CENTER for PRODUCE SAFETY

CPS 2019 RFP
FINAL PROJECT REPORT

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

Project Period
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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.*

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FINAL REPORT

Abstract
For decades, quaternary ammonium compounds-based sanitizers have been broadly used in food processing environments to control foodborne pathogens such as *Listeria monocytogenes*. Still, there is a lack of consensus on the likelihood and implication of reduced *Listeria* susceptibility to benzalkonium chloride (BC) that may emerge due to sublethal exposure to the sanitizers in food processing environments.

In this project, we attempted to fill multiple data gaps toward an integrative risk assessment of reduced BC susceptibility in *L. monocytogenes* during fresh produce processing. We substantiated the correlation between tolerance phenotypes and known genetic determinants of BC tolerance to facilitate the determination and prediction of tolerance and susceptible strains. We assessed BC selection on *L. monocytogenes* through a large-scale and source-structured genomic survey of 25,083 publicly available *L. monocytogenes* genomes from diverse sources in the United States. With the consideration of processing environment constraints, we monitored the temporal onset and duration of adaptive BC tolerance in both tolerant and susceptible *L. monocytogenes* isolates. Finally, we examined residual BC concentrations throughout a fresh produce processing facility at different time points during daily operation.

While genomic evidence supports elevated BC selection and the recommendation for sanitizer rotation in the general context of food processing environments, it also suggests a marked variation in the occurrence and potential impact of the selection among different commodities and sectors. For the processing of fresh fruits and vegetables, we conclude that properly sanitized and cleaned facilities are less affected by BC selection and unlikely to provide conditions that are conducive for the emergence of adaptive BC tolerance in *L. monocytogenes.*

Background
In the processing environments of ready-to-eat (RTE) foods, such as fruits and vegetables, *Listeria monocytogenes* is a food safety hazard of heightened concerns. As a soil saprotroph, *L. monocytogenes* is widely distributed in natural environments where contamination of food and other materials often occurs. The tainted materials can lead to reoccurring introduction and occasional persistence of the bacteria in food processing equipment and premises (1, 2). As an intracellular pathogen, *L. monocytogenes* can cause serious infections via contaminated foods, particularly threatening at-risk groups including pregnant women, newborns, elderly people, and individuals with weakened immune systems. In the United States, there have been at least 9 reported *Listeria* outbreaks linked to fresh produce since 2008, with the source of contamination all attributed to the processing or packing environment (3).

Food processing facilities rely on biocides to control *L. monocytogenes* and other harmful microorganisms. Quaternary ammonium compounds (QACs) such as benzalkonium chloride (BC) are among the most commonly used disinfectants or sanitizers in food processing environments. First registered with the US Environmental Protection Agency (EPA) in 1947 (4), BC-containing products have been increasingly applied in a wide range of industrial, agricultural, clinical, and domestic settings (5). Such widespread and prolonged use of BC-based disinfectants inevitably cause sublethal contact with various bacteria and consequent selection for BC tolerance (5). Historical concerns over the decrease in microbial susceptibility to BC can be traced to the 1960s when clinical (6) and laboratory (7) observations of tolerant bacteria were reported. Since then, BC tolerance has been documented in a variety of microorganisms, including common foodborne pathogens such as *Salmonella*, *Escherichia coli* O157, *Campylobacter*, and *L. monocytogenes* (5).

Reduced susceptibility to BC may result from either individual or combined effects of 1) selection for inherently more tolerant bacteria (BC selection), and 2) development of additional tolerance after adaptation to sublethal levels of BC (adaptive BC tolerance).

In the particular example of *L. monocytogenes* in food processing environments, there is still a lack of consensus on the likelihood and implication of reduced BC susceptibility that may develop upon sublethal exposure to the sanitizer. While multiple studies have shown the development of adaptive BC tolerance in *L. monocytogenes* under laboratory conditions (8-10), which can also cause reduced susceptibility to other disinfectants and antibiotics (11-13), the increased minimum inhibitory concentrations (MICs) of adapted *L. monocytogenes* were found still below the commonly applied concentration of a QAC-based disinfectant (18)

Rotational use of different sanitizers has been proposed to prevent the emergence of less susceptible *L. monocytogenes.* Sanitizer rotation was recommended in the 2017 FDA draft guidance on *L. monocytogenes* control in RTE foods (14) and the 2016 FSIS Sanitation Concerns in RTE Processing Environments (15). The recommendations are nonbinding and presumably precautionary. Despite laboratory evidence and government guidelines, the realistic impact of such tolerance on the food industry remains debated. Arguments against the concerns over tolerance selection or adaptive tolerance development have been focused on the real-world likelihood of such adaptive events and the practical efficacy of sanitizers in processing environments (16, 17).

Several factors have contributed to the debate. First, *L. monocytogenes* strains are often classified as either tolerant (resistant) or susceptible (sensitive) to BC. In many surveys, the classification relied on phenotypic assays using isolates from particular sources, creating variations among studies. According to the MICs of 200 isolates from Norway and other European countries related to fish or fish processing environments of Norwegian origin, BC-tolerant strains were defined as those having an MIC between 4 and 7 μg/ml (19). In another survey of a similar scale in France on isolates from smoked fish production, BC-tolerant strains had MICs from 7 to 15 μg/ml (20). However, later discoveries of mobile genetic element-conferred BC tolerance raised the MICs of tolerant strains above 30 μg/ml (21-23). Second, while the development of adaptive BC tolerance has been experimentally observed in *L. monocytogenes*, the observations were mostly derived from adaptation conditions that did not realistically mimic BC exposure scenarios in processing environments. For example, laboratory tolerance was often induced through repetitive subculturing under otherwise optimal growth conditions, including nutrient broth and non-refrigeration temperatures (7-10, 13, 18). Temporal patterns of tolerance development, such as how soon it emerges and how long it lasts, and the possibility of the development under typical environmental constraints during food processing remain poorly investigated. Such information is highly relevant to designing and managing sanitation programs. Finally, as a prerequisite for the emergence of adaptive BC tolerance, the actual levels and prevalence of sanitizer residuals at sublethal levels in processing environments are scarcely surveyed, creating a substantial challenge for risk assessment and sanitation optimization.

In this project, we attempted to address the aforementioned data gaps toward an integrative risk assessment of reduced BC susceptibility in *L. monocytogenes* during produce processing. We substantiated the correlation between tolerance phenotypes and known genetic determinants of BC tolerance to facilitate the determination and prediction of tolerant and susceptible strains. We performed a genomic survey on the prevalence and distribution of BC tolerance among more than 25,000 publicly available *L. monocytogenes* genomes from diverse sources in the United States. With the consideration of processing environment constraints, we explored the upper bounds and monitored the temporal onset and duration of adaptive BC tolerance in both tolerant and susceptible *L. monocytogenes* isolates. Finally, we surveyed residual BC levels throughout a fresh produce processing facility at different time points of daily operation.

Throughout the project and this report, we distinguish inherent BC tolerance as an intrinsic attribute (MIC without sublethal BC exposure) from adaptive BC tolerance as response to sublethal levels of BC (further increase of MIC through sublethal BC exposure). While we focused on BC, we also studied the inherent and adaptive tolerance of *L. monocytogenes* to chlorine as a control sanitizer.

Research Methods and Results

Methods

Isolates and genomes. *L. monocytogenes* isolates from produce or produce-related environments used for BC MIC assay were sampled during a survey in 30 retail produce departments across seven US states (24) or provided by the Center for Food Safety and Applied Nutrition, FDA. All these isolates had been previously sequenced. Strain H7750 and its plasmid-cured derivative H7550-Cd^s were originally from the 1998-1999 multistate outbreak linked to contaminated hot dogs (25). A total of 25,083 publicly available *L. monocytogenes* genomes and associated metadata were downloaded from National Center for Biotechnology Information (NCBI) on January 31, 2022. This set included all the US *L. monocytogenes* genomes deposited at NCBI as of the time of downloading that were sequenced by Illumina platforms (paired end reads) and assembled by the NCBI SKESA assembler (26) to include at least 2.5 Mb in the assembly.

Determination of BC MICs. Overnight cultures of isolates in Brain Heart Infusion broth (BHI) prepared from frozen stocks (-80°C) were transferred to Mueller Hinton Agar (MHA) with 5% defibrinated sheep blood (MHA-B) and incubated at 37°C for 72 h to enter the long-term survival (LTS) phase (27). For each strain, two single colonies of LTS cells were picked and suspended in 200 µl of Mueller Hinton Broth (MHB) (~10^9 CFU/ml), followed by serial dilutions in MHB to make a cell suspension at 10^6 CFU/ml. Suspensions of 48 isolates were transferred to a 48-well plate. From the plate, 10 µl of each suspension was picked by a 48-pin replicator and spotted onto MHA-B plates with 2% sheep blood containing different concentrations of BC (BC= 0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/ml), with duplicate plates for each concentration. After 48 h of incubation at 30°C, MICs were determined as the lowest assessed BC concentrations that completely prevented the growth of *L. monocytogenes* cells. All MICs were measured in three independent trials, each in duplicate. Strains H7550 and H7550-Cd^s (H7550 cured of pLM80) (21) were used as BC resistant (BC^r) and BC susceptible (BC^s) controls for determination of BC MIC.

Adaptation to sublethal concentrations of BC under the growth promoting condition. A third-generation quaternary ammonium compound (QAC) (alkyl distribution from C8H17 to C16H33, Acros Organics, NJ), which is a mixture of alkyl dimethyl benzyl ammonium chloride and alkyl dimethyl ethylbenzyl ammonium chloride, was used to adapt *L. monocytogenes.* Another compound (Decon-Quat 200C, Veltek Associates, PA), classified as a fifth-generation QAC for its synergistic combination of dual QACs, was also used to examine if *L. monocytogenes* responded to it in similar ways as to the third-generation QAC (17). LTS cells of H7550 and H7550-Cd^s were prepared as described above. Ten µl of cell suspension of each strain was plated on MHA with 2% defibrinated sheep blood containing 2.5 µg/ml (for H7550-Cds) or 15 µg/ml BC. The inoculated plates were incubated at 30°C for from 3 h up to 168 h. At each time point (3, 6, 9, 12, 15, 18, 21, 24, 48, 72, and 96 h), one plate was taken out of incubation to measure MIC of the adapted strain. Cells were collected by flushing the plate with 2 ml of MHB along with gentle scrubbing by a spreader. About 1.5 ml of cell suspension was collected and then vortexed, from which 20 µl was taken for the determination of MIC as described above.

After sublethal BC adaptation for 96 h, cells of each adapted strain were harvested as described above. Twenty µl of harvested cells were transferred to MHB without BC and incubated at 30°C for 24 h. The subculturing continued every 24 h for up to 4 days. MIC after each subculturing was determined as described above. Similar to adapting cells to the constant BC level at the half intrinsic MIC, adapting cells to increasing levels of MICs was also performed. Briefly, after 24 h adaptation to BC at a certain concentration, cells were harvested by flooding the plate as described above and 20 µl of harvested cells were transferred to MHA plates containing a higher BC concentration. At the end of adaptation at each BC concentration (2.5, 5, 10, 15, 20, 25, and 30 µg/ml for H7550-Cds; 15, 20, 25, 30, 35, 40, and 45 µg/ml for H7550), MIC was determined as described above.

Adaptation to sublethal concentrations of BC under growth limiting conditions. Adaptation of LTS cells of H7550 and H7550-Cd^{s} under growth limiting conditions was performed similar to the procedure described above, except BC-containing 0.1% buffered peptone water instead of MHA was used for the adaptation at 25°C or 4°C.

Determination of chlorine MIC. LTS cell suspensions in MHB were prepared as described above. An aliquot (100 µl) of the suspension (10^6 CFU/ml) was added to each well in a 96-well plate containing 100 µl of chlorine solution, with total chlorine ranging from 200 to 800 µg/ml, with 100 µg/ml increments. The final concentration of total available chlorine in each well was from 100 to 400 µg/ml with 50 µg/ml increments. Bacterial growth was observed by turbidity changes after incubation at 37°C for 24 h. Wells with *L. monocytogenes* cells but without chlorine and wells with Tryptic Soy Broth (TSB) alone were included as positive and negative controls, respectively. Chlorine MIC was determined as the lowest assessed concentration of total available chlorine that inhibited the visible growth of *L. monocytogenes* after 24 h of incubation at 37°C. Aliquots (100 µl) from wells without visible growth of *L. monocytogenes* were plated onto duplicate Tryptic Soy Broth with 0.6% Yeast Extract (TSBYE) plates to determine the minimum bactericidal concentration (MBC), the concentration of total available chlorine that kills all *L. monocytogenes* cells. Total chlorine concentrations in chlorine solutions were measured with a Kemio Disinfection device (Palintest, CO).

Source categorization of *L. monocytogenes* genomes. For each publicly available genome, the associated description of isolation source was retrieved from NCBI Short Reads Archive (https://www.ncbi.nlm.nih.gov/sra) and parsed by LexMapr (version 0.7.1, https://github.com/cidgoh/LexMapr) according to the Interagency Food Safety Analytics Collaboration (IFSAC) Food Categorization Scheme (https://www.cdc.gov/foodsafety/ifsac/projects/food-categorization-scheme.html). Source categories deduced by LexMapr were further curated and consolidated if needed. Specifically, environmental sources without any specified animal or plant vehicle were categorized as *environmental*. Sources deduced as *multi-ingredient* by LexMapr were merged into a single category of *multi-ingredient food*. Fish, shellfish and other aquatic animals were grouped as *aquatic animals*. All poultry, swine, bovine, dairy and meat sources were classified as *land food animals*. Genomes of unclassified or unknown sources were categorized into *other sources*.

Genomic survey of BC resistance genes. Genome data were indexed with Colorid (https://github.com/hcdenbakker/colorid), an implementation of BItsliced Genomic Signature Index (BIGSI). The presence or absence of BC resistance genes, including *bcrABC*, *qacH*, *qacC*, *qacA*, *emrE*, *emrC*, *mdrL*, and *lde*, was determined in each genome using the Colorid indices. The sequences of *bcrABC, qacH*, *emrE, mdrL*, and *lde* were retrieved from the *Listeria* database of Pasteur Institute. The sequences of *qacC* (NCBI accession: Z37964), *qacA* (NCBI

accession: X56628.1) and *emrC* (NCBI accession: LT732640.1:1575-1961) were retrieved from NCBI.

Statistical analysis. Comparison of *bcrABC* prevalence among source categories of *L. monocytogenes* was performed using Chi-squared tests, and *post-hoc* analysis with *Bonferroni* correction were performed with scipy (version 1.7.1) and statsmodels (0.13.0) Python packages.

Collection of sanitizer residues and measurement of BC concentrations. An approximately 10-15 ml sample of residual sanitizer was taken in duplicate at each sampling area by either transferring liquid with a 15ml transfer pipet (VWR International Radnor, PA) or by wiping the area with a Nasco “Speci-Sponge” (Nasco Madison,WI) until saturated and squeezing the liquid into a 50 ml conical tube. Runoff liquid that dripped from a particular processing equipment onto the floor underneath and near the equipment was sampled immediately after sanitation or cleaning. Liquid accumulation was directly sampled from the floor drain and the bottom shelf of a cart. Samples were transferred to a refrigerator within 1-2 h. Following the manufacturer instructions, residual sanitizer samples were titrated to determine BC concentration using the LaMotte 3043-DR-01 QAC kit (LaMotte Chestertown, MD).

Results

BC MICs in 359 selected fresh produce isolates correlate with known resistance genes. To establish the baselines of intrinsic BC MICs informed by the presence and absence of known BC tolerance genes, we assembled a set of 359 isolates representing diverse produce origins (22 commodities), different produce-related environments (packinghouses and retailers), and major genetic lineages of *L. monocytogenes* (three lineages and five serotypes). These isolates were sampled between 1994 and 2019 in 26 US states, Canada, Chile, Mexico, Peru and Egypt, including 18 isolates linked to six vegetable or fruit commodities from five outbreaks. The genomes of these isolates had been sequenced, with a substantial percentage (44.8%) carrying known BC tolerance genes, including the *bcrABC* gene cassette (n=154) (25), *qacH* (n=6) (23), and *emrE* (n=1) (22). The MICs of BC against these isolates assume a bimodal distribution (**Figure 1**). All the resistance gene-carrying isolates had MICs between 25 and 35 µg/ml, and nearly all the isolates (98.0%) devoid of any of these tolerance genes had an MIC of 5 µg/ml, except a few showing an MIC of 10 µg/ml. The tolerance levels conferred by the resistance genes are similar to those reported in previous studies, including 30-40 µg/ml for *bcrABC* (21) and 30 µg/ml for both *qacH* (23) and *emrE* (22). Therefore, despite the underrepresentation of isolates with *qacH* and *emrE*, MIC surveys from this and other studies that correlated BC MICs to BC genotypes collectively show a clear distinction between intrinsically BC tolerant (BC-tolerant, MIC ≤ 10 µg/ml) and susceptible (BC^s, MIC ≥ 25 µg/ml) strains of *L. monocytogenes*. Since the 359 isolates are phylogenetically diverse, the observed bimodal distribution of BC MICs likely holds for *L. monocytogenes* isolates from non-produce sources. The narrow peaks of the distribution and the strong correlation between tolerance pheno- and genotypes (Figure 1) also indicate that the level of intrinsic BC tolerance is highly predictable in *L. monocytogenes* by tolerance genes, especially in the case of *bcrABC*. This finding made our original plan to develop more sophisticated resistance prediction algorithm (Objective 5) unnecessary, because such prediction can be simply and reliably made by the presence/absence of the tolerance genes.

Uniform chlorine tolerance and no adaptive chlorine tolerance among produce isolates. In contrast with the bimodal distribution of BC MICs, chlorine MICs among the 359 produce isolates assumed a uniform distribution all at the same level of 150 µg/ml. Further

measurements of minimum bactericidal concentration (MBC) showed little variation as well, ranging from 150 to 183 µg/ml. Adaptation of three representative strains (each representing a major lineage) to sublethal concentrations of chlorine (50 µg/ml for 24 h, followed by 75 µg/ml for another 24 h) did not increase the MIC of the adapted cells. Further increasing the chlorine exposure level to 100 µg/ml killed all three strains.

Prevalence of *bcrABC* is associated with sequence type (ST) size and varies among isolate source categories. We assembled a set of 25,083 publicly available *L. monocytogenes* genomes from the US to survey the distribution of known genetic determinants of BC tolerance. The genomes were collected between 1931 and 2021 (70.8% after 2010) and from diverse sources including humans (n= 7,327), environmental (n=10,955), multi-ingredient food (n=2,081), fruits and vegetables (n=1,330), land food animals (n=1,736), aquatic animals (n=302), other animals (n=235), and other sources (n=309). Except for "other sources", all these source categories were deduced from publicly available description of isolation source associated with each genome according to the Interagency Food Safety Analytics Collaboration (IFSAC) Food Categorization Scheme (see Methods and Materials).

Thirty-one percent of these isolates were predicted to be BC tolerant by carrying any of the surveyed tolerance genes, of which *bcrABC* is the predominant resistance determinant, accounting for 93.6% of presumptive resistant isolates (**Table 1**). Compared with *bcrABC*, other resistance genes were rare, with only *qacH* exceeding 1% in prevalence (Table 1).

The presence of *bcrABC* in different lineages and multiple polyphyletic STs indicates independent acquisition of the cassette by *L. monocytogenes*, presumably through horizontal dissemination of the plasmid that harbors *bcrABC*. Similarly, transposon-borne *qacH* is scattered in different lineages and STs (**Figure 2a**), suggesting that horizontal gene transfer plays a role in its phylogenetic distribution.

We noticed an uneven distribution of *bcrABC* among lineages and STs of multilocus sequence typing (MLST) of the Pasteur Institute (**Figure 2**). It was present in 17.9% and 45.2% of isolates in Lineage I and Lineage II, respectively, but absent from other lineages. The largest ST in Lineage I (ST5) and the five most prevalent STs in Lineage II (ST321, ST7, ST155, ST9 and ST199) were all over-represented by *bcrABC*-positive isolates; these six STs account for 78.2% of such isolates in the entire set (Figure 2a). These STs featured isolates from diverse sources including vegetables and fruits, with environmental isolates being the predominant source category in each ST (**Figure 2b**). While environmental isolates were found in 620 STs, these six STs contributed 42.8% of all isolates and 79.4% of *bcrABC*-positive isolates in the environmental category.

We further observed an association between large ST size and high *bcrABC* prevalence. With the entire isolate set, we ranked STs by size (as a proxy measure of ST prevalence) and sampled isolates from larger to smaller STs until roughly half of the set were included. The prevalence of *bcrABC* was significantly higher among isolates from the largest STs than the other half of isolates from smaller STs (**Figure 3a**). The same observation was made in each source category (Figure 3a). Notably, such an association was largely attributable to the six aforementioned large STs in Lineage I (ST5) and Lineage II (ST321, ST7, ST155, ST9 and ST199) (Figure 3a), whereas linear regression between ST size and *bcrABC* prevalence across all STs did not show a correlation (data not shown).

By source category, fruits and vegetables exhibited a relatively low level of *bcrABC* prevalence (18.6%), which was statistically similar to that of land food animals (22.8%); significantly lower than those of multi-ingredient food (48.6%), environmental (41.2%), and aquatic animals (35.3%); and significantly higher only than that of humans (7.2%) (**Figure 3b**). When further compared with subcategories under land food animals, fruits and vegetable isolates ranked only above bovine and dairy by source-specific *bcrABC* prevalence (Figure 3b). Under the environmental category, subcategories associated with food processing

environments, including food and non-food contact surfaces and environmental swabs and sponges, feature much higher *bcrABC* prevalence than non-processing environments, such as water sediments and soil (Figure 3b).

Temporal patterns of adaptive BC tolerance. Because BC tolerance in US isolates was predominantly caused by *bcrABC*, we focused the characterization of the temporal patterns of adaptive BC tolerance on the gene cassette. We determined the onset and duration of adaptive tolerance to BC by continuously monitoring the MICs of a BC^r strain H7550 and its BC^s derivative H7550-Cd^s during and after sublethal exposure to BC. The two isolates differed only by a plasmid that harbors *bcrABC* to confer BC tolerance (21). The continuous monitoring was performed by exposing *L. monocytogenes* cells to BC at both growth-promoting (nutrient culture at 30°C) and growth-limiting (0.1% buffered peptone water at 25°C and 4°C) conditions.

We observed both similarities and differences in the temporal patterns of adaptive BC tolerance between H7550 and H7550-Cd^s. During sublethal BC exposure (half of the respective intrinsic MIC) under the growth-promoting condition, both strains exhibited two phases of tolerance increase divided by a plateau of no MIC change (**Figure 4**). Regardless of their intrinsic MICs, both strains gained 5 µg/ml of MIC after the first phase and peaked with another increase of 20 µg/ml by the end of the second phase (Figure 4). During the first phase, increase of MIC emerged between 6 and 9 h in both strains (Figure 4). Under the growth limiting conditions in 0.1% buffered peptone water at both 4°C (**Figure 5a, 5b**) and 25°C (**Figure 5c, 5d**), neither strain developed any adaptive tolerance upon exposure to BC at half of their intrinsic MIC (Figure 5). At 25°C, both strains did not survive after 6 h of exposure (Figure 5c, 5d).

In comparison to the synchronization in MIC increase during the first phase, the development of additional tolerance took substantially longer in H7550 than in H7550-Cd^s (Figure 4). The plateau after the first phase lasted 9 h in H7550 (9-18 h) compared with 3 h in H7550-Cd^s (9-12 h). Then it took 27 h for H7750 (21-48 h) to reach the maximum adaptive tolerance, compared with 3 h for H7550-Cd^s (15-18 h). Because of these prolonged adaptive responses in H7750, the full development of tolerance was more than 4 times as long in H7750 (66 h) as in H7750-Cd^s (15 h). Another notable difference between the two strains occurred upon removal of BC exposure after the full tolerance development. In H7550-Cd^s, the tolerance persisted throughout repeated sub-culturing without BC exposure (Figure 4a), which confirmed previous reports that the acquired tolerance in BC^s strains was hereditary (28). By contrast, acquired tolerance in H7550 dropped to where the MIC was after the first phase of tolerance development within 1 h after the removal of BC exposure (Figure 4b). The finding of hereditary adaptive tolerance suggests that the underlying mechanism of the tolerance is unlikely transcriptomic responses, because such responses cannot be inherited by offspring cells. Therefore, we did not proceed with the original plan to study the transcriptome (Objective 4).

In addition to H7550 and H7550-Cd^s, we examined adaptive BC tolerance in three other *bcrABC*-positive/BC-tolerant isolates and three other *bcrABC*-negative/BC-susceptible isolates using the same adaptation method. The two groups of isolates each represented three major genetic lineages and all exhibited similar temporal patterns of tolerance to H7550 and H7550-Cd^s, respectively.

Additionally, we tested if exposure to increasing levels of BC induced higher tolerance and if a newer generation of BC-based sanitizer acted similarly on tolerance development. We found that exposing H7750 and H7750-Cd^s to gradually increasing concentrations of BC from half of their intrinsic MICs to the fully developed tolerance induced similar levels of maximum tolerance to those caused by the exposure to constant levels of BC. We also found that a “fifth-generation” QAC that synergistically combines two older generations of QACs (17) induced similar adaptive tolerance responses in both H7750 and H7750-Cd^s to the third-generation sanitizer that was used in our previous assays.

Residual BC concentrations throughout a produce processing facility present a low likelihood for tolerance development. We surveyed residual BC concentrations throughout a fresh produce processing facility in the Southeast US. The facility processes fresh tomatoes, onions, jalapeno peppers, and cilantro by following a daily schedule that includes: 1) early morning sanitizing using a QAC product (BC concentration at 400 µg/ml), 2) production throughout the day, and 3) evening cleaning by tap water. The temperature of the facility was constantly maintained at around 4°C. Residual sanitizer samples were collected at two time points from 10 different locations in the facility daily for three consecutive days. The time points include post-sanitation (early morning) and post-cleaning (evening). The sampling locations included cart (bottom shelf), packing machine, conveyor belts (big, small, and inclined), floor drain, sorting machine, and processing machine. These locations were spread out in a raw product room and a processing room. The two rooms were connected by two processing lines. Two sampling locations associated with the inclined conveyor belt from two different lines in the raw product room were considered less accessible for sanitation and cleaning, because of their locations in tight corners and the elevated heights of the belts.

Right after QAC sanitation in the morning, nearly all sampling locations reached BC concentrations that were multiple times higher (**Table 2**) than the upper bound of adaptive BC tolerance of *L. monocytogenes* at 50 µg/ml, which was obtained by adapting a BC-tolerant strain under a growth-promoting condition (nutrient culture, 30°C). The exceptions were samples from the bottom shelf of a cart (below detection limit, Day 1 and Day 2), the packing machine (20 and 30 µg/ml, Day 1), and the processing machine (60 and 70 µg/ml, Day 1) (Table 2). These results suggest that the application of the QAC sanitizer that contained BC at 400 µg/ml should leave residual BC concentrations sufficient for effective inhibition of *L. monocytogenes* at the sampled locations prior to the start of daily production, even if the pathogen acquired maximum adaptive tolerance.

Right after cleaning that concluded daily operation, no detectable BC presence was found from all sampling locations on all three days (Table 2). These results suggest that between operations, the facility was free of residual BC that could provide sublethal exposure to *L. monocytogenes*.

Outcomes and Accomplishments

This project bridged important data gaps toward an integrative and realistic risk assessment of adaptive BC tolerance in *L. monocytogenes* during fresh produce processing. Findings from this project will be submitted for presentation at the 2023 IAFP annual meeting and for publication in *Applied and Environmental Microbiology*.

Summary of Findings and Recommendations

- Benzalkonium chloride (BC) resistance of *L. monocytogenes* in the US is mostly mediated by the *bcrABC* gene cassette and can be reliably predicted by its presence/absence in the isolate genome. Chlorine resistance is uniformly present in *L. monocytogenes*, with little variation among isolates.
- General selection for BC-tolerant strains of *L. monocytogenes* are associated with food processing environments. Compared with other source categories, including various food animals, such selection appears to be relatively moderate among isolates from fruits and vegetables.
- Under a growth promoting condition (nutrient culture and 30°C), the upper bound of adaptive BC tolerance was 25 µg/ml in the BC-susceptible (BC^s) strain and 50 µg/ml in the BC-tolerant strain. To reach the upper bounds, a two-phase process of tolerance development was required under sublethal exposure to BC, which took 21 h for the BC-susceptible strain and 72 h for the BC-tolerant strain. Once developed, the tolerance remained stable, not dependent on external BC, and hereditary in the BC-susceptible strain, but waned within an hour in the BC-tolerant strain after the removal of BC exposure.
- Recognition of the distinct temporal patterns of adaptive BC tolerance between BC-tolerant and BC-sensitive strains allows a differential assessment of their respective likelihood and risk regarding the tolerance. The long exposure requirement for full tolerance (50 µg/ml) development in BC-tolerant strains (72 h) makes it unlikely to occur under the frequency of daily sanitation. Even fully developed, the rapid loss of the tolerance after BC exposure makes a lingering concern unlikely. In comparison, the finding that BC-susceptible strains could develop full, stable and hereditary tolerance (25 µg/ml) within the interval of consecutive sanitation shifts (24 h) may warrant further mechanistic understanding of the tolerance.
- Under growth limiting conditions (0.1% buffered peptone water at 4 and 25°C) that more realistically mimicked growth constraints on *L. monocytogenes* in produce processing environments, neither the BC-susceptible nor the BC-tolerant strain developed any adaptive tolerance.
- No adaptive tolerance to chlorine was observed in *L. monocytogenes*.
- External constraints make the development of adaptive BC tolerance in *L. monocytogenes* unlikely in produce processing environments. A daily regime of pre-production sanitation by QAC (400 µg/ml BC) and post-production water rinse left no detectable level of BC residuals for *L. monocytogenes* to be exposed to in multiple sampled locations in a produce processing facility. Development of adaptive tolerance also requires optimal growth of the bacteria, which is unlikely to occur in produce processing environments.

For the processing of fresh fruits and vegetables, we conclude that properly sanitized and cleaned facilities are less affected by BC selection and unlikely to provide conditions that are conducive for the emergence of adaptive BC tolerance in *L. monocytogenes.*

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APPENDICES

Publications and Presentations

He et al. Genomic indictors, temporal characteristics, and produce processing environment constraints of *Listeria* adaptive tolerance to benzalkonium chloride. (in preparation).

Budget Summary

This project was awarded $186,733 in research funds and the majority of funds were spent.

Tables 1–2 and Figures 1–5 (see below)

Table 1. Prevalence of known BC resistance genes among publicly available *L. monocytogenes* genomes in the US.

Resistance determinant	All isolates (n=25,083)	BC^r isolates (n=7,665)
bcrABC	28.60%	93.58%
qacH	1.76%	5.75%
emrC	0.04%	0.13%
emrE	0.14%	0.44%
qacC	< 0.1%	< 0.1%
qacA	< 0.1%	< 0.1%

Table 2. Residual BC concentrations in a produce processing facility.

Sample location	Day 1		Day 2		Day 3	
	Post-sanitation	Post-cleaning	Post-sanitation	Post-cleaning	Post-sanitation	Post-cleaning
Cart (bottom shelf, PR[a])	0/0[b]	0/0	0/0	0/0	200/70	0/0
Ppacking machine (PR)	30/20	0/0	190/180	0/0	130/140	0/0
Line 1 conveyor belt (PR)	300/300	0/0	180/190	0/0	230/230	0/0
Floor drain (PR)	150/130	0/0	200/130	0/0	140/200	0/0
Line 2 small conveyor belt (PR)	230/250	0/0	300/290	0/0	300/240	0/0
Line 2 inclined conveyor belt (RR[c])	270/270	0/0	240/250	0/0	310/290	0/0
Line 2 sorting machine (RR)	170/160	0/0	370/380	0/0	280/270	0/0
Line 1 inclined conveyor belt (RR)	260/260	0/0	340/350	0/0	300/140	0/0
Line 2 large conveyor belt (PR)	190/200	0/0	210/200	0/0	150/140	0/0
Line 1 processing machine (PR)	60/70	0/0	240/270	0/0	270/260	0/0

[a] Processing room (PR)
[b] Replicate 1/Replicate 2 (µg/ml)
[c] Raw product room (RR)

Figure 1. MIC and BC resistance determinants of 359 *L. monocytogenes* strains

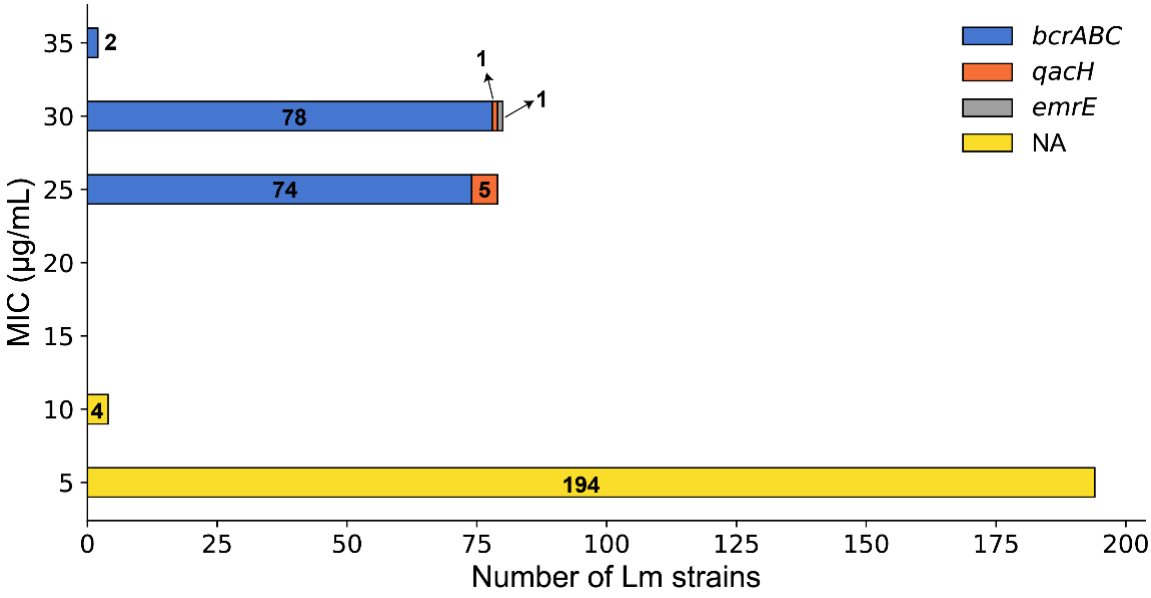

Figure 2. Genomic survey of BC tolerance in publicly available *L. monocytogenes* genomes in the US. **a.** A minimum spanning tree based on MLST of *L. monocytogenes* isolates marked by *bcrABC* and *qacH* prevalence in each ST. Major STs with high *bcrABC* prevalence are identified by ST numbers. **b.** A minimum spanning tree based on MLST of *L. monocytogenes* isolates marked by isolate source categories.

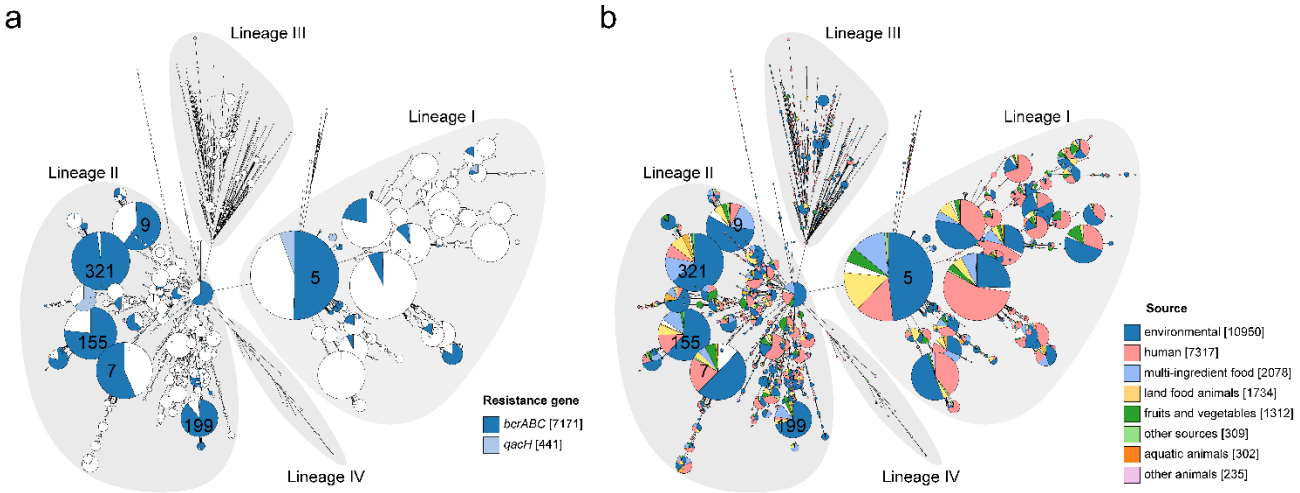

Figure 3. Comparison of *bcrABC* prevalence by source categories and STs.
Percentage above each bar is the *bcrABC* prevalence and n is the number of isolates in this group. **a.** *bcrABC* prevalence comparison between isolates from bigger STs and smaller STs by source categories. Isolates from the largest ST in Lineage I and the five largest STs in Lineage II were shown in hatches. **b.** Comparison of *bcrABC* prevalence by major source categories (middle). Further comparison is expanded to sub-categories under "environmental" (left) and "land food animals" (right). The left figure is the *bcrABC* prevalence among top *environmental* isolates. Difference letters above the bar indicate significant difference in *bcrABC* prevalence by chi-squared test ($p<0.05$).

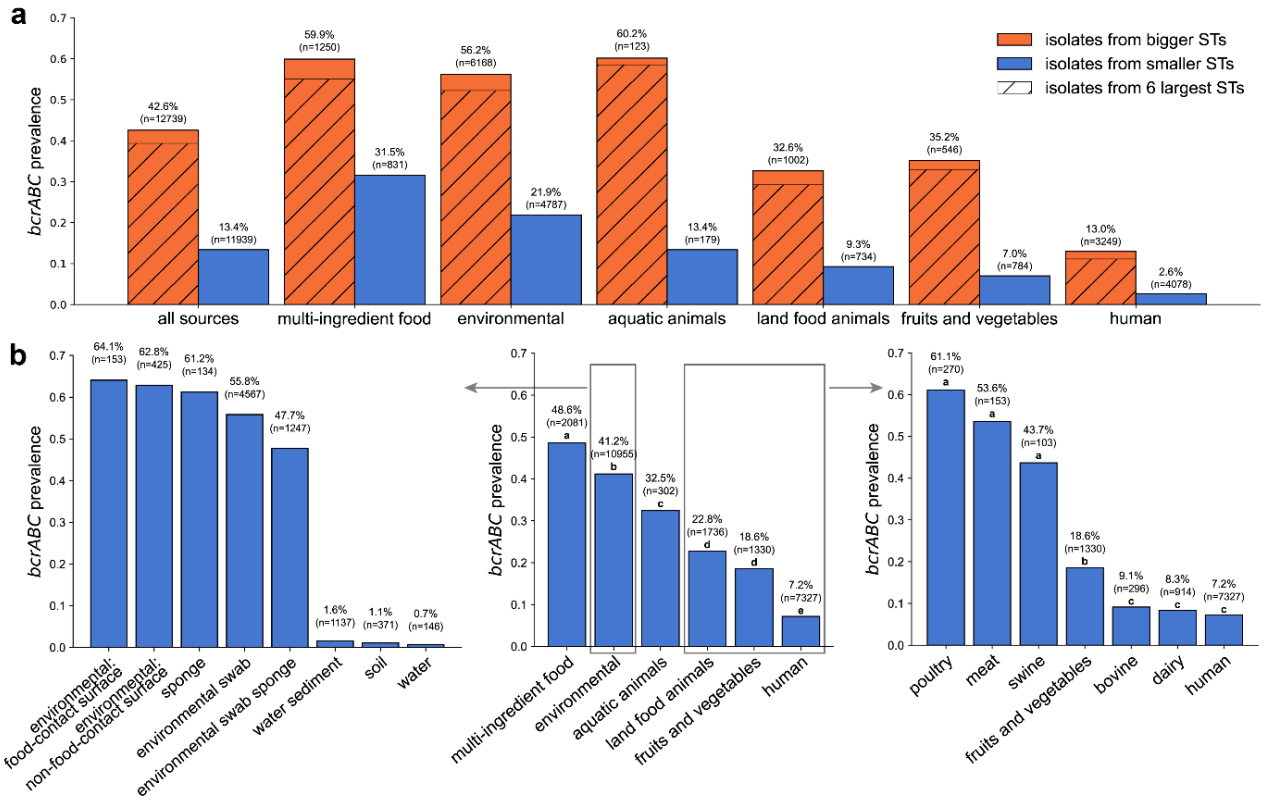

Figure 4. Temporal patterns of BC adaptive tolerance under growth promoting condition. Y axis on the left shows MIC of the isolates (H7550-CD^s, blue circles; H7550 orange circles). Y axis on the right shows BC concentrations in the cultures (solid horizontal lines). The two phases of tolerance development in both strains are labeled. **a.** Continuous monitoring of BC MIC in H7550-CD^s first under sublethal exposure to BC (5 µg/ml, half of its intrinsic BC MIC) on nutrient culture at 30 °C and then during sub-culturing without BC exposure. Five sub-cultures were monitored and each lasted 24 h (97, 121, 145, 169 and 193 h). **b.** Continuous monitoring of BC MIC in H7550 first under sublethal exposure to BC (15 µg/ml, half of its intrinsic BC MIC) on nutrient culture at 30 °C and then during sub-culturing without BC exposure. Five sub-cultures were monitored and each lasted 24 h (97, 121, 145, 169 and 193 h).

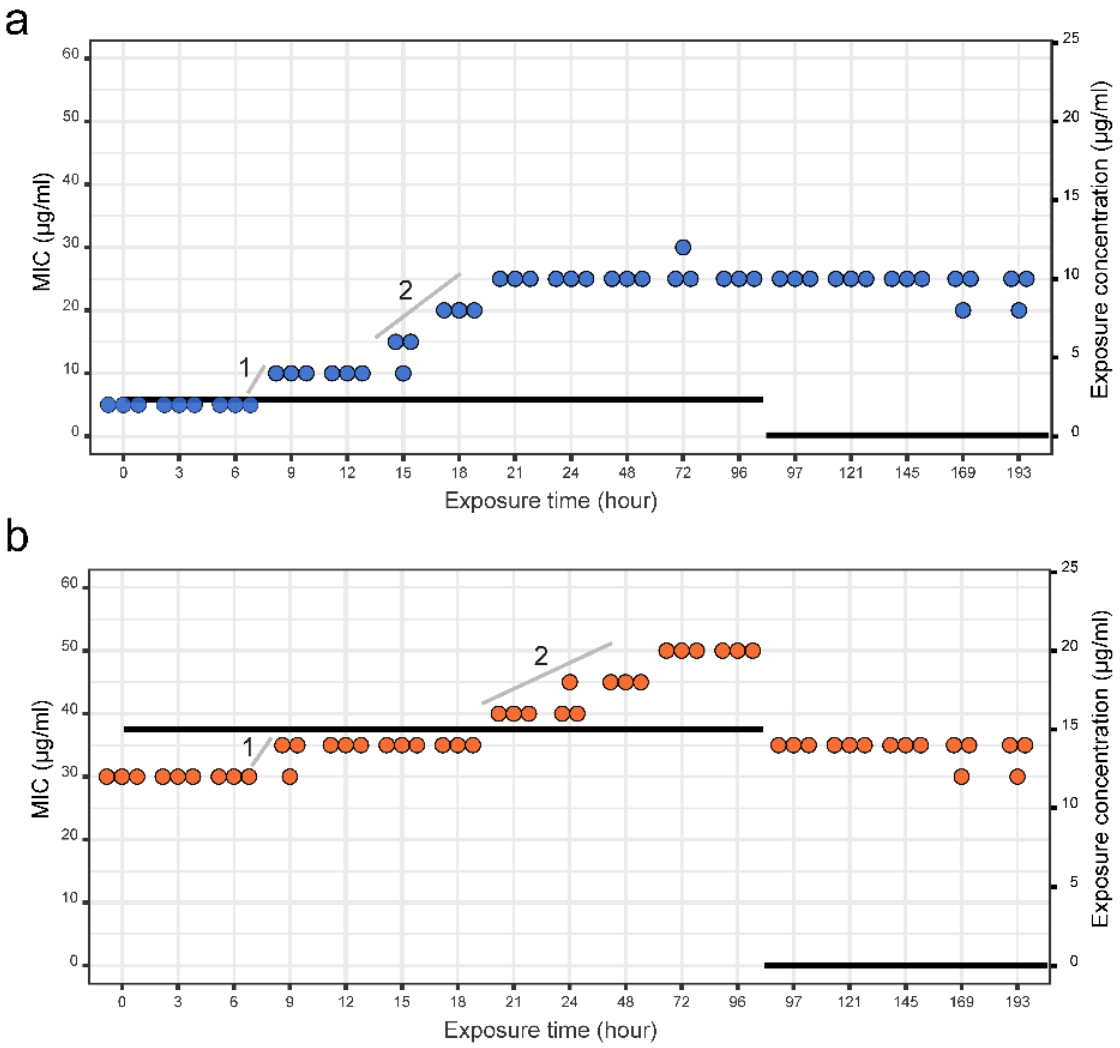

Figure 5. Temporal patterns of BC adaptive tolerance under growth limiting condition. Y axis on the left shows MIC of the isolates (H7550-CD^s, blue circles; H7550 orange circles). Y axis on the right shows sublethal concentrations of BC (half of the intrinsic BC MIC) in the cultures (solid horizontal lines). Empty circles represent unculturable cells. **a. and b.** Continuous monitoring of BC MIC in H7550-CD^s (a) and H7550 (b) in 0.1% buffered peptone water at 4°C. **c. and d.** Continuous monitoring of BC MIC in H7550-CD^s (c) and H7550 (d) in 0.1% buffered peptone water at 25°C.