PennState
College of Agricultural Sciences

PROPOSAL TRANSMITTAL

Date: 12/13/2018 **PSU Ref. No: 206091**

Title: Combating Listeria Monocytogenes Growth in Tree Fruit Packinghouse Biofilms

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Proposed Project **5/1/2019 - 4/30/2020** **Total Project Request: $16,486**

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Title: Combating *Listeria monocytogenes* Growth in Tree Fruit Packinghouse Biofilms

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Duration of Project: One year (5/1/19-4/30/20), new project.

Justification:
In this proposal, we are addressing the SHAP Research Priority "Listeria/ Pathogen Mitigation" by investigating the effectiveness of biocontrol strains to modify microbial ecology and inhibit growth of *Listeria monocytogenes* (Lm) in biofilms made of tree fruit packinghouse environmental microbiomes (i.e., all microorganisms present in the environment). Within the last 2 years, we have been working with tree fruit packers to determine the occurrence of Lm at locations within packing houses. Our results to date demonstrate that eradicating this pathogen with conventional sanitation measures is difficult, especially where there is unhygienic and difficult-to-clean equipment. Thus there is a need to evaluate novel treatments that could mitigate the problem. The results from this proposed project will provide new information on the feasibility of altering the microbial ecology of tree fruit (e.g., apple) packinghouses using added biocontrol strains as a means to reduce the occurrence of Lm. This promising new biocontrol approach has been proven effective in other food industries but has not yet been tested in produce packing facilities.

It is estimated that out of 1,591 cases of human listeriosis occurring each year in the US, over 15.9% are fatal[(1)]. Prior to a 2014 listeriosis outbreak[(2)], caramel apples were not considered to be risky foods due to the acidic pH (~3-5) of the fruit and low water activity (i.e., free water available for bacterial growth) of the caramel coating. However, subsequent studies have shown that puncturing apples with a stick followed by applying a caramel coating creates a microenvironment suitable for growth of Lm[(3)]. This has accelerated concerns within the tree fruit industry on the potential impact of recent government food safety regulations on the tree fruit industry. In particular, under the Food Safety Modernization Act (FSMA), tree fruit and other produce industries are required to comply with new food safety standards for growing and packing fresh produce. Since Lm is widespread in the soil, on vegetation, in water, on silage and in animal feces, it is commonly introduced into produce processing facilities. It is therefore critical to reduce levels and eliminate this pathogen from food processing environments in order to prevent its transmission to food[(2)]. Despite considerable efforts, this has proven to be challenging, since Lm is unique among human pathogens in that it can colonize indoor food processing facilities that are continuously moist and cool, and where an ample supply of nutrients (food debris) is available (e.g., produce packinghouses and long-term storage facilities). Lm is particularly challenging to eradicate with sanitizers at locations where it can hide in difficult to clean equipment parts and in the facility environment (e.g., roller brushes in tree fruit packinghouses, cracks in the floors). Lm can grow in protective biofilms where they are

supported by the presence of other environmental microorganisms. It is also known that that certain microbial species can suppress Lm growth. **We therefore propose to combat Lm in tree fruit packinghouses using a biological control strategy**. Specifically, we will **assess the effectiveness of certain bacterial strains that are known to inhibit Lm growth in processing facility environments.**

Despite the poor ability of Lm to form monoculture biofilms, it has recently been shown that other microorganisms can facilitate Lm biofilm formation in mixed culture. For example, *Pseudomonas fluorescens* can increase Lm attachment to surfaces (up to 1-2 log increases) and Lm can in turn enhance *P. fluorescens* extracellular matrix (slime) production[4]. Given that *Pseudomonas* spp. are abundant in food processing environments[5,6], *Pseudomonas* spp. may facilitate Lm persistence in such facilities by protecting it enclosed in environmental biofilms[7]. Once the biofilm composed of facility environment microbiome and Lm is formed, it is challenging to eliminate Lm using standard cleaning and sanitizing procedures[8–10]. The challenge with removal of biofilms is even greater when they form and persist in difficult to clean areas, such as roller brushes in tree fruit packinghouses, and in floor cracks or deep within equipment. We therefore aim to study the feasibility of using certain strains of other bacterial species to control Lm through a well-established microbiological principle known as competitive exclusion. Over time, these biofilm forming microbes can establish themselves in difficult to clean areas where they can compete for nutrients that would otherwise be available to support Lm growth, and/or produce secondary metabolites (e.g., bacteriocins) that can inhibit Lm. This approach has been proven to be successful in meat processing facilities[11–13], as well as a pet food processing facility (unpublished data reported by industry at the 2018 International Association of Food Protection annual meeting).

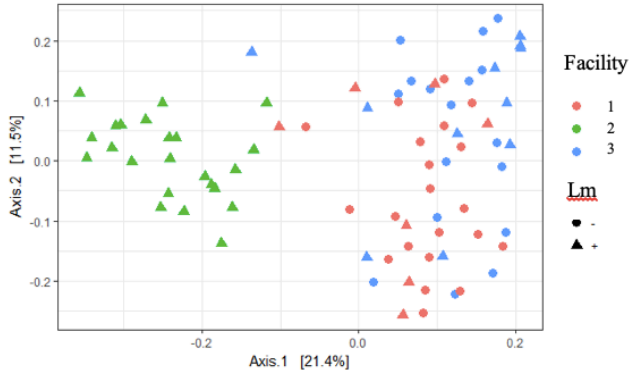

Fig. 1: Differences in microbiomes collected over 6 months in tree fruit packing facility with persistent clone of Lm (#2; green) and facilities with sporadic, transient Lm presence (#1; red, #3; blue).

Within the past 2 years, we have established trusted relationships with three tree fruit packinghouses in PA where we have been determining the occurrence of Lm and monitoring environmental microbiomes and their association with Lm. We found distinct environment microbiomes in facilities with persisting Lm (Facility 2) compared to those with sporadically detected Lm (Facilities 1 and 3; see Fig. 1). Microbiomes in Facility 2 also had lower overall microbiome diversity and had a higher abundance of microbial groups that are known biofilm formers (e.g., Pseudomonadaceae). This indicates that the microbiome in the food processing environment may play an important role in protecting Lm enclosed in a microbiome biofilm. We therefore aim to investigate the efficacy of specific nonpathogenic microbial strains that have a Lm reduction effect. This strategy, demonstrated in other food industries, could provide valuable data for the industry that could lead to alternative strategies for controlling this pathogen. The outputs of this project will be shared with industry members through Penn State Extension site visits, speaking events at industry conferences, and training workshops. If the results show merit in our *in vitro* study, these interventions may be tested in the future in actual facility environments.

Objective(s):
Obj. 1: Characterize biofilms grown from tree fruit packinghouses microbiome.
Obj. 2: Evaluate specific strains of nonpathogenic microbial species on their ability to inhibit growth of Lm.

Procedure(s):
Obj. 1: Characterize biofilms grown from tree fruit packinghouses microbiome.
1.1 Sample collection. Three apple packinghouses with which we are already collaborating will be visited twice to collect samples for biofilm assays and microbiome characterization. Five environmental samples will be collected per facility, in areas that had been previously identified as high-risk areas in terms of Lm contamination (e.g. areas underneath the brush rollers). One of the facilities has a persisting problem with Lm and two show only sporadic presence of transient Lm. One sample will be used for microbiome characterization before biofilm formation and four samples will be collected for biofilm growth and for testing the inhibitory effects on Lm growth in these biofilms (i.e., negative control, positive control, biocontrol strain 1 and strain 2). Microbiomes of biofilms grown as outlined in Obj. 2 will be characterized in the same way as microbiomes prior to biofilm formation.

Samples will be collected using 3M sponges hydrated with neutralizing buffer from 40cm X 40 cm areas. Samples collected for biofilm experiments will be processed within 24 h from collection. Samples will be stored on ice in a cooler during the transportation to the lab. Samples collected for the microbiome analyses will be processed as described in 1.2 and those collected for biofilm experiments will be processed as described in 2.2.
1.2 DNA extraction for microbiome analysis. Each environmental sponge sample collected for microbiome characterization will be homogenized with 50 ml of phosphate buffer containing 0.9% NaCl in a stomacher, for 7 minutes at 230 rpm. Fifty milliliters of the homogenate will be transferred to a sterile 50 ml conical tube and centrifuged at 11,000 g and 4 ºC for 20 minutes. After centrifugation, the supernatants will be discarded and pellets will be stored at -80 °C until DNA extraction. DNA will be extracted from approximately 0.25 g sample using Qiagen DNeasy PowerSoil DNA extraction kit. Approximately 0.25 g of sterile sponge (representative of a production lot) will also be processed, as a negative control, to confirm absence of microbial DNA contaminants on the sterile sponge. Concentration of DNA will be determined with both Nanodrop One and Qubit 3 using Qubit dsDNA High Sensitivity (HS) Assay Kit. DNA samples will then be shipped to the Penn State Genomics Core Facility for 16S rRNA (V4) gene amplification, library preparation and metagenomics sequencing in a single Illumina MiSeq that will provide enough 250 x 250 bp paired end reads per sample.
1.3 Sequence analyses and OTU normalization. Sequences will be analyzed with Mothur v1.39.5 pipeline that we are currently using in our lab. Paired end sequence reads will be assembled into contigs. Contigs will be filtered based on the sequence length. Chimera will be discarded using UCHIME algorithm and the remaining sequences will be assigned taxonomy using Silva database. Operational Taxonomic Units (OTUs) will be calculated using opticlust with 97% similarity threshold. Samples will be normalized by rarefying, to allow for unbiased alpha diversity calculation.
1.4 Statistical analyses of microbiome data. Alpha and beta diversities will be calculated based on rarefied OTUs. For alpha diversity, Shannon, Simpson, and Chao1 indices will be calculated for microbiomes from each facility. Weighted UniFrac distances will be calculated between

bacterial communities found in different samples, using R package Phyloseq. Principal coordinates analysis (PCoA) will be run based on the beta diversity. The reduced dimensionality of data will allow for visualization of potential clustering of microbiomes from different facilities and microbiomes inoculated with biocontrol strains and those without added biocontrol strains. PERMANOVA will be carried out to statistically characterize the differences between biofilms grown in presence of biocontrol strains and those grown without them. These data will provide information on the effects of biocontrol strains on formed microbiome biofilm and its association with Lm.

Obj. 2: Assess the effect of biocontrol strains in inhibiting growth of Lm in tree fruit packinghouse microbiome biofilms.

2.1 Biocontrol strains.

We will test the ability of two biocontrol strains to inhibit Lm grown in a biofilm made of microbiome present in environmental samples collected in facilities with persistent, sporadic or absent *Listeria* spp contamination (biofilm preparation described below in 2.2). The two biocontrol strains will be purchased through ATCC (*Lactococcus lactis* subsp. *lactis, Enterococcus durans*[11]). These isolates have previously been shown to be able to reduce Lm grown in biofilms in the meat processing facility drain where 10^7 cells/ml were applied to cover surface of drains using a foam formula[11].

2.2 Competitive exclusion biofilm assay.

To grow microbiome biofilms, we will homogenize the biomass collected from environmental sponge samples (collected in Obj. 1) in 100 ml of BHI. The biomass suspension will be aliquoted in a 24-well microtiter plate (V = 2 ml/well) and inoculated with 10^5 CFU/ml biocontrol strain and 10^5 CFU/ml of Lm. Negative controls will not be inoculated with biocontrol strains nor Lm, and positive controls will be inoculated just with Lm., but no biocontrol strains will be added. The Lm will be inoculated at 10^5 CFU/ml as this is the natural contamination level that we have detected in the apple processing facility with persisting Lm clone in our previous study using a sensitive Most Probable Number assay.

Each test will be performed in triplicates at 15°C and incubated for 5 days (120 h) to let the biofilm develop over a longer period of time. After 120 h incubation, planktonic cells will be removed, loosely attached cells will be washed off and biofilm will be resuspended by adding 2 mm sterile glass beads and shaking the plates on the benchtop microplate shaker following the methods optimized in our lab. Serial dilutions of resuspended biofilm biomass will then be quantified using a modify FDA BAM MPN protocol that we have optimized in a microtiter plate format for our ongoing project. Briefly, 90 ml of BLEB will be added to a bag containing the sponge sample, and hand massaged. Eight serial ten-fold dilutions of the resuspended biofilm will be prepared in BLEB. Three-tube MPN assay will then be carried out using undiluted sample and dilutions 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}. 40 µl BLEB supplement will be added to each tube after 4 hours of incubation at 30 ºC. Supplemented samples will be further incubated for 44 ± 2 hours**.** After 48 h incubation, a loopful of each dilution enrichment will be streaked onto ALOA and incubated for 24 to 48 hours at 37 ºC until obtaining visible colonies. Colonies appearing blue green with on ALOA will be considered as putative *Listeria* and those having a halo around a colony as Lm. We conclude that this medium is highly selective for *Listeria* and is therefore expected to produce very low level of false positive results. The MPN of Lm per ml of sample will be determined using an MPN calculator provided in the Excel file available for download in "BAM Appendix 2: Most Probable Number from Serial Dilutions" protocol. The significance of

difference among treatments and the negative control will be statistically evaluated using ANOVA.
This model will serve the purpose of growing biofilms to measure the efficacy of biocontrol strains in microbiome biofilms. The project will provide valuable data demonstrating the potential feasibility of this Lm biocontrol strategy that can be further tested in a real facility environment or a food safety pilot plant (available at Penn State) in a subsequent project.

Budget: Indicate projected costs with estimated annual costs. For multi-year projects, indicate estimated annual costs for subsequent years.
Salaries_____ Supplies and sequencing services ___ 9,000____
Hourly wages__ 6,480____ Travel____500_____
Fringe Benefits___ 506____ Miscellaneous____/_____
Total ___16,486___

Other Support:
None.

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