**AGRICULTURAL RESEARCH FOUNDATION**
**FINAL REPORT**
**FUNDING CYCLE 2020 – 2022**

**TITLE:** Generating new insights into antibiotic and sanitizer resistance development in *Listeria* species isolated from produce handling and processing environments in the Pacific Northwest

**RESEARCH LEADER:** Jovana Kovacevic (PI), Stephanie Brown (co-PI), Joy Waite-Cusic (co-PI)

**COOPERATORS:** None.

**EXECUTIVE SUMMARY:** When vulnerable populations, such as elderly, young children, pregnant women and their fetuses contract listeriosis disease, 20-40% cases lead to mortality unless they are promptly treated with antibiotics that are effective against *Listeria monocytogenes*, a causative agent of listeriosis. These microorganisms are commonly found in food processing facilities, where they become exposed to sanitizers. There is mounting evidence that a level of cross-resistance between antibiotics and sanitizers used in food processing (e.g., quaternary ammonium compounds [QACs]) exists. However, the mechanisms that lead to reduced susceptibility to sanitizers or antibiotics following exposure to one or the other (i.e., cross-resistance), in *Listeria* spp. are not well defined. If the repeated exposure to sanitizers leads to tolerance to sanitizers and cross-resistance to antibiotics, we will not be able to effectively control *L. monocytogenes* in these environments and treat invasive listeriosis infections. The aim of the proposed research was to investigate the mechanisms behind antimicrobial resistance (AMR) development in *L. monocytogenes* recovered from produce handling and processing environments. These findings provide further insight into the ecology and characteristics of *Listeria* isolates recovered from agricultural environments. These data also help further our understanding of cross-resistance in the context of produce handing and processing environments and provide insight into the future state of antibiotic use to treat listeriosis.

**OBJECTIVES:**
1) Determine prevalence of AMR genetic markers in *Listeria* spp. isolates (n=72) from produce processing and handling environments using whole genome sequencing (WGS);
2) Investigate the distribution of AMR genetic markers within clonal complexes of *Listeria monocytogenes* isolates (n=48) to guide hypothesis development around transfer risk;
3) Identify genomic changes that occur in *L. monocytogenes* following sanitizer exposure and their impact on antibiotic cross-resistance.

**PROCEDURES:**
**Whole genome sequencing of *Listeria monocytogenes.*** Genomic DNA was extracted from 72 *Listeria* spp. isolates using the DNeasy Blood and Tissue kit (QIAGEN, USA) according to the

manufacturer's instructions for Gram positive bacteria. Quality was measured using the HS dsDNA assay kit (Fisher; Waltham, MA, USA) on the Qubit fluorometer (Fisher; Waltham, MA, USA). The quality of genetic material was determined using the nanodrop spectrophotometer (Fisher; Waltham, MA, USA). DNA libraries were prepared at Oregon State University Center for Quantitative Life Sciences (Corvallis, OR, USA) using the PlexWell kit (seqWell; Beverly, MA, USA) according to the manufacturer's instructions. Libraries were sequenced using 2 × 150-bp paired-end sequencing on the Illumina HiSeq platform. (Illumina, San Diego, CA, United States). Raw reads were quality checked with FastQC (v 0.11.9; http://www.bioinformatics.babraham.ac.uk/projects/fastqc), followed by low-quality trimming using Trimmomatic (v 0.39). Trimmed reads were *de novo* assembled using SPAdes (v 3.14.1) optimized with Unicycler (v 0.4.8). Resulting assembly files were assessed for quality and annotated with Prokka (v 1.12).

**Screening for genetic markers of antimicrobial resistance (AMR).** Genome assemblies (n=72) were screened for the presence/absence of genes encoding for AMR using BLAST+, PATRIC and ResFinder databases. Genes associated with tolerance towards sanitizers (e.g., *bcrABC, emrE, emrC, tetR, tnpABC, qacC, qacH*) were screened using the BLASTN algorithm with a minimum nucleotide identify and alignment length coverage of 80% (Hurley et al., 2019).

**MLST and cgMLST.** Classical multi-locus sequence typing (MLST) data according to Ragon et al. (2008) were *de novo* extracted from the whole genome sequence of *L. monocytogenes* (n = 48) isolates. Assembled sequences of seven housekeeping genes (i.e., *abcZ, bglA, cat, dapE, dat, ldh and lhkA*) were uploaded to *L. monocytogenes* MLST database (http://www.pasteu r.fr/mlst), maintained by the Institut Pasteur. Each sequence of seven resulting alleles was assigned an MLST profile or sequence type (ST), after comparison to the online database. Novel alleles and novel sequence types were forwarded to Institut Pasteur for designation of allele and ST numbers. Grouping of STs into clonal complexes (CC) was based on the scheme set by the Institut Pasteur, where STs belonging to the same CC do not have more than one allelic mismatch (Ragon et al., 2008). Core genome MLST (cgMLST) was performed in the BIGSdb-*Lm* platform, using a scheme consisting of 1748 conserved core genes. After comparing isolates to cgMLST profiles already in the database, assemblies of all isolates were submitted to the *Listeria* Pasteur database to obtain cgMLST type (CT) assignments.

**Minimum inhibitory (MICs) and minimum bactericidal concentrations (MBCs).**
A microbroth dilution assay described by Bland et al. (2022) was used to determine both MIC and MBCs of a commercial quaternary ammonium compound (cQAC; 1–6 ppm; Professional Lysol No Rinse Sanitizer; EPA registration 675-30; Reckitt Benckiser, Parsippany, NJ, United States). Stock solution of the cQAC sanitizer was prepared in accordance with the manufacturer recommended concentration (MRC; 200 ppm), filter sterilized, and stored for up to 1 week at 4◦C.

**Experimental adaptations to sublethal concentrations of sanitizer.** A total of six *L. monocytogenes* isolates were selected for adaptation to a commercial cQAC sanitizer (Table 1). All isolates were previously serogrouped, multi-locus sequence typed and assessed for AMR using a standard disk diffusion assay (CLSI, 2015; Jorgensen et al., 2021). Isolates used for

**Table 1 |** Genetic diversity and AMR profiles of *Listeria monocytogenes* isolates selected for evaluation of cross-resistance.

| Isolate ID | Sequence type (ST) | Clonal complex (CC) | AMR profile[a] |
|---|---|---|---|
| WRLP354 | 2 | 2 | CLI |
| WRLP380 | 2 | 2 | AMP, CIP, PEN |
| WRLP394 | 219 | 4 | CLI |
| WRLP483 | 219 | 4 | PEN, NOV |
| WRLP530 | 6 | 6 | CLI |
| WRLP533 | 6 | 6 | CLI |

[a]AMP, ampicillin; CLI, clindamycin; NOV, novobiocin; PEN, penicillin.

adaptation were selected based on their AMR profiles and MLST. Specifically, up to two isolates belonging to each available hypervirulent clonal complex (CC) 2, CC4, and CC6 (Maury et al., 2016), and with unique AMR profiles (when available) were included. Isolates were stored at –80°C in trypticase soy broth (TSB; Acumedia, Neogen, Lansing, MI, United States) with 25% (v/v) glycerol. Prior to use, isolates were resuscitated on TSA with incubation at 35°C for 24 h and used for a maximum of two weeks.

All six strains were experimentally adapted to increasing concentrations of cQAC sanitizer. Strains were sub-cultured at 30°C with progressively higher concentrations of cQAC, alternating between TSB-YE (2 ml, 150 rpm shaking), and TSA-YE plates (Figure 1). Adaptations started in TSB-YE at a concentration of 1 ppm. Once the culture was visibly turbid, 10 µl was transferred into fresh TSB-YE and TSA-YE media with cQAC (2 ml total volume for TSB-YE and 15 ml for TSA-YE plates). Concentration of cQAC present in the media was increased by 1 ppm following stabilization of each incremental adaptation in TSB-YE. Once growth was observed on TSA-YE, a single colony was transferred to TSB-YE at the same concentration to stabilize the adaptation at each increment. Adaptations were stopped when no growth was visually observed after 5 days of incubation at 30◦C in TSB-YE. The adaptations scheme used in the isolates reported here is depicted in Figure 1. For each cQAC concentration adaptation (1, 2, and 3 ppm), strains were stabilized by five passages in TSB-YE with appropriate cQAC concentration. Adapted strains were frozen in TSB supplemented with cQAC at half the adapted concentration with 25% (v/v) glycerol. TSA-YE supplemented with 3 ppm cQAC was used to revive cQAC-adapted cultures from frozen stock for use in the following assays.

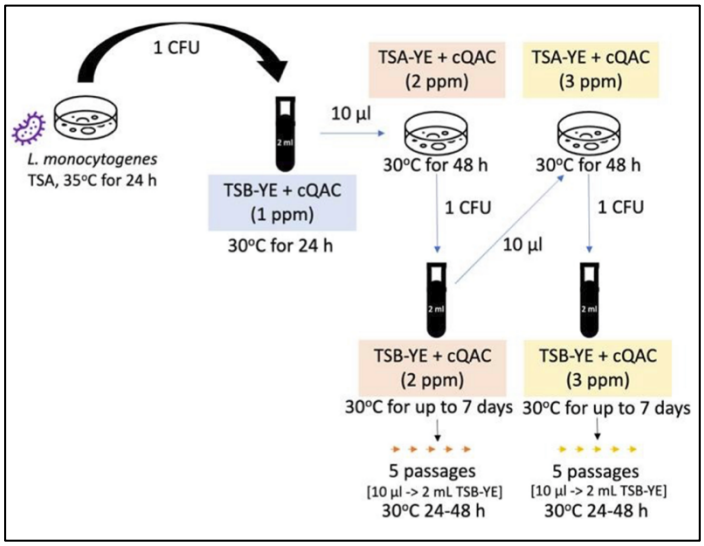



**Figure 1 |** Adaptation scheme of *L. monocytogenes* to minimal concentrations of cQAC.

**Antibiotic susceptibility disk diffusion assay.** Following adaptation to cQAC, disk diffusion assays were used to determine if and how the adaptation affected sensitivity to a panel of 17 antibiotics (BBL Sensi-Disk, BD Diagnostic, Sparks, MD, United States). Antibiotics tested

included: amikacin (AMK; 30 µg), ampicillin (AMP; 10 µg), cefoxitin (FOX; 30 µg), chloramphenicol (CHL; 30 µg), ciprofloxacin (CIP; 5 µg), clindamycin (CLI; 2 µg), erythromycin (ERY; 15 µg), gentamicin (GEN; 10 µg), kanamycin (KAN; 30 µg), novobiocin (NOV; 30 µg), penicillin G (PEN; 10 µg), rifampicin (RIF; 5 µg), streptomycin (STR; 10 µg), cotrimoxazole (SXT; 1.25/23.75 µg), imipenem (IMP; 10 µg), tetracycline (TET; 30 µg), and vancomycin (VAN; 5 µg). These assays were carried out as described in Jorgensen et al. (2021). In all assays *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC25923 were used as control strains. Resulting diameters were compared with previously determined zones of inhibitor for the WT strains described by Jorgensen et al. (2021).

**Whole genome sequencing of cQAC adapted *L. monocytogenes* isolates.** Following adaptation and susceptibility assays, adapted isolates were sequenced and evaluated for single nucleotide polymorphisms to determine genetic changes. DNA was extracted as described above. Whole genome sequencing libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States), according to the manufacturer's protocol. Paired-end sequencing (2 x 150 bp) was performed on the Illumina MiSeq at the Center for Quantitative Life Sciences (Oregon State University, Corvallis, OR, United States). Raw sequence reads were processed as described above for wild type strains. Mutations were identified by mapping the reads of the adapted isolates to draft assemblies of the respective wild-type following protocol described by Bland et al. (2022).

**SIGNIFICANT ACCOMPLISHMENTS:**

Despite the hurdles and delays presented by the Covid-19 pandemic, significant work has been accomplished, with data disseminated through both professional conferences and peer reviewed publications. The findings from this study have been published in three peer reviewed publications:


Bland, R., A. J., Weisberg, E. R. Riutta, J. H. Chang, J. G. Waite-Cusic, J. Kovacevic. 2022. Adaptation to a commercial quaternary ammonium compound sanitizer leads to cross-resistance to select antibiotics in *Listeria monocytogenes* isolates from fresh produce environments. *Front. Microbiol.* 12 (782920); https://doi.org/10.3389/fmicb.2021.782920.

Bland, R., J. D. Johnson, A. J., Weisberg, E. R. Riutta, J. H. Chang, J. G. Waite-Cusic, J. Kovacevic. 2021. Application of whole genome sequencing to understand diversity and presence of genes associated with sanitizer tolerance in *Listeria monocytogenes* from produce handling sources. *Foods.* 10(10), 2454; https://doi.org/10.3390/foods10102454.

Jorgensen, J., R. Bland, J. Waite-Cusic, J. Kovacevic. 2021. Diversity and antimicrobial resistance of *Listeria* spp. and *L. monocytogenes* clones from produce handling and processing facilities in the Pacific Northwest. *Food Control* https://doi.org/10.1016/j.foodcont.2020.107665.


Results have also been shared via posters presented at the International Association for Food Protection Annual Conference (2020 virtual format; 2021 Phoenix, AZ) and the American Society for Microbiology Next Generation Sequencing Conference (2020 virtual format).

**Objective 1:** *Determine prevalence of AMR genetic markers in* Listeria *spp. (n=72) from produce processing and handling environments by whole genome sequencing (WGS).*

Two *L. monocytogenes* (WRLP370 and WRLP410) isolates harbored the *bcrABC* cassette, which is known to assist in tolerance towards quaternary ammonium compounds. None of the other genes associated with sanitizer tolerance were observed in the tested isolates. Genes associated with cadmium resistance (*cadA1* and *cadA2*) were found in 33/48 of the isolates. The presence of the five-gene stress survival islet 1 (SSI-1), associated with increased tolerance to low pH and high salt stress, was found in 4/48 isolates (WRLP367, WRLP370, WRLP410, WRLP360).

**Objective 2:** *Investigate the distribution of AMR genetic markers within clonal complexes of* Listeria monocytogenes *isolates (n=48) to guide selection of isolates in final objective.*

*L. monocytogenes* isolates (n=48) were grouped into 10 MLST profiles (ST) belonging to 10 different clonal complexes (CC). Core genome MLST (cgMLST) further differentiated the 48 isolates into 16 cgMLST types (CT). Genetic indicators of tolerance towards quaternary ammonium compound (*bcrABC*) were only present in Lineage 2-ST155-CC155-CT9497 isolates. Cadmium resistant determinants were strictly found in lineage 1 isolates (33/48; CC2, CC4, CC6, CC388, CC688, CC345). Twenty-six isolates belonged to CC2, CC4 or CC6, previously described as hypervirulent clonal complexes (Maury et al., 2016).

Due to the high prevalence of hypervirulent CCs within the sample set and the clinical relevance of isolates that may have increased virulence, two isolates belonging to each of these CC's were chosen to further investigate cross-resistance potential.

*Results from objectives 1 and 2 are detailed in Jorgensen et al. (2021) and Bland et al. (2021) publications.*

**Objective 3:** *Identify genomic changes that occur following sanitizer exposure that could lead to cross resistance in* Listeria monocytogenes.

Following adaptation assays, all isolates were able to tolerate an additional 1 ppm of the commercial quaternary ammonium compound confirmed by MIC (increased from 2 to 3 ppm) and MBC (increased to 3 to 4 ppm). In the presence of reserpine, a known efflux pump inhibitor, the MIC and MBC of all cQAC adapted strains (qAD) revered back to the wild-type (WT) phenotype (Table 2). The effect of reserpine on the phenotypic tolerance of these

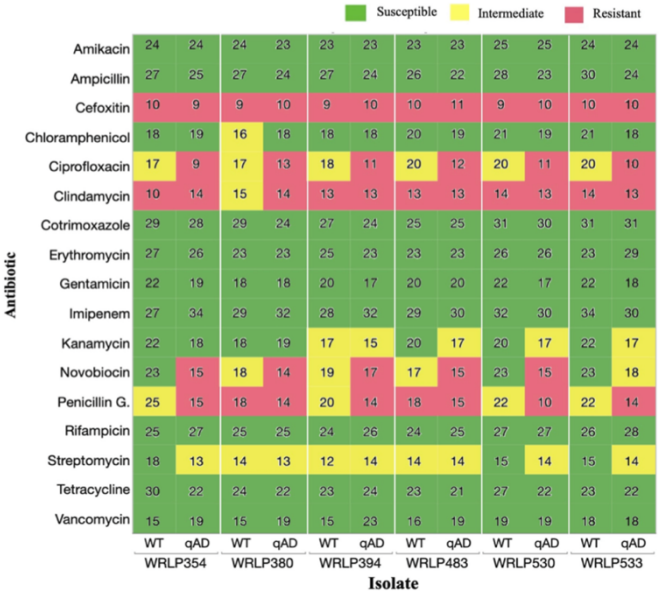
**Table 2 |** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of a commercial quaternary ammonium compound sanitizer (cQAC) for wild-type (WT) and cQAC-adapted (qAD; 3 ppm) *L. monocytogenes* strains in the absence/presence of reserpine (+R).

| Isolate no. | Concentration (ppm)[a] | | | |
|---|---|---|---|---|
| | WT[b] | WT + R[c] | qAD | qAD + R |
| WRLP354 | | | | |
| MIC[b] | 2 | <1 | 3 | 2 |
| MBC[b] | 3 | 3 | 4 | 3 |
| WRLP380 | | | | |
| MIC | 2 | <1 | 3 | 2 |
| MBC | 3 | 3 | 4 | 3 |
| WRLP394 | | | | |
| MIC | 2 | <1 | 3 | 2 |
| MBC | 3 | 3 | 4 | 3 |
| WRLP483 | | | | |
| MIC | 2 | <1 | 3 | 2 |
| MBC | 3 | 3 | 4 | 3 |
| WRLP530 | | | | |
| MIC | 2 | <1 | 3 | 2 |
| MBC | 3 | 3 | 4 | 3 |
| WRLP533 | | | | |
| MIC | 2 | <1 | 3 | 2 |
| MBC | 3 | 3 | 4 | 3 |

[a] *Manufacturer recommended concentration (MRC) for the cQAC is 200 ppm.*
[b] *Reported MIC and MBC tested in stepwise increments over three biological replicates.*
[c] *In the presence of reserpine, WT isolates did not grow at the lowest dose of 1 ppm cQAC.*

isolates to cQAC suggests the role efflux pumps play in inherent tolerance and adaptive tolerance towards quaternary ammonium compounds.

**Figure 2 |** Antibiotic susceptibility of wild-type (WT) and cQAC adapted (qAD) *Listeria monocytogenes* strains (n = 6) to 17 antibiotics. Values reported represent inhibition zone diameters measured in mm. For adapted isolates, the median of 2-3 independent replicates is reported.




A shift in antibiotic susceptibility to 7/17 tested antibiotics (CHL, CIP, CLI, KAN, NOV, PEN, and STR) was seen in isolates following adaption to cQAC (Figure 2). The observed shifts varied between each isolate with no obvious differences between the three CCs (CC2, CC4, and CC6) that were evaluated. WRLP530 and WRP533 exhibited changes in susceptibility to 5/17 antibiotics, WRLP354 had profiles changes for 4/17 antibiotics, and the last three isolates (WRLP380, WRLP394, and WRLP483) had susceptibility shifts for 3/17 antibiotics. Shifts in CIP susceptibility were seen across all six tested isolates prompting further investigation. The MIC of CIP was measured for both WT and adapted isolates to confirm the changes observed in the disk diffusion assay. Isolate pairs had CIP MIC differences ranging from 0.5 to 2 µl/ml and 1 to > 8 µl/ml, confirming that susceptibility to CIP was effected across all isolates following adaptation.

Following sequence comparison of each adapted and WT pair, between 0 and 4 nucleotide deletions and SNPs were identified. Some of these were located in non-coding regions on the genome. While WRLP354 was not found to have any high quality SNPs or deletions, the remaining five isolates had SNPs and/or deletions in the transcriptional regulator gene *fepR*. This gene is predicted to encode for a transcriptional regulator FepA, a multidrug efflux pump. In WRLP380, WRLP483, and WRLP533 the mutation was present as a SNP, leading to a change in an amino acid. In WRLP394 and WRLP483, the SNP led to a premature stop codon (PMSC) at position 141 and 175, respectively, truncating the protein by 55 and 21 amino acids, respectively. In three of the isolates ( WRLP394, WRLP530, and WRLP533), deletions between 1 to 33 bp were observed. The mutations found in *fepR* suggest that loss of *fepR* function plays an important role in *L. monocytogenes* adapting to cQAC; however, confirmation of its role would require deletion and complementation mutants.

*Results from Objective 3 are described in Bland et al. (2022) publication.*

**References:**

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol*, *23*(3-4), 277-294. https://doi.org/10.1016/0168-1605(94)90157-0

Bland, R. N., Johnson, J. D., Waite-Cusic, J. G., Weisberg, A. J., Riutta, E. R., Chang, J. H., & Kovacevic, J. (2021). Application of whole genome sequencing to understand diversity and presence of genes associated with sanitizer tolerance in *Listeria monocytogenes* from produce handling sources. *Foods*, *10*(10). https://doi.org/10.3390/foods10102454

Bland, R., A. J., Weisberg, E. R. Riutta, J. H. Chang, J. G. Waite-Cusic, J. Kovacevic. 2022. Adaptation to a commercial quaternary ammonium compound sanitizer leads to cross-resistance to select antibiotics in *Listeria monocytogenes* isolates from fresh produce environments. *Front Microbiol 12*(782920); https://doi.org/10.3389/fmicb.2021.782920.

Hurley, D., Luque-Sastre, L., Parker, C. T., Huynh, S., Eshwar, A. K., Nguyen, S. V., Andrews, N., Moura, A., Fox, E. M., Jordan, K., Lehner, A., Stephan, R., & Fanning, S. (2019). Whole-genome sequencing-based characterization of 100 *Listeria* monocytogenes isolates collected from food processing environments over a four-year period. *mSphere*, *4*(4). https://doi.org/10.1128/mSphere.00252-19

Jorgensen, J., Bland, R., Waite-Cusic, J., & Kovacevic, J. (2021). Diversity and antimicrobial resistance of *Listeria* spp. and *L. monocytogenes* clones from produce handling and processing facilities in the Pacific Northwest. *Food Control*, *123*(107665).

Maury, M. M., Tsai, Y. H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., Criscuolo, A., Gaultier, C., Roussel, S., Brisabois, A., Disson, O., Rocha, E. P. C., Brisse, S., & Lecuit, M. (2016). Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nat Genet*, *48*(3), 308-313. https://doi.org/10.1038/ng.3501

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., & Brisse, S. (2008). A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog*, *4*(9), e1000146. https://doi.org/10.1371/journal.ppat.1000146

**BENEFITS & IMPACT:**

Our work has shown that *L. monocytogenes* from produce handling environments remain sensitive to clinically relevant antibiotics. In addition, the overall prevalence of genes associated with tolerance towards QAC sanitizers was low across the tested isolates. However, our data demonstrated the potential for *L. monocytogenes* isolates to develop cross-resistance between a commercial QAC sanitizer and antibiotics representing different classes. While the increased minimum inhibitory and minimum bactericidal concentration of QAC-adapted isolates remained well below the manufacturer recommended concentration for the commercial product, it is not uncommon for bacterial cells to be exposed to lower or sublethal concentrations of sanitizers in the processing environment either by dilution, presence of organic matter (e.g., decreasing the efficacy of the sanitizer), or issues with the hygienic design of equipment or facility (e.g., resulting in microbial niches, biofilm formation or dilution effect of sanitizer). Our data highlight that sublethal exposures to cQACs could have deleterious effects if adapted *L. monocytogenes* strains become implicated in human illness that requires antibiotic treatment. The potential for *L. monocytogenes* to develop cross-resistance to

clinically relevant antibiotics following minimal adaptation to a formulated QAC product is especially concerning. In particular, this trend amongst genotypes that are classified as hypervirulent and frequently involved in cases of listeriosis highlights the need to better understand the effect that sanitizer exposures and low-level adaptations may have on the AMR development and a potential public health risk.

**ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:** USDA NIFA CARE (2021-2024).

**FUTURE FUNDING POSSIBILITIES:** Center for Produce Safety