but not in a general systemic reaction of the cow as described in previous models where usually 100 µg LPS were administered intramammarily [10]. Furthermore the controlled intramammary LPS infusion had the advantage of inducing “sterile” mastitis milk for the applied growth assay. In comparison to mastitis milk from naturally occurring intramammary infections there was no potential carryover of pathogens.

The *in vitro* growth assay of *E. coli* compared growth in whole milk to cell depleted skim milk and LB medium after LPS infusion. The assay showed that growth inhibition only occurred in cell rich milk from cows 24 h after LPS infusion (Table 2, Figure 1 top) whereas no statistical significant difference was detected between time points 0, 72 and 240 h (Figure 1). This overlaps in part with a study by Lohuis et al. (1990) who studied *E. coli* growth in milk 18 h after intramammary LPS infusion and found growth inhibition as well as bactericidal activity in whole and skim milk [10]. However they could not show an evident relation between cells in milk and bacterial growth. These differences may attribute to the fact, that LPS was administered in a 100 fold dose and caused acute clinical mastitis and the composition of milk was much more affected.

Milk serves as a good growth medium for *E. coli in vitro*. However, growth in LB was significantly greater than in milk preparations. Whether this observed difference is based on the different content of nutrients or the presence of antimicrobial factors in milk remains speculative. Within this study *E. coli* reached comparable levels of cfu/ml compared to another *in vitro*-study of Kornalijnslijper et al. (2003), who showed that after 6 h *E. coli* grew up to 10log 5.1 in whole milk and 10log

5.3 in skim milk [9].

Figure 1. *E. coli* growth over 24 h in whole milk (A) and cell depleted skim milk (B) collected 0 h, 24 h, 72 h (n=14) and 240 h (n=10) after intramammary LPS infusion. Data are expressed as mean and SEM.

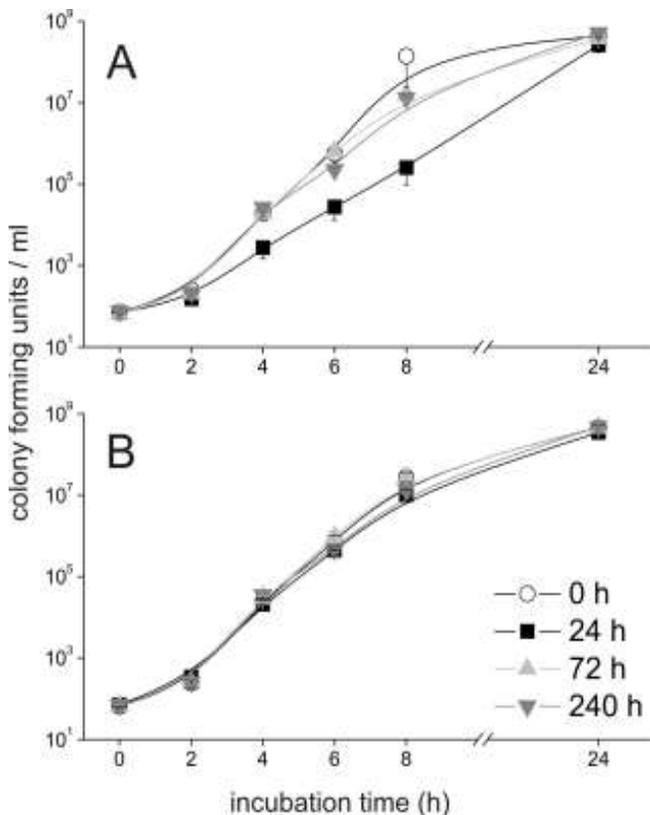
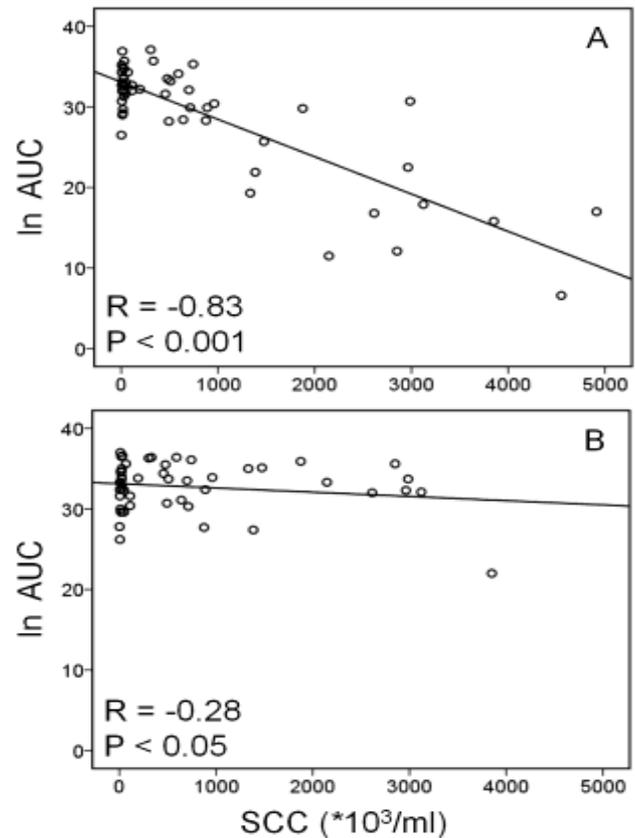


Figure 2 shows that SCC levels in whole milk and *E. coli* growth were negatively correlated. These data clearly indicate that the observed growth inhibition of *E. coli in vitro* was a cell mediated effect. It is most likely that the high amount of immigrated neutrophils (>90%) keeps up an antimicrobial environment by bactericidal activity (phagocytosis, reactive oxygen species, release of β -defensins). Furthermore the data show that the quantity of PMN in milk has to exceed a certain threshold so that bacterial growth is inhibited. In whole milk, collected 72 h after LPS infusion the initial ratio of pathogens and PMN was fairly high (about 1:6), still no growth inhibition could be detected. Furthermore the data show that the quantity of PMN in milk has to exceed a certain threshold so that bacterial growth is inhibited. In whole milk, collected 72 h after LPS infusion the mean SCC ranged around $0.6 \cdot 10^6$ /ml milk (Table 1) and individual samples even exceeded $0.9 \cdot 10^6$ /ml milk. The initial ratio of pathogens and PMN in these samples was fairly high (about 1:6), still no growth inhibition could be detected. The data indicate that no significant *in vitro* growth inhibition of *E. coli* in whole milk can be expected when the SCC is $< 0.6 \cdot 10^6$ /ml. Whole milk collected 24 h after LPS infusion showed a higher SCC (mean $2.6 \cdot 10^6$ /ml) and had an inhibitory effect on *E. coli* growth (Table 2). However the data showed a high vari-

ability thus a significant inhibitory threshold for SCC could not be shown within this study.

Figure 2. Interdependence of *E. coli* growth and somatic cell count (SCC) in whole milk (A) and cell depleted skim milk (B). Log transformed bacterial counts over 24 h are expressed as area under the curve (ln AUC).



The observation that growth inhibition in whole milk samples collected 24 h after LPS infusion only occurred over the first 8 h of incubation (Figure 1) might be due to an increasing loss of functional active PMN present in milk. Bovine blood PMN are known to have a rather short half life of approximately 9 h [14]. Additionally *in vitro* we face a lack of new immigrating PMN compared to *in vivo* conditions, where constantly PMN enter the site of infection [15]. Speed and quantity of PMN influx determine the extent of *E. coli* multiplication in the mammary gland and in consequence the severity of disease [3].

Conclusion

Somatic cells in milk transiently act as the main inhibiting factor for *E. coli* growth in mastitis milk *in vitro*. They are predominated by PMN which underpins the importance of these cells in the resolution of intramammary infection during bovine mastitis. Furthermore not alone the initial abundance of PMN but also their functionality is of key importance for *E. coli*

growth in milk. Thus a fast and constant immigration of PMN to the site of infection may be crucial for effective pathogen elimination.

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