CENTER for PRODUCE SAFETY

CPS 2017 RFP
FINAL PROJECT REPORT

Project Title
Preservation of stone fruits by spray application of edible coatings with antimicrobial properties

Project Period
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Objectives
1. *Develop a flow-through system to determine cleaning efficacy on surfaces with flat and topographical features and determine surface role and cleaning procedure in the possible pathogen contamination of stone fruits.*
2. *Evaluate novel fruit coating formulations with antimicrobial properties that can be developed as brush-independent (spray) applications and can replace traditional wax treatments to maintain fruit integrity and shelf life.*
3. *Determine efficacy and properties of selected coating formulations in challenge studies in controlled conditions.*

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FINAL REPORT

Abstract
Biofilm formation in peach packinghouses can be the source of pathogenic bacteria since they can form in hard-to-clean spaces. A flow-through system was developed and used to grow biofilms and determine efficacy of cleaning systems on surfaces such as brushes and rollers with different typographical characterization. Based on the concept that Listeria can exist on these surfaces, it was hypothesized that existing, as well as novel, fruit coatings could carry a bacteriophage (P100) that is targeted for *Listeria monocytogenes* (Lm) to reduce or prevent its presence. Results showed that biofilm attachment was greater on rough surfaces and for surfaces with mineral oil coating compared with vegetable oil coating. Chlorine, in general, was effective in inactivating the pathogen in treatments at 200 ppm concentration and longer exposure time (20 minutes). Chlorine was less effective at 20 and 100 ppm, with cells surviving most treatments. However, 200 ppm of chlorine applied for 20 minutes achieved only a 3-log reduction on Green PSE filaments coated with Prima Fresh® 220. Six different coating formulations were evaluated to determine their effectiveness as a carrier of P100. Gelatin- and pectin-based coatings were selected for comparison with industry standard coatings (vegetable and mineral oil coatings). Adjustment to a pH that allowed for optimal P100 functionality provided some challenges related to mixing time. All coatings tested were effective against *L. monocytogenes* when tested in solution, but testing on peaches was not conclusive. In general, all coatings tested were not negatively affected by incorporation of P100 based on peach weight loss, firmness, and observational sensory-liking evaluation.

Background
Biofilms are defined as a sessile bacterial community attached to a substrate or to each other. Bacterial attachment and biofilm formation are problematic for public health and constitute a legitimate concern for cross-contamination. The sloughing of cells and dispersal of the biofilm are problematic. Also, the biofilm's extracellular matrix provides a physical barrier and protects cells against environmental factors and makes them more resistant to sanitizers. Biofilms can form on various surface materials, such as stainless steel, glass, and synthetic polymers, and on the surfaces of containers used in harvesting and transporting, and on conveyer belts.

Edible coatings ("waxes") are a common industry practice for postharvest fruit processes to help control the interchange of oxygen and moisture, thereby improving shelf life while the product makes its way through the distribution chain to the consumer. Edible coatings are either sprayed or dipped onto the surface of fruit, providing them with a semi-permeable membrane. It is important that the coating does not detract from the appearance of the fruit and perhaps enhances the appearance by providing a shiny, smooth, fresh surface. In addition to control of gases (oxygen, carbon dioxide and ethylene) and moisture, edible coatings can carry functional components such as antioxidants, colorants, nutraceuticals, flavors and antimicrobials. Some of the desired characteristics of edible coatings are to be semi-permeable, have even surface coverage, retain desired optical properties that can be maintained throughout the distribution cycle, are easy to apply using existing equipment or with minimal equipment modification, are low cost, and are safe for human consumption.

Since stone fruits can serve as transmission vehicles for *L. monocytogenes*, more research is needed to investigate factors that can promote *L. monocytogenes* contamination during postharvest handling, domestic transportation, and distribution. Specifically, little is known about *L. monocytogenes* attachment, survival, and colonization on stone fruit and there is no complete characterization of postharvest risks in these commodities. The possible contamination is more problematic since the "hurdle technology" used in most ready-to-eat (RTE) foods (combinations

of low pH, reduced water activity, and presence of antimicrobials) is not possible in RTE produce. For this project, we hypothesized that *L. monocytogenes* survival on stone fruits can be inhibited by a coating exhibiting both antimicrobial and preservation properties. The overall aim of the project was to characterize the conditions favorable for *L. monocytogenes* contamination of stone fruit in the packinghouse and design measures to reduce pathogen survival and avoid cross-contamination.

Research Methods and Results

Methods:

Objective 1. Develop a flow-through system to determine cleaning efficacy on surfaces with flat and topographical features and determine surface role and cleaning procedure in the possible pathogen contamination of stone fruits.

Bacterial strains and culture conditions: *L. monocytogenes* Scott A (obtained from Dr. Xiuping Jiang, Clemson University), and *L. monocytogenes* Petite Scott A (ATCC 49594) were used throughout the experiments. Strains were transformed with a plasmid encoding for the green fluorescent protein (GFP) and were maintained as freezer stocks at -80°C in 25% (wt/vol) glycerol. Prior to experiments, strains were cultured in tryptic soy broth (TSB) supplemented with 10 μg ml^{-1} erythromycin for plasmid maintenance.

Media: For biofilm growth, a chemically defined media was used or Listeria Synthetic Medium (LSM). Briefly, the media were prepared by mixing the 8 concentrated stock solutions prepared in advance with sterile distilled water (Table 1) and then adding glutamine and cysteine, which were prepared and added as fresh stocks. *L. monocytogenes* was grown in TSB supplemented with erythromycin at 10 μg ml^{-1} (overnight at room temperature on a rotary shaker) and then subcultured twice in 2X LSM for biofilm inoculation. Biofilms were grown in 0.5X LSM supplemented with erythromycin at 10 μg ml^{-1}.

Biofilm apparatus: Two systems were developed: (1) a flow-through apparatus where biofilms can form on test surfaces under laminar flow conditions (**Figure 1A**), and (2) a batch system for surfaces, such as brushes and brush filaments, with atypical shapes (**Figure 1B**). The advantages of the two systems are that (i) there is more flexibility in testing methods for different surfaces, and (ii) these biofilms are formed in relatively constant shear forces. The flow-through system is composed 8 individual flow cells (Figure 1A), with an inlet for fresh media and an outlet for microbial waste, along with connecting tubing inlet for microbial inoculum. Surfaces to be tested are inserted between a custom-cut silicone gasket and a glass slide and the whole apparatus can be autoclaved. The batch reactor consists of two pumps that flow media in and out with the same flow rate (to maintain constant level of nutrients without generating shear force), and the test containers are placed on a rotary shaker (Figure 1B).

Preparation of surfaces: Hytrel™ food grade (HYT 3078) was obtained from Dupont, USA, and Compac, New Zealand. This material is used to manufacture rollers for stone fruit packing lines. Coupons were machined (1 inch x 3 inch x 3/32 inch) with either a smooth surface (smooth) or with 1.0 ± 0.3 μm^2 and 3.5 ± 0.5 μm^2 surface roughness. The coupons were manufactured with a rotary platform, double-head abrader and the roughness was verified with an Olympus LEXT OLS4100 surface profilometer. Before experiments, coupons were sonicated in an alkali detergent solution for 20 minutes in an ultrasonic water bath, rinsed with distilled water, and then sonicated in a 10% phosphoric acid solution for 20 minutes and rinsed again with distilled water. Coupons were sterilized by dipping in 70% ethanol for 5 minutes prior to use.

Brushes were tested in two types of experiments: (i) testing of individual brush filaments – Waxlon, synthetic filament; Red polyethylene 0.015-inch-thick (Red PSE); Green polyethylene

0.020-inch-thick (Green PSE); and horse hair filaments (HH); and (ii) testing of the whole brush samples, which were custom-made brushes – tufted brush 50/50 HH/black PSE, tufted Waxlon, tufted 0.020 Green PSE, and tufted 0.015 red PSE. (All brushes were 4 inches high, with a 3.75-inch outer diameter and with no ends.)

Individual brush filaments were mounted for biofilm formation as follows: 1-inch diameter holes were punched through 0.5-inch silicone sheets, creating a mold. The molds were filled with 2% agarose (around 4 ml), which was allowed to solidify, and then small holes were cut in the plugs with sterile syringe needles. Individual filaments, cut to obtain 3-cm long bristles, were sterilized by placing in 70% ethanol solution for 5 minutes and then placed individually in the agarose plug holes with sterile tweezers. This preparatory work was performed under a biosafety cabinet.

Whole brushes were sterilized by dipping in 70% ethanol for 20 minutes, and then were allowed to dry overnight in a biosafety cabinet while exposed to ultraviolet light. In some experiments the filaments or brushes were coated with commercially available coatings: Prima Fresh® 220 (Prima 220), a mineral oil emulsion, or Prima Fresh® 55 (Prima 55), a vegetable oil emulsion. Coating solutions were prepared as recommended (Mr. George Nickolich, personal communication). The sterilized brush filaments and whole brushes, as previously mentioned, were allowed to coat for 10 min prior to use in experiments.

Inoculation and biofilm formation: Early exponential growth phase cultures of *L. monocytogenes* Scott A (optical density 0.15) grown in 2X LSM were inoculated in the flow cells (10 ml each, with a sterile syringe through a separate inlet tubing) and the cells were allowed to attach for 1 hour. For the batch system, the inoculum was pumped in the containers through separate tubing than the media feed. Prior to starting the media feed, the containers of the batch reactor were emptied of the inoculum by activating the end pump. After the attachment phase, the media was pumped at a flow rate of 500 µl min^{-1} for the flow-through cells and 1 ml min^{-1} for the batch reactor. The batch reactor had double use for the brush filament experiments as well as testing of the whole brushes.

Biofilm survival in environmental conditions: Biofilms were grown for 48 hours, and then the coupons were placed in open petri dishes inside individual humidity cabinets where the relative humidity was maintained at 26±5, 55±5, and 95±5 %RH with saturated salt solutions. The RH and temperature were monitored with wireless dataloggers (Fisher Scientific). Samples were removed at specific time intervals, and live cells were determined by plate count on TSA supplemented with erythromycin at 10 µg ml^{-1}.

Treatment of biofilms with chlorine and benzalkonium chloride: Biofilms of *L. monocytogenes* Scott A were developed on coupons and brush filaments as described above. Each test surface was removed from the apparatus and placed in test tubes or sterile petri dishes containing the disinfectant solution. Chlorine solutions were prepared from a 6% stock of sodium hypochlorite (vol/vol, Ricca Chemical Company) by adding different volumes to phosphate buffer (pH 7.5) to obtain 20, 100, and 200 ppm. The concentration of chlorine was determined with a Hach kit. Benzalkonium chlorite (BK) solutions at 100 and 400 ppm were prepared from a 50% stock solution (w/w, Alfa Aesar) in sterile water. Surfaces were exposed to sanitizer for selected time intervals and then removed and placed in sterile tubes containing TSB supplemented with 1% sodium thiosulphate. Serial dilutions were made and plated on TSA-erythromycin plates and incubated at 37°C to determine the populations of the surviving cells.

Microscopy: Hytrel coupons and brush filaments were analyzed with a surface profilometer (Olympus LEXT OLS4100) and roughness measurements were determined with a 20X lens. Measurements were collected from 5 different parts of the sample and each material was analyzed with at least triplicate samples. Biofilms of *L. monocytogenes* were imaged with a confocal microscope (Leica TCS SPE).

Statistical analysis: All experiments were replicated 3 times or more. In testing of the disinfectants, two test surfaces were used in each replicate. Data sets were analyzed using the general linear model of the Statistical Analysis System (SAS). Duncan's multiple range test and proc *t* test were used to determine which conditions were significantly different. Significant differences in mean values are presented at a 95% confidence level ($P \leq 0.05$).

Objective 2. Evaluate novel fruit coating formulations with antimicrobial properties that can be developed as brush-independent (spray) applications and can replace traditional wax treatments to maintain fruit integrity and shelf life.

Coating Formulations: The goal was to develop a coating that was food grade, contained GRAS ingredients in the formulation, had a viscosity similar to existing commercial coatings, had similar visual effects on the fruits as existing coatings, and provided similar or improved shelf life in terms of sensory acceptability and moisture retention (weight loss). Pass/fail criteria were established to determine which coatings would be used for Objective 3.

Aloe vera – In 100mL distilled water, add 24g aloe vera, stir to dissolve and add 8g glycerol.

Whey protein isolate – In 150mL distilled water, add 6g whey protein isolate and stir for 20 min; add 3g glycerol and heat to 90°C for 30 min.

Methylcellulose – In 100mL 50:50 ethanol:distilled water, add 3g methylcellulose, heat to 75°C for 15 min.

Sodium alginate – In 100mL distilled water, add 2g sodium alginate, stir; in 10mL distilled water, add 0.1g calcium chloride and then add to sodium alginate until dissolved.

Gelatin – In 100mL distilled water, add 2.5g gelatin and 0.5g glycerol; bring to a boil on a hot plate and cool to room temperature; adjust pH to 6.44 using sodium hydroxide (1N).

Pectin – In 100mL distilled water, add 2.31g citrus pectin and 1.35g glycerol, heat to 75°C for 15 min; adjust to pH 7.68 with sodium hydroxide (1N).

Coating method: A batch of peaches was submerged for 5 min in a container holding 2L of coating, and then removed by using gloved hands. The coated peaches were spread out on bread racks lined with cheesecloth, and were allowed to dry at room temperature for 2 h and then refrigerated.

Dye coverage testing: Blue food coloring was added to the peach coatings and examined for smooth coverage of the coating. Defects such as clumps or roughness were also observed. Descriptions of coverage are provided in Table 4.

Zone of inhibition testing: Peach coating formulations were produced with and without incorporation of Listex™ P100 phage mixture, at a concentration of $2x10^8$ PFU/mL (PFU = plaque forming units). Coating solutions tested were methylcellulose (4000 cps), Aloe vera, whey protein isolate, and sodium alginate. In other testing, pectin and gelatin with nisin, P100 or combinations of both were evaluated. Spot-on-lawn assays were used: tryptic soy agar was seeded with a suspension containing 8 log (CFU/mL) of *L. monocytogenes* Scott A strain. Aliquots (10 µL) of each solution were placed onto the agar plates and incubated for 48h at 35°C. Phosphate buffer was used as the control and all tests were performed with six data points per treatment. Inhibition zones were measured with a digital caliper.

Objective 3. Determine efficacy and properties of selected coating formulations in challenge studies in controlled conditions.

Coatings: Coatings with or without phage (P100) for peaches were assigned the following codes: C-PP = Control; GC = Gelatin, no phage; GCP = Gelatin w/phage; MC = Mineral oil, no phage; MCP = Mineral oil w/phage; PC = Pectin, no phage; PCP = Pectin, w/phage; VC = Vegetable, no phage; VCP = Vegetable oil w/phage.

Inoculation procedure for coated peaches: *Listeria monocytogenes* strains Scott A and 15313 were used. For each culture, an overnight culture was centrifuged at 6,000 rpm for 10 min at 4°C; supernatant was discarded and then the pellet was gently re-suspended in the same volume of saline. Samples were centrifuged again at 6,000 rpm for 10 min at 4°C; supernatants were discarded and pellets were re-suspended in saline. The two strains of *Listeria* were combined in equal volumes in one flask. Optical density was measured at 600 nm to 0.5 and adjusted with sterile saline to 9 log CFU/mL. Peaches were soaked in inoculum for 30 min, and then placed in refrigerated storage. Two inoculation levels were used: 3 log and 6 log.

Sampling: Peaches were removed from refrigerated storage. Three peaches per treatment were used for each sampling period. Peaches were placed in a sterile stomacher bag and diluted 1:1 (w/w) with sterile 0.8% saline solution and then periodically agitated by hand over a 30-minute period. The rinsate from the peaches was sampled, and serial dilutions were made using phosphate buffer solution. Triplicate plates were used for each treatment. DRBC (dichloran rose bengal chlortetracycline) agar was used for enumeration of yeasts and molds, and PCA (plate count agar) for non-inoculated peaches. Plating on TSA with rifampicin (TSA-R) was used for isolation of *L. monocytogenes* on inoculated peaches. Results represent data from only one trial set of peaches.

Sensory Observation of Coated Un-inoculated Peaches: Thirty panelists evaluated un-inoculated peaches from each coating treatment. A 9-point hedonic scale was used, with 0 = neither like nor dislike, 1 = like slightly, 2 = like moderately, 3 = like very much, 4 = like extremely; -1 = dislike slightly, -2 dislike moderately, -3 dislike moderately, and -4 dislike extremely. Three peaches, one from each of three coating treatments, were presented on white plates, and panelists were asked to mark ballots without picking up the peaches although they were allowed to pick up the plate for closer observation. In addition to the hedonic liking scale, panelists were asked whether they would buy the peaches from each coating group and to provide any comments from their evaluation.

Texture: A Wagner Instruments fruit test (FT) handheld penetrometer was used for testing the firmness of all of the coated and uncoated peaches. There were 5 peaches per treatment, and 5 measurements made per peach; n = 25 per treatment.

Weight loss: 5 peaches from each treatment were weighed at three different time intervals. The % weight loss was calculated as: (weight day 0 – weight at time of testing)/ weight day x 100. .

Outcomes and Accomplishments

Objective 1:
We developed two test systems for biofilm formation. Experiments were performed for both reactors, and results were reproducible from cell to cell. We tested for reproducibility by microscopic observation of the biofilm coverage and thickness, and by cell plate counts. *Listeria monocytogenes* Scott A formed “mature” biofilms after 48-h growth in minimal media conditions; a biofilm with thick coverage right before cell sloughing was considered to be mature.

We tested pathogen long-term viability when exposed to dry conditions. Coupons with 48-h grown biofilms were stored in controlled temperature and RH conditions (26, 55 and 95% RH), along with inoculated droplets of cells from culture. Planktonic cells of the pathogen did not

survive drying for more than 6 h without adjuvants or osmoprotectants in all tested dry conditions. However, as biofilms, cells can survive in low RH conditions for more than 10 days (**Figure 7**), suggesting that favorable conditions and niches of *L. monocytogenes* should be avoided because exposure to desiccation does not necessarily get rid of the pathogen.

Biofilms developed on Hytrel coupons of smooth, 1.5 μm^2 and 3.5 μm^2 roughness and increased to 9.2, 9.8 and 10.4 log CFU/coupon, respectively. Roughness provided more surface for *L. monocytogenes* attachment and possible protection against shear forces. Treatment with 200 ppm chlorine for 20 min inactivated the pathogen on smooth and 1.5 um^2 rough coupons, and reduced the pathogen from 10.4 to 2.1 log CFU/coupon on the 3.5 μm^2 coupons.

Biofilms of *L. monocytogenes* Scott A were developed on brush filaments. Results showed that cells attached in significantly higher levels on Green PSE (average 5.1 log CFU/filament) and the lowest attachment was on the red PSE (average 3.6 log CFU/filament). The Green PSE filament is larger in diameter, and confocal microscopy and roughness measurements indicated that the Red PSE has a smoother surface compared with Green PSE (**Figure 2** and **Table 2**). Red PSE roughness consisted mostly of smooth pits whereas Green PSE roughness involved more rugged areas at the edges of the filament. From our sample set, Waxlon had the lowest general roughness. Confocal microscopy indicated that cells and biofilms were located only on the outside of the filaments (**Figure 3**). Interestingly, cell attachment was significantly higher ($P \leq 0.05$) when filaments were coated with Prima Fresh® 220 mineral oil coating, especially for Green PSE Prima 220 (average 6.5 log CFU cells/filament). Prima Fresh® 55, the vegetable oil–based coating had a similar effect but not significant compared to the non-coated filaments. The increased attachment was observed for all filaments, including Red PSE and horse hair.

Biofilms formed on brush filaments for 36 h were treated with 20, 100 or 200 ppm chlorine for 1, 5, 10, and 20 min. Chlorine, in general, was effective in inactivating the pathogen when treated at 200 ppm concentration and for longer exposure time (20 min). Chlorine was less effective at 20 ppm (**Figure 4**) and 100 ppm (**Figure 5**), with cells surviving most treatments. *However, 200 ppm of chlorine applied for 20 min achieved only a 3-log reduction on Green PSE Prima 220 filaments (coated with Prima Fresh® 220)* (**Figure 5**). The pathogen reduction was significantly higher on the other filaments tested but we isolated residual bacteria after treatment (1.3 log CFU/filament detection limit).

The same brush filament sample combinations were treated with 100 or 400 ppm benzalkonium chlorite (BK). The sanitizer proved more effective than chlorine ($P \leq 0.05$), and 400 ppm for 20 minutes reduced the pathogen by more than 4 log on Green PSE Prima 220 but it did not completely eliminate *L. monocytogenes* (**Figure 6**). The sanitizer was significantly more effective on the Red PSE non-coated sample compared with the rest of the filaments.

The same results were observed when biofilms formed for 36 h on whole brushes were treated with chlorine at 200 ppm or BK at 400 ppm for 20 min (**Table 3**). These experiments were designed as enriched samples, where survivors can grow after sanitizer treatment (positive versus negative or complete inactivation). We observed that BK is more effective in inactivating *L. monocytogenes* biofilms on whole brushes, including in the presence of coatings.

Objective 2:
Aloe vera and whey protein isolate coating solutions had significantly higher zones of inhibition compared with the sodium alginate, methyl cellulose, and methyl cellulose control (**Figure 8**). Methyl cellulose control was the coating solution without P100 bacteriophage, indicating that the ethanol in the coating solution provided the inhibitory effect rather than the bacteriophage since the phage is inactivated by the ethanol during the mixing process. Peptone was used as a

negative control and did not contain the bacteriophage. There was no zone of inhibition for the peptone, as expected.

The decision summary regarding selection of coatings for the challenge study is provided in **Table 4**. Two additional coating formulations were tested for effectiveness as a carrier for Listex™ P100: gelatin and pectin. Both gelatin and pectin have been used as antimicrobial coatings containing nisin in our laboratory for previous studies. To determine if nisin would have a synergistic effect, it was added to the study with and without incorporation of the P100 phage mixture and screened using a spot-on-lawn assay. As shown in **Figure 9**, pectin and gelatin with nisin and/or P100 were statistically similar in size of zones of inhibition. Pectin with P100 alone is not shown because it did not have zone of inhibition due to acidified water used to solubilize the pectin. Nisin did not show any synergistic effect with P100. All coatings required pH adjustment to allow P100 to perform optimally, which slowed the mixing of the coatings. Based on discussions with personnel at CPS and industry partners, it was determined that spray application could be pursued at a later time, after results of coating efficacy against *Listeria* were tested.

Objective 3:
Coating peaches with mineral oil containing P100 was significantly ($P < 0.10$) less effective for reducing *L. monocytogenes* on the surface of the peaches compared with no coating or coating peaches with mineral oil without P100. Pectin coating without P100, gelatin coating with P100 and vegetable oil coating without P100 had significantly lower *L. monocytogenes* after 14 days of storage compared with all other coatings (**Figure 10**). Phage assays indicated that the phage was still active in all coatings applied to peaches after 14 days of storage. It should be noted that these results only represent one set of data and there was no significant difference among *L. monocytogenes* counts from all of the tested peach coatings after 14 days of refrigerated storage. More testing should be done with additional replicates to obtain more reliable data from which conclusions may be drawn. Coatings containing phage (vegetable oil, gelatin and pectin-based) were all significantly more firm than peaches without phage (**Figure 11**). However, the presence of phage in the coatings did not significantly ($P > 0.10$) affect weight loss in peaches (**Figure 12**). Pectin-based coated peaches with and without phage had significantly more weight loss compared with peaches coated with vegetable or mineral oil (with and without phage). Based on sensory observation, panelists significantly liked peaches with the vegetable oil–based coating containing phage compared with the pectin (with phage) and gelatin-based coating (with and without phage). Liking scores for all coated peaches were within the range of neither like or dislike or slightly like or dislike (**Figure 13**), so from a practical standpoint, there was little difference among all of the peaches evaluated.

Summary of Findings and Recommendations
We would like to point out the resilience of *Listeria monocytogenes* in the environment, especially when forming biofilms. Although our experiments were designed to simulate the "worst-case" scenario by growing the mature biofilms for 48 hours, we recommend that possible niches of *L. monocytogenes* should be identified in packinghouses and eliminated. Desiccation or exposure to low relative humidity will not kill the pathogen, and it can detach and contaminate other surfaces and food products. Also noticeable is the difference in the attachment on different brush filaments, since smoothness of the filament seems to be important in holding the pathogen. When using a mineral oil coating such as Prima Fresh 220, *the brush should be cleaned thoroughly* to eliminate the coating, prior to sanitizer treatment. Alternatively, use a vegetable oil coating, which will provide a less hydrophobic environment. We also determined that in the case of brushes and their filaments, the cationic surfactant benzalkonium chloride

(BK), is more effective in disinfection than chlorine. However, in the case of brushes, the sanitizer treatment should be allowed to continue for a longer time (i.e., 20 minutes). Regarding coatings, the incorporation of P100 phage (Listex™ P100 phage mixture) was effective for reducing *L. monocytogenes* populations in solution but its effectiveness was inconclusive when tested on coated peaches. The process of mixing and pH adjustment would need more development to be industry ready, as the process was lengthy. Peaches with traditionally used coatings (mineral or vegetable oil) had less weight loss than those coated with gelatin or pectin (with and without phage) but were less firm. Based on sensory observations, all peaches were generally liked the same, indicating that the coatings did not affect the overall liking of the peaches. Overall, coatings traditionally used in industry were not negatively affected by the addition of P100 except for pH adjustment. In addition, gelatin and pectin-based coatings with and without phage had similar properties to industry standard coatings (vegetable and mineral oil based) and provided peaches with increased firmness. The project data shows there were no negative effects, thus further research is warranted.

APPENDICES

Publications and Presentations:

Manuscript in preparation: Claudia Ionita, Terri Bruce, Kay Cooksey, Paul Dawson, Julie Northcutt, and Justin Scott. Biofilm formation by *Listeria monocytogenes* on filaments and brushes used for coating: influence of material and coating.

Budget Summary

Total funds awarded were $97,040. All funds are expected to be used by the end of the project term. Remaining funds will be used for publication fees and travel to the 2019 CPS Research Symposium.

Tables 1–4 and Figures 1–13

(see below)

Table 1. Chemicals and concentration and stock solutions used for the preparation of Listeria Synthetic Medium. Stocks of MOPS, glucose, phosphate, magnesium stocks were autoclaved whereas the rest of the components were filter-sterilized.

Stock name	Stock Dilution Factor	Volume stock (ml)	Ingredient	Final Conc. in M or(uM)	MW (g/mol)	Final Conc. g/L	Total in stock (g)
MOPS (pH 7.5) adjust pH to 7.5 with 45 ± 2 ml 10N NaOH (800 ml water and 400g NaOH pellets the adjust to 1L)	10	1000	MOPS	0.1	209.3	20.93	209.3
Glucose	40	1000	Glucose	0.0555	180.2	10	400
Phosphate	100	500	KH2PO4	0.0048	136.1	0.656 3.096	32.8
			Na2HPO4 • 7H2O	0.0115	268.1		154.8
Magnesium	100	500	$MgSO_4$ • 7H2O	0.0017	246.5	0.409	20.45
Micro-Nutrients -dissolve all components in boiling water prior to filter sterilization	100	500	Biotin	(2.05)	244.3	0.0005	0.025
			Riboflavin	(1.33)	376.3	0.0005	0.025
			Para-Aminobenzoic Acid	(7.29)	137.1	0.001	0.05
			Lipoic Acid	(0.02)	206.3	0.000005	0.00025
			Niacinamide/Nicotinamide	(8.19)	122.12	0.001	0.05
			D-Pantothenic Acid (hemicalcium)	(4.20)	238.27	0.001	0.05
			Pyridoxal • HCl	(4.91)	203.62	0.001	0.05
			Thiamine • HCl	(2.96)	337.27	0.001	0.05
Minimum Amino Acidsc Dissolve the 8 minimum amino acids in hot 1N NaOH, this stock is the "minimum amino acid" stock	50	500	L-Arginine • HCl	0.0005	210	0.1	2.5
			L-Histidine • HCl • H2O	0.0005	209.6	0.1	2.5
			DL-Isoleucine	0.0008	131.17	0.1	2.5
			L-Leucine	0.0008	131.17	0.1	2.5
			DL-Methionine	0.0007	149.21	0.1	2.5
			L-Phenylalanine	0.0006	165.19	0.1	2.5
			L-Tryptophan	0.0005	204.23	0.1	2.5
			DL-Valine	0.0009	117.15	0.1	2.5
nine (dissolve in 40 ml of 0.2N HCl then dilute to the final concetration in wa	100	500	Adenine	(18.50)	135.13	0.0025	0.125
Trace Metals	100	500	FeCl2 • 4H2O	(5)	198.8	0.00099	0.05
			MnSO4 • H2O	(50)	169	0.00845	0.423
			ZnSO4 • 7H2O	(1)	287.6	0.00029	0.014
			CaCl2 • 2H2O	(10)	147	0.00147	0.074
			CuSO4 • 5H2O	(0.1)	249.7	0.00002	0.001
			CoCl2 • 7H2O	(0.1)	281.1	0.00003	0.001
			H3BO3	(0.1)	61.8	0.00001	0.0003
			Na2MoO4 • 2H2O	(0.1)	242	0.00002	0.001
			NaCl	0.008555784	58.44	0.5	25
			Sodium Citrate (Tri-Sodium Salt)	(100)	294.1	0.02941	1.471
Added Fresh Can't make stock of these , must be fresh			L-Cysteine * 2HCL	(634.44)	157.62	0.1	
			L-Glutamine	(4105.65)	146.14	0.6	

Table 2. Area roughness parameters for filament materials. The following parameters were measured and represent the average of 3 samples (measurements taken for each sample in 5 different spots): Sq, root mean square height; Ssk, skewness; Sku, kurtosis; Sp, maximum peak height; Sv, maximum pit height; Sz, maximum height; and Sa, arithmetical mean height. Measurements were collected with a profilometer with a 20x magnification lens.

Filament material	**Sq** [µm]	**Ssk**	**Sku**	**Sp** [µm]	**Sv** [µm]	**Sz** [µm]	**Sa** [µm]
Green PSE	4.33125	-1.00675	7.2635	15.705	27.7335	43.4385	3.11
Red PSE	4.38275	-4.28925	31.02075	8.195	40.29375	48.48875	2.27025
Waxlon	0.479	-1.307	3.95725	0.518	1.66575	2.18375	0.37575
Horse hair	0.89225	-2.32525	11.67275	1.74475	6.75925	8.50425	0.59825

Table 3. Inactivation of *L. monocytogenes* Scott A grown on whole brushes. Biofilms were allowed to develop for 36 hours, and then brushes were exposed to sanitizers. Brushes were subjected to enrichment in TSB with erythromycin and sodium thiosulphate. The assay detected positive/negative samples. Positive samples were plated on TSA erythromycin and Oxford medium for confirmation.

Brush type	Number of samples	Disinfectant	Number of positive samples
Green PSE	8	Chlorine 200 ppm	2
		BK 400 ppm	0
Green PSE Prima 220	8	Chlorine 200 ppm	7
		BK 400 ppm	3
Green PSE Prima 55	8	Chlorine 200 ppm	3
		BK 400 ppm	1
Red PSE	8	Chlorine 200 ppm	0
		BK 400 ppm	0
Red PSE Prima 220	8	Chlorine 200 ppm	2
		BK 400 ppm	0
Red PSE Prima 55	8	Chlorine 200 ppm	0
		BK 400 ppm	0
Waxlon	8	Chlorine 200 ppm	1
		BK 400 ppm	0
Waxlon Prima 220	8	Chlorine 200 ppm	3
		BK 400 ppm	1
Waxlon Prima 55	8	Chlorine 200 ppm	1
		BK 400	0
Horse hair /Black PSE	8	Chlorine 200 ppm	2
		BK 400 ppm	1
Horse hair/Black PSE Prima 220	8	Chlorine 200 ppm	3
		BK 400 ppm	1
Horse hair/Black PSE Prima 55	8	Chlorine 200 ppm	3
		BK 400 ppm	2

Table 4. Decision summary regarding selection of coatings for challenge study.

Coating	**Advantages**	**Disadvantages**	**Go/ No go Decision**
Aloe vera	Natural, good clarity, good inhibitory results	Lengthy mixing time, possible bitter taste,	No
Whey protein isolate	Natural, good inhibitory results, good mixing time	Possible allergen labeling due to whey protein, poor coverage on peach, lengthy mixing time.	No
Sodium alginate	Natural, good clarity in solution	Too thick, poor coverage on peach (clumps)	No
Methylcellulose	Natural, good clarity	Too thick, poor coverage on peach, ethanol deactivated phage	No
Methylcellulose +nisin	Natural, good clarity	Same as above plus nisin had no synergistic effect	No
Pectin	Natural, no off flavor, good coverage, good clarity	Needed pH adjustment for phage stabilization	Yes
Gelatin	Natural, no off flavor, good coverage, good clarity	Needed pH adjustment for phage stabilization	Yes

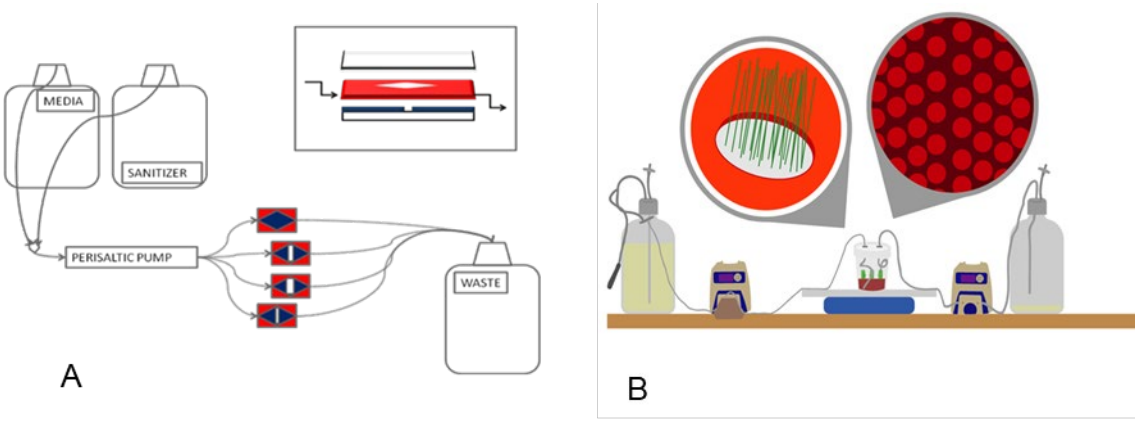

Figure 1. Schematic representation of the biofilm apparatus used in this study. (A) Flow-through system with inlet of fresh media and connecting tubing for pumping out waste. Insert shows the assembly of a typical flow cell. (B) Batch biofilm apparatus where biofilms are grown in containers with constant flow of media in and the same volume being pumped out. Inserts show testing of the individual brush filaments inserted individually in agarose plugs and testing where the whole brush is inserted in a container.

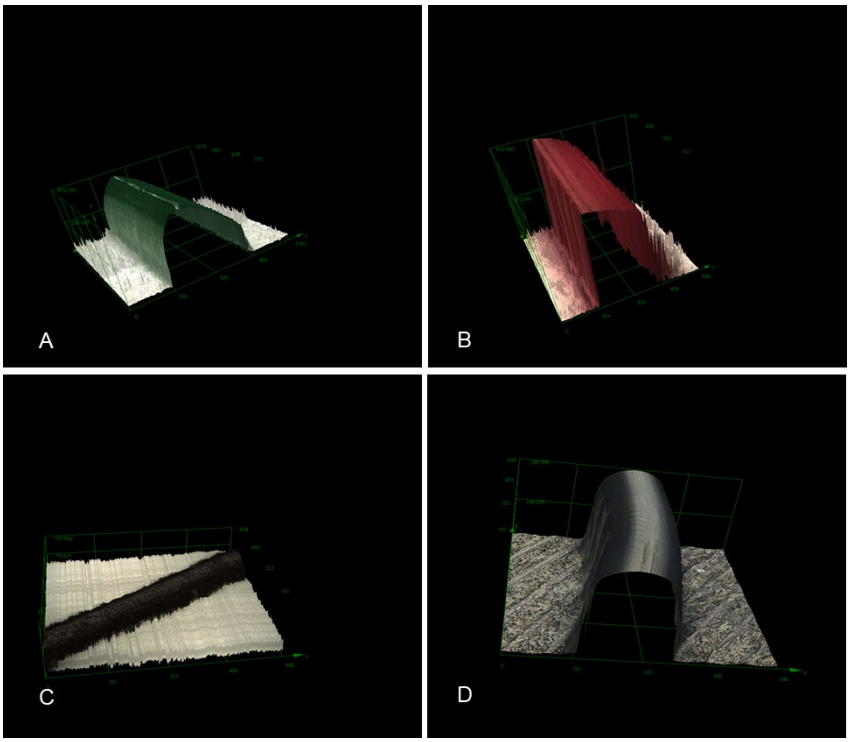

Figure 2. Confocal images of brush filaments. (A) Green PSE, (B) Red PSE, (C) Horse hair and (D) Waxlon. Green and Red PSE images were collected with 10X magnification and Waxlon and horse hair with 20X magnification. Images and roughness were collected with an Olympus LEXT OLS4100 surface profilometer.

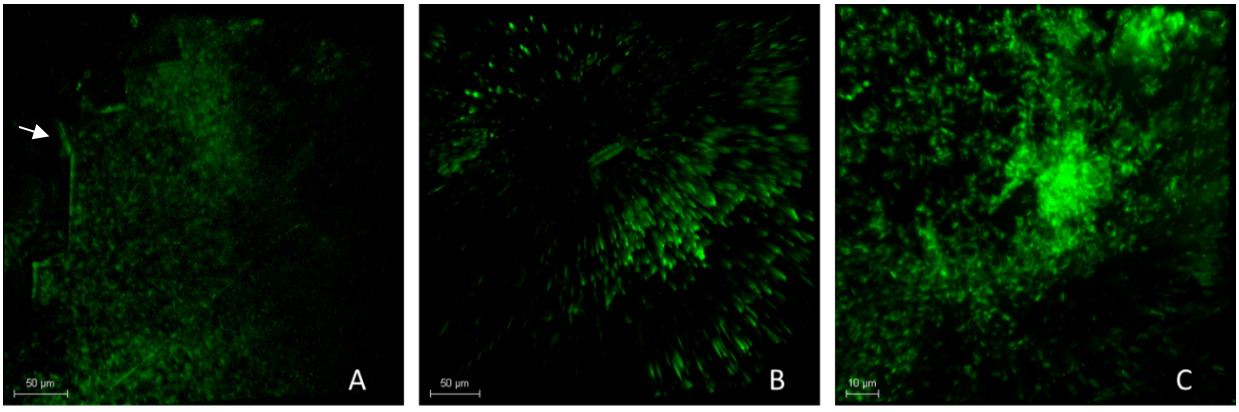

Figure 3. Confocal image of *L. monocytogenes* Scott A biofilms grown on (A) Green PSE, (B) Green PSE Prima 55, and (C) Green PSE Prima 220. Arrow points out the rough edges of the Green PSE filament where bacteria are located. The Green PSE Prima 220 consists of 'plumes' of cell aggregates. (A) and (B) were collected with a 20x magnification lens, and (C) with a 63x magnification lens.

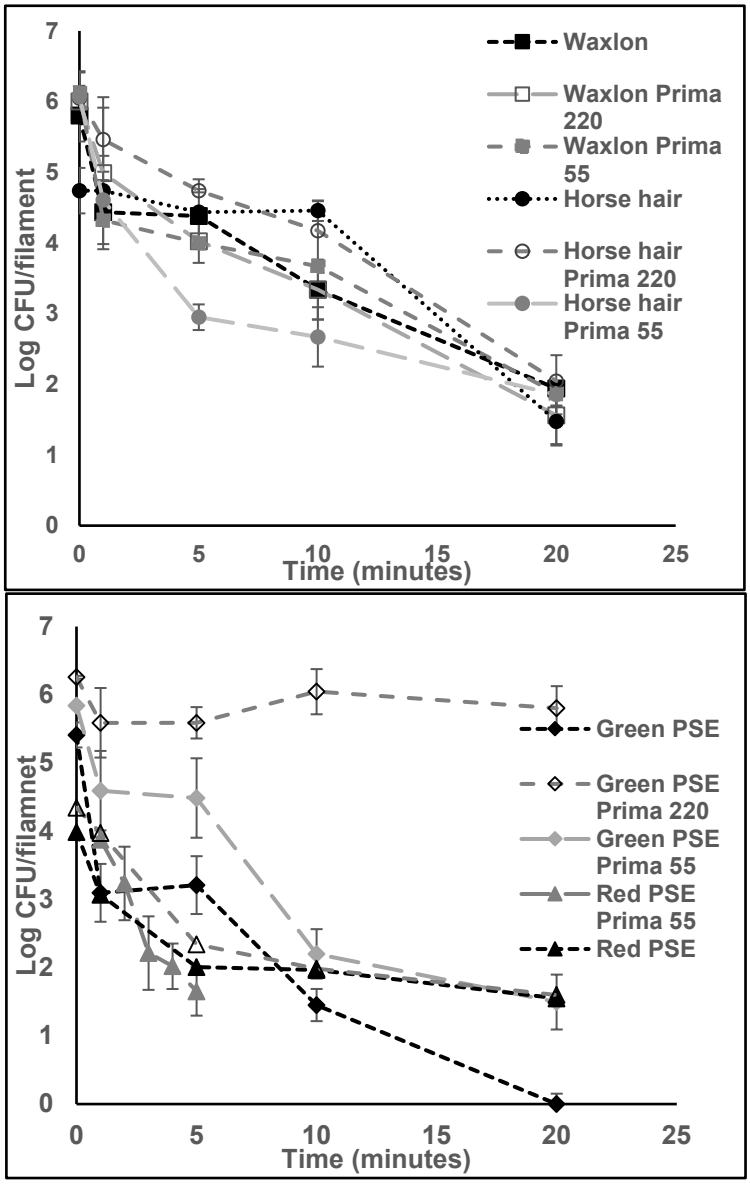

Figure 4. Inactivation of *L. monocytogenes* Scott A biofilms on brush filaments treated with 20 ppm chlorine. Vertical bars indicate standard deviation and the values are the means of 3 independent experiments each with triplicate samples.

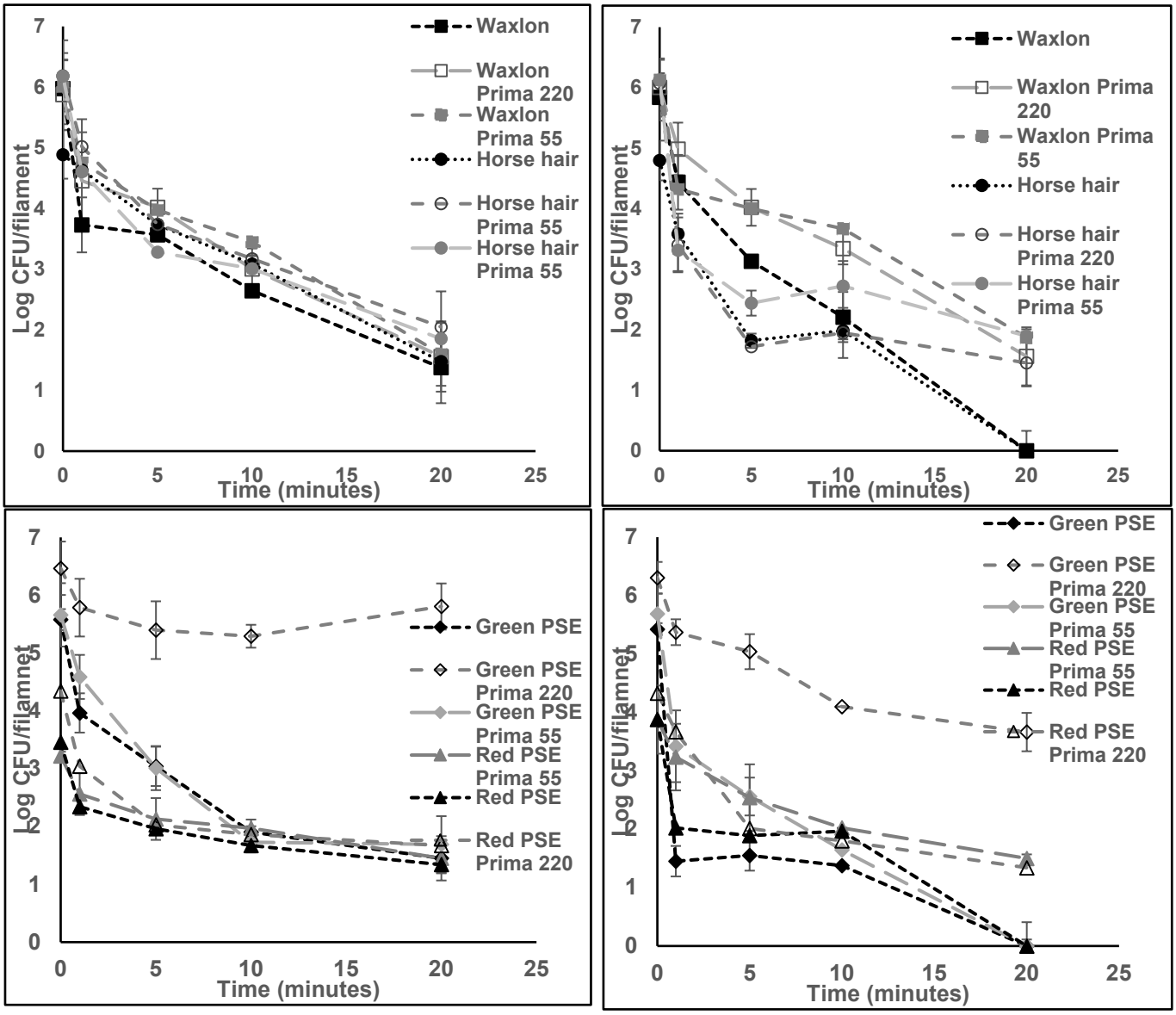

Figure 5. Inactivation of *L. monocytogenes* Scott A biofilms on brush filaments treated with 100 ppm chlorine (left column) and 200 ppm chlorine (right column). Vertical bars indicate standard deviation and the values are the means of 3 independent experiments each with triplicate samples.

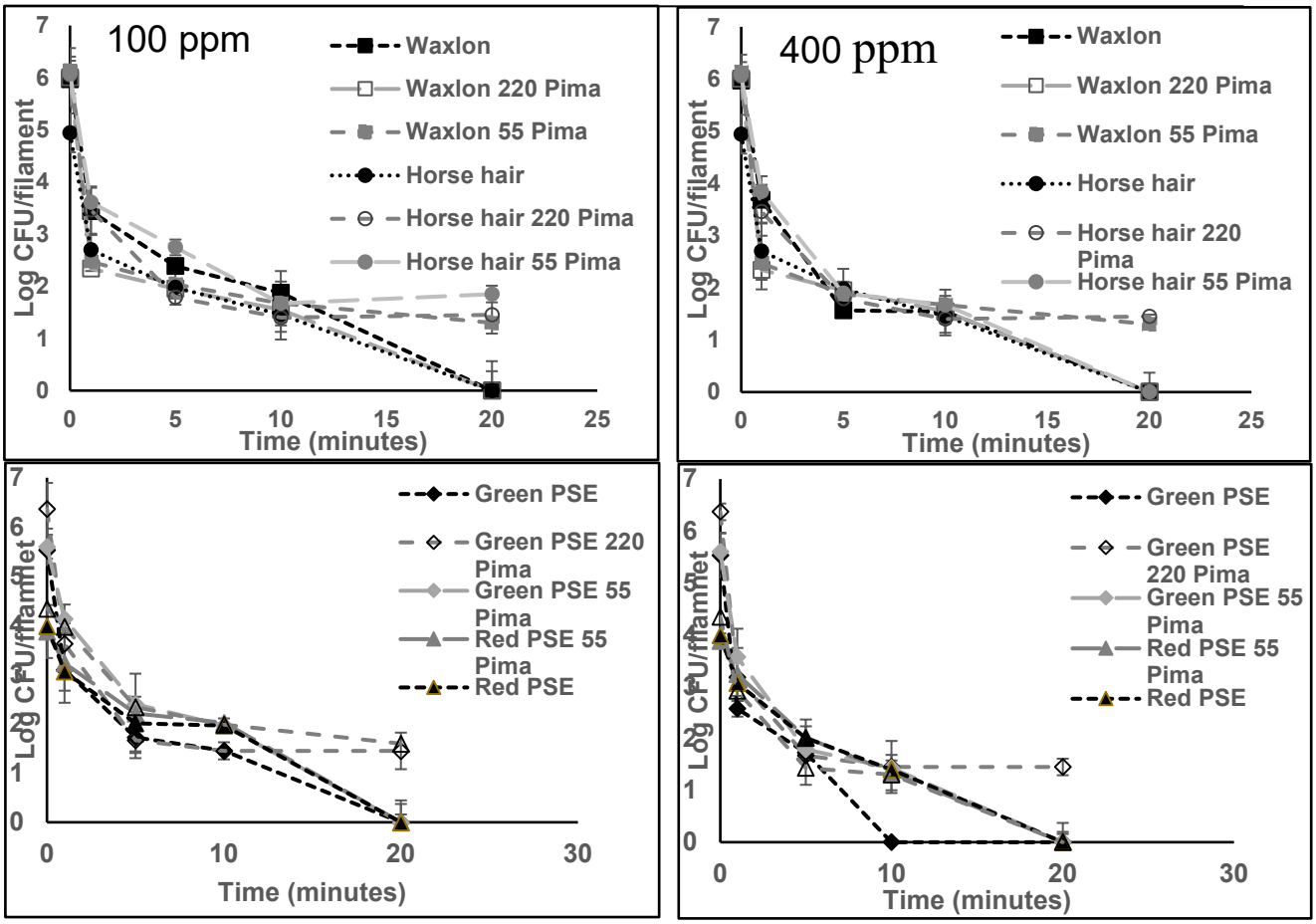

Figure 6. Inactivation of *L. monocytogenes* Scott A biofilms on brush filaments treated with 100 ppm benzalkonium chlorite (BK) (left column) and 400 ppm BK (right column). Vertical bars indicate standard deviation and the values are the means of 3 independent experiments each with triplicate samples.

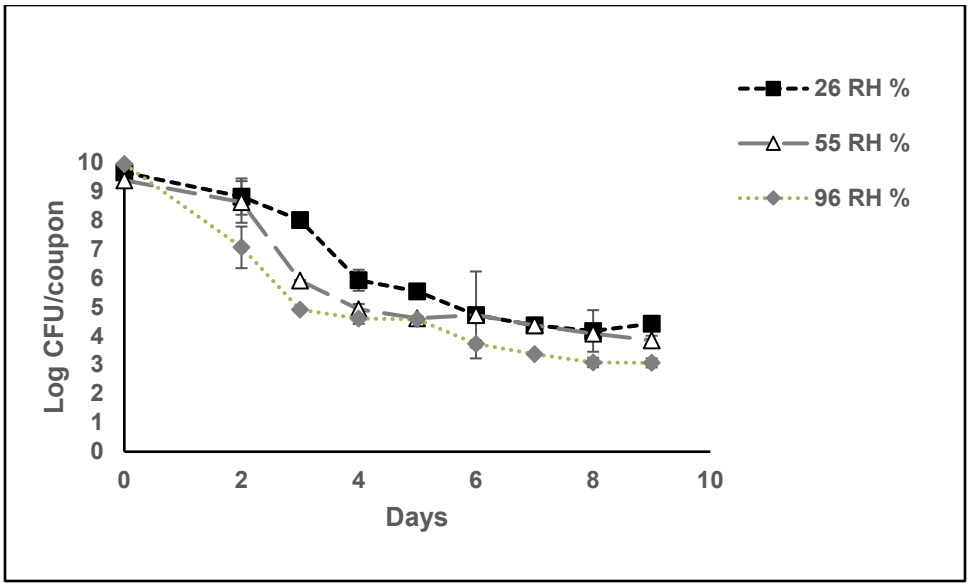

Figure 7. Survival of mature biofilms of *L. monocytogenes* Scott A stored at different relative humidity and 25°C. Data points are means of 2 independent experiments in triplicate samples and vertical bars are standard deviations.

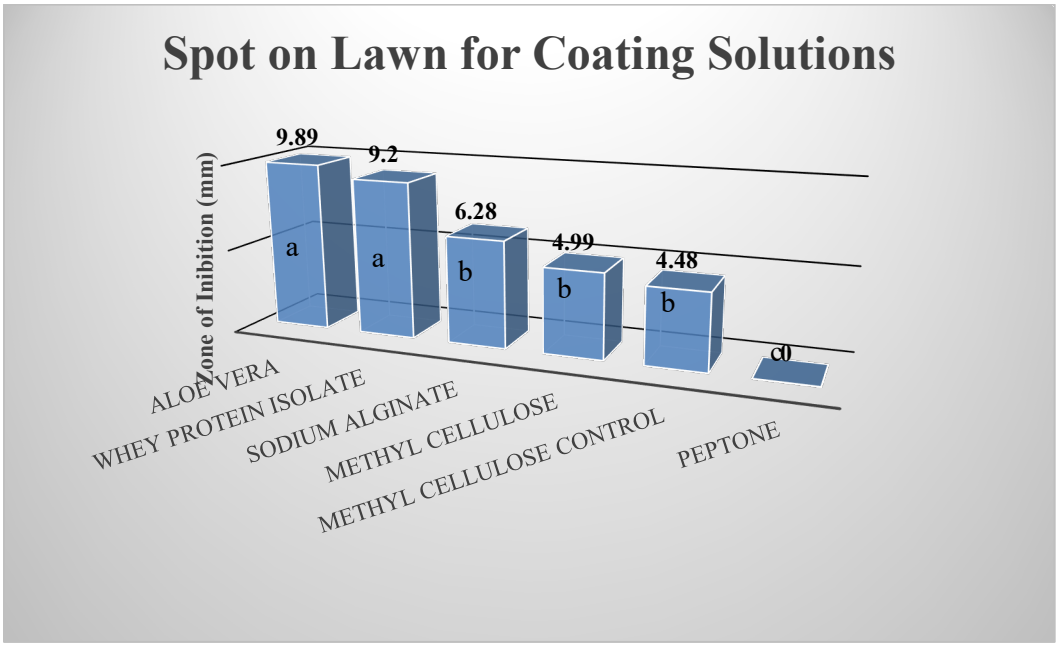

Figure 8. Zones of inhibition (mm) produced by coating solutions for *L. monocytogenes* Scott A.

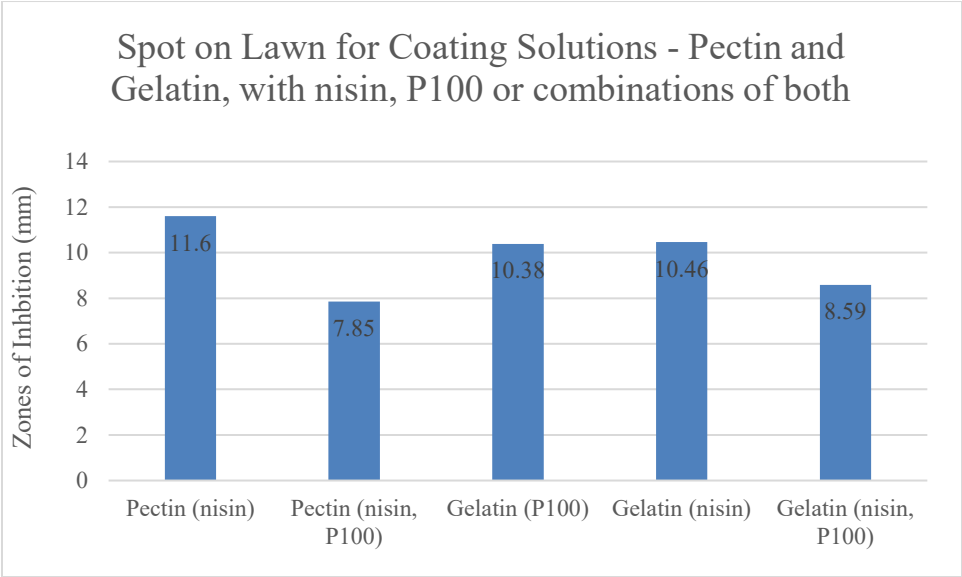

Figure 9. Zones of inhibition (mm) produced by coating solutions for *L. monocytogenes* Scott A using pectin with nisin or with nisin and phage (P100) and gelatin with phage, nisin, or nisin and phage.

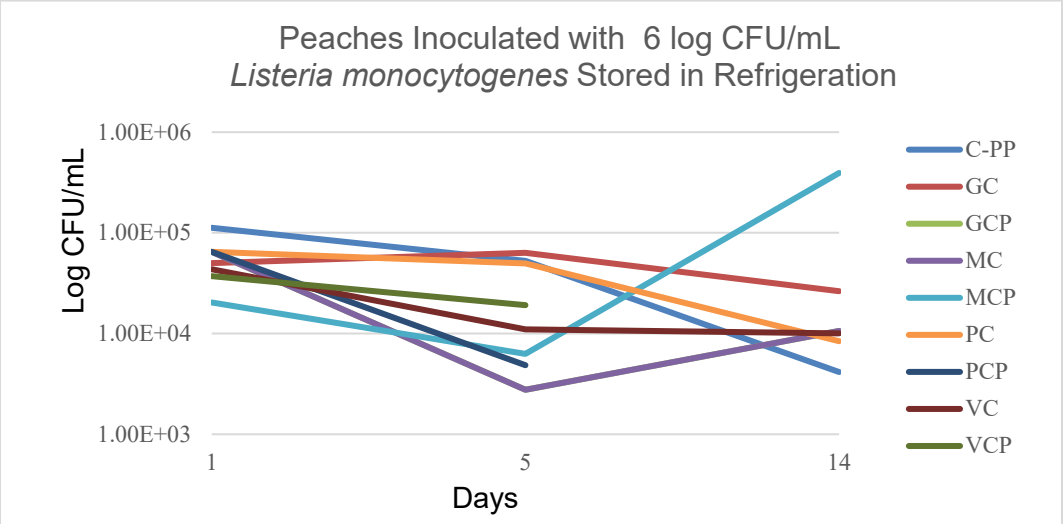

Figure 10. Reduction of Listeria monocytogenes inoculated (6 log) on peaches coated with different coatings with or without P100. (C-PP = Control; GC = Gelatin, no phage; GCP = Gelatin w/phage; MC = Mineral oil, no phage; MCP = Mineral oil w/phage; PC = Pectin, no phage; PCP = Pectin, w/phage; VC = Vegetable, no phage; VCP = Vegetable oil w/phage.)

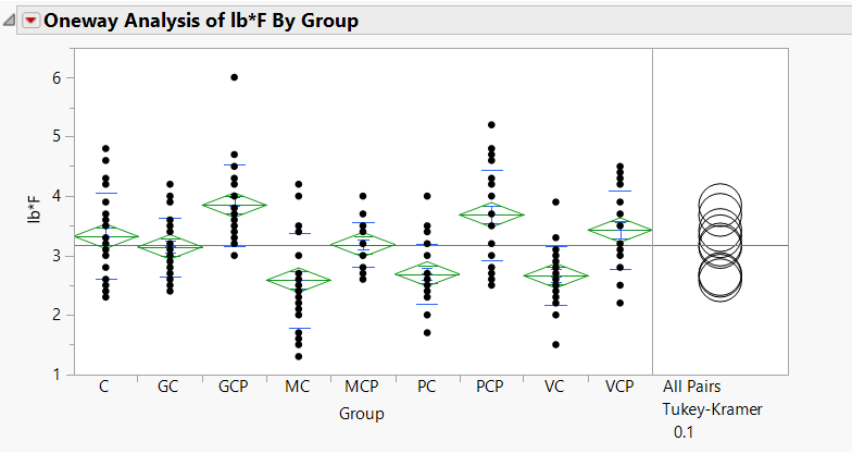

Figure 11. Firmness One Way ANOVA of coated peaches with and without P100 phage. (C-PP = Control; GC = Gelatin, no phage; GCP = Gelatin w/phage; MC = Mineral oil, no phage; MCP = Mineral oil w/phage; PC = Pectin, no phage; PCP = Pectin, w/phage; VC = Vegetable, no phage; VCP = Vegetable oil w/phage.)

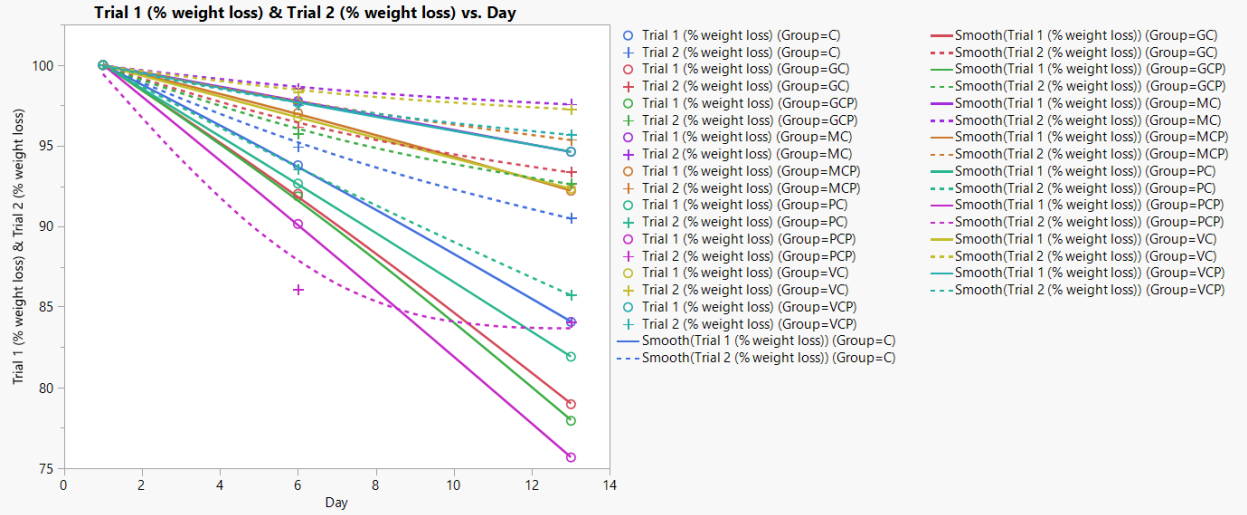

Figure 12. Weight loss vs. day for trial 1 and 2 coated peaches with and without P100.

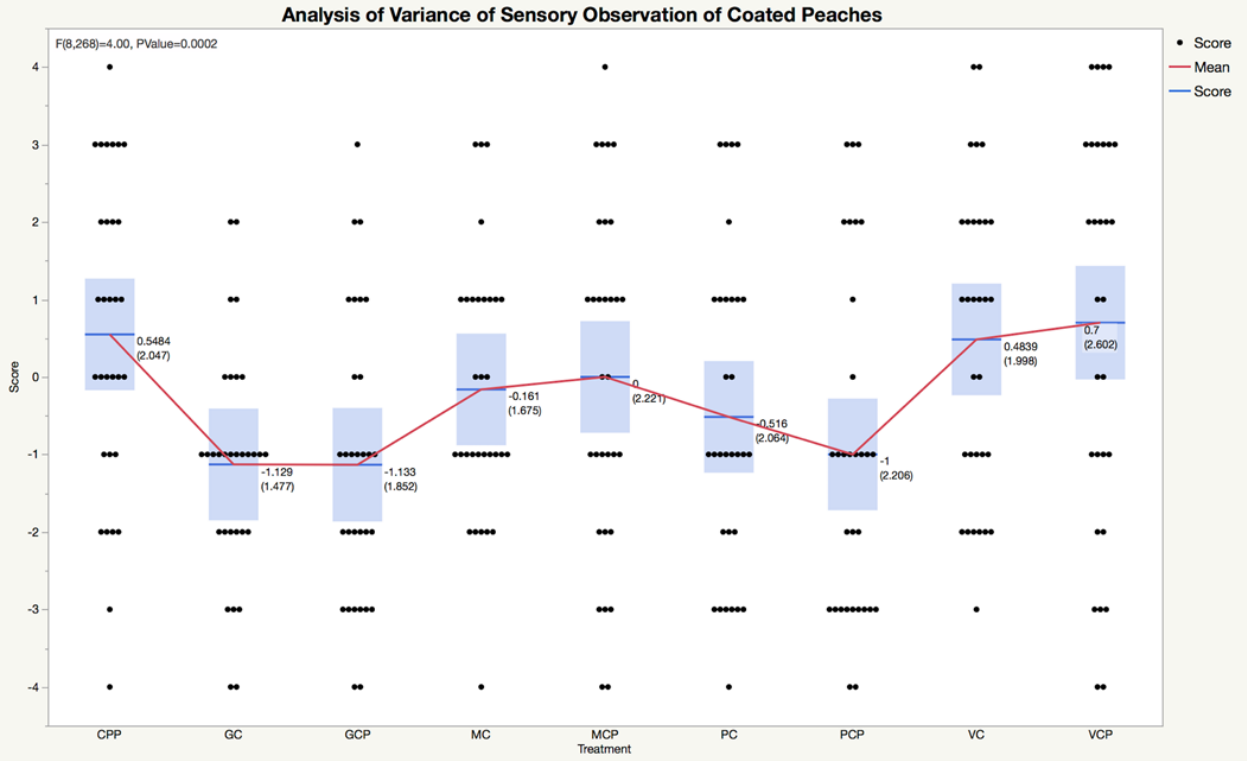

Figure 13. Sensory observations using Hedonic scale of liking.