# LACTIC ACID SENSITIZATION OF *SALMONELLA* TYPHIMURIUM DT104 AND *LISTERIA MONOCYTOGENES* IN NONACID (WATER) MEAT DECONTAMINATION WASTE FLUIDS

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## Background

Meatborne pathogens, such as *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes*, are neutrophiles in nature (4), including environments associated with live animals and fresh meat (1, 3). However, as an adaptive response to continuous acid stress during their life cycle, these pathogens may develop mutants of increased permanent acid resistance, cross-protection and virulence (4, 5). Thus, avoiding acid exposure of pathogens during processing of meat may be advantageous for food safety. Meat decontamination technologies have been shown to assist packing plants in passing the regulatory criteria in the U.S. (1, 3, 9) by reducing bacterial contamination of carcasses by 1 to 3 logs (8, 9). However, acid-based interventions may also increase the risk of development of acid-protected strains among those that may survive on the treated meat (2) or in the acidic waste fluids (6). Recently, we showed that *E. coli* O157:H7, the most acid-resistant among meat pathogens (5), may survive for at least 2 weeks in acidic washings while maintaining a high acid tolerance response (ATR) at 10°C (6). However, the pathogen was dramatically sensitized to acid when inoculated in nonacid washings also stored at 10°C (7), suggesting that meat decontamination can be monitored to avoid acid adaptation. In accordance, water-based meat decontamination technologies may also lead to acid sensitization of *S.* Typhimurium DT104 and *L. monocytogenes* after a period of habituation (growth) in nonacid meat environments.

## Objectives

The objective of this study was to assess acid tolerance response (ATR) of *S.* Typhimurium DT104 and *L. monocytogenes* following habituation/growth in nonacid (water) spray-washings from fresh meat incubated at 10°C. ATR of survivors was compared with that of pure cultures of the pathogens grown in broth with 1% (acid-adapted) or without (nonadapted) glucose at 10°C.

## Methods

A streptomycin (Sm)-resistant (800 µg/ml) derivative of *L. monocytogenes* N-7144 and the multi-drug resistant *Salmonella* Typhimurium DT104 strain ATCC 700408, also grown in 800 µg/ml Sm, were used. Strains were cultured twice in 10 ml of trypticase soy broth (BBL, Becton Dickinson Co., Sparks, MD) with 0.6% yeast extract (Difco, Becton) (TSBYE) at 30°C for 24 h prior to inoculating meat decontamination waste fluids (washings). Washings were prepared by spraying fresh (≤ 72 h post-mortem top round cuts (*ca.* 2 kg each) of beef with 2 l of cold (10°C) or hot (85°C) tap water. Spraying was accomplished with a model spray-washer (CHAD Company, Olathe, KS), as described previously (6). Portions (100 ml) of water (10°C or 85°C) washings were inoculated with approximately $10^5$ cells/ml of each of the strains and incubated statically at 10°C for 14 days. Flasks with 100 ml of sterile glucose-free tryptic soy broth (TSBYE-G; BBL) or 100 ml of this medium with 1% added glucose (Sigma, St. Louis, MO) (TSBYE+G) were also inoculated and incubated as above. This was done to compare growth at 10°C and subsequent ATR of the pathogens when in co-culture with natural flora in meat washings as compared to their pure cultures in broth. All samples were analyzed microbiologically and for pH at 0, 2, 4, 7 and 14 days. Samples (1 ml) were serially diluted in 9 ml of 0.1% buffered peptone water (BPW), and plated on tryptic soy agar (BBL) with 0.6% yeast extract and 800 µg/ml Sm (Sigma) (TSAYE+Sm) and PALCAM (for *L. monocytogenes*) or XLT4 (for *S.* Typhimurium DT104) agars (Difco). Colonies were counted after incubation at 30°C for 48 h. The pH was measured with a digital pH meter (Accumet 50, Fisher Scientific, Houston, TX) equipped with a glass electrode. Acid tolerance was assessed after 2 and 8 days of incubation at 10°C by exposure to TSBYE or new water washings acidified to pH 3.7 or 3.5, respectively, with lactic acid (DL-lactic acid, 85% wt/wt, Sigma). TSBYE (pH 7.2) or meat washings (pH 6.8) without pH adjustment were used as controls. One ml of culture diluted as appropriate in 0.1% BPW was pipetted into 9 ml of acidified media to give a concentration of *ca.* $10^5$ cells/ml. Samples (1 ml) were taken at 0, 60 and 120 min, serially diluted, and plated to determine surviving populations of the natural flora (TSAYE), *L. monocytogenes* (TSAYE+Sm; PALCAM) and *S.* Typhimurium (TSAYE+Sm; XLT4), respectively. Colonies on agar plates were enumerated after incubation at 30°C for 48 to 72 h. Experiments were performed twice.

## Results

Both *L. monocytogenes* and *S.* Typhimurium DT104 grew by 1 to 2 log cfu/ml in water (10 or 85°C) meat washings at 10°C (Table 1). Growth of the pathogens in TSBYE was much faster and greater compared to their growth in the washings. This behavior appeared to be due to the lower nutrient content of the washings and the competition by a gram-negative, mainly (>90% of colonies) oxidase-positive natural flora, which rapidly outgrew (>$10^8$ cfu/ml) the pathogens (data not shown). Under all conditions at 10°C, the growth of the pathogens on PALCAM or XLT4 (data not shown) was slightly (< 0.5 log cfu/ml) less pronounced than growth on TSAYE+Sm (Table 1).

The average pH of the meat washings increased from 6.71-6.85 at day-0 to 7.09-7.51 at day-14, an increase attributed to the accumulation of alkaline catabolic products by the predominant natural flora. As it was expected, the pH of pure TSBYE-G cultures of the pathogens during incubation at 10°C remained high (pH 6.79-7.01 by day-14), while major decreases in pH occurred in the corresponding TSBYE+G cultures due to the fermentation of glucose. The final pH of TSBYE+G cultures of *L. monocytogenes* after 14 days at 10°C was 5.04, while the pH of TSBYE+G cultures of *S.* Typhimurium increased from day-7 (pH 5.47) to day-14 (pH 5.98).

The ATR of *L. monocytogenes* and *S.* Typhimurium DT104 following growth in broth with 1% or without glucose or in water meat washings at 10°C was simultaneously pathogen-, medium- and incubation-time-dependent. At 2 days, reductions in viable populations of broth cultures of *L. monocytogenes* after a 120-min exposure at pH 3.7 in TSBYE were greater than the respective reductions in populations from washings, irrespective of glucose (Table 2). This could be due to a greater sensitivity of the exponential-phase cells in broth compared to the less rapidly growing cells in the washings. At 8 days, however, this response was reversed; pathogen populations from the washings became far more acid-sensitive compared to populations from broth cultures, especially the acid-adapted ones which showed the lowest reductions. Under all conditions tested, lower numbers of survivors were recovered on PALCAM compared to TSAYE+Sm (Table 2) due to acid injury. Interestingly, the ATR of *L. monocytogenes* could not be verified against acidified (pH 3.5) meat washings due to irreversible attachment of cells on meat particles in all treatments, including the controls, which resulted in unreliable microbiological data (data not shown).

*Salmonella* Typhimurium DT104 was found to be more sensitive to acid than *L. monocytogenes* after exposure to meat washings at 10°C. No pathogen survivors from meat washings were recovered on TSAYE+Sm following exposure to acidified (pH 3.5) washings for 60 min, and irrespective of time, 2 (data not shown) or 8 days (Table 3). Also, no survivors of *Salmonella* were detected in broth cultures at day-2, probably because of the high acid sensitivity of the pathogen when in its exponential phase (data not shown). At 8 days, however, survival of acid-adapted *Salmonella* in broth was high, while nonadapted populations became undetectable (< 1 log CFU/ml), but at a slower rate compared to populations from the meat washings (Table 3). Notably, no survivors of acid-challenged *Salmonella* were detected on XLT4, irrespective of type of culture, previous acid adaptation or incubation time (data not shown), indicating that conditions on this medium were harsh to allow for recovery of acid-injured populations. Nonacid-treated *Salmonella* were fully recovered on all treatments and media, including XLT4.

## Conclusions

• *Listeria monocytogenes* and *S.* Typhimurium DT104 may grow similarly in nonacid (water) waste fluids from meat decontamination at 10°C. Their growth may be restricted due to nutrient limitation and competition by the natural flora, but cell injury is limited.

• Stationary-phase cells of *L. monocytogenes* and *S.* Typhimurium DT104 transferred to water meat washings at 10°C and habituated to this natural environment for extended times may become more acid-sensitive compared to their pure cultures grown at the same temperature in broth. The presence of 1% glucose in broth increases ATR of the pathogens due to induction of acid adaptation at 10°C.

• The potential for *L. monocytogenes* and *S.* Typhimurium DT104 to reduce their ATR following survival/growth in nonacid (water) washings may be a means of lessening potential safety risks associated with acid-adapted pathogens in meat plants that use acidic meat decontamination technologies. The more intense use of water-based interventions in rotation with lactic acid treatments may assist in optimizing pathogen reduction due to acid on carcasses or primal cuts of meat, while minimizing the chances for survivors to develop or maintain high ATR.

## References

1. Bacon, R.T., Belk, K.E., Sofos, J.N., Clayton, R.P., Reagan, G.O., Smith, G.C. (2000) J. Food Prot. 63:1080-1086.
2. Berry, E.D., C.N. Cutter (2000) Appl. Environ. Microbiol. 66:1493-1498.
3. Elder, R.O., Keen, J.E., Siragusa, G.R., et al. (2000) Proc. Natl. Acad. Sci. USA 97:2999-3003.
4. Gahan, C.G.M., Hill, C. (1999) Int. J. Food Microbiol. 50:93-100.
5. Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N., Foster, J.W. (1996) Appl. Environ. Microbiol. 62:3094-3100.
6. Samelis, J., Sofos, J.N., Kendall P.A., Smith, G.C. (2002) J. Food. Prot. 65:33-40.
7. Samelis, J., Sofos, J.N., Ikeda, J.S., Kendall P.A., Smith, G.C. (2002) Lett. Appl. Microbiol. 34:7-12.
8. Smulders, F.J.M., Greer, G.G. (1998) Int. J. Food Microbiol. 44:149-169.
9. Sofos, J.N., Smith, G.C. (1998) Int. J. Food Microbiol. 44:171-188.

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**Table 1.** Growth* (log cfu/ml, n = 4) of inoculated ($10^5$ cfu/ml) pathogens in nonacid spray-washings from meat sprayed with cold (10°C) or hot (85°C) water, or in culture broth with 1% (TSBYE+G) or without (TSBYE-G) glucose, at 10°C

| Type of culture | *Listeria monocytogenes* (days at 10°C) | | | | | *Salmonella* Typhimurium DT104 (days at 10°C) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 7 | 14 | 0 | 2 | 4 | 7 | 14 |
| Water (10°C) washings | 5.5 | 5.6 | 6.7 | 6.8 | 6.5 | 5.1 | 5.7 | 6.3 | 6.7 | 7.1 |
| Water (85°C) washings | 5.5 | 5.8 | 6.7 | 7.0 | 6.9 | 5.0 | 5.6 | 6.4 | 6.6 | 7.0 |
| TSBYE+G | 5.1 | 8.2 | 9.4 | 9.4 | 9.0 | 5.1 | 7.8 | 9.1 | 9.1 | 8.9 |
| TSBYE-G | 5.1 | 8.0 | 9.0 | 9.1 | 8.8 | 5.2 | 8.0 | 9.1 | 9.3 | 9.2 |

Values are the means of two replicate experiments with two samples analyzed per replicate
*Growth was determined on TSAYE agar supplemented with 800 mg/l streptomycin (TSAYE+Sm).

**Table 2.** Reductions (log N/No)* in populations of *L. monocytogenes* in acidified TSBYE (pH 3.7/lactic acid) following habituation/growth for 2 or 8 days at 10°C in nonacid spray-washings from meat sprayed with cold (10°C) or hot (85°C) water, or in culture broth with 1% (TSBYE+G) or without (TSBYE-G) glucose.

| Type of culture | Cells exposed to acid after 2 days at 10°C | | | | Cells exposed to acid after 8 days at 10°C | | | |
|---|---|---|---|---|---|---|---|---|
| | TSAYE+Sm | | PALCAM | | TSAYE+Sm | | PALCAM | |
| | 60 | 120 | 60 | 120 | 60 | 120 | 60 | 120 |
| Water (10°C) washings | -2.6 | -3.7 | -3.5 | -3.8 | -1.6 | -3.1 | -2.4 | -4.9 |
| Water (85°C) washings | -2.6 | -3.7 | -3.7 | -3.9 | -0.9 | -2.5 | -2.1 | -4.3 |
| TSBYE+G | -4.6 | -4.9 | -4.9 | X | -0.3 | -0.5 | -0.6 | -1.1 |
| TSBYE-G | -4.9 | X | -4.9 | X | -0.3 | -1.7 | -0.3 | -2.9 |

*N, surviving populations (cfu/ml) after 60 or 120 min of exposure to acid; No, populations (cfu/ml) exposed to acid at time 0.
X, no further reductions at 120 min because surviving populations were undetectable (<1 log cfu/ml) after 60 min of exposure.

**Table 3.** Reductions (log N/No)* in populations of *S.* Typhimurium DT104 in acidified meat washings (pH 3.5/lactic acid) following habituation/growth for 8 days at 10°C in nonacid spray-washings from meat sprayed with cold (10°C) or hot (85°C) water, or in culture broth with 1% (TSBYE+G) or without (TSBYE-G) glucose.

| Exposure to acid (min) | Water (10°C) washings | Water (85°C) washings | TSBYE+G | TSBYE-G |
|---|---|---|---|---|
| 60 | -4.6 | -4.7 | -0.7 | -1.8 |
| 120 | X | X | -1.2 | -4.2 |

*Reductions were calculated on the basis of surviving populations of *Salmonella* on TSAYE+Sm. For symbols see Table 2.