**Growth and membrane fluidity of food-borne pathogen Listeria monocytogenes in the presence of weak acid preservatives and hydrochloric acid**

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**INTRODUCTION**

Listeria monocytogenes has been associated with a variety of food products, including dairy foods, meat, poultry, and seafood as well as fruits and vegetables (Farber and Peterkin, 2000; Mastronicolis et al., 2011). In 2008, 1,381 confirmed human cases of listeriosis were reported in the European Union and the reported case-fatality rate was 28.3% [European Food Safety Authority (EFSA), 2010].

Modification of membrane lipid composition is clearly an important adaptation mechanism in L. monocytogenes, which allows it to grow in a stressful environment such as low temperature (Annous et al., 1997; Mastronicolis et al., 2003), low pH (Giusti et al., 2007; Mastronicolis et al., 2010), presence of disinfectants (Biabiauddin et al., 2011), pressure, ion concentrations etc. (Beales, 2004). Changes in lipid composition can lead to changes in cytoplasmic membrane fluidity (Mykytczuk et al., 2007).

The term “membrane fluidity” is a convenient one to summarize a multifaceted phenomenon that has contributions from molecular packing (order) and molecular motions (viscosity; Russell, 2002). Membranes can exist in different phases and the most consistent phase transition is the one occurring when the membrane passes from a tightly ordered “gel” or “solid” phase to a liquid-crystal phase which is the active state of the membrane. A widely used method for determining the phase transition temperature (\(T_m\)) is calorimetry. The influence of hydrocarbon chain length, branching and unsaturation, as well as the head group of the membrane lipids on the value of \(T_m\) is considerable. In general, increasing the chain length, decreasing the branching or increasing the saturation of the chains increases the phase transition temperature (New, 1994; Mykytczuk et al., 2007).

Weak lipophilic acids can occur naturally in many fruits and vegetables and have been widely used to maintain microbial stability in low pH foods. Weak acid preservatives affect the cells’ ability to maintain pH homeostasis, disrupting substrate transport and inhibiting metabolic pathways (Beales, 2004). The effect of many weak acid preservatives is dependent on the fluidity and permeability of the cytoplasmic membrane, since it is the first barrier to encounter the stress and any sensing mechanism would be located within it (Beales, 2004; López et al., 2006). Changes in the lipid profile of the plasma membrane may alter membrane permeability and fluidity, which may in turn contribute to tolerance (Beales, 2004).

In our previous report on the effects of different acidic stresses such as hydrochloric, acetic, and lactic acid (pH 5.5) or benzoic...
acid (pH 7.3) on L. monocytogenes total, polar and neutral lipid compositional changes, our results suggest that only low pH value enhances the antimicrobial activity of an acid, though irrespective of pH, the acid adaptation response leads to a similar alteration in fatty acid composition, mainly originating from the neutral lipid class of adapted cultures (Mastroncelli et al., 2010). However, the effects of the aforementioned acidic antimicrobials on membrane fluidity in L. monocytogenes have not been determined and compared to date. The present work was intended to provide new data by determining and comparing modifications in Tm of L. monocytogenes membrane lipids (and thus alterations in membrane fluidity) in response to acid stress induced by acids such as hydrochloric, acetic, lactic, or benzoic acids and also to correlate the fatty acid compositional changes of each acid-adapted culture (from our previous data) with the lipid thermodynamic behavior in order to clarify if modifications in the membrane physical state of adapted cells act as a defense mechanism against acid stress.

MATERIALS AND METHODS

CULTURE OF THE ORGANISM

An avirulent strain L. monocytogenes, DP-L1044 (D. Portnoy, University of Pennsylvania) prepared by a transposon insertion (Camilli et al., 1991), in the parent strain Lm1040SS, was grown in brain heart infusion broth (BHI, Difco Laboratories) at 30 °C (24 h). A 10 mL aliquot of this was then inoculated into 1 L of BHI broth, which was then incubated at 30 °C (Lmcontrol) until early stationary phase. Four aliquots (10 mL) of the same stock were then inoculated, respectively, into 1 L BHI that were adjusted to pHinitial 7.3. An avirulent strain L. monocytogenes had been determined not to grow in BHI at pH 5.5. A 10 mL aliquot of this was then inoculated into 1 L of BHI broth, which was then incubated at 30 °C (24 h). A 10 mL aliquot of this was then inoculated into 1 L of BHI broth, which was then incubated at 30 °C until identical thermal scans were obtained, using a scanning rate of 10 °C min⁻¹. The temperature scale of the calorimeter was calibrated using indium (Tm = 136.6 °C) and dipalmitoylphosphatidylcholine from Avanti Polar Lipids Inc. (Alabaster, AL, USA) bilayers (Tm = 41.2 °C). The following diagnostic parameters in the observed endothermic events were recorded during the phase transition and are used for the study of lipids: Tm (maximum of the temperature peak), and ΔH (the area under the peak represents the enthalpy change during the transition).

The repeatability of the thermograms and reversibility of the transitions were checked after each run by re-heating the sample after cooling. All samples were scanned a minimum of three times.

STATISTICAL ANALYSIS

The results were evaluated by analysis of variance (ANOVA). t-test for unpaired observations was tested at a confidence level of 95%.

RESULTS

Growth of L. monocytogenes in BHI medium with time was determined for each treatment by measuring absorbance (OD) at 600 nm and shown in Figure 1. The presence of lactic, acetic, or hydrochloric acid at pH 5.5 was accompanied by low survival (P < 0.01), while cells grown at neutral pH in the presence of benzoic acid displayed little antilisterial activity (P < 0.05). The obtained OD600 values were at early stationary phase: Lmcontrol 0.811 ± 0.010, 10 h; LmLA 0.996 ± 0.028, 168 h; LmAA 0.217 ± 0.019, 72 h; LmHCl 0.320 ± 0.014, 24 h; and LmBA 0.694 ± 0.019, 10 h.

Lmcontrol CELLS

The DSC analysis revealed Tm value 25.78 ± 1.06 °C as well as enthalpy difference (ΔH) 8.99 ± 0.557 J g⁻¹ (Table 1 and Figure 2).

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**Table 1 | Data from differential scanning calorimetry analysis of L. monocytogenes total lipids before (Lmcontrol) and after acid stress exposure by lactic (LmLA), acetic (LmAA), hydrochloric (LmHCl), or benzoic (LmBA) acid.**

| Lmcontrol | LmLA  | LmHCl | LmBA  |
|-----------|-------|-------|-------|
| Tm1 (°C)  | 25.78 ± 0.106 | 29.35 ± 0.238 (Tm1) | 29.23 ± 0.213 (Tm1) | 30.25 ± 2.01* |
| ΔH (J·g⁻¹) | 8.90 ± 0.057 | 14.92 ± 0.168 | 8.246 ± 0.178 | 11.618 ± 0.40* |
| BCF/SSCFA | 8.3 | 1.6 | 2.1 | 2.6 |

* Tm, phase transition temperature; ΔH, enthalpy difference.
* Values statistically increased compared to Lmcontrol, P < 0.05.
* Values statistically increased compared to Lmcontrol, P < 0.05.
* Ratios of total branched-chain fatty acids, BCF, to total saturated straight chain fatty acids, SSCFA, of total lipid fatty acid profiles of cells. These data were derived from our previous study (Mastronicolis et al., 2010).
* The data for LmAA were: 127.8 ± 0.29°C for Tm1, 73.4 ± 0.41°C for ΔH, and 1.4 for BCF/SSCFA. One set of extracted lipids was utilized because the appropriate weight of lipids for DSC analysis was collected by harvesting five cultures.

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**DISCUSSION**

Other authors examined the antimicrobial effects of these acids. Ravichandran et al. (2011) observed that benzoic acid (5 g/L) demonstrated antimicrobial activity against L. monocytogenes after 72 h incubation at 37°C. Heavin et al. (2009) observed that benzoic acid was more effective at inhibiting growth of L. monocytogenes than acetic acid, in a medium with a pH of 6.4 (acidified with HCl). Hydrochloric, lactic, and acetic acids at pH 3.5 gave similar kill curves (Cypriano et al., 1993). Hydrochloric acid caused low survival of L. monocytogenes at pH 5 (Karatzas et al., 2010) and slight antibacterial action against L. monocytogenes was observed with acetic acid at pH 5 (Chavant et al., 2004). In contrast, Vasseur et al. (1999) observed that the antibacterial effect was: acetic acid > lactic acid > hydrochloric acid. Similar results were observed by Bonnet and Montville (2005) in L. monocytogenes growing at pH 3.5. Khan-Thabt et al. (2000) also found that acetic acid had a more deleterious effect on L. monocytogenes than hydrochloric acid did. Exposure to lactic acid at pH 4.0 totally inactivated L. monocytogenes, whereas exposure at pH 4.5 had inhibitory effect (at 5 or 10°C), therefore, even small differences in pH, such as 0.5 units, may have a major impact on the survival of pathogens and hence, on food safety (Tiganitas et al., 2009). The comparative study of acid habituation of L. monocytogenes, under the same experimental conditions is important for the identification of differences between the survival of the pathogen, as comparison between laboratories is difficult because of variation in the assay conditions used (exact pH value, bacterial strains, incubation temperatures, etc.).

This study provides a first approach to observing the role of phase transitions of membrane lipids (membrane fluidity) in the acid adaptation response of L. monocytogenes. We have previously studied the lipid composition of L. monocytogenes cells grown in the presence of various acids (hydrochloric, acetic, lactic, and benzoic acid) and the analysis of membrane lipids revealed that L. monocytogenes similarly altered its fatty acid composition by incorporation more straight (mostly C16:0 and C18:0) and fewer branched-chain fatty acids into its membrane independently of the acid utilized (Table 1; Marstonicolis et al., 2010). It is expected that these fatty acids changes lead to membranes with decreased fluidity and low permeability properties (Kaneda, 1991; Zhang and Rock, 2008). In the current study, the measured lipid Tm value of...
each set of adapted cells was increased compared to Lmcontrol and this observation is interpreted by the above fatty acid compositional changes. However, the increases in \( T_m \) values are not of equal extent and therefore are not absolutely reflected by the acyl chain compositional changes. This fact indicates that fatty acid changes may be crucial but they are not the sole mechanism by which L. monocytogenes perceives the acid stress (alters its membrane lipids). Furthermore, the growth of L. monocytogenes in the presence of hydrochloric, lactic, and acetic acid at pH 5.5 caused an increase of neutral lipid percentages (Mastronciolo et al., 2010).

Hydrochloric acid will be dissociated, whereas acetic (\( pK_a = 4.74 \)) and lactic acid (\( pK_a = 3.79 \)) will be undissociated at pH 5.5. The latter form of both organic acids is membrane-permeable and thus allows acetic and lactic acid to enter the microbial cell. In this work, when the cells were grown in the presence of acetic or hydrochloric acid, the highest \( T_m \) values and low survival were observed (Figure 1, Table 1), suggesting that the decrease in membrane fluidity was related to low survival. However, this tendency was reversed in the case of lactic acid, which caused the highest antimicrobial action (Alonso-Hernando et al., 2010). In sub-optimal pH, a decrease in membrane fluidity of L. ferrooxidans was observed and this is likely linked to the overall increase in saturated fatty acids at the expense of unsaturated fatty acids (Mykytczuk et al., 2010). Adaptation to acid and starvation stress increased net cell hydrophobicity and decreased membrane fluidity of L. innocua (Moorman et al., 2008). ATR(+) L. monocytogenes cells (cells exposed to mild acid (pH 5.5), which are subsequently able to resist severe acid (pH 3.5) conditions) had lower membrane rigidities than ATR(−) cells (cells subjected at pH 3.5 directly; Najjar et al., 2009). After exposure to oregano essential oil concentrations up to 0.50%, the membrane fluidity of L. monocytogenes was decreased presumably to block, or at least to reduce essential oil entrance and partition into the membrane (Serio et al., 2010). Growth in the presence of butyrate, leucine, valine, isovalerate, or iso-butyrate increased the calculated (theoretical estimation) transition temperature of L. monocytogenes, because of the decrease of branched-chain at the expense of saturated-chain fatty acids (Jablotok et al., 2010). Increase in phase transition temperatures was observed with increased osmotic pressure in Saccharomyces cerevisiae (Laroche et al., 2001). Decreased membrane fluidity was also observed in Bacillus subtilis subjected to osmotic pressure (López et al., 2009).

An understanding of phase transitions and fluidity of membranes is important, since the phase behavior of a membrane determines such properties as permeability, fusion, aggregation, and protein binding, affects critical biochemical reactions, transport systems, all of which can markedly affect the stability of membranes, and their behavior in the cell (New, 1994; Yak and Marshall, 2006). Acid habituation of pathogens may enhance survival in an acidic food or in the stomach and subsequently cause infection after ingestion. The resistance or adaptation of pathogens to such conditions affect food safety and thus is clearly of significance to the food industry (Reales, 2004).

Although the acid adaptation response of L. monocytogenes altered the fatty acid composition similarly, irrespective of the acid utilized (Mastronciolo et al., 2010), in the present study observed \( T_m \) values were increased but not equally. This suggests that the \( T_m \) value (membrane fluidity) of lipids does not depend only on the acyl constituent, but also on the total composition and nature of the lipid molecular structure (e.g., phospho-, glyco-, amino-head groups for polar lipids or the specific lipid molecule for neutral lipids, e.g., dicycloides, esters, waxes, etc.). Thus, understanding the physical chemistry of membrane lipids is important in the sense that the characteristics of lipid species, and their heterogeneity, all affect biological membranes. Our current understanding of the role of individual lipid species in a heterogeneous lipid matrix and the specific lipid-lipid and lipid-protein interactions is still far from comprehensive. Therefore, one conclusion of this study would support the in-depth identification of the membrane polar and neutral lipid molecules of L. monocytogenes cells in the presence of the acids utilized. Furthermore, in this study an avirulent...
In conclusion, in this study we observed that adaptive response of L. monocytogenes to weak or strong acid food preservatives includes an increase in the tolerance of fatty acids (in the case of hydrochloric or acetic acid) or as mild defense mechanism (in the cases of benzoic or lactic acid).

References

Alonso-Hernando, A., Almo-Calleja, C., and Capita, R. (2010). Effects of exposure to poulty chemical decontaminants on the membrane fluidity of Listeria monocytogenes and Salmonella enterica strains. J. Food Microbiol. 157, 130–136. doi: 10.1016/j.jfmi.2009.11.002

Amoo, B. A., Boateng, I. A., Borhis, D.-O., Labah, D. P., and Wilkinson, B. J. (1997). Critical role of anion-stimulated fatty acid in the growth of Listeria monocytogenes at low temperatures. Appl. Environ. Microbiol. 63, 3847–3849.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservation, low pH, and osmotic stress a review. Compr. Rev. Food Sci. Food Saf. 3, 1–20. doi: 10.1111/j.1541-4337.2003.tb00057.x

Bisbiboulas, P., Pyken, M., Birooulas, L., Diakogiannis, I., Berberi, A., and Mastronicolis, S. K. (2011). Adaptive changes in cell membrane phospholipids and fatty acid composition of the food pathogen Listeria monocytogenes as a stress response to disinfectant sodium benzoate and chloramphenicol. Lett. Appl. Microbiol. 52, 275–280. doi: 10.1111/j.1472-765X.2010.03959.x

Bosnet, M., and Monstrelle, T. J. (2005). Acid tolerance of Listeria monocytogenes persist in a model food system contaminated with lactic bacteria. Lett. Appl. Microbiol. 40, 237–242. doi: 10.1111/j.1472-765X.2005.02651.x

Camilli, A., Goldfine, H., and Portnoy, D. A. (1991). Z. Listeria monocytogenes mutants lacking phospholipid A of specific phospholipase C are avirulent. J. Exp. Med. 173, 751–754. doi: 10.1084/jem.173.5.751

Charron, T., Gaillard-Martineau, B., and Hébrard, M. (2004). Antimicrobial effects of outers of plant- and toxin- sensitive Listeria monocytogenes cells according to the growth phase. Int. J. Food Microbiol. 90, 241–248.

Chihhi, N.-E., Rebouco da Silva, M., Delattre, G., Leroche, M., and Fedorovich, M. (2008). Influence of different fatty acid pattern behaviours of two strains of Listeria monocytogenes Scott A and CNL 895807 under different fermentation and salinity conditions. FEMS Microbiol. Lett. 258, 155–166. doi: 10.1111/j.1574-6968.2005.00152.x

European Food Safety Authority (EFSA) (2010). The community summary report on trends, and sources of zoonoses, zoonotic agents, and foodborne outbreaks in the European Union in 2008. EFSA J. 8, 1–96. doi: 10.2903/j.efsa.2010.1496

Farber, J. M., and Potier, P. I. (2008). “Listeria monocytogenes” in The Microbiological Safety and Quality of Foods, ed B. M. Lund, T. C. Bund- Parker, and G. W. Gould (Amsterdam: Elsevier), pp. 179–212.

Fecht, J., Leus, M., and Staksen-Slinear, G. H. (1995). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 270, 14081–14087.

Genta, E. S., McDowell, D. A., Blais, I. S., and Wilkinson, B. J. (2007). Role of branched-chain fatty acid in pH stress tolerance in Listeria monocytogenes. Appl. Environ. Microbiol. 73, 997–1001. doi: 10.1128/AEM.00853-06

Harwood, S. R., Brennan, O. M., Morris- ley, J. P., and O’Herne, C. P. (2003). Inhibition of Listeria monocytogenes by acetate, benzoate, and sorbate: biochemical and cell wall effects. J. Food Prot. 66, 275–280. doi: 10.4315/0362-028X-66.4.275

Hojberg, C., and Wilkinson, B. J. (2007). Cold dependence of fatty acid profile of different lipid structures of Listeria monocytogenes. Food Microbiol. 24, 21–24. doi: 10.1016/j.fm.2006.08.002

Mastronicolis, S. K., Barbier, A., Diako- giannis, I., Petrou, E., Kaka, I., Baltis, T., et al. (2015). Alteration of the phospho- or neutral lipid content and fatty acid composition in Listeria monocytogenes due to acid adaptation mechanisms for hydrochloric, acetic, and lactic acids at pH 5.5 or benzoic acid at neutral pH. Antonie Van Leeuwenhoek 107, 307–316. doi: 10.1007/s10482-015-9979-x

Mastronicolis, S. K., Diakogiannis, I., Barbier, A., Bubolzulis, P., Soukoulis, C., and Tzia, C. (2011). Effect of cold adaptation on the survival of Listeria monocytogenes in ice-cream formulations during long-term frozen storage. Antonie Van Leeuwenhoek 100, 393–397. doi: 10.1007/s12047-011-0216-7

Mastronicolis, S. K., German, J. B., Mogoulas, N., Petrou, E., Pika, P., and Smith, G. M. (1998). Influence of cold shock on the fatty-acid composi- tion of different lipid classes of the food-borne pathogen Listeria mono- cytogenes. Food Microbiol. 15, 299–306. doi: 10.1016/S0741-7300(97)00170-9

Mastronicolis, S. K., Tzovas, J. T., Petrou, G. D., and Lasko, L. G. (2010). Cytoskeletal membrane fluidity and fatty acid composition of Acidobacterium fermentans in response to pH stress. Extremophiles 14, 427–441. doi: 10.1007/s00792-010-0319-2

Mektezuz, N. C. S., Tzovas, J. T., Lasko, L. G., and Ferroni, G. D. (2007). Haemophilus parainfluenzae in study of bacterial cytoskeletal membrane fluidity under environ- mental stress (review). Prog. Biochem. Mol. Biol. 95, 80–82. doi: 10.1016/j.phmb.2007.02.002

Najjar, M. Z. B., Chikindas, M. L., and Mortellaro, T. A. (2009). The acid tolerance response alters membrane fluidity and induces main resistance in Listeria monocytogenes. Probiot. Antimicrob. Proteins 1, 133–135. doi: 10.1007/s12874-009-0025-8

New, B. R. C. (1986). Lipids: A Practical Approach. New York: Oxford University Press.

O’Donnell, B., Gahan, C. G. M., and Hill, C. (1996). Adaptive acid tolerance response in Listeria monocytogenes: isolation of an acid- tolerant mutant which demonstrates increased virulence. Appl. Envi- ron. Microbiol. 62, 1689–1696. doi: 10.1128/JEM.62.6.1689-1696.1996

Phan-Thanh, L., Mahmoud, F., and Allige, E. (2008). Influence of hydrochloric acid on the cold adaptation of different lipid classes Listeria monocytogenes. Int. J. Food Microbiol. 121, 125–128. doi: 10.1016/j.ijfoodmicro.2007.11.008

Rothschild, M., Hettiarachchy, S. K., Gomot, V., Baka, S. C., and Singh, S. (2011). Enhancement of antimicro- bial activities of naturally occurring phenolic compounds by nanoscale
diagnosis against Listeria monocytogenes. Escherichia coli O157:H7 and Salmonella typhimurium in broth and chicken meat system. J. Food Saf. 31, 467–471. doi: 10.1111/j.1745-4565.2011.00322.x

Russell, N. J. (2002). Bacterial membranes: the effects of chill storage and food processing: an overview. Int. J. Food Microbiol. 79, 27–34. doi: 10.1016/S0168-1605(02)00176-9

Serio, A., Chiarini, M., Tettamanti, E., and Paparella, A. (2010). Electronic paramagnetic resonance investigation of the activity of Origanum vulgare L. essential oil on the Listeria monocytogenes membrane. LWT-Food Sci. Technol. 43, 149–157. doi: 10.1016/j.lwt.2009.08.008

Yuk, H.-G., and Marshall, D. L. (2006). Effect of trisodium phosphate adaptation on changes in membrane lipid composition, rottenin secretion, and acid resistance of Escherichia coli O157:H7 in simulated gastric fluid. Int. J. Food Microbiol. 106, 39–44. doi: 10.1016/j.ijfoodmicro.2005.05.009

Zhang, Y.-M., and Rock, C. O. (2014). Membrane lipid homoeostasis in bacteria. Nat. Rev. Microbiol. 6, 222–233. doi: 10.1038/nrmicro1819

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