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Adaptive acid tolerance response of *Listeria monocytogenes* strains under planktonic and immobilized growth conditions

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ABSTRACT

The acid resistance of *Listeria monocytogenes* was evaluated: (i) after short (shock) or long-term (adaptation during growth) exposure to reduced (5.5) or neutral (7.2) pH in a liquid (broth) medium or on a solid surface (agar), and (ii) after growth on the surface of ham and turkey slices or in homogenates of these products. Three *L. monocytogenes* strains (serotypes 1/2a, 1/2b and 4b) were individually inoculated at: (i) 10^4–10^5 CFU/ml in tryptic soy broth with 0.6% yeast extract (TSBYE) or on tryptic soy agar with 0.6% yeast extract (TSAYE) at pH 7.2 with 1% (+G) or without (−G) glucose of or TSBYE and TSAYE with 0.25% glucose at pH 5.5 (lactic acid) and incubated at 20 °C, and (ii) 10^2–10^3 CFU/cm^2 on ham and turkey slices (pH 6.39–6.42; formulated with potassium lactate and sodium diacetate) or in their homogenates (1:4 and 1:9; representing viscous [slurry] and liquid residues [purge], respectively), and stored at 10 °C. The acid resistance of each strain was assessed in TSBYE of pH 3.5 (lactic acid) for strains growing in broth or on agar surfaces, and in TSBYE of pH 1.5 (HCl) for strains growing on ham and turkey slices or in their homogenates. Habituation at pH 5.5 for 3 or 24 h at 20 °C increased acid (pH 3.5) resistance of all strains compared to the control (pH 7.2). Cells grown on the surface of TSAYE-G (pH 7.2 or 5.5) showed higher resistance than cells grown in broth (TSBYE-G), whereas the opposite was observed for cells grown on TSAYE + G or in TSBYE + G. Growth of *L. monocytogenes* on meat product slices was markedly slower than in homogenates. Pathogen reductions following exposure to pH 1.5, after 10 and 27 days of storage were strain-dependent and in the ranges of 0.5–2.5, 1.3–4.5 and 4.0–7.6 log units for cells grown on product slices in 1:4 and 1:9 homogenates, respectively. The results suggest that *L. monocytogenes* cells growing on food surfaces or in viscous matrices may show higher resistance to lethal acid conditions than cells growing in liquid substrates.

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1. Introduction

Listeria monocytogenes is an invasive pathogen considered responsible for various outbreaks associated with consumption of contaminated ready-to-eat (RTE) liquid or solid foods, such as butter, milk, cheese, deli meats, cantaloupes, etc. (CDC, 1999, 2002, 2008, 2011; Fretz et al., 2010; Maijala et al., 2001; Olsen et al., 2005). Physical properties (e.g., microstructure) and food composition, especially fat content, affect growth of *L. monocytogenes* during storage and subsequent survival of the organism during gastrointestinal (GI) passage in the host (Barmpalia-Davis et al., 2009; Stopforth et al., 2005; Wonderling and Bayles, 2004). Survival in the GI tract is primarily associated with the acid resistance of invasive pathogens against the low pH of the stomach (1.0–2.0).

Growth of *L. monocytogenes* on solid surfaces is slower than in liquid media (Lebert et al., 2004; Meldrum et al., 2003); however, it has been found that the thermotolerance and osmotolerance of bacteria are reduced in liquid (e.g., purge) compared to solid (e.g., meat muscle) substrates (Murphy et al., 2000). Specifically, survival of *Salmonella* at low a_w on meat carcasses is higher compared to liquid cultures (Kinsella et al., 2007). Furthermore, Gawande and Bhagwat (2002a) reported that solid surfaces protected *Salmonella* from the lethal effect of acid, suggesting that immobilization on surfaces increases the resistance of pathogens to stress compared to growth in liquid suspensions.

Emulsified structured foods rich in fat, such as sausages, dressings, mayonnaise, etc., or solid surfaces, such as those of apples, tomatoes and cucumbers have been suggested as protecting pathogens, including *L. monocytogenes* and *Salmonella* against low pH (2 to 3.0) (Barmpalia-Davis et al., 2009; Gawande and Bhagwat 2002a,2002b). Similarly, the heat resistance of *Salmonella* was higher on whole muscle than in ground beef or chicken and even higher than in broth (Murphy et al., 2000; Orta-Ramirez et al., 2005).

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The resistance of attached cells to stresses is a contact-mediated phenomenon presumably associated with *de novo* protein synthesis (Gawande and Bhagwat, 2002a). However, it is unclear whether such physiological changes may induce further adaptation to other stresses during storage of foods. Growth of *L. monocytogenes* on turkey meat (as a whole or sliced), in slurry (1:4) of whole muscle meat, or on sliced processed turkey meat at 10 °C resulted in higher resistance against simulated gastric fluid (SGF) of pH 3.5 to 7.0 than growth in Brain Heart Infusion Broth, regardless of pH of the growth substrate (Peterson et al., 2007). Kinsella et al. (2007) demonstrated that osmotic (a_w 0.79–0.96) and cold (4 °C) shock caused less injury to *Salmonella* immediately after attachment on meat surfaces than suspended in meat solution, but the opposite was observed after 72 h of incubation at 4 °C. The authors suggested that surface attachment provided mechanical protection to cells from the initial cold and osmotic stress, but only planktonic cells could adapt to these stresses, because attached cells were prevented from contact with the stress factor.

Stress adaptation may be the result of bacteria being exposed to certain food components during storage of foods. Such components may be introduced to raw materials, e.g., organic acids in the form of their salts may be added as ingredients in meat product formulations, as is the case of sodium diacetate (Stopforth et al., 2005; Wonderling and Bayles, 2004), or gradually released as a result of fermentative metabolism of bacteria (Buchanan and Edelson, 1996). The latter also resembles the microenvironments formed in fermented foods by starter cultures. Nonetheless, growth of bacteria on surfaces causes accumulation of acidic products around and within colonies, thereby reducing the surrounding pH (Malakar et al., 2000). Such a phenomenon may either trigger acid adaptation mechanisms of the cells residing within colonies, or sensitize them due to perturbation of pH homeostasis and metabolic exhaustion of cells (Calicioglu et al., 2002; Ryu and Beuchat, 1998).

Given that acidity is the first and most critical barrier of human defense against bacterial infection, the investigation of whether acid resistance of *L. monocytogenes* is affected, not only by surface-contact, but also by growth in environments of different physical properties is of importance. Providing such information for strains of different serotypes and/or lineage may also explain their potential for persistence in the processing environment or survival in the gastrointestinal tract. The objective of the present study was to evaluate the acid resistance of *L. monocytogenes* cells: (i) after short (shock) or long-term (adaptation during growth) exposure to reduced (5.5) or neutral (7.2) pH in a liquid (broth) or on a solid surface (agar), and; (ii) after growth on the surface of ham and turkey slices or in homogenates of these products.

2. Materials and methods

2.1. Strain selection

Based on a preliminary screening of 18 *L. monocytogenes* strains for their ability to grow at 5 and 30 °C, 3% NaCl (w/v) and pH 5.0 (data not shown), the following three strains were chosen in order to represent isolates of different serotypes: strain FSL N1-227 (serotype 4b) and strain FSL R2-499 (serotype 1/2a), both epidemic strains included in the so-called "five-human and disease diversity cocktail" (Fugett et al., 2006), as well as the slow-growing strain 11 (serotype 1/2b) based on the comparisons between growth rates of various strains at 4 and 30 °C by Lianou et al. (2006). Stock cultures of each strain were maintained and activated as described in previous studies (Skandamis et al., 2008, 2009). Strains were subcultured individually for 24 h at 30 °C in tryptic soy broth (TSB; Difco, Becton Dickinson Co., Sparks, MD) supplemented with 0.6% (w/v) yeast extract (TSBYE) and then in glucose-free TSBYE (TSBYE-G) before preparation of inocula for use in the study.

2.2. Experimental design

In the present study, two types of substrates were used for the growth of *L. monocytogenes*: (i) TSBYE without agar or with 1.5% agar (i.e., TSAYE, w/w) simulating liquid or solid matrices, respectively, and (ii) homogenates and slices of RTE ham and turkey. TSBYE and TSAYE were prepared without (−G) or with 1% (+G) glucose (w/v and w/w for TSBYE and TSAYE, respectively) at pH 7.2, or with 0.25% glucose at pH 5.5, adjusted with 85% lactic acid (v/v for TSBYE or v/w for TSAYE; Purac® FCC 88; Purac America, Lincolnshire, IL).

2.3. Ready-to-eat (RTE) food matrices

Commercially-manufactured turkey and ham (pH 6.39–6.42), both formulated with potassium lactate and sodium diacetate were used to prepare homogenates in water at 1:4 dilution, representing viscous RTE residues (slurry) and 1:9 dilution, representing liquid residues (e.g., purge). For preparation of homogenates, 100 g of each product was blended (Waring Commercial Laboratory Blender 7012G, Torrington, CT) with sterile water (200 ml) and filtered through sterile cheesecloth. The volume of filtrate was increased up to 400 or 1000 ml by adding sterile water in order to obtain filtered homogenates of 1:4 and 1:10 strength, respectively. It should be noted that the homogenate strength is indicative of the dilution in water and not necessarily the exact amounts of solids in each preparation. Both RTE products received at the lab had been cut in slices of 3 mm thickness by the manufacturer. They were further cut in 5×5 cm slices at the laboratory (same thickness), for use in the experiments.

2.4. Experimental procedure

Individual strains were grown in TSBYE-G, harvested at stationary phase (30 °C, 18 h) and each strain was added at the level of 10^{4-5} CFU/ml or cm^2 in the test tubes containing 10 ml of TSBYE or on the surface of TSAYE, with glucose content and pH adjusted as described in Section 2.2. Inoculated media were stored at 20 °C, for up to 96 h aerobically, shaken by vortexing for 10 s every 24 h. Conditions between consecutive vortexing, could be characterized as hypoxic (Noriega et al., 2008), i.e., with a potential oxygen gradient formed inside the test tubes bearing the TSBYE. Such conditions were thought to affect the maximum population density of *L. monocytogenes*, but not the growth rate, nor the subsequent acid resistance of the organism (Noriega et al., 2008). The high inoculation level was chosen so that growth to stationary phase under the present conditions (i.e., pH 5.5 or 7.2 and 0.5% NaCl [w/v or w/w] at 20 °C) occurs faster than desiccation of plates.

Experiments were performed in three independent repetitions with two samples analyzed per treatment each time. Homogenates were dispensed (25 ml) in plastic centrifuge tubes, inoculated with 10^{2-3} CFU/ml of each *L. monocytogenes* strain, placed in anaerobic jars (AnaeroGen, OXOID Ltd, Basingstoke, Hampshire, UK) and stored at 10 °C, for up to 63 days. The selection of 10 °C instead of the recommended refrigeration temperature of 4 °C aimed to ensure that remarkable growth of *L. monocytogenes* would have occurred in a product with antimicrobials. In addition, 10 °C simulated a common temperature abuse scenario, applied in previous relevant studies with RTE meat products (Barmpalia et al. 2005; Stopforth et al. 2005). The initial population of *L. monocytogenes* tested is a common inoculation level in challenge studies with RTE meat products (Augustin et al., 2011) and high enough not to be outcompeted by the natural microflora. Product samples were vacuum-packaged (2 samples per bag) and stored at 10 °C. Experiments with RTE foods and their homogenates were performed in two independent experiments with two samples analyzed in each experiment.

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