CENTER for PRODUCE SAFETY

CPS 2018 RFP
FINAL PROJECT REPORT

Project Title

Fate of different *Listeria monocytogenes* strains on different whole apple varieties during long-term simulated commercial storage

Project Period

January 1, 2019 – December 31, 2020 (extended to July 31, 2021)

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Objectives

1. *Assess the impact of apple variety, production region, growing season, and storage atmosphere on the survival of different Listeria monocytogenes strains on unwaxed apples.*
2. *Characterize the impact of three different industry-relevant, apple-waxing scenarios on survival of Listeria monocytogenes on Granny Smith, Gala or Honeycrisp apples during atmospheric storage.*

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FINAL REPORT

Abstract

The 2014 caramel apple outbreak rendered *Listeria monocytogenes* (*Lm*) a concern for fresh, whole apples. The past recalls and outbreaks for apples contaminated with *Lm* have been linked to the post-harvest packing environment where *Lm* can persist in environmental niches, likely as biofilms. This study focused on the possible differences in *Lm* survival as affected by *Lm* strain, apple cultivar, grower region, harvest year, storage conditions, waxing, and whether the organism was grown in a broth (planktonic) or on a surface (biofilm) prior to inoculation.

Unwaxed Gala (G), Granny Smith (GS) and Honeycrisp (HC) apples were received from growers in three different regions and dip-inoculated in an 8-strain (~6.5 log CFU/ml) *Lm* cocktail of planktonically-grown cell suspensions. Michigan-grown apples were also dip-inoculated with 48-h biofilm cultures. After inoculation, the apples were dried and subjected to air or controlled atmosphere (1.5% O_2, 1.5% CO_2) storage at 2°C. At monthly intervals, apples were removed from storage for *Listeria* enumeration. Additionally, inoculated apples were waxed with Shield-Bright AP-40 shellac (Pace International, Rochester, MN) after 3 or 7 months and then air-stored at 2°C. Additional non-inoculated apples were similarly stored and later inoculated and waxed after 3 or 7 months.

After 90 days of storage at 2°C, no significant difference in *Listeria* survival ($P > 0.05$) was seen between apples air-stored or under a controlled atmosphere, regardless of harvest year. As expected, *Listeria* populations decreased over time, with the pathogen still quantifiable in most samples after 7 months of storage. The year of harvest significantly impacted *Listeria* survival, with greater survival seen in harvest year 1 ($P < 0.05$) as compared to harvest year 2. The cultivar of apple significantly impacted *Listeria* survival ($P < 0.05$) during both harvest years. Regardless of harvest year, Gala exhibited significantly greater reductions in *Listeria* ($P < 0.05$). The type of inoculum significantly impacted *Listeria* survival ($P < 0.05$), regardless of harvest year. *Listeria* cells previously grown as a biofilm survived significantly better ($P < 0.05$) compared to planktonically grown cells. *Listeria* survival on waxed apples was influenced by the time of inoculation before waxing and the type of inoculum – planktonic or biofilm. These findings will inform the industry of the presently unknown risks associated with different components of apple production and packing and aid in the design and validation of *Lm*-targeting interventions to better ensure apple safety.

Background

Until 2014, outbreaks of listeriosis related to fresh produce had involved raw vegetables or whole cantaloupe (*10, 22*). However, in 2014-2015 a large multistate outbreak in the US was traced to caramel apples, while some outbreak patients reported only consuming whole apples (*1, 5*). A second listeriosis outbreak linked to caramel apples occurred in the US in 2017 (*20*). Fortunately, only three people were involved in this outbreak (*20*) versus the 2014-2015 listeriosis outbreak, which had affected 35 people in 12 different states (*1*). Traceback investigations of the contaminated apples from the 2014-2015 outbreak led to one apple packing facility in California (*1, 4*). Samples collected from the apple packing plant, the caramel apple

production facility, and apples in the supply chain confirmed the presence of *Listeria monocytogenes* (*Lm*) isolates that were closely related to the clinical isolates from the outbreak (*1*). This was the first time that *Lm* was considered a pathogen of concern for caramel apples or fresh whole apples (*1, 10*).

Whole fresh apples have been implicated in *Lm* outbreaks, even though their surface does not support the growth of *Lm*, possibly due to lack of nutrients and moisture (*19, 24, 29, 31*). *Lm* growth at room temperature has only been observed on caramel apples (*11, 29*), fresh-cut apples (*2, 9*), and when *Lm* is forcibly internalized via the stick into caramel apples (*11, 13, 29*). The Zhu team at Washington State University (WSU) reported that inoculation of two apple varieties (Fuji, Granny Smith) with a cocktail of *Lm* led to a limited decline in *Lm* numbers during 3 months of cold storage, which could not ensure safety. Both apple varieties were from the same region (West Coast) and yielded similar results, but, unexpectedly, the low inoculum resulted in a slight, transient increase in *Lm* populations on the apples, which was not observed with the high inoculum (*31*). Additional studies supported the survival of *Lm* populations on whole fresh apples, with limited reductions even after 5 months of cold storage (*19, 24*).

Most studies have examined *Lm* on apples from unidentified production regions (*19, 29*). Hence, data are needed for apples from other major apple-growing regions in the US, such as the Northeast and Midwest. Our project therefore included apples that originated from three major apple-growing regions. Also, our *Lm* survival experiments included Gala and Honeycrisp apples during long cold storage (*18, 24, 29, 31*).

The 2014 caramel apple outbreak involved two unrelated serotype 4b strains (*1*), one of which was an emerging clone (ST382) later involved in two other, unrelated outbreaks via stone fruit and green salads (*8, 10*). Our project was designed to assess the survival of these and other *Lm* strains that have been linked with major outbreaks and have common or emerging genotypes. A cocktail of different genetically-tagged *Lm* strains was employed to investigate the behavior of *Lm* on apples and allow, via next generation sequencing (NGS), assessment of differential strain survival during extended storage.

The reservoir for the *Lm* strains that caused the caramel apple and other produce-associated outbreaks of listeriosis remains unknown. However, epidemiological analyses tend to show that *Lm* genotypes from the packing environment usually match those from post-harvest and from the outbreak patients, proposing that the source of contamination is the packinghouse environment, where *Lm* can persist as sessile cells in biofilms (*10*). Several studies by the Ryser laboratory at MSU have clearly documented the potential for *Lm* transfer from contaminated equipment to fresh produce (*15, 30*). Research at the FDA has also suggested the potential for contamination of fresh produce during the washing step in dump tanks (*18*). *Lm* could detach from surfaces where it survives in biofilms and further contaminates the product during packing. Moreover, a recent study showed that *Listeria* spp. in apple packing facilities are most prevalent in the waxing area (*28*) where they may grow in biofilms on the equipment surface and become protected by layers of wax residue.

Apple fruit are harvested by hand, placed into plastic or wooden bins and transported directly to storage facilities. In some cases, they are presorted prior to storage, a process that involves floating the fruit from the bins, sorting them by color and/or size, and returning them to bins for storage. The storage process typically involves stacking the bins in cold rooms maintained between 0 and 3°C. Once a room is full and fruit temperature reduced substantially, the fruit are often gassed with the ethylene action inhibitor 1-methylcyclopropene (1-MCP), a plant growth regulator used to suppress fruit ripening. Treatment with 1-MCP enables long-term storage (3 to 12 months) and marked retention of fruit textural properties valued by consumers (*3*). Following 1-MCP treatment, long-term stored apple fruit are also stored in a modified atmosphere low in O_2 and with slightly elevated CO_2. This process, termed controlled atmosphere (CA) storage, also minimizes the impact of ethylene and retards the ripening process. Upon completion of the storage period, the fruit are unloaded from the bins, typically by floating the fruit out of the bins, waxed, and sorted for quality prior to packing and shipping.

Since waxing is one of the final steps in the handling process for apples and covers the entire exterior of the fruit, it has the potential to impact microbial populations on the fruit skin. Thus far, only one study demonstrated that waxing decreased the population of *Lm* immediately after waxing, however *Lm* survival on the same waxed apples was enhanced during subsequent storage (*19*). The initial drop in the *Lm* population after waxing could be attributed to certain chemical components of the wax, such as isopropyl ethanol, while the wax could protect *Lm* against environmental stresses and encourage better survival on apples (*19*). Since apple waxing is a major step during processing and an important source of contamination, it was considered essential to test the impact of different waxing scenarios on the fate of *Lm* on the apples.

Research Methods

Overall Experimental Design. The overall experimental design is diagrammatically summarized in Figures 1 and 2. Industry collaborators in three major US apple-growing regions kindly provided freshly harvested, unwaxed, and unsorted apples of three different varieties (Gala, Granny Smith, and Honeycrisp). The study included two independent trials, using apples from two successive harvest years. The project monitored *Listeria* survival exclusively on apples inoculated in the laboratory.

Survival of different *Lm* strains was assessed on apples that were dip-inoculated with two different types of inoculum (planktonic cells, and cells derived from biofilms formed on a polystyrene [100 mm x 15 mm] petri dish) and then subjected to either air or controlled atmosphere (CA; 1.5% O_2, 1.5% CO_2) storage at 2°C. The impact of waxing on *Lm* survival was further assessed during 3 months of air and CA storage. Two different industry-relevant apple-waxing scenarios were examined: (i) waxing of previously stored inoculated apples, and (ii) waxing of previously stored, uninoculated apples immediately after dip-inoculation and drying.

Objective 1: Assess the Impact of *Lm* Survival on Unwaxed Apples

Study design. Variables examined included three apple varieties, three different apple production regions, two harvest seasons, and eight different strains of *Lm* grown both planktonically and as a biofilm for apple inoculation.

Apples. Fresh, unwaxed apples of three different varieties—Gala, Granny Smith, and Honeycrisp—measuring 2.5 to 3.0 inches in diameter were shipped to MSU by industry collaborators from three major US apple-growing regions during August to October in years 2019 and 2020 of the study. Immediately upon arrival, apples were placed in a walk-in cooler at 2°C (Fig. 4). After removing any damaged or undersized fruit measuring <2.5 inches in diameter, the apples were treated with 1 µL/L 1-MCP for 24 h at 2°C to suppress ripening and preserve fruit quality.

Bacterial strains. Eight different strains representing the major *Lm* serotypes 1/2a, 1/2b and 4b and major clones were selected from the Kathariou laboratory strain collection based on relevance to apple-related hazards and other outbreaks. Thus, our panel included both lineage I (serotype 1/2b and 4b) and lineage II (serotype 1/2a) strains (Table 1). This panel of eight strains included one from each of the two clones implicated in the 2014-2015 caramel apple outbreak, i.e., clonal complex (CC) 1 and sequence type (ST) 382, both of serotype 4b. Unless otherwise indicated, all strains were grown in Brain Heart Infusion broth. Strains were preserved at -80°C.

Strain barcoding. All strains were barcoded with unique DNA sequences for subsequent metagenomic analysis. These strain barcodes were short (30 bp) DNA sequences stably incorporated into the chromosome, as previously described (*35*) (Fig. 3). Specifically, *Escherichia coli* SM10λpir with plasmid pTZ200.mix, was kindly provided by Dr. M. Waldor and Dr. P. Zhang, and was used as the donor of pTZ200.mix that was electroporated into each of the strains. Transformants were selected on Brain Heart Infusion (BHI) agar containing chloramphenicol (plasmid marker; 10 µg/mL). PCR and sequencing were done for individual colonies to identify uniquely barcoded isolates. For one of the strains, 4b1, barcoding was based on chromosomal *gfp* sequences.

Analysis of allelic differences between parental strains and their barcoded derivatives. All strains were sequenced by collaborator Yi Chen at the FDA, and multilocus allele differences in the whole genome (wgMLST) and core genome (cgMLST) were identified via the BIGSdb PasteurMLST Genome Comparator (*14*).

Phenotypic comparisons of parental strains and their barcoded derivatives. Each of the constructed barcoded strains was compared to its parental strain for certain basic phenotypic characteristics, such as the ability to form biofilms, hemolytic activity, motility on soft agar, and virulence.

Biofilm formation was assessed using the crystal violet assay in 96-well polystyrene plates (Greiner Bio-One, Monroe, NC, USA) and by measuring absorbance at 580 nm as described (*25*). Each strain was tested in eight wells per trial and two independent trials.

Hemolytic activity was assessed by streaking each barcoded *Lm* strain onto half a tryptic soy agar plate with 5% sheep blood and its parental strain on the other half of the same plate (Remel Inc., Lenexa, KS, USA). The plates were incubated at 37°C for up to 48 h. At least three independent trials were performed using duplicate plates for each trial.

Motility was assessed by spot-inoculating 5 µl cell suspensions on plates of tryptic soy broth supplemented with 0.4% agarose (TSA; Becton, Dickinson & Co.), incubating at 25°C and visually comparing the diameter of the spots at 24 and 48 h. Each plate had four spots of the tagged strain in the top row and four spots of the parental counterpart in the bottom row (Fig. 8). Three independent trials were performed.

Differences in virulence between the strains were tested using the *Galleria mellonella* model. Overnight cell cultures were washed in phosphate buffered saline (PBS) and inoculated (10^5 and 10^6 CFU per larva) into the larvae of the greater wax moth *Galleria mellonella*, as described (*25*). The cell suspensions were also serially diluted and plated to confirm the inoculum levels of each strain. Larvae were incubated at 37°C and survival was monitored over seven days. Ten larvae were used for each assessment per trial. Two independent trials were performed.

Preparation of planktonic and biofilm-derived *Lm* inoculum for the apples. Two different types of inoculum were used: planktonic and biofilm-derived cells. Biofilm-associated cells can exhibit pronounced physiological differences from their planktonic counterparts, with contamination of produce likely to occur when *Lm* is shed from biofilms on equipment or environmental surfaces during apple packing (*10*). Although most inoculation studies use cells derived from planktonic cultures, the inclusion of biofilm-derived cells is especially relevant in simulating likely industry scenarios and thus in this study, both types of cells (planktonic and biofilm-derived) were used as inoculum.

For the planktonic inoculum, the eight *Lm* strains were individually grown overnight in tryptic soy broth containing 0.6% yeast extract (TSBYE) at 37°C. Each strain was then measured for optical density (OD_{600}) and adjusted to ensure that the strains were combined in equal proportions. Cells were then washed twice by centrifugation for 15 minutes each. After the first centrifugation, TSBYE was decanted and the cell pellet was resuspended in PBS. After the second centrifugation, new PBS was added to the cell pellet which was resuspended before being diluted in deionized (DI) water and used as a cocktail to dip-inoculate the apples.

For the biofilm inoculum, the same eight barcoded strains were similarly grown individually overnight, diluted to the same OD_{600} value, and combined in equal proportions to obtain an 8-strain cocktail. Approximately 80 sterile petri plates (150 mm diameter) each containing 19.6 mL TSB were inoculated with 400 µL of the multi-strain cocktail and incubated at 37°C for 48 h. After removing the media, the plates were rinsed twice with 1 mL of sterile distilled water using a pipette, to remove any loosely adherent cells. Triplicate sterile cotton-tipped swabs (Puritan) moistened in 0.85% saline were used to swab and remove the attached *Lm* cells. The three swabs from each plate were transferred to a 50-ml conical tube containing 20 ml PBS which were vortexed vigorously for 1 minute. After removing the swabs, the suspensions

from the ~80 tubes were combined to obtain the final suspension of *Lm* biofilm cells for inoculation.

Apple inoculations and subsequent treatment/storage. Recognizing that some apples were stored for up to 7 months and an additional 14 days after storage, apples of each cultivar/region were dip-inoculated to yield ~10^5 CFU/apple, with this inoculation level based on previous *Lm* survival data from WSU (*31*). After dip-inoculation, Gala, Granny Smith, and Honeycrisp apples were air-dried at room temperature and placed under air or CA (1.5% O_2, 1.5% CO_2) storage at 2°C for 3 or 7 months, respectively (Fig. 4). At no time were uninoculated apples tested for presence of any pathogens, including *Listeria*.

Sampling and bacterial enumerations. Inoculated apples were sampled immediately after air-drying (day 0), weekly from weeks 1–4 and then monthly for the next 6 months. At each sampling time, two composite samples of three inoculated apples were randomly removed from storage. The top and bottom portion of the apples were first removed with a sterilized knife. Then the skin portion from the outside of the apple was removed using an electric apple peeler (Starfrit Rotato Express, Electric Peeler 093209-006-BLCK). The top and bottom portions were added to one Whirl-pak bag, with the peel added to a second bag. The samples were combined in Whirl-pak bags, diluted based on the sample weight (1:5) in sterile PBS, and then homogenized in a stomacher for 1 minute at 300 rpm.

Lm was enumerated by plating appropriate PBS dilutions on non-selective/differential (tryptic soy agar with 0.6% yeast extract, esculin and ferric ammonium citrate, mTSAYE) (*30*) and selective/differential media (Modified Oxford, MOX). Following incubation, all black colonies on mTSAYE and MOX were counted as *Listeria*. Counts from the stem and calyx and the peel were combined and expressed as CFU/apple.

Objective 2: Impact of different industry-relevant apple-waxing scenarios on survival of *Lm* on Granny Smith, Gala and Honeycrisp apples during atmospheric storage

Study design. Two different industry-relevant, apple-waxing scenarios were assessed for their impact on *Lm* survival: (1) waxing of inoculated apples after storage, and (2) waxing of stored uninoculated apples immediately after dip-inoculation and drying. Scenario 1 simulates contamination during pre-harvest, harvest, or early post-harvest stages, whereas scenario 2 simulates contamination from the processing line (e.g., flume washing) shortly before waxing.

Apples. For the first scenario, inoculated apples (equal numbers with planktonic and biofilm-derived inocula) were removed after 3 and 7 months of air or CA storage, respectively. Half of the apples were waxed, with the other half remaining unwaxed (control). All of the apples were air-stored for 12 weeks at 2°C. For the second scenario, fresh unwaxed Gala, Granny Smith, and Honeycrisp apples from the same sources were stored, but without inoculation. These uninoculated apples were then removed after 3 and 7 months of air or CA storage, respectively, and inoculated and waxed as follows.

Inoculation. After 3 or 7 months of storage, uninoculated apples were dip-inoculated (equal numbers with each inoculum type) with the eight-strain *Lm* cocktail, air-dried, and waxed as described above. A control batch was similarly inoculated, but not waxed.

Apple waxing. For waxing, a shellac-based apple wax (Shield Brite AP-40, Pace International, Wapato, WA) was applied at full strength as recommended by the supplier. A 10 ml tube with an attached atomizer was used to mimic commercial wax application. The apples were sprayed once on the calyx end and placed on a rotating table. While turning on the turntable, three sprays (~0.15 ml/spray) were applied to evenly distribute the wax on the surface. After waxing, the apples were dried in a biosafety hood at room temperature and an airflow ~ 10 m/s (Fig. 5).

Apple storage, sampling, and microbial analyses. After air-drying, all waxed apples were air-stored for 14 days at 2°C. Populations of *Lm*, mesophilic aerobic bacteria, and yeast/mold were enumerated weekly or until signs of spoilage using the previously described methods except that the peelings from two composite samples of 3 apples each were placed in a Whirl-pak bag containing PBS and 1% Tween 20 warmed to 42°C for removal of the wax during stomaching. Appropriate dilutions were then surface-plated on mTSAYE and MOX for enumeration of *Lm* cells, as in Objective 1.

Data analysis. Two independent apple storage trials were conducted in years 1 and 2. Two composite samples of three apples each per cultivar and growing region were analyzed at each time point. Analysis of variance applying Tukey tests were used (SAS, Inc., Cary, NC) to determine statistical significance of the impact of apple cultivar, region, growing season, inoculum type (planktonic vs. biofilm-derived) and storage atmosphere on adherence and survival of *Listeria* on the apples. Significance was set at $P < 0.05$. The impact of waxing on *Lm* survival was statistically analyzed using the Student t-test (SAS, Inc.).

Research Results

The barcoded strains each had a unique strain differentiating them (Fig. 3) and were used as a cocktail for all inoculations, as described above. After inoculation and drying on day 0, *Listeria* populations grown planktonically ranged from 3.22 to 5.59 and 4.20 to 5.50 log CFU/apple in harvest years 1 and 2, respectively. When the inoculum was grown as a biofilm, *Listeria* populations ranged from 5.97 to 6.23 and 6.00 to 6.60 log CFU/apple in harvest years 1 and 2, respectively.

Storage atmosphere. After 90 days of storage at 2°C, no significant difference in *Listeria* survival ($P > 0.05$) was seen between apples air-stored or under a controlled atmosphere, regardless of the harvest year. Figure 6 shows the comparison between air and controlled atmosphere storage during harvest year 1. Populations of *Lm* on Gala and Honeycrisp apples decreased ~1.5 to 2.5 log CFU/apple after 90 days for both air and controlled atmosphere. Additionally, *Lm* populations on Granny Smith apples decreased less (~0 to 1 log CFU/apple) from 30 to 90 days during both air and CA storage (Fig. 6). During harvest year 2, Lm

populations on Gala and Granny Smith apples initially decreased ~2 to 2.5 log CFU/apple during 14 to 90 days of air and CA storage (Fig. 7), with populations on Honeycrisp apples initially decreasing ~2 log CFU/apple between 30 to 90 days of storage.

Extended storage. As expected, *Listeria* populations decreased over time, with the pathogen still quantifiable in most samples after 7 months of storage. Some planktonic (Figs. 8 and 9) and biofilm (Figs. 10 and 11) inoculated apples still had *Listeria* populations of 4.6 log CFU/apple after 210 days of storage. For harvest year 1, Lm survived better on Granny Smith apples from all growing regions during long-term storage (Fig. 8). For harvest year 2, apples from Region C supported better survival of *Lm* (log reduction ~1 to 2 CFU/apple) compared to Regions A and B (log reduction ~2 to 3 CFU/apple). *Listeria* populations decreased to near the limit of detection (1.6 log CFU/apple) in some planktonic-inoculated Gala samples from Region B during both years 1 and 2 (Figs. 8 and 9).

Harvest year. The year of harvest significantly ($P < 0.05$) impacted *Listeria* survival, with greater survival seen in harvest year 1 (Fig. 8) as compared to harvest year 2 (Fig. 9). Less survival was seen on Granny Smith apples from harvest year 1 ($P < 0.05$) compared to harvest year 2 (Fig. 8 and 9). After long-term storage, *Listeria* populations on Granny Smith apples decreased within the range of 0.0 to 0.64 and 1.4 to 3.85 log CFU/apple for harvest years 1 and 2, respectively.

Apple cultivar. The cultivar of apple significantly impacted *Listeria* survival ($P < 0.05$) during both harvest years. During harvest year 1, Granny Smith apples exhibited a significantly lower reduction in *Listeria* ($P < 0.05$) compared to Gala and Honeycrisp (Fig. 8). During harvest year 2, Honeycrisp had a significantly lower reduction in *Listeria* ($P < 0.05$) compared to Gala and Granny Smith (Fig. 9). After 210 days of storage, *Listeria* populations on Granny Smith apples decreased 0.0 to 0.64 and 1.4 to 3.85 log CFU/apple, for harvest years 1 and 2, respectively (Figs. 8 and 9). Additionally, *Listeria* populations on Honeycrisp apples decreased 1.20 to 3.15 and 0.93 to 3.05 log CFU/apple, for harvest years 1 and 2, respectively (Figs. 8 and 9). Regardless of harvest year, Gala exhibited significantly greater reductions in *Listeria* ($P < 0.05$). After 210 days of storage, *Listeria* populations on Gala apples decreased 0.34 to 2.29 and 1.85 to 3.43 log CFU/apple, for harvest years 1 and 2, respectively (Figs. 8 and 9).

Inoculum type. The type of inoculum significantly impacted *Listeria* survival ($P < 0.05$), regardless of harvest year. *Listeria* cells previously grown as a biofilm survived significantly better ($P < 0.05$) compared to planktonically grown cells (Figs. 10 and 11). After 210 days of storage, *Listeria* populations previously grown planktonically decreased 0.03 to 2.55 and 0.93 to 3.95 log CFU/apple for harvest years 1 and 2, respectively (Figs. 10 and 11). After long-term storage, *Listeria* populations previously grown as a biofilm changed by -0.01 to 1.36 and 1.50 to 2.76 log CFU/apple in harvest years 1 and 2, respectively, on apples after 210 days of storage (Figs. 10 and 11).

Waxing. *Listeria* survival was influenced by the time from inoculation to waxing and the type of inoculum – planktonic or biofilm. Even though biofilm-derived cells survived significantly better than planktonic-derived cells before waxing, they survived significantly less ($P < 0.05$) than

planktonic cells on waxed apples that were inoculated, air-stored for 90 days, and then waxed (Table 2). Storing inoculated apples for 210 days before waxing significantly increased the survival of biofilm-grown cells of *Listeria* compared to when similarly stored apples were inoculated immediately before waxing ($P < 0.05$) (Table 2). Regardless of inoculum type or time, *Listeria* populations generally decreased <1.5 log CFU/apple on waxed apples during 14 days of refrigerated storage. Most apples were spoiled just 14 days after waxing.

Genetic barcoding – Phenotypic and genomic analysis of the barcoded strains. Genetic barcoding of the strains did not generally impact the phenotypic traits. More specifically, no significant differences were observed between the tagged and parental strains regarding biofilm formation at 25 and 37°C (Fig. 12). Furthermore, no significant differences were found between the tagged and parental strains for two representative strain pairs after assessing their virulence with the *Galleria mellonella* model (Fig. 13), with similar findings obtained for the remaining strains. Lastly, motility was not impacted by inserting the chromosomal tags (Fig. 14), with tagged and parental strains growing similarly at 4, 25 and 37°C (data not shown). Lastly, regarding hemolytic activity of the strains, no reproducible differences in hemolytic activity were found between the tagged and corresponding parental strains.

Analysis of the whole genome sequence data of each strain with the BIGSdb PasteurMLST Genome Comparator showed only a small number of differences between the genomes of all the tagged and parental strains (Table 3). It should be noted that wgMLST targets 3,270 different loci of *Listeria*, and none of the tagged strains demonstrated more than three loci with differences. This tool can only identify allele differences, not the type of the difference. For example, a difference in only one nucleotide could lead either to a different amino acid and potentially a significant difference or in a silent mutation. Three pairs of tagged and parental strains demonstrated differences in loci of the core genome. In each case, a single-locus difference was noted among the 1,748 loci of the core genome (Table 3). For two of the barcoded strains, F2365-2 and the CFSAN073872-6, the differences in the core genome loci were attributed to a single nucleotide change that also resulted in an amino acid change. A single amino acid change might not have a major impact on the functionality of the corresponding protein and fitness of the strain might not be affected. It should also be pointed out that a single nucleotide change could sometimes represent a sequencing error. Thus, overall, the whole genome sequence data confirm that the barcoded strains are extremely similar to their parental counterparts.

Outcomes and Accomplishments

Both apple storage and waxing studies from years 1 and 2 have been completed as proposed, with genomic analysis of the *Lm* strains to be completed by the end of 2021.

Summary of Findings and Recommendations

- Storage atmosphere does not impact survival of *Listeria*
- Surface-grown *Listeria* cells from biofilms in packinghouses may survive longer on apples
- Harvest year and apple cultivar impact *Listeria* survival
- The timing of apple contamination and waxing impact *Listeria* survival – waxing reduced survival when *Listeria* contamination occurred before long-term CA storage
- Recommend cleaning and sanitization to reduce established biofilms within facilities, as biofilm-derived cells tended to persist longer on apples
- Findings do not warrant changes in waxing or use of air and CA storage

APPENDICES

Publications and Presentations

Great Lakes Fruit, Vegetable and Farmers Market Expo 2018
Listeria and apples – The 'Core' concerns. Dr. Elliot Ryser, Michigan State University

Poster at CPS Research Symposium 2019
Fate of different Listeria monocytogenes strains on different whole apple varieties during long-term simulated commercial storage.
Elliot Ryser, Sophia Kathariou (Co-PI) Randy Beaudry (Co-PI), Cameron Parsons, Duncan Matthews, Rania Raftopoulou

Poster at the 20th ISOPOL Conference 2019, Toronto, Canada
Sequence tagging of *Listeria monocytogenes* strains for monitoring relative fitness on fresh apples. C. Parsons, E. Ryser, R. Raftopoulou, S. Kathariou

Great Lakes Fruit, Vegetable and Farmers Market Expo 2019
Stalking *Listeria* in apple packinghouses: best handling practices to avoid an outbreak.
Dr. Elliot Ryser, Grand Rapids

CPS Virtual Site Visit 2020
Fate of *Listeria monocytogenes* on apples.

Poster at IAFP 2020, Virtual
Survival of Planktonic- and Biofilm-grown *Listeria monocytogenes* on Apples as Affected by Apple Variety, Grower Region, and Storage Conditions.
Natasha Sloniker[1], Ourania Raftopoulou[2], Sophia Kathariou[2], Randy Beaudry[3] and Elliot Ryser[1]
[1]Dept. of Food Science and Human Nutrition, [3]Dept. of Horticulture, Michigan State Univ, East Lansing, MI and [2]Dept. of Food Science, North Carolina State Univ., Raleigh, NC

Presentation at CPS Research Symposium 2020
Fate of *Listeria monocytogenes* strains on different whole apple varieties during long-term simulated commercial storage. Elliot Ryser

Presentation at New York Horticulture Expo 2020
Fate of different *L. monocytogenes* strains on different whole apple varieties during long-term simulated commercial storage. E. Ryser, R. Beaudry, S. Kathariou

Poster at ASM Microbe Online Summer 2020
Sequence tagging of *Listeria monocytogenes* strains can have unexpected phenotypic impacts.
O. Raftopoulou, E. Ryser, C. Parsons, D. Elhanafi, S. Kathariou

Oral Presentation for the Genetics and Genomics Initiative Flash Talks 2020, NCSU
Sequence tagging of *Listeria monocytogenes* strains may lead to unexpected phenotypic impacts.
O. Raftopoulou, E. Ryser, C. Parsons, D. Elhanafi, S. Kathariou

Poster at Plant and Microbial Biology L.W. Parks Annual Event 2020, NCSU
Sequence tagging of *Listeria monocytogenes* strains may impact their hemolytic activity, colony size and motility/ chemotaxis. O. Raftopoulou, E. Ryser, C. Parsons, D. Elhanafi, S. Kathariou

Presentation at Michigan State University Food Science and Human Nutrition, FSC 892 Seminar, 2021
Survival of Planktonic- and Biofilm-grown Listeria monocytogenes on Apples as Affected by Apple Variety, Grower Region, and Storage Conditions. N. Sloniker

Poster at IAFP 2021, Phoenix, AZ
Predicting the Survival of *Listeria monocytogenes* on Apples and Detection Using Ultraviolet Spectroscopy. O. Raftopoulou, M. Kudenov, E. Ryser, R. Beaudry, S. Kathariou

Poster at IAFP 2021, Phoenix, AZ
Survival of Planktonic and Biofilm-grown *Listeria monocytogenes* on Apples as Affected by Waxing and Storage Conditions.
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Presentation at CPS Research Symposium 2021
Fate of different *Listeria monocytogenes* strains on different whole apple varieties during long-term simulated commercial storage. Elliot Ryser

Budget Summary

Total research funds awarded to this project were $346,054: $228,260 from the Specialty Crop Block Grant Program through the Washington State Department of Agriculture (WSDA SCBGP grant# K2546) and $117,794 from CPS Campaign for Research funds. The research team expects to use all the funds by the end of the project term.

Tables and Figures – see below

Tables 1–3 and Figures 1–14

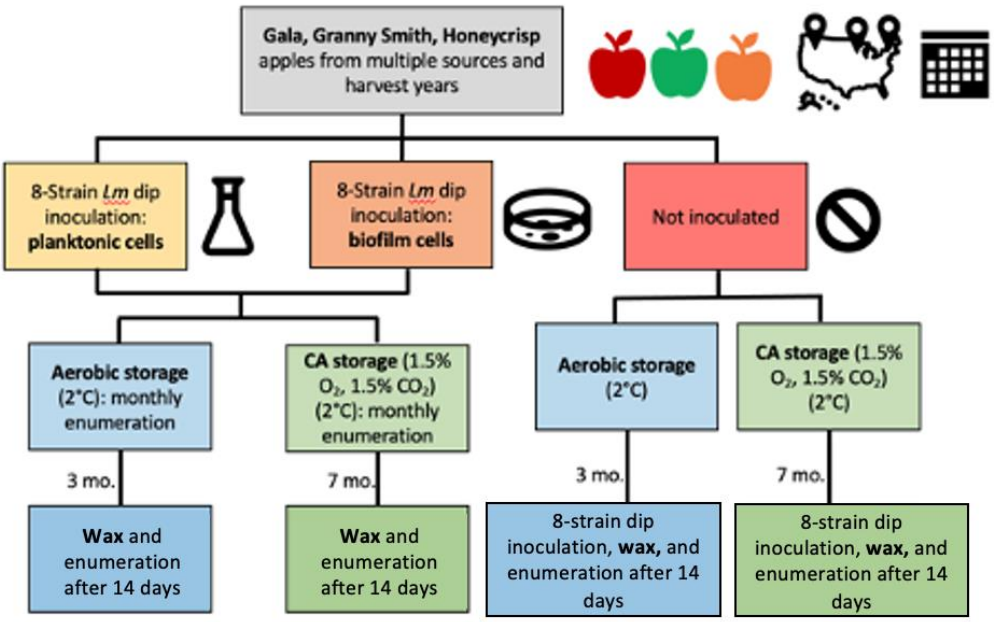

Figure 1. Experimental design.

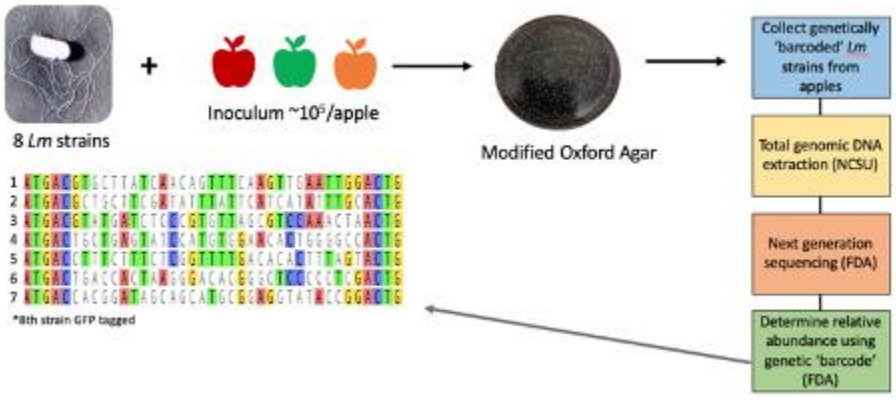

Figure 2. *Lm* strain differentiation of survivors from the apple surface.

Table 1. Diverse panel of *Lm* strains used for barcoding.

Serial number	Strain	Serotype	Genotype	Source
1	2014L-6680	4b	ST1 (CC1)	Caramel apple outbreak, 2014-2015
2	H7858	4b	ST6 (CC6)	Hot dog outbreak, 1998-99
3	F2365	4b	ST1 (CC1)	California cheese outbreak, 1985
4	2014L-6695	4b	ST382	Caramel apple outbreak, 2014-2015
5	CFSAN023957	4bv-1	ST554	Mung bean sprouts outbreak, 2014
6	2010L-1723	1/2a	ST378	Celery outbreak, 2010
7	CFSAN073872	1/2b	ST581	Apples, 2017
8	4b1	4b	ST145 (CC2)	Human listeriosis, 1962

Figure 3. Nucleotide alignment of the unique 30-nt tags barcodes that were chromosomally inserted into the *Lm* strains. The four nucleotides at each end of the sequence are conserved and flank the variable barcode. The number in front of each nucleotide sequence corresponds to the strain serial number in Table 1.

Figure 4. Storage room containing the storage chambers for apples in the Michigan State University Plant and Soil Sciences Building.

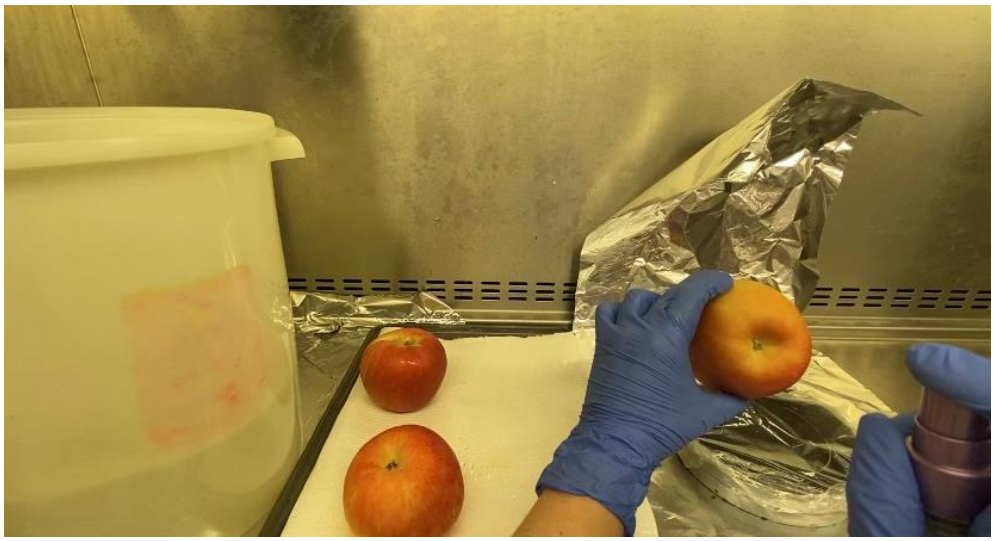

Figure 5. Apples being waxed in the biosafety cabinet.

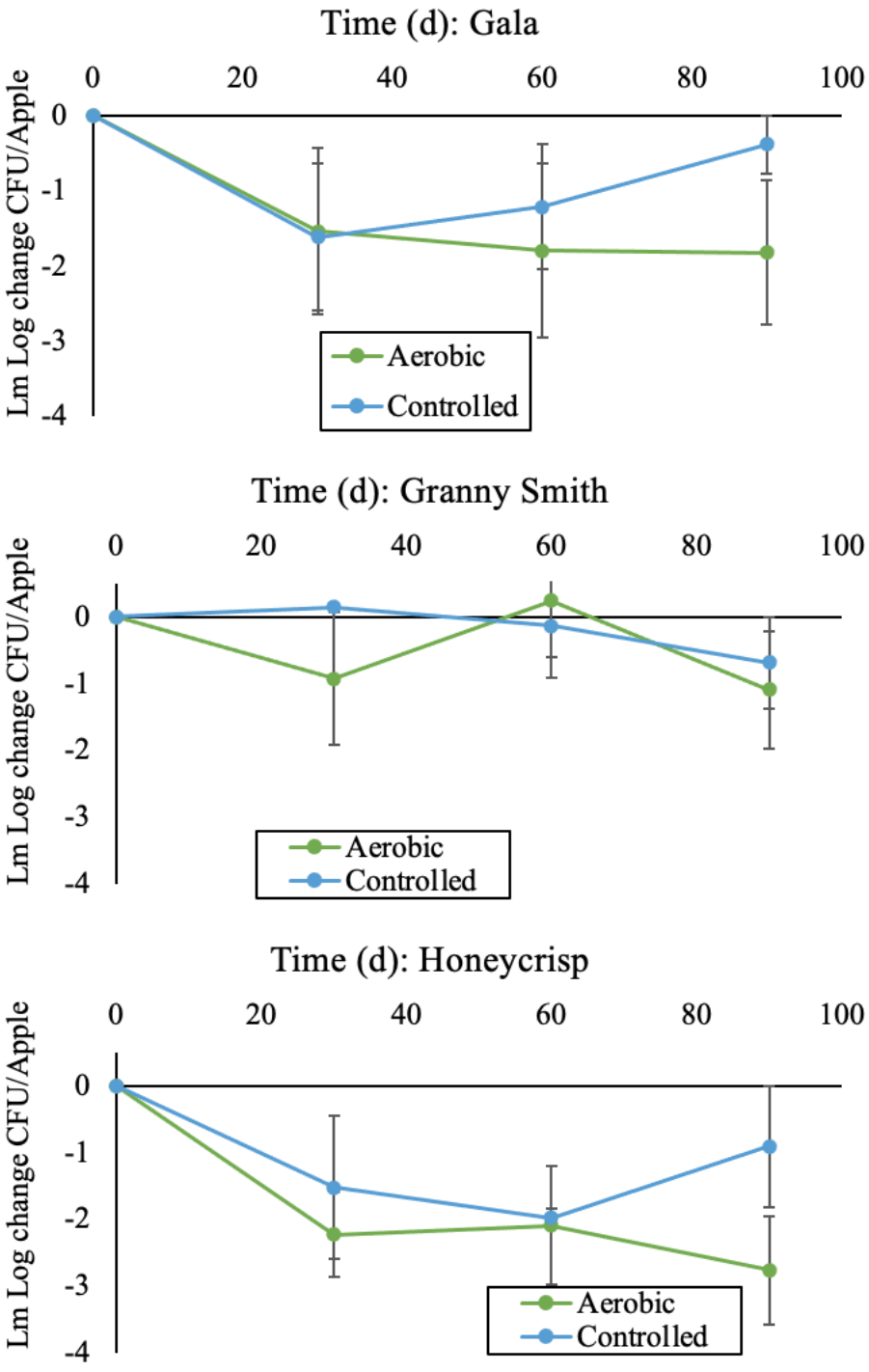

Figure 6. Average (± SD) *Lm* log (CFU/apple) reductions for Harvest Year 1 on unwaxed Gala, Granny Smith, and Honeycrisp apples that were inoculated with the *Lm* planktonic cocktail and subjected to air (green) and controlled atmosphere (blue) storage.

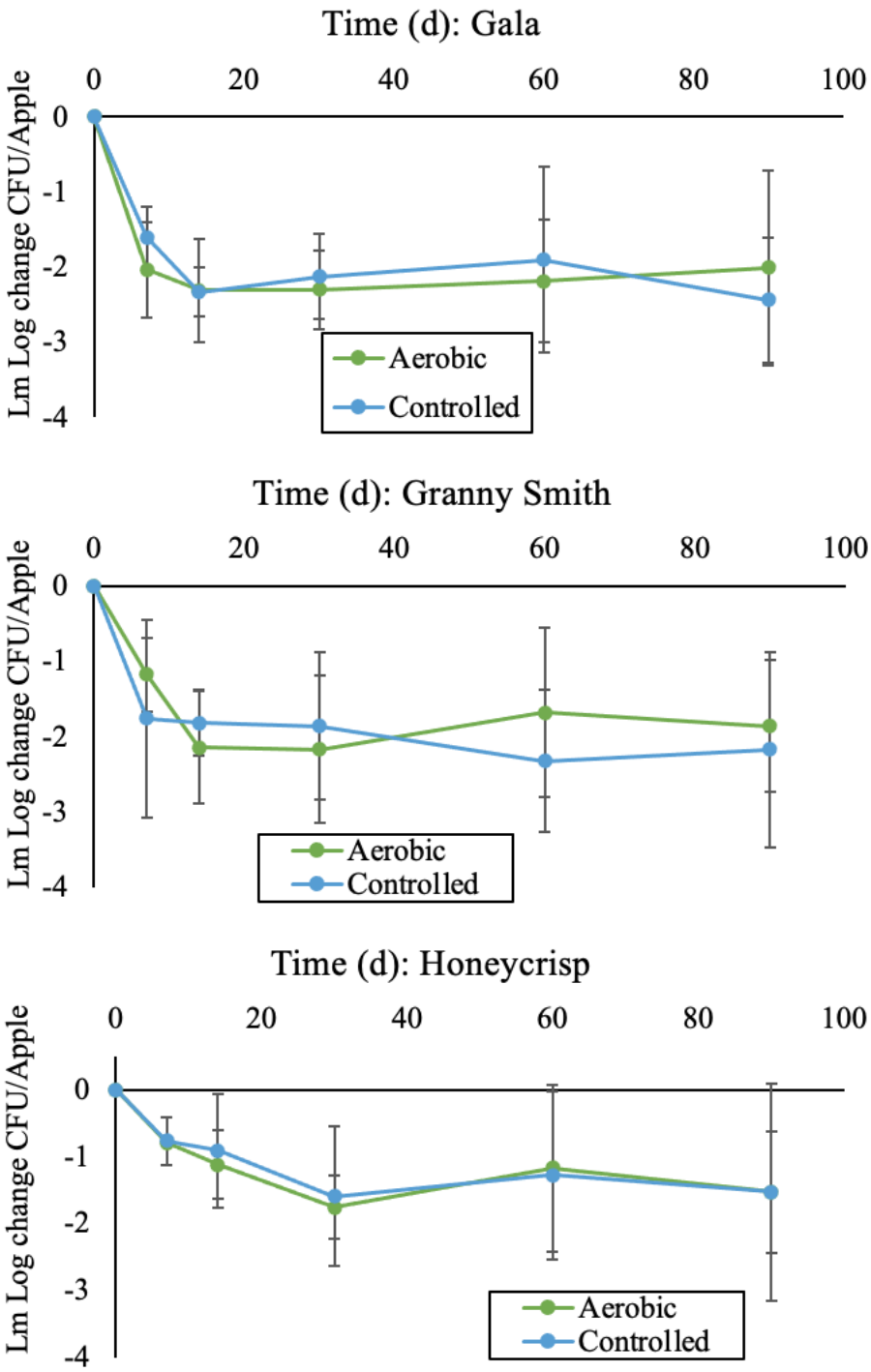

Figure 7. Average (± SD) *Lm* log (CFU/apple) reductions for Harvest Year 2 on unwaxed Gala, Granny Smith, and Honeycrisp apples that were inoculated with the *Lm* planktonic cocktail and subjected to air (green) and controlled atmosphere storage (blue).

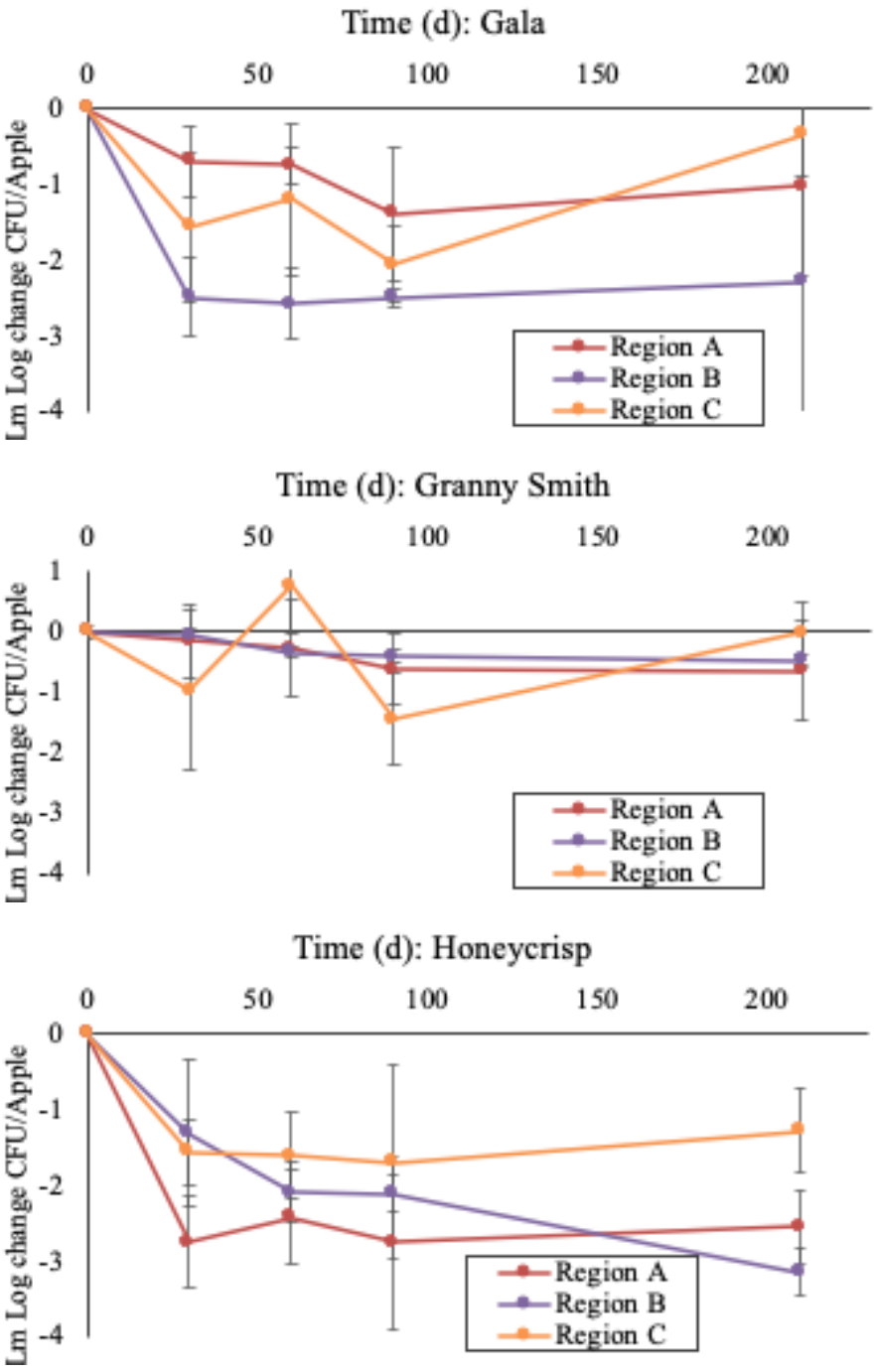

Figure 8. Average (± SD) *Lm* log (CFU/apple) reductions for Harvest Year 1 on unwaxed Gala, Granny Smith, and Honeycrisp apples from Region A (red), B (purple) and C (orange) that were inoculated with the *Lm* planktonic cocktail and subjected to air and controlled atmosphere storage.

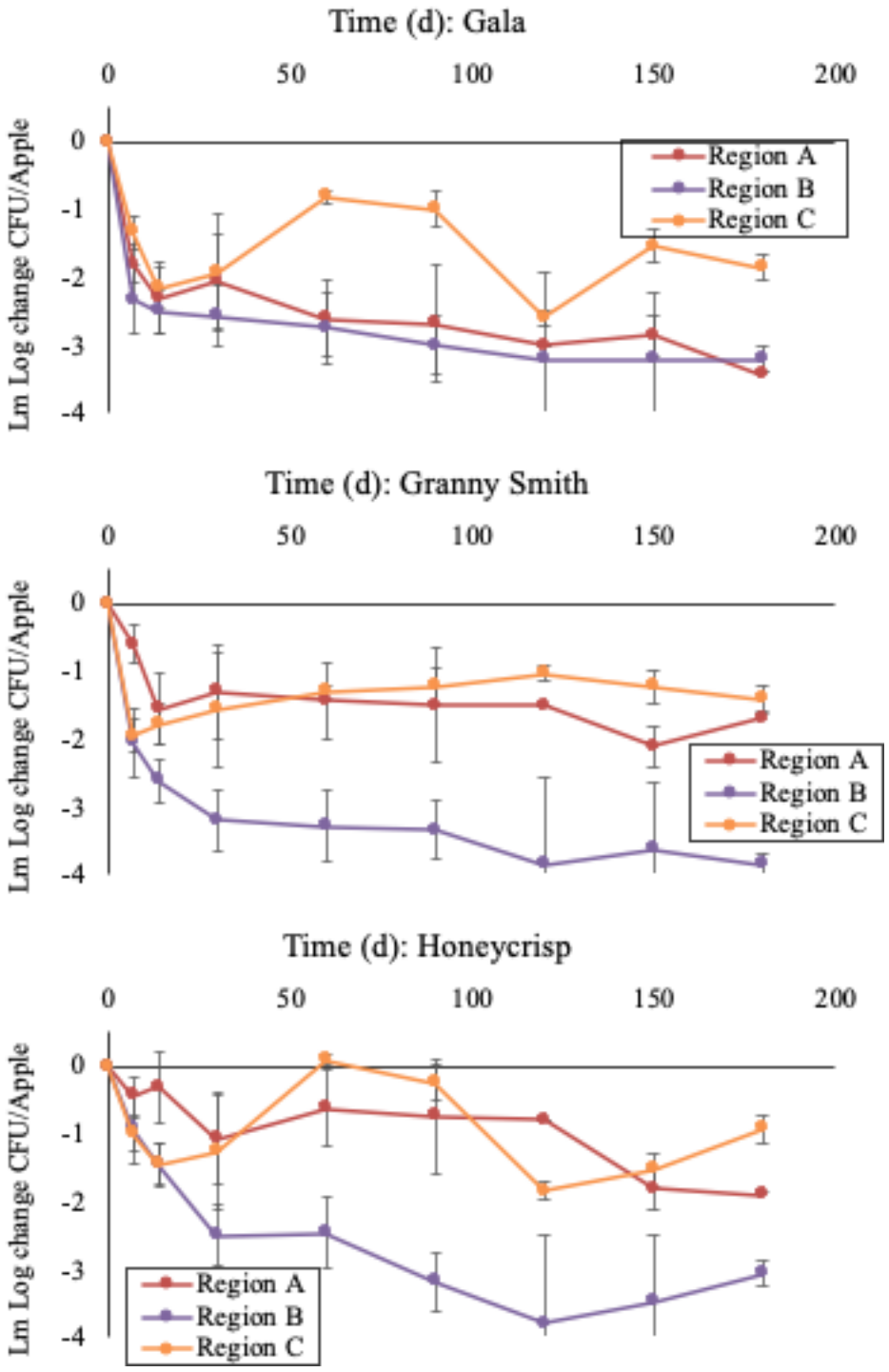

Figure 9. Average (± SD) *Lm* log (CFU/apple) reductions for Harvest Year 2 on unwaxed Gala, Granny Smith, and Honeycrisp apples from Region A (red), B (purple) and C (orange) that were inoculated with the *Lm* planktonic cocktail and subjected to air and controlled atmosphere storage.

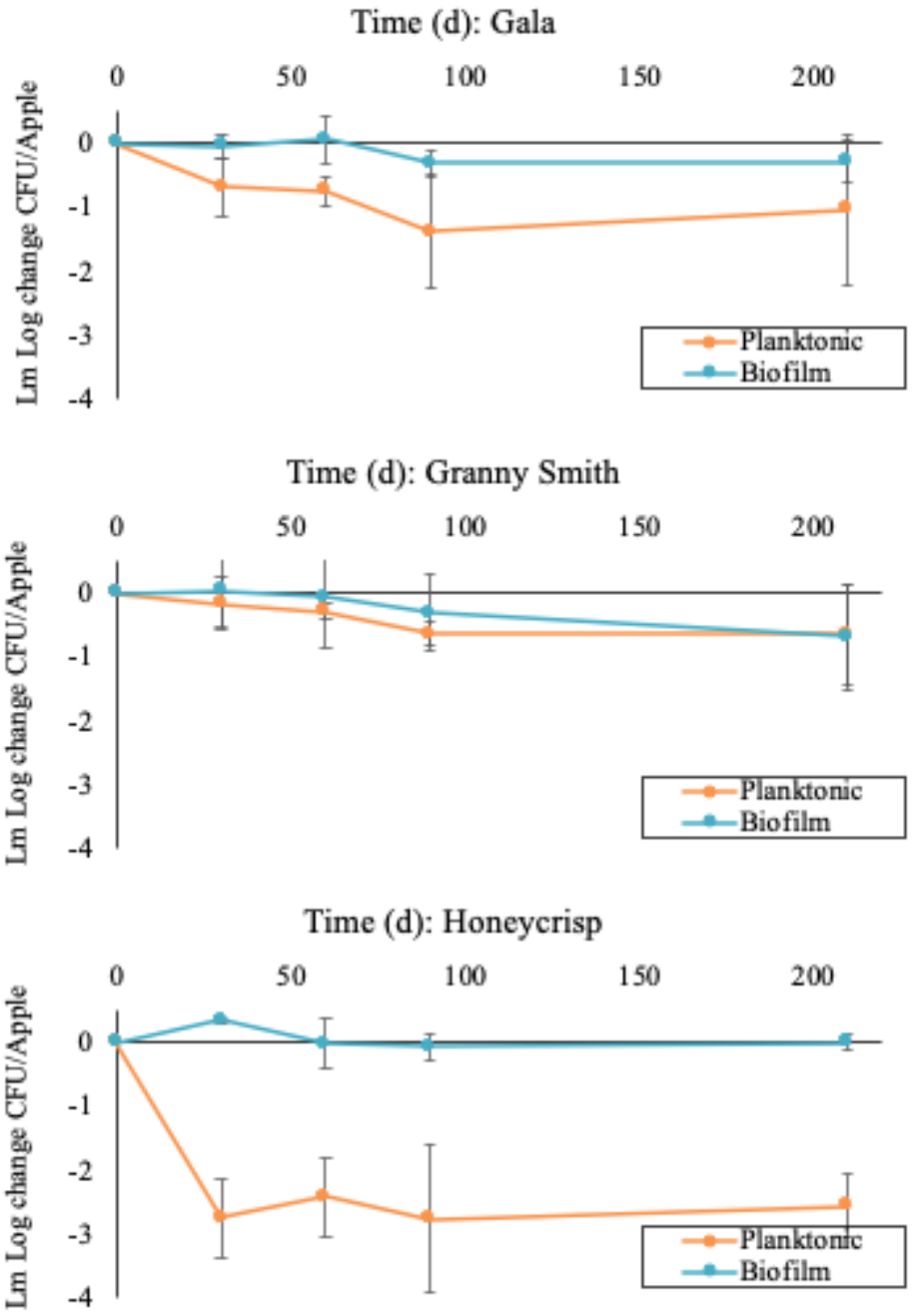

Figure 10. Average (± SD) *Lm* Log (CFU/apple) reductions for Harvest Year 1 on unwaxed Gala, Granny Smith, and Honeycrisp apples from Region A that were inoculated with the *Lm* planktonic (orange) or biofilm (blue) cocktail and subjected to air and controlled atmosphere storage.

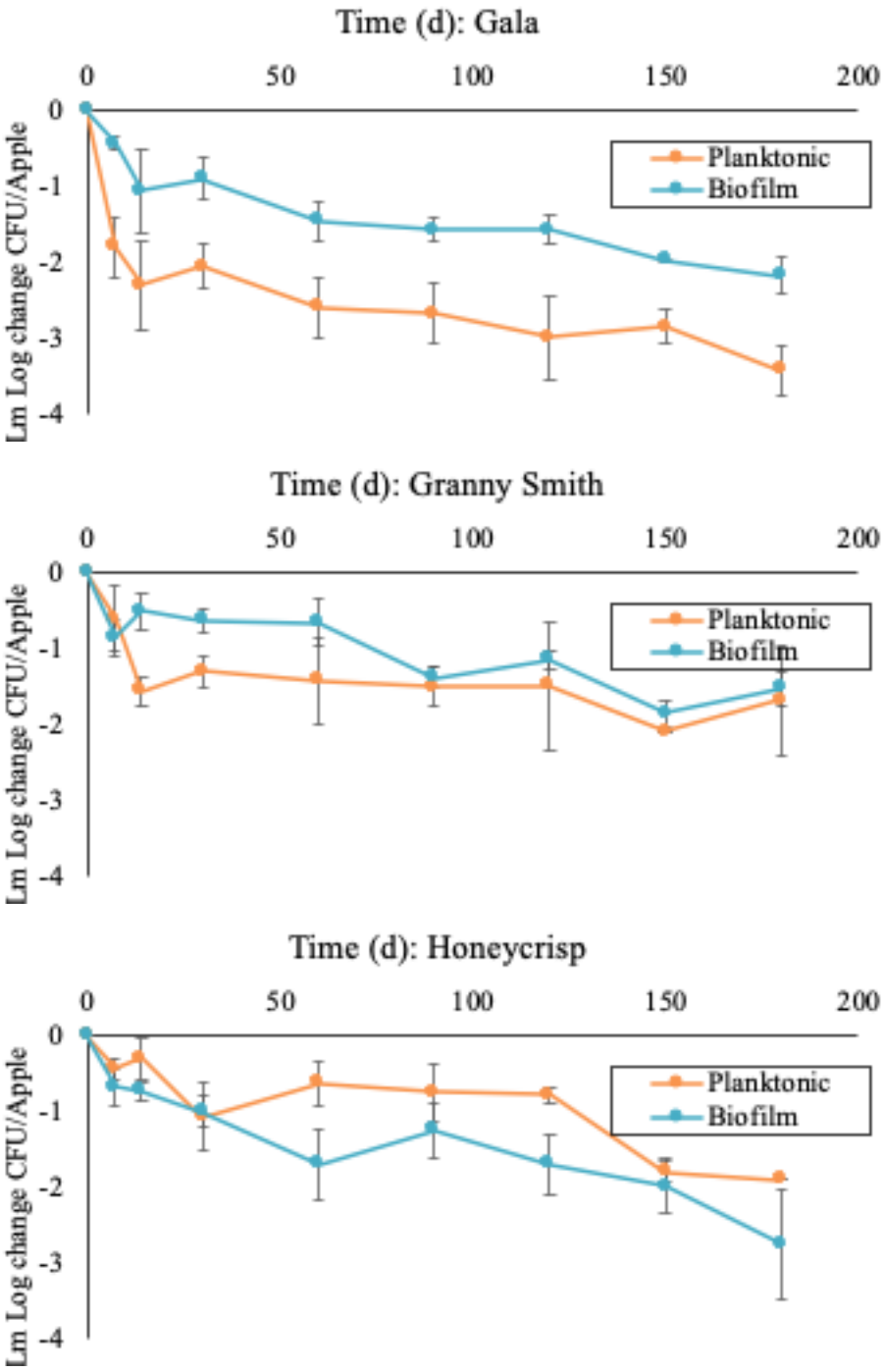

Figure 11. Average (± SD) *Lm* Log (CFU/apple) reductions for Harvest Year 2 on unwaxed Gala, Granny Smith, and Honeycrisp apples from Region A that were inoculated with the *Lm* planktonic (orange) or biofilm cocktail and subjected to air and controlled atmosphere storage.

Table 2. *Lm* reductions on apples for different storage, inoculation time, and waxing time.

Scenario 1: Inoculated month 0, aerobic storage 3 months, waxed month 7												
	G	**SD**	**G Wax**	**SD**	**GS**	**SD**	**GS Wax**	**SD**	**HC**	**SD**	**HC Wax**	**SD**
Planktonic	-1.3	0.18	-1.3	0.21	-1.2	0.55	-1.1	0.08	-0.55	0.08	-2.33	0
Biofilm	-0.04	0.08	0.16	0.19	0.02	0.01	-0.83	0.12	-0.61	0.19	-0.98	0.31
Scenario 2: Aerobic storage 3 months, inoculated month 3, waxed month 3												
	G	**SD**	**G Wax**	**SD**	**GS**	**SD**	**GS Wax**	**SD**	**HC**	**SD**	**HC Wax**	**SD**
Planktonic	0.68	0	-0.62	0.75	-1.57	0.16	-0.99	0.5	-1.07	0.23	-1.64	0.98
Biofilm	-1.24	0.06	-3.11	0.58	-0.51	0.27	-0.08	0.5	-0.5	0.25	-1.32	0.06
Scenario 3: Inoculated month 0, controlled atmosphere storage 7 months, waxed month 7												
	G	**SD**	**G Wax**	**SD**	**GS**	**SD**	**GS Wax**	**SD**	**HC**	**SD**	**HC Wax**	**SD**
Planktonic	-1.42	0.21	0.9	0.85	-1.76	0.65	-0.17	0	NA	NA	NA	NA
Biofilm	-1.32	0.6	-0.97	0.23	-0.34	0.2	-0.2	0.88	-0.72	0.37	0.38	0.33
Scenario 4: Controlled atmopshere storage 7 months, inoculated month 7, waxed month 7												
	G	**SD**	**G Wax**	**SD**	**GS**	**SD**	**GS Wax**	**SD**	**HC**	**SD**	**HC Wax**	**SD**
Planktonic	0.41	1.05	0.35	0.50	-0.37	0.14	1.58	0.50	-0.84	0.26	0.39	0.84
Biofilm	-2.25	0.46	-1.52	0.72	-2.97	0.92	-1.31	0.03	-0.92	0.30	-1.74	1.23

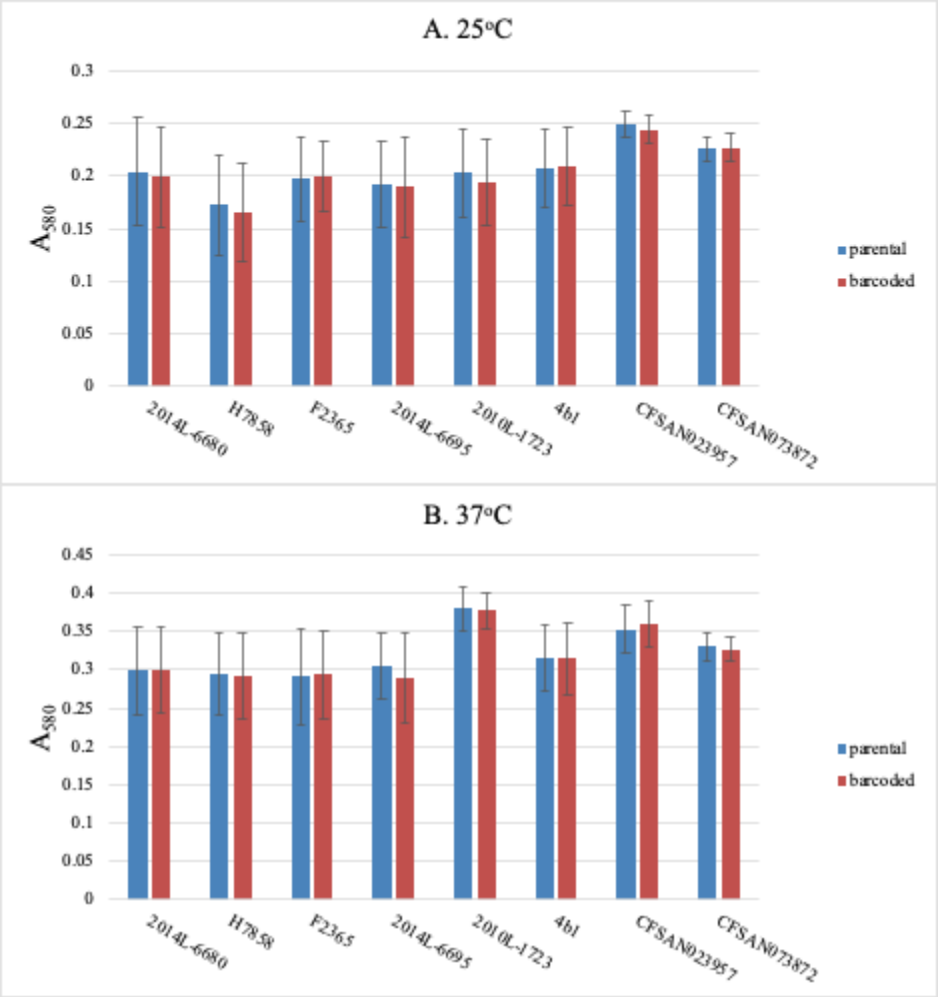

Figure 12. Lack of significant impact of strain barcoding on the ability to form biofilms on polystyrene. Biofilm formation of barcoded strains (red) and their parental counterparts (blue) was determined at (A), 25°C and (B), 37°C. The absorbance (A_{580}) of medium (tryptic soy broth) was subtracted from each experimental value.

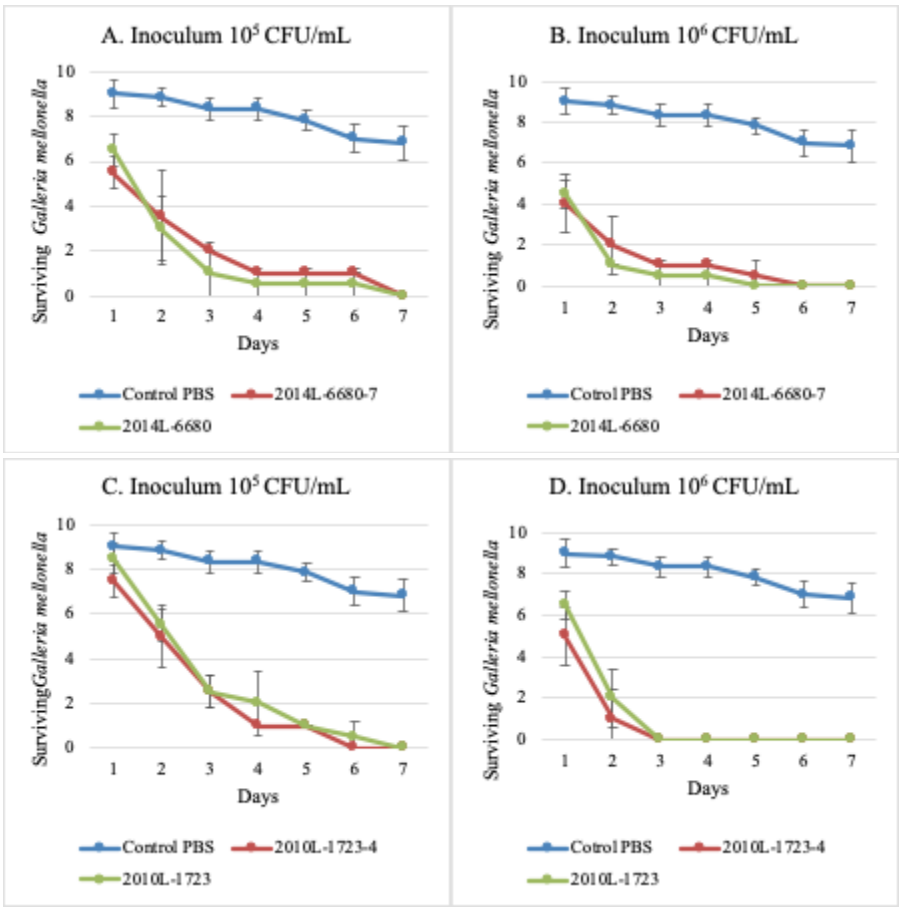

Figure 13. Lack of significant impact of strain barcoding on virulence based on survival assays with *Galleria mellonella*. Larvae (n=10) were inoculated with the indicated strain at 10^5 CFU/larva (A and C) or 10^6 CFU/larva (B and D) and survival at 37°C monitored over seven days. Two different barcoded / parental strains are shown here as representatives of all the strains. A and B, barcoded strain 2014L-6680-7 strain and its parental strain 2014L-6680; C and D, barcoded strain 2010L-1723-4 strain and its parental strain 2010L-1723. Parental strains are in gray, and the bar-coded derivatives in orange. Controls (blue) were injected with the same volume of sterile PBS and incubated similarly to the inoculated larvae.

Figure 14. Lack of noticeable impact of strain barcoding on motility. The tagged strain 2014L-6695-5 and its parental counterpart are spotted on the top and bottom row, respectively. The soft agar plate was incubated at 25°C for 48 h.

Table 3. Allelic variation between parental strains and their barcoded derivatives, according to BIGSdb PasteurMLST Genome Comparator, using cgMLST and wgMLST.

Barcoded Strains	Serotype, Genotype	Loci with differences (wgMLST)
2010L-1723-4 VS 2010L-1723	1/2a, ST378	*lmo0330* *lmo0828* *lmo1232* (core gene-recombination and DNA strand exchange inhibitor protein)
F2365-2 VS F2365	4b, ST1	*lmo1040* (core gene-molybdenum ABC transporter permease) *lmo2841*
2014L-6680-7 VS 2014L-6680	4b, ST1	*lmo0459* *lmo0464* *lmo1136*
2014L-6695-5 VS 2014L-6695	4bv-1, ST382	*lmo0461* *lmo2767*
H7858-1 VS H7858	4b, ST6	*lmo0426* *lmo1136*
CFSAN073872-6 VS CFSAN073872	1/2b, ST581	*lmo0669* (core gene- oxidoreductase)
CFSAN023957-A10 VS CFSAN023957	4bv-1, ST554	*lmo1482*

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