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Behaviour of *Listeria monocytogenes* isolates through gastro-intestinal tract passage simulation, before and after two sub-lethal stresses

J. Barbosa, S. Borges, R. Magalhães, V. Ferreira, I. Santos, J. Silva, G. Almeida, P. Gibbs, P. Teixeira*

CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200 - 072 Porto, Portugal

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ABSTRACT

The effects of previous exposure to sub-lethal acidic and osmotic stresses on the survival of *Listeria monocytogenes* during exposure to gastro-intestinal (GI) tract simulation, was investigated. Six *L. monocytogenes* strains isolated from cheeses were selected and exposed to high salt concentrations or acidic conditions and their viability compared in quick and slow digestions. The results demonstrated that (i) all isolates were more sensitive to the exposure to acidic than to osmotic sub-lethal conditions (ii) significant differences ($p < 0.05$) between the two types of digestion were observed; in slow digestion, the log reduction was higher for all the tested isolates (iii) all isolates were inhibited in the presence of bile salts for both types of digestion (iv) differences between quick and slow digestion were not observed ($p > 0.05$) after exposure to either osmotic or acidic stress (v) a higher cellular inactivation ($p < 0.001$) was observed during the passage through the GI tract simulation after exposure to osmotic than to acidic stresses and (vi) neither osmotic nor acidic sub-lethal stresses conferred resistance to simulated GI tract conditions.

1. Introduction

Listeria monocytogenes is a Gram-positive, food-borne pathogen, which causes disease in both animals and man. Infections of humans mainly occur in immuno-compromised individuals, elderly, pregnant women and neonates (McLauchlin, 1996; Schlech, 2000), as a result of occasional contamination of ready-to-eat (RTE) and raw food products, and also other prepared foods such as coleslaw, milk, pâté, soft cheese, meat and seafood products (Guerra et al., 2001; Mena et al., 2004; Vitas et al., 2004; Francis and O'Beirne, 2006).

Some hurdles used in food processing and preservation to prevent bacterial growth in foods may not be sufficient to inhibit *L. monocytogenes* since, as an ubiquitous organism, it easily adapts to different environmental conditions to ensure survival in nature. The common application of organic acids to reduce pH, and salt to reduce the water activity (Farber and Peterkin, 1991), does not prevent growth of *L. monocytogenes*, which may be isolated from many food products of low pH and containing high amounts of salt (Ferreira et al., 2007a; 2007b). This ability to adapt to several individual stresses makes this microorganism more resistant to further and different stresses, including those encountered in the human body, e.g. those encountered during gastro-intestinal passage. After ingestion, the first hurdle is the acid pH of the stomach. Many studies refer to the resistance of food pathogens to low pH (Merrell and Camilli, 2002; Paramithiotis et al., 2006). In the small intestine, the pathogens need to tolerate exposure to bile salts in order to survive and colonize the intestine. Since *L. monocytogenes* is known to colonize and invade the human organism causing disease, it follows that this pathogen is able to resist high levels of bile salts (Olier et al., 2004).

The aim of this study was to evaluate previous exposure to sub-lethal acid and salt stresses, frequently applied in food preservation, on the survival of six isolates of *L. monocytogenes* during simulated gastro-intestinal passage.

2. Materials and methods

2.1. Origin of isolates

From the *Listeria* culture collection of *Escola Superior de Biotecnologia*, six cheese isolates of *L. monocytogenes* were selected for this study: 1079 (serotype 1/2b-3b); 1055/4 (serotype 4b-4d-4e); 1486/1 (serotype 1/2b-3b); 1509/2 (serotype 1/2c-3c); 1592/2 (serotype 1/2b-3b) and 1743 (serotype 4b-4d-4e). Pulsed Field Gel Electrophoresis (PFGE) profiles of isolates 1079 and 1055/4 match clinical PFGE profiles of two strains isolated from clinical cases of listeriosis occurred in Portugal.

* Corresponding author. Tel.: +351 22 5580095; fax: +351 22 5580111/5090351.
E-mail address: pcteixeira@esb.ucp.pt (P. Teixeira).

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2.2. Growth and storage conditions

Stock cultures were grown on Tryptic Soy Agar (Pronadisa, Madrid, Spain) supplemented with 0.6% (w/v) of Yeast Extract (Lab M, Lancashire, United Kingdom) (TSAYE) at 37 °C for 24 h and preserved at −80 °C in Tryptic Soy Broth supplemented with 0.6% (w/v) of Yeast Extract (TSBYE) containing 30% (v/v) of glycerol (Sigma, Steinheim, Germany); stock cultures were sub-cultured twice before use in assays.

2.3. Inoculum

The preparation of the inoculum was done according to Ramalheira et al. (2010). One colony was transferred, from TSAYE incubated at 37 °C for 24 h, to 10 ml of TSBYE and incubated at the same conditions. For the final inoculum, 0.1 ml of the last culture was transferred to 10 ml of TSBYE (1:100) and incubated at 37 °C for 24 h to reach stationary phase. Each isolate was harvested by centrifugation (8877 x g, 10 min, 4 °C; Rotina 35R, Hettich, Germany), re-suspended in 10 ml of sterile quarter strength Ringer's solution (Lab M) and mixed to obtain an inoculum of approximately 10^7 CFU/ml.

2.4. Survival of sub-lethal stress conditions

Various sub-lethal pH values and high salt concentrations were tested. Aliquots of 0.5 ml of inoculum were placed into glass flasks with 49.5 ml of Buffered Peptone Water (BPW, LabM) adjusted to various pH values (from 2.0 to 4.0) with 1 M lactic acid (José M. Vaz Pereira, Lda, Lisbon, Portugal) for acid stress, or BPW containing various percentages of sodium chloride (from 7 to 40% (w/v)) (NaCl, Panreac, Barcelona, Spain) for osmotic stress. The flasks were held at 37 °C and samples were taken at time 0 (time of inoculation) and times 5, 10, 15, 20, 30, 45 and 60 min. All assays were done in duplicate.

Serial decimal dilutions of each sample were made using sterile quarter strength Ringer's solution and plated for enumeration.

For each experiment an aliquot of 0.5 ml of inoculum was placed into glass flasks with 49.5 ml of BPW at pH 7.0 and used as control. All controls were performed in duplicate.

2.5. Simulated gastro-intestinal conditions

The simulation was achieved according to Madureira et al. (2005). Aliquots of 0.5 ml of inoculum (prepared as described in section 2.3) were placed into glass flasks with 49.5 ml of BPW adjusted to pH 2.5 with Hydrochloric Acid (1 M HCl, Pronalab, Lisbon, Portugal) and with 1000 units/ml of a filter sterilised solution of pepsin (Sigma). The glass flasks were kept at 37 °C and samples were taken at time 0 (time of inoculation) 30 min until a total of 60 min (quick gastric transit simulation) or for a total of 120 min (slow gastric transit simulation). After this, a filter sterilised solution of sodium hydroxide (1 M NaOH, José M. Vaz Pereira, Lda) was added to each glass flask in order to increase the pH from 2.5 to 7.0 and a sterile solution of bile salts (Pronadisa) was also added to achieve a final concentration of 0.3% (w/v). The flasks were held at 37 °C and samples were taken at time 0 (time of bile salts addition) and every 30 min for a total of 60 min (quick digestion simulation) or for a total of 120 min (slow digestion simulation). All assays were done in triplicate.

Serial decimal dilutions of each sample were made in sterile quarter strength Ringer's solution and plated for enumeration.

For each experiment five controls were done: an aliquot of 0.5 ml of inoculum was placed into glass flasks with: 49.5 ml of BPW at pH 7.0; 49.5 ml of BPW at pH 2.5; 49.5 ml of BPW at pH 7.0 with 1000 units/ml of pepsin; 49.5 ml of BPW at pH 7.0. After 60 min, a bile salts solution was added (0.3% (w/v) of final concentration) and 49.5 ml of BPW at pH 7.0 and after 120 min, a bile salts solution was added (0.3% (w/v) of final concentration). All controls were performed in duplicate.

2.6. Gastro-intestinal conditions after sub-lethal stresses

Aliquots of 0.5 ml of inoculum were placed into glass flasks with 49.5 ml of BPW adjusted to pH 3.5 with lactic acid (1 M) and BPW containing 30 or 40% (w/v) of NaCl, kept at 37 °C during 1 h, and samples were taken at time 0 (time of inoculation) and time 60 min. The control for each sub-lethal stress was done by placing an aliquot of 0.5 ml of inoculum into glass flasks with 49.5 ml of BPW at pH 7.0.

After the exposure to sub-lethal stresses, each suspension was harvested by centrifugation (8877 x g, 10 min, 4 °C; Rotina 35R), and the pellet re-suspended and placed into glass flasks with a BPW adjusted to pH 2.5 with HCl and with 1000 units/ml of a filter sterilised solution of pepsin. All the further steps were done as described above (2.5. *Simulated Gastro-intestinal conditions*), as well as the same five control experiments. For each control experiment during gastro-intestinal simulation, an aliquot of 0.5 ml of inoculum was initially placed into glass flasks with 49.5 ml of BPW with acid or salt, to simulate the exposure to each sub-lethal stress, and then treated as described above.

2.7. Enumeration

To carry out the enumeration, the drop count technique was used (Miles and Misra, 1938). Each dilution prepared in sterile quarter strength Ringer's solution was plated on TSAYE in duplicate. After incubation at 37 °C for 48 h, the colonies were counted and the CFU/ml calculated.

2.8. Statistical analysis

Three replicates were conducted for each experiment. Microbial counts were transformed to logarithmic reduction using the equation: log (N/N0), where N is the microbial cell density at a particular sampling time and N0 is the initial cell density.

An analysis of variance (one-way ANOVA) was performed to test any significant effects of slow and quick gastric transit simulations and digestions, both without sub-lethal stresses as well as with each sub-lethal stress condition, on the survival of *L. monocytogenes* in simulated GI tract. All calculations were carried out using the software Kaleidagraph (version 4.4, Synergy Software, Reading, USA).

3. Results and discussion

After several acidic or osmotic conditions tested (data not shown), the values selected were pH 3.5 (adjusted with lactic acid) and saturated solutions of 30% (w/v) (only for isolates 1592/2 and 1743) or 40% (w/v) of NaCl. The survival of the six *L. monocytogenes* isolates to the selected sub-lethal acidic or osmotic stress conditions, are presented in Fig. 1. It is clear that all isolates were more sensitive to the exposure to acidic than to osmotic conditions, which was already reported (Tiganitas et al., 2009). While one hour of exposure caused approximately 1 or 2 log-unit reduction at pH 3.5, for osmotic stress reductions of 0 log-unit (isolate 1079); <0.5 log-unit (isolates 1055/4 and 1486/1), approximately 1 log-unit (isolates 1592/2 and 1743) and 1.2 log-unit (isolate 1509/2), were obtained. The choice of this two stresses were based on the fact that