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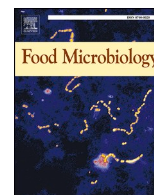

Listeria monocytogenes cross-contamination during apple waxing and subsequent survival under different storage conditions

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ABSTRACT

This study evaluated *Listeria monocytogenes* cross-contamination between inoculated fruits, waxing brush, and uninoculated fruits during apple wax coating and investigated the fate of *L. monocytogenes* on wax-coated apples introduced via different wax coating schemes. There were 1.8–1.9 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* on PrimaFresh 360, PrimaFresh 606, or Shield-Brite AP-450 coated apples introduced before wax coating after 6 weeks of ambient storage (22 °C and ambient relative humidity). *L. monocytogenes* showed a similar trend ($P > 0.05$) on waxed apples under cold storage (1 °C and ~ 90% relative humidity); there were 1.8–2.0 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* during the 12 weeks of cold storage regardless of wax coating type. For cross-contamination study, a waxing brush was used to wax one inoculated apple (6.2 $\log_{10}$ CFU/apple); then, this brush was used to wax five uninoculated apples in a sequence. There were 3.7, 3.5, 3.3, 2.9, and 2.7 $\log_{10}$ CFU/apple and 3.6 $\log_{10}$ CFU/brush of *L. monocytogenes* transferred from the inoculated apple to uninoculated apple 1 to apple 5, and the waxing brush, respectively. The die-off rate of *L. monocytogenes* on wax-coated apples contaminated during wax coating was not significantly different from that contaminated on apples before wax coating, and 1.8–1.9 $\log_{10}$ CFU/apple reductions were observed during the 12 weeks of cold storage. The application of wax coatings, regardless of wax coating type, did not impact the survival of endogenous yeasts and molds on apples during ambient or cold storage. *L. monocytogenes* transferred onto waxing brushes during wax coating remained relatively stable during the 2-week ambient holding. Fungicide application during wax coating reduced ($P < 0.05$) yeast and mold counts but had a minor impact ($P > 0.05$) on the survival of *L. monocytogenes* on apples after 12 weeks of cold storage. Collectively, this study indicated that a high cross-contamination risk of *L. monocytogenes* during apple waxing, and *L. monocytogenes* on wax-coated apples introduced via different scenarios is stable during subsequent cold storage, highlighting the need for potential intervention strategies to control *L. monocytogenes* on wax-coated apples.

1. Introduction

Fresh apples have been implicated in the listeriosis outbreaks and multiple recalls due to *Listeria monocytogenes* contamination (Angelo et al., 2017; Fda, 2017, 2019). *Listeria* spp. is widely distributed in the processing and packing environment and is more prevalent in the wax area of apple packing facilities (Ruiz-Llacsahuanga et al., 2021; Simonetti et al., 2021). This presents a potential risk of *Listeria* contamination of fresh apples during postharvest handling in packing plants. The packing equipment and environment are also recognized as an important source of contamination of apples and other produce implicated in *L. monocytogenes* outbreaks (Angelo et al., 2017; FDA, 2011). Once *L. monocytogenes* is introduced to fresh apples, it can be persistent on fresh unwaxed apples during subsequent storage (Sheng et al., 2017).

Apples marketed in the United States are commonly applied with food-grade wax coatings as a finishing step of postharvest packing to

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improve glossiness, minimize moisture loss, and extend shelf life during subsequent handling and short-term storage in distribution and retailing (Bai et al., 2003; Saftner, 1999). Two FDA-approved natural carnauba-based waxes, PrimaFresh 360 and PrimaFresh 606, and a shellac-based wax, Shield-Brite AP-450, are the primary wax coatings used in the apple packing plants= in the state of Washington. Currently, limited information is available on the fate of *L. monocytogenes* on these wax-coated apples. One recent study showed that the population of *L. monocytogenes* on apples decreased immediately after waxing with Shield-Brite AP-40 (Macarisin et al., 2019). However, the survival of *L. monocytogenes* on the Shield-Brite AP-40 coated apples was higher than that on unwaxed apples during subsequent 160 days of cold storage at 3 °C (Macarisin et al., 2019). It was also reported that *Salmonella* Muenchen on apples coated with Shield-Brite AP-40 showed a much higher reduction than on unwaxed apples during 12 weeks of cold storage at 2 °C (Kenney and Beuchat, 2002).

Antimicrobial interventions such as chlorine and peroxyacetic acid are often used to minimize *L. monocytogenes* cross-contamination on apples during commercial packing; however, postharvest sanitizer treatments reduce but cannot eliminate *L. monocytogenes* on apples (Shen et al., 2019; Sheng et al., 2020). Thus, *L. monocytogenes* can be potentially present on apples after antimicrobial interventions and further contaminate polishing brushes during wax coating application. In commercial apple packing plants, *Listeria* spp. was prevalent on polishing brushes and plastic flaps of wax coating operation unit as high as 23.6% during the apple packing in-process and remained at 10.9% even after sanitation (Ruiz-Llacsahuanga et al., 2021). It was also reported that 5.5% of samples, including the surfaces of polishing brushes, drying brushes, and conveyors, isolated from the apple packing facility involved in the 2014–2015 caramel apple listeriosis outbreak were positive for *L. monocytogenes* (Angelo et al., 2017). These findings indicate the potential risk of *L. monocytogenes* cross-contamination from contaminated brushes to uncontaminated apples during wax coating application. However, little is known about the potential of *L. monocytogenes* cross-contamination from contaminated apples to waxing brushes and from contaminated brushes to uncontaminated apples during wax coating. Neither is known about the behavior of *L. monocytogenes* on wax-coated apples introduced via different scenarios.

The objective of this study was to 1) evaluate the cross-contamination of *L. monocytogenes* from apple to waxing brush and from the waxing brush to apple; 2) examine the fate of *L. monocytogenes* on wax-coated apples contaminated before or during wax-coating application under different storage conditions; 3) assess the persistence of *L. monocytogenes* on waxing brushes contaminated during wax coating application.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Three *L. monocytogenes* outbreak strains, NRRL B-57618 , NRRL B-33053, and NRRL-33466 were obtained from the USDA-ARS collection (National Center for Agricultural Utilization Research (NRRL), Peoria, IL) and maintained at −80 °C in trypticase soy broth (Becton, Dickinson, and Company (BD), Sparks, MD) supplemented with 0.6% yeast extract (Fisher Scientific, Fair Lawn, NJ) (TSBYE) and 20% (v/v) glycerol (J. T. Baker, Phillipsburg, NJ). Each *L. monocytogenes* strain was activated in TSBYE at 37 °C for two consecutive days and mixed in equal proportions to prepare a three-strain cocktail inoculum.

2.2. Fruit wax coating solutions

A carnauba-based wax, PrimaFresh 360 (PF 360), a morpholine-free carnauba-based wax, PrimaFresh 606 (PF 606), and a shellac-based wax, Shield-Brite AP-450 (AP-450), were obtained from Pace International (Wapato, WA).

2.3. Survival of L. monocytogenes in wax coating solutions

Ten milliliters of each wax solution (22 °C) were inoculated with the above-prepared three-strain *L. monocytogenes* cocktail at 7.7 $\log_{10}$ CFU/ml and incubated at 22 °C on a shaker at 350 rpm (Orbit, Lab-Line Instruments Inc., Melrose Park, IL) for 24 h. Wax solution post 0, 0.5, 1, 4, 12, and 24-h contact was sampled and serially diluted with sterile phosphate-buffered saline (PBS, pH 7.4) and plated, in duplicate, onto trypticase soy agar (BD) supplemented with 0.6% yeast extract (TSAYE) plates. The resulting colonies were enumerated after incubation at 37 °C for 24 h. Three samples were collected per treatment at the respective sampling point and experiments were repeated independently three times. Sterile deionized water inoculated with *L. monocytogenes* was used as a negative control.

2.4. Apple inoculation

Fresh unwaxed Fuji apples with a uniform size of (220–240 g) were selected and dip-inoculated with the three-strain *L. monocytogenes* cocktail to obtain the inoculation level of ~6 $\log_{10}$ CFU/apple or ~8 $\log_{10}$ CFU/apple as previously described (Shen et al., 2019) and dried in ambient air for 18 h at 22 °C before wax application.

2.5. Waxing application of pre-inoculated apples

Apples inoculated with ~6 $\log_{10}$ CFU/apple of *L. monocytogenes* were coated with PF 360, PF 606, or AP-450. Fungicides are incorporated into wax solutions during commercial apple processing to control the decay of fruits (Aror and Narasimham, 1988). To examine the potential impacts of fungicide application on the survival of *L. monocytogenes* on waxed apples, two fungicides commonly used during wax coating, fludioxonil-based fungicide Shield-Brite FDL-230 (Pace International, Wapato, WA) at 212 ppm and natamycin (Sigma-Aldrich, St. Louis, MO) at 90 ppm, were incorporated into PF 360 wax coating at manufacturer's recommended concentration. Each wax solution was manually spread on the surface of the apples evenly and dried for 5 h before being subjected to subsequent storage.

2.6. Transfer of L. monocytogenes from apple-to-brush and brush-to-apple during the wax coating process

One hundred microliters of PF 360 or AP-450 wax solution was spread evenly on an inoculated apple at 6 or 8 $\log_{10}$ CFU/apple using a horsehair brush (National Novelty Brush Company, Lancaster, PA), which is the commonly used material for wax polishing brushes in apple packing facilities. Then, this waxing brush was used to coat five uninoculated apples consecutively, which were labeled apples 1 to 5 (Fig. 1A). One inoculated apple along with five uninoculated apples was considered as one experimental unit. There were 12 sets of apples for each wax coating and inoculation combination.

To prepare waxed apples contaminated with 6 $\log_{10}$ CFU/apple of *L. monocytogenes* during wax coating application for storage studies, one horsehair brush was used to coat an inoculated apples (8 $\log_{10}$ CFU/apple) and then uninoculated apples in a sequence, which was repeated for 10 times (Fig. 1B). The resulting waxed apples were dried at 22 °C for 5 h before being subjected to subsequent storage.

2.7. Survival of L. monocytogenes on waxed apples contaminated during different waxing scenarios

The waxed apples, regardless of wax coating type, fungicide application, and contamination mode, were stored at 22 °C and ambient relative humidity (RH) and/or 1 °C and ~ 90% RH for up to 12 weeks and sampled at 2-, 4-, 6-, 9-, and 12-week for *L. monocytogenes*

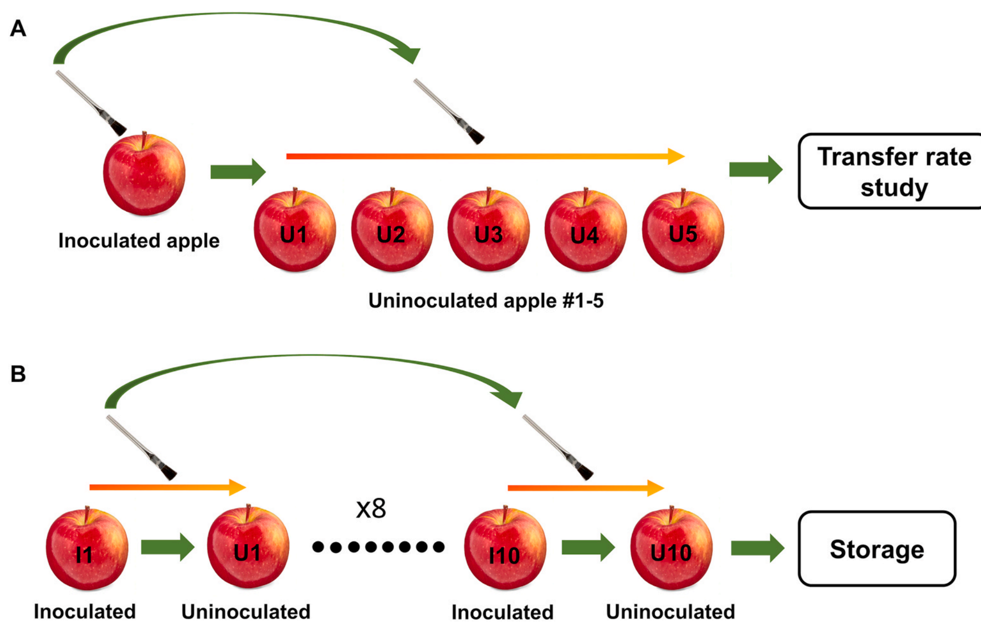

Fig. 1. Illustration for the preparation of waxed apples contaminated with *Listeria monocytogenes* during wax coating. A. Wax-coated apples for the apple-to-brush and brush-to-apple transfer rate study. B. Wax-coated apples for the storage study. I: inoculated apple; U: uninoculated apple.

enumeration. There were 40 apples per treatment at each sampling point.

2.8. Microbial analyses of apples

Each apple was transferred to a stomacher bag (Fisher Scientific) containing 10 ml of sterile PBS with 0.1 g/l sodium bicarbonate (J. T. Baker) and hand-rubbed for 80 s (Shen et al., 2019). Rub solutions were 10-fold serially diluted with sterile PBS, plated in duplicate on TSAYE plates, and incubated at 37 °C for 4 h for the recovery of any injured cells, then TSAYE plates were overlaid with modified Oxford agar (BD) to discern *L. monocytogenes* from apple resident background flora, and incubated at 37 °C for additional 48 h (Kang and Fung, 1999; Shen et al., 2019).

Rub solutions from selected treatments were also plated on duplicate Potato Dextrose Agar (PDA, BD) plates supplemented with 0.1 g/l chloramphenicol (MP biomedicals, Solon, OH) for yeast and mold counts. PDA plates were incubated at ambient temperature (22 °C) for 5 days.

2.9. Persistence of L. monocytogenes on waxing brushes

Waxing brushes contaminated with *L. monocytogenes* from the apple-to-brush and brush-to-apple transference study were stored at 22 °C for 14 days, and sampled at 3-, 7-, and 14-day for *L. monocytogenes* enumeration. There were three brushes per treatment at each sampling point. Waxing brushes used for wax coating of *L. monocytogenes* inoculated apples were stored at 22 °C for 12 weeks and sampled at 1-, 2-, 4-, 6-, 9-, and 12-week for *L. monocytogenes* enumeration. There were four brushes per treatment at each sampling point.

To enumerate *L. monocytogenes*, the horsehair of each brush was collected in 10 ml of sterile PBS supplemented with 0.1 g/l sodium bicarbonate (J. T. Baker), vortexed at full speed for 5 min using a vortex mixer (VWR, Radnor, PA). The detached solution was serial diluted and plated onto TSAYE plates overlaid with modified Oxford agar (BD) to enumerate *L. monocytogenes.*

2.10. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using IBM SPSS 19.0 (Chicago, IL, US). $P < 0.05$ was considered significant.

3. Results

3.1. Survival of L. monocytogenes in wax coating solutions

Populations of *L. monocytogenes* decreased ($P < 0.05$) in three commonly used apple wax coatings during 24-h incubation (Fig. 2). Populations of *L. monocytogenes* in wax solutions were reduced by 2.1–2.2 $\log_{10}$ CFU/ml in 1-h contact regardless of wax type (Fig. 2). 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. 2).

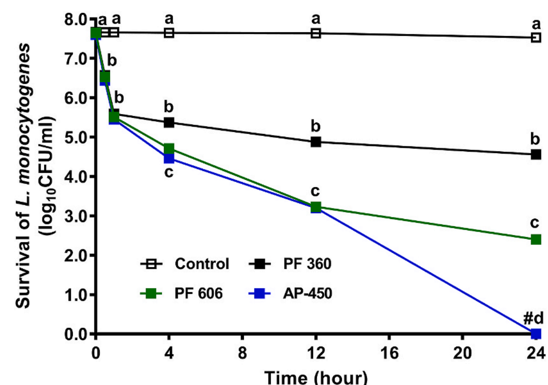

Fig. 2. Survival of Listeria monocytogenes in apple wax coating solutions at 22 °C. Control: deionized water. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360; PF 606: PrimaFresh 606. #: the limit of detection was 1 CFU/ml. Mean ± SEM, n = 9. [a-d] Mean at each sampling point without a common letter differ significantly ($P < 0.05$).

3.2. 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. 3A). After waxing of the 5th uninoculated apple, 3.6 log_{10} CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 3B). 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. 3C). After waxing of the 5th uninoculated apple, 5.5 log_{10} CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 3D). 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. 3).

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

To represent wax applications at apple packing plants, 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* on waxed apples introduced to apples before waxing decreased by 1.8–1.9 log_{10} CFU/apple during six weeks of ambient storage (Fig. 4A). *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. 4B). The application of wax coating had a minor impact on the survival of *L. monocytogenes* on apples regardless of storage temperature (Fig. 4A and B).

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. 4C, 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} CFU/apple on PF 360 coated apples regardless of fungicide application after 12 weeks of cold storage (Fig. 4C).

Given the prevalence of *Listeria* species in waxing areas (Ruiz-Llacsahuanga et al., 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. *L. monocytogenes* was reduced by 1.8 log_{10} CFU/apple after 12 weeks of cold storage (Fig. 4D), which has a similar trend as *L. monocytogenes* introduced to apples before waxing application

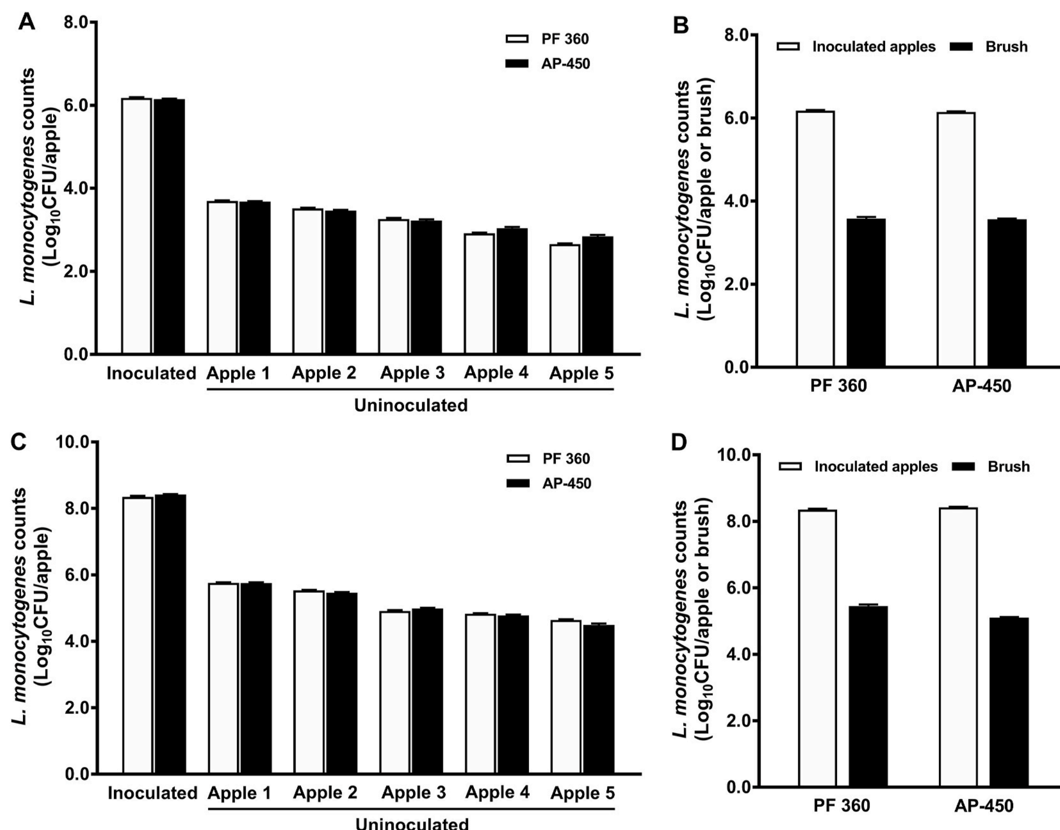

Fig. 3. Transfer of *Listeria monocytogenes* from inoculated apples to uninoculated apples and waxing brushes during wax coating. A. Transfer from inoculated apples to uninoculated apples, source apples were loaded with ~6 log_{10} CFU/apple; B. Transfer from inoculated apples to waxing brushes, source apples were loaded with ~6 log_{10} CFU/apple. C. Transfer from inoculated apples to uninoculated apples, source apples were loaded with ~8 log_{10} CFU/apple; D. Transfer from inoculated apples to waxing brushes, source apples were loaded with ~8 log_{10} CFU/apple. 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.

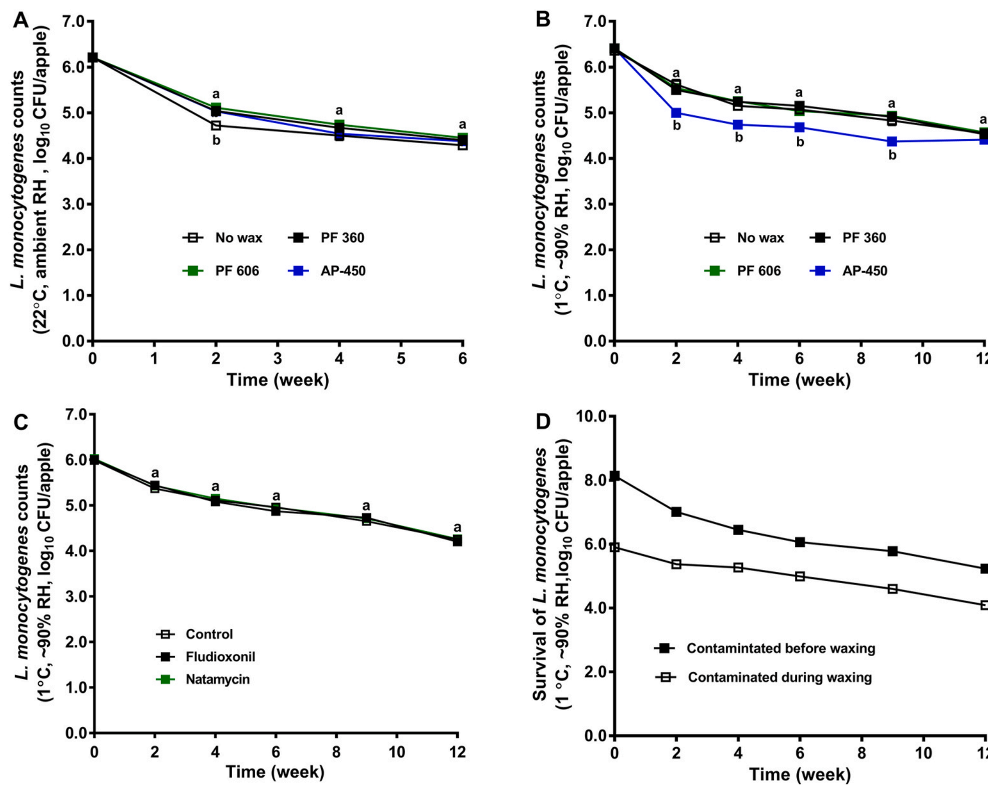

Fig. 4. The fate of *Listeria monocytogenes* on wax-coated apples contaminated before or during wax coating application for up to 12-week storage. A. 22 °C and ambient RH. B. 1 °C and ~90% RH; No wax: unwaxed control apples; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. C. Fungicide application in combination with PF 360 coating; Control: PF 360 without fungicide; Fludioxonil: PF 360 with fludioxonil; Natamycin: PF 360 with natamycin. D. *L. monocytogenes* introduced to apples during wax coating; source apples were inoculated with ~8 $\log_{10}$ CFU/apple of *L. monocytogenes* before wax coating. Data were presented with mean ± SEM, n = 40. [a-b] Means at each sampling point without a common letter differ significantly ($P < 0.05$).

whether apple had an initial population of ~6 $\log_{10}$ CFU/apple (Fig. 4B) or ~8 $\log_{10}$ CFU/apple (Fig. 4D).

3.4. Yeast and mold counts on wax-coated apples

The initial yeast and mold counts on apples immediately after wax coating were 4.7–4.8 $\log_{10}$ CFU/apple (Fig. 5). Application of wax coatings, regardless of wax type, did not impact ($P > 0.05$) the survival of yeasts and molds on apples during 6-week ambient or 12-week cold storage (Fig. 5A and B). Yeast and mold counts of waxed apples under 6-week ambient storage gradually increased by 0.3–0.4 $\log_{10}$ CFU/apple with time (Fig. 5A). The yeast and mold population of apples under cold storage increased by 0.4–0.5 $\log_{10}$ CFU/apple during the first 4-week and maintained at 5.2–5.5 $\log_{10}$ CFU/apple during the subsequent 8 weeks of storage (Fig. 5B). 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. 5C). Fludioxonil and natamycin had similar effectiveness ($P > 0.05$) in controlling yeasts and molds on waxed apples (Fig. 5C).

3.5. Survival of L. monocytogenes on brushes contaminated during wax coating

There were 3.6 and 5.5 $\log_{10}$ CFU/brush of *L. monocytogenes* transferred to the waxing brush after it was used to wax one inoculated apple at 6.2 $\log_{10}$ CFU/apple and 8.4 $\log_{10}$ CFU/apple, respectively, followed by waxing five uninoculated apples with PF 360 (Fig. 6A). *L. monocytogenes* remained relatively stable on waxing brushes during 2 weeks of holding at 22 °C; there were 0.5 and 1.3 $\log_{10}$ CFU/brush reduction, respectively, on waxing brushes with the initial contamination level of 3.6 and 5.5 $\log_{10}$ CFU/brush (Fig. 6A). A similar

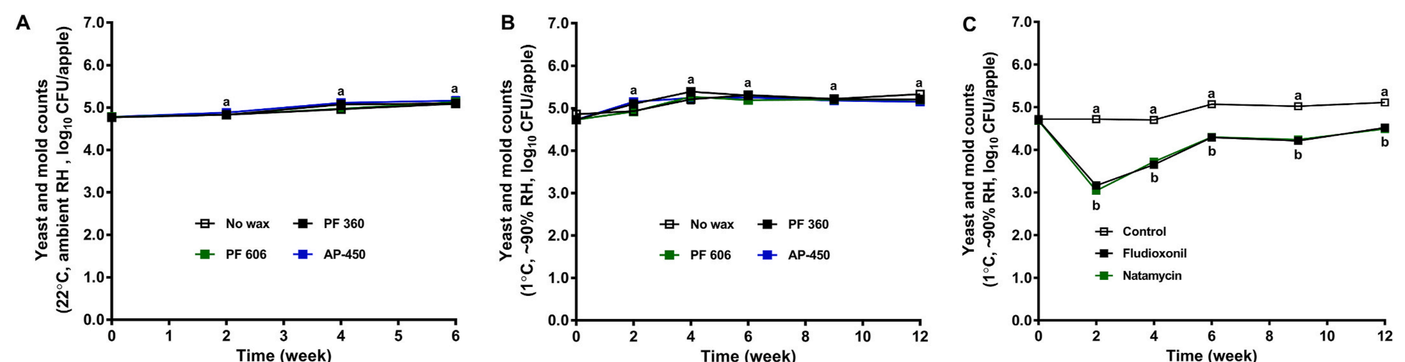

Fig. 5. Yeast and mold counts on wax-coated apples contaminated before wax coating during up to 12-week storage. A. 22 °C and ambient RH. B. 1 °C and ~90% RH; No wax: unwaxed control apple; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. C. Fungicide application in combination with PF 360 coating; Control: PF 360 without fungicide; Fludioxonil: PF 360 with fludioxonil; Natamycin: PF 360 with natamycin. Data were presented with mean ± SEM, n = 40. [a-b] Means at each sampling point without a common letter differ significantly ($P < 0.05$).

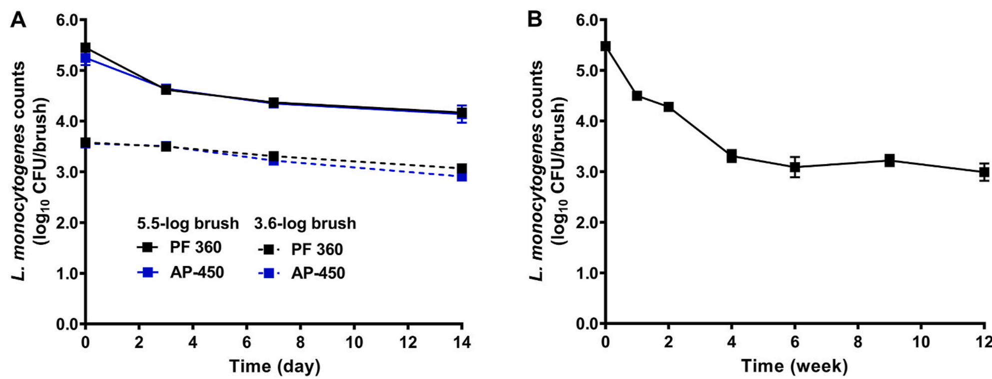

Fig. 6. Persistence of *Listeria monocytogenes* on waxing brushes. A. 14-day ambient holding of waxing brushes contaminated at low (3.6 $\log_{10}$ CFU/brush) and high (5.5 $\log_{10}$ CFU/brush) levels, where one brush was used to coat one inoculated apple followed by five uninoculated apples. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. Data were presented with mean ± SEM, n = 6. B. 12-week ambient holding of waxing brushes, where one brush was used to coat ten inoculated apples and ten uninoculated apples in a sequence of coating an inoculated apple followed by an uninoculated apple. Data were presented with mean ± SEM, n = 4.

contamination level and die-off rate ($P > 0.05$) of *L. monocytogenes* was found on waxing brushes used to put AP-450 wax coating to apples with the same method (Fig. 6A).

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 further subjected to 12 weeks of ambient holding, where each brush was used to coat ten apples inoculated with 8.1 $\log_{10}$ CFU/apple of *L. monocytogenes* and ten uninoculated apples in a sequence of an inoculated apple followed by an uninoculated apple (Fig. 1B). The average transfer of *L. monocytogenes* onto each brush at the end of the wax coating was 5.5 $\log_{10}$ CFU/brush (Fig. 6B). There was 1.2 $\log_{10}$ CFU/brush *L. monocytogenes* reduction after two weeks of holding, then remained relatively stable at ~3 $\log_{10}$ CFU/brush during the subsequent eight weeks of holding (Fig. 6B).

4. Discussion

4.1. Survival of L. monocytogenes in wax solutions

The tested wax coating solutions showed bactericidal effects against *L. monocytogenes*, causing a 2.1–2.2 $\log_{10}$ CFU/ml reduction within 1 h exposure. Jo et al. (2014) reported a 2.0 log reduction of *L. monocytogenes* right after exposure to the carnauba-shellac wax coating solution, supporting the findings of our work. The antimicrobial activity of AP-450 was superior when compared with PF 360 and PF 606 solutions during prolonged incubation. This is likely due to isopropyl alcohol in the AP-450 formula, which was reported to have an antimicrobial effect against *L. monocytogenes* (Pendieton et al., 2012). PF 606 has a similar formula as PF 360 except for the morpholine composition, however, PF 606 had a higher antimicrobial effect against *L. monocytogenes* in pure wax solution. The exact reason remains unknown, which might be due to the different viscosity of each wax coating solution, warranting future investigation.

4.2. Transfer of L. monocytogenes from inoculated apples to waxing brushes and from waxing brushes to uninoculated apples during wax application

A significant amount of *L. monocytogenes* was transferred from the contaminated apples to waxing brushes and uncontaminated apples during wax coating application no matter which wax coating was used. Pathogen cross-contamination from inoculated produce to the waxing brush and from the contaminated waxing brush to uninoculated produce during wax coating was also found during the cucumber processing (Jung and Schaffner, 2021). Wang and Ryser (2014) studied the transfer of *Salmonella* from inoculated tomatoes to different roller conveys and reported as high as 2.1–2.4 $\log_{10}$ CFU/100 cm^2 of *Salmonella* transfer from inoculated tomatoes (~3 $\log_{10}$ CFU/g before conveying) to foam roller convey. Our finding indicates the potential risk of *L. monocytogenes* cross-contamination from contaminated apples to waxing brushes and from contaminated brushes to uncontaminated apples. The data highlight the importance of adopting more effective antimicrobial interventions to possibly eliminate *L. monocytogenes* from apples during postharvest processing and handling before wax coating application and routinely cleaning waxing brushes.

4.3. Fate of L. monocytogenes on wax-coated apples introduced during different waxing schemes

The immediate application of the wax coating, regardless of wax type, did not impact the survival of *L. monocytogenes* on apples. This finding was supported by a recent study by Mendes-Oliveira et al. (2022) that the populations of *L. monocytogenes* were not impacted by the petroleum-based wax-coated apples immediately after waxing. An earlier study also showed that the populations of *Escherichia coli* O157: H7 and *S.* Muenchen on apples were not significantly impacted 30-min after waxing with Shield-Brite AP-40 under 21 °C (Kenney and Beuchat, 2002). Similarly, the application of carnauba-based wax coating did not impact the population of *Salmonella* inoculated on mangoes (Mathew et al., 2018). In contrast, the application of Shield-Brite AP-40 wax coating solution on inoculated apples caused 1.2 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* immediately after wax coating (Macarisin et al., 2019).

The population of *L. monocytogenes* decreased by 1.5 $\log_{10}$ CFU/apple on unwaxed Fuji apples after 2 weeks of ambient storage, which is consistent with our previous finding (Sheng et al., 2017). Application of wax coating, regardless of type, did not impact the survival of *L. monocytogenes* introduced to fresh apples before wax coating during 6 weeks of ambient storage. However, the counts of *L. monocytogenes* on petroleum-based wax-coated Gala apples were 1.5 $\log_{10}$ CFU/g higher compared with unwaxed control after 10 days of 22 °C storage (Mendes-Oliveira et al., 2022). *S.* Muenchen were decreased by 0.9 and 0.5 $\log_{10}$ CFU/apple on unwaxed and Shield-Brite AP-40 coated apples, respectively, after 6 weeks of 21 °C storage (Kenney and Beuchat, 2002). The difference in antimicrobial effects might be due to the difference in strain, apple variety, wax coating, and other factors.

The reductions of *L. monocytogenes* on unwaxed apples during 6 weeks of cold storage were significantly lower when compared with unwaxed apples under ambient storage (1.3 $\log_{10}$ CFU/apple vs 1.9 $\log_{10}$ CFU/apple). Similarly, populations of *S.* Muenchen were decreased by 0.2 and 0.9 $\log_{10}$ CFU/apple, respectively, on unwaxed apples under 6 weeks of 2 °C and 21 °C storage (Kenney and Beuchat, 2002). There was 1.2–1.4 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on PF 360 and PF 606 coated apples during 6 weeks of cold storage, while a higher reduction, i.e., 1.7 $\log_{10}$ CFU/apple , was found on AP-450 coated apples. Though the application of Shield-Brite AP-40 coating resulted in an instant 1.2 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on Fuji apples when compared to unwaxed control apples, Shield-Brite AP-40 coating application facilitated the survival of *L. monocytogenes* on apples when compared to the unwaxed control

apples during prolonged storage at a 3 °C (Macarisin et al., 2019). The observed difference in behaviors warrants further well-controlled studies.

There were 1.8–2.0 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* on Fuji apples regardless of wax coating application and type of wax coating after 12 weeks of cold storage. This is consistent with our previous finding on unwaxed Fuji apples (Sheng et al., 2017). Similarly, there was ~2.5 $\log_{10}$ CFU/g tissue reduction of *Salmonella* found on the stem scar area of unwaxed and PrimaFresh Ultra 2000-coated cherry tomatoes after 2 weeks of storage at 10 °C (Yun et al., 2015). Populations of *E. coli* O157:H7 were comparable on unwaxed and carnauba wax-coated plums during 4 weeks of cold storage at 4 °C (Kim et al., 2013).

L. monocytogenes present on apples before or during wax coating may experience different micro-environments. *L. monocytogenes* contaminated on apples before wax coating are exposed to low oxygen or an anaerobic environment between the coating layer and the fruit epidermis after the waxing (Bai et al., 2002; Miranda et al., 2021). However, *L. monocytogenes* on wax-coated apples introduced during wax coating with 12 weeks of cold storage was not significantly different from that introduced before wax coating and stored under the same condition. This might be because the survival of *L. monocytogenes,* a facultative anaerobe, was less impacted by the atmospheric environment. Data obtained in our study, collectively, indicated that the commercial wax coatings barely resulted in any reduction of *L. monocytogenes* on apples immediately after wax coating application and after 12 weeks of cold storage. Furthermore, *L. monocytogenes* introduced to waxed apples regardless of waxing schemes can be persistent for a prolonged time, highlighting the need for alternative wax coating with anti-*Listeria* effects for apples.

4.4. Potential impacts of fungicide application on the fate of L. monocytogenes on wax-coated apples

Besides preserving fruit quality, the final wax coating also serves as a carrier for fungicide application. The fungicides fludioxonil and natamycin have been incorporated into wax coatings during commercial apple wax coating application (Delves-Broughton et al., 2005; Jiang et al., 2019). Fludioxonil is listed in the Conventional Reduced Risk Pesticide Program as posing less risk to human health and the environment compared with other conventional alternatives (EPA, 2018). However, it might still cause cytotoxicity and genotoxicity to human cells as low as 5 ppm (Graillot et al., 2012). Natamycin is a natural fungicide produced by different *Streptomyces* species (Elsayed et al., 2019). Natamycin is considered a Generally Recognized as Safe compound and approved as an additive in beverages by the FDA due to its low toxicity to humans (USDA, 2015). Fludioxonil and natamycin are effective against apple decay pathogens including *Botrytis cinerea* and *Penicillium expansum* (Errampalli, 2004; Errampalli et al., 2007; He et al., 2019). Our study demonstrated that the addition of fludioxonil or natamycin in the fruit wax coating did not impact ($P > 0.05$) the survival of *L. monocytogenes* on waxed apples during cold storage. In agreement with our finding, the application of fludioxonil or propiconazole fungicide during Prima Fresh 220 wax coating did not impact the survival of *L. monocytogenes* on peaches during 30 days of cold storage at 1-2 °C (Kuttappan et al., 2021).

4.5. Yeast and mold counts on wax-coated apples under different storage regimes

The application of the wax coating, regardless of wax type, had no impact ($P > 0.05$) on the survival of yeasts and molds on apples under ambient or cold storage. Similarly, the application of Shield-Brite AP-40 had no impact on the survival of molds on apples after 12 weeks of cold storage at 2 °C (Kenney and Beuchat, 2002). Populations of yeasts and molds were comparable on dates coated with or without a carnauba-derived wax solution after 21 days of storage at 25 °C (Mehyar et al., 2014).

The addition of fungicide, regardless of type, in wax coating significantly reduced yeast and mold counts on apples during 12 weeks of cold storage. In support of our finding, natamycin at 200 ppm in gum arabic coating reduced the yeast and mold counts on shiitake mushrooms after 16 days in a 4 °C storage (Jiang et al., 2013). The application of 300 ppm fludioxonil on conidia of *P. expansum* reduced the incidence of blue mold by 100% on unwaxed apples after 6 days of storage at 20 °C compared with untreated conidia inoculated apples (Errampalli and Crnko, 2004). However, the efficacies of fungicides were reduced during the extended storage, which might be due to the dissipation of fungicides in wax solutions during the storage (Fenoll et al., 2009; Ladaniya, 2011).

4.6. Fate of L. monocytogenes on waxing brushes during the ambient holding

In commercial apple packing lines, waxing brushes are commonly made with horsehair or a mixture of horsehair and nylon. Horsehair brush is porous and can absorb the wax solution in the bristles, which results in better distribution of wax on the fruit surface (Njombolwana et al., 2013) and brings higher glossiness to fruit after wax coating application (Marmur et al., 2013; Njombolwana et al., 2013). However, due to these distinct characteristics, *L. monocytogenes* contaminated on brushes made with horsehair are difficult to clean (Ruiz-Llacsahuanga et al., 2022). Our study showed *L. monocytogenes* transferred from inoculated apples to waxing brushes was relatively stable during 2 weeks of ambient storage no matter how *L. monocytogenes* were introduced to waxing brushes. This might be correlated with the porous structure of the horsehair brush that trapped *L. monocytogenes* in the bristles and protected the organism from desiccation stress in the subsequent storage. In support of our finding, *Listeria* spp. had a prevalence of 19.6% on waxing brushes made with nylon and horsehair mix (horsehair and polyethylene) among all food contact surfaces swabbed in commercial apple packing facilities (Ruiz-Llacsahuanga et al., 2021) and *Listeria* attached to waxing brushes made with horsehair mix were more difficult to clean compared with nylon waxing brushes (Ruiz-Llacsahuanga et al., 2022).

A higher reduction of *L. monocytogenes* was found on waxing brushes at an initial contamination level of 5.3–5.5 $\log_{10}$ CFU/brush compared to the waxing brushes with an initial contamination level of 3.6 $\log_{10}$ CFU/brush during 2 weeks of ambient storage introduced within the same method. Similarly, *L. monocytogenes* showed a higher reduction on polystyrene surfaces at ~9 $\log_{10}$ CFU/coupon compared to that at ~7 $\log_{10}$ CFU/coupon during 2 days of 4 °C storage (de Candia et al., 2015).

5. Conclusion

We observed a high cross-contamination risk of *L. monocytogenes* from inoculated apples to waxing brushes and from contaminated brushes to uninoculated apples during wax coating application. The fate of *L. monocytogenes* on wax-coated apples introduced before or during waxing was similar ($P > 0.05$). Specifically, there was about 1.8–2.0 $\log_{10}$ CFU/apple reduction during 6 weeks of ambient storage or 12 weeks of cold storage, regardless of wax coating type. Wax coating had no impact ($P > 0.05$) on yeast and mold counts on apples, regardless of coating type and storage temperature. Fungicide application during wax coating reduced ($P < 0.05$) decay yeast and mold counts but had a minor impact ($P > 0.05$) on the survival of *L. monocytogenes* on apples. *L. monocytogenes* transferred onto waxing brushes during wax coating application remained relatively stable during 2 weeks of holding at ambient temperature. The data collected in this study will inform the apple industry about *L. monocytogenes* safety risks on apples and waxing brushes during wax coating application and provide quantitative data about the fate of *L. monocytogenes* on wax-coated apples during prolonged storage.

Declaration of competing interest

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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