CENTER for PRODUCE SAFETY

CPS 2011 RFP
FINAL PROJECT REPORT

Project Title
Distribution of *Salmonella* in pistachios and development of effective sampling strategies

Project Period
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Objectives
1. To determine the prevalence, concentration and distribution of *Salmonella* in U.S. pistachios for 2 crop years.
2. To develop cost-effective sampling strategies that could be used by the pistachio industry to evaluate the microbial status of raw pistachios.

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FINAL REPORT

Abstract
Nuts and other low-moisture foods have generally been considered low-risks for foodborne illness because they are consumed in a dry state where water activity (available moisture) is too low to support microbial growth. Over the past 14 years outbreaks associated with consumption of raw almonds, hazelnuts, pine nuts, and peanut butter have been documented in the U.S. and elsewhere. In 2009 and 2013 there were recalls of pistachios after *Salmonella* was isolated from U.S. product. These recalls lead to an increased interest in identifying sources and routes of contamination of this organism in pistachios. Immediately after hulling, pistachios are passed through a float tank containing water that separates nuts on the basis of density. About 85% of pistachios are fully formed with nuts that fill the shell; these nuts sink and are called "sinkers." The remaining 15% include pistachios that are smaller, less developed nuts and those with insect damage or adhering hull; these nuts float and are called "floaters." Raw inshell floater and sinker pistachios from the 2010, 2011, and 2012 harvests were collected within 2 months of harvest from seven collaborating pistachio processors representing 98% of California pistachio production. Twelve approximately 1-kg samples were collected from each storage silo holding between 1 to 1.5 million pounds of pistachios; 3,968 samples were collected over the 3 years. The overall prevalence of *Salmonella* in 100-g subsamples was 2% for floaters and 0.4% for sinkers, with an overall combined weighted prevalence of 0.6%. Levels of *Salmonella* in positive samples were low; an average of 1 MPN (or cells)/100 g was detected in floater samples (range 0.1 to 8 MPN/100 g) and 0.2 MPN/100 g was detected in sinker samples (range 0.1 to 0.6 MPN/100 g). In 2012, samples were further categorized into those that were harvested in the "first shake," "second shake", or were "mixed" (combination of first and second shake). All of the positive samples collected in 2012 came from silos containing "second shake" or "mixed" pistachios. At the prevalence and levels of *Salmonella* found in this study, processors can increase the probability of detecting *Salmonella* in pistachio floater samples by enriching 250 to 375 g of sample that has been collected from throughout a silo. In contrast three to four 375 g enrichments would be needed to reliably detect *Salmonella* in positive sinker samples. A relatively narrow range of *Salmonella* serovars (nine total) were isolated from pistachios over the 3 years of this study; several serovars were isolated in more than 1 year and often across multiple samples within a year. These findings suggests that contamination of pistachios may occur during postharvest handling, and that some strains of *Salmonella* may be resident in the environment in and around some pistachio hulling and storage facilities. The data generated by this research are being used to update and improve a Quantitative Microbial Risk Assessment (QMRA) for pistachios. We are also working with the pistachio industry to identify opportunities to reduce prevalence and levels of *Salmonella* in pistachios particularly in the floater stream.

Background
Nuts and other low-moisture foods are generally considered low-risks for foodborne illness because they are consumed in a dry state where water activity (available moisture) is too low to support microbial growth. However, it is increasingly recognized that many foodborne pathogens can cause illness at very low concentrations, such that microbial growth is not required. In the past decade, foodborne illness outbreaks associated with consumption of raw almonds, pine nuts, hazelnuts, and peanut butter have been documented in the U.S. In 2009 there was a large recall of pistachios after *Salmonella* Montevideo was isolated from commercial products, and in 2013 a smaller recall was also triggered by isolation of *Salmonella* Senftenberg from pistachios that was also epidemiologically linked to several illnesses across the U.S. However, with the exception of almonds, very little is known about the prevalence, levels, and distribution of *Salmonella* in nut production and processing environments; these data are important to develop robust quantitative microbial risk assessment (QMRA) and for developing scientifically-sound product sampling schemes for verification of food safety plans.

One objective of this research was to determine the prevalence (in 100-g samples), levels, and distribution of *Salmonella* in U.S. pistachios over a 2-year period. *Salmonella* isolates were characterized as a means of providing insight into potential routes of contamination, with the long-term goal of identifying appropriate mitigation strategies. A second objective was to use these data to evaluate cost-effective sampling strategies that could be applied by the pistachio industry to evaluate the microbial status of raw pistachios.

Research Methods and Results

Prevalence of *Salmonella* in California pistachios
Raw inshell pistachio samples from the 2011 and 2012 harvests were collected within 2 months of harvest and stored at 4°C. Previous studies have demonstrated that *Salmonella* does not decrease on inshell pistachios stored under these conditions (Kimber et al., 2012). Seven collaborating pistachio processors representing the majority of California pistachio production (about 98%) participated in the survey. The number of samples collected from each collaborator roughly corresponded to their proportion of the approximate crop volume. Pistachios were coded to blind the samples, and then stored and processed for *Salmonella* by the American Council for Food Safety and Quality (ACFSQ) (Fresno, Calif.).

During the harvest process, pistachios are removed from the tree, transported to a hulling facility where the hulls are removed and the nuts are then dried and stored. Immediately after hulling but before drying, pistachios pass through a float tank that separates nuts on the basis of density – nuts that fill the shell are heavier and sink while smaller nuts and those with insect damage or adhering hull float. Nuts that sink are called "sinkers" (~85% of the typical crop) and nuts that float are called "floaters" (~15% of the typical crop). Sinker and floater nuts are handled independently after initial separation and are dried and stored separately. An evaluation of data from a 2010 survey (generated prior to receiving the current grant funding) indicated a potentially higher prevalence of *Salmonella* in floater samples. Therefore, we chose to analyze a disproportionate number of floaters in 2011 and 2012 (35% and 27% of samples, respectively). Pistachios are stored in silos that hold 1 to 1.5 million pounds of product and have from three to 10 sampling ports (88% have five to eight sampling ports). With a couple of exceptions, a total of 12 samples of ~1 kg each were taken from each silo. For two silos in 2012 a total of 24 samples were collected and from one silo a total of 28 samples were collected.

A total of 2,816 pistachio samples were collected and analyzed in 2011 and 2012. Because the data from 2010 (1,152 samples) were important to the overall evaluation of the survey data they are included throughout the results and discussion. Thus a total of 3,968 samples were analyzed and a total of 32 positive samples (21 floaters and 11 sinkers) were identified over the 3-year survey. The average prevalence of *Salmonella* in 100-g samples of pistachios was 0.62% based on a weighted average in relative proportion to the amount of floaters and sinkers produced each year (Table 1). The prevalence of *Salmonella* was significantly higher in floater pistachios (average 2%) than sinker pistachios (average 0.4%).

Levels of *Salmonella* in positive pistachio samples
Each positive pistachio sample was evaluated using a three-tube MPN technique (3 X 50 g, 3 X 5 g, and 3 X 0.25 g). The average level of *Salmonella* in the 21 positive floater samples was 3.8 MPN/100 g with a range of 0.47 MPN/100 g (nine samples) to 48 MPN/100 g (one sample). None of the MPN tubes were positive for *Salmonella* in any of the sinker samples; the MPN for all 11 of the sinker samples was 0.47 MPN/100 g.

Distribution of *Salmonella* in positive pistachio samples
The remaining pistachios from the positive samples were subsampled in 50-g portions (from four to 15 portions in 2010 or 10 portions in 2011 and 2012) and each subsample was separately enriched for *Salmonella*. The majority of initially-positive sinker samples were negative upon retesting (9 of 11; Table 2); two sinker samples had four additional positive subsamples (4 of 17 and 4 of 14) in 2010. The number of additional *Salmonella*-positive subsamples for initially-positive floater samples ranged from 0 (2 of 21) to 7 (1 of 21). These additional samples were used to recalculate MPN values. In almost all cases the calculated MPN decreased with subsequent retesting. The average level of *Salmonella* in the 11 positive sinker samples was 0.23 ± 0.18 MPN/100 g, with a range of 0.10 MPN/100 g (one sample) to 0.62 MPN/100 g (one sample). The average level of *Salmonella* in the 21 positive floater samples was 1.1 ± 1.7 MPN/100 g, with a range of 0.14 MPN/100 g (two samples) to 8 MPN/100 g (one sample).

In 2012, on the recommendation of one of our industry partners, we also made note of whether or not the pistachios were harvested from the first or second shake of the tree. Pistachio trees are not always shaken twice during harvest, but "second shake" nuts often have softer hulls because it is later in the season and the fruit is more mature. A silo was considered positive if any of the initial 12 samples collected from that silo were positive. One of 84 (~1%) sinker silos evaluated yielded a positive sample; 31% (10 of 32) of the floater silos yielded one or more positive samples. All the positive floater samples came from silos that held second shake or mixed (first and second shake) nuts, and the single positive sinker sample also came from a second shake silo. First shake sinker and floater silos made up 46% and 25%, respectively of the total sampled.

Distribution of *Salmonella* in initially-negative pistachio samples
Initially-negative pistachio samples were also retested in 50-g portions. In 2010, the decision to retest samples was made at the end of the survey after most of the samples had been discarded. Ten sinker samples were each retested by enrichment of 10 50-g portions. Of the 10 sinker samples retested, nine were negative for all 10 subsamples, and one yielded a single positive.

A random number generator was used to select 10 or 20 initially-negative samples for retesting in 2011 and 2012, respectively. A total of 10 subsamples were enriched for *Salmonella* from each of these samples. In 2011, four of these samples were floaters and six were sinkers. Of the 40 floater subsamples tested, one was positive; none of the 60 sinker subsamples was positive on retesting. In 2012, four of the initially-negative samples were floaters and 16 were sinkers. Of the 40 floater subsamples tested, one was positive; none of the 160 sinker subsamples was positive on retesting.

***Salmonella* serovars isolated from pistachios**
Three isolated colonies from each initially-positive enrichment were purified and serotyped. All initial three isolates from a sample were the same serotype, with the exception of one sinker sample in which a second serovar was identified. Isolates from each positive MPN tube were also serotyped as well as any isolates from secondary 50-g subsample enrichments. The serovars isolated from MPN subsamples were often, but not always, the same as the initial isolate. More than one serovar of *Salmonella* was isolated from 14 of the 32 positive samples; three or four different serovars of *Salmonella* were isolated from eight different pistachio samples. All isolates over the three years were one of nine different serovars (Table 3). *Salmonella* Montevideo was isolated from 66% of the samples (45%, 50%, and 87% of samples in 2010, 2011, and 2012).

Pulsed Field Gel Elecrophoresis (PFGE) patterns (fingerprints) for *Salmonella* isolates
PFGE patterns were determined to provide further information on the diversity of the *Salmonella* isolates. There were two PFGE patterns of *Salmonella* Montevideo isolated in each of the 3 years (of 11 isolates evaluated) and two PFGE patterns of *Salmonella* Worthington (of 10 isolates evaluated) over 2 years. *Salmonella* Enteritidis PFGE patterns were the same within each of the three phage types identified (PT9c, PT37, and RDNC). Three different *Salmonella* Liverpool PFGE patterns were noted for the eight isolates. Two PFGE patterns differing by a single band were noted for the eight *Salmonella* Senftenberg isolates.

Cost-effective sampling strategies for testing raw floater pistachios
To evaluate sampling strategies for testing raw pistachios some assumptions needed to be made. Specifically it was assumed that the specificity of the analytical method for detecting *Salmonella* in 100 g of pistachios is 99.9% accurate due to culture confirmation, hence, the probability of a false positive is only 0.1%. It was also assumed that the analytical methods used are capable of reliably detecting one or more cells of *Salmonella*, that the *Salmonella* are Poisson distributed throughout the samples, and that the overall MPN values estimated represent the mean of this Poisson distribution. Based on these assumptions, the sensitivity of detecting *Salmonella* contamination in 100 grams of pistachios is 56% when an unweighted arithmetic mean of all samples of 0.82 MPN/100 g was used (floaters and sinkers combined), and 36% when an unweighted median of all samples of 0.45 MPN/100 g was used (floaters and sinkers combined) for the average concentration of *Salmonella* contamination. Based on these numbers the sensitivity of detecting *Salmonella* would be over 90% if 300 g were analyzed (0.82 MPN/100 g) or 600 g were analyzed (0.45 MPN/100 g) (Table 4).

A second approach to determining appropriate sample size was also taken. The International Commission on the Microbiological Criteria for Foods has an online spreadsheet that can be used for such purposes (http://www.icmsf.org/main/software_downloads.html). The data generated here for levels of *Salmonella* in positive floater and sinker samples were used to evaluate an appropriate sample size based on the desired probability of finding *Salmonella* should the organism be present. They assume that any positive sample would be contaminated at the levels found in this study.

Both the initial MPN/g calculated (see Levels of *Salmonella* in Positive Samples above) as well as the MPN calculated using all samples (see Distribution of *Salmonella* in Positive Samples above) were used. It is usually recommended that a sampling plan achieve at least a 95% probability of detecting a positive in a positive lot (5% or lower values in the Table 5). For floater silos contaminated at levels reported here, a 250 g or greater subsample would provide an estimated 97% likelihood of detecting a positive. For sinker samples, three 375 g samples would be needed to provide a 95% assurance of finding *Salmonella* in a positive lot.

Outcomes and Accomplishments
The data generated by this project are being used to complete a QMRA for pistachios that was initiated in a previously funded CPS project. In addition, on July 17, 2013, the Food and Drug Administration posted a formal request for raw data that will be used to develop a tree nut risk assessment (risk of salmonellosis associated with eating tree nuts) over the next 1 to 2 years. We worked closely with the pistachio industry to organize the data generated from this grant to include in their formal data submission to the docket that was due December 16, 2013 ((Docket No. FDA-2013-N-0747) Assessment of the Risk of Human Salmonellosis Associated with the Consumption of Tree Nuts; Request for Comments, Scientific Data and Information).

Summary of Findings and Recommendations
The overall weighted prevalence of *Salmonella* in raw California inshell pistachios in 100-g samples determined for nearly 4,000 samples collected over three harvests (2010, 2011, and 2012) was 0.6%. This finding is similar to the prevalence of *Salmonella* observed for other tree nuts: e.g. almonds at 1% prevalence (Lambertini et al., 2012), and inshell pecans at 0.8% prevalence (Danyluk personal communication). The overall levels of *Salmonella* determined for positive samples (average 0.8 MPN/100 g or 0.008 MPN/g) were also similar to those reported in other nuts and other low moisture foods.

However, there was a significant difference in the overall prevalence of *Salmonella* in sinker (0.4%) and floater pistachios (2%). The average level of *Salmonella* in sinkers (0.2 MPN/100 g) was also significantly lower than that of floaters (1 MPN/100 g). In addition, more than one positive sample was not typically identified from the 12 samples collected from a single sinker silo. The sinker stream makes up roughly 85% of the total volume by weight of the annual crop (Figure 1). The majority of sinkers (90%) are inshell product with small amounts of kernel (3%), shell (5%), and inedible kernel (2%). In contrast, the 15% floater stream is largely inedible kernel (50%) with relatively small amounts of inshell (10%) and kernel (15%).

The data suggest that the higher prevalence and levels of *Salmonella* in floater pistachios is driven by a subset of silos that are much more contaminated than the rest. Data from 2012 suggest that this contamination is strongly associated with pistachios that are "second shake." For these floater silos the prevalence of *Salmonella* among the samples analyzed ranged from 14 to 100% (average 48% positive 100-g samples from 11 silos). The level of *Salmonella* in these silos was the same as the calculated overall average (0.008 MPN/g). These silos each contain 1 to 1.5 million pounds of pistachios and even though only 25% of the weight is edible product, the amounts are significant. It is not known if the prevalence of *Salmonella* from these silos would be similar after the product was sorted and shelled. While most pistachios are treated by one or more processes that have been validated to reduce *Salmonella*, large volumes of pistachios, even when contaminated at low levels, pose a risk of contaminating the both the equipment and facility in which they are handled. This increases the risk of a post-processing recontamination event.

To maintain optimum quality, pistachio processors target short times between shaking nuts from the tree to the time the hull is removed. Based on data from our earlier CPS-funded study *Salmonella* can grow on harvested pistachios under temperatures and humidities that can be achieved in harvest trailers that are held for several hours. Increases in levels of *Salmonella* are significant after 6 hours. *Salmonella* can also multiply in hulled pistachios when there are delays between hulling and drying. Because the prevalence of *Salmonella* was lower in sinker pistachios it is likely that the cause of the higher prevalence in a number of floater silos occurred after the float tank. The association with second shake pistachios may be related to the condition of the hulls that adhere to a greater portion of hulled floaters but also suggests the possibility of delays between hulling and drying.

The isolation of a narrow range of *Salmonella* serovars and PFGE patterns over the 3 years of this study suggests that several strains of *Salmonella* may have established resident and persistent populations at one or more of the pistachio handlers that participated in this study. It is possible that several silos are contaminated with these *Salmonella*. Cleaning and sanitizing silos is challenging and it is possible that the reoccurring contamination of floaters is due to an introduction of *Salmonella* after the pistachios are dried and as they are loaded into these silos. However, this explanation does not explain the strong association of positive samples with second shake pistachios and especially with floaters.

Pistachios were sampled through silo sample ports using a sample trier. The sampling was not supervised by UC Davis but was undertaken by a contract laboratory. Although the triers are sprayed with a 70% ethanol solution between each sample, the construction of each triers are complex making sanitation a challenge. It is possible that the sanitation step was inadequate and resulted in some cross-contamination among samples. This might explain why some of the floater silos had a higher prevalence of *Salmonella* but would not fully explain why the positives were consistently associated with floater samples in each of the 3 years since the same triers were used to collect both floater and sinker samples.

Although all of the pistachios from this survey have already been processed and distributed, it is strongly recommended that the participants in this study closely evaluate their floater stream for future crops. Particular attention should be given to those silos that contain second shake or mixed pistachios. Analyzing a single 250 to 375-g sample of pistachios per silo by enrichment (assuming subsamples are taken from throughout the silo and well mixed) should give a reasonable likelihood of finding *Salmonella* if present at the levels observed in 2012. If positives are found, these facilities should handle pistachios from these silos with caution and they should consider performing a root cause analysis with the goal of developing an action plan to 1) reduce prevalence of *Salmonella* in floater pistachios and 2) eradicate potentially resident populations of *Salmonella*.

The overall results from sinker pistachios provide substantial data demonstrating that production of pistachios with low prevalence and levels of *Salmonella* is possible. By focusing on identifying the root cases for contamination of second shake floater pistachios the U.S. pistachio industry should be able to implement targeted mitigation strategies that will further reduce the overall prevalence of *Salmonella* in this commodity.

Cited References

Kimber, M. A., H. Kaur, L. Wang, M. D. Danyluk, and L. J. Harris. 2012. Survival of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at –19, 4, and 24°C. J. Food Prot. 75:1394–1403.

Lambertini, E., M. D. Danyluk, D. W. Schaffner, C. K. Winter, and L. J. Harris. 2012. Risk of salmonellosis from consumption of almonds in the North American market. Food Res. Int. 45:1166–1174

APPENDICES

Publications and Presentations (required)

No publications to report.
No presentations specific to these data to report.

Budget Summary (required)

The funds were spent as outlined in the original budget. A significant amount of the funds were used to process the initial samples for *Salmonella*. UC Davis staff determined the levels and distribution of *Salmonella* in initially-positive and in initially-negative samples. Staff at UC Davis processed all of the *Salmonella* isolates including adding them to the culture collection, ensuring they were serotyped, phage typed when necessary, and that PFGE patterns were determined.

The funds were sufficient to implement the project as proposed.

Tables and Figures (optional)

Table 1. Prevalence of *Salmonella* 2010-2012

Year	No. samples (sinkers/floaters)	No. positive samples (sinkers/floaters)	% positive sinkers/floaters	Total % positive proportionate to crop volume[1]
2010	1152 (984/168)	11 (7/4)	0.71/2.38	0.96
2011	1380 (900/480)	6 (3/3)	0.33/0.63	0.38
2012	1436 (1052/384)	15 (1/14)	0.1/3.64	0.63
Total/Average	3968 (2935/1033)	32 (11/21)	0.37/2.03	0.62

[1]Assumes 85% sinkers and 15% floaters.

Table 2. Additional positive 50-g subsamples identified in initially-positive pistachio samples in 2011 and 2012 (30 samples total)

Number of additional positive samples	Sinker	Floater	Total
0	9	2	11
1	0	6	6
2	0	5	5
3	0	3	3
4	2	2	4
5	0	1	1
6	0	1	1
7	0	1	1

Table 3. *Salmonella* serotypes isolated from positive pistachio samples (2010 through 2012).

Salmonella serotype	Number of the 32 samples with serotype (% of total samples with serotype)	Year when serotype isolated (no. of samples with serovar)
Agona	2 (6)	2010 (1), 2012 (1)
Enteritidis Phage Type: RDNC	3 (9)	2010 (1), 2011 (1), 2012 (1)
Enteritidis Phage Type: 9c	2 (6)	2010 (1), 2011 (1)
Enteritidis Phage Type: 37	1 (3)	2010 (1)
Liverpool	8 (25)	2011(1), 2012(7)
Montevideo	21 (66)	2010 (5), 2011 (3), 2012 (13)
Senftenberg	8 (25)	2010 (2), 2012 (6)
Tennessee	1 (3)	2010 (1)
Worthington	12 (38)	2010 (4), 2012 (8)
Total	57 isolates	

Table 4: Sensitivity to detect *Salmonella* contamination in samples of floater pistachios as a function of the number of replicate 100-g aliquots that are tested per batch

No. of 100-g aliquots	Sensitivity (arithmetic mean)[1]	Sensitivity (median)[1]
1	56%	36%
2	81%	59%
3	91%	74%
4	96%	83%
5	98%	89%
6	99%	93%

[1] Calculations of sensitivity based on using either the arithmetic mean of 1.1 MPN/100 g or the median of 0.63 MPN/100 g for the typical concentration of *Salmonella* contamination.

Table 5: Probability of a negative *Salmonella* test result when the sample is actually positive.

						375 g			
Pistachio	Mean	Sigma	100 g	200 g	250 g	1	2	3	4
Floater	-1.42[1]	0.52	2.56%	0.07%	0.01%	0.00	ND	ND	ND
	-1.94[2]	0.42	23.17%	5.37%	2.58%	0.42%	0.00	ND	ND
Sinker	-2.33[1]	0.05	62.47%	39.03%	30.85%	17.13%	2.93%	0.50%	0.09%
	-2.64[2]	0.26	76.33%	58.26%	50.90%	36.31%	13.19%	4.79%	1.74%

[1]Mean and Sigma from initial MPN calculations
[2]Mean and Sigma from MPN calculated using all samples tested

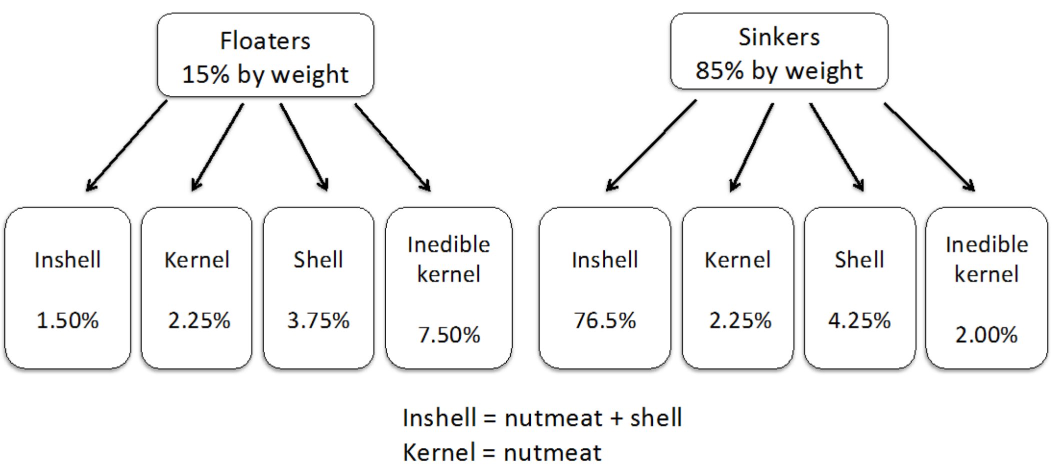

Figure 1. Proportion of edible product in sinker and floater streams of pistachios.

Suggestions to CPS (optional)
None.