Project/Proposal Title: Fate of *Listeria* on fresh apples as affected by commercial apple waxes

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Cooperators: Stemilt Growers LLC.; Hansen Fruit; Allan Brothers; Pace International LLC.; Jones-Hamilton Co.

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 83,842
Total Project Request for Year 2 Funding: $ 85,841
Total Project Request for Year 3 Funding: $ 86,419

WTFRC Collaborative Costs:

Item	2020-2021	2021-2022	2022-2023
Salaries			
Benefits			
Wages	2,104	2,135	2,168
Benefits	1,089	1,093	1,110
RCA Room Rental	2,250	2,316	2,385
Shipping		275	275
Supplies	250	275	275
Travel	350	375	400
Plot Fees			
Miscellaneous			
Total	6,043	6,469	6,613

Footnotes:

Budget 1
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Item	2020-2021	2021-2022	2022-2023
Salaries	32,667	33,974	35,333
Benefits	6,632	6,898	7,173
Wages			
Benefits			
Equipment			
Supplies	28,500	28,500	28,500
Travel	2,000	2,000	2,000
Miscellaneous	8,000	8,000	6,800
Plot Fees			
Total	77,799	79,372	79,806

Footnotes:

OBJECTIVES

1. Examine the fates of *Listeria,* resident bacteria, and yeast/mold on apples applied with commercial apple wax under subsequent cold storage.
2. Evaluate the fates of *Listeria* on waxed apples contaminated during wax application under subsequent cold storage.
3. Investigate the serotype-specific survival of *Listeria* on waxed apples and the killing effects of residual sanitizers on the fates of *Listeria* and resident microbes on waxed apples during subsequent cold storage

SIGNIFICANT FINDINGS

1. The dry temperature, whether at 22 °C (72 °F), 45 °C (113 °F), or 60 °C (140 °F), had no discernible impact on the survival of *Listeria innocua* on wax-coated apples from the selected varieties.
2. The population of *L. innocua* on unwaxed apples decreased by 1.9 $\log_{10}$ CFU/apple over the course of 18 weeks of refrigerated air storage.
3. *L. monocytogenes* reduced by 1.8-2.0 $\log_{10}$ CFU/apple on waxed apples during 12-week cold storage, regardless of the type of wax coating.
4. The fate of *Listeria* on wax-coated apples was similar to that on unwaxed apples.
5. The fate of *L. innocua* on Granny Smith apples exhibited comparable trends to those observed on Fuji apples, irrespective of the specific type of wax coating.
6. A significant risk of cross-contamination of *L. monocytogenes* occurred during the wax coating application process, from inoculated apples to waxing brushes, and from contaminated brushes to uninoculated apples, highlighting the importance of waxing station sanitation.
7. The die-off rate of *L. monocytogenes* on wax-coated apples contaminated during wax coating process was not significantly different from those contaminated before wax coating.
8. Different *L. monocytogenes* serotypes, including 1/2a, 1/2b, and 4b, exhibited distinct survival profiles on Granny Smith apples.
9. Serotype 1/2a displayed the highest resilience, maintaining a high population on Granny Smith apples throughout storage. In contrast, serotype 4b, linked to the caramel apple outbreak, exhibited the lowest survivability, with a rapid decline observed within 48 h of attachment at 22 °C (72 °F).
10. A 30-sec treatment with peroxyacetic acid (PAA) at 80 ppm resulted in a ~1.4 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on apples but had no residual killing effect on *L. monocytogenes* or resident microbes on wax-coated apples during the subsequent 16 weeks of cold storage. The count of *L. monocytogenes* recovered from wax-coated apples treated with 80 ppm PAA was significantly lower than that of control apples washed with tap water.
11. *L. monocytogenes* remained viable on waxing brushes during 12 weeks of ambient holding.
12. Including fungicides in the wax coating effectively reduced yeasts and molds on wax-coated apples; however, it did not impact *L. monocytogenes* survival.
13. The wax coating did not affect the survival of yeasts and molds on apples, irrespective of the apple cultivars; an increase of 0.4-0.5 $\log_{10}$ CFU/apple was observed after 18 weeks of cold storage, independent of the type of wax treatment applied.
14. Wax coating increased the glossiness of apples, regardless of wax treatment.

15. The firmness of apples decreased after 18 weeks of commercial storage, regardless of whether a wax coating was applied or the types of coating used. However, wax coating reduced the firmness loss in Granny Smith apples across all coating types.
16. Total soluble solids (TSS) were maintained in both unwaxed and wax-coated apples after 18 weeks of cold storage. Titratable acidity (TA) decreased in both unwaxed and wax-coated Fuji apples, while wax coating reduced TA loss in Granny Smith apples.
17. The application of the wax coating, irrespective of type, had no impact on interior and exterior disorders on Fuji and Granny Smith apples, but it significantly reduced internal browning in Granny Smith apples.

METHODS

1. Strain selection

L. monocytogenes strains for BSL2 lab storage: A panel of *L. monocytogenes* serotypes consisting of serotypes 1/2a, 1/2b, and 4b was selected and used in this study (Table 1). To confirm the serotype-specific survivability of *L. monocytogenes* strains, we also used an additional set of strains covering these three serotypes, including NRRL B-57618, NRRL B-33466, and NRRL B-33053 (Table 1).

Table 1. Detailed information on *L. monocytogenes* strains used in this study.

Strain No.	Serotype	Source	Antibiotics resistance
LS808	1/2a	Linked to a celery outbreak	Erythromycin at 2.5 mg/l
LS810	1/2b	Linked to a cantaloupe outbreak	Erythromycin at 2.5 mg/l, Rifampicin at 100 mg/l
LS1062	4b	Linked to apple outbreak	Streptomycin at 1000 mg/l
NRRL B-57618	1/2a	2011 cantaloupe outbreak isolate	None
NRRL B-33466	1/2b	Processing plant environmental isolate	None
NRRL B-33053	4b	1983 Coleslaw outbreak isolate	None

L. innocua strains employed for commercial cold storage: *L. innocua*, a widely used surrogate for *L. monocytogenes,* was used to investigate the fates of *Listeria* during commercial cold storage. A 3-strain cocktail of *L. innocua* isolates, sourced from an apple packing facility and other fresh produce processing plants, was prepared using our established methodology.

2. Apple inoculation

Apples were contaminated with *Listeria* prior to the waxing application: Washed and unwaxed apples of the selected varieties without cuts or bruises were individually and separately inoculated to establish 1×10^6 CFU/apple of a 3-strain cocktail of *L. monocytogenes* or *L. innocua* per our well-established method. Additionally, to rule out confounding effects from potential antagonistic interactions among strains, each serotype from the LS808, LS810, and LS1062 set, as well as from the *L. monocytogenes* NRRL B-57618, B-33466, and B-33053 set, was used to prepare individual inocula. The resulting inoculum was used separately for apple inoculation. The inoculated apples were held at 22 °C (72 ºF) for 24 h before the wax coating was applied.

Apples were contaminated during waxing application: To test the potential of *L. monocytogenes* cross-contamination from apple-to-brush and brush-to-apple, one waxing brush was used to coat one *L. monocytogenes* inoculated apple; then, this contaminated brush was used to wax five uninoculated apples in a sequence (Fig. 1).

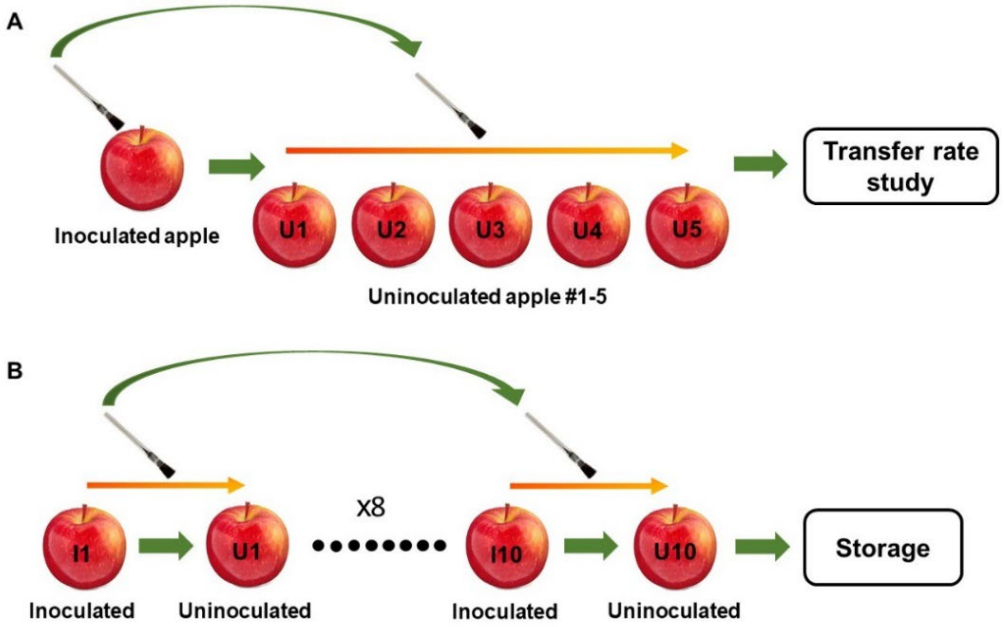

Fig. 1. **Illustration for the preparation of waxed apples contaminated with *Listeria monocytogenes* during wax coating.** A. Wax-coated apples for the apple-to-brush and brush-to-apple transfer rate study. B. Wax-coated apples for the storage study. I: inoculated apple; U: uninoculated apple.

3. Waxing application

Wax selection: Three commercial apple fruit waxes, namely Prima Fresh 360 HS (PF360), Prima Fresh 606 EU (PF606) or Shield Brite AP-450 (AP-450) were used in the proposed studies.

Waxing application: Each wax solution was manually applied evenly to both inoculated and uninoculated apple surfaces of the selected cultivars unless specified. To assess the fate of *Listeria* on waxed apples subjected to cross-contamination during the waxing process, brushes contaminated with *L. monocytogenes* were used for manual wax application. This enables the cross-contamination of *L. monocytogenes* to uninoculated apples (Fig. 1).

4. Wax coating drying

To evaluate the impacts of wax coating drying conditions/temperatures on the survival of *Listeria* on waxed apples, apples immediately following wax coating were subjected to different drying temperatures (~22 °C/72 °F, 45 °C/113 °F, or 60 °C/140 °F) for 2 min, followed by an additional 5 h drying at room temperature (~22 °C/72 °F) before being subjected to cold storage (Fig. 2).

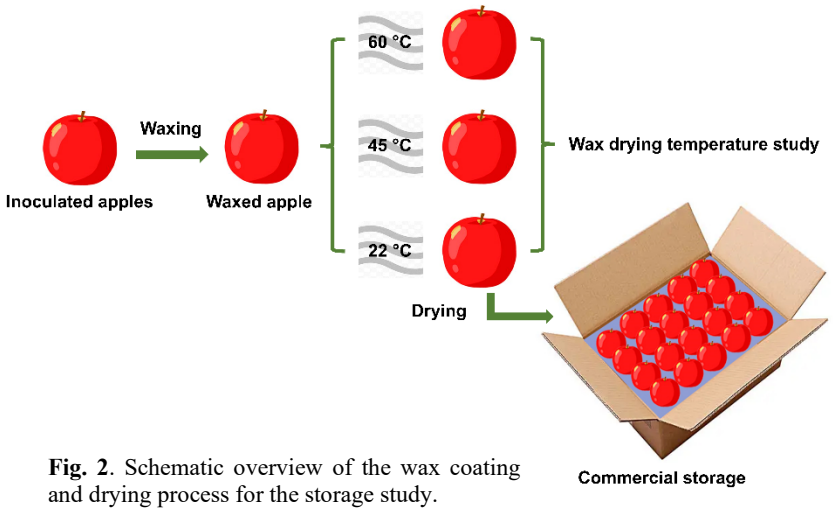

Fig. 2. Schematic overview of the wax coating and drying process for the storage study.

5. Cold storage treatments and sampling

BSL2 lab cold storage: Uninoculated or inoculated apples (with or without wax coating) from the selected cultivars were subjected to 1 °C (34 °F) or ambient temperature storage for up to 16 weeks. Samples were taken at designated intervals to enumerate *L. monocytogenes* or resident microbiota (background bacteria or yeast/mold). Two independent and sequential trials were conducted, each using a different lot of apples. In each independent trial, 20 apples per treatment were sampled on each sampling day.

Commercial facility storage: Uninoculated apples and apples inoculated with a 3-strain *L. innocua* cocktail and coated with different wax coatings were stored at 1 °C (34 °F) for up to 18 weeks in refrigerated air (RA) room within a commercial packing facility. Apples of each treatment combination were sampled during storage to enumerate the survival of *L. innocua* and yeast/mold. The study was conducted over two consecutive years. Four sets of 10 apples were used for each wax treatment on each sampling day.

6. Residual killing effects of antimicrobial interventions

Fresh, unwaxed Granny Smith apples of uniform size (220-240 g) were selected and dip-inoculated with a three-strain *L. monocytogenes* cocktail as previously described. The inoculated apples were treated with 80 ppm peroxyacetic acid (PAA), a commonly used sanitizer in spray bar interventions, for 30 seconds. After treatment, the apples were dried at room temperature for 3 h and then manually wax-coated with PrimaFresh 360 HS as outlined above. Apples inoculated and treated with tap water served as controls.

The wax-coated apples were subsequently dried at room temperature for 4 h before being stored at 1 °C (34 °F) and ~90 % relative humidity for up to 16 weeks. Temperature and humidity levels were monitored daily using a hygro-thermometer (Extech Instruments) throughout the storage period. A sample size of 40 apples per sampling time and treatment was used.

7. Survival microorganism analysis

Listeria enumeration: At each sampling day, *Listeria* survival on waxed apples under the respective storage (BSL2 or commercial facility) was detached and serially diluted. Appropriate dilutions were plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) plates overlaid with modified Oxford agar per our established method. For the serotype-specific survival profile analysis, the detached microbial suspensions were plated onto TSAYE plates with erythromycin, erythromycin and rifampicin, and streptomycin for the enumeration of serotype 1/2a serotype 1/2b, and serotype 4b, respectively.

All plates were incubated at 35 °C (95 °F) for 48 h and subsequently enumerated. If the survival of *Listeria* on apple fruit fell below the enumerative detection limit, the suspension was assessed for presence/absence after 48 h of enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaked onto a selective *Listeria* agar plate. Presumptive positive colonies were further confirmed by PCR (FDA, 2015).

Resident microbiota: Microbial suspension at appropriate dilutions was also plated on duplicate Potato Dextrose Agar plates supplemented with 0.1 g/l chloramphenicol for yeast and mold counts. The PDA plates were incubated at room temperature (~22 °C/ 72 °F) for 5 days.

8. Fruit quality analysis

At harvest or 18-week storage, fruit quality parameters such as firmness, total soluble solids, and titratable acidity, as well as external and internal disorders, including superficial scald and lenticel decay, were assessed at the end of cold storage by the WTFRC quality lab using established methods (Shen et al., 2021). A sample size of 10 apples per replicate with 4 independent replicates per wax type was used for internal and external disorder assessment.

9. Glossiness measurement

The gloss index of apples was determined at 60° with a gloss meter (Novo-Curve, Rhopoint Instrumentation, East Sussex, UK). The gloss units (GU) were directly measured on the fruit surface with 10 randomly selected spots per fruit. A total of 10 apple fruits per treatment condition was used for gloss analysis.

10. Statistical analysis.

Data were analyzed with IBM SPSS 19.0 (Chicago, IL). Mean differences were assessed through a one-way analysis of variance (ANOVA), followed by a Tukey multiple comparison test. *P* values less than 0.05 were considered significant differences.

RESULTS AND DISCUSSION

1. Survival of *L. monocytogenes* in wax coating solutions

L. monocytogenes in wax solutions were reduced by ~2 $\log_{10}$ CFU/ml in 1-h contact regardless of wax type (Fig. 3). AP-450 showed a superior antimicrobial efficacy against *L. monocytogenes*, followed by PF 606 and PF 360. *L. monocytogenes* in AP-450 wax solution dropped to below the limit of detection (1 CFU/ml) after 24-h incubation compared with 5.3 and 3.1 $\log_{10}$ CFU/ml reductions observed in PF 606 and PF 360 wax solutions (Fig. 3).

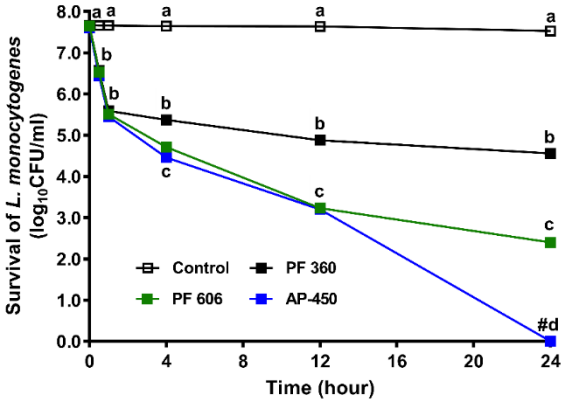

Figure 3. Survival of *L. monocytogenes* in apple wax coating solutions at RT. Control: water. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360; PF 606: PrimaFresh 606. Mean ± SEM, n = 9. [a-d] Mean at each sampling point without a common letter differ significantly ($P < 0.05$).

2. Effects of wax drying temperatures on survival of *L. innocua*, yeast and mold counts, and the glossiness of the waxed apples

During the commercial apple packing line process, wax coatings of apples are dried as they pass through a heated air dryer (42-45 °C/ 108-113°F). To simulate this commercial drying process in our study, we first evaluated the impact of wax coating drying temperatures of 22 °C (72 °F), 45 °C (113 °F), and 60 °C (140 °F) on the survival of *L. innocua* on apples and the glossiness of waxed apples in our BSL2 lab. Data in Fig. 4 indicated that the drying temperature did not affect the

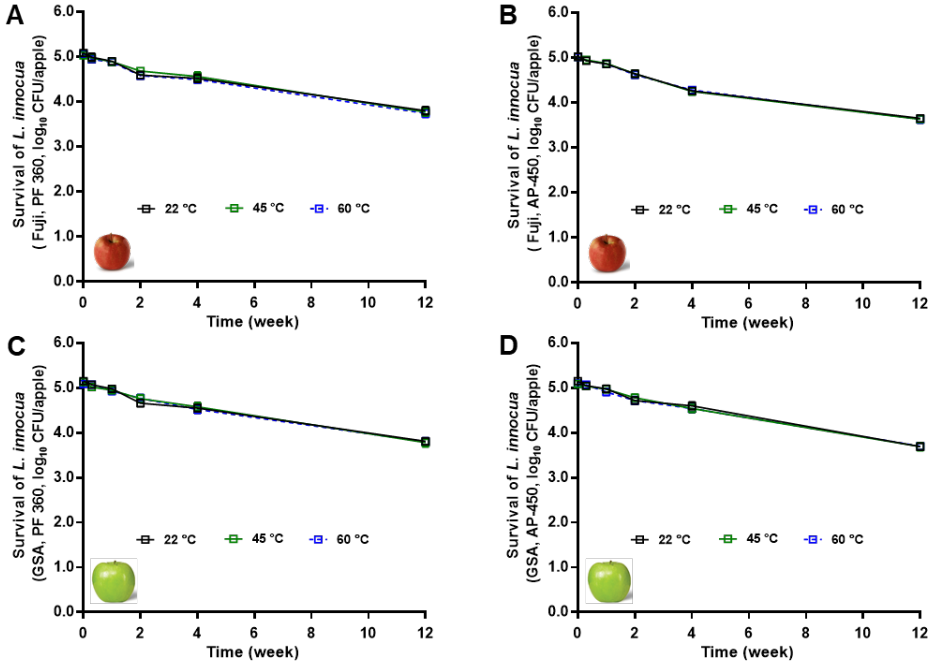

Figure 4. Impact of wax drying temperature on *L. innocua* on waxed apples during 12 weeks of storage at 1 °C (33 °F). A. Fuji apples coated with PrimaFresh 360 HS (PF 360); B. Fuji apples coated with Shield-Brite AP 450 (AP 450); C. Granny Smith apples coated with PF 360; D. Granny Smith apples coated with AP 450 Mean ± SEM, n = 12.

fate of *L. innocua* on apples during the 12 weeks of storage at 1 °C (34 °F). Populations of *L. innocua* on waxed apples gradually declined, resulting in 1.4-1.5 $\log_{10}$ CFU/apple reduction of *L. innocua* by the end of storage, regardless of apple varieties, drying temperature, and wax type (Fig. 4). Similarly, the application of a shellac-based wax at 25 °C (77 °F), 50 °C (122 °F), and 60 °C (140 °F), for 2 min caused comparable reductions of *E. coli* on the stem scar area of oranges (Pao, Davis, Kelsey, & Petracek, 1999). However, another study found that applying Shield-Brite AP-40 on apples at 55 °C (131 °F), drying caused additional 1.0 and 0.4 $\log_{10}$ CFU/apple reduction of *Escherichia coli* O157:H7 and *Salmonella*, respectively, compared to drying at 21 °C (70 °F), (Kenney & Beuchat, 2002). These different observations may be due to variations in pathogens and the wax coating formulations.

Similarly, yeast and mold count on PF 360- or AP 450-coated Fuji apples (Fig. 5AB) and GSA (Fig. 5CD) were not affected by drying temperature, showing 0.5-0.6 $\log_{10}$ CFU/apple increase during the initial 4 weeks of cold storage and remaining stable over subsequent 8 weeks of cold storage (Fig. 5).

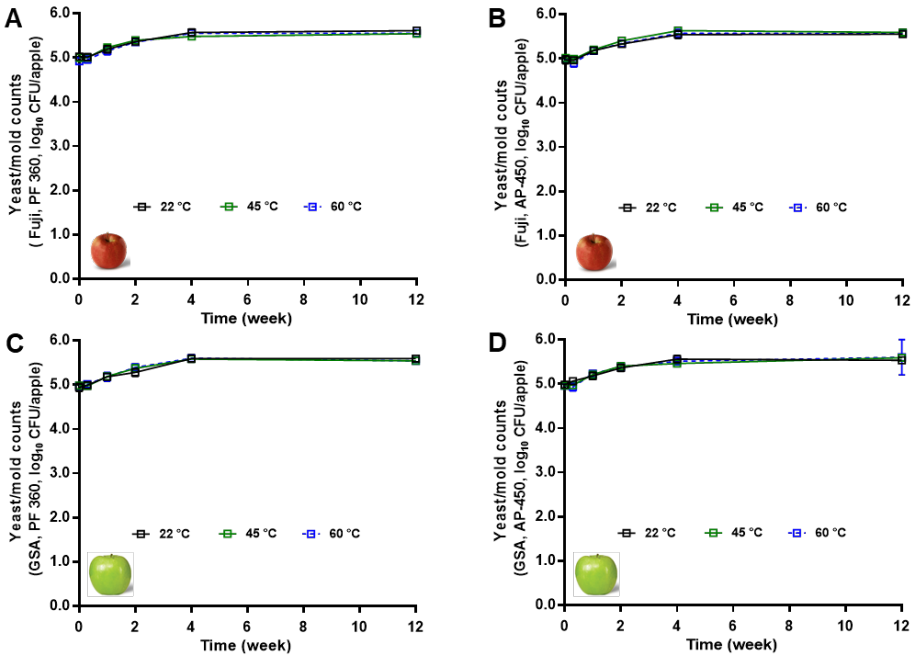

Figure 5. Impact of wax drying temperature on yeast and mold counts on waxed apples during 12 weeks of storage at 1 °C (34 °F). A. Fuji apples coated with PrimaFresh 360 HS (PF 360); B. Fuji apples coated with Shield-Brite AP-450 (AP-450); C. Granny Smith apples coated with PF 360; D. Granny Smith apples coated with AP-450. Mean ± SEM, n = 12.

The gloss indices of apples increased after coating with wax solutions, with an increase of 11.2-12.2 GU observed for apples coated with PF 360 and 18.7-20.0 GU for those coated with AP-450, compared to unwaxed apples (Su et al., 2023). The drying temperature had no impact on gloss indices, regardless of the wax type and apple varieties. Based on these findings, a drying temperature of 22 °C (72 °F) was selected for preparing wax-coated apples during the subsequent studies.

3. Transfer of *L. monocytogenes* from apple-to-brush and brush-to-apple during wax application

To test the potential of *L. monocytogenes* cross-contamination from apple-to-brush and brush-to-apple, one waxing brush was used to coat one *L. monocytogenes* inoculated apple; then, this contaminated brush was used to wax five uninoculated apples in a sequence (Fig. 1A). During PF 360 wax coating application, there were 3.7, 3.5, 3.3, 2.9, and 2.7 $\log_{10}$ CFU/apple of *L. monocytogenes* transferred from the inoculated apple (6.2 $\log_{10}$ CFU/apple) to uninoculated apple 1 to apple 5, respectively (Fig. 6A). After waxing the 5th uninoculated apple, 3.6 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 6B). Similarly, for apples with a higher contamination level (8.4 $\log_{10}$ CFU/apple), 5.8, 5.6, 5.0, 4.8 and 4.6 $\log_{10}$ CFU/apple of *L. monocytogenes* were transferred to uninoculated apple 1 to apple 5 during wax coating application (Fig. 6C). After waxing of the 5th uninoculated apple, 5.5 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 6D). A similar transfer rate of *L. monocytogenes* from the inoculated

apple to the waxing brush and uninoculated apples was found for AP-450, regardless of the initial contamination level (Fig. 6).

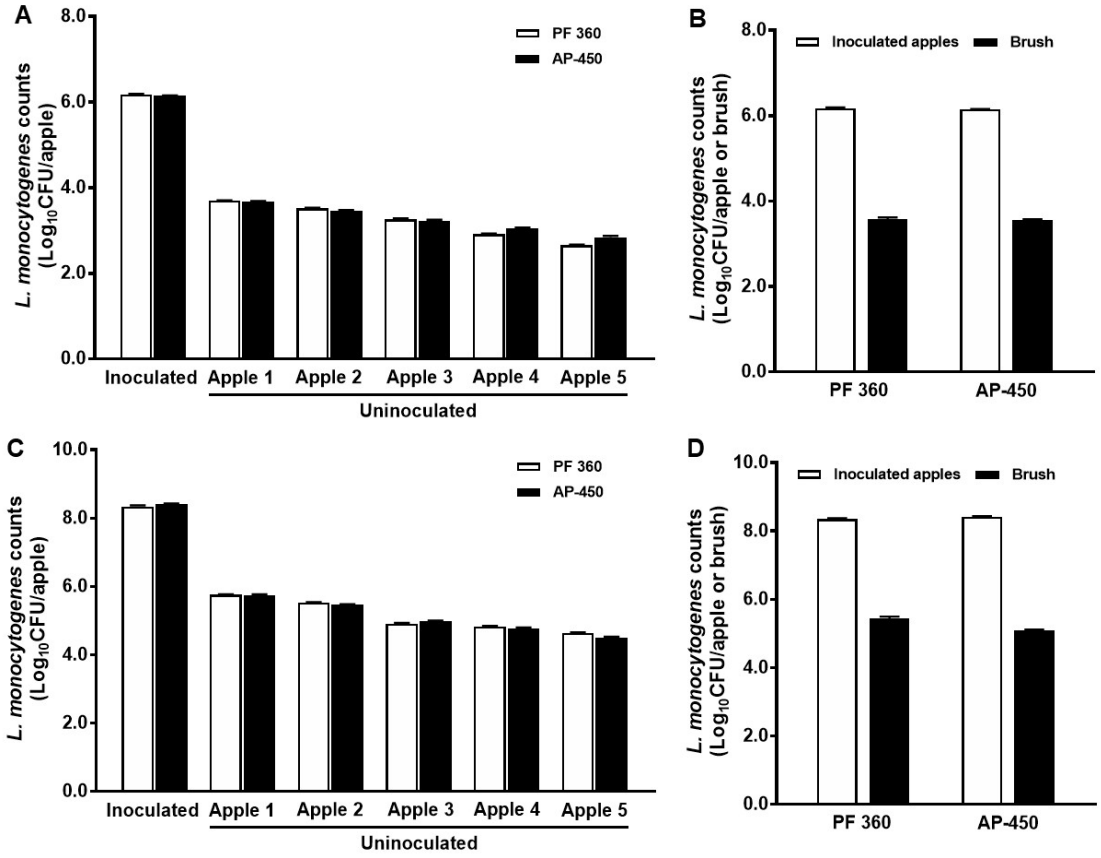

Figure 6. Transfer of *L. monocytogenes* from inoculated apples to uninoculated apples and waxing brushes during wax coating. A. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to uninoculated apples; B. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to waxing brushes. C. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to uninoculated apples; D. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to waxing brushes. Apple 1-5: *L. monocytogenes* on uninoculated apples transferred from contaminated waxing brushes. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. Data were presented with mean ± SEM, n = 24.

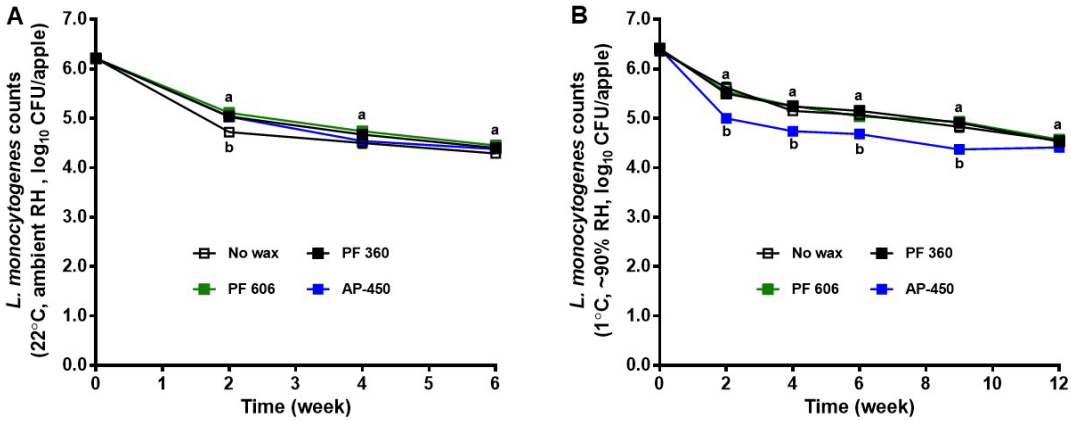

Figure 7. Fates of *L. monocytogenes* on wax-coated apples contaminated before wax coating application for up to 12 weeks of storage. A. ambient T and RH. B. 1 °C (34 ºF) and ~90 % RH; No wax: unwaxed control apples; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

3. Survival of *L. monocytogenes* on waxed apples contaminated during different waxing schemes

To represent wax applications at apple packinghouses, three commonly used fruit wax coatings, PF 360, PF 606, and AP-450 were applied to the inoculated fruits, followed by up to 12-week storage. *L. monocytogenes* showed a similar trend on waxed apples under cold storage; there were 1.8-2.0 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* on apples during 12 weeks of cold storage regardless of wax coating type, though the reduction on AP-450 waxed apples was higher ($P < 0.05$) at 2-9 weeks of storage (Fig. 7B). The application of wax coating had a minor impact on the survival of *L. monocytogenes* on apples regardless of storage temperature (Fig. 7).

Given the prevalence of *Listeria* species in waxing areas (Ruiz-Llacsahuanga, Hamilton, Zaches, Hanrahan, & Critzer, 2021; Simonetti et al., 2021), it is likely that *L. monocytogenes* can be introduced to wax-coated apples during the wax-coating process. Therefore, we next examined the fate of *L. monocytogenes* on PF 360 coated apples introduced during wax coating with the same contamination level as pre-contaminated apples at ~6 $\log_{10}$ CFU/apple (Fig. 1B). *L. monocytogenes* was reduced by 1.8 $\log_{10}$ CFU/apple after 12 weeks of cold storage (Fig. 8), which had a similar trend as *L. monocytogenes* introduced to apples before waxing application whether apples had an initial population of ~6 $\log_{10}$ CFU/apple (Fig. 7B) or ~8 $\log_{10}$ CFU/apple (Fig. 8).

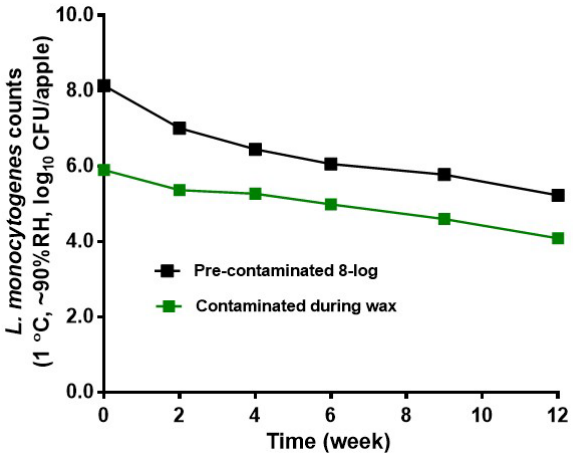

Figure 8. Fates of *L. monocytogenes* on wax-coated apples introduced during PrimaFresh 360 coating application for up to 12 weeks of storage. Source apples were inoculated with ~8 $\log_{10}$ CFU/apple of *L. monocytogenes* before wax coating (black line). Mean ± SEM, n = 40.

4. Impacts of fungicide application in PrimaFresh 360 coating on fates of *L. monocytogenes* and endogenous yeasts and molds on waxed apples during 12 weeks of cold storage

Fungicides can be incorporated into wax coating solutions under commercial apple waxing. To evaluate the potential impacts of fungicide applications during wax coating on the fate of *L. monocytogenes* on waxed apples, PF 360 wax coating was further applied in combination with two widely used fungicides, fludioxonil, and natamycin, followed by 12 weeks of cold storage. As shown in Fig. 9A, fludioxonil or natamycin in the fruit wax coating did not impact ($P > 0.05$) the behavior of *L. monocytogenes* on waxed fruits. Populations of *L. monocytogenes* decreased by 1.7-1.8 $\log_{10}$

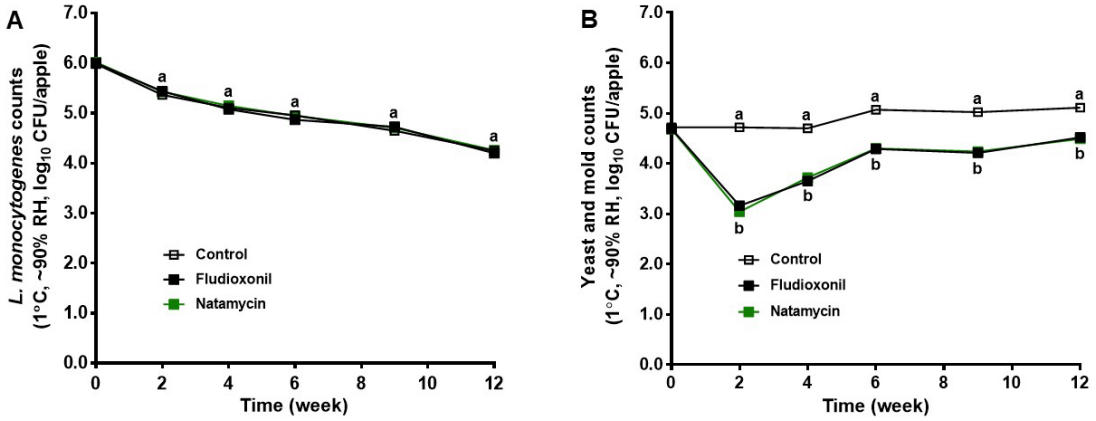

Figure 9. Impacts of fungicide application in PrimaFresh 360 coating on fates of *L. monocytogenes* (A) and endogenous yeasts and molds (B) on wax-coated apples during 12 weeks of cold storage. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

CFU/apple on PF 360-coated apples regardless of fungicide application after 12 weeks of cold storage (Fig. 9A). Including fungicides in a wax solution reduced yeast and mold counts on waxed apples by 1.5-1.6 $\log_{10}$ CFU/apple at 2-week cold storage, but the counts then gradually increased to 4.5 $\log_{10}$ CFU/apple at 12-week cold storage (Fig. 9B). Fludioxonil and natamycin had similar effectiveness ($P > 0.05$) in controlling yeasts and molds on waxed apples.

5. Serotype-specific survival of Granny Smith apples during 48 h of attachment

The initial inoculation level of the *L. monocytogenes* 3-strain cocktail on apples was ~5.3 log CFU/apple, with ~4.9 $\log_{10}$ CFU/apple for each serotype (Fig. 10A). After 48 h of the establishment at RT, the counts of *L. monocytogenes* cocktail, LS808 (1/2a), and LS810 (1/2b) on GSA increased by 0.44-0.5 $\log_{10}$ CFU/apple (Fig. 10A-B, $P < 0.05$), while the culturable count of LS1062 (4b) significantly decreased by 1.36 $\log_{10}$ CFU/apple ($P < 0.05$) (Fig. 10A). To rule out the possibility that the reduction in the 4b strain was due to the potential antagonistic interactions among strains, each serotype was individually inoculated on apples, and the survival patterns of each serotype on apples mirrored those observed in the cocktail environments (Fig. 10). To further confirm serotype-specific survival and attachment, an additional set of *L. monocytogenes* strains linked to fresh produce outbreaks (Table 1) was examined individually on apples during 24 and 48 h of attachment. Their survival again mirrored the LS808, 810, and 1062 set (Data not shown).

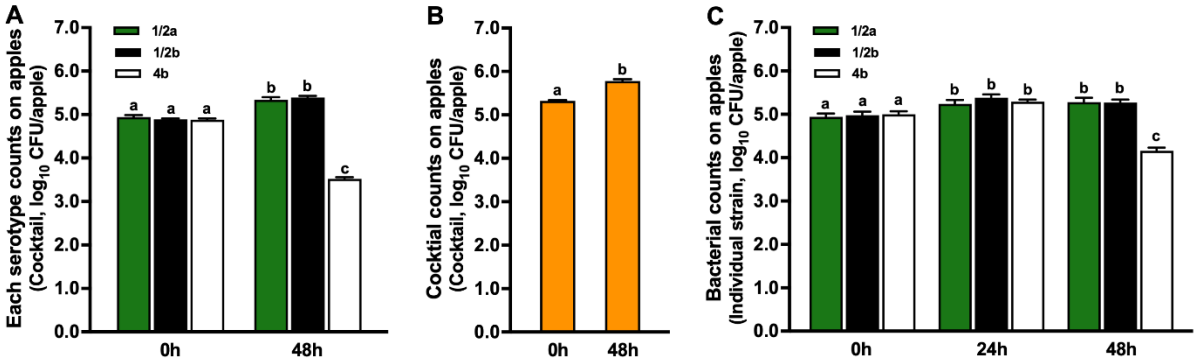

Figure 10. Counts of *L. monocytogenes* on unwaxed Granny Smith apples during 48 h attachment at 22 °C. A. Counts of each serotype in the cocktail inoculated on apples, immediately after inoculation (0 h) and 48 h post-inoculation (48 h). B. Counts of the *L. monocytogenes* cocktail inoculated on apples at 0 h and 48 h post-inoculation. C. Counts of each serotype individually inoculated on apples 0 h, 24 h, and 48 h post-inoculation. Mean ± SEM, n = 20. Different

Over 12 weeks of 1 °C (34 °F) or RT storage, the total counts of the 3-strain *L. monocytogenes* recovered from waxed GSA, regardless of wax coating application or the type of wax coating, remained relatively stable (Figs. 11-12). Each serotype exhibited a unique survival profile during 1 °C (34 °F) storage. After a sharp decrease post 48 h ($P < 0.05$), serotype 4b remained relatively low (3.5 $\log_{10}$ CFU/apple) but stable counts on GSA over the subsequent 12 weeks of 1 °C (34 °F) storage, irrespective

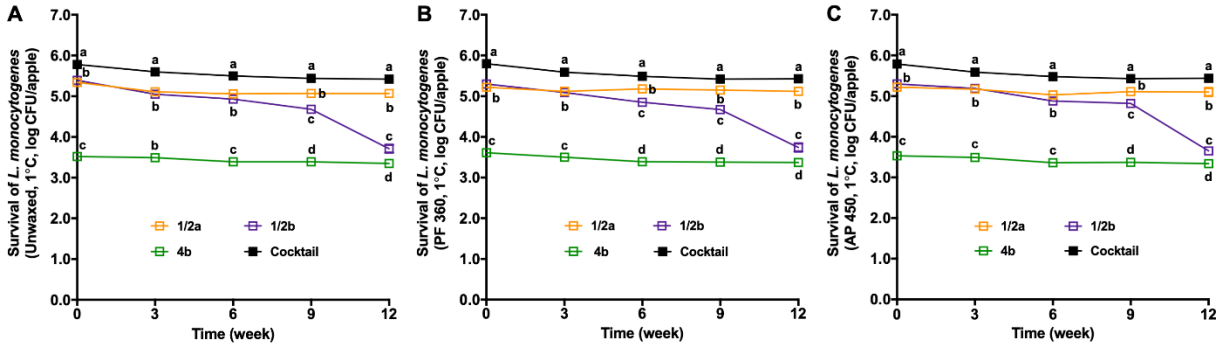

Figure 11. Survival of *L. monocytogenes* serotypes on Granny Smith apples during 12 weeks of storage at 1 °C (33 °F). A. Unwaxed apples; B. PrimaFresh 360 (PF 360) coated apples; C. Shield-Brite AP-450 (AP-450) coated apples. Mean ± SEM, $n = 40$. Different letters (a-d) indicate significant differences at each sampling point ($P < 0.05$).

of wax coating or the type of wax (Fig. 11). Among the serotypes tested, serotype 1/2a was the most resilient, maintaining a high population level (5.3 $\log_{10}$ CFU/apple) throughout the entire storage duration, regardless of the type of wax coating. Serotype 1/2b counts on GSA remained relatively stable during the first 9 weeks of storage but exhibited a drastic reduction (1.7 $\log_{10}$ CFU/apple) ($P < 0.05$) by the end of the 12-week storage. The fate of each serotype strain was comparable to unwaxed GSA (Fig. 11), indicating that the wax coating had minimal impact on the survival of distinct serotypes ($P > 0.05$).

The fate of each *L. monocytogenes* serotype as well as the 3-strain cocktail, on waxed GSA during ambient temperature storage showed similar trends to those observed under cold storage conditions (Fig. 12). Serotype 4b exhibited a swift decline, while serotype 1/2a became the dominant serotype on the surface of GSA.

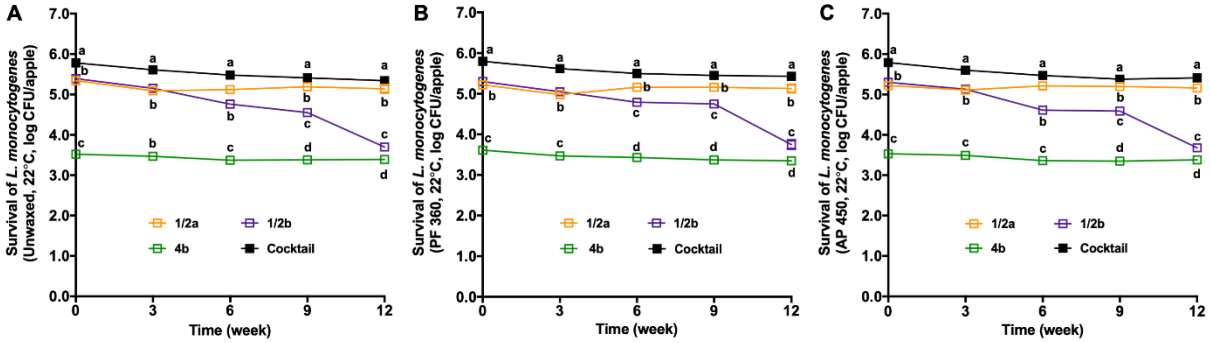

Figure 12. Survival of *L. monocytogenes* serotypes on Granny Smith apples during 12 weeks of storage at 22 °C (72 °F). A. Unwaxed apples; B. PrimaFresh 360 (PF 360) coated apples; C. Shield-Brite AP 450 (AP 450) coated apples. Mean ± SEM, $n = 40$. Different letters (a-d) indicate significant differences at each sampling point ($P < 0.05$).

6. Residual killing effects of antimicrobial interventions

PAA is one of the most frequently used sanitizers in the Washington apple industry and can be applied at 80 ppm without the need for further rinsing (FDA, 2017). A 30-sec treatment with PAA at 80 ppm resulted in a ~1.4 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on apples before wax coating. However, PAA did not exhibit a residual killing effect on *L. monocytogenes* (Fig. 13A) or endogenous yeast and mold counts (Fig. 13B) on wax-coated apples during the subsequent 16 weeks of cold storage. The count of *L. monocytogenes* (Fig. 13A) and yeasts and molds (Fig. 13B) recovered from wax-coated apples treated with 80 ppm PAA was significantly lower than those on control apples washed with tap water.

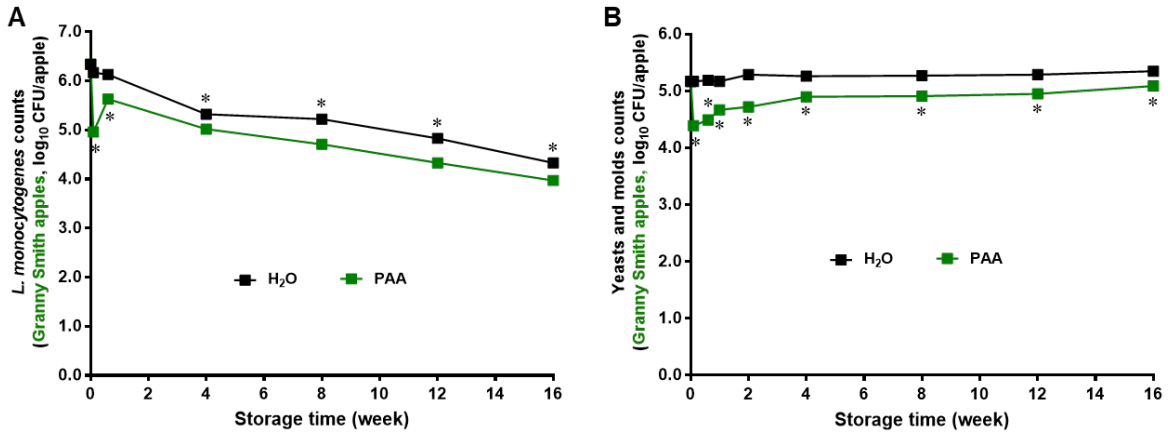

Figure 13. The population of *L. monocytogenes* (A) and yeasts and molds (B) on PrimaFresh 360 (PF 360) coated Granny Smith apples washed with or without 80 ppm peroxyacetic acid (PAA) during 16 weeks of simulated cold storage at 1 °C (33 °F). Mean ± SEM, $n = 40$. *: indicate significant differences at each sampling point ($P < 0.05$).

7. Persistence of *L. monocytogenes* on wax coating brushes

L. monocytogenes remained relatively stable on waxing brushes during 2 weeks of holding at 22 ºC (72 ºF). A 0.5 and 1.3 $\log_{10}$ CFU/brush reduction was observed on waxing brushes with initial contamination levels of 3.6 and 5.5 $\log_{10}$ CFU/brush, respectively (Fig. 14A). A similar contamination level and die-off rate ($P > 0.05$) of *L. monocytogenes* were found on waxing brushes used to apply AP-450 wax coating to apples using the same method (Fig. 14A). To evaluate the fate of *L. monocytogenes* on waxing brushes under long-term holding, the waxing brushes used to prepare PF 360-coated apples for the storage study were subjected to 12 weeks of ambient holding. After two weeks, a 1.2 $\log_{10}$ CFU/brush reduction in *L. monocytogenes* was observed, with populations remaining relatively stable at ~3 $\log_{10}$ CFU/brush during the subsequent ten weeks of holding (Fig. 14B).

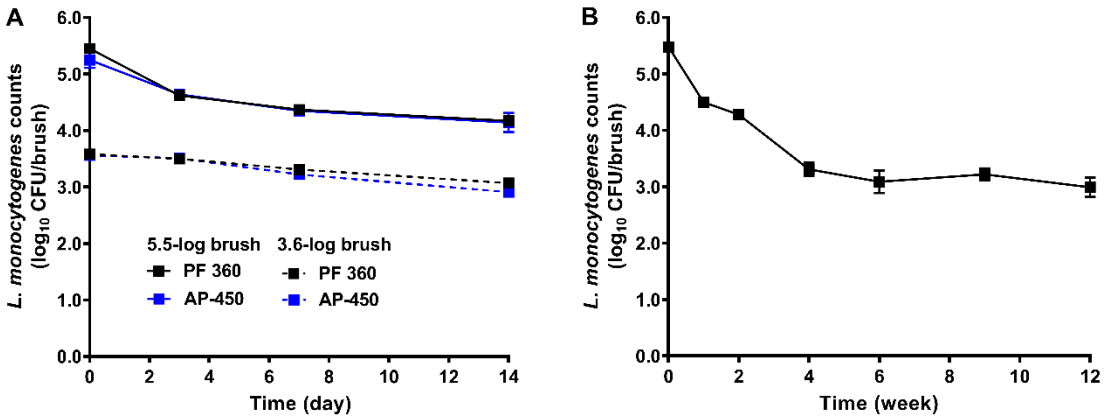

Figure 14. Persistence of *L. monocytogenes* on waxing brushes during holding at ambient temperature. (A) 14-day ambient holding of waxing brushes contaminated at low (3.6 $\log_{10}$ CFU/brush) and high (5.5 $\log_{10}$ CFU/brush) levels. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. (B) 12-week ambient holding of waxing brushes coated with PF 360. Data are presented as mean ± SEM, n = 4-6. Brushes were contaminated with *L. monocytogenes* during the wax

8. Survival of *L. innocua* on different wax-coated apples during commercial cold storages.

The initial *L. innocua* counts on waxed and unwaxed Fuji (Fig. 15A) and Granny Smith (Fig. 15B) apples before storage was 5.6 $\log_{10}$ CFU/apple. During the first 6 weeks of cold storage, *L. innocua* counts decreased by 0.7-0.8 $\log_{10}$ CFU/apple on both unwaxed and wax-coated apples, regardless of the coating types and apple varieties (Fig. 15). Over the following 12 weeks of storage, *L. innocua* on

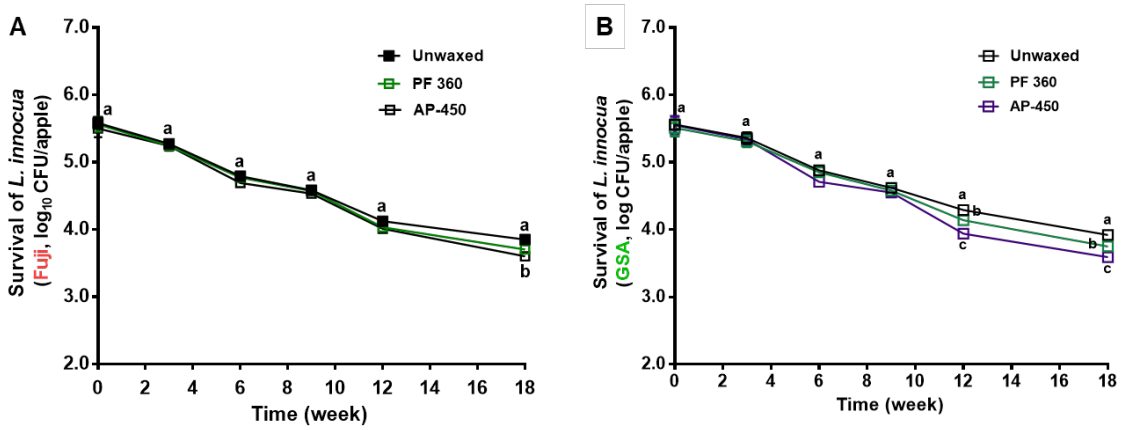

Figure 15. Fates of *L. innocua* on Fuji (A) and Granny Smith (B) apples coated with or without wax during 18 weeks of commercial refrigerated air storage. PF 360: PrimaFresh 360 HS; AP 450: Shield-Brite AP 450. Mean ± SEM, n = 40. Different letters (a-c) at each sampling point indicate significant differences ($P < 0.05$).

waxed apples further decreased by 1.8-2.0 $\log_{10}$ CFU/apple (Fig. 15). In summary, *L. innocua* survival was similar on unwaxed and waxed apples; however, wax coatings, regardless of type, slightly enhanced *Listeria* die-off during the 18 weeks of cold storage.

9. Yeast and mold count on wax-coated apples during commercial cold storage

The initial populations of yeasts and molds on Fuji (Fig. 16A) and Granny Smith (Fig. 16B) apples were 4.8-5.0 $\log_{10}$ CFU/apple (Fig. 16). Over 18 weeks of cold storage at 1°C (34°F), these populations gradually increased by 0.3-0.6 $\log_{10}$ CFU/apple (Fig. 16). The application of wax did not impact the survival of yeasts and molds during this period, with similar trends observed for both Fuji and Granny Smith apples. Similarly, a 0.5 $\log_{10}$ CFU/apple increase in yeasts and molds was noted on unwaxed Fuji apples after 24 weeks of storage at 1°C (34°F) (Sheng et al., 2018). Populations of yeast and mold on unwaxed Saltanat apples increased by 0.6-0.9 $\log_{10}$ CFU/cm^2 after 5 months of 2 °C (36 °F) storage (Juhneviča, Skudra, & Skudra, 2011).

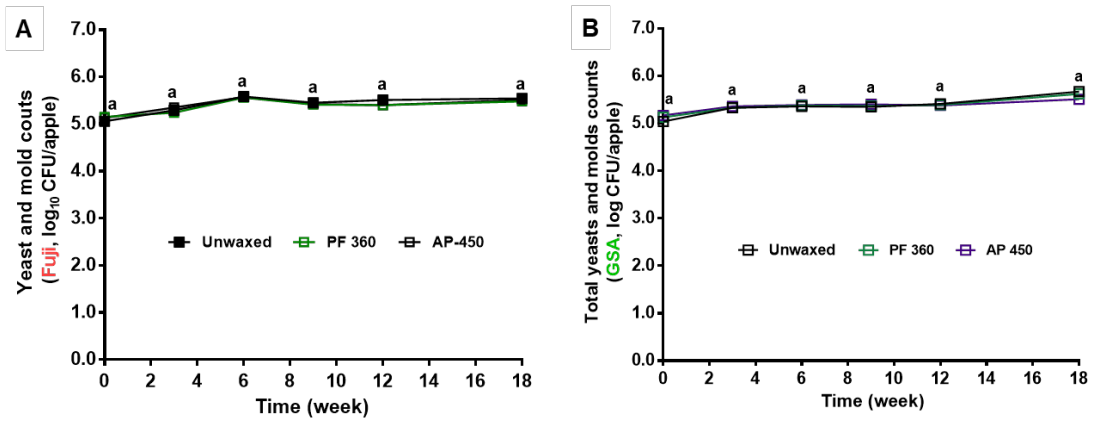

Figure 16. Yeast and mold counts on Fuji (A) and Granny Smith (B) apples coated with or without wax during 18 weeks of commercial refrigerated air storage. PF 360: PrimaFresh 360 HS; AP 450: Shield-Brite AP 450. Mean ± SEM, n = 40. Different letters (a-c) at each sampling point indicate significant differences ($P<0.05$).

10. Quality attributes of wax-coated apples during commercial cold storage

Wax coating significantly increased the glossiness of apples, regardless of the type of wax applied. After 18 weeks of commercial storage, the firmness of apples decreased in both waxed and unwaxed treatments, although wax coatings helped reduce the firmness loss in GSA across all coating types (Shen et al., 2025; Su et al., 2023). Total soluble solids (TSS) remained stable in both unwaxed and wax-coated apples during the 18-week cold storage period. Titratable acidity (TA) decreased in both unwaxed and wax-coated Fuji apples, but wax coating helped reduce TA loss in GSA apples (Su et al., 2023). The application of wax coatings, regardless of type, had no impact on interior and exterior disorders in Fuji and GSA, but it significantly reduced internal browning in GSA apples (Shen et al., 2025; Su et al., 2023). In agreement with our findings, 1% beeswax coating suppressed the increase of TSS in Generos, Starkrimson, Idared, and Jonagold apples during 4 months of cold storage at 2 °C (Anghel, 2011). TA decreased in both unwaxed and wax-coated GSA after 18 weeks of cold storage, consistent with our earlier finding on unwaxed Granny Smith apples over 24 weeks cold storage (Sheng et al., 2022).

References:

Anghel, R. M. (2011). The influence of wax protection film on apple fruits, in order to maintain their quality during cold storage. *Agronomy, 54*(1), 87-90.

FDA. (2015). BAM Protocol: Simultaneous Confirmation of *Listeria* species and *L. monocytogenes* isolates by real-time PCR. Retrieved from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm279532.htm

FDA. (2017). 21CFR173.315; Chemicals used in washing or to assist in the peeling of fruits and vegetables. *https://www.accessdata.fda.gov/SCRIPTs/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=173.315&SearchTerm=chemicals*.

Juhneviča, K., Skudra, G., & Skudra, L. (2011). Evaluation of microbiological contamination of apple fruit stored in a modified atmosphere. *Environ Exp. Biol, 9*, 53-59.

Kenney, S. J., & Beuchat, L. R. (2002). Survival of *Escherichia coli* O157: H7 and *Salmonella* Muenchen on apples as affected by application of commercial fruit waxes. *Int J Food Microbiol., 77*(3), 223-231.

Pao, S., Davis, C. L., Kelsey, D. F., & 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*(2), 359-362.

Ruiz-Llacsahuanga, B., Hamilton, A., Zaches, R., Hanrahan, I., & Critzer, F. (2021). Prevalence of *Listeria* species on food contact surfaces in Washington State apple packinghouses. *Appl Environ Microbiol, 87*, e02932-02920. doi:10.1128/AEM.02932-20

Shen, X., Su, Y., Hua, Z., Chiu, T., Wang, Y., Mendoza, M., . . . Zhu, M. J. (2025). Evaluating serotype-specific survival of *Listeria monocytogenes and Listeria innocua* on wax-coated Granny Smith apples during storage. *Int J Food Microbiol, 427*, 110964. doi:10.1016/j.ijfoodmicro.2024.110964

Shen, X., Su, Y., Hua, Z., Sheng, L., Mendoza, M., He, Y., . . . Zhu, M. J. (2021). Effectiveness of low-dose continuous gaseous ozone in controlling *Listeria innocua* on Red Delicious apples during 9-month commercial cold storage. *Front Microbiol, 12*(2612), 712757. doi:10.3389/fmicb.2021.712757

Sheng, L., Hanrahan, I., Sun, X., Taylor, M. H., Mendoza, M., & Zhu, M. J. (2018). Survival of *Listeria innocua* on Fuji apples under commercial cold storage with or without low dose continuous ozone gaseous. *Food Microbiol, 76*, 21-28. doi:10.1016/j.fm.2018.04.006

Sheng, L., Shen, X., Su, Y., Xue, Y., Gao, H., Mendoza, M., . . . Zhu, M. J. (2022). Effects of 1-methylcyclopropene and gaseous ozone on *Listeria innocua* survival and fruit quality of Granny Smith apples during long-term commercial cold storage. *Food Microbiol, 102*, 103922. doi:10.1016/j.fm.2021.103922

Simonetti, T., Peter, K., Chen, Y., Jin, Q., Zhang, G. D., LaBorde, L. F., & Macarisin, D. (2021). Prevalence and distribution of *Listeria monocytogenes* in three commercial tree fruit packinghouses. *Front Microbiol, 12*, 652708. doi:10.3389/fmicb.2021.652708

Su, Y., Shen, X., Hua, Z., Zhu, H., Chiu, T., Wang, Y., . . . Zhu, M. J. (2023). Fate of *Listeria innocua* on wax-coated Fuji apple surfaces under commercial refrigerated air storage. *Postharvest Biol Technol, 198*, 112236.

EXECUTIVE SUMMARY

Project Title: Fate of *Listeria* on fresh apples as affected by commercial apple waxes

Key words: *Listeria monocytogenes*; serotype, apples; wax-coating; drying temperature waxing brush; cross-contamination; commercial storage.

Abstract

This research examines the fate of *Listeria monocytogenes* on apples contaminated before and during wax coating, its persistence on contaminated waxing brushes, cross-contamination risks, and the serotype-specific survival of *L. monocytogenes* on wax-coated apples. The study further investigates the fate of *Listeria* and resident yeasts and molds on apples treated with commercial wax under cold storage, using *Listeria innocua* as a surrogate. Findings indicate that while wax coatings enhance apple glossiness, help maintain weight, and slightly reduce firmness loss, they offer limited antimicrobial effects against *Listeria*. Specifically, a reduction of about 1.9 $\log_{10}$ CFU/apple was observed over 18 weeks of commercial refrigerated air storage - comparable to that on unwaxed apples. The survival dynamics of *L. monocytogenes* also varied by serotype, with serotype 1/2a exhibiting higher resilience than others, such as serotype 4b, which declined more rapidly. There was also a notable cross-contamination risk among contaminated apples, uncontaminated apples, and waxing brushes during the simulated waxing process. *L. monocytogenes* remained viable on waxing brushes for up to 12 weeks at ambient temperature, with an initial 1.2 $\log_{10}$ CFU/brush reduction in the first two weeks, followed by stable populations at ~3 $\log_{10}$ CFU/brush during the subsequent ten weeks of holding. Additionally, the wax coating did not impact the survival of yeasts and molds on apples, with an increase of 0.4–0.5 $\log_{10}$ CFU/apple observed after 18 weeks of cold storage, regardless of wax type. Including fungicides in the wax coating effectively reduced yeasts and molds on wax-coated apples; however, it did not impact *L. monocytogenes* survival. Overall, this research underscores that while wax coatings offer some benefits for fruit quality, they are insufficient for controlling *Listeria* contamination risks during apple storage and pose a cross-contamination risk during the waxing process. The findings highlight the need for enhanced sanitation strategies and the development of wax coatings with antimicrobial properties.