CPS 2019 RFP
FINAL PROJECT REPORT

Project Title

Listeria develops reduced sanitizer sensitivity but not resistance at recommended sanitizer use levels

Project Period

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Objectives

1. *Screen >500 Listeria spp. and L. monocytogenes isolates from packinghouses and fresh-cut operations for reduced sensitivity to key sanitizers, including quaternary ammonium compounds, sodium hypochlorite and peroxyacetic acid.*
2. *Perform whole genome sequencing (WGS) of Listeria spp. and L. monocytogenes strains identified as showing reduced sanitizer sensitivity to identify mutations and gene acquisitions responsible for reduced sanitizer sensitivity.*
3. *Expose selected strains with reduced sanitizer sensitivity to increasing sanitizer concentrations to determine the potential of these strains to become resistant to sanitizer levels close to the recommended use levels.*

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Abstract

Survival and persistence of *Listeria* spp. and *Listeria monocytogenes* in packinghouses and fresh-cut produce processing facilities continues to be a major concern. While "*Listeria* issues" in these facilities can typically be traced back to survival of these organisms in locations that cannot be (easily) reached by sanitizers (so called niches), *there is also a concern about emergence of sanitizer resistant Listeria* spp. and *L. monocytogenes*. We hypothesize that *Listeria* isolates can develop increased resistance to low-level concentrations of sanitizer but not tolerance at recommended sanitizer use levels.

Overall, our data indicate that wildtype produce-associated *Listeria* and *L. monocytogenes* isolates can show a wide range of sanitizer susceptibility, with some isolates showing no reduction after short (30 sec) exposure to peroxyacetic acid (PAA) and sodium hypochlorite (NaOCl), while all isolates showed at least 2.7 log reduction after exposure to quats. Considerable concerns have been raised about quat resistance and presence of quat resistance genes in *Listeria*; our data suggest that previously reported quat or oxidative stress resistance genes, e.g., *bcrABC, qacH* or SSI-2, do not predict *Listeria* survival at use-level concentration of sanitizer. In addition, increased resistance at low concentrations of benzalkonium chloride (BC) is due to genetic adaptation but does not confer a survival advantage at use level concentrations. Our findings suggest that quat resistance of *Listeria* is not a major issue for the produce industry and that concerns about "*quat resistant Listeria superbugs*" may not be warranted. Our data also indicate that caution should be warranted before implementing sanitizer rotation or changes that reduce use of quats, as quats provided for more consistent *Listeria* reduction as compared to PAA and NaOCl.

As to more specific results, a collection of 588 produce-associated *Listeria* was assembled, comprising 414 *L. monocytogenes* (56 pre-harvest, 272 post-harvest, 86 retail) and 174 *L.* spp. (29 pre-harvest, 145 post-harvest). *Listeria* spp. included *seeligeri* (n=71), *innocua* (n=59), *welshimeri* (n=33), *marthii* (n=6) and *ivanovii* (n=5). All isolates were (i) phenotypically characterized by exposure to sanitizer (i.e., 300ppm benzalkonium chloride [BC], 80ppm peroxyacetic acid [PAA] or 500ppm sodium hypochlorite [NaOCl]) for 30 sec and assessed for their survival (Obj. 1) and (ii) genotypically characterized by whole genome sequencing (WGS) for presence and absence of previously described sanitizer resistance genes e.g., *bcrABC* and *qacH* (Obj. 2). Log reduction for BC ranged from 2.7 to 5.7 log, for PAA from 0.0 to 6.1 log, and for NaOCl 0.0 to 8.1 log. Retail isolates were significantly more tolerant to BC, PAA and NaOCl than isolates from the pre- or post-harvest environment, which could indicate a selection along the food supply chain for more tolerant isolates. For PAA, *L. monocytogenes* isolates were significantly more tolerant than *L.* spp. Phylogenetic lineages of *L. monocytogenes* isolates were not associated with sanitizer tolerance. Retail isolates were more likely to carry *bcrABC* than post- or pre-harvest isolates (47.7%, 15.8%, 5.9%, respectively [$P<0.0001$]). However, presence of known sanitizer resistance genes was not associated with log reductions when exposed to either sanitizer.

A subset of 68 *Listeria* isolates was assessed for their ability to grow at low concentrations of BC; concentrations of BC that inhibited growth ranged from 1–6ppm. Isolates carrying resistance genes (*bcrABC* or *qacH*) showed growth at significantly higher concentrations than isolates lacking these genes ($P<0.001$). When isolates were serially passaged in increasing concentrations of BC, isolates were able to grow at concentrations up to 18 mg/L BC, but acquired resistance was reduced after removal of selective pressure (substreaking on BHI agar, "substreaked isolates"). However, the majority of substreaked isolates was still able to grow at higher concentrations as compared to prior to serial passaging, suggesting a genetic mechanism

of adaptation. High-quality single nucleotide polymorphism analysis identified point mutations in a locus for substreaked isolates that encodes for a tetR family transcriptional regulator, which suggests a common mechanism of adaptation (Obj. 3). To determine if genetic adaptation increases the likelihood of survival when exposed to use level concentrations of BC, substreaked and parental isolates were exposed to BC at use level concentrations. No significant difference was detected for substreaked and parental isolates after survival of BC (paired t-test, P=0.942). To assess the possibility of horizontal gene transfer, combinations of strains were co-cultured and assessed for their growth at low concentrations of BC. Co-cultures were also serial passaged in increasing concentrations of BC. Co-cultures did not show growth at higher concentrations of BC than a single culture indicating a low risk for horizontal gene transfer.

Background

In addition to known sanitizer resistance mechanisms (i.e., *bcrABC* and *qacH* for quaternary ammonium compound sanitizer), there are concerns that additional unknown mechanisms can lead (i) to resistance to sanitizers other than quats or (ii) to resistance to higher levels of sanitizers, as compared to the current quat resistance mechanisms, which typically only confer reduced sensitivity to quat levels considerably below recommended use levels. Importantly, even reduced susceptibility to sanitizers (to concentrations considerably below recommended use levels) can be of practical concern, as it may not be unusual for *Listeria* to be exposed to reduced sanitizer concentrations in produce operations, for example due to high organic loads, which reduces sanitizer efficacy even if applied at appropriate concentrations.

This project aims to provide industry with better tools (i) to assess the risk of reduced sanitizer susceptibility in *Listeria*, (ii) to more rapidly and reliably identify *Listeria* with reduced sanitizer susceptibility, and (iii) to control *Listeria* with reduced sanitizer susceptibility. To achieve these goals, we provide baseline data on the frequencies of *Listeria* spp. and *L. monocytogenes* with reduced sensitivity to different sanitizers; these data will not only help industry assess whether resistance to sanitizers other than quat is a concern, but we also provide information on the levels of reduced sensitivity that could be expected for *Listeria* in produce facilities. We identified resistance genes and resistance mechanisms (e.g., single nucleotide polymorphisms) that provide resistance to a sanitizer and we determined the level of increased resistance achieved for a strain through serial passaging.

Research Methods and Results

Bacterial strains

A collection of 588 produce-associated *Listeria* was assembled comprising 414 *L. monocytogenes* (56 pre-harvest, 272 post-harvest, 86 retail) and 174 *L.* spp. (29 pre-harvest, 145 post-harvest). Isolates from the pre-harvest environment were conveniently selected from previously published studies to include (i) *L.* spp. (Weller et al., 2015) and (ii) *L. monocytogenes* isolates representing each pulsed-field gel electrophoresis (PFGE) subtype identified among all samples collected (Harrand et al., 2020). Post-harvest isolates included (i) representative isolates (i.e., from a single sample representing a different *sigB* allelic type [AT]) from a longitudinal study conducted by Estrada et al. (2020), and *Listeria* isolates were collected from eleven produce packinghouses in three U.S. states, and associated produce commodities included microgreen, peach, apple, tomato, broccoli, cauliflower, and cucumber (Estrada et al., 2020), (ii) a submission from the FDA specifically for this project with isolates from different single nucleotide polymorphism (SNP) clusters, (iii) isolates available in the Food Safety Lab, including isolates from bean sprouts, broccoli sprouts, coleslaw and RTE salad, (iv) isolates from other research labs, including isolates associated with onion, apple, cantaloupe, and

mushroom, (v) representative *Listeria* isolates from a study conducted by Sullivan et al. (2020) from three produce packinghouses and five fresh-cut facilities (Sullivan & Wiedmann, 2020), (vi) *Listeria* isolates selected from a study conducted by Bardsley et al. (2021) (*unpublished*) to include one of each *sigB* AT isolated from a produce packing house, and (vii) produce-associated *L. monocytogenes* from a previously established produce strain collection (Harrand et al., 2019). Produce retail isolates from a study by Burnett et al. (2020) were selected to include representative isolates from each sequence type isolated from each of the seven states included in this study (Burnett et al., 2020). All isolates were collected in the United States. All *Listeria* isolates were stored in brain heart infusion (BHI; Difco, Becton Dickinson, Sparks, Md.) with 15% glycerol at −80°C. Isolates were streaked from the −80°C glycerol stocks onto BHI agar plates and incubated at 37°C for 24h. Streaked plates were stored at 4°C for at least 24h but no longer than 7 days prior to experiments.

Bacterial growth curves

Bacterial growth curves were generated for all isolates to estimate the time point at which the isolates reach early stationary phase (T_{max}) at 22°C in BHI broth. For bacterial culture preparation, 5 ml of BHI broth was inoculated with a single colony from a cold room stored plate and incubated non-shaking at 22°C for ~40 h. The cultures were diluted 1:1000 to reach ~10^5 CFU/mL and 300uL of each diluted culture was transferred into a 96-well flat bottom plate. The plates were incubated for 48h at 22°C in a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, VT, USA) and absorbance was measured at OD600 every 10min. Bacterial growth curves were group based on their estimated T_{max} value, using Growthcurver package in R (Sprouffske & Wagner, 2016), where T_{max} values within one group differed by a maximum of 2.5h. Isolates were divided into seven subgroups based on cut-off values for incubations (i.e., 36h, 33.5h, 31h, 28.5h, 26h, 23.5h, 21h). An isolate was incubated for the time that the estimated T_{max} value was closest to but higher (e.g., an isolate with an estimated T_{max} value of 27h would be incubated for 28.5h).

Sanitizer treatment at use level concentrations

Sanitizer solutions were prepared 1 hour prior to use. In brief, three sanitizer solutions were prepared in phosphate-buffer saline (PBS), including 80ppm PAA in PBS pH5.0, 300ppm BC in PBS pH8.0 and 500ppm NaOCl in pH6.0.

Bacterial cultures were grown to early stationary phase in a 96-well flat-bottom plate. A 200uL aliquot from each well was transferred into a 96–deep well plate and spun down for 10min at 4,000rpm. Supernatant was removed and 12 isolates at a time were resuspended by pipetting up and down eight times with 200uL of either (i) PBS and sanitizer solution for treated cells or (ii) PBS for untreated control cells. Cells were incubated for 30sec and sanitizer was deactivated by adding 400uL of 1.43x Dey-Engley neutralizing broth (D/E), resuspending the solution eight times, followed by incubation for 5min. Deactivated cultures were serial diluted 1:10 in D/E to achieve a final dilution of 10^{-9}. All dilutions were spot plated in 10uL volume on BHI agar plates and plates were incubated for 24h at 37°C, followed by colony enumeration.

Reserpine experiments, which were conducted to determine involvement of efflux pump activities by blocking efflux pump activity with the reserpine molecule, were performed as described above; treatment included (i) BC, (ii) BC and 20mg/L of reserpine, (iii) DMSO, solvent for reserpine and (iv) PBS.

Minimum inhibitory concentration assay, serial passaging and co-culture

For MIC experiments, cultures were pre-grown by inoculating 5mL BHI broth with a single colony, followed by incubation for 35h at 22°C, followed by subculturing 1:1000 in 5mL BHI

broth and an additional incubation of 35h at 22°C. Bacterial cultures were subsequently diluted 1:1000 and inoculated into each target concentration of BHI-BC (i.e., 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 12 mg/L BC) to a final concentration of ~10^6 CFU/mL per well. Broth microdilution method was used to determine the initial minimum inhibitory concentration (MIC) of isolates to BC in a 96-well microtiter plate with 200µL volume. BC (Sigma-Aldrich, St. Louis, MO) was prepared in BHI broth to achieve appropriate target concentrations. Absorbance of cultures was measured at 600nm (OD600) in a microplate reader (Biotek Instruments, Winooski, VT) before (T0) and after incubation of 24h at 22°C (T24). Difference in absorbance before and after 24h incubation >0.100 (ΔOD600) represented isolate growth.

For serial-passage experiments, all *Listeria* isolates were serial passaged in increasing concentrations of BHI-BC in a 96-well microtiter plate with 200µL volume. Bacterial cultures grown to stationary phase were diluted 1:1000 to achieve a final concentration of ~10^6 CFU/mL in initial concentration of 0.25 mg/L BHI-BC, and cultures were incubated up to 48h at 22°C to allow for growth. Growth was assessed at 24h; isolates were incubated for an additional 24h if no growth was observed after 24h. After exposure to the initial BHI-BC concentration of 0.25 mg/L, cultures that showed growth were exposed to BC concentrations that were increased to 0.5, 1 and 2 mg/L and continued to increase by 2 mg/L. If growth was detected, isolates were subcultured 1:10 in the next incremental concentration of BHI-BC. When isolates failed to grow, dilutions were plated on BHI agar for enumeration and plates were incubated for 24h at 37°C. The concentration at which they failed to show growth based on optical density was recorded as "serial passaged MIC".

Serial passaged isolates were assessed for their stability of acquired resistance to BC. Enumeration plates from the serial passage experiment were kept at 4°C. Single colonies from enumeration plates were substreaked on BHI agar and plates were incubated at 37°C for 24h. After incubation, colonies were substreaked again for a series of seven times. MIC assays were performed on seven-time substreaked isolates and on colonies obtained directly from the initial enumeration plate.

For co-culture experiments, four strains were mixed together by adding 1mL of culture of each strain in a tube, followed by thorough vortexing and spinning for 10min at 4,000rpm. The cell pellet was then resuspended in 200uL BHI broth and the resuspension was transferred onto a 0.45µm filter placed on a BHI agar plate, followed by incubation for 48h at 22°C; this step was used to allow for horizontal transfer of resistance genes. Single cell control cultures were plated in 200uL volume on a filter placed on BHI agar and incubated for 48h at 22°C. After incubation, each filter was removed with forceps and transferred into a 50mL Falcon tube and washed with 5mL BHI by pulse vortexing. Filters were aseptically removed, and cultures were diluted to achieve OD_{600} of ~0.2. OD-adjusted cultures were used for an initial MIC and serial passaging experiments.

Whole genome sequencing and raw data processing

DNA was extracted using DNA extraction kits (DNeasy Blood and Tissue kit and DNeasy Powersoil, Qiagen, Valencia, CA) following the manufacturer's instructions. Genomic DNA was sequenced using Illumina Miseq and Hiseq platform (Illumina, Inc. San Diego, CA) with 2x250bp paired-end reads. The adapters from raw sequencing data were removed using Trimmomatic v 0.36 (Bolger et al., 2014) followed by quality assessment using FastQC v 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Sequences were assembled *de novo* with SPAdes version 3.14.0 (Bankevich et al., 2012). Quality control of assemblies was performed with QUAST v 5.0.2 (Gurevich et al., 2013) and average coverage was determined using SAMtools v 1.11 (Li et al., 2009). Genomes with a minimum of 40x coverage were included in genomic analysis. Contigs smaller than 500bp were removed and contigs were blasted using Kraken (Wood & Salzberg, 2014) to confirm strain identity.

Genomic analyses

The SNP based analysis was performed using kSNP3 v 3.1 with an estimated optimal kmer size of 19 determined using Kchooser for each species including, *monocytogenes*, *innocua, ivanovii, seeligeri, marthii* and *welshimeri* (Gardner et al., 2015). A maximum-likelihood phylogeny based on core genome SNP was generated with 1000 bootstrap repetitions in RaxML.

For *Listeria monocytogenes*, sequence types (ST) and clonal complex (CC) were assigned based on identified allelic types for each of the 7 housekeeping genes using Pasteur MLST *L. monocytogenes* database (http://bigsdb.web.pasteur.fr/listeria/listeria.html) and were grouped into clonal complexes. Genomes have been annotated with PROKKA v 1.14.5 (Seemann, 2014). The pangenome was constructed using Roary v 3.12.0 (Page et al., 2015). Roary output was used in treeWAS for genome wide association studies (GWAS) (Collins & Didelot, 2018) to identify association of genes and sanitizer survival.

Genomes were searched with blastn using a maximum e-value of 1e-20 for presence of previously described quat tolerance genes, including *bcrABC, qacA, qacC, qacH, qacE, emrE*, as well as the stress survival islet 2 (*lin0464, lin0465*), previously described to confer tolerance to oxidative stress. Sequences of these genes were downloaded from Pasteur database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef&page=downloadAlleles) used to create a local nucleotide BLAST database. Genes were identified as present if they exhibited at least 95% coverage and 85% identity.

Genomic data were also analyzed using Platon to identify plasmid presence and absence (Schwengers et al., 2020).

Genomes of serial passaged and substreaked isolates were compared with the respective parental, non-serial passaged genome to identify high quality SNPs using the CFSAN SNP Pipeline v 2.2.1 (Davis et al., 2015). If SNPs were located in an open reading frame they were classified into synonymous and nonsynonymous. The sequence of the open reading frame was searched against the BLASTX database using default parameters (States & Gish, 1994).

Statistical analyses

Data were analyzed in R, version 4.0.4 (R Core Team, Vienna, Austria). A logistic equation was fitted to the growth curve data using the SummarizeGrowth function in the growthcurver package, version 0.3.0 (90), to obtain the time point a bacterial culture reached early stationary phase. Linear mixed-models were used to determine (i) the effect of gene presence and absence on survival of sanitizer at use level concentrations, (ii) the effect of 'isolate type' and species on MIC, (iii) the interaction effect of tolerance gene presence and 'isolate type' on MIC, and (iv) the effect of *L. monocytogenes* vs. any other species of *Listeria* on MIC using R lme4 package (Bates et al., 2015). For these analyses, factors of isolate type (parent, serial passaged, notsubstreaked, substreaked), resistance gene (presence or absence), species were considered fixed effects and isolate ID was considered a random effect. Model assumptions of normality and homogeneity of variance were assessed. Post-analysis of means was performed with Tukey's (honestly significant difference) HSD using the emmeans package in R (Lenth, 2016). For exposure to use level concentration of BC experiments, paired student t-tests were used to compare the log reductions of adapted isolates to parent isolates for each species. Use level experiments were conducted with two biological replicates per isolate. P values of ≤0.05 were considered statistically significant. The detection limit for bacterial colony enumeration was 2 log CFU/ml. In cases where no colonies were observed, the limit of detection was set to 2 log CFU/ml for statistical analyses. All sanitizer exposure trials were conducted in duplicate. If the log reduction from the first to second trial was larger than 2 log a third replicate was performed. MIC assays were also conducted in duplicate.

Results

Objective 1 (*Screen >500 Listeria spp. and L. monocytogenes isolates from packinghouses and fresh-cut operations for reduced sensitivity to key sanitizers, including quaternary ammonium compounds, sodium hypochlorite and peroxyacetic acid.*)

Listeria produce-associated strain collection

A collection of 588 produce-associated *Listeria* was assembled, comprising 414 *L. monocytogenes* (56 pre-harvest, 272 post-harvest, 86 retail) and 174 *L.* spp. (29 pre-harvest, 145 post-harvest). *Listeria* spp. included *seeligeri* (n=71), *innocua* (n=59), *welshimeri* (n=33), *marthii* (n=6) and *ivanovii* (n=5). *Listeria* isolates from this collection were associated with produce including broccoli, sprouts, microgreens, mushrooms, apples, cantaloupe, cabbage, cauliflower, tomato, peach, celery, lettuce, cucumber, onion, carrot, and avocado. *L. monocytogenes* isolates represented lineage I (n=202), II (n=152) and III (n=60) with 74 different clonal complexes (CC) based on the 7-gene MLST scheme. The ten most common CC included CC9 (10%), CC5 (7%), CC6 (5%), CC1 (5%), CC4 (3%), CC155 (3%), CC388 (3%), CC7 (3%), CC37 (3%), CC369 (2%).

Sanitizer survival at use level concentrations

The log reduction ranged from 2.7 log to 5.7 log for BC, with a mean log reduction of 4.2 and standard deviation of 0.7 log (**Figure 1A**). For PAA, log reductions ranged from 0.0 to 6.1 log, with a mean log reduction of 2.4 and a standard deviation of 1.7 log (**Figure 1B**). For BC and PAA, a total of 588 isolates were included in the sanitizer screen. For NaOCl, 129 *Listeria* isolates were included, and log reductions ranged from 0.0 to 8.1 log, with a mean log reduction of 4.9 and a standard deviation of 1.6 log (**Figure 1C**). Regression analysis showed that retail isolates are significantly more tolerant than pre- or post-harvest isolates for all three sanitizers ($P<0.005$). *L. monocytogenes* was significantly more tolerant than *L.* spp. when exposed to PAA or NaOCl, and no significant difference was detected when exposed to BC (Figure 1A-C). *Listeria* lineages did not significantly differ in their response to sanitizer exposure.

Objective 2 (*Perform whole genome sequencing (WGS) of Listeria spp. and L. monocytogenes strains identified as showing reduced sanitizer sensitivity to identify mutations and gene acquisitions responsible for reduced sanitizer sensitivity.*)

Prevalence of QUAT sanitizer tolerance genes and stress survival islet 2

The two quat tolerance genes identified (*bcrABC* and *qacH*) were present in 19.0% and <0.0% of all genomes, respectively. *L. monocytogenes* genomes had a higher prevalence of *bcrABC* (24.6%) than did *L.* spp (5.7%). Among all *Listeria* genomes, genomes from isolates associated with the retail environment had the highest prevalence for *bcrABC* (47.7%), as compared to *Listeria* genomes associated with the post-harvest (15.8%) and pre-harvest (5.9%) environment. For *L. monocytogenes*, lineage II had significantly more isolates encoding *bcrABC* than did lineage I or III (**Figure 2**). The one isolate carrying *qacH* also belonged to lineage II.

Plasmidome analysis revealed that 28.7% of all *Listeria* isolates carried at least one plasmid; while 31.4% of *L. monocytogenes*, 14.1% of *L. seeligeri*, 22% of *L. innocua*, 39.4% of *welshimeri*, 50% of *marthii* carried at least one plasmid, no plasmids were found in *ivanovii*. Twelve *L. monocytogenes* isolates carried two plasmids. Retail isolates were more likely to carry a plasmid than pre- or post-harvest isolates (53.5%, 9.4%, 17.5%, respectively).

Stress survival islet 2 (SSI-2) was only found in 96.6% of *L. innocua* isolates and 4.8% of *L. monocytogenes* isolates, predominantly in lineage III (26.7% of lineage III isolates carried SSI-2) (**Figure 3**).

Phenotypic sanitizer response and genomic analysis

Regression analysis did not support any associations of quat tolerance genes presence/ absence and phenotypic response to BC. The stress survival islet 2, previously described to be involved with oxidative stress response, was also not associated with phenotypic response when *Listeria* were exposed to PAA or NaOCl.

Small-scale experiments with reserpine, a small alkaloid that is known to block efflux pump activity, further supported the lack of efflux pump involvement in survival when exposed to BC at use level concentrations. Isolates with *bcrABC* or *qacH* did not differ in their log reduction when exposed to BC and reserpine compared to exposure to BC alone.

A treeWAS analysis did not identify significant associations in genes from the *L. monocytogenes* pangenome and the phenotypic response, log reduction after BC exposure.

Objective 3 (*Expose selected strains with reduced sanitizer sensitivity to increasing sanitizer concentrations to determine the potential of these strains to become resistant to sanitizer levels close to the recommended use levels (e.g., 200 ppm quat or above).*)

MIC and serial passaging

68 *Listeria* isolates were selected for serial passaging experiments; these isolates represented the 10% most tolerant and the 10% most sensitive isolates from each species based on log reductions obtained in the sanitizer screen. For *L. monocytogenes,* isolates were selected to not only include the 10% most tolerant and sensitive isolates, but to also include the nine most common CC from lineage I and II and three most common CC from lineage III.

The initial MIC of isolates ranged from 1–6 mg/L BC. Isolates carrying tolerance genes (*bcrABC* or *qacH*) showed significantly higher MIC than isolates lacking these genes ($P<0.001$). Through serial passaging, isolates grew at concentrations up to 18 mg/L BC. Isolates carrying tolerance genes were more likely to achieve growth under higher BC concentrations.

After selection pressure was removed by (i) plating on BHI for enumeration (“not substreaked”) and (ii) by substreaking colonies from enumeration plates seven times on BHI agar (“substreaked”), isolates were assessed by MIC assays for maintaining their acquired increased MIC. Isolates showed a lower MIC for “not substreaked” and “substreaked” as compared to the maximum concentration under which they showed growth during the serial passaging experiments. However, the MIC for 53 substreaked isolates remained higher than compared to their initial MIC, indicating an adaptation mechanism that allows for improved survival at low concentrations of BC. In addition, substreaked *L. monocytogenes* isolates showed a significantly higher MIC compared to substreaked *Listeria* spp. isolates ($P<0.001$), and presence of tolerance genes (i.e., *bcrABC* and *qacH*) was no longer significantly associated with a higher MIC for substreaked isolates (**Figure 4**). Substreaked isolates were also assessed for their survival of BC at use level concentrations but no significant difference was detected between parental and substreaked isolates (paired t-test, $P=0.942$).

SNP analysis

In order to determine whether certain mutations accounted for the increased resistance to low-level concentrations of BC, we performed WGS on the two isolates for each species that showed the highest increase in minimum inhibitory concentration (MIC) after substreaking (as compared to initial MICs). Isolates were selected randomly if multiple isolates had the same increase in MIC after substreaking. In addition, if not already included through the selection process, all isolates carrying the sanitizer resistance genes *bcrABC* were included if the MIC increased by more than two-fold through the subculturing. In total, seventeen isolates were

selected for WGS. In eleven genomes from substreaked isolates, single point mutations were identified in a gene encoding for a tetR family transcriptional regulator. In eight sequences, these SNPs led to a premature stop codon; in three sequences the SNPs led to a nonsynonymous mutation (**Figure 5**). These data suggest that continuous exposure of *Listeria* isolates to low-level concentrations of BC can lead to selection of mutations that enhance the ability of *Listeria* to survive low-level BC exposure.

Co-culture

Isolates were selected for co-culture experiments to include isolates that (i) had a high initial MIC, (ii) represented relevant genotypes (i.e., *bcrABC* or *qacH* presence and absence), and (iii) represented each lineage and plasmid incompatibility type. Seven isolates were selected, and four strains were co-cultured in six combinations (**Figure 6**).

Co-culture experiments showed that initial MIC of co-cultures was not higher than any MIC of the single cultures. After serial passaging, the MIC of co-cultures was also not higher than the MIC of single cultures. This indicates that horizontal gene transfer, the exchange of resistance genes among isolates, is not a major contributor to the acquisition of BC resistance genes.

Outcomes and Accomplishments

Overall, the project provided critical data on (i) frequency of tolerance to three key sanitizers at use level concentrations (300ppm benzalkonium chloride, 80ppm peroxyacetic acid, 500ppm sodium hypochlorite) in produce-associated *Listeria*, and (ii) quat resistance and stress resistance genes presence and association with phenotypic resistance. The 588 isolates represented the pre-harvest, post-harvest and retail environments, providing frequencies to sanitizer tolerance along the produce supply continuum. The genotypic and phenotypic characterization did not identify a correlation in between previously described sanitizer and stress resistance genes and survival at use level concentrations, suggesting that these genes do not play a role in isolate survival when sanitizer is applied at use level concentrations.

The study provides industry with important data on (i) maximum concentrations of sublethal concentrations of BC that support *Listeria* growth when continuously exposed, and (ii) the spread of sanitizer resistance genes through horizontal gene transfer. A key finding of this study is the discovery of a novel adaptation mechanism that allows *Listeria* to grow at low-level concentrations of BC through a single point mutation, suggesting that independent of acquisition of sanitizer resistance genes through horizontal gene transfer, *Listeria* is able to grow at increased low-level concentrations of BC if continuously exposed to sublethal concentrations. Most importantly, key information from this study is scientific data that supports the finding that adaptation to low concentrations of BC does not increase the likelihood of survival at use level concentrations.

Our data also indicate that wildtype produce-associated *Listeria* and *L. monocytogenes* isolates can show a wide range of sanitizer susceptibility, with some isolates showing no reduction after short (30 sec) exposure to PAA and NaOCl, while all isolates showed at least 2.7 log reduction after exposure to quats. Caution is warranted before implementing sanitizer rotation or changes that reduce use of quats, as quats provided for more consistent *Listeria* reduction as compared to PAA and NaOCl.

Summary of Findings and Recommendations

Listeria isolates showed a range of responses to sanitizers at use level concentrations, with BC showing a minimum reduction of 2.7 log for all isolates compared to PAA or NaOCl where some isolates reach zero reduction after 30 sec of exposure, indicating application of BC is more likely to achieve target reductions for *Listeria*.

L. monocytogenes is more tolerant than *Listeria* spp. when exposed to PAA or NaOCl. Differences between *L. monocytogenes* and *Listeria* spp. were not observed for BC, suggesting equal likelihood of reduction of all species when BC is used.

Prevalence of sanitizer and stress resistance genes, i.e., *bcrABC, qacH* and SSI-2, are not correlated with sanitizer survival when sanitizer is applied at use level concentrations.

Listeria can grow at concentrations of BC up to 18ppm when continuously exposed to sublethal concentrations, supporting the importance of ensuring proper quat concentrations at all locations in a facility.

There is a limited risk of sanitizer resistance genes spreading through horizontal gene transfer. *Listeria* can adapt to sublethal concentrations of BC through point mutations. Sanitizer resistance genes, e.g., *bcrABC* and *qacH*, play a limited role in adaptation to sublethal concentrations of BC. Importantly, (i) acquired resistance to low concentrations of BC, or (ii) the presence of sanitizer resistance genes does not increase the survival at use level concentrations. This underlines the importance of well written and followed sanitary standard operation procedures (SSOPs) to ensure sanitizer is applied at target concentrations.

APPENDICES

Publications and Presentations

No publications to date.

Presentations:
NYS Dairy Inspectors, March 2021. “Sanitizer tolerance in *Listeria* isolates.”

CPS Research Symposium, June 2020. “*Listeria* develops reduced sanitizer sensitivity but not resistance at recommended sanitizer use levels.” [Poster]

Accepted abstract submissions to IAFP 2021:

1. “Genotypic and phenotypic characterization of sanitizer susceptibility of produce-associated *Listeria* isolates” – Anna Sophia Harrand, Renato Orsi, Jordan Skeens, Martin Wiedmann.

2. “Adaptation of *Listeria* isolates to increasing sanitizer concentrations” – Samantha Bolten, Anna Sophia Harrand, Jordan Skeens, Martin Wiedmann.

Budget Summary

A total of $187,874 in research funds was awarded, and as of April 21, 2021, $186,975 has been spent. This includes Salaries and Wages ($102,933), Employee Benefits ($40,833), Compensation, Graduate & Sabbatical ($12,110), Materials and Supplies ($15,597), Services ($5,255), and Indirect Costs ($10,216).

Figures 1–6

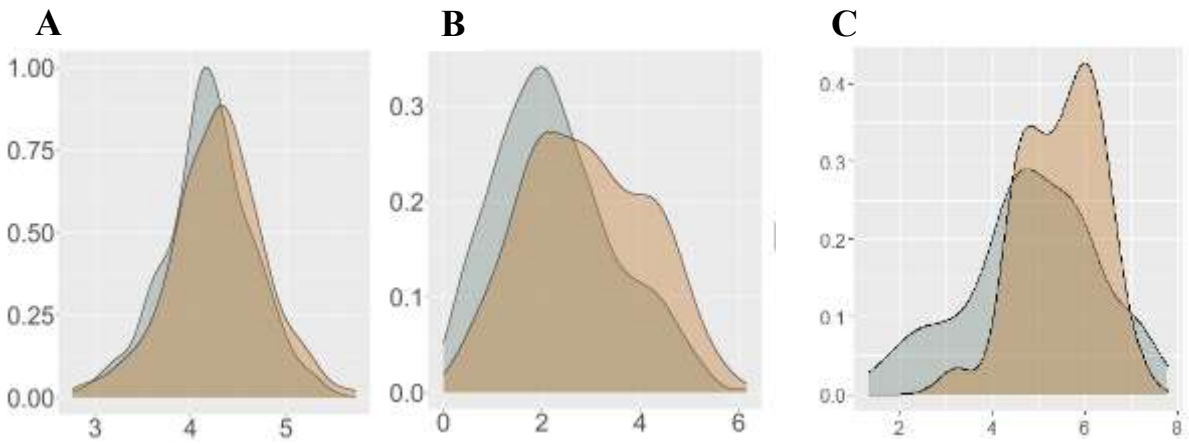

Figure 1: Density plot on average log reduction for 588 *Listeria* isolates (x-axis) after exposure to BC (A) and PAA (B) and average log reduction for 129 *Listeria* isolates (x-axis) after exposure to NaOCl (C). Grey shaded area represents *L. monocytogenes* and brown shaded area represents *Listeria* spp.

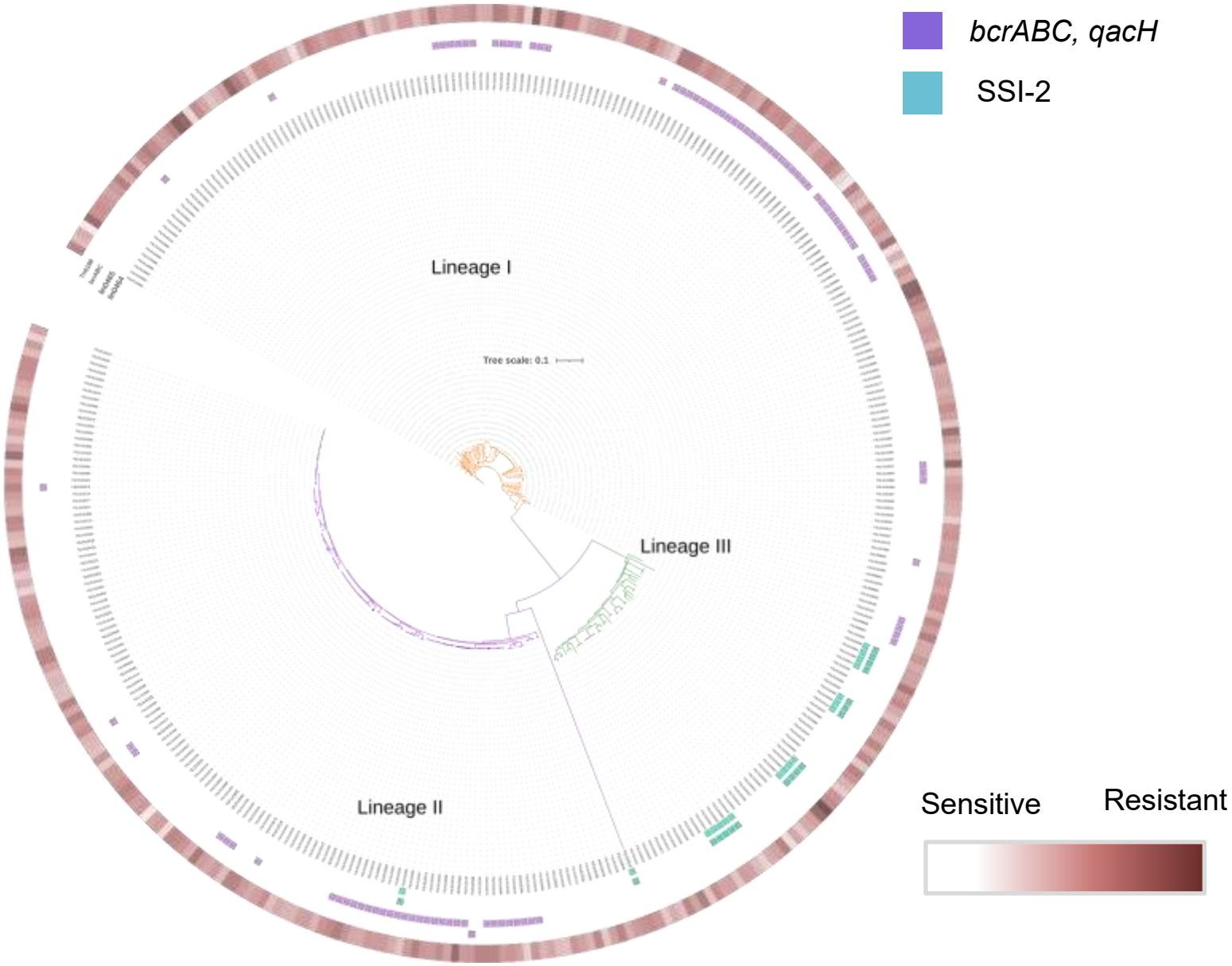

Figure 2: *Listeria monocytogenes* maximum-likelihood tree based on core SNP analysis using kSNP. The phylogeny for strains was inferred using RAxML and tree was rooted by midpoint. The tips are labeled with Food Safety Lab strain ID (FSL) and are followed by squares to indicate gene presence/absence (green represents SSI-2, and purple represents quat tolerance genes *bcrABC* and *qacH*). Outer circle represents the range of phenotypic response of an isolate to 300ppm BC; the darker the shade the less log reduction was observed. The bar indicates 0.1 substitutions per site. The data displayed suggests that presence or absence of quat resistance genes does not predict an isolate's survival at use level concentration.

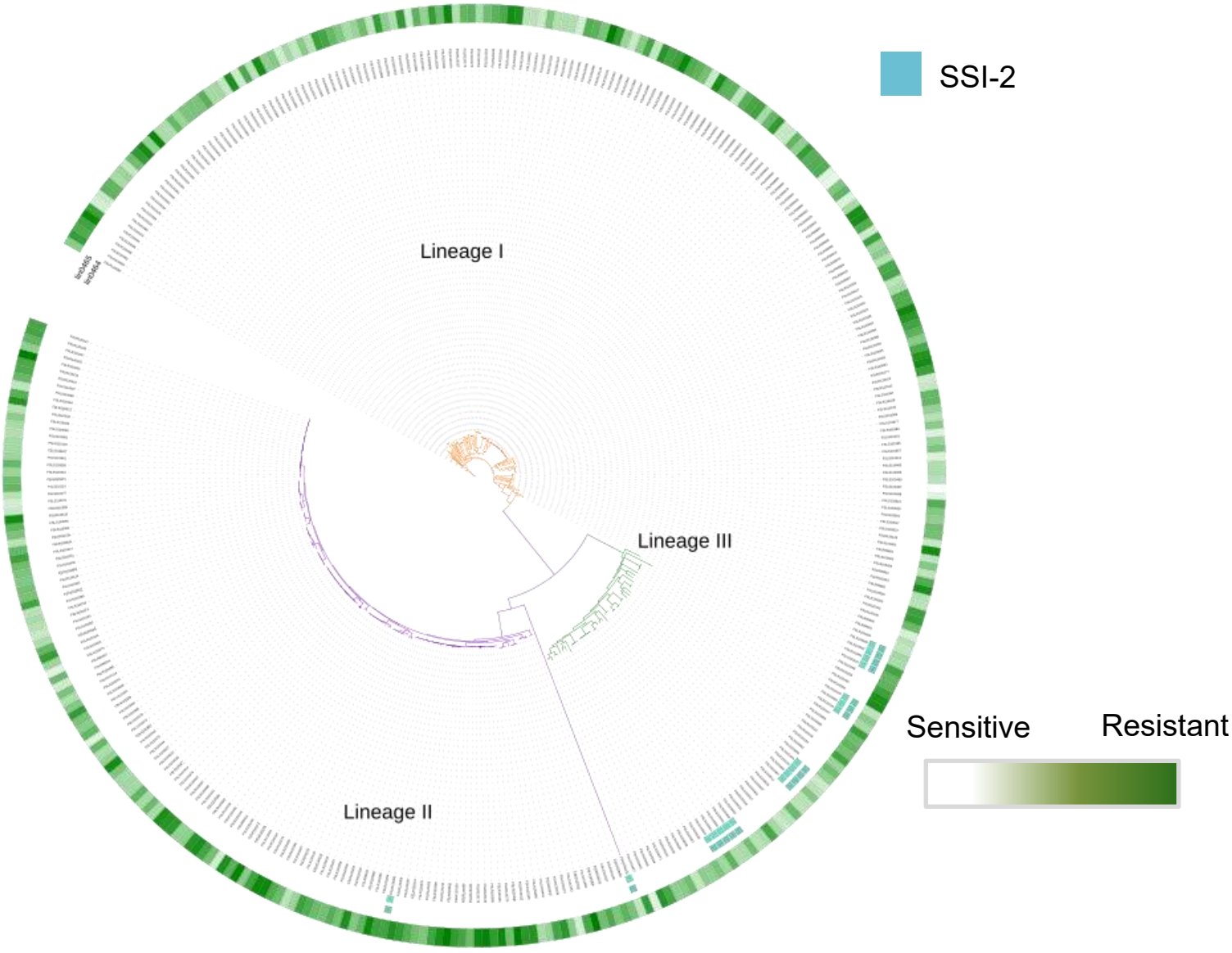

Figure 3: *Listeria monocytogenes* maximum-likelihood tree based on core SNP analysis using kSNP. The phylogeny for strains was inferred using RAxML and tree was rooted by midpoint. The tips are labeled with Food Safety Lab strain ID (FSL) and are followed by squares to indicate gene presence/absence (green represents SSI-2, and purple represents quat tolerance genes *bcrABC* and *qacH*). Outer circle represents the range of phenotypic response of an isolate to 80ppm PAA; the darker the shade the less log reduction was observed. The data displayed suggests that presence or absence of oxidative stress resistance genes SSI-2 does not predict an isolate's survival at use level concentration.

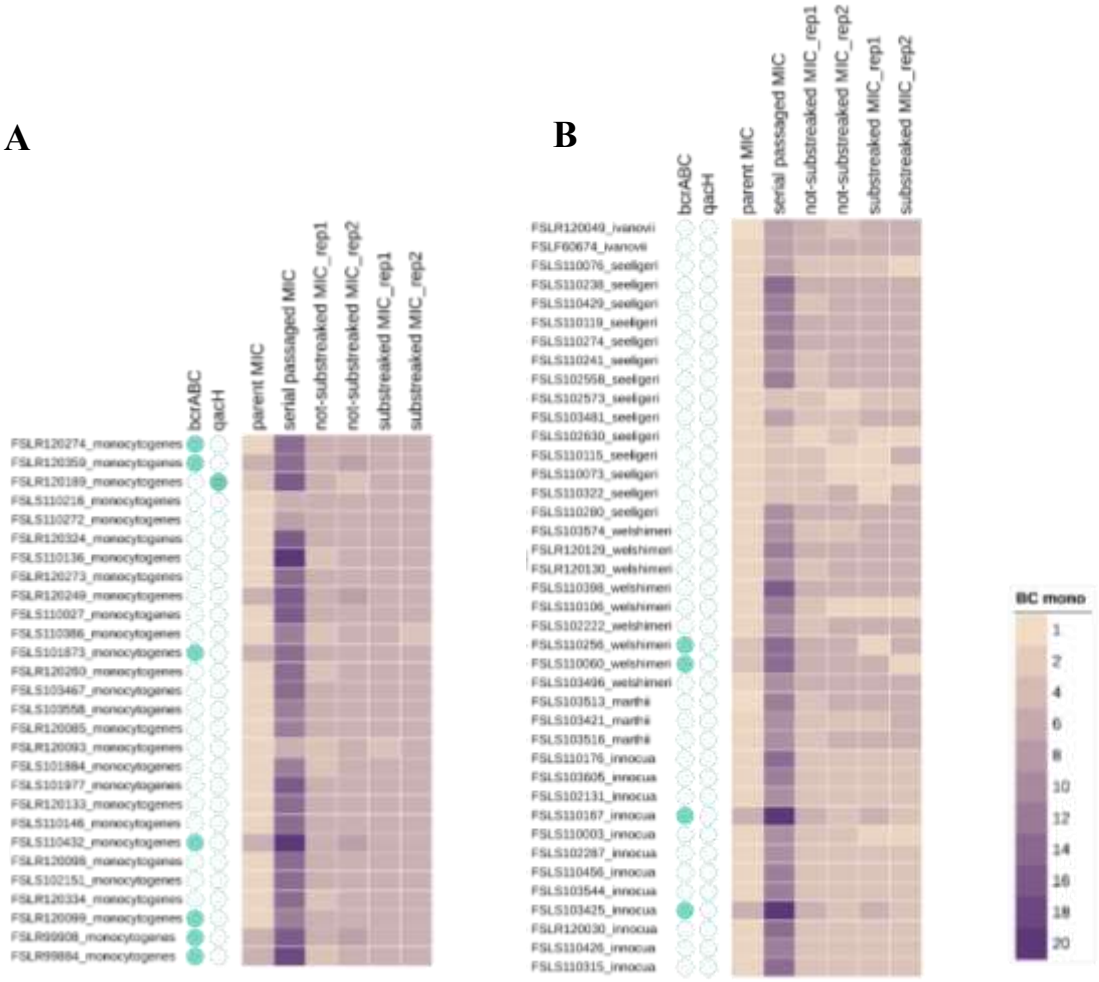

Figure 4: MIC values for *L. monocytogenes* on the left (A) and *Listeria* spp. on the right (B) isolates grown in BHI and BC. *BcrABC* and *qacH* presence is indicated by filled circles. BC concentrations are provided by legend on the right in mg/L; darker shaded areas indicate growth at higher concentration of BC.

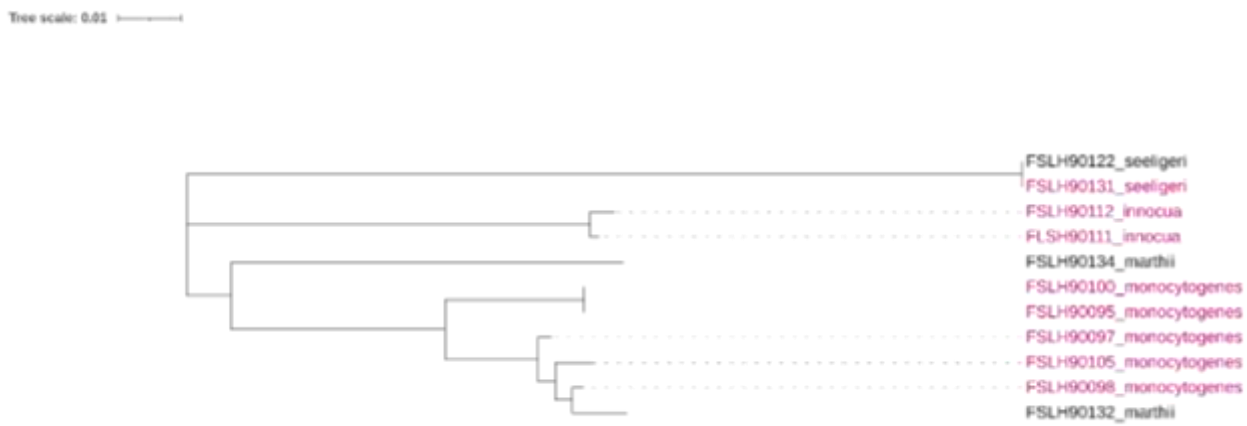

Figure 5: Neighbor-joining tree built with nucleotide sequences encoding for tetR from substreaked *Listeria* isolates in which single nucleotide mutations were identified. Pink tip label indicates premature stop codon in *tetR*.

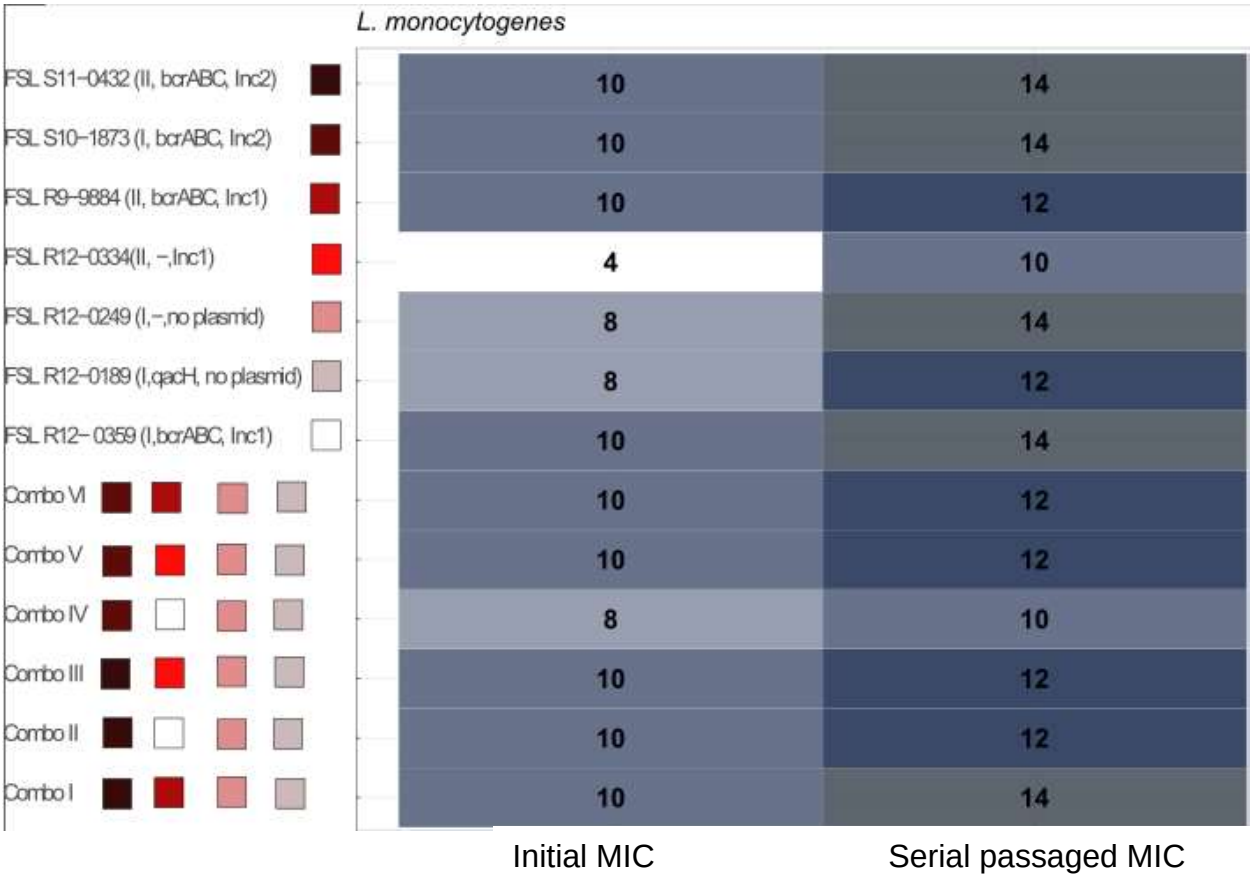

Figure 6: Seven *Listeria* were assessed in single culture for their initial MIC (first seven rows and first column). Strains are labeled with Food Safety Lab ID, information in parenthesis indicate lineage, genotype (*bcrABC, qacH* or absence indicated by "-") and plasmid incompatibility or plasmid absence. Six combination of four strains were incubated together; colors indicate strain combinations. Single cultures and co-cultures were serial passaged and MIC values are listed in the right column.

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