Survival of Thirteen *Listeria monocytogenes* Strains in a Dynamic Model of the Stomach and Small Intestine

Colorado State University

Ioanna M. Barmpalia-Davis, Ifigenia Geornaras, Patricia A. Kendall*, and John N. Sofos

Center for Meat Safety & Quality, Department of Animal Sciences, Colorado State University, Fort Collins, CO 80523, USA

ABSTRACT

Being able to survive passage through the human gastrointestinal tract may be linked to the ability of *Listeria monocytogenes* to establish infection. We examined differences in survival among thirteen *L. monocytogenes* strains (including 10403S and its Δ*sigB* mutant, A1-254), representing different serotypes (1/2, 1/2a, 4a, 4b) and three genotypic lineages, using a dynamic gastrointestinal model. *L. monocytogenes* counts were determined (in triplicate; PALCAM, tryptic soy agar plus 0.6% yeast extract) during the gastrointestinal challenge (gastric, for 120 min; intestinal, for 240 min; 37ºC) following mixing of 4 or 16-hour cultures (tryptic soy broth without dextrose plus 0.6% yeast extract) with artificial saliva. Parameters included gastric emptying and gastrointestinal fluid secretion rates, gradual gastric acidification (pH reduction to 2.00 within 88 min), and intestinal pH maintenance (6.50±0.30). Inactivation curves in each gastrointestinal compartment were fitted using the Baranyi model. Initial *L. monocytogenes* counts were 7.0-8.0 log CFU/ml (4-hour cultures) or 8.0-9.0 log CFU/ml (16-hour cultures). Strain gastric inactivation rates (log CFU/ml/min) ranged from 0.002±0.001 to 0.262±0.022 and from 0.009±0.001 to 0.221±0.013, for 4 and 16-hour cultures, respectively. Trends in gastric survival were similar between 4 and 16-hour cultures, except for strains N1-227 (4b, lineage 1), A1-254 (1/2a, lineage 2), and J1-158 (4b, lineage 3) that exhibited greater (P < 0.05) acid-sensitivity as 16-h cultures. Differences in gastric survival among strains were observed mainly after 120 min of exposure. Counts reaching the intestine 30 min after digestion were 5.4±0.4 to 8.1±0.3 log CFU/ml (4-hour cultures) and 7.0±0.5 to 9.1±0.1 log CFU/ml (16-hour cultures). Intestinal inactivation rates (log CFU/ml/min) were 0.004±0.005 to 0.016±0.004 (4-hour cultures) and 0.000±0.003 to 0.021±0.013 (16-hour cultures). Although strains C1-056 and Scott A displayed the highest acid-sensitivity, levels recovered from the intestine were >5 log CFU/ml, even after the 240-min challenge. No serotype or lineage-related trends were identified (P≥0.05) in this study. Results suggest that *L. monocytogenes* levels on a food may affect cell numbers in the gastrointestinal tract more than differences in acid-resistance among strains.

OBJECTIVE

The objective of this study was to examine differences in gastrointestinal (GI) survival among 13 *L. monocytogenes* strains, representing different serotypes and three genotypic lineages (Wiedmann et al., 1997), using a dynamic model of the human stomach and small intestine.

INTRODUCTION

Listeria monocytogenes, the etiological agent of listeriosis, is a ubiquitous microorganism, frequently isolated from various food products (Farber and Peterkin, 1991). Although the occurrence of foodborne listeriosis is evidently affected by the immune status of the human host (Gellin and Broome, 1989), characteristics of particular pathogenic strains are also thought to be involved in pathogenesis (Ryser, 1999). Indeed, epidemiological data, invasion assays, and food surveys have indicated that different serotypes and genetic groups of the pathogen display great diversity in virulence and environmental distribution (Wiedmann et al., 1997; Gray et al., 2004).

Variations among *L. monocytogenes* strains, relative to their responses under stressful conditions may contribute, to some extent, to the virulence heterogeneity described above. That is because tolerating stressful conditions is essential for bacterial survival within the human digestive tract (Gahan and Hill, 2005). Under normal conditions, the acidic environment of the stomach is one of the most important defense barriers against foodborne infection (Smith, 2003). Subsequently, pathogens that survive in the gastric environment and reach the small intestine in a viable state must withstand the presence of bile and volatile fatty acids, and high osmolarity (Gahan and Hill, 2005). Strain differences have been identified in terms of resistance to stresses encountered within the human host, such as acidity (Lianou et al., 2006), high osmolarity (Faleiro et al., 2003) and bile (Olier et al., 2004). However, to our knowledge, no studies have examined strain-to-strain variation specific to the GI stages of the *L. monocytogenes* infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

- Thirteen strains of *L. monocytogenes* were tested (Table 1).
- Each strain was activated by transferring a loopful of the culture in 10 ml tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 0.6% yeast extract (Acumedia, Baltimore, MD; TSBYE; incubation at 30ºC for 24 h).
- From the resultant culture, 1 ml was transferred in 100 ml TSBYE without dextrose (Difco) and incubated (30ºC) for 4 or 16 h.
- The initial cell density was 6.9 to 7.2 log CFU/ml.

Simulated GI fluids

Artificial saliva (Minekus et al., 1995)

- 6.2 g NaCl, 2.2 g KCl, 0.22 g $CaCl_2$, 1.2 g $NaHCO_3$ added to 1 liter of distilled water.
- Sterilized by autoclaving and cooled to approximately 25ºC before use.

Simulated gastric fluid (Naim et al., 2004)

- 0.4 g/liter glucose, 3.0 g/liter yeast extract, 1.0 g/liter Bacto Peptone (Difco), 4.0 g/liter porcine mucin (Sigma-Aldrich, St. Louis, MO), 0.5 g/liter cysteine, 0.08 g/liter NaCl, 0.4 g/liter $NaHCO_3$, 0.04 g/liter K_2HPO_4, 0.04 g/liter KH_2PO_4, 0.008 g/liter $CaCl_2$-$2H_2O$, 0.008 g/liter $MgSO_4 \cdot 7 H_2O$, 1.0 g/liter xylan (Sigma-Aldrich), 3.0 g/liter soluble starch (Sigma-Aldrich), 2.0 g/liter pectin (Sigma-Aldrich), and 1 ml/liter Tween 80.
- Autoclaved and cooled to approximately 25ºC.
- Addition of 3 g/liter pepsin from porcine stomach mucosa (Sigma-Aldrich) and pH adjustment to 2 (5N HCl) before use.

Artificial intestinal fluid (Koo et al., 2001)

- 0.1 g/liter trypsin from porcine pancreas (type IX-S; Sigma-Aldrich) and 3.5 g/liter pancreatin from porcine pancreas (Sigma-Aldrich).
- Filter-sterilized (0.45-μm pore-diameter cellulose filter; Millipore Corp., Bedford, MA).

Biliary secretions (Minekus et al., 1995)

- Bile (porcine bile extract, Sigma-Aldrich) solutions (2% and 4%) prepared in distilled water and filter-sterilized before use.

GI challenge

- Cultures (4- or 16-h) of individual strains were diluted (1:1, vol/vol) with artificial saliva.
- During the challenge, the pH of the GC was adjusted manually with 5N HCl (Dressman et al., 1990) to reproduce *in vivo* human gastric pH values: pH 5 at 10 min, pH 4 at 28 min, pH 3 at 58 min, and pH 2 at 88 min.
- The intestinal pH was maintained (0.3 M $NaHCO_3$) at 6.5±0.3 (Minekus et al., 1995).

Microbiological analyses

- Populations of *L. monocytogenes* were assessed before mixing the culture with saliva (0 min) and during the GI challenge (15, 30, 60, 90, and 120 min in the GC and 30, 60, 90, 120, and 240 min in the IC) by plating on tryptic soy agar (Difco) supplemented with 0.6% yeast extract (TSAYE) and PALCAM agar (Difco).
- All plates were incubated at 30ºC for 48 h.

Statistical analyses

- The GI challenge was conducted three times for each 4-h or 16-h culture.
- Cell counts were divided by dilution factors (DF; Koo et al., 2001):

$$\text{DF (GC)} = \frac{\text{Remaining sample (ml)}}{\text{Remaining sample (ml)} + \text{HCl (ml)} + \text{Gastric fluid (ml)}}$$

$$\text{DF (IC)} = \frac{\text{Remaining sample (ml)}}{\text{Total IC contents (ml)} + \text{Gastric contents (ml)} + NaHCO_3\text{ (ml)} + \text{Bile (ml)} + \text{Intestinal fluid (ml)}}$$

- Numbers obtained were converted into log CFU/ml and analyzed using the Glimmix Procedure of SAS version 9.1 (SAS Institute, Cary, NC).
- *L. monocytogenes* counts were used to determine inactivation rates (IR) for each strain in each GI compartment with the model of Baranyi and Roberts (1994) and the DMFit program (Institute of Food Research, Norwich, UK).

RESULTS

pH of cultures

- Initial (before mixing with saliva) pH values: 6.97±0.10 to 7.29±0.21 (4-h cultures) and 6.46±0.10 to 6.65±0.03 (16-h cultures).
- Artificial saliva did not cause considerable changes (<0.2 units) in pH of cultures.

Gastric survival

- Microbial counts on PALCAM agar and TSAYE were similar (< 0.3 log CFU/ml difference), throughout the gastric challenge.
- *L. monocytogenes* strains reached 6.9 to 8.0 log CFU/ml and 8.6 to 9.1 log CFU/ml within 4 and 16 h of incubation (30ºC), respectively.
- The Δ*sigB* mutant, A1-244, exhibited no apparent changes in populations during the 4-h incubation (0 min counts: 6.9 log CFU/ml), while the parental strain, 10403S, reached 7.7 log CFU/ml within 4 h, suggesting that disruption of the *sigB* gene affected growth at 30ºC.
- For most strains, significant (P < 0.05) reductions in initial populations were observed mainly at 120 min of the gastric challenge (exposure at pH 2 for 30 min).
- Reductions in populations after the 120-min challenge ranged from 0.4 (strain R2-501) to 7.6 log (strain C1-056) log CFU/ml (4-h cultures) and from 1.3 (strain R2-500) to 8.0 (strain C1-056) log CFU/ml (16-h cultures).
- Gastric IR (log CFU/ml/min) of *L. monocytogenes* 4-h and 16-h cultures varied from 0.002±0.003 (strains R2-500 and 10403S) to 0.262±0.022 (strain C1-056) and from 0.009±0.001 (strain R2-500) to 0.221±0.013 (strain C1-056), respectively.
- Strain C1-056 had the highest IR among strains, followed by another clinical isolate, Scott A, that had an IR of 0.209 and 0.197 log CFU/ml/min as a 4 and 16-h culture, respectively.
- A valid comparison between the gastric resistance of 4-h cultures of the Δ*sigB* mutant strain, A1-254, and that of the wild-type strain, 10403S, may not be feasible, since, as already mentioned, the slow growth of the mutant strain prevented it from being in the same physiological state (i.e., exponential phase) as the other *L. monocytogenes* strains.
- As a 16-h culture, the Δ*sigB* mutant strain was more (P < 0.05) acid-sensitive than the parental strain 10403S (IR: 0.048 log CFU/ml/min), as it displayed an IR of 0.188 log CFU/ml/min.
- No significant (P ≥ 0.05) differences were observed between IR of food and human isolates linked to the same outbreak/sporadic case (Fugett et al., 2006).

A, B: 500-ml Erlenmeyer flasks representing the gastric (GC) and intestinal compartment (IC), respectively (Koo et al., 2001); C, D: pH meters (Ultra Basic, Denver Instrument, Arvada, CO) used for monitoring the pH in the GC and IC; E, F, G peristaltic pumps (Variable speed Pump Low Flow, Fisher Scientific) used for delivering gastric fluid (flow rate: 0.33 ml/min) in the GC and intestinal fluid (flow rate: 0.33 ml/min) and 2 or 4% bile (flow rate: 0.5 ml/min) in the IC; H: peristaltic pump (205U, Watson-Marlow Limited, Cornwall, England) used for transferring (flow rate: 1.1 ml/min) the gastric contents in the IC; I: shaking water bath (Shaking Water bath 50, Precision Scientific, Chicago, IL) stabilized at 37ºC.

Schematic diagram of the dynamic GI model

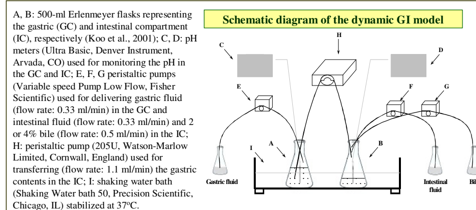


Table 1. *Listeria monocytogenes* strains used in this study

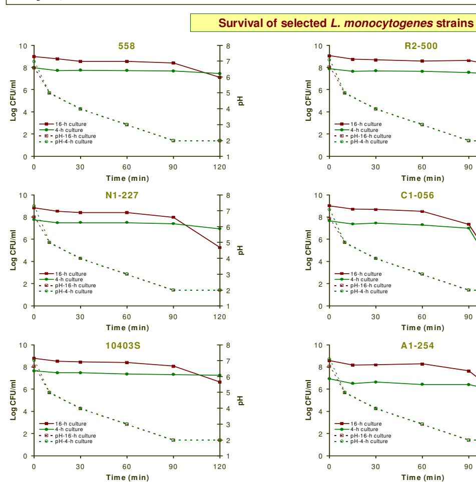
Strain	Serotype	Lineage	Origin
558	1/2	NK	Pork meat
R2-500[1]	4b	1	Food, epidemic, North Carolina (2000)
R2-501[1]	4b	1	Human, epidemic, North Carolina (2000)
Scott A	4b	1	Human
N1-225[1]	4b	1	Human, epidemic US (1998-99)
N1-227[1]	4b	1	Food, epidemic US (1998-99)
C1-056[1]	1/2a	2	Human, sporadic case
N3-031[1]	1/2a	2	Food, sporadic case (1989)
J1-101[1]	1/2a	2	Human, sporadic case (1989)
10403S[2]	1/2a	2	NK
A1-254[2]	Δ*sigB* mutant of 10403S (Wiedmann et al. (1998)		
J1-158[1]	4b	3	Goat
J1-168[1]	4a	3	Human, sporadic case

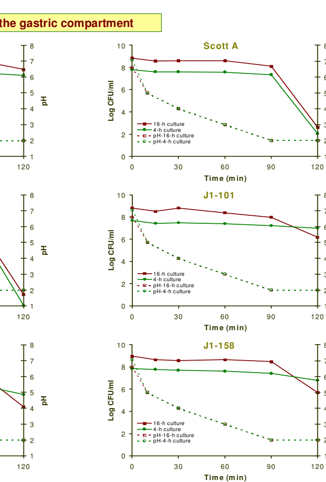
NK: not known

Strains were kindly provided by [1]Dr. Martin Wiedmann (Fugett et al., 2006) and [2]Dr. Kathryn J. Boor (Department of Food Science, Cornell University, Ithaca NY)

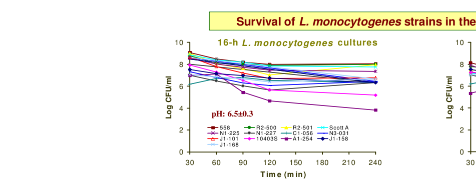
Serotype and lineage designation is as provided by donor (except for 558 and Scott A)

Survival of selected *L. monocytogenes* strains in the gastric compartment

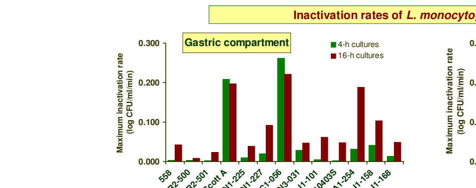




Survival of *L. monocytogenes* strains in the intestinal compartment



Inactivation rates of *L. monocytogenes* strains



ACKNOWLEDGEMENTS

The study was supported by the National Integrated Food Safety Initiative of the United States Department of Agriculture Cooperative State Research, Education and Extension Service (agreements 2004-51110-02160 and 2005-51110-03278), and by the Colorado State University Agricultural Experiment Station.

*Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, CO 80523

Intestinal survival

- Transfer of gastric contents in the IC began while the gastric pH was still high and, thus, viable populations of the pathogen transferred from the GC to the IC within the first 30 min of gastric emptying were comparable to the initial (0 min) levels of each strain.
- Initial (30 min) *L. monocytogenes* populations in the IC ranged from 5.4 to 8.1 log CFU/ml and 7.0 to 9.1 log CFU/ml, for 4- or 16-h cultures, respectively.
- Subsequent reductions in *L. monocytogenes* strain populations during the intestinal challenge were slight as indicated by calculated IR (log CFU/ml/min) that varied from 0.004 (strains R2-501 and C1-056) to 0.016 (strain A1-254), for 4-h cultures, and from 0.000 (strain R2-500) to 0.021 (strain A1-254), for 16-h cultures.
- The estimated IR of the Δ*sigB* mutant strain, A1-254, (4 or 16-h culture) was slightly higher (P ≥ 0.05) than the corresponding IR of the wild-type strain (10403S), suggesting that the contribution of *sigB* in the intestinal survival of this strain was probably small.

CONCLUSIONS

- Under the conditions of this study, no clear serotype or lineage-related trends in GI survival were identified (P ≥ 0.05).
- The gastric resistance of 4-h cultures was generally higher than that of 16-h cultures, suggesting that either *L. monocytogenes* cells were still at exponential phase after 16 h of incubation or that after 4 h they maintained, to some degree, the high acid-resistance of the stationary phase inoculum used to initiate the culture.
- Considerable strain-to-strain variation was observed in terms of gastric survival; however, numbers of pathogenic cells reaching the IC in a viable state during the first stages of the GI challenge were similar for most strains, considering that gastric emptying started when gastric pH was still high.
- Thus, the increased survival of acid-sensitive *L. monocytogenes* clinical isolates C1-056 and Scott A during the initial stages of the gastric challenge may help explain their implication in human illness, particularly if high contamination levels were involved.
- Overall, all *L. monocytogenes* strains survived the *in vitro* GI passage, highlighting the importance of simulating the dynamics of gastric emptying and gradual acidification in studies designed to investigate gastrointestinal aspects of the *L. monocytogenes* infection.

REFERENCES

- Baranyi, J., and T. A. Roberts. 1994. Int. J. Food Microbiol. 23: 277-294
- Dressman, J. B., R. R. Berardi, L. C. Dermentzoglou, T. L. Russell, S. P. Schmaltz, J. L. Barnett, and K. M. Jarvenpaa. 1990. Pharm. Res. 7: 756-761
- Faleiro, M. L., P. W. Andrew, and D. Power. 2003. Int. J. Food Microbiol. 84: 207-216
- Farber, J. M., and P. I. Peterkin. 1991. Microbiol. Rev. 55:476-511
- Fugett, E., E. Fortes, C. Nnoka, and M. Wiedmann. 2006. J. Food Prot. 69: 2929-2938
- Gahan, C. G. M., and C. Hill. 2005. J. Appl. Microbiol. 98: 1345-1353
- Gellin, B. G., and C. V. Broome. 1989. JAMA 261: 1313-1320
- Gray, M. J., R. N. Zadoks, E. D. Fortes, B. Dogan, S. Cai, Y. Chen, V. N. Scott, D. E. Gombas, K. J. Boor, and M. Wiedmann. 2004. Appl. Environ. Microbiol. 70: 5833-5841
- Koo, J., D.L. Marshall, and A. DePaola. 2001. Appl. Environ. Microbiol. 67: 2895-2902
- Lianou, A., J. D. Stopforth, Y. Yoon, M. Wiedmann, and J. N. Sofos. 2006. J. Food Prot. 69: 2640-2647
- Minekus, M., P. Marteau, R. Havenaar, J.H.J. Huis In't Veld. 1995. Altern. Lab. Anim. 23: 254-258
- Naim, F., S. Messier, L. Saucier, and G. Piette. 2004. Appl. Environ. Microbiol. 70: 6637–6642
- Olier, M., S. Rousseaux, P. Piveteau, J.-P. Lemaitre, A. Rousset, and J. Guzzo. 2004. Int. J. Food Microbiol. 93: 87-99
- Ryser, E. T. 1999. Dairy, Food Environ. San. 19: 828
- Smith, J.L. 2003. J. Food Prot. 66: 1296-1303
- Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor. 1998. J. Bacteriol. 180: 3650-3656
- Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. A. Batt. 1997. Infect. Immun. 65: 2707-2716