

# Differential inactivation of *Listeria monocytogenes* by D- and L-lactic acid

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## ABSTRACT

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**Aims:** To determine inactivation of *Listeria monocytogenes* by the two lactic acid isomers.

**Methods and Results:** The survival of four strains with varying sensitivity to acid was determined following treatment with L- or D-lactic acid at 100 mmol l<sup>-1</sup> (pH 3.7) or HCl at pH 3.37. There was some, but not complete, similarity in the relative sensitivity of the four strains to the two types of acid. All strains were most sensitive to D-lactic acid, which gave 0.6–2.2 log units greater reduction than L-lactic acid midway in the inactivation curves. Even very low concentrations of the two isomers had an immediate effect on pH<sub>i</sub> which was identical for the two isomers.

**Conclusions:** The results show that *L. monocytogenes* is more sensitive to D- than to L-lactic acid; however, this difference is less than the strain variation in L-lactic acid sensitivity.

**Significance and Impact of the Study:** This work has implications for the application of lactic acid for food preservation as well as for the understanding of the antibacterial mechanisms of weak organic acids.

**Keywords:** fluorescence ratio-imaging microscopy, hydrochloric acid, intracellular pH, lactic acid isomers, strain variation.

## INTRODUCTION

Lactic acid is the main metabolic end-product of lactic acid bacteria during fermentation of a variety of food products and beverages, and plays a key role for flavour and texture, shelf-life and safety of the products. The produced isomer depends on the bacterial species, for example, *Lactococcus* and *Carnobacterium* produce L-lactic acid, while *Leuconostoc* produces D-lactic acid (Liu 2003).

*Listeria monocytogenes* is a relatively acid-tolerant pathogen. Depending on the specific conditions, lactic acid can either kill or limit growth of *L. monocytogenes*. These effects have been characterized in detail for certain strains (e.g. Buchanan *et al.* 1993; Young and Foegeding 1993; Tienun-goon *et al.* 2000), and lactic acid is included in growth

prediction models for this pathogen (see e.g. <http://www.ifr.ac.uk/Safety/GrowthPredictor/>). The response of *L. monocytogenes* to acid stress has also been characterized extensively (Cotter and Hill 2003). In comparison, the inhibitory mechanisms of weak organic acids are still poorly understood. Under acidic conditions, undissociated organic acids are generally believed to freely cross the membrane to the cytoplasm, where the more neutral pH will result in release of protons, acidification of the cytoplasm, and dissipation of the membrane pH gradient. This mechanism does not, however, seem to account entirely for their activity, and accumulation of the anion also has a toxic effect (Russell 1992). The presence of a specific interaction not solely related to intracellular pH is supported by observations of differences in the activity of the lactic acid isomers. At sublethal concentrations, growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* was inhibited more by L- than by D-lactic acid (Benthin and Villadsen 1995a). Similarly, McWilliam Leitch

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and Stewart (2002) reported that the inactivation of *Escherichia coli* strains was considerably higher for L-lactic acid than for the D-isomer.

The aims of the present work were to determine whether the two lactic acid isomers had differing activity against strains of *L. monocytogenes* with varying acid sensitivity. Additionally, we determined the kinetics of intracellular pH of single cells to further characterize the antibacterial mechanism of the two isomers of this weak organic acid.

## MATERIALS AND METHODS

### Strains and growth conditions

The four strains of *L. monocytogenes* used in this study were a meat isolate, strain 412 (Gravesen *et al.* 2000) (denoted strain 4140 in some studies, e.g. Budde and Jakobsen 2000; Shabala *et al.* 2002), two human isolates, strains 22 (Gravesen *et al.* 2002) and LO28 (Vicente *et al.* 1985), and the sequenced animal isolate, strain EGDe (Glaser *et al.* 2001). The strains were cultured in brain–heart infusion broth (BHI; Scharlau Chemie, Barcelona, Spain) at 37°C with vigorous agitation (225 rev min<sup>-1</sup>). Colony-forming units (CFU ml<sup>-1</sup>) were enumerated by spotting 10 µl portions of a 10-fold serial dilution in peptone saline onto tryptone soya agar (TSA; Scharlau Chemie) plates. From each dilution, two aliquots were spotted onto the plates, which were subsequently incubated at 37°C for ca 40 h. The lactic acid isomer produced by the four strains was determined with a D-/L-lactic acid enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

### Inactivation with D-lactic acid, L-lactic acid or HCl

Overnight cultures of the *L. monocytogenes* strains were treated with 100 mmol lactic acid l<sup>-1</sup> at a final pH (pH<sub>ex</sub>) of 3.7 by adding appropriate volumes of 1 mol l<sup>-1</sup> stocks of D-lactic acid (96% pure; Sigma) or L-lactic acid (98% pure; Sigma). In order to ensure that exactly the same amount of each stereoisomer was used, the relative concentrations of the two stock solutions were determined by HPLC (Nissen *et al.* 2003), and the used volumes were adjusted to compensate for minor differences in concentration. The lactic acid-treated cultures were incubated at 37°C with agitation, and CFU ml<sup>-1</sup> was determined at suitable time intervals for up to 8 h. Inactivation by HCl was determined at a pH<sub>ex</sub> of 3.37. Two independent experiments were made with lactic acid treatment of strain 412.

### Determination of intracellular pH

The intracellular pH, pH<sub>i</sub>, was determined using fluorescence ratio-imaging microscopy (Siegumfeldt *et al.* 1999) for

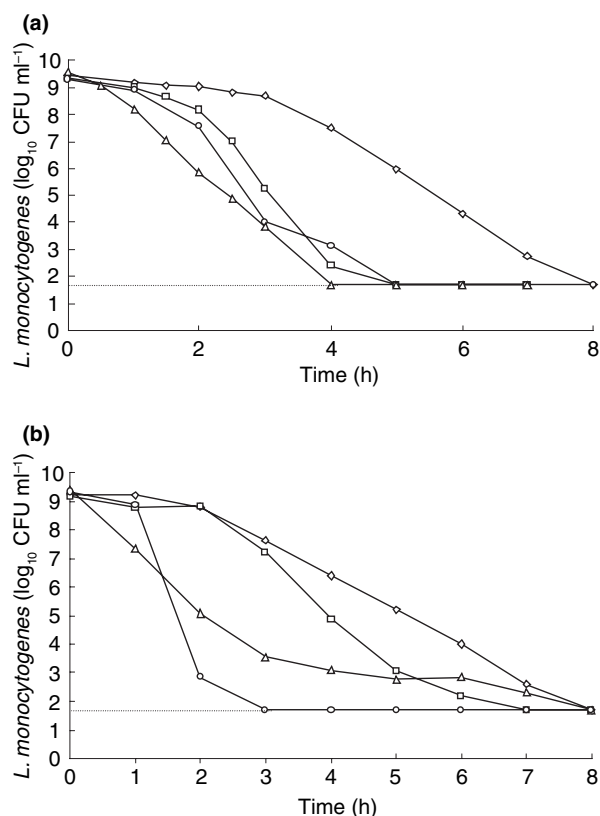
time-lapse studies of single cells following treatment with either D-lactic acid or L-lactic acid. Briefly, cells of an overnight culture (see above) of strain 412 were harvested by centrifugation, and stained with 5(6)-carboxyfluorescein diacetate succinimidyl ester (cFDASE) as described (Siegumfeldt *et al.* 1999; Budde and Jakobsen 2000) with the following modifications: the concentration of cFDASE was 44.4 µmol l<sup>-1</sup>; pH of the potassium phosphate buffer was adjusted to pH 6.0; staining and energizing of the cells were performed simultaneously by incubation for 60 min. For immobilization, a poly-L-lysine-coated glass cover slip (Shabala *et al.* 2002) was assembled with the perfusion chamber, and a sample of stained cells was added; unattached cells were removed by rinsing with buffer. The pH<sub>i</sub> of immobilized cells was determined from R<sub>490/435</sub>, the concentration-independent ratio between emission following excitation at 490 and at 435 nm, and a calibration curve as previously described (Budde and Jakobsen 2000).

An image of the immobilized cells was recorded corresponding to time zero. Lactic acid in phosphate buffer containing glucose (10 mmol l<sup>-1</sup>) was added at final concentrations of 7.3, 5 or 2 mmol l<sup>-1</sup>, giving pH<sub>ex</sub> values of 4.2, 4.9, and 5.7, respectively, and images were recorded for 7 min at 1-min intervals. A control experiment was made using HCl at a pH<sub>ex</sub> of 4.2, and pH<sub>i</sub> was measured after 4 min. Two samples were made of each treated culture and of the untreated control. In each sample, pH<sub>i</sub> was determined as the average of the values for 20 cells, and the same cells were analysed at each time point. However, for the culture treated with 5 mmol l<sup>-1</sup> of L-lactic acid, data was obtained for only one sample. We therefore made an independent repeat experiment, where two samples treated with 5 mmol l<sup>-1</sup> of each lactic acid isomer were analysed.

## RESULTS

### Strain variations in sensitivity to organic and inorganic acid

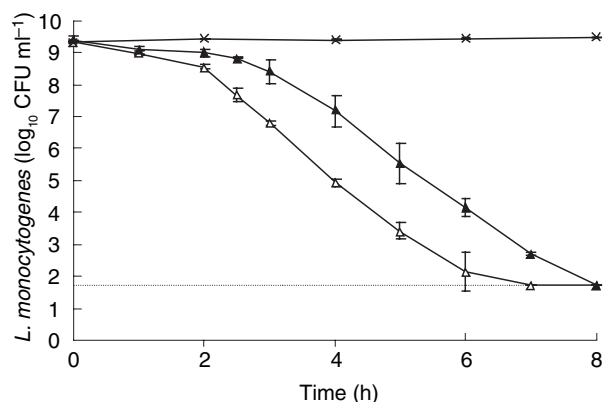
The viability of the four tested strains of *L. monocytogenes* following treatment with L-lactic acid or HCl is shown in Fig. 1. The employed levels of the two acids, resulting in pH<sub>ex</sub> of 3.7 and 3.37, respectively, were chosen such that similar ranges of inactivation times were obtained. Strain 412 was least sensitive to both types of acid, and this was especially pronounced for treatment with L-lactic acid (Fig. 1a). Strain 22 was almost as resistant to HCl as strain 412 (Fig. 1b). *Listeria monocytogenes* EGDe had somewhat atypical viability curves compared with the other strains. The inactivation of strain EGDe commenced more or less immediately after addition of both types of acid, while the inactivation of the three other strains was delayed 1–3 h, giving an initial shoulder on the viability curves.



**Fig. 1** Viability of stationary aerobic cultures in BHI at 37°C of *Listeria monocytogenes* 412 (diamonds), 22 (squares), LO28 (circles) and EGDe (triangles) following treatment with (a) 100 mmol l<sup>-1</sup> of L-lactic acid at a final pH of 3.7 or (b) HCl at a final pH of 3.37. The dashed lines represent the detection limit

### Difference in antilisterial effect of the lactic acid stereoisomers

The viability of the four *L. monocytogenes* strains following treatment with 100 mmol l<sup>-1</sup> D-lactic acid or L-lactic acid was compared. The strains were more sensitive to D-lactic acid than to L-lactic acid, which was primarily because of differences in the lengths of the shoulders of the inactivation curves; the maximum inactivation rates were almost identical for the two isomers. The difference was most pronounced for strain 412 (Fig. 2). Because of the difference in inactivation times (Fig. 1a), we chose to evaluate the strain variation in relative sensitivity to the two lactic acid isomers as the cell counts midway in the inactivation curve – i.e. after *ca* 4–5 log units reduction – for treatment with D-lactic acid (after 4 h of treatment for strain 412, 3 h for strain 22 and 2 h for strains LO28 and EDGe). At these times, the cell count for the D-lactic acid-treated cultures was 2.3 log units lower than that for L-lactic acid for strain 412 (Fig. 2), and 2.2 and 1.0 log units lower for strain LO28



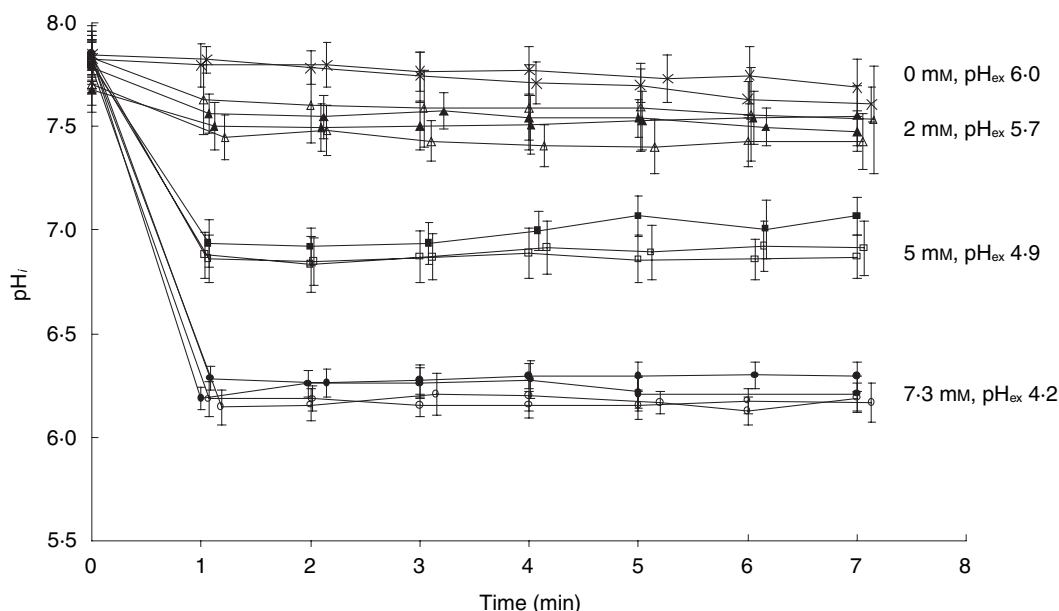
**Fig. 2** Viability of stationary aerobic cultures in BHI at 37°C of *Listeria monocytogenes* 412 following treatment with 100 mmol l<sup>-1</sup> of L-lactic acid (closed triangles) or D-lactic acid (open triangles) at a final pH of 3.7. The untreated control is represented by crosses. The data are the averages of two independent experiments, and the error bars show the standard deviations. The detection limit is shown by a dashed line

and EGDe, respectively; for strain 22, the difference was marginal, 0.6 log units (results not shown).

The four strains exclusively produced the L-lactic acid isomer, at concentrations of 3.7, 3.3, 3.6 and 5.2 mmol l<sup>-1</sup> in overnight cultures of *L. monocytogenes* 412, LO28, EGDe and 22, respectively, which gave total concentrations of lactic acid in the range 6.9–8.8 mmol l<sup>-1</sup> (BHI contained 2.7 mmol l<sup>-1</sup> L-lactic acid and 0.9 mmol l<sup>-1</sup> D-lactic acid).

### Effect of D- and L-lactic acid on intracellular pH

The effect of lactic acid treatment on pH<sub>i</sub> was determined for strain 412. Preliminary experiments using 100 mmol L-lactic acid l<sup>-1</sup> showed that pH<sub>i</sub> extremely quickly decreased to below the detection limit (pH<sub>i</sub> < 5.5–6.0, Budde and Jakobsen 2000). In order to enhance the detection of putative differences between the effects of the two isomers, we therefore chose lower concentrations for the following experiments, as shown in Fig. 3. There was no difference between the effect of treatment with L-lactic acid and D-lactic acid. The pH<sub>i</sub> was affected by lactic acid at a concentration as low as 2 mmol l<sup>-1</sup> (pH<sub>ex</sub> 5.7), and was reduced more by higher concentrations (5 and 7.3 mmol l<sup>-1</sup>, pH<sub>ex</sub> 4.9 and 4.2 respectively). The reduction was apparent already at the first time point (*ca* 1 min after acid addition), and no further change occurred within the next 6 min. The average pH<sub>i</sub> values were 7.7, 7.5, 6.9 and 6.2 for cells following treatment with 0, 2, 5 and 7.3 mmol l<sup>-1</sup> lactic acid. In comparison, cells treated with HCl at pH<sub>ex</sub> 4.2 had a decrease in pH<sub>i</sub> of 0.6. The identical effect on pH<sub>i</sub> of



**Fig. 3** Intracellular pH of cells of *Listeria monocytogenes* 412 treated with L-lactic acid (closed symbols) or D-lactic acid (open symbols) at the indicated concentrations and final pH-values, determined by fluorescence ratio-imaging microscopy. The untreated controls are represented by crosses. Each data point is the average value for 20 cells, and the error bars show the standard deviations

5 mmol l<sup>-1</sup> of each isomer was confirmed in an independent experiment (results not shown).

## DISCUSSION

In this study, we show that the antilisterial activity of lactic acid is stereospecific, however, while *E. coli* and *L. delbrueckii* subsp. *bulgaricus* previously were reported to be most susceptible L-lactic acid (Benthin and Villadsen 1995a; McWilliam Leitch and Stewart 2002), *L. monocytogenes* was most sensitive to D-lactic acid. D- and L-lactic acid had identical effect on pHi in *L. monocytogenes* (this work) and in *E. coli* (McWilliam Leitch and Stewart 2002), showing that the penetration of the lactic acid across the membrane is identical for the two isomers. The observations indicate that, in addition to the nonstereospecific pH-related detrimental effect of lactic acid, there is an additional antimicrobial mechanism that involves specific interaction with a chiral molecule. The three tested organisms are least sensitive to the isomer that they intrinsically produce: *E. coli* and *L. delbrueckii* subsp. *bulgaricus* produce D-lactic acid (Benthin and Villadsen 1995b; Bunch *et al.* 1997), while *L. monocytogenes* is a L-lactic acid producer (this work). This correlation implies that the additional mechanism is a consequence of self-protection against the intrinsically produced isomer, and could involve an enzymatic reaction, e.g. metabolization of the intracellular lactate, or the presence of a stereospecific efflux system for the produced

isomer. *Listeria monocytogenes* can utilize lactate as carbohydrate source (Kouassi and Shelef 1996), and the presence of stereospecific lactate carriers or permeases has been indicated in eucaryotic and procaryotic systems (Russell 1992; Carpenter and Halestrap 1994). Further research may test this hypothesis.

The sensitivity to lactic acid of the four tested strains of *L. monocytogenes* varied considerably, and corresponded more or less to their relative sensitivity to HCl. The varying acid sensitivity did not correlate to intrinsically synthesized levels of L-lactic acid. The strain variation in sensitivity to L-lactic acid was larger than the difference between the antilisterial effects of the two isomers. In addition, the difference in the antimicrobial effect of the two isomers against *L. monocytogenes* was less than the previously reported difference in inactivation of *E. coli* (McWilliam Leitch and Stewart 2002). The observations indicate that the stereospecific antimicrobial activity of lactic acid does not have substantial practical importance regarding *L. monocytogenes*. However, the present knowledge suggests considerable species-specific variation in relative sensitivity to the two isomers, which may differentially influence the development of beneficial and unwanted bacteria, and thereby shift the composition of a complex microflora. A better understanding of the involved mechanisms could enhance optimal use of lactic acid or lactic acid producers in food production and preservation.

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