THE INCIDENCE AND PERSISTENCE of *Listeria monocytogenes* IS ASSOCIATED WITH ENVIRONMENT MICROBIOTA IN TREE FRUIT PACKING FACILITIES

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AN INCREASE IN THE IMPLICATION OF TREE FRUITS IN OUTBREAKS AND RECALLS DUE TO CONTAMINATION BY FOODBORNE PATHOGENS

2011 Multistate Outbreak of *Salmonella* Agona Infections Linked to Imported Papayas (106 cases)

2012 Multistate Outbreak of *Salmonella* Braenderup Infections Linked to Mangoes (127 cases)

2012 Outbreak of *Salmonella* Illness Linked to Mangoes in Canada (21 cases)

2014 Outbreak of Listeriosis Linked to Stone Fruit in the USA (2 cases)

2015 Multistate Outbreak of listeriosis linked to prepackaged caramel apples (35 cases)

2017 Multistate Outbreak of *Salmonella* Urbana Infections Linked to Maradol Papayas (7 cases)

2017 Outbreak of Listeriosis Linked to Caramel Apples (3 cases)

2017 Multistate Outbreak of *Salmonella* Newport and *S. infantis* Infections Linked to Maradol Papayas (4 cases)

2017 Multistate Outbreak of *Salmonella* Anatum Infections Linked to Maradol Papayas (20 cases)

2019 Recall of whole avocados due to *L. monocytogenes* contamination

2019 Multistate Recall of Imported Stone Fruits Due to *L. monocytogenes* contamination

2019 Multistate Outbreak of *Salmonella* Infections Linked to Cavi Brand Papayas (71 cases)
SOURCES AND ROUTES OF TREE FRUIT CONTAMINATION

- **Demonstrated:** Knowledge gap
- **Suspected:** ~5%
- **Unknown:** ~95%

Whole apples used in preparation of caramel apples were the ingredient contaminated by *L. monocytogenes*.

All whole apples were contaminated at a single packing facility in California.
WHY *L. monocytogenes*?

### Pathogen | Estimated annual # illnesses | Estimated annual # of deaths
---|---|---
*Listeria monocytogenes* | 1,600 | 255 (16% of cases)
*Salmonella* | 1.0 million | 378 (0.03% of cases)
Norovirus | 5.5 million | 149 (0.002% of cases)

FEW CASES, SEVERE

EXTREME SURVIVOR IN FOOD AND FOOD PRODUCTION ENVIRONMENTS

- Grows at -1.5°C and 45°C
- Higher virulence of cultures grown at 4°C comparing to those grown at 37°C
- Extreme halotolerance, survives over one year in 23.8% NaCl
- Superior barotolerance, survives over 150s at 400 MPa
APPROACH AND PREVALENT KNOWLEDGE GAPS

- Longitudinal surveillance of apple and stone fruit production environments for *L. monocytogenes* occurrence
  - Pre-harvest
  - Post-harvest
    - Seasonal variations
    - Processing/packing plants
    - Areas of a plant
- Whole genome sequencing of all *L. monocytogenes* isolates
  - Persistent genotypes
  - Characterize inter-zone transfer in fruit packing facilities
  - Identify the development of AR in *L. monocytogenes*
- Microbiome analysis of post-harvest environmental samples
- Evaluate the role/risk of convectional postharvest practices on *L. monocytogenes* survival/transmission in apple production continuum
MICROBIOME STUDY DESIGN

- Three Apple Fruit Packing Facilities
- 13 standardized sample location (non-food contact surfaces)
- 3 sampling time points
SEASONAL DIFFERENCES IN L. monocytogenes OCCURRENCE

A significant (p < 0.05) association of season and L. monocytogenes incidence in facilities

**SEASONAL DIFFERENCES:**

- **Fall**
  - Increased volume of apples due to local harvest
  - Increased organics and microbial load introduced into the apple packing facilities

- **Winter**
  - Moderate volumes of apples processed from CA storage
  - Due to lower temperatures, less competition with background microbiota for L. monocytogenes

- **Spring**
  - Decreasing volumes of apples packed due to lack of supply
  - Decreasing availability of organic and moisture due to non-operation status
WHOLE GENOME SEQUENCING AND SEQUENCE ANALYSIS

APPROACH

- WGS was performed using a MiSeq (Illumina, Inc.) with the version 2 kit (2_250 bp)
- Core genome multi locus sequence type (cgMLST) was performed to analyze the genotypic diversity of the isolates
- Strain: cgMLST cluster + 12 allele difference

DEFINING THE CRITERIA AND DETERMINING THE NUMBER OF ISOLATES

- Isolates of the same strain from the same sample are counted as 1 unique isolate
- Isolates of the same strain from different samples are all counted
- Year 1 (2016-2017) generated 616 isolates
SUMMARY OF THE PRESENCE (+) AND ABSENCE (-) OF *L. monocytogenes* IN ENVIRONMENTAL SAMPLES COLLECTED FROM 3 APPLE PACKING FACILITIES

Chi-square test of *L. monocytogenes* occurrence among processing sections

<table>
<thead>
<tr>
<th>L. monocytogenes occurrence</th>
<th>Sectiona</th>
<th>Dry</th>
<th>Wash</th>
<th>Wax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td></td>
<td>18</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>21</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>Chi-Square</td>
<td></td>
<td>4.38</td>
<td>2</td>
<td>0.112</td>
</tr>
<tr>
<td>Likelihood</td>
<td></td>
<td>4.454</td>
<td>2</td>
<td>0.108</td>
</tr>
</tbody>
</table>

aDF, degree of freedom.

Chi-square test of *L. monocytogenes* occurrence among facilities

<table>
<thead>
<tr>
<th>L. monocytogenes occurrence</th>
<th>Facilitya</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td></td>
<td>28</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>11</td>
<td>39</td>
<td>16</td>
</tr>
<tr>
<td>Chi-Square</td>
<td></td>
<td>4.38</td>
<td>2</td>
<td>0.112</td>
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<td></td>
<td>4.454</td>
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</tbody>
</table>

aDF, degree of freedom.

BACTERIAL ALPHA DIVERSITY DISTRIBUTIONS DETERMINED FOR SAMPLES COLLECTED FROM PACKING FACILITIES, F1, F2 AND F3.

MYCOBIOME ALPHA DIVERSITY DISTRIBUTIONS DETERMINED FOR SAMPLES COLLECTED FROM PACKING FACILITIES, F1, F2 AND F3.

CLUSTERING OF BACTERIAL COMMUNITIES BASED ON THE UniFrac DISTANCES CALCULATED USING 16S rRNA V4 GENE SEQUENCES

CLUSTERING OF FUNGAL COMMUNITIES BASED ON THE UniFrac DISTANCES CALCULATED USING ITS2 SEQUENCES

RELATIVE ABUNDANCES OF BACTERIAL FAMILIES IN SAMPLES COLLECTED IN THREE APPLE PACKING FACILITIES

In association with presence (+) or absence (-) of *L. monocytogenes*

In function of the location (washing, fan drying and waxing) within facilities

RELATIVE ABUNDANCES OF FUNGAL FAMILIES IN SAMPLES COLLECTED IN THREE APPLE PACKING FACILITIES

In association with presence (+) or absence (-) of *L. monocytogenes*

In function of the location (washing, fan drying and waxing) within facilities

**SUMMARY**

- *L. monocytogenes* sporadically occurs in apple packing houses; however, in some facilities the contamination can become widespread and persistent.

- Higher bacterial and fungal diversity is associated with reduced occurrence of *L. monocytogenes* in packing houses. Whereas lower alpha diversity is associated with higher occurrence and persistence of *L. monocytogenes* in fruit packing facilities.

- Increased abundance of *Pseudomonadaceae* and *Dipodascaceae* organisms positively correlated with *L. monocytogenes* persistence in fruit processing environments.

- Current study provides baseline data needed for further in-depth investigation of microbial interactions between non-pathogenic and pathogenic microorganisms found in food processing environments.

- Further research in the area may lead to the optimization of pathogen control strategies and the development of novel, complementary, biocontrol methods to improve food safety.
HYPOTHESIS:

If apples become contaminated by *L. monocytogenes* during or after washing operations, wax coating may facilitate the survival of this pathogen on whole fruit during subsequent storage.

OBJECTIVES:

- Determine if apple coating with wax can facilitate the survival of *L. monocytogenes* on whole apples during long-term (< 5 months) storage
- Determine the effect of apple cultivar on the survival of *L. monocytogenes* in apple calyces and stem areas during prolonged cold storage
- Compare the survival of *L. monocytogenes* strains from the caramel apple outbreak, other outbreaks, tree fruit production environments and apples during prolonged cold storage
Red Delicious, Granny Smith and Fuji apples were spot-inoculated in calix and stem areas with a six-strain cocktail of *L. monocytogenes*. Half of inoculated fruits were subjected to coating with shellac wax. *L. monocytogenes* populations in waxed and un-waxed apples were enumerated in the course of prolonged storage at 4°C.
Changes in *L. monocytogenes* populations in waxed (—○—) and un-waxed (—●—) apples were assessed by direct plating (LOD 1.39 log CFU/apple; dotted line) and by the MPN analysis (—△— in waxed and —▽— in un-waxed apples). Data represent the means ($n = 10$) ± s.e. Asterisks (black for direct plating and red for MPN) indicate values that are statistically significantly different ($p < 0.05$) in waxed from corresponding values in un-waxed apples.
MODERATE BUT SIGNIFICANT EFFECT OF CULTIVAR

Table 1. The effect of apple cultivar on *Listeria monocytogenes* survival on un-waxed apples

<table>
<thead>
<tr>
<th>Enumeration Method</th>
<th>Cultivar</th>
<th>Storage time (days)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>16</th>
<th>30</th>
<th>62</th>
<th>93</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>16</td>
<td>30</td>
<td>62</td>
<td>93</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Granny Smith</td>
<td>a 3.85 (0.09)</td>
<td>a 3.93 (0.10)</td>
<td>a 3.74 (0.10)</td>
<td>a 3.48 (0.08)</td>
<td>a 3.06 (0.22)</td>
<td>a 2.97 (0.27)</td>
<td>a 2.79 (0.41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red Delicious</td>
<td>a 3.99 (0.07)</td>
<td>ab 3.74 (0.05)</td>
<td>ab 3.61 (0.07)</td>
<td>ab 3.20 (0.14)</td>
<td>a 2.83 (0.21)</td>
<td>b 2.53 (0.17)</td>
<td>b 1.89 (0.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fuji</td>
<td>b 5.13 (0.06)</td>
<td>ac 3.85 (0.04)</td>
<td>ac 3.83 (0.04)</td>
<td>ac 3.65 (0.05)</td>
<td>b 3.51 (0.03)</td>
<td>a 3.20 (0.05)</td>
<td>a 2.60 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Granny Smith</td>
<td>2.01 (0.53)</td>
<td>a 1.22 (0.28)</td>
<td>a 0.62 (0.50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red Delicious</td>
<td>1.76 (0.13)</td>
<td>ab 0.17 (0.18)</td>
<td>b 0.41 (0.30)</td>
<td>a 0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fuji</td>
<td>2.54 (0.14)</td>
<td>ac 0.97 (0.20)</td>
<td>a 1.30 (0.42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent the means (n = 10) and numbers in parenthesis indicate the standard error (s.e.). For direct plating data, values in the same column that are preceded by a different letter are significantly (p < 0.0167) different from each other. For MPN data, values in the same column that are followed by a different letter are significantly (p < 0.0167) different from each other.
**MODERATE BUT SIGNIFICANT EFFECT OF CULTIVAR**

**Table 2. The effect of apple cultivar on *Listeria monocytogenes* survival on waxed apples**

<table>
<thead>
<tr>
<th>Enumeration Method</th>
<th>Cultivar</th>
<th>Storage time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Direct plating, log CFU/apple</td>
<td>Granny Smith</td>
<td>a 3.39 (0.09)</td>
</tr>
<tr>
<td></td>
<td>Red Delicious</td>
<td>b 2.94 (0.12)</td>
</tr>
<tr>
<td></td>
<td>Fuji</td>
<td>c 3.92 (0.15)</td>
</tr>
<tr>
<td>MPN, log CFU/apple</td>
<td>Granny Smith</td>
<td>2.32 (0.19) a</td>
</tr>
<tr>
<td></td>
<td>Red Delicious</td>
<td>2.32 (0.14) a</td>
</tr>
<tr>
<td></td>
<td>Fuji</td>
<td>2.48 (0.17) a</td>
</tr>
</tbody>
</table>

Values represent the means (n = 10) and numbers in parenthesis indicate the standard error (s.e.). For direct plating data, values in the same column that are preceded by a different letter are significantly (p < 0.0167) different from each other. For MPN data, values in the same column that are followed by a different letter are significantly (p < 0.0167) different from each other.
Survival of outbreak, food, and environmental strains of Listeria monocytogenes on whole apples as affected by cultivar and wax coating

Dumitru Macarisin1, Ishani Sheth1, Minji Hur1, Anna Wooten1, Hee Jin Kwon1, Zhujun Gao1, Antonio De Jesus1, Wayne Jurick II2 & Yi Chen1

The 2014–2015 U.S. nationwide outbreak of listeriosis linked to apples used in commercially produced, prepackaged caramel apples was the first implication of whole apples in outbreaks of foodborne illnesses. Two case patients of this outbreak didn’t consume caramel apples but did eat whole apples, suggesting that contaminated whole apple may serve as a vehicle for foodborne listeriosis. The current study evaluated the effect of conventional fruit coating with wax and that of apple cultivar on the survival of outbreak-associated and non-outbreak L. monocytogenes strains on Red Delicious, Granny Smith and Fuji apples during 160 days under simulated commercial storage. L. monocytogenes survived in calyxes and stem ends of apples of all 3 cultivars through the duration of the experiment. After 2 months of storage, significantly (p < 0.05) larger L. monocytogenes populations were recovered from apples coated with wax than those un-waxed, regardless of the cultivar. No differences in survival amongst L. monocytogenes strains (serotypes 1/2a and 4b) from clinical, food, and environmental sources were observed. The observation that coating with wax facilitates prolonged survival of L. monocytogenes on whole apples is novel and reveals gaps in understanding of microbiological risks associated with postharvest practices of tree fruit production.
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