Listeriolyisin O Secretion by *Listeria monocytogenes* in Broth Containing Salts of Organic Acids

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**ABSTRACT**

Antilisterial effects of salts of organic acids have been documented, but there is little information on listeriolyisin O (LLO) secretion in the presence of these salts during aerobic and anaerobic incubation. LLO secretion and populations of *Listeria monocytogenes* were studied in broth containing potassium sorbate (0.05 to 4%), sodium lactate, citrate, acetate, or propionate (0.1 to 8%) after aerobic or anaerobic incubation for 24 h at 35 and 20°C. The order of the antilisterial effects of the salts was propionate > sorbate > acetate > lactate > citrate. Cell proliferation was suppressed during incubation under anaerobic conditions but LLO secretion was enhanced. Citrate, acetate, and lactate enhanced LLO secretion during incubation at 35°C, whereas sorbate suppressed it. Overall, effects of the acids at 20°C were similar to those observed at 35°C, but only acetate and citrate enhanced LLO secretion. The observation that salts of specific organic acids enhance LLO secretion may suggest increased virulence of *Listeria monocytogenes*. Combinations of sorbate, an LLO blocking agent, with propionate or lactate, which inhibit proliferation of *Listeria monocytogenes*, may prolong the shelf life and increase the safety of foods.

Key words: *Listeria monocytogenes*, listeriolyisin O, organic acids

*Listeria monocytogenes* is a gram-positive, aerobic to facultatively anaerobic, intracellular bacterium that grows over broad ranges of temperature and pH. Because of its wide distribution in nature and its pathogenicity, the organism is of concern to the food industry. It is responsible for serious illnesses in the elderly, newborns, and pregnant women, with a mortality rate of about 30% in outbreaks (2).

All virulent *Listeria monocytogenes* strains produce listeriolyisin O (LLO), a sulfhydryl-activated protein with a molecular weight of 55,000 to 60,000 (17, 18). This highly potent lytic agent is regarded as an essential virulence factor of the organism, and membrane cholesterol is thought to be the toxin binding site at the eukaryotic cell surface (5). The activity and secretion of the toxin are affected by environmental factors such as pH (9, 22), temperature (7, 8, 21, 23, 26), growth media (4, 7, 8, 17, 18, 23, 24), and oxygen availability (3). LLO secretion of 6 µg/mg of protein at 26°C and 384 µg/mg at 37°C was reported (7). McKeillar (23) reported maximum LLO production after 184 and 50 h at 10 and 30°C, respectively, while Myers and Martin (26) did not detect any LLO activity at 4 and 25°C. Studies on the effect of sodium chloride are conflicting, with one reporting stimulation of LLO secretion with 2.5% NaCl (6) and another reporting its inhibition at concentrations above 1% (23). A sharp increase in LLO secretion was seen when *L. monocytogenes* was grown in charcoal-treated broth compared with untreated medium (18). Increased hemolytic activity was observed under reduced aeration in one study (3), while no changes were reported in another study even though there was a significant growth inhibition (23).

There are several reports on the antilisterial effects of organic acids such as acetic, lactic, citric, propionic, and sorbic acid or their salts, which are used as food preservatives (12, 13, 19, 28). Naturally present in plant foods (sorbate, citrate), or produced by microorganisms (acetate, lactate, propionate), these acids or their salts are generally recognized as safe substances for miscellaneous and general-purpose usage (10, 25). The majority of studies with *L. monocytogenes* have focused on survival and proliferation of the organism in the presence of these compounds, but not on LLO secretion. It is not clear whether compounds that stimulate or suppress LLO secretion by listeriae may influence the safety of foods. Therefore, this study was undertaken to investigate the effect of salts of sorbic, lactic, citric, acetic, and propionic acids on growth and LLO secretion at neutral pH during aerobic and anaerobic incubations.

**MATERIALS AND METHODS**

**Bacterial culture and media**

*L. monocytogenes* Scott A (serotype 4b) was used in the study. The culture was maintained on tryptic soy agar slants at 5°C. Before use, the culture was activated in tryptic soy broth supplemented with 0.6% yeast extract (TSBY). All media were from Difco Laboratories, Detroit, MI.

**Chemicals**

Potassium sorbate and sodium propionate were obtained from Aldrich Chemicals, Milwaukee, WI, sodium citrate dihydrate from Fisher Scientific, Pittsburgh, PA, sodium lactate from Purac, Lincolnshire, IL, and sodium acetate anhydrous from Dr. Paul Lohmann, Emmerthal, Germany.

**Growth measurement and LLO assay**

Proliferation of *L. monocytogenes* in TSBY was measured after incubation for 24 h at 20 or 35°C by absorbance readings at 600 nm...
(Perkin-Elmer Lambda 3B spectrophotometer, Norwalk, CT). Listeriolysin O activity in the supernatant fluid was assayed as described by Geoffroy et al. (18). Cell suspensions (5 ml) were centrifuged at 10,000 rpm (Sorvall Instruments, Hoffman Estate, IL) for 10 min at 5°C. Volumes of the supernatant fluid (0.005 to 0.5 ml) were added to phosphate-buffered saline, pH 6.5 (PBS; 75 mM NaH2PO4, 75 mM Na2HPO4, 75 mM NaCl), supplemented with cysteine HCl (Sigma, St. Louis, MO) to 20 mM final concentration. The total volume (1 ml in microcentrifuge tubes) was incubated for 10 min at room temperature. Defibrinated sheep blood (0.5 ml of 2.25%) (Remel, Lenexa, KS) was added, and tubes were incubated in a water bath at 37°C for 1 h. Absorbance at 541 nm was measured after centrifugation for 30 s at 10,000 rpm (Sorvall MC 12V). The hemolytic activity was estimated graphically by plotting percentage lysis versus volume of culture extract on log-probit graph paper (16). One hemolytic unit (HU) was defined as the amount of LLO required to release half of the hemoglobin of the erythrocytes. Absorbance of 100% hemolysis was determined by adding 1 ml distilled water to 0.5 ml blood. PBS, cysteine HCl, and 0.5 ml blood served as blank. At least two trials were performed for each experiment.

**Relationship between cell density and LLO secretion**  
*L. monocytogenes* cells (0.1 ml of a 24-h culture, about 5 x 10^6 CFU) were added to sterile TSBY (6 ml). The tubes were incubated at 35°C, and cell density and percent hemolysis were determined after 0, 4, 6, 8, 10, 12, 18, and 24 h. For anaerobic conditions, 0.5% Oxyrase™, an oxygen-reducing membrane from *E. coli* (Oxyrase Inc., Mansfield, OH), was added to the medium in screw-capped tubes. The tubes were stored for 30 min at room temperature before inoculation as previously described. Samples incubated at 20°C were analyzed in a similar manner after 0, 8, 10, 12, 14, 16, and 24 h.

**Effect of the salts of the organic acids**  
Tubes containing TSBY (6 ml) and 0.05, 0.1, 0.4, 1.2, and 4% sorbate or 0.1, 0.5, 1.2, 4, 6, and 8% lactate, citrate, acetate, or propionate were prepared and sterilized (15 min at 121°C). After the media were cooled to room temperature, 0.1 ml of a 24-h culture of *L. monocytogenes* was added to each tube, and samples were incubated at 35°C for 24 h. A similar experiment was conducted using an incubation temperature of 20°C with concentrations of 0.5, 1.2, or 4% sorbate, lactate, citrate, acetate and propionate. Medium pH was measured but not adjusted since all salt-containing media had a pH of 7.2 to 7.4. Cell density and LLO secretion were determined after 24 h of incubation.

**Effect of sorbate in combination with lactate, citrate, acetate, or propionate**  
TSBY samples (6 ml) containing 0.1, 0.5, 1.2, 4, and 6% lactate, citrate, acetate, or propionate were prepared and potassium sorbate added to provide a final concentration of 0.4%. The media were sterilized, cooled, and inoculated as described before. Cell density and LLO secretion were determined after incubation for 24 h at 35°C.

**RESULTS**

The relationship between cell density (A_600) and LLO secretion, expressed as percent hemolysis, under aerobic and anaerobic conditions at 35°C is shown in Fig. 1A. Maximum populations were reached after 8 h under both conditions. While cell density was about 20% lower under anaerobic conditions, 50% hemolysis was reached after 6 h under anaerobic conditions and 8 h under aerobic conditions. Maximum LLO secretions were seen after about 8 h (80% hemolysis) and 10 h (68% hemolysis) under anaerobic and aerobic conditions, respectively, and these values corresponded to late exponential growth phase. Addition of Oxyrase™ to the assay system did not affect LLO secretion. When listerial cells were incubated at 20°C, cell density increased faster and A_600 values were higher under aerobic conditions (Fig. 1B). Changes in cell density and LLO secretion were slower and LLO secretion lower at 20°C than at 35°C, particularly during aerobic incubation. Overall, cell density and LLO activity remained stable for 24 h of incubation at both temperatures after reaching maximum levels.

The effects of potassium sorbate, sodium lactate, sodium citrate, sodium acetate, and sodium propionate on cell density of *L. monocytogenes* at 35°C are summarized in Table 1. In general, the effect of these salts was concentration dependent, and 50% inhibition relative to control samples in TSBY under aerobic conditions required about 3.9, 2.4, 2.1, and 0.65% lactate, acetate, sorbate and propionate, respectively. Citrate was the least inhibitory, and 50% inhibition could not be
reached with the highest concentration tested (8%) (Table 1). Anaerobic conditions did not alter the inhibition pattern of the salts observed during aerobic incubation. The lowest sorbate concentration that reduced proliferation of listeriae, 0.4%, (Table 1), was used to test its effects in combination with each of the other salts. Incubation of the organism in broth containing these salt combinations under aerobic conditions generally resulted in about 10% suppression of cell proliferation, similar to the effect of 0.4% sorbate alone (Table 1).

Sorbate caused a sharp reduction in LLO secretion. No activity was detected at a concentration of 2% after incubation for 24 h at 35°C under aerobic or anaerobic conditions (Fig. 2). Effects of lactate, citrate, acetate, and propionate on the hemolytic activity during aerobic and anaerobic incubation at 35°C and effects of combinations of 0.4% sorbate with each of the salts in the growth media are illustrated in Fig. 3. Enhanced hemolytic activity was seen during aerobic incubation in the presence of lactate, citrate, and acetate, with maxima at 2, 4, and 2% of the salts, respectively (Fig. 3A,B,C). The activity remained unchanged with up to 2% propionate (Fig. 3D). The hemolytic activity declined rapidly during anaerobic incubation with increase in lactate and propionate concentrations (Fig. 3A and 3D), increased sharply and then declined sharply with 1% acetate followed by a sharp decrease.

### Table 1. Cell density of L. monocytogenes incubated aerobically or anaerobically in TSBY containing potassium sorbate and/or sodium lactate, citrate, acetate, and propionate after 24 h at 35°C

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration</th>
<th>Aerobic (%)</th>
<th>Anaerobic (%)</th>
<th>Aerobic plus 0.4% potassium sorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (TSBY)</td>
<td>0 0.1 0.5 1 2 4 8</td>
<td>100 97 97 89 83 68 95</td>
<td>78 72 71 68 68 68 68</td>
<td>95 72 71 68 68 68 68</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>0 0.1 0.4 1 2 4 6 8</td>
<td>97 97 87 73 52 47 32 9</td>
<td>72 71 69 45 31 35 28 6</td>
<td>72 71 69 45 31 35 28 6</td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>0.1 0.5 1 2 4 6 8</td>
<td>95 89 83 68 68 55 9 5</td>
<td>72 68 59 51 35 46 5 2</td>
<td>78 72 68 57 39 46 5 2</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.1 0.5 1 2 4 6 8</td>
<td>98 94 88 85 74 70 55 55</td>
<td>73 68 62 59 60 55 55 55</td>
<td>90 90 85 73 57 46 55 55</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.1 0.5 1 2 4 6 8</td>
<td>85 81 72 55 30 24 91 91</td>
<td>70 64 53 44 26 15 70 70</td>
<td>76 69 61 51 40 34 66 66</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td>0.1 0.5 1 2 4 6 8</td>
<td>91 52 47 29 29 29 9</td>
<td>70 43 34 23 23 23 6 6</td>
<td>66 44 41 15 15 10 6 6</td>
</tr>
</tbody>
</table>

* Cell density as percentage of control incubated aerobically in TSBY; A600 of 1.18 = 100%.

* nd, not determined.
produced 50% inhibition was calculated. Expressed in millimoles, the values were between 0.164 and 0.994 (propionate, 0.311; sorbate, 0.434; acetate, 0.994, and lactate, 0.164). While the concentrations of propionate, sorbate, and lactate were <0.5 mM, the higher concentration of acetate could have been caused by evaporation losses during storage of the compound and during heat sterilization of the media. Comparison of the inhibitory effects of acetate following hot or cold sterilization showed that at concentrations above 4% the latter treatment was 5 to 20% more inhibitory. Other factors may explain the antimicrobial effects of salts of organic acids at neutral pH, and the nature and accumulation of anions have to be taken into consideration as well (11, 19, 27).

The highest LLO secretion was reported at neutral pH and at late exponential growth phase (9, 22). In our study, after reaching its maximum, LLO secretion remained unchanged during the 24-h incubation period at 35 and 20°C, in contrast to reports of a sharp decline in hemolytic activity after the bacterial exponential growth phase ended (18). LLO secretion was suppressed or completely inhibited at reduced incubation temperature, confirming reports of an increase in the virulence gene Hly of L. monocytogenes when cells were incubated in TSBY containing potassium sorbate. Cells were incubated for 24 h at 35°C under aerobic (O) and anaerobic (●) conditions.

![Figure 2. Hemolytic activity of Listeria monocytogenes in TSBY containing potassium sorbate. Cells were incubated for 24 h at 35°C under aerobic (O) and anaerobic (●) conditions.](image)

Cell proliferation and LLO secretion in the presence of the five salts were also determined during incubation at 20°C (Table 2). The order of the inhibitory effects of the salts was propionate > sorbate > acetate > lactate > citrate, the same as during incubation at 35°C. Hemolytic activity at 20°C in TSBY alone was about 40% of the activity at 35°C. Enhanced hemolytic activity, albeit lower than in samples at 35°C, was seen at 20°C in media containing citrate and acetate, whereas propionate and sorbate inhibited LLO secretion at 20°C.

### DISCUSSION

The inhibitory effect of organic acids on microbial proliferation is attributed to damage caused by the hydrogen ion liberated inside the cell following permeation of the acid in its undissociated form. Acidification of the cytoplasm uncouples both substrate transport and oxidative phosphorylation from the electron transport system (14). Consequently, the antimicrobial activity of weak acids generally correlates with their pKₐ values and increases with a decrease in medium pH. Although our study was conducted at neutral pH, and all media pH ranged from 7.2 to 7.4, the data confirm that the higher the pKₐ, the higher the inhibition (pKₐ values are 4.9, 4.8, 4.75, 3.86, and 3.1 for propionate, sorbate, acetate, lactate, and citrate, respectively). Concentrations of weak acids in the protonated form are very low at neutral pH. These values were calculated from the Henderson-Hasselbalch equation at the measured pH of media containing the salt levels that produced 50% inhibition. The concentrations were (%): propionate, 0.457; sorbate, 0.31; acetate, 0.34, and lactate, 0.047. Since acid concentrations to achieve 50% inhibition varied, being considerably lower, e.g., for propionate than for lactate, the total concentration of the protonated form of each acid that

**Table 2. Cell density and hemolytic activity of L. monocytogenes in TSBY in the presence of potassium sorbate or sodium lactate, citrate, acetate, or propionate after 24 h at 20°C**

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration (%)</th>
<th>Cell density (% of control)</th>
<th>Hemolytic activity (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium sorbate</td>
<td>0.5</td>
<td>71</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>0.5</td>
<td>89</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>70</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>53</td>
<td>1.8</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.5</td>
<td>99</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>99</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>82</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>62</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.5</td>
<td>85</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td>0.5</td>
<td>42</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>18</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cell density (A₆₅₀) in control was 1.17.

* Hemolytic activity (A₄₉₂) in control was 1.4. A hemolytic unit (HU) is the amount of LLO required to release half the erythrocyte hemoglobin.
Figure 3. Hemolytic activity of Listeria monocytogenes in TSBY containing sodium lactate (A), citrate (B), acetate (C), or propionate (D) after incubation for 24 h at 35°C. Aerobic incubation (○), anaerobic incubation (●), aerobic incubation with 0.4% potassium sorbate (□).

Inhibition of LLO secretion with potassium sorbate was observed at each concentration studied (0.05 to 4%). Selective inhibition of LLO secretion by sorbate, but not of cell proliferation of *L. monocytogenes*, has been reported (20, 23).

More recently, the addition reaction between sorbate and cysteine involving thiols was shown to have a stoichiometry of 1:1, resulting from a biomolecular attack at position 5 of sorbic acid (29). Addition of another thiol at position 3 was proposed by the authors, who also speculated that thiol ion is the most powerful nucleophile in the food system.
be inhibited by sorbate in a similar manner. Since listeriolysin, tetanolysin, streptolysin, and botulinolysin are all sulphydryl-activated toxins (1), their secretion could be suppressed or prevented by sorbate. The present study also indicates stimulation of LLO secretion in the presence of low concentrations of salts of short-chain saturated organic acids. Carbohydrates in the growth medium were reported to influence the respiratory virulence of L. monocytogenes for the mouse and guinea pig (15). While the combination of sorbate with the saturated salts in the present study blocked secretion of LLO, pathogenicity testing in the mouse model will demonstrate whether virulence is influenced by the growth medium from which cells are harvested.

REFERENCES


