Growth of *Escherichia coli* in Whole and Skim Milk from Endotoxin-Induced Mastitic Quarters: In Vitro Effects of Deferoxamine, Zinc, and Iron Supplementation


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ABSTRACT

A marked growth inhibition of *Escherichia coli* 0101 K99 F41 was observed in whole and skim milk collected from inflamed quarters 18 and 36 h after intramammary administration of .1 mg *E. coli* lipopolysaccharide. Individual cow variation in the ability of milk from endotoxin-infused quarters to inhibit growth of *E. coli* was found. Growth inhibition of *E. coli* was observed in milk from endotoxin-infused quarters and was most pronounced in skim milk sampled at postinfusion h 18, and incubated at 38°C. The mechanism by which bacterial growth was depressed was probably of noncellular origin.

Addition of Fe (45.5 μg/ml) and Zn (2.7 μg/ml) to whole and skim milk sampled from inflamed quarters at 18 h after endotoxin infusion resulted in a growth-promoting effect. Addition of deferoxamine (6 mg/ml) depressed bacterial growth. Effects of Fe, Zn, and deferoxamine on bacterial growth did not differ in whole and skim milk. No clear relationship was observed between reduction in Zn concentrations in skim milk from inflamed quarters at 18 h after endotoxin infusion and growth inhibition of *E. coli* in the same samples.

INTRODUCTION

Marked decreases in plasma Zn and Fe concentrations occur in cattle after intravenous and intramammary (i.m.m.) administration of endotoxins (24, 25). Zinc and Fe deprivation inhibited growth of a number of gram-positive and gram-negative bacteria (4, 13, 27), especially at febrile temperatures (13). These changes in trace elements may result in an effective, albeit nonspecific, antibacterial host defense mechanism (4, 13, 21, 27).

In addition to the effect on plasma Zn concentrations, i.m.m. administration of endotoxin [0.04 mg lipopolysaccharide (LPS) of *E. coli*] resulted in a marked decrease in Zn in whole and skim milk samples from LPS-infused quarters compared to uninfused (23). Moreover, Zn was lower (*P<.05*) in skim milk from LPS-infused quarters than in skim milk from uninfused ones between 11 and 19 h after administration. However, Zn in skim and whole milk from uninfused quarters was not markedly different (23). These findings demonstrate that decreased Zn concentrations in skim milk from the quarters infused with LPS cannot be explained fully by the decrease in plasma Zn concentrations but should be attributed partly to a local redistribution of Zn within the inflamed quarter (23).

It seems likely that changes in trace elements in mastitic quarters may play an important role in local, nonspecific antibacterial defense mechanisms of the udder (23). Therefore, the purpose of our study was to determine if bacterial growth was inhibited in whole and skim milk from LPS-treated quarters and the effect of incubation temperature (38 and 41°C) on bacterial growth. Furthermore, we were interested in determining if bacterial growth in milk from LPS-infused quarters was affected by addition of Zn and Fe, or the chelating agent, deferoxamine.
MATERIALS AND METHODS

Animals

Twenty-three clinically healthy cows of different breeds, ages, weights, and stage of lactation were used. Before each experiment, the udder was examined clinically and quarter foremilk samples were obtained for bacteriological examination and SCC (11, 15). Only cows with quarter SCC below 500,000/ml and negative bacteriological examination were used.

Endotoxin-Induced Mastitis

Purified LPS obtained from \textit{E. coli} 0111 B4 (Lot 61438, Difco, Detroit, MI) was used. Just before administration, LPS was dissolved in sterile, pyrogen-free saline. Endotoxin-induced mastitis was produced in all experiments by i.m. administration of .1 mg LPS in the left or right rear quarter as described (23). During intervals between samplings, LPS-infused quarters were not milked.

Experimental Design

In the first experiment (three cows), bacterial growth was analyzed in whole and skim milk from LPS-infused rear quarters and in whole milk from uninfused rear quarters. Milk was sampled immediately prior to LPS infusion (PIH 0) and at postinfusion h (PIH) 18 and 36 from LPS-infused and uninfused quarters. Analysis of bacterial growth was at 38 and 41°C to determine the effect of incubation at febrile temperature on bacterial growth.

In additional experiments (12 cows), growth analysis was done only in whole and skim milk from LPS-infused quarters at PIH 0 and 18. The samples were only incubated at 38°C, because growth inhibition was more pronounced at this temperature.

In another series of experiments (12 cows), the effects of supplementation with Zn and Fe on bacterial growth (8 cows) and the effects of the chelating agent, deferoxamine (4 cows), on bacterial growth in milk from LPS-treated quarters were studied. Milk was collected at PIH 18 from LPS-infused quarters. Bacterial growth was analyzed in whole and skim milk at 38°C. In controls these substances were replaced by double distilled water (DDW).

Sample Handling

Milk samples were collected in containers (Sterilon®, Continental Pharma, Zutphen, Neth). After arrival at the laboratory, part of each sample was kept at 4°C (whole milk) and part was used for skim milk preparation. To prepare skim milk, samples were centrifuged for 20 min at 3000 rpm; the fat layer was discarded and the skim milk removed. Bacterial growth was analyzed immediately after skim milk preparation.

Analysis of Bacterial Growth

\textit{Bacteria}. A strain of \textit{E. coli} 0101 K99 F41, isolated from a clinical case of bovine mastitis, was used. The strain was maintained on nutrient agar CM3 (Oxoid Ltd., Basingstoke, Hampshire, Engl.) at 4°C and was passaged to fresh nutrient agar every 3 wk to ensure viability and purity. Characterization was after each passage by colony morphology and biochemical reactivity (8). The \textit{E. coli} strain was harvested from nutrient agar and suspended in 5 ml brain-heart infusion broth (BHI) CM 225 (Oxoid) and grown at 37°C. Prior to each assay a culture was prepared in BHI containing approximately 1 x 10^9 cfu/ml. Actual bacterial counts were determined in each experiment by enumerating viable counts on violet red bile glucose agar (VRBG) CM 485 (Oxoid) after 18 to 24 h incubation at 37°C. Viable counts ranged from 6.7 x 10^8 to 1.4 x 10^9/ml.

Analysis of Bacterial Growth Without Supplement

The culture containing 1 x 10^9 cfu/ml was diluted in saline to approximately 1 x 10^6 cfu/ml immediately prior to each growth experiment. Aliquots of 1 ml from this diluted culture were transferred to 9 ml whole or skim milk and incubated in a waterbath at 38 or 41°C. Bacterial growth in milk was monitored by viable counts on VRBG (7). Culture samples (1 ml) were collected after 0, 2, 4, 6, and 8 h of incubation, transferred to 9-cm petri dishes in appropriate dilutions, and 15 ml of medium were added. After the medium solidified it was overlaid with 10 ml of the same medium. Enumeration took place after 18 to 24 h at 37°C.

Assay System for Monitoring Effects of Zinc, Iron, and Deferoxamine on Bacterial Growth
Growth. Iron concentration was minimized in all experiments using acid-washed glassware as well as chemicals of highest purity. Glassware was rinsed twice with a chelating solution [prepared by dissolving 500 mg deferoxamine (Desferal®, Ciba-Geigy N. V., Groot Bijgaarden, Belgium) in 1000 ml DDW], rinsed two times with DDW, dried, and autoclaved.

Stock solutions of FeCl₃ (4000 µg/Fe/ml) and ZnCl₂ (4000 µg Zn/ml) were prepared dissolving FeCl₃ (Art. No. 3943, E. Merck, Darmstadt, FRG) and ZnCl₂ (Art. No. 8816, E. Merck) in DDW. Prior to the experiments, solutions were diluted with DDW to yield 1000 µg Fe/ml, and 60 µg Zn/ml, respectively. Deferoxamine solution was prepared by dissolving 500 mg deferoxamine in 5 ml DDW and diluted to a final concentration of 66.7 mg/ml. Solutions were sterilized by membrane filtration [.20 µ pore size (Millipore Corp., Bedford, MA)].

The assay system consisted of 9 ml whole or skim milk, 1 ml inoculum containing 10⁴ cfu/ml, and 1 ml growth effector. Two growth effector systems were tested. One consisted of a .5 ml solution of FeCl₃ (1000 µg Fe/ml) and .5 ml of a solution of ZnCl₂ (60 µg Zn/ml); the other consisted of 1 ml of a solution containing 66.7 mg deferoxamine/ml. Thus, final concentrations of Fe and Zn in the assay system were 45.5 and 2.7 µg/ml, respectively. Concentration of deferoxamine was 6 mg/ml. In controls, growth effectors were replaced by 1 ml DDW. Each assay was duplicated. Monitoring of bacterial growth was performed by viable counts, as described, after 0, 2, 4, 6, and 8 h incubation at 38°C.

Zinc Analysis

Zinc was determined in skim milk samples, taken from LPS-infused quarters of 9 cows at PIH 0 and 18. Samples were kept overnight at 4 to 6°C, and Zn concentrations determined spectrophotometrically (Model 305 B, Perkins-Elmer Corp, Norwalk, CT) after thorough sample homogenization.

Somatic Cell Count

To evaluate skim milk preparation, somatic cells in skim milk samples were counted electronically in a Coulter Counter (Coulter Electronics, UK) (11). Cell counts in skim milk did not exceed 6000/ml (results not shown).

Statistical Analysis

Analyses used a linear regression model (1) where the logarithm of the viable count of colony-forming units was the dependent variable. However, in every analysis cow effects and linear and quadratic terms for incubation time were forced into the model. For example, to test the effect of addition of Fe and Zn in whole milk, the regression model was as follows:

\[
\log\text{cfu} = \alpha + b_{1-7} \times \text{cow}_{1-7} + b_8 \times \text{time} + b_9 \times \text{time}^2 + b_{10}
\]

where:
- \(\alpha\) = intercept.
- \(b_{1-7}\) = coefficients for cow effects.
- \(b_8, b_9\) = coefficients for linear and quadratic effects of incubation time.
- \(b_{10}\) = coefficient, indicating effects of Zn and Fe addition on log colony-forming units.

The \(b_{10}\) was divided by its standard error. This ratio has a Student \(t\) distribution. Significance was defined at \(P<.05\). All calculations were performed with the microcomputer package Statistix® (NH Analytical software, Roseville, MN 1986).

RESULTS

Bacterial Growth Without Supplement

The results of the growth experiments in whole milk samples from LPS-infused and uninfused quarters and in skim milk from LPS-infused quarters of 3 cows are in Figure 1. Growth inhibition did not occur in whole milk samples drawn from uninfused quarters at PIH 18 as compared with those of controls sampled prior to LPS infusion (Figure 1A). However, growth inhibition at 38 and 41°C was moderate (\(P<.003\)) in whole milk samples collected at PIH 36 from the uninfused quarters.

Growth inhibition (\(P<.0001\)) occurred in whole milk drawn from LPS-infused quarters at PIH 18 and 36 (Figure 1B). The skim milk samples collected from LPS-infused quarters at PIH 18 also showed marked inhibition of bacterial growth (\(P<.0001\)) at 38°C but not at 41°C (Figure 1C). Figure 2 illustrates results of
Figure 1. Growth (mean ± SEM) of Escherichia coli 0101 K99 F41 in whole milk from untreated rear quarters (A) and in whole milk (B) or skim milk (C) from lipopolysaccharide (LPS)-infused rear quarters of three cows. Milk was sampled at 0 h (solid lines), 18 h (dashed lines), and 36 h (thin lines) after intramammary infusion of .1 mg E. coli LPS in one rear quarter. Incubation was at 38 or 41°C.

growth experiments in skim milk drawn from LPS-infused quarters of 11 cows. A clear growth inhibition was observed in skim milk of 3 cows sampled 18 h after LPS infusion. This depressed bacterial growth was either absent or much less in skim milk from 8 other cows.

Zinc concentrations in skim milk, sampled at PIH 0 and 18 from LPS-infused quarters of 9 cows, ranged from 14 to 62 μmol/L and from 7 to 37 μmol/L, respectively (Table 1). A reduction (P<.05) in Zn concentrations of 50.8 ± 3.16% (mean ± SEM) was observed in samples collected at PIH 18. No relationship was observed between bacterial growth inhibition in skim milk from LPS-treated quarters and Zn concentration or Zn reduction in skim milk.

Effect of Zinc and Iron on Bacterial Growth

The effect of Zn and Fe (2.7 μg Zn/ml and 45.5 μg Fe/ml) on bacterial growth is in Figure...
GROWTH OF ESCHERICHIA COLI IN MASTITIC MILK

TABLE 1. Concentrations and reduction of zinc and bacterial growth inhibition in skim milk from lipopolysaccharide-(LPS)-infused quarters.

<table>
<thead>
<tr>
<th>Cow no.</th>
<th>Zn Concentration at(^1) (µmol/L)</th>
<th>Zn Reduction(^2) of baseline value (%)</th>
<th>Inhibition of bacterial growth(^3) at PIH 18</th>
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\(^1\) Concentrations of Zn (µmol/L) in skim milk from LPS-infused quarters prior to (PIH 0) and 18 h after (PIH 18) infusion of .1 mg Escherichia coli LPS in the left or right rear quarter of nine cows.

\(^2\) Reduction of Zn at PIH 18 was calculated as a percentage of Zn concentrations at PIH 0.

\(^3\) Growth of E. coli 0101 K99 F41 was analyzed in skim milk collected from LPS-infused quarters at PIH 18. Incubation was at 38°C.

3. Growth was analyzed in whole milk and skim milk collected at PIH 18 from LPS-infused quarters. Figure 3 refers only to milk samples in which growth inhibition was observed (8 cows). From these growth curves it is apparent that after several hours incubation, growth of E. coli was enhanced in presence of Zn and Fe (P<.0001). Increase in bacterial growth did not differ in whole and skim milk (P>.79).

Effect of Deferoxamine on Bacterial Growth

The effect of deferoxamine on growth of E. coli is in Figure 4. Results refer only to milk samples in which growth inhibition did not occur (4 cows). Milk was sampled from LPS-infused quarters at PIH 18. Incubation of milk samples in presence of deferoxamine resulted in depressed bacterial growth compared to controls (P<.0001). Growth inhibition in whole and skim milk did not differ (P>.81).

DISCUSSION

The present study demonstrated diminished bacterial growth of E. coli in whole and skim milk collected from LPS-infused quarters at PIH 18 and to a lesser extent at PIH 36 (Figures 1 and 2). Several antibacterial mechanisms play a role in removal of bacteria from the udder (9, 16) [e.g., polymorphonuclear phagocytosis, immunoglobulins, complement, lactoferrin (Lf), transferrin (Tf), lysozyme, and the lactoperoxidase/thiocyanate/hydrogenperoxide system]. In this context it seems strange that growth inhibition in cell-free skim milk from LPS-treated quarters at PIH 18 was at least equal to the growth inhibition in whole milk (Figure 1B, C); however, phagocytosis is less effective in whole milk (20).

In addition, presence of bacterial growth inhibition in cell-free skim milk from inflamed quarters and not in skim milk from uninfamed quarters of the same cow (Figures 1 and 2), suggests an inflammation-dependent humoral antibacterial mechanism. In vitro antibacterial properties of skim milk from LPS-infused quarters have been demonstrated, whereas bacterial growth was not depressed in skim milk from uninflamed quarters (5). At present the mechanism is not known.

The clinical relevance of growth inhibition in milk from LPS-infused quarters may be substantiated by the finding that a small amount of LPS (10 µg) administered i.m.m. 16 h before challenge with Streptococcus agalactiae in the same...
quarter, prevented the establishment of mastitis (3). However, mastitis developed in the collateral quarter, which was not pretreated with LPS and which received the same inoculum (3).

As shown in Figure 2, individual variation existed in the ability of milk from LPS-infused quarters of different cows to inhibit growth of E. coli. Apparently individual variation among cows exists not only in recruitment of polymorphonuclear leukocytes, the ability of milk to support phagocytosis, and the ability of polymorphonuclear leukocytes to phagocytose (18), but also in the antibacterial properties of cell-free skim milk.

Growth of E. coli in skim milk drawn from LPS-infused quarters at PIH 18 was inhibited when incubated at 38°C, whereas growth inhibition was not present at 41°C (Figure 1C). These findings differ from the results of studies in which Aeromonas hydrophila and Pasteurella
multocida (cultured in trypticase soy broth and BHI, respectively) showed diminished growth at 41°C, unless growth media were supplemented with Fe (13). Diminished growth rates of these bacteria at 41°C may be due to less efficient production of siderophores, which are essential for microorganisms to obtain Fe (26).

It appeared that addition of Fe and Zn resulted in a marked growth-promoting effect (Figure 3) that did not differ in skim and whole milk \((P<.79)\). Addition of deferoxamine, an Fe-binding substance, resulted in depressed bacterial growth in whole and skim milk sampled from LPS-infused quarters at PIH 18.

Although not quantified in this study, concentrations of Fe, Lf, and Tr have been found to increase in milk from LPS-infused quarters (10). The determination of Fe saturation of Lf and Tr in whole and skim milk proved unreliable due to the binding of Fe to other components in skim milk (e.g., casein) (2, 12). Thus, a proper evaluation of the role
of Fe and Zn in the growth inhibition of E. coli in milk from LPS-infused quarters is not possible. The concentration of free ionic Fe in body fluids lies far below requirements of most microorganisms (4, 27) due to presence of high affinity Fe-binding proteins, such as Lf (14), and Tr (27). Therefore, exogenous Fe was found to promote bacterial growth in vivo and in vitro (4). In addition, bacteriostatic effects of Fe-binding substances have been shown in vitro (6, 17, 19, 22).

A marked reduction in Zn concentrations of 50.8 ± 3.16% was observed in skim milk collected at PIH 18 from LPS-infused quarters (Table 1). These data agree with Zn reduction in skim milk observed in previous experiments (23). Effects of Zn on bacterial growth do not show a linear dose-response effect; microbial metabolism is inadequate without trace amounts of Zn and is also altered by the presence of Zn concentrations exceeding the normal physiological range (21). Little is known of the role of Zn in combination with Fe in the interaction of nonspecific host defense mechanisms and microorganisms.

It can be concluded that growth inhibition of E. coli may occur in whole and skim milk from LPS-infused quarters at PIH 18 and to a lesser extent at PIH 36. There appeared to be individual variation in the ability of milk from inflamed quarters of different cows to inhibit growth. Growth inhibition was most pronounced in skim milk collected from LPS-infused quarters at PIH 18, when incubated at 38°C. Mechanism by which bacterial growth in skim milk from LPS-infused quarters was depressed was restricted to the inflamed quarter and probably was of noncellular origin.

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REFERENCES


