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

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Received: 10-09-2015
Accepted: 11-25-2015
Published: 12-04-2015

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*10^4/ml a SCC increase (>2.5*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.
Milk SCC was determined every 12 h by Fossomatic 5000® in Milk analysis every 12 h after trial start. then every 12 h. Milk samples (10 ml) were taken aseptically temperature, blood leukocyte counts and general condition were tightly monitored for signs of inflammation. Rectal body temperature was measured internally via the teat canal. Over the following 240 h animals strain 1303 (in 5 ml 0.9% sterile, pyrogen free saline) intrac- nellinol) of every teat orifice all animals received 1 µg LPS of a concentration of 0.2 µg/ml and kept stored in 5 ml aliquots for the LPS was diluted in 0.9% sterile, pyrogen-free saline to the purification step with triethylamine and deoxycholate [12]. Final- ly low SCC (<50,000 cells/ml) and had been repeatedly di- agnosed free from bacteria in milk of all udder quarters. The 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 pu- rification 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% etha- nol) 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.

**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.

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-negative 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 ana- lyzed 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 laminar 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 pre- pared 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 cal- culating 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.

E. coli the number of the first 8 h of incubation (Figure 1). After 24 h incubation time after LPS infusion we could only detect growth inhibition over whole milk (Figure 2). In whole milk samples collected 24h after LPS infusion we could only detect growth inhibition over whole milk but not in cell depleted skim milk. 

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

<table>
<thead>
<tr>
<th>time after LPS inoculation (h)</th>
<th>whole milk (In AUC)</th>
<th>cell depleted skim milk (In AUC)</th>
<th>LB1 (In AUC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n mean</td>
<td>SD</td>
<td>n mean</td>
<td>SD</td>
</tr>
<tr>
<td>0</td>
<td>14</td>
<td>32.4 ± 2.9</td>
<td>32.4 ± 3.1</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>19.8 ± 7.4</td>
<td>31.8 ± 3.9</td>
</tr>
<tr>
<td>72</td>
<td>14</td>
<td>32.2 ± 2.9</td>
<td>33.4 ± 2.7</td>
</tr>
<tr>
<td>240</td>
<td>10</td>
<td>32.7 ± 1.5</td>
<td>32.9 ± 2.4</td>
</tr>
</tbody>
</table>

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

significant differences to 0 h bold (P< 0.001)

<table>
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</table>

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.

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.

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

Escherichia coli can be expected when the SCC is $< 0.6 \times 10^6/\text{ml}$. Whole milk collected 24 h after LPS infusion showed a higher SCC (mean $2.6 \times 10^6/\text{ml}$) and had an inhibitory effect on *E. coli* growth (Table 2). However, the data showed a high variability 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.

**Acknowledgments**

This work was funded by the Wilhelm-Schaumann-Stiftung.

**References**


