Effect of NaCl pretreatment on the growth and survival of *Listeria monocytogenes* at high osmolarity

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**SUMMARY**

*Listeria monocytogenes* is a food-borne pathogen widely distributed in the environment and potentially found in many kinds of fresh and processed foods. The ability of this microorganism to tolerate salt is important. Seven strains...
of *L. monocytogenes* were tested in order to determine their survival capacities in presence of NaCl. The study has shown that some strain could be cultivated in media containing up to 11% NaCl and that the growth delay observed varied according to the salt concentration and the strains. The second part of this study was carried out on *L. monocytogenes* LO28, a reference strain well characterised by many authors. In order to increase the NaCl resistance of this strain, shifts from low to higher NaCl concentrations were applied to growing cells. None of the tested conditions was able to increase the salt resistance of the LO28 strain.

**Key words:**
Listeria monocytogenes; salt; stress.

### 1 – INTRODUCTION

*L. monocytogenes* is a pathogen of public health significance causing occasional outbreaks and sporadic cases of food-borne illness. This organism is of particular concern because of its ability to survive and grow under a wide range of adverse conditions, such as low temperature, low pH and high osmolarity (BARKER and PARK, 2001). For instance, during sausage fermentation, most of the bacteria are stressed by the addition of salt, and the production of lactic acid by the starters generally used. Surviving to these hurdles demands the adaptation of bacteria to these conditions, which are far from those existing in optimal growth conditions, that is generally a neutral pH and isotonic salt concentrations. For that purpose, bacteria can activate mechanisms allowing them to increase their resistance to a more important stress, which sometimes can be different from the first applied.

Traditionally, salt (NaCl) is used to preserve many food products, in order to inhibit the growth of spoilage and pathogenic bacteria. *L. monocytogenes* is a salt-tolerant organism, and some strains can grow up to 12% NaCl or are surviving 150 days in pure salt at ambient temperature (GALDIERO *et al.*, 1997; GNANOU BESSE *et al.*, 2000). This important survival ability is one of the reasons, which may explain the difficulty to control *L. monocytogenes* in a number of salted food products. In this study, the growth of seven *L. monocytogenes* strains (reference strains and strains isolated from industrial plants) was evaluated at high salt concentration. Additionally, the influence of a pretreatment at low salt concentration on the further resistance of *L. monocytogenes* LO 28 to high NaCl concentrations was investigated.
2 – MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Seven strains of *L. monocytogenes* were used in this study: the LO28 strain (serotype 1/2c) and the EGD strain (1/2a) were obtained from P. Cossart (Institut Pasteur, Paris, France). Strains A (serotype 4b), B (4b), C (1/2a), D (4b) and E (1/2a) were isolated from industrial plants: strains A, B and D from three dairy plants, and strains C and E from one meat plant. The strains were grown in MCDB medium (MCDB 202, Cryobiosystem, L’Aigle, France), a chemically defined medium (HEBRAUD and GUZZO, 2000) supplemented with 1% yeast nitrogen base (YNB, Difco Laboratories, Detroit, Mich., USA) and 3.6 g/l glucose. The pH of this medium was adjusted to 7.3. The original MCDB medium contains 0.75% NaCl.

In all experiments, bacteria from frozen stocks were grown overnight on BHI agar (Difco Laboratories) slopes at 37°C in order to inoculate a flask containing MCDB medium to an initial optical density of 0.1 at 600 nm (OD\textsubscript{600}). After an overnight growth at 37°C with shaking, the cultures obtained were used to inoculate another culture of the same medium at an OD\textsubscript{600} of 0.05.

2.2 Growth delay of *L. monocytogenes* at different concentrations of salt

For the 7 strains, NaCl was added at final concentrations raising up to 11% (w/v) in the culture (OD\textsubscript{600} of 0.05), and bacteria were incubated at 37°C without shaking. The growth delay was defined as the time necessary to observe a colour change of the medium, which was also associated with an increase of the opacity of the growth medium.

2.3 Salt shocks of *L. monocytogenes* LO28 in exponential phase

Cultures were grown aerobically at 37°C until they reached exponential phase (OD\textsubscript{600} of 0.3). Salt treatments were performed on these cultures dispersed into Erlenmeyer flasks and NaCl was added at final concentrations ranging from 2 to 25% NaCl. The time of the shift was set to zero. The growth curves were followed by optical density measurements (OD\textsubscript{600}) at different times. Then, to test the survival abilities of the strains, final concentrations of salt ranging from 6 to 25% have been tested. In this context, after a serial dilution in tryptone-salt solutions viable cells were streaked on Tryptic Soy Agar (TSA, Difco Laboratories) plates incubated for 24h at 37°C and enumerated. Each experiment was performed in duplicate.

2.4 Salt pretreatment of *L. monocytogenes* LO28 in exponential phase

The cultures were grown aerobically at 37°C until they reached exponential phase (OD\textsubscript{600} of 0.3), cells were enumerated in order to calculate the number of initial bacteria corresponding to No. Salt pretreatments were performed by dispensing the culture into Erlenmeyer flasks containing different quantities of salt in order to obtain a final concentrations of 3.5%, 4% or 7% NaCl. After two hours, NaCl were added in some cultures containing 3.5% and 7% of salt to get...
higher final concentrations of 25% NaCl. In the same way, NaCl were added in the cultures containing 4% of salt to get a final concentration of 6% and 8% NaCl. Controls without salt were used in each experiments. Viable cells (corresponding to N) were enumerated as described previously. To calculate the ratio of surviving cells or the bacterial growth, the N/No ratio was calculated. Each experiment was performed in duplicate.

3 – RESULTS AND DISCUSSION

3.1 Growth delay of several L. monocytogenes strains at different concentrations of salt

For the seven strains, elevated concentration of NaCl caused a concentration-dependent growth delay (Table 1). All strains exhibited a similar behaviour up to 8% NaCl. The growth delay varied at 9% NaCl in the range of 3 to 8 days for EGD and E strains, respectively. The LO28, the A and D strains were inhibited above 10% NaCl, as no growth was observed during the time of the experiment (11 days); other strains were more tolerant to such osmotic conditions. The C and E strains were able to grow in a medium containing 11% NaCl. These results are in good agreement with the literature which indicated that some strains are able to grow up to 12% NaCl (GALDIERO et al., 1997). The C and E strains were isolated from salted meat curing plant. This ecological niche could explain why these strains are able to grow at a higher NaCl concentration.

Table 1

Growth delay of the L. monocytogenes strains after addition of NaCl

<table>
<thead>
<tr>
<th>Strains</th>
<th>0% NaCl</th>
<th>6% NaCl</th>
<th>7% NaCl</th>
<th>8% NaCl</th>
<th>9% NaCl</th>
<th>10% NaCl</th>
<th>11% NaCl</th>
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<tbody>
<tr>
<td>EGD</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>nd*</td>
</tr>
<tr>
<td>LO28</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>nd*</td>
<td>nd*</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>nd*</td>
<td>nd*</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>nd*</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>nd*</td>
<td>nd*</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

*nd, no growth detected

3.2 Growth and survival of L. monocytogenes LO28 at different salt concentrations

The L. monocytogenes LO28 strain was chosen, because this strain is well characterized (MICHEL and COSSART, 1992; MARRON et al., 1997), and has already shown adaptation mechanisms to various stresses (O’DRIS-COLL et al., 1996).
In this study, low concentrations of NaCl did not influence the growth of *L. monocytogenes* LO28, in media containing up to 3% NaCl (Figure 1). A slower growth was observed in concentrations ranging from 4% to 6% NaCl. This observation is in agreement with previous studies (GALDIERO et al., 1997; VASSEUR et al., 1999). When the concentrations of salt were above 8% NaCl, growth ceased. Exposure to high salt concentrations (6 to 25%) resulted in a decrease of cultivability (Figure 2), but bacteria were not immediately killed as already shown by Shahamat et al. (1980) who noticed that culturability of *L. monocytogenes* slowly decreased within 5 days when it was exposed in culture media containing 25.5% NaCl.

### 3.3 Adaptation treatments to salt of *L. monocytogenes* LO28

In order to show if a pretreatment at low salt concentration could increase the resistance of the *L. monocytogenes* LO28 strain to higher salt concentrations, two experiments were carried out (Table 2). In the first, bacteria were pretreated with a salt concentration (3.5% and 7% NaCl) decreasing the growth of the microorganism and then incubated in a more concentrated medium (25% NaCl) (Figure 3). The survival curves in a medium containing 25% NaCl were similar for all the conditions tested independently of the salt concentration during the pretreatment. In the second experiment, a 4% NaCl medium were used in the pretreatment, which were followed by treatments with 6 or 8% NaCl (Figure 4). All the growth curves at 6% and 8% of salt were similar, even if the cells were adapted to a medium containing 4% NaCl. In these two experiments, controls were carried out by a pre-treatment without the addition of salt before the treatment in a more concentrated salt medium. Some adaptation tests were realized in classical growth medium (BHI) but none of the conditions tested allow an
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Increasing of the *L. monocytogenes* resistance to salt (data not shown). For these specific conditions of pretreatment, adaptation of *L. monocytogenes* to high salt concentrations was not observed, in contrast to other microorganisms. For instance, *Enterococcus faecalis* exhibited a higher survival at 28.5% NaCl, if the cells were subjected to a moderate salt shock (6.5% NaCl) (FLAHAUT et al., 1996). In the same way, *Bacillus subtilis* was able to survive toxic concentration of NaCl (6%) by pretreatment with 2% NaCl (VÖLKER et al., 1992).

**Table 2**

The two experimental conditions applied to *L. monocytogenes* LO28 in MCDB medium (Figure 3 and Figure 4).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Pretreatment (2 h)</th>
<th>Final treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0% NaCl</td>
<td>0% NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5% NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7% NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25% NaCl</td>
</tr>
<tr>
<td></td>
<td>3.5% NaCl</td>
<td>25% NaCl</td>
</tr>
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<td>7% NaCl</td>
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<td></td>
<td></td>
<td>6% NaCl</td>
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<tr>
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<td></td>
<td>8% NaCl</td>
</tr>
<tr>
<td></td>
<td>4% NaCl</td>
<td>6% NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8% NaCl</td>
</tr>
</tbody>
</table>
Figure 3
Survival of *L. monocytogenes* LO28 in MCDB medium. Without additional NaCl (●), 25% NaCl (★) and after pretreatment for 2 hours with 3.5% (■) or 7% NaCl (▲) followed by treatment with 25% NaCl

Figure 4
Survival of *L. monocytogenes* LO28 in MCDB medium. Without additional NaCl (●), 6% (▲), 8% NaCl (□) and after pretreatment for 2 hours with 4% followed by treatment with 6% (●) and 8% NaCl (■)
The resistance of *L. monocytogenes* to salt is varying with the strains, some of them being able to grow in media containing 11% NaCl. In the case of the *L. monocytogenes* LO28 strain, a slower growth was observed above 4% NaCl and none of the pretreatments tested allowed an increase of this strain resistance to higher salt concentrations. However, a previous study has shown that the *L. monocytogenes* Scott A strain can adapt to salt and ensure a cross protection against other lethal stresses. Thus, an exposure to 7% NaCl for 1 hour increased the resistance of *L. monocytogenes* to a lethal concentration of H$_2$O$_2$ (0.1%), and a pretreatment with 5% ethanol or heat shocking at 45°C for 1 hour increased its resistance to 25% NaCl (LOU and YOUSEF, 1997). Adaptation of this microorganism was only shown for cross protection experiments with the Scott A strain. Complementary studies are necessary to determine if the lack of adaptation after a pretreatment with low concentration of NaCl is strain dependent or due to the conditions tested.

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REFERENCES


