FINAL PROJECT REPORT

Project Title: Assessment of Apple Packing for *Listeria* Risk

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Cooperators: Multiple industry partners. As of August 2015, Ines Hanrahan assumed PI status, since Dr. Killinger left WSU to take a position with FDA. Dr. Killinger remains as a co-PI on the project. The assistance of Tonia Green, Lauren Walter, Kyu Ho Jeong, and Andy Liao are acknowledged and greatly appreciated. Special thanks to Dr. Meijun Zhu (WSU) for help with oversight of WSU based budgets upon Dr. Killingers departure.

Other funding sources: None

Total Project Funding:

Budget History:

Item	**2015**
Salaries	23,525
Benefits	10,499
Wages	1,500
Benefits	33
Equipment	
Supplies	25,000
Travel	6,000
Plot Fees	
Miscellaneous	
Total	66,557

OBJECTIVES

1) Examine current industry standard practices for control of *Listeria* in packing environments and compare sampling and detection methods for *Listeria* spp.

2) Examine the prevalence of generic *Listeria* spp. and *Listeria monocytogenes* associated with Fuji apples stored under refrigerated storage and controlled atmosphere storage with and without ozone application

SIGNIFICANT FINDINGS

- The results from a single facility indicated that damaged walls and floors present challenges for normal cleaning procedures. Furthermore, bumpers and HVAC systems also appeared to be difficult to clean. After an aggressive cleaning, floors, bumpers, and damaged walls had reduced observations of presumptive positives for generic *Listeria* spp..

- Items associated with cleaning, including floor scrubbers and foggers, may also serve as sources of cross-contamination. This demonstrates the need for regular maintenance, cleaning and sanitizing of equipment and items used to clean facilities. A chemical fogger and floor scrubber both tested presumptive positive for generic *Listeria*.

- Results indicated that items such as wooden pallets and forklifts that move from Zone 4 (outside production areas) into cold storage or packinghouses areas (Zone 3) could serve as sources of contamination. A forklift was sampled and found to be presumptive positive for generic *Listeria*, 87.5% (7/8) positive and wooden pallets were also (100%).

- Apples that were directly contacted by open surface irrigation water had a higher percentage positive (12.3%) for generic *E. coli* compared to apples that were not contacted by irrigation water (3.3%).

- Further examination of refrigerated and controlled atmosphere storage on microbial levels is warranted as well as the use of ozone during controlled atmosphere storage.

METHODS

Objective 1: Environmental Listeria spp. risks in tree fruit packing operations

Partnering organizations and packing houses were identified for preliminary meetings to assess potential high risk areas for *Listeria* spp. contamination. Consideration was given to equipment design, materials used as food contact surfaces as well as cleaning and sanitation regimes. Data collected at each packinghouse were blinded for confidentiality. Each packinghouse was interviewed to understand current practices related to cleaning and sanitation practices as well as environmental monitoring to prioritize areas for environmental sampling.

Three facilities were interviewed for participation; based on responses, two were selected for sampling. Selection of target organisms, *Listeria* spp., total coliforms and generic *E. coli*, was discussed. The scope of sampling was explored, examination of food contact surfaces (Zone 1) as well as areas immediately adjacent to food contact surfaces (Zone 2), production areas (Zone 3) and areas outside of production (Zone 4). The approach taken for sampling and study design were different based on input from the facility and their specific needs. Aggressive environmental sampling was conducted in each facility on multiple days. In one facility, an evaluation of *Listeria*

prevalence was performed in the packing operation and cold storage rooms, including Zone 1-4 testing prior to cleaning and sanitation.

In one facility, a comparison of sanitation practices was performed. Two cold storage rooms were designated for the study. Cold storage rooms were sampled before and after typical sanitation versus before and after a more, aggressive sanitation protocol. Samples were collected from Zone 3 and some Zone 4 locations. In some cases, samples were composited to reduce the number of samples processed and increase the number of areas examined in the cold storage rooms. For example, all high wall samples were composited and all the low wall samples were compostied into one sample to create only two samples. Samples prior to cleaning and sanitation were collected from both rooms on one day. In another facility, samples were collected prior to sanitation from Zones 1-4 to evaluate areas that may require aggressive sanitation and potential routes of microbial contamination.

Samples were collected using PUR-Blue ™ swabs (World Bioproducts), Quick swabs ™ (3M™), Swab Samplers™ (3M™) or EZ Reach ™ (World Bioproducts) sponge samplers that were appropriate for each type of sampling site. Previous results from our laboratory indicated that some surfaces, such as wood bins, would benefit from aseptic sampling with small pieces of 3M Scotch-Brite™ pads, so in some cases, samples were collected with commercially available swabs and using treated 3M Scotch-Brite™ pads. Efforts were made for most commerical sampling supplies to use Dey-Engley (D/E) neutralizing broth, but in some cases, supplies using letheen broth were used. Environmental samples were collected and held on ice for transport to the laboratory. Liquid from the sampling device was collected and brought up to a 10ml volume of D/E neutralizing broth to align sampling volumes among swabbing devices.

Several methods of analysis were used for isolation of presumptive positive generic *Listeria* spp. The FDA-BAM method (FDA-BAM, 2011) with some modifications was used. The same pre-enrichment steps were used for all methods; samples were pre-enriched in Buffered *Listeria* Enrichment Broth (BLEB) for 24 hours at 30°C; after 4 hours of incubation, acriflavin HCL (10mg/L), nalidixic acid (40mg/L) and natamycin (25mg/L) were added to reduce background flora levels. Serial dilutions of the pre-enriched sample were inoculated onto Environmental *Listeria* Petrifilm™ (3M™) for determination of generic *Listeria* spp.. At 24 and 48 hours incubation, the pre-enriched sample was plated onto Modified Oxford *Listeria* selective agar. At 24 hours incubation, the pre-enriched sample was plated onto HardyChrom *Listeria* agar for differentiation of generic *Listeria* spp. from *Listeria monocytogenes* and *ivanovii*. Results from these methods should be considered presumptive positive, not confirmed, for generic *Listeria* or *Listeria monocytogenes*. Some isolates remain to be tested for further confirmation using methods described below.

Selected samples were processed as duplicate swabs for comparison and examination of other methods, including the use of HardyChrom *Listeria* agar, a proprietary test for *Listeria* spp., qPCR and immunomagnetic separation followed by qPCR.

Objective 2: Examine the prevalence of generic Listeria and Listeria monocytogenes on Fuji apples stored under differing conditions

There is a need to understand the prevalence of *Listeria* spp. on fruit upon arrival from the orchard and after storage. Apples were harvested from WSU orchards and were not intended for the commercial market, so there were no negative commercial implications associated with the results. Microbial tests performed at harvest in October of 2014 did not include *Listeria* spp., but did examine fruit that was directly contacted by water and fruit that was not directly contacted by irrigation water from an open surface water source. Apples were examined for the presence of total coliforms, generic *E. coli* using 3M™ E. coli/Coliform Petrifilms™. The need to include *Listeria* spp. was identified and approved for funding, which allowed for examination of apples held under refrigerated storage and controlled atmosphere storage with and without ozone treatment. With input from post-harvest experts, a chemical supplier and a partnering facility, apples were stored refrigerated storage

for 2 months and 3 months and in controlled atmosphere storage with and without ozone application for 6 months and 8-9 months. The presence of presumptive *Listeria* spp. and *Listeria monocytogenes* were examined as described above. Apples were also examined for pathogenic *E. coli* O157:H7 and *Salmonella*. The isolation of *E. coli* O157 was performed using immunomagnetic separation (IMS), standard plating techniques, and latex agglutination (LeJeune et al., 2001; Wright et al., 1994). *Salmonella* spp. were isolated by standard plating techniques and latex agglutination (FDA-BAM 2011). Confirmation of presumptive positive samples for *E. coli* O157, *Salmonella* spp., generic *Listeria* and *Listeria monocytogenes* will be performed by third-party laboratory serotyping.

RESULTS AND DISCUSSION

Objective 1. Examination of Current Sanitation Practices for control of Listeria.

The examination of cleaning and santizing procedures at one facility focused on cold storage rooms. The facility requested that interpretation of presumptive positives be reported as positive if any media (1 of 3 media utilized) presented presumptive positive colonies; in this way, the most conservative approach to potential comtamination was captured. Samples were collected after a typical cleaning strategy and samples were collected after a more aggressive cleaning and sanitizing procedure. Two rooms were sampled on the same day before cleaning. Areas sampled were categorized into the following: walls, damaged walls, floors, damaged floors, bumpers, door and HVAC systems. Prior to cleaning, the cold storage rooms had some similarities and some differences regarding prevalence of generic *Listeria*. Both rooms had a high prevalence of generic *Listeria* (greather than 80%) for both floors and damaged floors. In one room, both door samples (100%) were positive for generic *Listeria*, while one sample from the other door was positive (50%). However, one room had 100% (2/2) presumptive positive wall samples and the other had none detected (0/2) for wall samples. One room had over 87.5% of bumpers test positive for generic *Listeria* while the other room had a lower percentage (62.5%). Before cleaning, 100% of wooden pallets (4/4) were presumptive postive for generic *Listeria*.

After the normal cleaning procedure, the walls exhibited the the greatest difference in generic *Listeria* prevalence; from 100% (2/2) positive before cleaning to 6.3% (1/16) detected after cleaning (Figure 1). However, generic *Listeria* prevalence on the floors were only sligthly affected by cleaning (from 100% (8/8) before cleaning to 75% (9/12) after. Furthermore, some areas had more observed generic *Listeria* after cleaning, including damaged floors, HVAC, and bumpers. The results from a single facility indicated that damaged walls and floors present challenges for normal cleaning procedures. Furthermore, bumpers and HVAC systems also appeared to be difficult to clean. Cleaning equipment was also examined after normal cleaning, and appeared to be a potential source of cross-contamination. The chemical fogger and floor scrubber both tested presumptive positive for generic *Listeria* (100%; 3/3 and 75%; 9/12, respectively. A forklift was also sampled and found to be presumptive positive for generic *Listeria*, 87.5% (7/8) positive.

In addition to some equipment in Zone 3, outdoor areas (Zone 4) were also examined. The docking areas immediately outside of the rooms were sampled, and some distant outdoor area where culled apples are kept and vehicle traffic was relevant. High traffic loading dock areas infront of both cold rooms were 100% (8/8) positive and the high forklift traffic in front of the packinghouse were 100% (4/4) positive. All samples taken in or near the culled apples were also 100% (8/8) positive; trucks travel through these areas toward the packinghouses and cold room loading docks.

The cold storage room designated for aggressive cleaning had some differences in presumptive generic *Listeria* spp. presence before cleaning. Presumptive *Listeria* spp. were not detected on the walls prior to cleaning. After an aggressive cleaning, floors, bumpers, and damaged walls were greatly affected. The observation of presumptive generic *Listeria* spp. associated with floors was lower after aggressive cleaning (87.5% before versus 7.7% after). For bumpers, a similar

obervation was made (87.5% before versus 5.3% after). Also, damaged walls had a lower observation of presumptive generic *Listeria* spp. after aggressive cleaning (50% to none detected). There was a moderate decrease in positives for the door (50% to none detected) and damaged floor samples (100% before to 37.5% after). The HVAC systems sampled had 25% presumptive generic *Listeria* spp. before cleaning, and decreased to 13.3% after cleaning. Although aggressive cleaning was performed, visual observations noted challenges with cleaning, as the presence of fruit debris in difficult to clean areas of the cold rooms was noted during sampling (between bumpers and the wall). Items associated with cleaning were also found to be presumptive positive for generic *Listeria*, including a chemical barrel (100%; 1/1).

In the facility where the packing line and cold storage rooms were examined for *Listeria* prevalence prior to cleaning and sanitation, for any individual sample, results varied when methods were compared within laboratories for presumptive generic *Listeria* (Tables 2-3). Table 3 provides presumptive positive results in three main areas of the facility for presumptive generic *Listeria* and *Listeria monocytogenes* using three methods. Zones 1-4 demonstrated presumptive positive results prior to cleaning and sanitation. Some sampling sites were swabbed in adjacent locations and the duplicate swab was examined for comparison and examination of other methods as well as confirmation. The results of twenty-five samples involved in duplicate testing and confirmation are provided (Table 2). Although individual sample results varied by method, in 6 of 9 categories (line equipment, non-line equipment, line support, drain, drencher, drencher parts and cold room floors) presumptive positive generic *Listeria* results were confirmed by qPCR or immunomagnetic separation followed by qPCR. Furthermore, samples testing presumptive positive for generic *Listeria* or *Listeria monocytogenes* were confirmed positive for *Listeria monocytogenes* in the following categories: non-line equipment, drain, drencher and drencher parts.

Objective 2. Examination of the prevalence of Listeria on Fuji apples during storage.

Fuji apples were harvested and apples that were contacted by surface water from under-tree irrigation were observed and collected for comparison with apples that were not directly contacted by surface water. The apples were stored for 2 and 3 months in refridgerated atmosphere (RA) storage and in controlled atmosphere (CA) storage for 6 and 8-9 months, with and without ozone. At harvest, *Salmonella* spp. and *E. coli* O157:H7 were not detected, *Listeria* spp. and *Listeria monocytogenes* were not tested at that time (Table 1). Apples that were directly contacted by open surface irrigation water had a higher percentage positive (12.3%) for generic *E. coli* compared to apples that were not contacted by irrigation water (3.3%).

Apples in 2 months RA storage were presumptive postive for *Salmonella* (7%), *E. coli* O157 (3%), *Listeria* spp. (11-21%), and *Listeria monocytonegenes*(5%) (Table 1); these results are not confirmed for *Listeria* spp. or pathogen presence. Additional testing will be performed for confirmation. After 6 months in CA storage, observed total coliform levels were lower in both the untreated and ozone treated apples. At 8-9 months CA storage, observed total coliforms were lower from the ozone treated apples (14% versus 29.6%). Further examination of refrigerated and controlled atmosphere storage on microbial levels is warranted as well as the use of ozone during controlled atmosphere storage.

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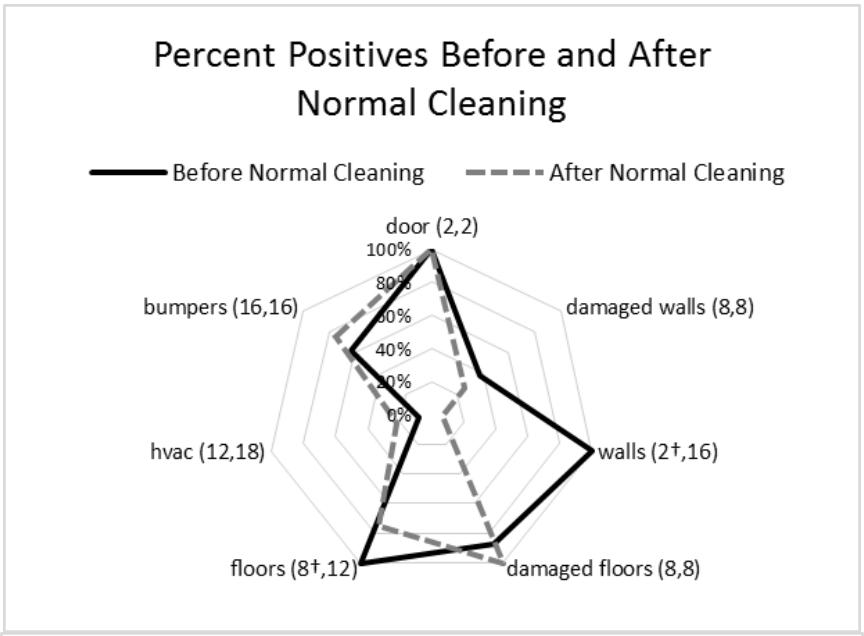
Figure 1: Percentage of presumptive positive samples for generic *Listeria* before and after normal cleaning and aggressive cleaning of apple storage cold rooms. Samples were organized within categoreis and number of samples collected before and after each type of cleaning provided in parenthesis. † denotes samples that were composited (8 samples into each sample). Samples were considered presumptive positive if any media (1 of 3 media utilized) presented presumptive positive colonies.



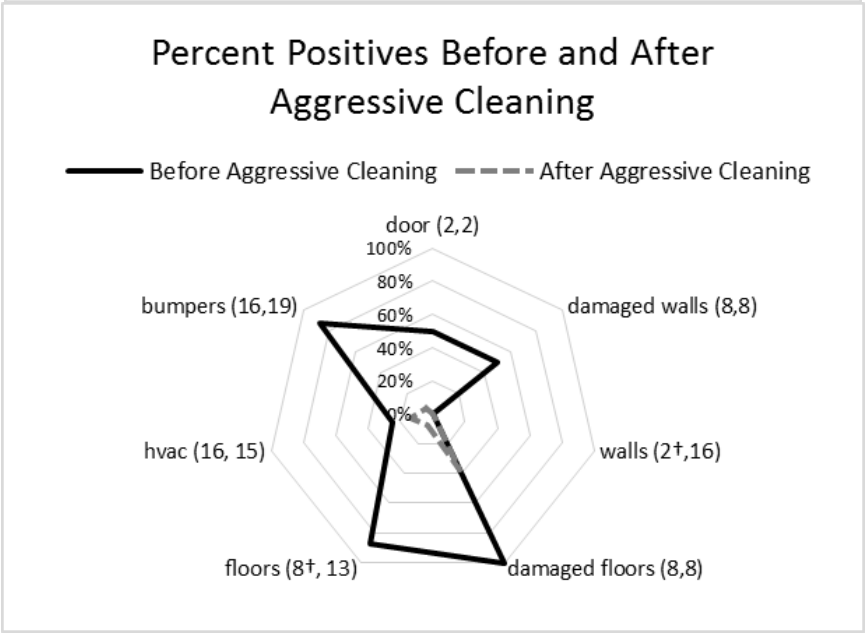


Figure 2: Percent positives of supplementary Zone 3 and 4 samples taken in addition to the before and after cleaning samples. Cleaning equipment includes a fogger, a riding floor scrubber, and chemical barrel used to clean the rooms. Outdoor samples include ground samples taken in the loading dock area outside of each room as well as infront of a high traffic forklift door of the packing house and an apple cull area. Other includes wooden pallets, forklifts, and fruit debris.

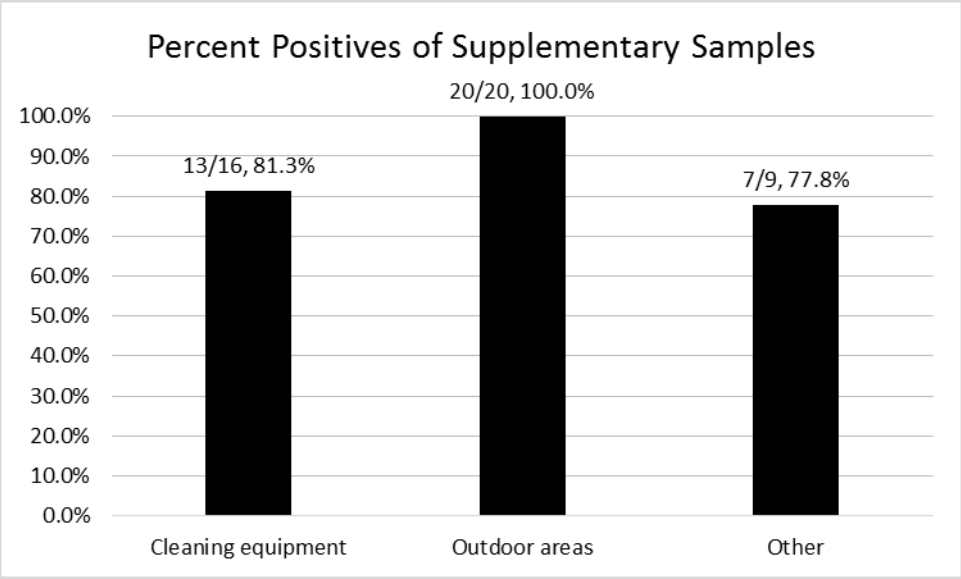


Table 1. Percentage of positive and presumptive positive samples among apples tested at harvest (with and without direct water contact from a surface irrigation source) after refrigerated atmosphere (RA) storage (2 and 3 months) and controlled atmosphere (CA) storage (with and without ozone, 8-9 months).

Microorganism Type	**Harvest** No direct water contact (n=181)	**Harvest** Direct surface water contact (n=155)	**RA** 2 months (n=100)	**RA** 3 months (n=100)	**CA** 6 months No Ozone (n=100)	**CA** 6 months With Ozone (n-118)	**CA** 8 months No Ozone (n=98)	**CA** 8 months With Ozone (n=100)
Coliform	61.3%	61.9%	21%	19%	12%	10.2%	29.6%	14%
Generic *E. coli*	3.3%	12.3%	2%	ND	ND	ND	1%	ND
Generic *Listeria**	NT	NT	11-21%	20-21%	9-30%	2.5 – 25%	ND – 23%	ND – 5%
E. coli O157:H7*	ND	ND	3%	1%	ND	ND	ND	ND
*Salmonella**	ND	ND	7%	ND	ND	ND	ND	ND

*Presumptive positive test results, further confirmation necessary and being performed.
n=number of samples collected
ND= none detected
NT= not tested

Table 2. Percentage of positive samples within sampling categories by testing media or method for presumptive (†) or confirmed generic *Listeria* or *Listeria monocytogenes*.

Category (number of samples)	*Listeria* spp. MOX†	*Listeria* spp. Petri film†	Proprietary *Listeria* spp. †	*L. mono* CHR 1†	*L. mono* CHR 2†	*Listeria* spp. qPCR	*L.* mono qPCR	*Listeria* spp. IMM qPCR	*L.* mono IMM qPCR
Packingline									
Line equipment (2)	50%	50%	50%	ND	50%	50%	ND	NT	NT
non-line equipment (3)	33%	67%	33%	ND	100%	33%	33%	NT	NT
drain (5)	60%	60%	100%	20%	100%	100%	100 %	NT	NT
line support (1)	100%	ND	100%	ND	ND	ND	ND	NT	ND
walls (1)	100%	ND	100%	ND	ND	NT	NT	ND	NT
Outdoors									
drencher (4)	50%	100%	100%	25%	75%	75%	75%	NT	ND
Cold Rooms									
drencher parts (5*)	100%	ND	100%	ND	20%	NT	NT	60%	33%
floors (2)	50%	50%	100%	ND	ND	NT	NT	50%	0%
HVAC (1)	ND	ND	100%	ND	ND	NT	NT	ND	NT

*One sample was composited and the composite was positive**.**

ND= none detected

NT= not tested

Table 3. Percentage of positive samples within sampling categories by testing media or method for presumptive (†)generic *Listeria* or *Listeria monocytogenes*. Further confirmation testing is being performed.

	Listeria spp. MOX†	*Listeria* spp. Petrifilm†	*L. mono* CHROM†
Packingline			
line-equipment (96†)	22%	35%	ND
line support (9)	89%	56%	ND
floors (4)	100%	50%	ND
non-line equipment (8)	38%	38%	ND
drain (4)	50%	100%	ND
walls (12)	50%	50%	8%
Outdoors			
loading area (10)	60%	100%	ND
drencher (2)	100%	100%	ND
Cold Rooms			
floors (15)	13%	20%	7%
drencher parts (8†)	88%	13%	13%
walls (22†)	36%	14%	ND
door (4)	25%	25%	ND
hvac (11)	ND	9%	ND
non-line equipment (1)	ND	ND	ND

†Some samples were composited to reduce the total number of samples.
ND= none detected

EXECUTIVE SUMMARY

This study identified several areas of apple packinghouses and cold storage rooms that are important to evaluate in environmental monitoring programs. Floors, damaged floors, and concrete bumpers were challenging to clean unless aggressive cleaning and sanitizing practices were followed. Although HVAC systems did not frequently test positive for presumptive generic *Listeria*, they appeared to be challenging to clean with normal cleaning procedures. Loading and drenching areas may harbor generic *Listeria* or *Listeria monocytogenes*; therefore, consideration of vehicle and worker traffic patterns are important to consider to reduce contamination risk in production areas. Results indicated that items such as wooden pallets and forklifts that move from Zone 4 (outside production areas) into cold storage or packinghouses (Zone 3) could serve as sources of contamination. Items associated with cleaning, including floor scrubbers and foggers, may also serve as sources of cross-contamination. This demonstrates the need for regular maintenance, cleaning and sanitizing of equipment and items used to clean facilities. This may also explain why some areas had more observed positives for generic *Listeria* after cleaning, especially floors and damaged floors. Other equipment in Zone 3 also appeared to harbor generic *Listeria* or *Listeria monocytogenes*, emphasizing the importance of cleaning and sanitizing not only direct food contact surfaces but also other equipment in production areas as well as drains. Incoming fruit may also harbor microbial contamination. Apples that were directly contacted by open surface irrigation water had a higher percentage positive (12.3%) for generic *E. coli* compared to apples that were not contacted by irrigation water (3.3%). After 2 months in refrigerated storage, a low percentage of the apples tested presumptive positive for pathogens; however, presumptive positive samples for pathogens were not observed with further controlled atmosphere storage (6-8 months) of apples harvested at the same time. After 8 months of controlled atmosphere storage, percentage positive for total coliforms were lower among apples treated with ozone. Further examination of refrigerated and controlled atmosphere storage on microbial levels is warranted as well as the use of ozone during controlled atmosphere storage. The potential exists for microbial contamination to enter packinghouses on fruit at a low prevalence rate or through routes of contamination associated with vehicles, equipment and worker foot traffic. Therefore, emphasis on cleaning and sanitizing in production areas is necessary to prevent establishment of biofilms and long-term sources of *Listeria* contamination.