Influence of Bovine Lactoferrin on the Growth of Listeria monocytogenes

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ABSTRACT

The influence of bovine lactoferrin (LF) and Apo-LF on growth of Listeria monocytogenes in Ultra-High Temperature (UHT) 2% fat milk was determined. The effect of LF was dependent upon both the degree of iron saturation and concentration. Before iron removal, LF was found to be approximately 52% saturated with iron, and at 23 and 46 mg/ml LF, minimal growth inhibition of L. monocytogenes was observed. Following dialysis, Apo-LF iron saturation was reduced to approximately 18%. At 15 and 30 mg/ml Apo-LF, a bacteriostatic effect against L. monocytogenes was observed. Inhibition of growth associated with Apo-LF was abolished when ferric ammonium citrate was added to saturate the iron binding sites of the Apo-LF.

Antimicrobial agents naturally present in foods have gained attention because of the demand for "all-natural" food products (5). There are several proteins and enzymes present in milk which have bacteriostatic properties including lactoferrin, lysozyme, and lactoperoxidase (5,12). Lactoferrin (LF) is an iron-binding protein present in mammalian milk, other physiological fluids, and polymorphonuclear leukocytes (21). Each molecule of LF binds two ferric iron cations with two bicarbonate anions (77). Lactoferrin is believed to participate in the natural immune defense system present in mammalian milk (19,27). In vitro studies on the role of human milk in protecting breast-fed infants against enteric infections from enteropathogenic Escherichia coli concluded that the interaction of LF and specific immunoglobulins plays a significant part in the natural defense system (8,29,30). Postpartum human milk has approximately 1 mg/ml LF, whereas postpartum bovine milk has approximately 0.3 mg/ml LF (36). The activity of LF is dependent upon its concentration, its degree of iron saturation (35), and the interaction of other milk components (8,19,30). The role of LF in prevention of new coliform intramammary infections in the bovine mammary gland has recently been studied (6,7,24,26).

Because infant formulae lack natural defense components of human milk, Reiter (27) suggested fortifying infant formula with LF and immunoglobulins. The major drawback in fortification with LF is complicated and inefficient isolation techniques (17). Several recent studies (1,2,17,37) investigated the use of affinity chromatography for large-scale isolation of LF from cheese whey. Kawakami et al (17) found, using a one-step immunoaffinity column, that pasteurization (65°C for 30 min) of Cheddar cheese whey did not significantly affect recovery or iron binding capacity of LF.

Previous studies (10,31,32) showed a marked increase in growth of L. monocytogenes when synthetic growth medium was supplemented with iron. Increased growth of L. monocytogenes in an iron-supplemented medium suggests that LF could act as an effective natural antimicrobial agent against L. monocytogenes. The iron requirement for growth of L. monocytogenes, the result of LF withholding iron from microorganisms, and improved LF isolation techniques all contribute to the potential for LF being used as a natural antimicrobial agent in dairy products. The purpose of this study was to determine the effect of LF on growth of L. monocytogenes.

MATERIALS AND METHODS

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

The purity of LF was determined using a 10% sodium dodecyl sulfate (SDS) acrylamide slab mini gel electrophoresis procedure (18). The LF was dissolved in a solubilizing buffer (20mM Tris acetate, 20% [v/v] glycerol, 20% [w/v] SDS, and 2% [v/v] beta mercaptoethanol), and 5 μg were loaded onto the stacking gel. Proteins were separated on the slab gel by electrophoresis at 18 milliamps for 1.5-2.0 h in the presence of a 1% SDS buffer. SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were also run with each LF sample. Standards included: rabbit muscle phosphorylase (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). The slab gel was silver-stained (Bio-Rad Laboratories).

Lactoferrin preparation

Lactoferrin isolated from bovine colostrum (Sigma Chemical Co., St. Louis, MO) was desaturated using the method of Mazurier and Spik (22). Acetic acid-sodium acetate buffer (pH 3.8; ionic strength, 0.2 M) containing 40 mM EDTA was used...
to dissolve LF. A 1% solution of LF and buffer was equilibrate overnight at 4°C. After equilibrate, the solution was dialyzed for 3 d against deionized-distilled water. Dialysis tubing had a molecular weight cut-off of 12,000-14,000 daltons (Spectrum Medical Industries, Inc., Los Angeles, CA). The sample was then lyophilized and stored at 4°C.

The amount of iron present in the desaturated sample was measured by an atomic absorption spectrophotometer (Instrumental Laboratories Inc., Wilmington, MA). A 3.0 ml sample was digested in 30.0 ml nitric acid and 3.5 ml perchloric acid, then diluted to a 10.0 ml final volume for measurement. The amount of iron present after dialysis was divided by the theoretical saturation level 1.396 μg Fe/ml protein (20) to determine degree of iron saturation.

Test microorganisms

Six strains of L. monocytogenes: two raw milk isolates; F5027 (serotype 1a), F5069 (serotype 4b); a heat resistant isolate, Scott A; and three reference cultures; ATCC 19114 (serotype 4a), ATCC 19115 (serotype 4b), ATCC 19117 (serotype 4d) were all used in the microtiter assay. Escherichia coli originally isolated from bovine milk was also used as a positive control. Listeria monocytogenes strains were provided by C. W. Donnelly, Dept. of Animal Science, The University of Vermont, Burlington. Each strain was grown in 5 ml tryptose phosphate broth (TPP; Difco laboratories, Detroit, MI) and then streaked on tryptose phosphate agar plate (TPA; Difco) to obtain individual colonies. For each strain, two colonies from the TPA plates were inoculated into 30 ml of UHT milk (Farm Best lowfat milk, Dairymen, Inc., Savannah, GA). Listeria monocytogenes strains were incubated at 35°C for 12 h and E. coli was incubated in a shaking water bath at 32°C for 6 h. Cells were collected by centrifugation, and each strain resuspended in 15 ml UHT milk. One-ml aliquots of bacterial cells were mixed with 1 ml of a 50% glycerol solution in a freezer vial and stored at -70°C for up to 2 months. Viable cell counts for each vial were determined by decimal dilution in 0.1% peptone buffer (pH 6.8) and plating on TPA plates, then incubating at 35°C for 18 h.

Iron concentration

To establish initial iron levels, both media (UHT milk and 0.1% peptone diluent) used in the microtiter assay were measured for iron concentration using an atomic absorption spectrophotometer. To minimize iron contamination, all glassware was soaked in 6 N hydrochloric acid bath for 4 h and rinsed 15 times with deionized-distilled water.

Microassay of bacterial growth

The microtiter assay system has been described previously (7,13,24,25). Growth inhibition studies were conducted in 96-well, round-bottom microtiter plates (Corning Glass Works, Corning, NY). Each well had a 300 μl capacity. For each test, a total volume of 250 μl was used consisting of 115 μl UHT milk, 125 μl Apo-LF, and 10 μl bacterial inoculum. Samples were measured and dispensed aseptically with micropipettes (Rainin EDP pipetter, Rainin Instrument Co., Inc., Brighton, MA). The stock solution of Apo-LF was dissolved in 0.1% peptone buffer and filter-sterilized using an Aerodisc (0.2 μm; Gelman Science, Ann Arbor, MI).

It should be noted that the ability of LF to pass through the filter unit changed after iron was removed. No LF was lost when the 52% iron-saturated protein was passed through the filter, whereas approximately 37% Apo-LF was lost during filtration.

This change may have been caused by expansion of the protein structure as the iron was released from the binding sites (3). Rosseau-Motreff et al. (28) reported that transferrin had a significant conformational change when iron was bound to the protein, making the structure more compact. Bishop et al. (6) and Nonnecke and Smith (24) used a 0.45 μm filtration system.

The concentration (mg/ml) of the LF stock solution was determined by measuring the absorbance at 280 nm and dividing by the extinction coefficient 1.45 (9). The bacterial inoculum consisted of 50-100 CFU/10 μl of UHT milk.

Prepared microtiter plates were covered with a sterile lid and incubated at 35°C for 18 h. After incubation, the number of viable cells in each well was determined in triplicate by the drop method (23) on TPA plates. All L. monocytogenes strains were incubated 18 h. Escherichia coli was incubated 12 h. All bacterial strains were incubated at 35°C. Sterile 0.1% peptone buffer was used as diluent.

Statistical analysis

For each experiment, a randomized complete-block design was used with growth measured as log CFU/ml. Each replicate represented one block. Duncan’s multiple-range test was used to compare growth means by strain and by concentration. The model statement was:

\[ G = C + S + (C \times S) + B \]

where:

- \( G \) = Bacterial growth (log CFU/ml) in UHT milk
- \( C \) = Concentration of Apo-LF
- \( S \) = Strain of L. monocytogenes or E. coli
- \((C \times S)\) = Interaction between the concentration and strain
- \(B\) = Blocks on replications of each assay

All analyses were conducted using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

RESULTS

Purity of the Apo-LF was analyzed by SDS-PAGE. The major component from the bovine colostrum LF (Sigma Chemical Co.) was the LF protein (Fig. 1). After measuring the amount of iron present in each LF sample, iron saturation of LF was determined theoretically using 0.025 μmoles Fe/ml of LF (20).

Studies were conducted to investigate the influence of LF on growth of L. monocytogenes before and after iron was removed from LF. Initial studies examined the effect of LF concentration before iron removal on growth of two L. monocytogenes strains, Scott A and ATCC 19115. Approximately 52% of the LF was iron bound. Final concentrations of LF in the assay were: 46, 23, 4.6, and 0.46 mg/ml. The initial inoculum for both strains was approximately 3 log CFU/ml (Table 1). The control for both strains, UHT milk without LF, reached an average cell count of 10.5 log CFU/ml after 18 h incubation at 35°C. There was no difference (P>0.01) in effect of LF on either Listeria strain within each LF concentration. Samples with the lower concentrations, 4.6 and 0.46 mg/ml LF, had viable cell counts equivalent to the control. The two higher concentrations, 46 and 23 mg/ml LF, in comparison to the control, reduced the growth of both Listeria strains 16% and 14%, respectively. There was no
Viable cell counts being <10 CFU/ml. Growth in Listeria strains was observed at concentrations above 3.5 mg/ml. Escherichia coli was inhibited at 30 mg/ml Apo-LF. A Lactoferrin (Sigma Chemical Co.) on a 10% sodium dodecyl sulfate mini-slab gel. (A) Lactoferrin before dialysis; (B) Lactoferrin after dialysis; (C) Molecular weight standards in kDa.

**Figure 1.** Purity of bovine lactoferrin (Sigma Chemical Co.) on a 10% sodium dodecyl sulfate mini-slab gel. (A) Lactoferrin before dialysis; (B) Lactoferrin after dialysis; (C) Molecular weight standards in kDa.

**TABLE 1.** Influence of 52% iron saturated lactoferrin (LF) on growth of Listeria monocytogenes in UHT Milk.

<table>
<thead>
<tr>
<th>Strain (serotype)</th>
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<td>8.7^a</td>
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<tr>
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<tr>
<td>ATCC 19115 (4b)</td>
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<table>
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**TABLE 2.** Influence of 18% iron saturated Apo-Lactoferrin (Apo-LF) on growth of Listeria monocytogenes in UHT Milk.

<table>
<thead>
<tr>
<th>Strain (serotype)</th>
<th>Apo-LF (mg/ml)</th>
<th>SEM</th>
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<td>F5027 (1a)</td>
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<td>0.03</td>
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<tr>
<td>F5069 (4b)</td>
<td>2.0^a</td>
<td>0.17</td>
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<td>0.05</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
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<tr>
<td>E. coli</td>
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<td>0.03</td>
<td>0.02</td>
<td>0.08</td>
<td>0.02</td>
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1 Data expressed as mean ± standard error of the mean (SEM) log CFU/ml.
2 Mean based on n = 2 for 30 mg/ml and n = 6 for all other concentrations.
3 Initial inoculum.
4 Means between strains and within a concentration differ (P<0.01).
5 ^a,bMeans within strains and among concentrations lacking an identical superscript differ (P<0.01).
6 ^a,b^cMeans within strains and among concentrations lacking an identical superscript differ (P<0.01).

The next study examined the effect of LF concentration on growth inhibition of L. monocytogenes after iron removal (Apo-LF) on UHT milk. The initial inoculum was approximately 3.0 log CFU/ml; data are expressed as mean ± standard error of the mean (SEM) log CFU/ml.

**TABLE 3.** Influence of Apo-Lactoferrin (Apo-LF) on growth of Listeria monocytogenes in UHT Milk.

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1 Data expressed as mean ± standard error of the mean (SEM) log CFU/ml.
2 Mean based on n = 2 for 30 mg/ml and n = 6 for all other concentrations.
3 Initial inoculum.
4 Means between strains and within a concentration differ (P<0.01).
5 ^a,bMeans within strains and among concentrations lacking an identical superscript differ (P<0.01).

The effect among the four strains of L. monocytogenes was tested. There was no difference (P=0.20) in replication of the assay.

To demonstrate that growth inhibition of L. monocytogenes was related directly to iron availability in the medium, iron was added to the growth medium in an attempt to abolish inhibition by Apo-LF. The amount of iron added to the medium was determined by calculating the theoretical number of iron binding sites for 50 mg/ml LF. Apo-LF (30 mg/ml) was added to both UHT milk and UHT milk with 0.125 M ferric ammonium citrate. The pH of the UHT milk was 6.4 without iron supplementation and 6.7 with the addition of iron. Because there was no difference in the four strains of L. monocytogenes tested, there was no difference (P=0.20) in replication of the assay.

The next study examined the effect of LF concentration on growth inhibition of L. monocytogenes after iron removal (Apo-LF) on growth of L. monocytogenes in UHT milk. The initial inoculum was approximately 3.0 log CFU/ml; data are expressed as mean ± standard error of the mean (SEM) log CFU/ml.

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The 52% iron saturated LF at 46 mg/ml theoretically had 518 nmoles iron-binding sites per ml, which was 81 times the amount of iron present in the milk. Even though iron removal did not change the theoretical iron binding capacity of the LF, inhibitory effects were different. Nonnecke and Smith (24) also found iron-saturated LF to be ineffective against growth of coliform bacteria, but the degree of iron saturation was not reported.

Previous studies have demonstrated bacterial growth inhibition in the presence of Apo-LF. (6,24). Nonnecke and Smith (24) found that Apo-LF at concentrations of 15 and 30 mg/ml were less inhibitory than 5 mg/ml. Bishop et al. (6) found 5 mg/ml Apo-LF to be the most effective concentration. However, both group used a synthetic medium which contained 1.8-2.7 μM iron, whereas in the present study UHT milk (7.5 μM iron) was used as the growth medium. Bishop et al. (6) calculated the theoretical number of iron binding sites for 5 mg/ml at 107 nmoles of iron-binding sites per ml, or 50 times the amount of iron present in the synthetic medium. With 7.5 μM iron in UHT milk, concentrations of 15 and 30 mg/ml Apo-LF were required to provide an iron-binding capacity of 41 and 81 times the amount of iron present in the UHT milk. Therefore, the higher concentration of Apo-LF had the same iron binding capacity as the lower concentrations used in previous studies (6,24).

There are several other proteins and enzymes present in milk that have bacteriostatic properties (27) and could influence bacteriostatic behavior of LF. Immunoglobulins are a part of the antimicrobial system in milk. They have been shown in vitro to enhance the bacteriostatic effect of LF (25,29,30). Stephens et al. (30) demonstrated that this in vitro effect was based on antibody specificity for a microbial pathogen. Bovine IgG1 in combination with LF enhanced inhibition against a strain of E. coli that was pathogenic to calves, but bovine IgG1 and LF tested against human pathogenic strains had no synergistic effect.

Lysozyme is also present in milk and can be effective in inhibiting microbial growth (27). Milk lysozyme has been shown to be more effective than albumin lysozyme unless a chelator is added to the albumin (33). Hughey and Johnson (16) used albumin lysozyme against four strains of L. monocytogenes and found no inhibitory action without the addition of 1 mM EDTA. The required presence of a chelating agent for albumin lysozyme to lyse microbial cells suggests that the combination of milk lysozyme and lactoferrin could provide a synergistic antimicrobial effect.

The survival of microorganisms in the presence of iron-binding proteins such as LF depends upon their ability to synthesize iron-sequestering compounds. Such compounds are generally termed siderophores. There are over 200 natural siderophores that have been classified into two general categories, hydroxamates and phenolates (31). The ability of microorganisms to produce a siderophore is considered to be a virulence factor (14,34). Another iron-sequestering system similar to siderophores is the citrate-mediated system found in E. coli (4). Citrate is not a very effective chelating agent unless
present in high concentrations. However, it is considered a high-affinity iron carrier for \textit{E. coli} because of the specific receptor on the outer membrane of the bacteria (4). The antimicrobial action of LF has been shown to be dependent upon the molar ratio of citrate to LF for \textit{L. monocytogenes} (6.24:26). Oliver and Bushe (26) found the molar ratio of citrate to LF to be >1000 in both normal bovine milk and colostrum and then decrease to approximately 10 in dry secretions. Griffiths and Humphreys (15) were able to neutralize the citrate-mediated system in vitro with an excess concentration of bicarbonate anions. The specific mechanism of iron acquisition for \textit{L. monocytogenes} is not known. Cowart and Foster (10) were not able to identify an iron transport system, but they only assayed for two general compounds, hydroxamates and phenolates.

Results of the present study indicate Apo-LF is bacteriostatic against \textit{L. monocytogenes} grown in UHT milk. Further investigations could improve inhibitory effects against \textit{L. monocytogenes} when other milk components such as immunoglobulins and lysozyme are included with Apo-LF. A major draw back to further evaluation is cost of LF and the limited commercial availability (17). Improving the cost efficiency of LF isolation and development of low-cost LF sources, such as cheese whey, would enhance the potential use of lactoferrin as a future natural antimicrobial agent against \textit{L. monocytogenes}.

**Acknowledgments**

Recognition is given to Barbara Gillespie, Mark Lewis, and Linda Miller for their technical assistance.

**References**