Title:

Prevalence of *Listeria* species on food contact surfaces in Washington State apple packinghouses

Running Title:

*Listeria* spp. on food contact surfaces in apple packinghouses

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ABSTRACT

The 2014 caramel apple listeriosis outbreak was traced back to cross-contamination between food contact surfaces (FCS) of equipment used for packing and fresh apples. For Washington State, the leading apple producer in the U.S with 79% of its total production directed to the fresh market, managing the risk of apple contamination with Listeria monocytogenes within the packing environment is crucial. The objectives of this study were to determine the prevalence of Listeria spp. on FCS in Washington State apple packinghouses over two packing seasons, and to identify those FCS types with the greatest likelihood to harbor Listeria spp. Five commercial apple packinghouses were visited quarterly over two consecutive year-long packing seasons. A range of 27 to 50 FCS were swabbed at each facility to detect Listeria spp. at two timings of sampling, (i) post-sanitation and (ii) in-process (three hours of packinghouse operation), following a modified protocol of the FDA’s Bacteriological Analytical Manual method. Among 2,988 samples tested, 4.6% (n=136) were positive for Listeria spp. Wax coating was the unit operation from which Listeria spp. were most frequently isolated. The FCS that showed the greatest prevalence of Listeria spp. were polishing brushes, stainless steel dividers and brushes under fans/blowers, and dryer rollers. The prevalence of Listeria spp. on FCS increased throughout apple storage time. The results of this study will aid apple packers in controlling for contamination and harborage of L. monocytogenes and improving cleaning and sanitation practices of the most Listeria-prevalent FCS.

IMPORTANCE

Since 2014, fresh apples have been linked to outbreaks and recalls associated with post-harvest cross-contamination with the foodborne pathogen L. monocytogenes. These situations drive both public health burden and economic loss and underscore the need for continued
scrutiny of packinghouse management to eliminate potential *Listeria* spp. niches. This research assesses the prevalence of *Listeria* spp. on FCS in apple packinghouses and identifies those FCS most likely to harbor *Listeria* spp. Such findings are essential for the apple packing industry striving to further understand and exhaustively mitigate the risk of contamination with *L. monocytogenes* to prevent future listeriosis outbreaks and recalls.
INTRODUCTION

Washington State is the leading producer of apples in the United States (U.S) (1), with 79% of its total apple production destined for fresh consumption (2). Because of the ubiquitous nature of the foodborne pathogen *Listeria monocytogenes* and its ability to grow under a wide array of environmental conditions (3), contamination of fresh apples can occur during pre-harvest, harvest, and post-harvest (4), resulting in an increased risk of outbreaks of listeriosis. Cross-contamination during post-harvest handling has been identified as the likely root cause of *L. monocytogenes* contamination in the 2011 cantaloupe and the 2014 caramel apple listeriosis outbreaks (5, 6), highlighting the need for robust environmental monitoring programs within packinghouses (7). In both outbreaks, *L. monocytogenes* contamination of food contact surfaces (FCS) associated with packing equipment was identified during environmental assessments (8–10).

In 2011, the listeriosis outbreak that was linked to cantaloupe resulted in 147 infected people and 33 deaths across 28 states (6). Of 39 environmental samples that were collected in the facility in Colorado, 13 tested positive for *L. monocytogenes*. Twelve of these 13 isolates matched outbreak-related strains and were taken from FCS, including brush and felt rollers (9).

In 2014, the multistate caramel apple listeriosis outbreak was the first outbreak related to whole fresh apples. This outbreak infected 35 people and left seven dead across 12 states (5). Of the 31 patients interviewed, 28 reported having eaten commercially prepackaged caramel apples, whereas three people reported having consumed only whole apples (8). In the FDA traceback investigation, six of the seven environmental samples positive for *L. monocytogenes* that matched outbreak strains were isolated from packing equipment FCS such as brushes, conveyor...
belts, and a wooden bin; these results demonstrated the likely role cross-contamination of FCS played in subsequent apple contamination (8).

Since then, a listeriosis outbreak in 2017, with caramel apples as the presumptive source of contamination, infected three people (11). In addition, four voluntary recalls have been reported in the U.S after random final product samples of whole apples (12, 13) and apple slices (14), or equipment surfaces (15) tested positive for *L. monocytogenes*. These outbreaks and recalls further emphasize the persistent risk of cross-contamination during packing of fresh apples. Therefore, identifying strategies to control for contamination and harborage of *L. monocytogenes* is crucial.

As part of the Food Safety Modernization Act (FSMA), the FDA instated the implementation of a *Listeria* spp. environmental monitoring program (EMP) as a preventive measure to further reduce the potential for foodborne outbreaks related to ready-to-eat (RTE) foods such as apples (16). Currently, the FDA does not recommend testing for a specific pathogen (e.g., *L. monocytogenes*) on FCS as part of an EMP. However, testing for indicator organisms on FCS is suggested. As indicator organisms, *Listeria* species (spp.), can be used to identify the potential presence of *L. monocytogenes* by evaluating all the six *Listeria* spp. of the *sensu stricto* group including *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. marthii* (16). The detection of *Listeria* spp. on a surface does not necessarily indicate the presence of *L. monocytogenes*, but rather that the conditions are suitable for the establishment and proliferation of *L. monocytogenes* (16).

Research evaluating the prevalence of *Listeria* spp. in different types of produce packing facilities has been largely focused on both non-food contact surfaces (NFCS) (17–24), and the combined results of NFCS and FCS (25–31). However, research related to *Listeria* prevalence...
specifically on FCS is scarce; only two studies have been focused on FCS (32, 33); neither was performed in apple packinghouses.

Thus, the goals of this research were (i) to determine the prevalence of *Listeria* spp. on FCS in Washington State apple packinghouses over two packing seasons, and (ii) to identify in apple packing facilities those FCS types and design features with the greatest likelihood to harbor *Listeria* spp.

**RESULTS AND DISCUSSION**

**Prevalence of *Listeria* spp. in apple packinghouses**

The prevalence of *Listeria* spp. specifically on FCS was assessed over two packing seasons in Washington State apple packinghouses. *Listeria* spp. were isolated from all five packinghouses during both packing seasons. Among all tested samples (n=2,988), 136 (4.6%) were confirmed positive for *Listeria* spp. (Table 1). To compare these results with those of previous studies that assessed the prevalence of *Listeria* spp. in different types of produce processing facilities after three hours of packinghouse operation (Table 2), the value of the in-process prevalence (7.2%) was used. Seven studies found similar results with our data even though different commodities and types of surfaces (NFCS vs FCS) were tested, with prevalence ranging from 5.5 to 10.8% (18, 19, 25, 29, 31–33). Only two studies specifically assessed FCS, which was the focus of this study, both finding similar rates of *Listeria* spp. isolation in tomato (10.8%) (32) and frozen pepper (10.7%) (33) facilities.

Reported *Listeria* spp. prevalence was highest in packinghouses that processed frozen vegetables (82.2%) (28), potatoes (50.7%) (24), and mushrooms (23.9%) (23), (15.7%) (27). Unlike tree fruit, such commodities grow directly in contact with soil, which may increase the...
likelihood of finding *Listeria*. Other studies have reported a greater prevalence of *Listeria* spp. probably due to having evaluated either both NFCS and FCS or only NFCS such as floors and drains, where *Listeria* isolation is more likely.

While this study did not specifically target *L. monocytogenes* given that FCS were tested, in studies which did, rates of isolation generally fell between 1.2 and 9.5%. Higher rates of *L. monocytogenes* were reported in three studies, targeting NFCS primarily in fresh cut mushrooms (18.8%) (23), frozen vegetables (41.3%) (28), and tree fruits (56.4%) (17). Differences in frequency and stringency of sanitation programs, implementation of environmental monitoring programs targeting *Listeria*, and growing region may be significant divers of *Listeria* isolation rather than commodity type. Other factors, such as experimental design, sampling methods, and the size and age of packinghouses tested may have also impacted outcomes (19).

**The prevalence of *Listeria* spp. was affected by unit operation and FCS type**

The prevalence of *Listeria* spp. in each unit operation is displayed in Table 1. *Listeria* spp. were most frequently isolated from the wax coating unit operation (17.3%; n=110) (p<0.001). Furthermore, throughout the apple packing process the four FCS that showed the greatest prevalence of *Listeria* spp. were polishing brushes (19.6%; n=92), dividers under fans/blowers (17.4%; n=46), dryer rollers (10.5%; n=143), and brushes under fans/blowers (9.7%; n=206) (p<0.001) (Table 3).

In the wax coating unit operation, polishing brushes were the FCS most commonly implicated. These findings suggested a deficiency of routine sanitation procedures at this sampling site (16), and the ability of these FCS to trap wax residues and *Listeria* cells within polishing brush bristles. In the 2014 caramel apple listeriosis outbreak, polishing brushes were
one of the FCS that \textit{L. monocytogenes} was isolated from (8). Likewise, similar findings were discussed in an annual Fruit and Vegetable Convention (34), where a higher prevalence of \textit{Listeria} spp. was reported in the wax coating area, indicating that wax residues are related to an increase of \textit{Listeria} spp. persistence on both FCS and NFCS.

Studies that support our results have reported a greater long-term survival of \textit{L. monocytogenes} on waxed apples than unwaxed apples due to moisture retention over time (34), ultimately theorizing that entrapment of \textit{L. monocytogenes} cells and moisture within a wax coating was conducive for forming a microenvironment that enhances the survival of \textit{Listeria} in apples (35), and \textit{E. coli} O157:H7 cells that were found embedded to wax platelets on apples (36).

Another factor that could explain our results is the pH level of commercial waxes (6.7 to 8.6) (37). The optimal pH level for \textit{Listeria} to grow is 7.0 (38); therefore, if sufficient water activity, nutrients, and temperature are maintained, wax residues on FCS and NFCS may support the growth of \textit{Listeria} spp. if not otherwise removed.

Conversely, other studies have reported the immediate antibacterial activity of wax application on apples against \textit{L. monocytogenes} (34), \textit{E. coli} O157:H7 (37), and \textit{Salmonella Muenchen} (37), possibly because of one of the components of commercial wax, either isopropyl or ethyl alcohol (34). However, the concentration of these components in wax ranges from 15 to 23\% (39) which otherwise volatilizes rapidly and does not have an antimicrobial effect over time. In an \textit{in vitro} study, commercial wax did not show bactericidal activity against \textit{L. monocytogenes} (40).

In contrast, a study performed on NFCS in tree fruit packinghouses reported that the incidence of \textit{L. monocytogenes} under brush-beds, first drying, and wax coating areas was not
significantly different (17). These differences may be a result of NFCS being very interconnected between these unit operations, many times sharing drains.

Ultimately, future studies are warranted to further elucidate the mechanisms best suited for cleaning polishing brushes and determining if there are conditions where *Listeria* growth can be supported in this unit operation. Whether driven by wax accumulation during packing or *Listeria* growth events, the waxing unit operation is one that should be more closely scrutinized in order to limit cross contamination of apples during packing.

The second highest prevalence of *Listeria* spp. was obtained from both the first drying operation (9.4%; n=394), and the second drying (tunnel dryer) (8.2%; n=304) unit operations (p<0.001). In the first drying unit operation, dividers and brush rollers located underneath fans/air blowers (NFCS) were the FCS that showed the greatest prevalence of *Listeria* spp. Migration of pathogens from zones 2 or 3 (NFCS) to zone 1 (FCS) has been reported in previous studies (41, 42). As fans and air blowers circulate air, they also spread pathogens contained on the blades, motor, and cover of the fan, leading to cross-contamination of the dividers and brush rollers.

Moreover, repeated isolation of *Listeria* spp. has been shown on fans over brush beds in produce packinghouses (20), and on freezer fans in meat facilities (43). These devices represent potential niches for *L. monocytogenes* (44) and are recommended to be scheduled into daily cleaning and sanitation programs (7).

In the second drying unit operation, dryer rollers were the FCS that were most implicated. Tunnel dryer operating temperatures of 30-50 °C may create opportunities for *Listeria* growth in niches if other growth conditions are met. The optimal growth temperature of *L. monocytogenes* is 30-37 °C (45), and it can also grow at temperatures up to 50 °C (3). Packinghouses in this study often operated within the range of the optimal growth temperatures, thus increasing the...
potential proliferation of *Listeria* over time. Correspondingly, the survival of *L. innocua* (46), *E. coli* O157:H7 (37), and *Salmonella* Muenchen (37) has been reported on apples that were exposed to similar drying conditions.

Other explanations included visible dried leaves buildup in the inlet and outlet of the tunnel dryer. Also, in some packinghouses, a brush roller that was in the interior of the dryer to clean out leaves from the dryer rollers was considered a point of cross-contamination. Stainless steel dryer rollers were wrapped with different materials including vinyl or Teflon. Cracks and worn edges were observed in most of these FCS. One packinghouse used non-wrapped rollers and *Listeria* spp. were never isolated from this surface. Further research is warranted to determine growth/no-growth conditions within the tunnel drying unit operation and if surface type for rollers plays a significant role.

The third highest prevalence of *Listeria* spp. was obtained from the sorting unit operation (3.8%; n=1,254). Bristle rollers (8.8%; n=160), plastic interlocking chain conveyor belts (5.1%; n=256), Teflon transfer points and tape (4.6%; n=304), plastic flaps and transfer points (4.2%; n=427), side edges (3.3%; n=123), sorter cups (2.6%; n=76), solid conveyor belts (1.6%; n=186) and plastic guide rails (1.6%; n=128) were the FCS that were most implicated.

Interlocking belts are hard to clean due to their continuous length and joints in belts links causing entrapment of bacteria more easily (47, 48). Similarly, the rough polymeric material and hygienic design of solid conveyor belts support the growth and bacterial adhesion (49). Studies performed in a blueberry packing line (48), a minimally processed vegetable plant (49), and a sandwich processing plant (50) have reported that solid conveyor belts in their sorting areas were the major source of microbial contamination. Also, different species of *Listeria* were isolated from conveyor belts including *L. ivanovii* in frozen pepper packinghouses (33), and *L.*
monocytogenes, L. grayi, and L. innocua in a cabbage-packing facility (31). In 2013, the FDA evaluated the prevalence of Listeria spp. in 17 cantaloupe packing facilities, and two samples collected from conveyors were positive for L. monocytogenes at one facility (51).

Plastic flaps used to slow fruit down and transfer points used to bridge movement between one conveyor surface to the next are commonly made of polyvinylchloride (PVC), polyurethane, or Teflon which have a hydrophobic nature, allowing for easier microbial attachment (48, 52).

In addition, at the sorting unit operation, a significant increase in the prevalence of Listeria spp. was observed in the last sampling period (Q₄) during the in-process sampling (16.1%, n=161, p<0.05) (Figure 1). This result could be mainly attributed to cross-contamination with incoming apples that were stored for a longer period (10 to 12 months), which results in high rates of apple decay and potentially higher populations of Listeria coupled with the fact that wax may still be solidifying up until this unit operation.

Lastly, the lowest prevalence of Listeria spp. was obtained from the washing (0.7%; n=285), washing/sanitizing/rinsing (1.2%; n=331), and packing (0.3%; n=310) (p<0.001) unit operations. Sites which were exposed to sanitizers throughout production [brushes under spray bars (0.9%; n=227), dump tank/flume (0.9%; n=108)], as well as side edges (3.3%; n=123), sorter cups (2.6%; n=76), solid conveyor belts (1.6%; n=186), sorting guide rails (1.6%; n=128), traction belting (1.5%; n=66), PVC rollers (0.8%; n=123), packing tables and plastic crates (0.0%; n=64), sorting brushes (0.0%; n=193), and cup droppers (0.0%; n=60) had the lowest occurrence of Listeria spp.

The low prevalence of Listeria spp. obtained in the dump tank and flume (washing unit-operation) was attributed to the use of sanitizers (chlorine or peracetic acid (PAA)) within the...
water in the dump tank and connected flume. These sanitizers ensure the adequate microbial quality of wash water and reduce the likelihood of cross-contamination by inactivating foodborne pathogens that are introduced into the water (53, 54). The efficacy of chlorine (55–57) and PAA (58, 59) to avoid cross-contamination with *L. monocytogenes* has already been demonstrated in produce wash water. Although, during packing operations, incoming organic matter such as soil, leaves, and decaying fruit can reduce the efficacy of the sanitizer over time (60, 61). In the 2011 cantaloupe listeriosis outbreak, the use of municipal water without sanitizer in the wash water was one of the factors that was identified as a likely cause of cross-contamination of *L. monocytogenes* to cantaloupe (9).

Packinghouses that participated in this study used test strips or titration kits to monitor the concentration of these sanitizers within the dump tank and flume systems in addition to continuous monitoring and dosing systems. Dump tanks and flumes were all made of stainless steel. In equipment made of this type of material, *L. monocytogenes* cells have shown the least resistance to sanitizers, including PAA, chlorine dioxide, and acidic quaternary ammonia (62).

*Listeria* spp. isolation continued to be relatively low once product was conveyed out of the flume to the brush bed (washing/sanitizing/rinsing unit operation) where soap and sanitizers (e.g., PAA, chlorine, ozone, and chlorine dioxide) were applied via spray bars. The incorporation of sanitizer at this unit operation, similar to that of the dump tank and flume, safeguards against cross-contamination between apples and brush rollers; thus, low recovery should be anticipated. The use of PAA has been shown to reduce cross-contamination between apples inoculated with a surrogate of *L. monocytogenes* and brush beds of commercial apple packinghouses (63). Chlorine (64), chlorine dioxide (61), and ozone (61) have been demonstrated to reduce *L. monocytogenes* counts on apples.
Brush rollers have been identified as common harborage sites for *Listeria* because of their complex hygienic design (44, 65). These FCS can entrap apple debris and retain moisture within their bristles, creating the right environment for *Listeria* to grow. In the 2011 listeriosis cantaloupe outbreak brush rollers were one of the FCS where *L. monocytogenes* was isolated (9). However, no specification of the location of the brush beds was provided. Brush rollers that were evaluated within this unit operation were located in between spray bars that dispensed sanitizers and soap and were composed of polypropylene or polyethylene with a staple set cylinder configuration. Only two sites (1%; n=197) were positive for *Listeria* spp. at this unit operation during the in-process sampling, demonstrating that any shortcomings of brush sanitary design can be controlled for through regular introduction of sanitizers during production.

The low prevalence of *Listeria* spp. reported at the packing unit operation could be explained by less carryover of *Listeria*. As demonstrated in the previous unit operations, wax residues and *Listeria* microbial load of incoming apples were able to cross-contaminate FCS until the sorting unit operation, with wax set by the time apples are transferred to packing lines, potentially allowing for less transfer from apples to packing surfaces.

**Prevalence of *Listeria* spp. was affected by the timing of sampling (Post-sanitation, in-process)**

Of the 1,497 post-sanitation samples, 1.9% were positive for *Listeria* spp., compared to 7.2% of the 1,491 in-process samples (Table 1). Among all the positive *Listeria* spp. samples 21% (n=28) were detected during the post-sanitation sampling, whereas 79% (n=108) were detected during the in-process sampling.
In addition, timing of *Listeria* spp. isolation was also evaluated for each site amongst all the positive samples which were positive during a sampling event based upon three scenarios, 1) the sampling site testing positive post-sanitation and negative in-process, 2) the sampling site testing negative post-sanitation and positive in-process, or 3) the sampling site testing positive during both post-sanitation and in-process, to determine the frequency of each (Table 4). The outcomes of each scenario were significantly different from each other (p < 0.001), with *Listeria* spp. positive sites most frequently positive only for the in-process sampling (Scenario 2) (75.9%). This could be explained by allowing *Listeria* to come out of niche sites and subsequently contaminate FCS during the three hours of packinghouse operation (16). Another rationale is that incoming crop got to the packing line with a *Listeria* load capable of cross-contaminating the FCS. Similarly, in a study performed in an avocado packinghouse, which assessed the prevalence of *Listeria* spp. over two timings of sampling (25) as performed in this research, a higher prevalence of *Listeria* spp. during the in-process sampling was attributed to cross-contamination with the avocados during processing (25). More attention should be paid to this question in order to identify the most opportune approach which can be applied to detect harborage within packing equipment by running the equipment without any crop.

Isolation from surfaces in both sampling events (Scenario 3) occurred 17.2% of the time and is evidence of a deficiency of cleaning and sanitation procedures. This scenario was mostly reported on surfaces such as brushes (45%), including polishing brushes, bristle rollers, and brush rollers under the fan. Secondly, 15% of the cases were reported in dryer rollers, with many showing increased wear at this unit operation, resulting in cracks.

The low prevalence of *Listeria* spp. found in scenario 1 (6.9%) was attributed to a sample collection of different sampling sites (such as rollers inside the tunnel dryer). Other explanations
include removal during prior sampling or during packing, or application of sanitizers (such as from spray bars and fruit) which inactivated initial contamination.

The prevalence of *Listeria* spp. increased throughout crop storage time

Overall, an increase in the prevalence of *Listeria* spp. on FCS was observed throughout crop storage time and during both timings of sampling (Figure 1). The highest prevalence of *Listeria* spp. was obtained during the last quarter of sampling (Q₄) in the in-process sampling (38.2%; p≤0.05). After Q₂ a significantly higher prevalence of *Listeria* spp. was observed at both timings of sampling.

However, the increasing prevalence of *Listeria* spp. throughout crop storage time (quarters) differed by unit-operation. For the wax coating unit operation, a greater prevalence of *Listeria* spp. was obtained during all four quarters at both timings of sampling and it did not significantly increase over time. The only unit-operation where the prevalence of *Listeria* spp. increased during the post-sanitation sampling was the tunnel drying (from Q₁=0% to Q₃=13.9%; p≤0.05), and the three unit operations that accounted for the increase of the in-process prevalence of *Listeria* spp. over storage time were fan drying, tunnel drying, and sorting. These unit-operations showed significantly higher frequencies of isolation after the first quarter of sampling.

According to our findings, the prevalence of *Listeria* spp. increased after six months and three months of storage time for the post-sanitation and in-process samplings, respectively. One factor that could have influenced the increase during the post-sanitation sampling is the type of *Listeria* species that persisted in the packing equipment (66), affecting the efficacy of the cleaning and sanitation procedures. On the other hand, the increase in the prevalence of *Listeria* spp. during the in-process sampling was principally attributed to cross-contamination between
apples and FCS. Throughout storage, some of the most common apple post-harvest decay
diseases caused by the fungus *Botrytis cinerea*, *Penicillium expansum*, and *Mucor piriformis* (67)
can increase microbial pathogen growth (68). After harvest, apple bins go through a fungicide
drenching step before being stored for up to 12 months (54). Nevertheless, drenching methods
can cause cross-contamination with pathogens including *L. monocytogenes* due to the reuse of
fungicide solution (54). In addition, there is not a culling step (to eliminate bruised or damaged
apples) before the storage. Punctures, wounds, or damaged skin caused during harvest and
transportation facilitate the spread and growth of bacteria and fungus (69). Fungal growth
surrounding bruised tissues degrade the protective epidermal layer (70) and produce a pH
gradient (due to the use of organic acids) neutralizing the apple flesh (4), and leading to the
potential for survival and growth of *Listeria*. Thus, it has been hypothesized that as the storage
time increases so does the fungal growth and internal fruit pH, and when combined, these two
factors lead to an increase of the *Listeria* microbial load. However, further investigation
regarding the relationship between the survival of *Listeria* and fungal post-harvest disease is
required in a longer-term storage setting.

Moreover, *Listeria* can grow under refrigerated temperatures (71) employed for both regular
atmosphere (RA) and long-term controlled atmosphere (CA) storage of apples. *L.
monocytogenes* uses different cold adaptation mechanisms such as the stress response gene
sigma factor B (*sig B*) induced by refrigerated temperatures (72). This gene promotes the
formation of cryoprotectants (i.e., glycine betaine and carnitine), which stimulate cell
proliferation under cold stress (72, 73). Another mechanism is the alteration of the cellular
membrane lipid composition, in which the amount of unsaturated fatty acids increases under
refrigerated temperatures to ensure the optimum membrane fluidity, enzyme activity, and transportation of solutes necessary for *Listeria* survival (73, 74).

Studies that evaluated the survival of *Listeria* on apples throughout different long-term storage scenarios reported the survival of *L. monocytogenes* on apples after three months (75) and five months (34) of RA storage. Also, after seven months of either RA or CA storage, *L. innocua* survived on Fuji apples (76). It has been reported that CA storage reduces aerobic bacterial growth due to a reduced availability of oxygen (77), though facultative anaerobic bacteria such as *Listeria* cannot be inhibited under these conditions. Seven months of CA storage resulted in a greater reduction of *L. innocua* populations than RA storage (76). However, CA treatment did not significantly influence populations of *L. monocytogenes* (68) and *L. innocua* (76).

The findings provide science-based information on the FCS which require the most attention in order to not become a source of *Listeria* spp. contamination in apple packinghouses. Such results will provide a better understanding of how to control for contamination of *L. monocytogenes* to prevent future foodborne outbreaks and recalls associated with fresh apples through the improvement of EMPs, as well as enhanced cleaning and sanitation procedures on the most *Listeria*-prevalent FCS. Lastly, several areas of future research have been identified in order to determine the ability of *Listeria* to survive and grow in wax and the complex nature of *Listeria* survival and growth on apples throughout storage considering the interconnectedness to decay causing organisms.
MATERIALS AND METHODS

Apple packinghouses selection and layout

Five commercial apple packing facilities located in eastern Washington State, U.S., with varying line design and cleaning practices were chosen for this study. The distance range between apple packinghouses was from approximately 14 to 190 km. The selection of packinghouses was based on the packers’ willingness to participate in learning about their operation without added cost. The identity of packinghouses was kept confidential.

The product flow within each packinghouse was diagramed. Wet and dry areas were identified based on the presence of water during operations. The apple packing process was divided into seven unit operations as described as follows: washing, washing/sanitizing/rinsing, fan drying, wax coating, tunnel drying, sorting, and packing (78) (Supplemental Figure 1).

Sample sites

Food contact surfaces (zone 1) on different apple packinghouse equipment were selected from each unit operation (see Table 1 and Supplemental Table 1). In accordance with recommendations of the FDA’s Guidance for Industry: Control of Listeria monocytogenes in ready-to-eat foods, sampling sites were chosen based on environmental conditions and operations that support the growth of Listeria (i.e., hygienic design features, material type, efficacy and frequency of cleaning and sanitation procedures, and packer needs). Between 27 and 50 sites were sampled at each facility. Exact sampling sites for each packinghouse were photographed and described in detail to ensure consistency during all sampling events.
Sample Collection

Each packinghouse was visited four times over each of two packing seasons for a total of eight data collection points per facility. The purpose of these visits was to obtain data about FCS throughout the year-long packing season. Generally, apples are stored in cold storage rooms up to 12 months before packing. Packing season 1 (Apple crop 2018) included apples harvested from September through November 2018 and stored until July 2019, whereas packing season 2 (Apple crop 2019) included apples harvested from September through November 2019 and stored until July 2020.

Sampling periods were divided into four quarters. For instance, quarter one (Q1) represented apples stored for one to three months, quarter two (Q2) represented apples stored for four to six months, quarter three (Q3) represented apples stored for seven to nine months, and quarter four (Q4) represented apples stored for ten to 12 months. Typically, commercial apple packing facilities in Washington State use refrigerated regular atmosphere (RA, 0-2 °C) for apples that are stored up to three months, while controlled atmosphere (CA, 0-2 °C, O₂: 1-4%, CO₂: 0-2%) is used for apples stored up to 12 months. 1-Methylcyclopropene (1-MCP) is employed on all varieties of apples, regardless of storage treatment, except organically produced fruit.

Sample collection occurred at two timings of sampling: (i) after cleaning and sanitation procedures (post-sanitation) and (ii) after three hours of packinghouse operation (in-process). A 0.93 m² (30.5 cm x 30.5 cm) surface area was sampled with a premoistened sponge sampling stick [EZ-Reach sponge samplers with 10 ml of Dey/Engley (D/E) neutralizing broth; World Bioproducts LLC, Woodinville, WA]. Sampling sites with smaller surface areas were swabbed entirely. Samples were collected against standard product flow from dry areas to wet areas to avoid cross-contamination.
All collected samples were transported in a refrigerated cooler and analyzed within 24 h of collection in the Food Microbiology Laboratory of the Irrigated Agriculture Research and Extension Center at Washington State University, Prosser, WA.

Isolation, detection, and confirmation of *Listeria* spp.

The isolation, detection, and confirmation of *Listeria* spp. were conducted following a modified FDA bacteriological analytical manual (BAM) method (79). Each sampling sponge was hand-massaged and enriched with 90 ml of Buffered Listeria Enrichment Broth (BLEB; Difco, Becton Dickinson Co., Sparks, MD) for 4 h at 30°C. In order to select for *Listeria* spp., 1 ml of each of the following antibiotics, previously rehydrated and filter-sterilized, was added to the broth: 10 mg/L acriflavin monohydrochloride (Acros Organics, Fair Lawn, NJ), 40 mg/L nalidixic acid (Alfa Aesar, Ward Hill, MA), and 50 mg/L cycloheximide (Acros Organics, Fair Lawn, NJ). Samples were enriched for an additional 44 h at 30°C. Ten μl of enrichment was streaked in duplicate onto Modified Oxford Medium (MOX; Difco, Becton Dickinson Co., Sparks, MD) containing the Modified Oxford Antimicrobic Supplement (Bacto, Becton Dickinson Co., Sparks, MD) and incubated for 48 h at 35°C. Based on characteristic esculin hydrolysis (black halo formation), presumptive *Listeria* spp. colonies were selected for DNA extraction and PCR confirmation. Selected colonies were suspended in 0.5 ml of Tris-EDTA 1X buffer solution (Fisher Scientific, Fair Lawn, NJ), and stored at 4°C until the extraction of DNA. DNA extraction from presumptive *Listeria* spp. colonies was conducted using a Gen Elute Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, MO). A PCR amplification of a 1300 base pair (bp) target region in the *iap* gene was performed in the DNA extracted from presumptive positive *Listeria* spp. colonies. A pair of primers for the isolation of *L.* monocytogenes, *L.* ivanovii, *L.* innocua, *L.* seeligeri, and *L.* welshimeri was utilized (forward...
sequence: 5' ATATGAAAAAGCAACTATCGC 3' and reverse sequence: 5' AGAATACTAAATCACCAGGTTTTGC 3'; Thermo Fisher Scientific, Foster City, CA (78).

PCR assay was conducted using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Foster City, CA) in a 50 μl reaction mixture. Each 50 μl reaction mixture contained 25 μl of DreamTaq Green PCR Master Mix, 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 18 μl of molecular-grade water, and 5 μl of template DNA. An isolate of Listeria innocua 33090 (American Type Culture Collection, Manassas, VA) and molecular-grade water (Sigma Aldrich, St. Louis, MO) were used as positive and negative controls, respectively. All components were added to low profile 8-tube strips (0.2 ml) with individually attached caps (Greiner bio-one, Germany). Thermocycling was performed in the Mastercycler Nexus (Eppendorf, Germany). A 35-cycle program was run at 95°C for 30 s (denaturation), 62°C for 30 s (annealing), and 72°C for 1 min (elongation), followed by a 4°C hold until amplified products were evaluated. PCR products were analyzed by gel electrophoresis and visualized in E-Gel EX 1.0% agarose gel (Thermo Fisher Scientific, Foster City, CA). Each cell of the gel contained 5 μl of amplified DNA and 15 μl of molecular-grade water (Sigma Aldrich, St. Louis, MO). A 1 Kilobase (Kb) DNA-molecular ladder (Thermo Fisher Scientific, Foster City, CA) was included for comparison of amplicon size. Electrophoresis was carried out for 10 min at 48V, 90W. A positive result for Listeria spp. was indicated by the presence of characteristics bands at 1300 bp. This approach was used to identify only Listeria sensu stricto as a group (Listeria species including: L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, and L. welshimeri). Further evaluation of isolates was not conducted as agreed upon by participants in the survey.
Statistical analysis

A chi-square test or Fisher’s exact test (when expected observations were lower than 5) were used to analyze the categorical data of the presence or absence of *Listeria* spp. based upon the following categorical variables: unit operations (washing, washing/sanitizing/rinsing, fan drying, wax coating, tunnel drying, sorting, and packing), timing of sampling (post-sanitation and in-process), sampling periods (Q₁, Q₂, Q₃, and Q₄), and type of FCS (e.g., brushes under fans, polishing brushes, dryer rollers, bristle rollers, dump tank, and plastic flaps). A post-hoc pairwise comparison was used to compare the levels of each categorical variable when a significant difference was observed. The significance level for all tests was α=0.05. Statistical analysis was performed in R (Version 4.0.2) using RStudio (Version 1.3.1056) (RStudio, Inc., Boston, MA, USA).

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Table 1. Prevalence of *Listeria* spp. (%) by unit operation and timing of sampling.

| Unit operation                        | Examples of surfaces tested                               | Timing of sampling | Total prevalence $^d$ |
|---------------------------------------|-----------------------------------------------------------|--------------------|-----------------------|
|                                       |                                                            | N $^a$             | Post-sanitation $^b$  |
|                                       |                                                            | (n=1,497)          | In-process $^c$       |
|                                       |                                                            |                    |                       |
| Washing (Dump tank/flume)             | Dump tank, flumes, PVC rollers, traction belting.         | 285                | 0 (a)$^1$             |
| Washing/Sanitizing/Rinsing (Spray bars) | Brush rollers, plastic flaps, side edges.               | 331                | 0.6 (a)               |
| First drying (Fan and/or blower)      | Brush rollers, dividers.                                 | 394                | 4.6 (b)               |
| Wax coating                           | Polishing brushes, plastic flaps, transfer points.       | 110                | 10.9 (b)              |
| Second drying (Tunnel dryer)          | Dryer rollers, bristle rollers, transfer points.         | 304                | 4.6 (b)               |
| Sorting                               | Sorter cups, interlocking conveyor belts, solid conveyor belts, plastic | 1,254              | 0.8 (a)               |

$^a$ N

$^b$ Post-sanitation

$^c$ In-process

$^d$ Total prevalence

\[ 0.7 \text{ (a)} \]
guide rails, side edges, Teflon tapes, transfer points.

| Packing                      | Packing tables, solid conveyor belts, plastic crates, plastic flaps. |
|------------------------------|---------------------------------------------------------------------|
| Total                        | 2,988                                                               |

|     | 310  | 0 (a) | 0.7 (a) | 0.3 (a) |
|-----|------|-------|---------|---------|

- Number of samples tested.
- Statistical analysis post-sanitation prevalence: Fisher’s exact test, p<0.001.
- Statistical analysis in-process (3h of production) prevalence: Fisher’s exact test, p<0.001.
- Statistical analysis total prevalence: Chi-square test, p<0.001.
- Polyvinylchloride.
- Values within a column that are not followed by the same letter are significantly different (p<0.001).
Table 2. Prevalence of *Listeria* spp. and *L. monocytogenes* on food contact surfaces (FCS) and non-food contact surfaces (NFCS) in different types of produce packinghouses or processing facilities.

| Type of produce                          | Prevalence (%) | Type of surface tested | References |
|-----------------------------------------|----------------|------------------------|------------|
|                                         | *Listeria* spp. | Only (FCS, NFCS, both) |            |
|                                         |                |                        |            |
| Tree fruits                             | N/A            | 56.4                   | NFCS       |
| Cabbage, beets, parsnips                | 6.8            | 4.0                    | NFCS       |
| Microgreens, peach, apple, tomato, broccoli, cauliflower, cucumber | 5.8 | 3.2 | NFCS |
| Packinghouses and fresh-cut facilities | 3.4            | 3.0                    | NFCS       |
| Fresh-cut vegetables                    | N/A            | 7.9                    | NFCS       |
| Vegetables                              | N/A            | 9.5                    | NFCS       |
| Mushrooms                               | 25.1           | 18.8                   | NFCS       |
| Potatoes                                | 50.7           | 3.0                    | NFCS       |
| Avocadoes                               | 8.7            | N/A                    | Both       |
| Fresh-cut vegetables                    | N/A            | 4.4                    | Both       |

*References:*

17. (17)
18. (18)
19. (19)
20. (20)
21. (21)
22. (22)
23. (23)
24. (24)
25. (25)
26. (26)
| Food Item                                                                 | First Prevalence | Second Prevalence | Combination | Reference |
|---------------------------------------------------------------------------|------------------|-------------------|-------------|-----------|
| Mushrooms                                                                 | 15.7             | 1.6               | Both        | (27)      |
| Frozen vegetables (e.g. cauliflower, mushrooms, broccoli, carrot, zucchini) | 82.2             | 41.3              | Both        | (28)      |
| Frozen vegetables (e.g. tomato, broccoli, carrot, spinach, artichoke)     | 7.8              | 1.2               | Both        | (29)      |
| Pre-packaged salad, canned vegetables                                     | N/A              | 5.4               | Both        | (30)      |
| Cabbage                                                                   | 5.5              | 2.1               | Both*       | (31)      |
| Tomatoes                                                                  | 10.8             | N/A               | FCS         | (32)      |
| Frozen peppers                                                            | 10.7             | 0                 | FCS         | (33)      |

* N/A: Information not available.

* Results of *Listeria* prevalence are not combined for both types of surfaces. Values account for FCS only.
Table 3. Frequency of *Listeria* spp. isolation by food contact surface

| Food contact surfaces                                                                 | Na  | Frequency (%) |
|--------------------------------------------------------------------------------------|-----|---------------|
| Polishing brushes (e.g., polyethylene, polypropylene, nylon, horsehair mix)           | 92  | 19.6 (a)      |
| Stainless steel dividers under fan/blowers*                                          | 46  | 17.4 (ab)     |
| Dryer rollers (e.g., stainless steel roller wrapped with vinyl or Teflon)              | 143 | 10.5 (abc)    |
| Brushes under fan/blower (e.g., polyethylene, polypropylene)                         | 206 | 9.7 (abc)     |
| Bristle rollers (e.g., polyethylene, polypropylene)                                  | 160 | 8.8 (bcd)     |
| Plastic interlocking chain conveyor belts (e.g., polypropylene, polyethylene)        | 256 | 5.1 (cde)     |
| Teflon transfer points and tape*                                                      | 304 | 4.6 (cde)     |
| Plastic flaps and transfer points (e.g., polyvinylchloride (PVC), polyurethane)*     | 427 | 4.2 (de)      |
| Side edges (e.g., Painted-steel or high-density polyethylene)                         | 123 | 3.3 (cdef)    |
| Sorter cups*                                                                          | 76  | 2.6 (cdef)    |
| Solid conveyor belts (e.g., PVC, polyurethane, polyester nylon)                      | 186 | 1.6 (ef)      |
| Sorting plastic guide rails                                                          | 128 | 1.6 (ef)      |
| Traction belting (e.g., polyurethane, polyester nylon)*                               | 66  | 1.5 (cdef)    |
| Brushes under spray bars (e.g., polyethylene, polypropylene)                        | 227 | 0.9 (f)       |
| Stainless steel dump tank and flume                                                  | 108 | 0.9 (ef)      |
| PVC rollers                                                                           | 123 | 0.8 (ef)      |
Packing tables and plastic crates 64 0.0 (ef)
Cup droppers (e.g., painted steel)* 60 0.0 (ef)
Sorting brushes (e.g., polyethylene, polypropylene) 193 0.0 (f)

\(^a\) Number of samples tested.

\(^b\) Values within a column that are not followed by the same letter are significantly different (p<0.001).

\(^c\) Food contact surfaces which had a surface area smaller than 0.93 m\(^2\).
Table 4. Frequency of *Listeria* spp. isolation for a specific sampling site based on timing of sampling during a sampling event.

| Timing of sampling | N<sup>a</sup> | Frequency (%)<sup>b</sup> |
|--------------------|--------------|-------------------------|
| Post-sanitation    | In-process   | (n=116)                 |
| Scenario 1<sup>c</sup> | Positive | Negative | 8 | 6.9 (a)<sup>f</sup> |
| Scenario 2<sup>d</sup> | Negative | Positive | 88 | 75.9 (c) |
| Scenario 3<sup>e</sup> | Positive | Positive | 20 | 17.2 (b) |

<sup>a</sup> Number of sampling sites represented in each scenario.

<sup>b</sup>Total number of sampling sites with at least one positive detection of *Listeria* spp. From the total of positive samples (n=136), 20 sampling sites belonged to scenario 3, thus total n=116.

<sup>c</sup>Sampling sites in which *Listeria* spp. were detected only in the post-sanitation sampling.

<sup>d</sup>Sampling sites in which *Listeria* spp. were detected only in the in-process sampling.

<sup>e</sup>Sampling sites in which *Listeria* spp. were detected in both post-sanitation and in-process samplings.

<sup>f</sup>Values within a column that are not followed by the same letter are significantly different (p<0.001).
Post-sanitation bars within each unit operation that are not followed by the same lowercase letter are significantly different (p<0.05).

In-process bars within each unit operation that are not followed by the same uppercase letter are significantly different (p<0.05).

Figure 1. Prevalence of *Listeria* spp. is affected by unit operation and crop storage time