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Habituation to organic acid anions induces resistance to acid and bile in *Listeria monocytogenes*

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ABSTRACT

We evaluated the intrinsic and inducible resistance of four human pathogenic strains of *Listeria monocytogenes* to acid and bile, factors associated with virulence. Cells were grown in media at pH 7.4, or in media at pH 6.0 containing 0 (HCl control) or 4.75 mM of different organic acids, harvested at stationary or mid log phase, and challenged for 1 h in acid or bile. Stationary phase cells were intrinsically more resistant to either challenge than log phase cells, and large differences between strains were evident among the latter. Compared to the HCl control, habituation to log phase with organic acids induced significant ($p < 0.05$) and meaningful (≥1 log) increases in acid resistance of three of four strains tested, and in bile resistance of two strains suggesting that exposure to organic acid anions may enhance virulence in *L. monocytogenes*.

1. Introduction

Organic acids are widely used in meat industry to control pathogens such as *Escherichia coli*, *Salmonella* and *Listeria monocytogenes*. Specifically, lactic and acetic acids are employed as washes to decontaminate meat surfaces (Berry & Cutter, 2000), and their salts are added to processed meats to prevent outgrowth of bacteria (Anonymous, 2003). Levulinic acid (4-oxopentanoic acid) has been investigated recently for similar applications (Carpenter, Smith, & Broadbent, 2011; Thompson, Carpenter, Martini, & Broadbent, 2008). *L. monocytogenes* is a particularly serious public health concern, as the fatality rate from listeriosis infections in the United States is estimated to be as high as 28% (Mead et al., 1999; Ramaswamy et al., 2007).

Virulence in *L. monocytogenes* has been linked to acid and bile resistance, because each of these factors contributes to pathogen survival in the gastrointestinal tract (Conte et al., 2000; Conte et al., 2002; Saklani-Jusforgues, Fontan, & Goossens, 2000; Skandamis, Gounadaki, Geornaras, & Sofos, 2012; Werbrouck et al., 2009). Several prior studies have shown exposure to mild acid conditions (pH ≤ 5.5) may induce resistance to acid or bile in *L. monocytogenes* (Begley, Gahan, & Hill, 2002; Chorianopoulos, Giaouris, Grigoraki, Skandamis, & Nychas, 2011; Davis, Coote, & OByrne, 1996; Garner, James, Callahan, Wiedmann, & Boor, 2006; King, Ferenci, & Szabo, 2003; Kroll & Patchett, 1992; Melo, Andrew, & Faleiro, 2013; O'Driscoll, Gahan, & Hill, 1996; Phan-Thanh, Mahouin, & Alige, 2000; Skandamis et al., 2012; Werbrouck et al., 2009), but little is understood regarding the response of *L. monocytogenes* to the weakly acidic conditions that characterize ready-to-eat or enhanced meats. Because widespread use of organic acids in the meat industry may have unintended consequences in *L. monocytogenes* virulence, it is important to develop a greater understanding of the effects of organic acid salts on the physiology of this important pathogen. Thus, this research investigated the intrinsic resistance to acid and bile of four human-pathogenic strains of *L. monocytogenes*, as well as the effect of organic acid habituation at pH 6.0 on induced acid or bile resistance in these strains.

2. Material and methods

2.1. Bacterial strains

The four strains of *L. monocytogenes* used in this study are described in Table 1. Original cultures were stored as frozen (−80 °C) stock cultures. Working cultures were prepared from frozen stocks by two successive transfers (1% inoculum, v/v) into tryptic soy broth (TSB, pH 7.4; Becton, Dickinson and Company, Sparks, MD) and incubated at 37 °C overnight (~18 h) with shaking (220 rpm).

2.2. Growth curves

Growth curves were determined for each *L. monocytogenes* strain in each of the broth formulations ("treatments") used in this study. The baseline control treatment was 120 ml of standard TSB (pH 7.4). Other treatments included 120 ml TSB lacking dextrose and adjusted

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Table 1
Listeria monocytogenes strains used in the study.

Strain	Ribotype	Lineage	Serotype	Source
FSL R2-499	DUP-1053A	II	1/2a	Human isolate associated with the US outbreak linked to sliced turkey, 2000
FSL C1-056	DUP-1030A	II	1/2a	Isolated from human sporadic case
FLS N1-227	DUP-1044A	I	4b	Food isolate associated with the US outbreak, 1998–1999
FSL N3-013	DUP-1042B	I	4b	Food epidemic, UK, 1988–1990

to pH 6.0 with HCl and containing 0 (HCl control) or 4.75 mM of L-lactic acid (Sigma Chemicals, St. Louis, MO), levulinic acid (Sigma Chemicals, St. Louis, MO) or acetic acid (A Johnson Matthey Company, Ward Hill, MA). Overnight working cultures of each strain were prepared as described, individually collected by centrifugation (2500 ×*g* for 10 min; Sorvall RT1, Thermo Scientific, Germany) at 4 °C, then diluted to an optical density at 600 nm (OD_{600}) of 0.030 in TSB. A 1% inoculum (v/v) was transferred into each treatment medium, and the culture was incubated at 37 °C for up to 12 h with shaking. Duplicate 2 ml samples were collected hourly. The OD_{600} was measured on one sample, while the other was spread plated onto tryptic soy agar (TSA) (Sigma Chemicals, St. Louis, MO). Colonies on the plates were counted after 48 h incubation at 37 °C.

2.3. Intrinsic acid and bile resistance

Intrinsic resistance of each *L. monocytogenes* strain to acid or bile was determined using mid-log and stationary phase cells grown in TSB. Based on the previously determined growth curves, mid-log phase cells of all strains were collected by centrifugation after 4 h (cell density approx 10^7 CFU/ml). Early stationary phase cells of R2-499, N1-227 and N3-013 were collected after 11 h, and after 9 h for strain C1-056 (cell density was approx 10^9 CFU/ml). After centrifugation, the cells were washed once in 10 ml peptone water (0.1%, w/v), centrifuged again, and the pellet suspended in 1 ml peptone water to yield test suspensions of approximately 10^9 CFU/ml.

Duplicate 0.1 ml samples of each test culture were transferred into TSB adjusted to deliver an acid or bile challenge, while 1 ml samples were serially diluted then plated on TSA. The acid challenge was 10 ml of TSB without dextrose and acidified with HCL to pH 3.0, 2.5, 2.0 or 1.5. Bile challenge for log-phase cells was performed in 10 ml TSB lacking dextrose and containing 0.1%, 0.2%, 0.3% or 0.4% bile salts (sodium cholate: sodium deoxycholate [1:1], Sigma Chemicals, St. Louis, MO). Stationary phase cells were challenged at these same bile concentrations and with 0.5% bile salts. Cells were incubated under challenge conditions for 1 h at 37 °C with shaking at 220 rpm, and then the samples were collected, serially diluted in peptone water, and plated on TSA. The percent survival was calculated as follows: Percent survival $= N_c / N_0 * 100$; where N_c is the number of cells after each challenge and N_0 is the number of cells in the inoculum used for the challenge medium. Two independent trials of each experiment were performed.

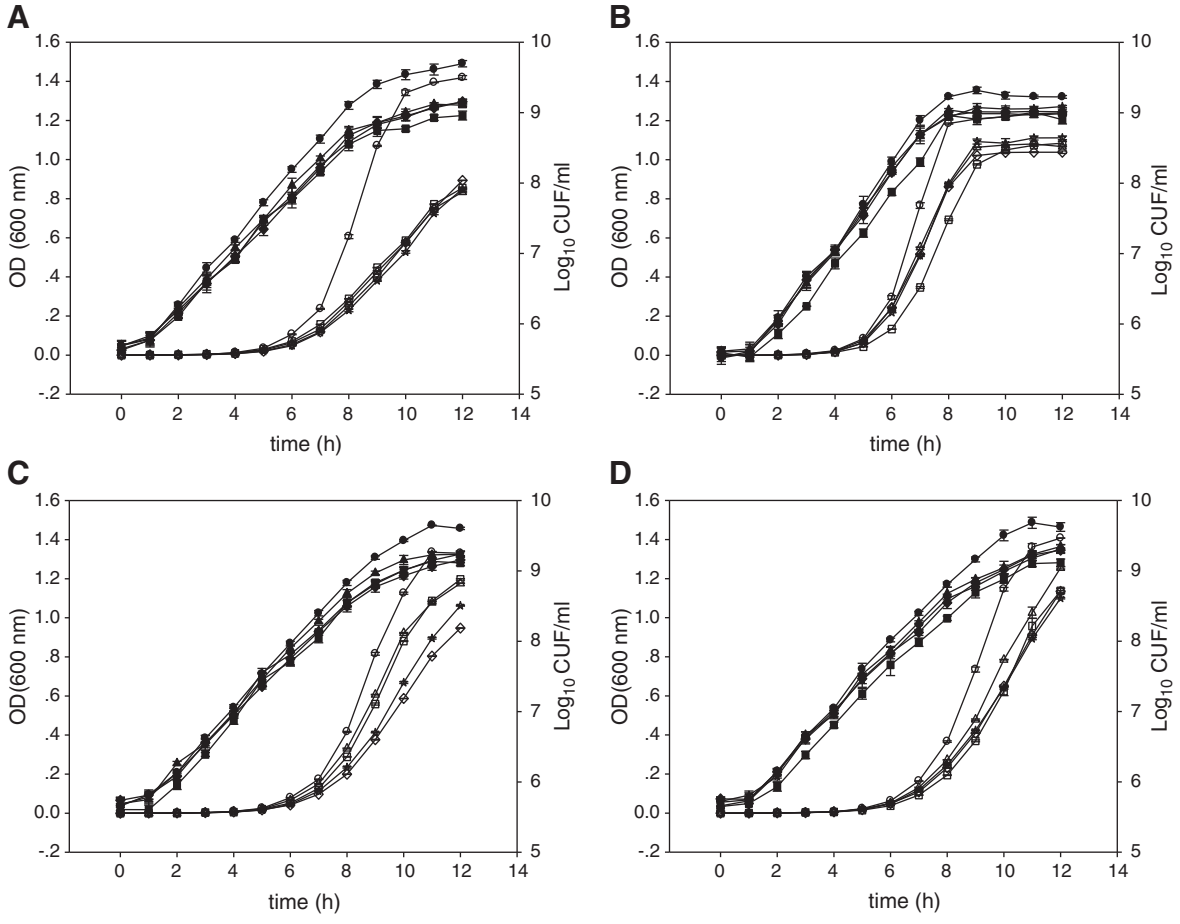

Fig. 1. Comparison of growth curves for *Listeria monocytogenes* strains in the various media used for this study. Open symbols are OD_{600} while filled symbols are plate count numbers. Symbols: circle, TSB at pH 7.4; square, TSB w/o dextrose pH 6.0; triangle TSB w/o dextrose pH 6.0 plus 4.75 mM L-lactic acid; diamond, TSB pH 6.0 plus 4.75 mM levulinic acid; star TSB pH 6.0 plus 4.75 mM acetic acid. Panel A: strain FSL R2-499, serotype 1/2a; B: strain FLS C1-056, serotype1/2a; C: strain FLS N1-227, serotype 4b; and D: strain FLS N3-013, serotype 4b. Error bars indicated the standard error of the mean for duplications.