Possibility, duration, and molecular predictors of sanitizer tolerance in *Listeria monocytogenes*

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Summary

We obtained a comprehensive set of 360 *L. monocytogenes* isolates for sanitizer tolerance screening. These isolates represent diverse produce origins (22 different commodities), produce-related environments (packinghouse and retailer), known sanitizer resistance determinants, and major phylogenetic lineages (three lineages and five serotypes) of the pathogen. These isolates had been collected from 27 US states and five other countries from 1994 to 2019, and included 18 isolates linked to six produce commodities from five outbreaks. Genomes of all these isolates have been sequenced and assembled. Using a subset of isolates (n=7) that cover a wide spectrum of benzalkonium chloride (BC) tolerance, we developed a high-throughput method for minimum inhibitory concentration (MIC) measurement. The strain collection and the method will be used to identify genetic markers for sanitizer tolerance prediction.

Benefits to the Industry

Further investigation built upon our work so far will help fill critical knowledge gaps regarding *L. monocytogenes* tolerance to chlorine and quaternary ammonium compounds. Our results will provide valuable prerequisite information for determining if sanitizer rotation is necessary for preventing the development of Lm tolerance to sanitizers. Our results will provide the industry with a WGS-aided tool for evaluation and risk assessment of sanitizer tolerance in *L. monocytogenes* as well as scientific evidence for substantiating, better implementing, or justifiably shelving sanitizer rotational programs.

Objectives

1. Survey of residual sanitizer concentrations in selected locations in two different produce processing facilities between sanitation shifts.
2. Measurement of intrinsic tolerance to sodium hypochlorite and benzalkonium chloride in 200–300 strategically selected *Listeria monocytogenes* (Lm) strains.
3. Evaluation of how different levels of sanitizers and lengths of sanitizer exposure affect the degree and duration of acquired sanitizer tolerance in selected Lm strains.
4. Characterization of transcriptomic shifts that accompany the waning of acquired sanitizer tolerance.
5. Whole genome sequencing (WGS) analyses of Lm to (1) develop machine-learning classifiers for intrinsic sanitizer tolerance prediction, and (2) search for evolutionary evidence for intrinsic tolerance development.

For the first term of the project, aims included: i) collect a comprehensive set of produce-related isolates to investigate sanitizer tolerance in *L. monocytogenes*, ii) assemble genomes of all the isolates; and iii) develop a high-throughput assay for measuring sanitizer MIC.

Methods

Seven out of 360 isolates were selected for the high-throughput method development, together with strains CFSAN028738 and CFSAN080788 used as benzalkonium chloride (BC)-resistant (BC^r) and BC-susceptible (BC^s) controls, respectively. Bacteria were grown in BHI broth and isolated on TSAYE. A single colony was either suspended in Mueller-Hinton (MH) broth or restreaked on MH agar with 5% sheep blood, which were incubated at either 30 or 37°C for 48, 72, and 168 h. Either 200 µL liquid culture or two colonies from MH agar suspended in 200 µL MH broth were transferred into a 48-well plate. Cultures were then transferred onto MH agar containing various concentrations of BC and 2% sheep blood by using a 48-pin replicator (**Figure 1**); plates were incubated at 30°C for 3 days.

Results to Date

A total of 360 *Listeria monocytogenes* isolates were obtained from FDA and Purdue University. Among these isolates, 127 were sampled from 22 produce commodities, 232 were collected from packinghouses and retailers, and one was isolated from a clinical sample. Phylogenetic analysis using WGS data shows that all three major lineages are represented by these isolates and 46% of them harbor antimicrobial resistance genes (**Figure 2**).

Preliminary results of the high-throughput assay show:

- *L. monocytogenes* cells grown on agar display more consistent tolerance to BC than those cultured in broth (**Figure 3A**)
- Temperature (30°or 37°C) does not affect the BC tolerance (**Figure 3B**)
- Cells grown on agar for 72 and 168 h (long-term-survival phase) show higher and more consistent BC tolerance (**Figure 3C**)

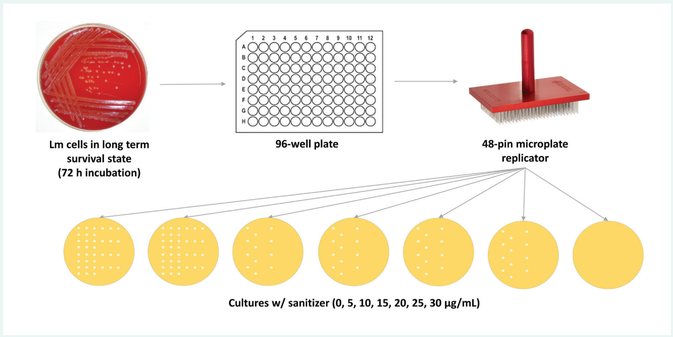

Figure 1. Sanitizer tolerance assay

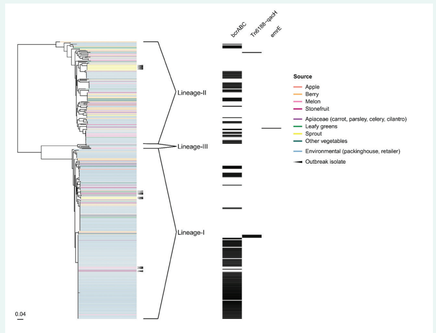

Figure 2. Phylogeny of the 360 *L. monocytogenes* isolates

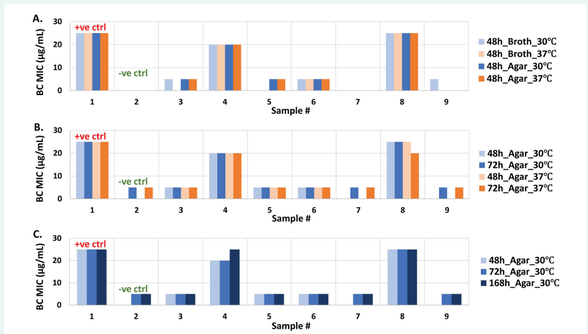

Figure 3. Minimum inhibitory concentration (MIC) of benzalkonium chloride (BC) for a subset of *L. monocytogenes* isolates (sample #3–9) incubated: (**A**) in MH broth or on MH blood agar at 30 and 37°C for 48 h; (**B**) on MH blood agar at 30 and 37°C for 48 h; (**C**) on MH blood agar at 30°C for 48, 72, and 168 h. Sample #1 was BC-resistant (BC^r) control and sample #2 was BC-susceptible (BC^s) control.