CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
Waxing of whole produce and its involvement in and impact on microbial food safety

Project Period
January 1, 2021 – December 31, 2022 (extended to February 28, 2023)

Principal Investigator
Luxin Wang, PhD
University of California, Davis
Department of Food Science and Technology
One Shields Avenue
Davis, CA 95616
T: 530-752-7552
E: lxwang@ucdavis.edu

Co-Principal Investigator
Linda J. Harris, PhD
University of California, Davis
Department of Food Science and Technology
T: 530-754-9485
E: ljharris@ucdavis.edu

Objectives

1. *Evaluate the microbial and chemical properties of currently available citrus waxes, and determine the potential for human pathogens to survive in these waxes under simulated packinghouse storage conditions.*
2. *Investigate the behavior of Listeria monocytogenes (LM) and Salmonella inoculated onto citrus fruit surfaces when storage waxes are subsequently applied and fruit is stored.*
3. *Characterize and evaluate the pathogen control efficacy of different finishing waxes, the heated drying steps, and subsequent storage by conducting in-lab and pilot-scale packinghouse trials.*

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Abstract
Many fresh fruits and vegetables are washed and waxed after harvest to prevent premature rotting and extend shelf life. Over the past decade, the influence of commercially available waxes on fruit and vegetable quality has been investigated and reviewed; however, only a few studies have investigated the impact of waxes on microbial food safety. In this project, the microbial and chemical properties of a wide range of citrus storage and finishing waxes were evaluated. In addition, the behavior of the common foodborne pathogens *Listeria monocytogenes* (LM) and *Salmonella* in pure waxes were studied (**Obj. 1**). A cocktail of LM or *Salmonella* was first artificially inoculated into full-strength storage and finishing waxes, and the inoculated waxes were stored at the ambient temperature (22 °C) and 4 °C for 24 h. Results indicated that populations of LM declined more slowly and to a lesser degree than populations of *Salmonella* in both storage and finishing waxes over 24 h, regardless of storage temperatures. Thus, LM was selected for the 10-day storage study in full-strength and diluted storage waxes as well as for the 135-day storage study with full-strength storage and finishing waxes. LM and *Salmonella* could survive in storage (full-strength or diluted) and finishing waxes under simulated packinghouse storage conditions. In general, survival of LM was better at 4 °C than at 22 °C, and better in diluted storage waxes than in undiluted storage waxes. In **Obj. 2**, the survival of LM on lemon surfaces after the application of diluted storage waxes followed by 87 days of storage at 12 °C was investigated. Reductions of LM were observed not only after the application of diluted waxes but also during the subsequent storage; the magnitude of the reduction was influenced by wax type and dilution ratio. In **Obj. 3**, the bacterial control efficacy of the application of finishing waxes and the following heated drying step were studied through lab-scale and pilot-scale studies. Lemons were first used to compare the reductions of LM and *Salmonella* during the lab-scale waxing and drying. Results showed that the reduction of pathogens during drying at ambient temperature (22 °C), or heated drying (60 °C) was influenced by wax type and temperature. Greater reductions were observed for LM than for *Salmonella*. Therefore, the survival of *Salmonella* on citrus fruit after lab-scale waxing and heated drying was further evaluated by storing the inoculated and waxed fruits at 4 and 22 °C. Greater population reductions were observed on waxed than on unwaxed lemon and orange surfaces after 30 days of storage, regardless of the fruit type or storage temperature. Reductions in populations of *Enterococcus faecium* (NRRL-B2354) (EF) were 0.2 to 0.8 log CFU/lemon lower than for *Salmonella* after application of finishing waxes to lemon surfaces followed by drying at 22, 50, and 60 °C. The observed bactericidal effect under laboratory conditions was further validated via pilot-scale studies at the Lindcove Research and Extension Center using the wax that gave the greatest reductions under laboratory conditions (2.72 to 2.87 log CFU/lemon). Under pilot-scale conditions, minimum reductions of EF ranged from 1.64 to 2.12 log CFU/lemon. However, 80% of uninoculated lemons tested positive after waxing when 20 inoculated and 130 uninoculated lemons were waxed at the same time. Outcomes of this study provide the critical information on the food safety role played by waxes and the waxing application steps in packinghouses, as well as bridging several knowledge gaps associated with storage and finishing waxes. The findings should provide useful information to establish and implement packinghouse food safety plans.

Background
Waxing is a process of covering fruits and vegetables with natural (carnauba, shellac, or resin) or artificial waxing materials (e.g., petroleum-based waxes). While providing the required gloss for cosmetic purposes, waxing can extend the shelf life of produce by reducing respiration and protecting the skin from environmental stresses that cause dehydration. For certain produce,

wax may also act as a carrier for fungicides or different bio-agents used in controlling plant pathogens, thus reducing premature rotting. In the fresh produce industry, many fruits and vegetables are waxed before they are moved to short- or long-term storage or before being delivered to retail markets. Examples include apples, avocados, citrus fruits, cucumbers, eggplants, pears, peppers, and tomatoes.

The wax application point may differ from one produce type to another. While many (e.g., apples) are waxed once before they are shipped, some produce, e.g., citrus fruits, are waxed twice during the process of washing, storage (storage wax), and packing (finishing wax). Storage waxes are typically applied to fruit surfaces without a heated drying step; fruits receiving storage waxes will be stored wet for up to 6 months before they are re-washed, rinsed, and waxed again with finishing wax for shipping and sale. When finishing waxes are applied, there is typically a heated drying step at temperatures ranging from 90-140 °F (32-40 °C) for up to 2 min.

Given the wide applications of wax in fresh produce, the influence of commercially available waxes on fruit and vegetable quality have been investigated and reviewed (Singh et al., 2019; Petracek et al., 1998; Meighani et al., 2015; Ramírez et al., 2015; Tietel et al., 2010). As discussed in these papers, the application of wax can effectively extend the shelf life of fruits and retain nutritional quality attributes. Unfortunately, few studies have investigated the impact of waxes on microbial food safety. Of particular interest is the ability of pathogens to survive in the waxes themselves, as might occur if waxes were contaminated in the packinghouse holding tanks, as well as the impact of wax application on pathogens that might already be on the fruit surface.

Pao and Brown (1998) and Pao et al. (1999) evaluated the impact of finishing waxes and waxing steps on microbial populations on fruit surfaces. The application of finishing wax to oranges followed by heat drying reduced the total aerobic plate counts and coliform counts from 3.7 log CFU/cm^2 and 35.2 most probable number (MPN)/cm^2 to 2.6 log CFU/cm^2 and 1.4 MPN/cm^2, respectively (Pao and Brown, 1998). By using a non-pathogenic *Escherichia coli* strain, Pao et al. (1999) showed that up to 5-log reduction in the mid-section of the orange could be achieved when wax dipping temperatures were ≥50 °C/122 °F and the pH of the wax solution was greater than pH 10. However, such reduction was not observed in the stem scar area nor from waxes with pH 7 or 8. Macarisin et al. (2019) showed that significantly ($P < 0.05$) higher numbers of LM were recovered from apples coated with finishing waxes than those that remained unwaxed.

While these previous studies provided useful information about the potential bacterial control efficacy of waxes and wax application, they are outdated. The waxes or wax formulas tested by Pao and Brown (1998) and Pao et al. (1999) are no longer available. A more comprehensive evaluation of current waxes and wax application methods on the microbial safety of fresh produce was needed. Currently available wax formulations have been modified due to environmental safety concerns, and are significantly different from the ones tested in the previously published studies. Information on the impact of storage waxes on the microbial safety of fresh produce was also lacking. Thus, by conducting both in-lab and pilot plant studies and by working with both pathogenic bacteria (LM and *Salmonella*) and a surrogate (EF), this project investigated the impact of storage and finishing waxes on the survival of pathogens in wax formulations and after application to fruit surfaces. Potential bactericidal effects of the application of finishing waxes and the heated drying step were studied in laboratory settings first and then validated through pilot-scale trials.

Research Methods and Results

1. Methods

Bacterial strains and inoculum preparation

Rifampin-resistant strains of *L. monocytogenes* (LM) and *Salmonella* were used in the study. Their parent strains, associated with fresh produce or tree nuts, are described in **Table 1**. In addition, a rifampin-resistant mutant of the surrogate strain *Enterococcus faecium* (NRRL-B2354) (EF) was used in the laboratory and packinghouse trials. All strains were stored in tryptic soy broth (TSB) supplemented with 15% glycerol (Fisher Scientific) at -80 °C.

To activate the bacteria from frozen stocks, frozen stock cultures (LM, *Salmonella*, or EF) were streaked onto tryptic soy agar (TSA) supplemented with rifampin (Biosynth International Itasca, IL) at 50 µg/mL (TSAR) and incubated at 37 ± 1 °C for 24 ± 2 h. An isolated colony from each culture was transferred into TSB supplemented with rifampin at 50 µg/mL (TSBR) and incubated at 37 ± 1 °C for another 24 ± 2 h. After that, the overnight culture (LM, *Salmonella*, or EF) was spread onto four TSA plates (250 µl/plate), dried in the biosafety cabinet for 30 min, and then incubated at 37 ± 1 °C for 24 ± 2 h. The resulting bacterial lawn was collected by adding 5 mL of 1× phosphate buffered saline (PBS, pH 7.4) onto the plate and scraping the lawn off by using L-shape disposable spreaders. Resuspended cells in 1 × PBS were pipetted into a 15-mL Falcon tube (Jackson Dickinson and Company). To prepare the LM or *Salmonella* cocktail inoculum, equal volumes (1 mL) of each strain were combined. Serial dilutions were then made in 1× PBS to achieve target populations (ca. 8 log CFU/mL) before inoculating into waxes or onto fruit surfaces.

Waxes and inoculation of waxes (Obj. 1)

Citrus storage waxes (labeled S1, S2, S3, and S4) and finishing waxes (labeled F1-F15) were obtained from commercial suppliers and held under ambient conditions (22 ± 0.6 °C; 28–63% relative humidity [RH], median 54% RH) before use. Upon receipt of these waxes, their background aerobic bacteria, yeast and mold were determined as described below in the microbiological analysis section. The pH of each wax was measured with a pH meter (Mettler Toledo, Thermo Fisher Scientific).

For storage waxes, waxes were used at full-strength or they were diluted with sterile ultrapure water (Milli-Q Advantage A10, MilliporeSigma) according to the manufacturers' recommendations. The dilution factors (wax:ultrapure water, v/v) were 1:9 and 1:39 for S1, 1:1 and 1:5 for S2, 1:10 and 1:60 for S3, and 1:9 for S4. Finishing waxes were used only at full-strength. To inoculate the wax samples, 0.2 mL of the prepared inoculum of either LM or *Salmonella* was added to 19.8 mL of wax to achieve target inoculation levels of 6 log CFU/mL or 4 log CFU/mL.

Storage conditions for storage and finishing waxes (Obj. 1)

Inoculated waxes were stored in sealed 50-mL Falcon tubes at refrigeration (4 ± 0.4 °C; 71–102% RH, median 96% RH) or ambient (22 ± 0.6 °C; 28–63% RH, median 54% RH) conditions. To compare the behavior of inoculated LM and *Salmonella*, full-strength storage and finishing waxes were sampled immediately after inoculation (0 h) and at 0.5, 1, 4, and 24 h during storage. To evaluate the fate of LM in diluted storage waxes, bacterial populations were determined on days 0, 1, 2, 4, 7 and 10. For long-term storage of the full-strength storage or finishing waxes, bacterial populations were determined on days 0, 2, 5, 10, 20, 30, 45, 60, 75, 90, 105, 120, and 135.

Inoculation of lemons and the application of storage waxes (Obj. 2)

Lemons were used as the model fruit for storage wax studies, as oranges do not receive storage waxes. Unwashed lemons (Eureka) were obtained from the Lindcove Research and

Extension Center. Upon receipt of lemons, the background aerobic bacteria and yeast and mold counts were determined as described below in the microbiological analysis section. A preliminary experiment was conducted to determine the best inoculation method for lemons. When LM was inoculated onto the midsection or the stem scar of each lemon, results showed that LM behaved similarly in both areas. Therefore, the lemon midsection was chosen as the inoculation area in subsequent studies. One circle (2.0 cm in diameter) was drawn on the midsection surface of each lemon to define the inoculation area. The prepared inoculum of LM was applied in 10 1-µL drops over each marked area to achieve an initial inoculation level of ca. 6 log CFU/circle. Inoculated lemons were held overnight in a biosafety cabinet, after which point no visible liquid was observed in the inoculated area. During waxing, each inoculated lemon was placed on top of a 118-mL (4-oz) sterile specimen container without lid (Thermo Fisher Scientific), and the diluted storage wax was then applied for 10 s using an airbrush compressor (Central Pneumatic) at ca. 10 cm above the lemon. The lemon was manually rotated 180 degrees and sprayed with the diluted storage wax for another 10 s. This application method delivered a total ca. 0.8 mL of wax per lemon. Lemons that were inoculated but unwaxed, or inoculated and sprayed with water, were used as controls.

Storage conditions for inoculated and waxed lemons (Obj. 2)

Inoculated lemons were stored under typical commercial storage conditions (12 ± 0.1 °C; 93–104% RH, median 101% RH) in a refrigerated incubator. LM populations on lemon surfaces were determined immediately after inoculation (day −1), after overnight drying (day 0), and after waxing on days 0, 1, 3, 7, 14, 28, 45, 60, 75, and 87.

Lab-scale evaluation of the bacterial control efficacy of finishing wax application (Obj. 3)

Unwashed lemons (Eureka) and oranges (Valencia) were obtained from the Lindcove Research and Extension Center. Four finishing waxes (F4, F6, F8, and F15) were selected, representing waxes in which different LM and *Salmonella* survival was observed in Objective 1. Lemons were used first to determine the reduction of LM and *Salmonella* on the fruit surface after finishing wax application. Finishing waxes were applied to LM- or *Salmonella*-inoculated lemons (6 log CFU/lemon) as described above and held at 22 °C or in a convection oven at 60 °C for 7 min (consisting of 3 min of temperature come-up time and 4 min of drying time). To evaluate the survival of *Salmonella* on citrus fruit after the lab-scale finishing wax application, *Salmonella*-inoculated lemons or oranges (6 log CFU/fruit) waxed with F6 (the wax that gave the lowest reductions of *Salmonella*; 1.60 to 1.65 log CFU/lemon) were stored under refrigeration and ambient conditions for 30 days. *Salmonella* populations on citrus fruit surfaces were determined immediately after inoculation (day −1), after waxing and drying (day 0), and on days 0, 1, 3, 5, 10, 20, and 30 during storage.

Pilot-scale trials for evaluating bacteria control efficacy of finishing wax application (Obj. 3)

Before starting the pilot trials, the behaviors of *Salmonella* and its surrogate microorganism, EF, were compared in laboratory settings. Lemons were inoculated with *Salmonella* or EF at 6 log CFU/lemon and commercial finishing waxes were then applied onto the inoculated lemons using an airbrush compressor as described above and dried at 22, 50, or 60 °C for 7 min. Changes of the *Salmonella* or EF populations on lemon surfaces were determined after inoculation, after wax application, and after drying. Results showed that EF could be used as a conservative surrogate for *Salmonella* for the validation of the finishing wax application and the heat drying treatment.

For the pilot-scale trials, finishing wax F4, which gave the greatest reductions under laboratory conditions (2.72 to 2.87 log CFU/lemon), was chosen. Lemons were selected based on the availability of citrus fruits. To evaluate the bactericidal efficacy, rifampin-resistant EF-inoculated lemons (6 log CFU/lemon) were labeled with markers. The schematic design of the

pilot trials is shown in **Figure 1**. Four pilot visits with three trials were performed for this project. In each trial, a total of three runs were conducted, with 100 to150 lemons used per run. The waxing line was operated at 87 L/h wax application rate and ~ 50 °C drying temperature. Among the three runs for each trial, the first run consisted of 100% uninoculated lemons, the second run 20% EF-inoculated lemons and 80% uninoculated lemons, and the third run 100% uninoculated lemons (Figure 1). After the first run, 15 to 20 lemons (uninoculated) were randomly sampled and enriched for EF to determine the residential rifampin-resistant EF in the waxing and drying lines. For the second run, a subset of 15 to 20 lemons were randomly sampled from the EF-inoculated lemons to enumerate the surviving EF after pilot waxing and drying. After the second and third runs, the survival and transfer of inoculated EF to a subset of 15 to 20 uninoculated lemons were determined by standard plating and enrichment methods, as described below. In addition, the spread of inoculated EF to equipment surfaces was determined by swabbing key equipment and fruit contact surfaces (waxing brush, waxing exit, dryer exit, and conveyor belt) before and after the runs using standard environmental sponge swab methods.

Microbiological analysis

Upon receipt of waxes from the suppliers, 1 mL of each wax was diluted in 9 mL of Dey-Engley (D/E) neutralizing broth, and then proper dilutions of wax samples were spread onto plate count agar (PCA) for enumeration of aerobic bacteria or onto dichloran Rose Bengal chloramphenicol agar (DRBC) for enumeration of yeasts and molds. To determine the background aerobic bacteria and yeast and mold counts of lemons or oranges, each whole fruit was placed into a 1,627-mL (55-oz) Whirl-Pak bag and combined with 20 mL of D/E broth. Sample bags were shaken by hand for 30 s, rubbed for 1 min, and shaken again for 30 s (referred herein as shake-rub-shake method) before plating the broth onto PCA and DRBC. PCA plates were incubated at 37 °C for 48 h, and DRBC plates were incubated at ambient conditions for 3 to 5 days.

Inoculated wax (1 mL) was taken at each sampling point, transferred into 9 mL of D/E neutralizing broth, mixed by vortexing at maximum speed for 10 s, and then spiral plated onto TSAR and xylose lactose tergitol-4 (XLT4) agar (*Salmonella*) or modified Oxford medium (MOX) (LM). When counts enumerated on TSAR were expected to be near or below the limit of detection (LOD; 1.0 log CFU/mL), 5 mL of the D/E mixture (corresponding to 0.5 mL of inoculated wax sample) was mixed with 5 mL of double strength enrichment broth (lactose broth for *Salmonella* and buffered *Listeria* enrichment broth for LM) and then incubated at 37 °C for 24 h. The enriched samples were spiral plated onto XLT4 or MOX to confirm the presence of targeted pathogens (LOD of enrichment: 0.3 log CFU/mL).

To determine whether the waxing step led to the movement of LM from the inoculation area, the inoculated marked circle on each lemon was aseptically excised with a sterile scalpel to about 1 mm thickness (yellow rind with minimal pith) from lemons after inoculation, after overnight drying, and after the application of waxes. Each excised lemon rind sample was placed in a tube containing 10 mL of D/E broth and held for 10 min, and then mixed by vortexing at maximum speed for 30 s, before the broth was spiral plated onto TSAR or MOX. LM on the remainder of each whole lemon was recovered by the shake-rub-shake method before plating the broth onto TSAR or MOX. When colony counts enumerated from TSAR were expected to be near or below the LOD by plating (1.0 log CFU/circle or 1.3 log CFU/lemon), 5 mL of the D/E mixture was mixed with 5 mL of 2 × buffered *Listeria* enrichment broth and incubated for 24 h at 37 °C, and then streaked onto MOX to confirm the presence of LM (LOD of enrichment: 0.3 log CFU/circle or 0.6 log CFU/lemon).

Microbial populations on the whole lemons or oranges (including the inoculated marked circle) were enumerated using the shake-rub-shake method followed by spiral plating onto TSAR for inoculated LM, *Salmonella*, and EF, or MOX (LM) or XLT4 (*Salmonella*). When colony

counts for TSAR were expected to be near or below the LOD by plating (1.0 log CFU/circle or 1.3 log CFU/lemon), 5 mL of the D/E mixture was mixed with 5 mL of double strength enrichment broth (buffered *Listeria* enrichment broth for LM, lactose broth for *Salmonella,* and TSBR for EF) and then incubated at 37 °C for 24 h, followed by streaking onto the selective agar or TSAR to determine the presence of target microorganisms.

Statistical analysis

All experiments were repeated twice with three replicates in each trial (n = 6). When results were negative after enrichment, the bacterial concentration was assigned a value of 0.00 log CFU/mL for analysis. For each inoculated pathogen, statistical analysis was performed across time points. Analysis of variance and t-tests was performed with the JMP 11 software package (SAS Institute, Cary, NC). Differences between means were considered significant when P values are < 0.05.

2. Results

pH and background microflora of citrus storage and finishing waxes (Obj. 1)

Objective 1 aimed to evaluate the microbial and chemical properties of currently available citrus waxes and determine the potential for human pathogens to survive in these waxes under simulated packinghouse storage conditions. The pH of waxes S1, S2, S3, and S4 ranged from 8.05 to 13.15. Initial populations of native mesophilic microbiota recovered on PCA and yeast and mold counts recovered on DRBC were below the limit of detection (1.00 log CFU/mL) for S1–S3 and 4.33 ± 0.27 and 3.22 ± 0.02 log CFU/mL, respectively, for S4. The pH of finishing waxes ranged from 8.8 to 10.1. Counts of finishing waxes on PCA ranged from <1.0 log CFU/mL to 4.40 log CFU/mL, while fungi counts of all finishing waxes were below the limit of detection.

Survival of LM and *Salmonella* in storage and finishing waxes (Obj. 1)

The behavior of LM and *Salmonella* in storage and finishing waxes was first monitored at 4 and 22 °C for 24 h. Initial populations of LM or *Salmonella* in the waxes were 6 log CFU/mL. During 24-h of storage, populations of LM declined more slowly and to a lesser degree than populations of *Salmonella* in full-strength waxes, regardless of storage temperature. Thus, the survival of LM was further evaluated as a function of inoculation levels, wax types/formulas, and storage temperatures. In addition, based on the 24-h results, waxes in which the reduction of LM after 24 h of storage at 4 °C was less than or equal to 1 log CFU/mL (**Figure 2**) were selected for following in longer storage studies, including four storage waxes (S1, S2, S3, and S4) and six finishing waxes (F1, F2, F3, F6, F12, and F15).

In commercial citrus packinghouses, storage waxes are typically applied in diluted forms and used in about 1 to 7 days, depending on the size of the packinghouse and the season. Therefore, a 10-day storage study was first conducted to monitor LM survival in full-strength and diluted storage waxes held at 4 and 22 °C [(NACMCF) National Advisory Committee on Microbiological Criteria for Foods, 2010]. To mimic the long-term storage of full-strength waxes at packinghouses, the survivability of LM in full-strength storage and finishing waxes held at 4 and 22 °C was evaluated for up to 135 days. In addition, the impact of inoculation level (4 or 6 log CFU/mL) on the survival of LM in waxes was also investigated.

Short-term storage of LM-inoculated full-strength and diluted storage waxes: LM survived better over 10 days at 4 °C than at 22 °C, and better in diluted storage waxes than in undiluted storage waxes. For example, LM in undiluted S1 fell below the limit of detection (LOD, 1.0 log CFU/mL) after one day of storage at 4 °C, while populations of LM in declined by 1.5 (TSAR) and 2.2 (MOX) log CFU/mL after 10 days. Increasing the storage temperature to 22 °C resulted in more rapid die-off of LM. LM could not be recovered by enrichment in 1:9 diluted S1 after 4 days of storage.

Long-term storage of LM-inoculated full-strength storage waxes: The survivability of LM in full-strength storage waxes S2, S3, and S4 for up to 135 days was evaluated. The survival of LM was dependent on individual waxes and storage temperatures. At 4 °C, no LM was recovered by enrichment in wax S2 after 90 days of storage. LM was reduced by 2.85 and 2.91 log CFU/mL (TSAR) in waxes S3 and S4, respectively, by the end of storage. Increasing the storage temperature to 22 °C facilitated the more rapid die-off of LM. No LM was recovered by enrichment in waxes S2, S3, or S4 after 30, 10, or 105 days of storage, respectively.

Long-term storage of LM-inoculated finishing waxes: The long-term survival of LM in citrus finishing waxes was also determined by individual waxes and storage temperatures. Faster die-off was observed in most waxes at 22 °C than at 4 °C. At 4 °C, LM populations in most waxes (F1, F3, F12, and F15) fell below the LOD after 30 days. In F6, LM gradually decreased to below the LOD by the end of storage. In F2, LM populations were reduced by 3.6 log CFU/mL after 135 days of storage at 4 °C. At 22 °C, no LM was recovered by enrichment after 20 days of storage except in F2. A rapid reduction of 2.5 log CFU/mL was observed in F2 during the first 10 days of storage. After that, LM populations increased to 5.7 log CFU/mL by Day 20 and then gradually decreased to 3.6 log CFU/mL at the end of storage (Day 135).

Impact of the inoculation levels Given that LM survived better in waxes S4 and F2 than in the other waxes tested, S4 and F2 were selected for investigating the impact of inoculation levels (4 or 6 log CFU/mL) on the survival of LM in waxes during long-term storage (135 days) at 4 °C or 22 °C. For the storage wax (S4), LM decreased faster at 22 °C than at 4 °C regardless of the inoculation level. With the 4 log CFU/mL inoculation level, the LM population decreased by 2 log after 135 days of storage at 4 °C, while LM could no longer be recovered even by enrichment after 10 days of storage at 22 °C. When inoculated at 6 log CFU/mL, at 4 °C, a decline of 2.29 log CFU/mL was detected on TSAR by the end of storage (135 days). At 22 °C, no LM could be recovered by enrichment after 105 days of storage. In the finishing wax (F2), LM gradually decreased to undetectable levels after 75 days of storage at 4 °C, regardless of inoculation level. At 22 °C, an initial decrease of 2 or 3.4 log was observed from Day 0 to Day 10 for the 4- or 6- log inoculation levels, respectively. Culturable LM numbers increased by approximately 3 logs from Day 10 to Day 20 for both inoculation levels. After Day 20, LM levels were stabilized at 5.5 to 5.1 log CFU/mL for 85 days and then gradually decreased to 3.6 log CFU/mL by Day 135 for both inoculation levels. Thus, inoculation levels impacted survival of LM in storage wax at 22 °C, but not at 4 °C or in finishing waxes at either temperature.

Behavior of LM on lemon surfaces that are coated with storage waxes (Obj. 2)

Objective 2 aimed to investigate the survival of LM on lemon surfaces after application of storage waxes and during subsequent fruit storage. LM was selected for fruit inoculation as it survived better than *Salmonella* in storage waxes in Objective 1, regardless of the storage temperature. Two commercial storage waxes (S1 and S2) were selected to represent wax types in which distinct behavior patterns of LM were observed in Objective 1. Waxes were diluted prior to application according to the manufacturer's directions. A preliminary test was performed to determine the impact of the waxing on the movement of inoculum.

Movement of LM on lemon surfaces caused by the application of storage waxes: The background microbiota of lemons used in this study was 4.32 ± 0.41 log CFU/lemon for total aerobic bacteria and 5.03 ± 0.58 log CFU/lemon for total yeast and mold counts (n = 12). After inoculation, waxing, and excising the inoculated area of the lemons, LM could be detected on the remainder of whole lemons. This finding indicated the migration of inoculated LM during waxing, drying, and/or excising of the inoculation area. A total reduction of up to 2.17 log CFU/sample (circle or remaining lemon) was observed after application of the diluted storage waxes, and the magnitude of reduction was determined by the type of waxes and the dilution ratio.

Survival of LM on lemons held at 12 °C for 87 days: Reductions of LM were observed after the application of diluted waxes and during subsequent storage; the magnitude of reduction was influenced by wax type and dilution ratio. LM was recovered at 5.82 log CFU/lemon from control lemons on day 0. Spraying lemons with water or 1:39 diluted wax S1 had no significant impact on LM populations immediately after inoculation. When sprayed with the other wax formulations (1:9 diluted S1, 1:5 diluted S2, and 1:1 diluted S2), LM populations on lemons were reduced by 1.62, 0.79, and 0.64 log CFU/lemon, respectively. During storage, the bacterial populations gradually declined regardless of the treatment. On day 75, LM could not be recovered from control lemons by plating methods, but all samples were enrichment-positive through the 87-days of storage. Similar declines of LM were observed on lemons treated with water, 1:39 diluted S1, and 1:5 and 1:1 diluted S2. LM could be recovered by plating or enrichment on days 28 and 87, respectively for lemons treated with 1:9 diluted S1.

Lab- and pilot-scale evaluation of the bactericidal effect of finishing wax application (Obj. 3)

Objective 3 aimed to determine the combined pathogen control efficacy of the application of finishing waxes followed by a heated drying step, as well as during subsequent storage in laboratory and pilot-scale packinghouse trials. Four finishing waxes (F4, F6, F8, and F15) were selected to represent waxes for which different survival of LM and *Salmonella* were observed in Objective 1. To select the pathogen used for the lab-scale analysis, the reduction of LM and *Salmonella* on lemon surfaces was compared after application of finishing waxes and a heated drying step. *Salmonella* survived better than LM and was thus selected for characterizing the pathogen control efficacy of different finishing waxes combined with the heated drying steps and during subsequent storage. The fate of *Salmonella* and a non-pathogenic surrogate microorganism (EF) on lemon surfaces was then compared after lab-scale finishing wax application.

Reductions in LM and *Salmonella* after application of finishing waxes and the heated drying step: Finishing waxes were applied to LM- or *Salmonella*-inoculated lemons and held at 22 °C or 60 °C for 7 min. The application of finishing waxes led to 2.0 to 3.1 and 2.8 to 4.4 log CFU/lemon reduction of LM, and 0.5 to 2.6 and 1.7 to 3.7 log CFU/lemon reduction of *Salmonella* at drying temperatures of 22 °C and 60°C, respectively. Significantly ($P < 0.05$) greater reductions were observed for LM than for *Salmonella* for all treatments. During the drying step, the magnitude of pathogen reduction was determined by wax type and drying temperature. The difference in pathogenic bacteria counts obtained from non-selective and selective agar indicated the formation of injured cells during waxing and drying.

Characterization of the pathogen control efficacy of finishing wax and the heated drying steps as well as during subsequent storage: The background microbiota of the oranges used in this study was 4.92 ± 0.52 log CFU/orange for total aerobic bacteria and 5.20 ± 0.51 log CFU/orange for total yeast and mold counts ($n = 12$). Finishing wax F6 was selected to mimic the worst scenario, because *Salmonella* showed the least reduction after waxing and drying using this wax. The application of F6 followed by heated drying at 60 °C led to reductions of 1.68 and 1.70 log CFU/fruit for lemons and oranges, respectively. No significant difference in *Salmonella* reduction due to waxing and drying was observed for these two types of citrus fruit. During 30 days of storage, greater reductions of *Salmonella* were observed on waxed lemon and orange surfaces compared to unwaxed fruit surfaces, regardless of the fruit type or storage temperature. Specifically, at 4 °C, reduction of *Salmonella* on waxed lemons or oranges was 1.29 to 1.41 and 0.9 to 1.04 log higher compared to unwaxed citrus fruits, respectively (based on TSAR results). At 22 °C, reduction of *Salmonella* on waxed lemons or oranges was 0.14 to 0.28 and 0.57 to 0.52 log higher compared to unwaxed citrus fruits, respectively.

Comparison of the fate of *Salmonella* and a non-pathogenic surrogate microorganism (EF) on lemon surfaces after the lab-scale finishing wax application: The reduction of *Salmonella* and EF on lemon surfaces after the application of finishing waxes as

well as after drying at 22, 50, and 60 °C was compared in laboratory settings. Greater reductions were observed for *Salmonella* than for EF. When ca. 0.3- to 3.7-log reduction of *Salmonella* was observed, ca. 0.1- to 2.9-log reduction of EF was observed depending on the drying temperature and the treatment (control or different types of waxes). This indicates that EF would serve as a conservative surrogate microorganism for *Salmonella* for application of waxes with a drying step.

Pilot trials: Temperature and relative humidity of the pilot plant ranged from 14.0 °C to 16.7 °C and 57.3% to 68.6%, respectively. Applying the wax that gave the greatest reductions (2.72 to 2.87 log CFU/lemon) under laboratory conditions achieved reductions ranging from a minimum of 1.64 to 2.12 log CFU/lemon. No inoculated EF was recovered from uninoculated lemons from the first run, confirming that the waxing equipment was not contaminated. When uninoculated lemons were waxed with inoculated lemons in the second and third runs, EF was recovered by enrichment from 80% (44 of 55) and 83.6% (46 of 55) of the uninoculated lemons, respectively. In addition, the EF could be recovered from the wax brush, the wax exit, the dryer exit, and the conveyor belt after waxing and drying.

Outcomes and Accomplishments

- Survival and die-off data of LM and *Salmonella* cocktails in various storage and finishing waxes were evaluated at ambient temperature and 4 °C.
- Information about how storage waxes impact the survival of LM on lemon surfaces during long-term storage was determined.
- The bactericidal efficacy of the application of finishing coupled with a subsequent heated drying step was investigated in both lab-and pilot-scale studies.
- Results were communicated to the citrus packinghouse industry and other audiences via presentations and scientific meetings.

Summary of Findings and Recommendations

This project has generated significant new findings, relative to factors affecting pathogen reduction in citrus waxes and during the waxing process. Key findings and recommendations:

- Common foodborne pathogens (LM and *Salmonella*) survive in storage and finishing waxes.
- The survival of common foodborne pathogens in full-strength storage and finishing waxes was influenced by wax type and storage temperature.
- LM survived better than *Salmonella* in storage waxes, with greater survival observed at 4 °C (39 °F) than at 22 °C (72 °F) and better in diluted than in undiluted storage waxes.
- The application of storage waxes led to LM reduction during storage. The differences in LM reduction between waxed and unwaxed lemons were influenced by wax type and dilution. When compared to unwaxed lemons, the application of select diluted storage wax (1:9 diluted S1) facilitated reduction in LM populations, whereas other waxes did not.
- The application of finishing waxes and a heated drying step led to higher reduction of LM than *Salmonella*.
- There was no significant difference in the fate of *Salmonella* on waxed lemons and oranges.
- Greater reductions of *Salmonella* were observed at 4 °C (39 °F) than at 22 °C (72 °F) when inoculated fruits were waxed and stored for 30 days.
- Greater reductions of *Salmonella* were observed on waxed lemon and orange surfaces when compared to unwaxed fruit surfaces, regardless of fruit type and storage temperature.
- Although the application of finishing waxes coupled with a heated drying step reduced the number of pathogens on fruit surfaces, if the initial contamination level is high, cross contamination between contaminated and uncontaminated fruits can happen during the waxing step.

References cited

Meighani, H., Ghasemnezhad, M., and Bakhshi, D. 2015. Effect of different coatings on post-harvest quality and bioactive compounds of pomegranate (*Punica granatum L.*) fruits. J. Food Sci. Technol. 52(7), 4507-4514.

NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. J Food Prot 73, 140-202.

Pao, S., Davis, C. L., Kelsey, D. F., and Petracek, P. D. 1999. Sanitizing effects of fruit waxes at high pH and temperature on orange surfaces inoculated with *Escherichia coli*. J. Food Sci. 64, 359-362.

Pao, S., and Brown, G. E. 1998. Reduction of microorganisms on citrus fruit surfaces during packinghouse processing. J. Food Prot. 61, 903-906.

Petracek, P. D., Dou, H., and Pao, S. 1998. The influence of applied waxes on postharvest physiological behavior and pitting of grapefruit. Postharvest Biol. Technol. 14, 99-106.

Ramírez, E., Sánchez, A. H., Romero, C., and Brenes, M. 2015. Combined use of nitrogen and coatings to improve the quality of mechanically harvested manzanilla olives. Food Chem. 171, 50-55.

Singh, S., Singh, B., and Alam, T. 2019. Evaluation of shelf-life, antioxidant activity and nutritional quality attributes in carnauba wax coated eggplant genotypes. J. Food Sci. Technol. 56(11), 4826-4833.

Tietel, Z., Bar, E., Lewinsohn, E., Feldmesser, E., Falllik, E., and Porat, R. 2010. Effects of wax coatings and postharvest storage on sensory quality and aroma volatile composition of “Mor” mandarins. J. Sci. Food Agric. 90(6), 995-1007.

APPENDICES

Publications and Presentations

Dissemination of research findings to stakeholders:

June 21–22, 2022. The PI attended the CPS Annual Symposium, gave a 5-minute presentation, and attended the poster session describing the work to date.

June 23, 2022. The PI, postdoctoral fellow, and one graduate student visited the University of California Lindcove Research and Extension Center. A tour was provided by the Director, and research strategies for next steps were discussed.

November 9, 2022. The PI Attended the CPS video conference meeting and gave a presentation on the update of the project progress.

Presentations:

June 21–22, 2022. The PI and postdoctoral fellow attended the 2018 CPS Research Symposium and gave oral and poster presentations.

October 26–28, 2020. The postdoctoral fellow presented a poster at the 2020 IAFP Annual virtual meeting (funding to attend the meeting provided from other sources). Lina Sheng, Linda J. Harris, and Luxin Wang. 2020. Survival of Foodborne Pathogens in Citrus Storage and Finishing Waxes. Annual IAFP Meeting 2020.

July 31–August 3, 2022. The postdoctoral fellow presented a poster at the 2020 IAFP Annual virtual meeting (funding to attend the meeting provided from other sources). Lina Sheng, Hongye Wang, Linda J. Harris, and Luxin Wang. 2020. Survival of *Listeria monocytogenes* and *Salmonella* in Citrus Storage Waxes. Annual IAFP Meeting 2022, Pittsburgh, PA.

Publications:

Three manuscripts for publication in scientific journals are in preparation.

Budget Summary

This research project was awarded $229,536 in grant funds. Overall, the project expenditures have followed the budget closely, and most funds were spent.

Table 1. Strain designations and sources for bacteria used in current project

Organism	Original strain designation	Harris culture collection | rifampin-resistant strain designation	Isolation source (original strain source)	Reference
Listeria monocytogenes	LIS0234	LJH1422 | LJH1541	Isolated from raw, diced yellow onions associated with 2012 recall (provided by the U.S. Food and Drug Administration)	US FDA, 2012
Listeria monocytogenes	LIS0235	LJH1423 | LJH1542	Isolated from raw, diced yellow onions associated with 2012 recall (provided by the U.S. Food and Drug Administration).	US FDA, 2012
Listeria monocytogenes	LIS0133	LJH1424 | LJH1543	Environmental isolate from a celery processing facility associated with 2010 outbreak (provided by the U.S. Food and Drug Administration)	Gaul et al., 2013
Listeria monocytogenes 1/2b	LIS0110	LJH1425 | LJH1544	Isolated from whole cantaloupe facility associated with a nationwide listeriosis outbreak in the U.S. during 2011 (provided by the U.S. Food and Drug Administration)	McCollum et al., 2013
Listeria monocytogenes 1/2a	LIS0087	LJH1426 | LJH1545	Isolated from cantaloupe and cantaloupe-processing facility associated with a nationwide listeriosis outbreak in the U.S. during 2011 (provided by the U.S. Food and Drug Administration)	McCollum et al., 2013
Listeria monocytogenes 1/2a	LIS0077	LJH1427 | LJH1546	Isolated from cantaloupe and cantaloupe-processing facility associated with a nationwide listeriosis outbreak in the U.S. during 2011 (provided by the U.S. Food and Drug Administration)	McCollum et al., 2013
Salmonella Enteritidis PT 30[a]	ATCC BAA-1045	LJH0608 | LJH0636	Isolated from raw almonds associated with 2000 to 2001 outbreak (provided by Silliker laboratories). Deposited to ATCC by Harris, L. J.	Isaacs et al., 2005
Salmonella Montevideo	G4639	LJH 519 | LJH614	Human isolate from 1993 tomato outbreak (provided by Dr. Larry Beuchat, University of Georgia, Griffin)	Zhuang et al., 1995

Salmonella Gaminara	F2712	LJH 518| LJH1220	Isolated from 1995 unpasteurized orange juice outbreak (provided by Dr. Larry Beuchat, University of Georgia, Griffin)	Cook et al., 1998
Salmonella Rubislow	F2833	LJH 588 | LJH1333	Isolated from 1995 unpasteurized orange juice outbreak (provided by Dr. Randy Worobo, Cornell University, Cornell)	Cook et al., 1998
Salmonella Muenchen		LJH 592 | LJH1337	Isolated from 1999 unpasteurized orange juice outbreak (provided by Dr. Larry Beuchat, University of Georgia, Griffin)	CDC, 1999

[a] PT, phage type.

References for strain table (Table 1)

Centers for Disease Control and Prevention (CDC). 1999. Outbreak of *Salmonella* Serotype Muenchen Infections Associated with Unpasteurized Orange Juice -- United States and Canada, June 1999. *Morb. Mortal. Wkly. Rep.* 48(27);582-585.

Cook KA, Dobbs TE, Hlady WG, Wells JG, Barrett TJ, Puhr ND, Lancette GA, Bodager DW, Toth BL, Genese CA, Highsmith AK. Outbreak of *Salmonella* serotype Hartford infections associated with unpasteurized orange juice. *JAMA* 1998 280(17):1504-9.

Gaul, L. K., N. H. Farag, T. Shim, M. A. Kingsley, B. J. Silk, and E. Hyytia-Trees. 2013. Hospital-acquired listeriosis outbreak caused by contaminated diced celery—Texas, 2010. *Clin. Infect. Dis.* 56:20–26.

Isaacs, S., J. Aramini, B. Ciebin, J. A. Farrar, R. Ahmed, D. Middleton, A. U. Chandran, L. J. Harris, M. Howes, E. Chan, A. S. Pichette, K. Campbell, A. Gupta, L. Y. Lior, M. Pearce, C. Clark, F. Rodgers, F. Jamieson, I. Brophy, and A. Ellis. 2005. An international outbreak of salmonellosis associated with raw almonds contaminated with a rare phage type of *Salmonella* Enteritidis. *J. Food Prot.* 68:191–198.

McCollum JT, Cronquist AB, Silk BJ, Jackson KA, O'Connor KA, Cosgrove S, Gossack JP, Parachini SS, Jain NS, Ettestad P, Ibraheem M. Multistate outbreak of listeriosis associated with cantaloupe. *New England J. Medicine* 2013 Sep 5;369(10):944-53.

U.S. Food and Drug Administration (US FDA). 2012. Gills Onions expands voluntary recall of diced and slivered red and yellow onions, and diced onion and celery mix because of possible health risk. Available at: https://wayback.archive-it.org/7993/20170406110507/https://www.fda.gov/Safety/Recalls/ArchiveRecalls/2012/ucm313399.htm.

Zhuang RY, Beuchat LR, Angulo FJ. Fate of *Salmonella* montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Applied and Environmental Microbiology* 1995 Jun;61(6):2127-31.

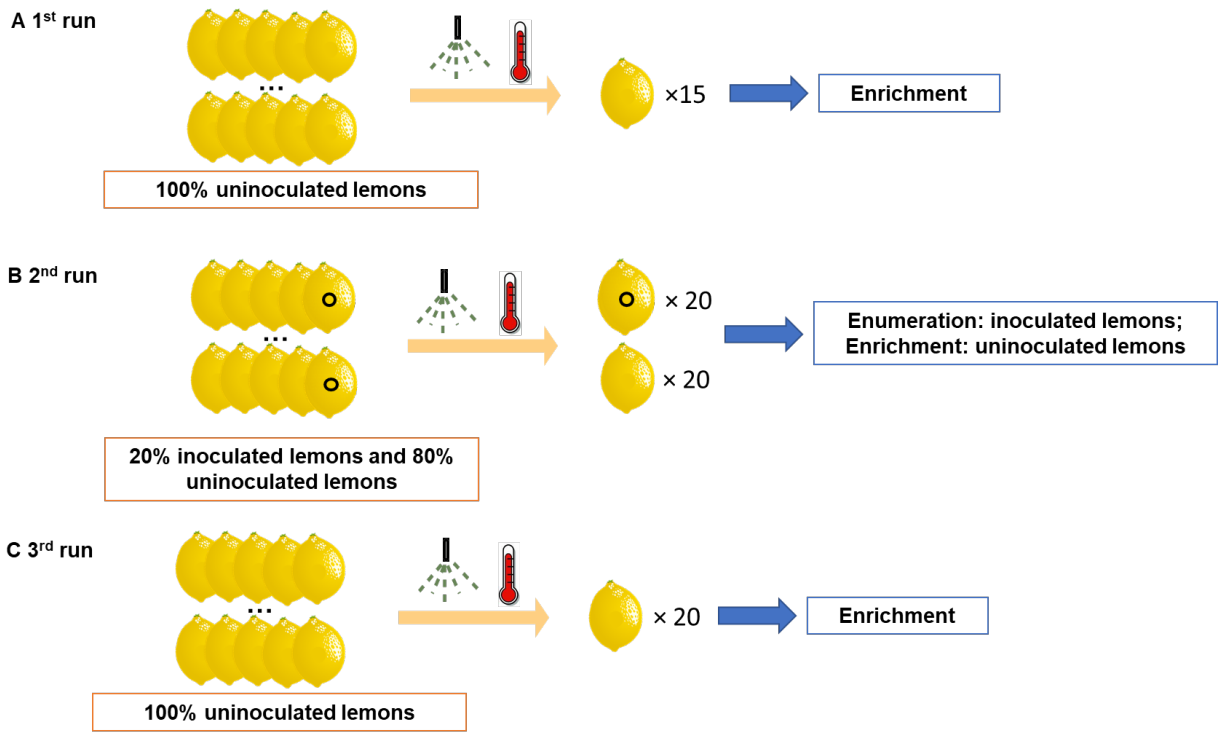

Figure 1 Schematic design of the pilot-scale waxing and drying (A, B, and C for 1st, 2nd, and 3rd run, respectively). The waxing application rate is 87L/h. The inlet temperature of the dryer is 50.7 °C (123.3 °F), and the outlet temperature of the dryer is 43.6 °C (110.5 °F). Lemons marked with circle are representing the rifampin-resistant *Enterococcus faecium* (NRRL-B2354) inoculated lemons.

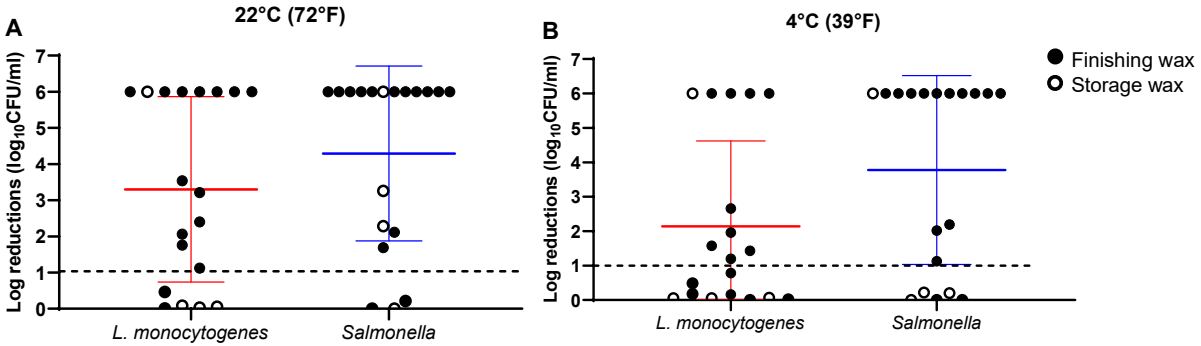

Figure 2 Log reductions of *L. monocytogenes* and *Salmonella* in artificially inoculated full-strength storage and finishing waxes when stored at 22 °C (72 °F) (A) and 4 °C (39 °F) (B) for 24 h. The red and blue lines represent the mean with SD. The dash lines separate waxes that resulted in <1-log reduction of pathogens after 24 h of storage from those that caused resulted in a >1-log reduction of pathogens after 24 h of storage.