CPS CENTER for PRODUCE SAFETY

CPS – SUMMER 2009 RESEARCH PROGRAM

FINAL PROJECT REPORT

Project Title

Impact of Almond Moisture, Almond Cultivar, and *Salmonella* Serovar on the Desiccation, Persistence, and Heat Resistance of *Salmonella* in Almonds

Project Period

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Objectives

Objective 1: To determine the impact of almond moisture and variety on the heat sensitivity of *Salmonella* Enteritidis PT30 inoculated onto almonds.

Objective 2: To evaluate differences in desiccation, storage and heat sensitivity among difference *Salmonella* isolates.

FINAL REPORT

Abstract
The purpose of this study was to evaluate the impact of almond moisture and cultivar on the heat sensitivity of *Salmonella* Enteritidis PT30 inoculated onto almonds and to evaluate differences in desiccation, storage and heat sensitivity among different *Salmonella* isolates. The heat resistance of *Salmonella* Enteritidis PT30 was determined on almonds equilibrated to moisture levels from 4 to 9%. Whole Mission and Nonpareil almonds inoculated with *Salmonella* Enteritidis PT30 were dried at 23°C for 3 days to moisture and water activity levels comparable to uninoculated almonds (4-5% moisture, 0.40-0.50 Aw). After drying, *Salmonella*-inoculated almonds were held at 23°C in the presence of saturated KI, NaCl, KCl or K_2SO_4 salts for 3 days. Almonds (10 g) were heated in 121°C hot oil for 1 min or in a laboratory oven at 135°C for 40 min. For almonds stored under ambient (30% RH) or saturated KI (68% RH), NaCl (75% RH), KCl (80% RH), or K2SO4 (90% RH), moisture and water activity levels were 5.1, 6.1, 6.5, 7.1, or 8.6% and 0.48, 0.60, 0.63, 0.70, or 0.77, respectively. Corresponding reductions in the populations of *Salmonella* on the surface of these almonds were 2.2, 3.5, 4.0, 4.7, or 5.1 log CFU/g, respectively, after the hot oil treatment. In contrast, no significant difference was observed in the reduction of *Salmonella* at 5 or 7% moisture when almonds were exposed to dry heat. No significant difference ($P > 0.05$) was observed in the reduction of *Salmonella* between Mission and Nonpareil varieties when moisture levels were the same. Relative humidity rapidly impacts the percent moisture and water activity of almonds and can significantly impact the efficacy of a thermal treatment. Moisture levels of inoculated almonds should be monitored in thermal validation studies. Moisture levels in almonds (5 or 7%) did not impact survival of *Salmonella* at room temperature over a 30-day period. *Salmonella* Enteritidis PT30 is more desiccation tolerant than other *Salmonella* isolates evaluated. At 121°C the heat sensitivity of four *Salmonella* isolates was similar in oil roasting. Survival of a six-strain cocktail of *Salmonella* on almonds stored at room temperature was similar to survival observed for *Salmonella* Enteritidis PT30. No reductions in population of *Salmonella* were observed at 4 or -20°C. These data indicate that risk assessments for almonds that are based on data derived for *Salmonella* Enteritidis PT30 are more broadly applicable to *Salmonella* spp.

Background
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. However, it is increasingly recognized that many foodborne pathogens can cause illness at very low concentrations, such that microbial growth is not required. Three outbreaks of salmonellosis associated with consumption of raw almonds have been documented in 2001, 2004, and 2006. Since 2001 significant advances have been made in understanding the ecology of *Salmonella* in the almond production and processing environment. Much of the work has been done with *Salmonella* Enteritidis Phage Type 30, the strain associated with a 2000-2001 outbreak and with the single almond cultivar Nonpariel. Although moisture levels are known to impact survival of *Salmonella* during thermal processing, a systematic evaluation of raw almond moisture that occurs in practice (3 to 8%) and heat resistance of *Salmonella* has not been made. The study sought to evaluate the impact of almond moisture and cultivar on the heat sensitivity of *Salmonella* Enteritidis PT30 inoculated onto

almonds and to evaluate differences in desiccation, storage and heat sensitivity among different *Salmonella* isolates.

Research Methods and Results

Almonds. Raw whole (untreated) Mission and Nonpareil almond kernels (size 25/27 or 27/30; 25 to 27 or 27 to 30 almonds per 28 g) were provided by Blue Diamond Growers (Sacramento, CA). Almonds were stored in sealed polyethylene bags (30.5 x 30.5 cm; Bitran, Com-Pac International, Carbondale, IL) inside a tightly sealed plastic tub at ambient temperature (23 ± 2°C) until inoculation.

Bacterial Strains. *E. coli* K12 was used to inoculate almonds for determination of moisture levels and water activity. *Enterococcus faecalis* (almond validation study surrogate) was used for some studies. *Salmonella* Enteritidis PT 30 (LJH 608,) *Salmonella enterica* serovars Enteritidis 9C (2004 raw almond outbreak), Enteritidis PT30 (ATCC BAA-1045; 2001 raw almond outbreak), Tennessee (peanut butter outbreak), Oranienburg (pecan isolate), Anatum (almond survey isolate), and Montevideo (pistachio isolate) were used. A spontaneous nalidixic acid resistant mutant was isolated and used for the inoculum cocktail (storage study). When nalidixic acid strains were used, 50 µg/ml nalidixic acid was incorporated into plating media. Isolates were stored at -80°C in tryptic soy broth (TSB; BD, Franklin Lakes, NJ) supplemented with 15% glycerol.

Preparation of inoculum. Methods that were developed previously were used to prepare the inoculum (Danyluk et al., 2005). Briefly, isolates were streaked onto tryptic soy agar (TSA; BD, Franklin Lakes, NJ) and incubated at 37°C for 24 ± 2 h. A single isolated colony was cultured overnight two times in TSB and incubated at 37°C for 24 ± 2 h. The overnight culture (1 ml) was spread over large 150 mm × 15 mm TSA plates to produce a bacterial lawn after incubation at 37°C for 24 ± 2 h. After incubation, 8 to 9 ml of 0.1% peptone was added to each TSA plate and the bacterial lawn was collected by scraping the slurry with sterile spreaders. Before inoculating the almonds, and where appropriate, inoculum preparations were pooled in equal volumes and thoroughly mixed. For the storage survival study, inoculum cocktail was diluted to 10^6 CFU/g with 0.1% peptone. Inoculum concentration was determined by plating the inoculum onto TSA and Bismuth Sulfite Agar (BSA).

Inoculation and storage of almonds. Almonds were inoculated as previously described (Danyluk et al., 2005) at a ratio of 400 g of almonds to 25 ml of inoculum. After mixing for 1 min to distribute the inoculum, almonds were spread onto two sheets of 46 x 57 cm filter paper (Fisher Scientific, Pittsburgh, PA) that was folded in half. The filter paper was placed on a metal rack inside a large plastic bin. Almonds were held with the lid slightly ajar for 3 days at 23 ± 2°C to moisture and water activity levels comparable to uninoculated almonds (4-5% moisture, 0.40-0.50 Aw). Levels of *Salmonella* were determined on the wet and dry almonds.

For the storage study, the dried inoculated nuts were stored in plastic zipper bags at room temperature for another 4 days (total 7 days at room temperature. Almonds were then transferred to a freezer (-20°C), refrigerator (4°C), or kept at room temperature (23°C) and sampled approximately monthly over 6 months to evaluate the survival of the pathogen.

Equilibration of moisture content using saturated salt solutions. Desiccator jars were modified to serve as humidity control chambers. The relative humidity in the jars was modified by using saturated solutions of KI, NaCl, KCl and K_2SO_4 and $MgCl_2$ salts. Almond samples (40 g) in duplicate were distributed in a single layer on weigh dishes that were placed in the sealed jars containing saturated salt solutions and equilibrated for 3 days at 23 ± 2°C. Saturated salt solutions were prepared by dissolving appropriate amount of salt in distilled water. Temperature and humidity were measured using monitors that were placed in each chamber (Sensitech, Inc.).

Hot oil treatment. Almonds were treated in hot oil as previously described (Du et al., 2010). Briefly, inoculated almonds (10 g) were placed in a wire mesh basket and immersed in safflower oil maintained at 121°C. The basket was moved slowly up and down in the oil to allow the equal distribution of temperature. Almonds were heated in oil for up to 2.5 min. The time for roasting was noted from the moment that mesh basket with almonds was immersed in hot oil. The oil was changed after heating approximately 50 samples.

Recovery of and enumeration of *Salmonella* on almonds. For the storage study, 10 g of almonds were added to 20 ml of 0.1% peptone. For the oil roasting study, almonds were removed from the oil bath after the treatment and drained for 10 s. Almonds were transferred immediately into a two-chamber filter bag (whirl-Pak, Nasco, Modesto, CA) that contained 20 ml of cold (4°C) TSB. Samples were stomached for 2 min at high speed in a two-chamber filter bag (Whirl-Pak, Nasco, Modesto, CA) using a Stomacher Lab Blender Model 400 (Seward).

Samples were serially diluted in BPB and plated in duplicate onto TSA and BSA. Plates were incubated for 24 ± 2 h at 37°C (TSA) and 48 ± 2 h at 37°C (BSA). Results were reported as the log of the number of survivors per gram of almonds. All the colonies on TSA plates were counted. BSA is a selective medium for *Salmonella*, only black centered colonies were counted on BSA plates.

Measuring the moisture content and water activity of almonds. Forty grams of *E. coli* K12-inoculated almonds and uninoculated almonds were homogenized for 20 s in food processor (Waring 2.5 Qt, Pro Food Processor, Torrington, CT) and sieved through a standard #12 testing sieve (1.7 mm, Fisher Scientific, Pittsburgh, PA). Four grams of the ground sample was placed on foil pan (0.6 X 10.2 cm) and moisture content was measured using a Halogen Moisture Analyzer (Mettler Toledo HG 63 Halogen Moisture Analyzer, Columbus OH). The ground, homogenized kernel samples were also used to measure water activity (Decagon Devices, AquaLab model 4TE, Pullman, WA). Moisture and water activity were determined for three separate samples and the results were averaged.

Outcomes and Accomplishments

Objective 1: To determine the impact of almond moisture and variety on the heat sensitivity of *Salmonella* Enteritidis PT30 inoculated onto almonds.

Moisture and water activity of uninoculated and E. coli *K12-inoculated almonds*
Almond moistures and corresponding water activity were successfully manipulated by storing the almonds in sealed jars with different saturated salt solutions. While there was some

variability, in general, almond moistures equilibrated after 3 days in the chamber. Humidity of approximately 30 to 98% were achieved in the chambers with corresponding almond moistures of 5 to 9% and water activities of 0.5 to 0.8, respectively (Table Appendix-1).

Moisture and water activity determinations require that the almonds are ground to a powder. This is difficult to safely do with pathogen-inoculated nuts. Therefore, we use *E. coli* K12 inoculated almond held under the same conditions as pathogen-inoculated nuts to measure moisture levels. The moisture and water activity for almonds stored under five different relative humidities was monitored over 3 days. No significant difference ($P > 0.05$) was observed between the uninoculated almonds and *E. coli* K12-inoculated almonds demonstrating the inoculation procedure does not adversely impact almond moisture levels and that uninoculated almonds may be used to determine the moisture levels in inoculated almonds held under the same conditions.

Impact of almond variety
No significant difference ($P > 0.05$) in reduction of *Salmonella* Enteritidis PT30 was observed in hot oil for Mission or NonPareil almonds equilibrated to either 4.8 or 8.4% moisture after heating in hot oil (121°C) for 1 min. Reductions on both cultivars of 1.7 and 4.8 log CFU/g were observed at 4.8 or 8.4% moisture, respectively. Reductions at 8.4% moisture were significantly ($P < 0.05$) greater than at 4.8% moisture.

Impact of moisture
Significant differences ($P < 0.05$) were observed in reduction of *Salmonella* Enteritidis PT30 on Mission almonds equilibrated to different moisture levels and heated in 121°C oil for 1 min. Reductions of 2.2, 3.4, 4.0, 4.6 and 5.0 log CFU/g were observed at 5.1, 6.1, 6.5, 7.1, and 8.6% moisture, respectively (Figure 1).

These differences were primarily due to difference in reduction observed upon initial introduction of the almonds in the oil (this non-linear curve was previously described by Du et al., 2010). After initial reduction, the rate of decline was very similar at the two moisture levels (Figure 2).

Impact of moisture during oil roasting vs dry roasting
Mission almonds were equilibrated to 5 or 7% moisture and heated in 121°C oil for 1 min or in a laboratory oven set at 135°C for 40 min (dry heat). Significantly greater reductions were observed at 7% moisture when the almonds were heated in hot oil but not with dry heat. Reductions in hot oil and under dry heat were 2.3 and 4.2 log CFU/g and 4.2 and 4.2 at 5 and 7%, respectively.

Impact of strain on survival of Salmonella *on almonds exposed to hot oil.*
Over 20 *Salmonella* isolates were screened for heat tolerance on almonds exposed to hot oil for 1 min. Strains with the lowest reduction after 1 min were selected for further study. Four isolates (*Salmonella* Enteritidis PT30 (2001 almond outbreak), *Salmonella* Enteritidis 9c (2004 almond outbreak), *Salmonella* Oranienburg (pecan isolate) and *Salmonella* Anatum (almond survey isolate) were selected for further study. Also included was the *Enterococcus faecalis* isolate approved as a surrogate for use in almond validation studies. Difference in survival during

drying after inoculation were noted. Survival curves were similar but not identical over the 4-minute oil treatment. After 3 minutes of heating reductions were not significantly different among the isolates tested.

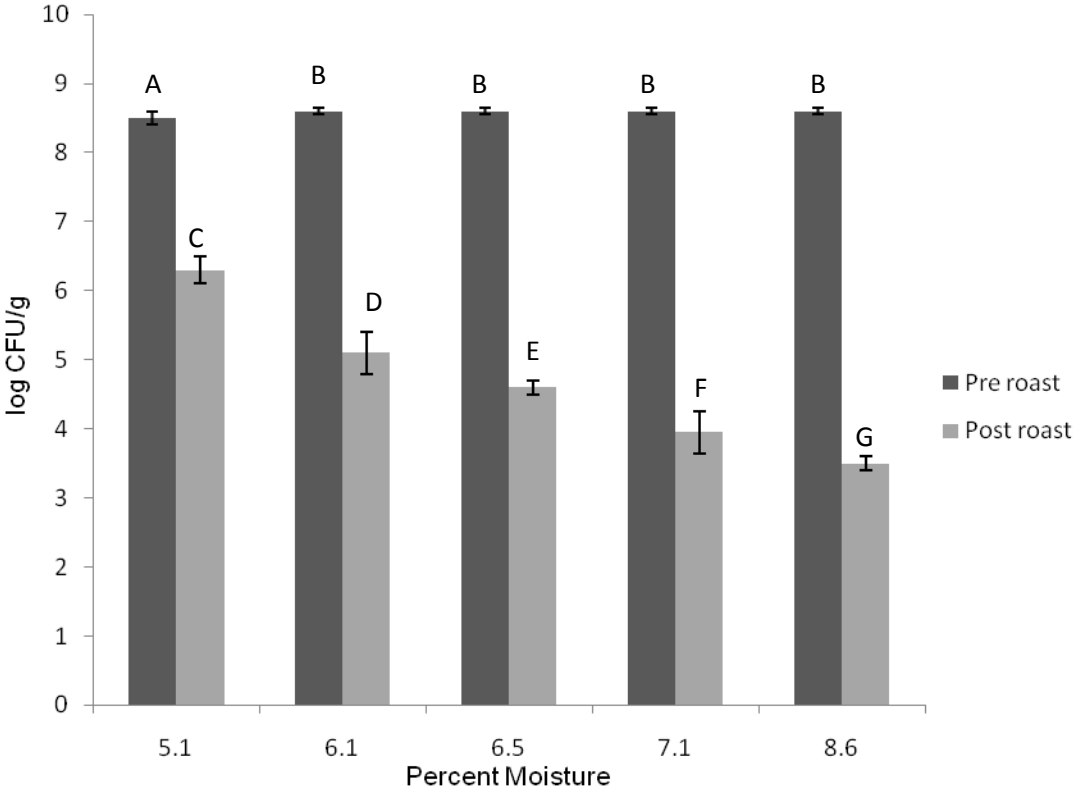

Figure 1. Reduction of *Salmonella* Enteritidis PT30 on Mission almonds stored for 3 days with different saturated salts and heated in hot oil at 121°C for 1 min. TSA and BSA were not significantly different; BSA shown (n=6), Within each level of moisture, bars that are not labeled by the same letter are significantly different ($P<0.05$).

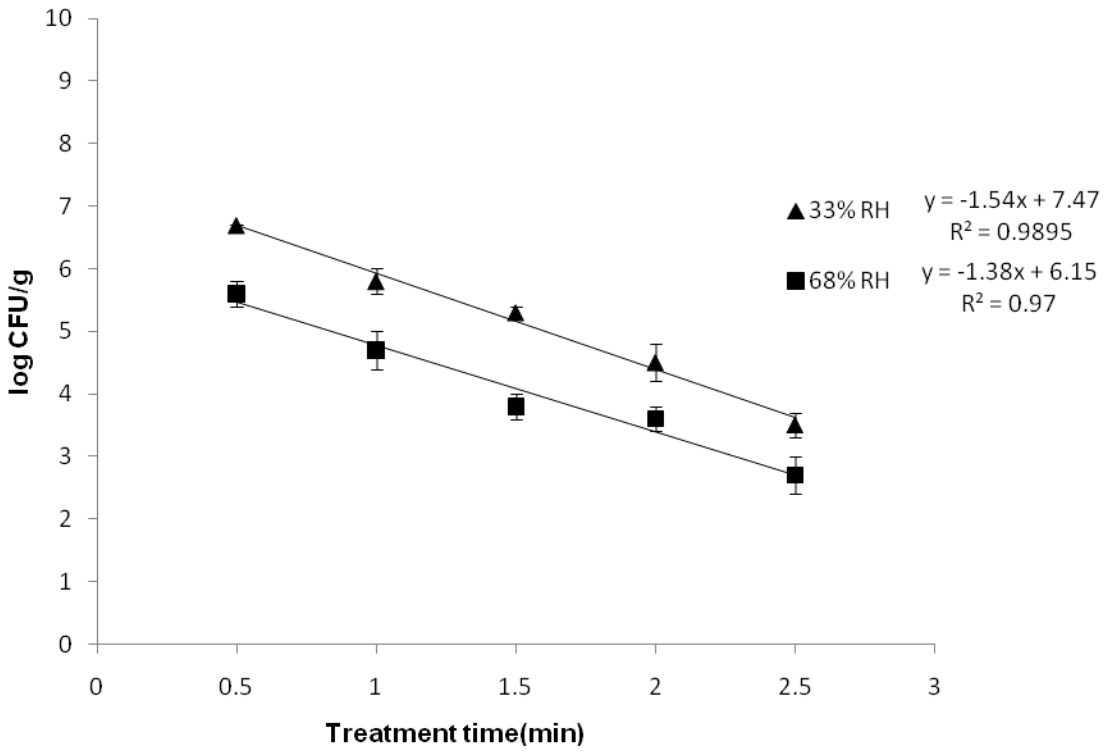

Figure 2. Survival of *Salmonella* Enteritidis PT30 on Nonpareil almonds stored at ambient (33% RH) or with saturated KI (68% RH) and heated in hot oil at 121°C for different times (BSA data shown).

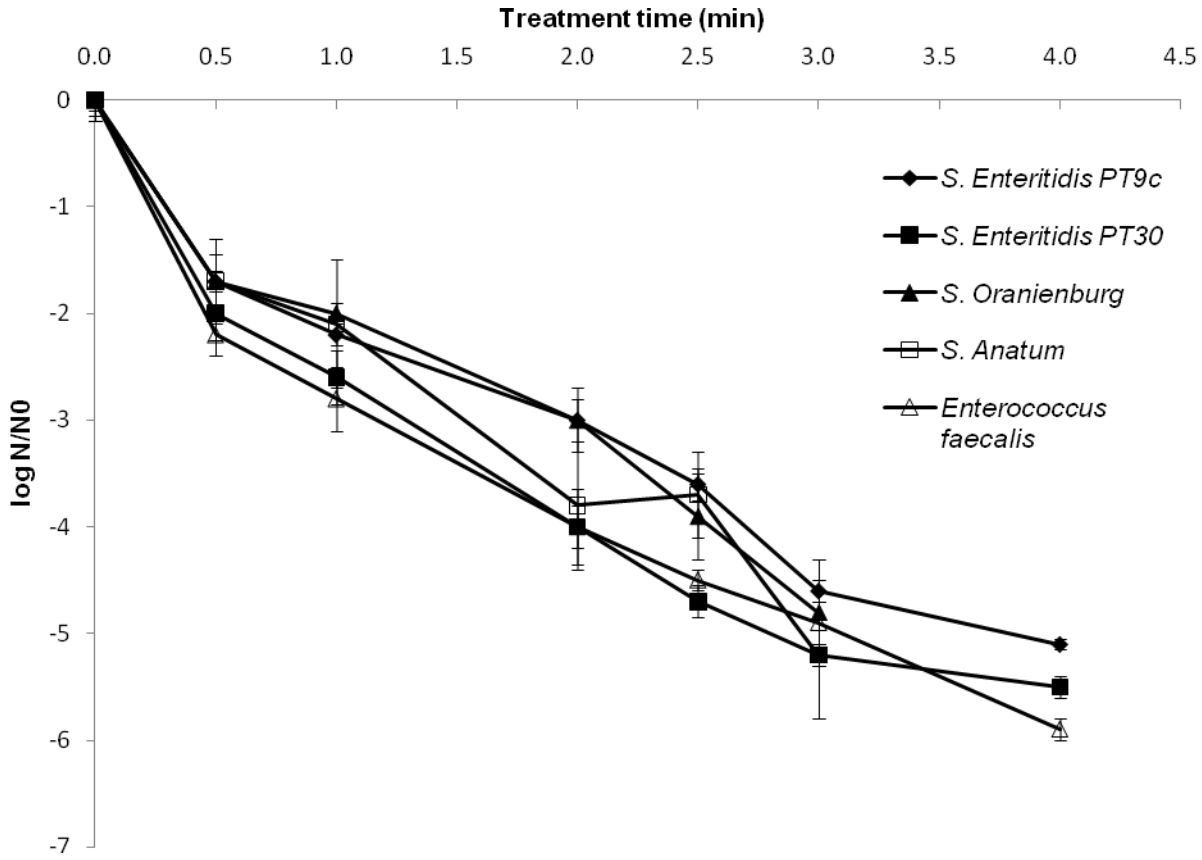

Figure 3. Impact of serovar on the heat resistance of *Salmonella* and *Enterococcus faecalis* inoculated on to Nonpareil almonds stored at ambient (33% RH) and heated in hot oil at 121°C for different times.

Objective 2: To evaluate differences in desiccation, storage and heat sensitivity among difference *Salmonella* isolates.

Desiccation tolerance among Salmonella *serovars*
Significant differences in reduction of *Salmonella* (as much as 1 log CFU/g) were observed among different *Salmonella* isolates (Figure 4). The underlying reasons for these differences are currently being explored in a separate study with a wider range of isolates.

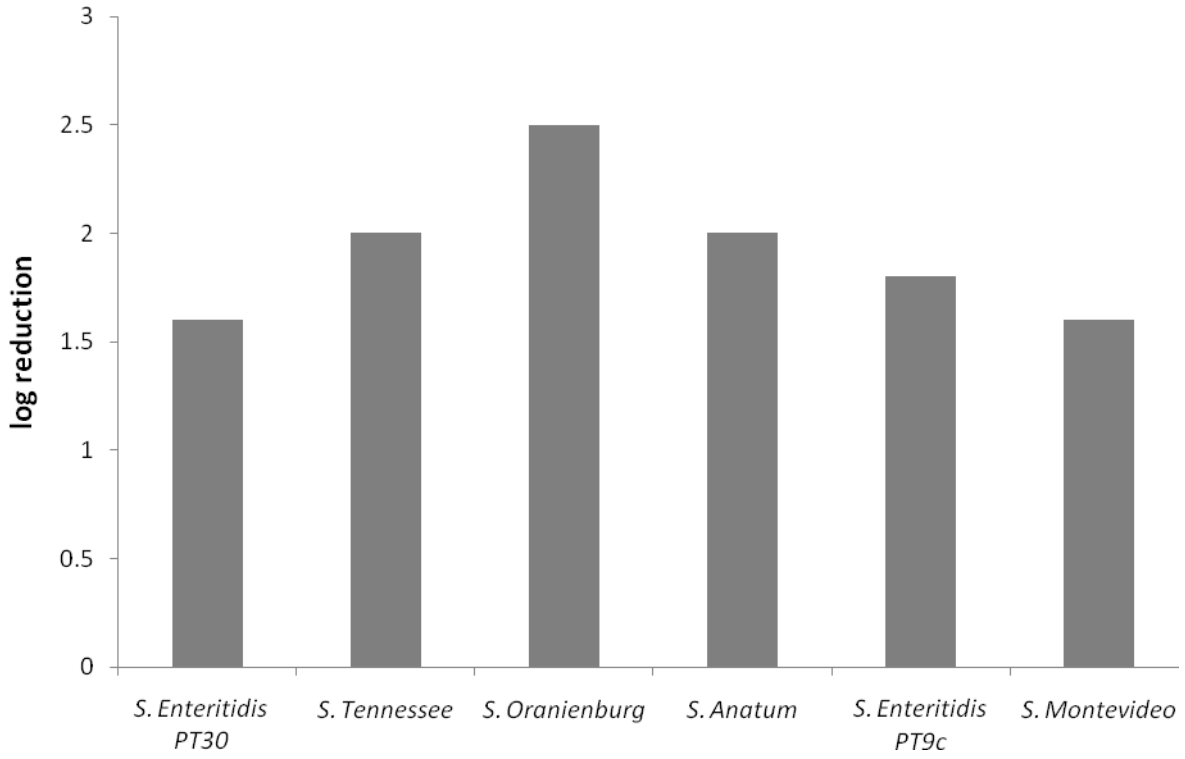

Figure 4. Survival of *Salmonella* serovars on inoculated almonds after 72 h of drying at ambient temperature.

Survival of Salmonella *Enteritidis PT30 and a cocktail of six strains of* Salmonella *on almonds.*
Data generated previously for survival of *Salmonella* Enteritidis PT30 were re-evaluated for this study. Six separate previously generated survival curves at 23°C, three at 4°C and one at -20°C were fit using both a linear model and exponential curve. The exponential model fit well for most but not all of the curves at 23°C. However, significant (P<0.001) linear relationships were observed for all survival curves at 23°C. Rates of decline ranged from 0.16 log CFU/month to 0.32 log CFU/month with an average of 0.23 ± 0.05 log CFU/month. The data generated in the current study with a six-strain cocktail fell within this range (reduction of 0.29 log CFU/month) confirming that data generated for *Salmonella* Enteritidis PT30 may be appropriately used to predict the behavior of other *Salmonella* on stored almonds. Similar to previous studies at 4 and -20°C, no reduction of the *Salmonella* cocktail was observed over a 6-month period.

Impact of humidity of survival of Salmonella *Enteritidis PT30 on almonds*
No difference in survival was observed for *Salmonella* Enteritidis PT30 on almonds stored for 30 days at ambient, 36, or 70% relative humidity.

Summary of Findings and Recommendations

Relative humidity rapidly impacts the percent moisture and water activity of almonds. In a short storage study (30 days) humidity (36 or 70%) did not impact the storage survival of *Salmonella*. However, moisture levels significantly impacted the efficacy of oil roasting with significantly

greater reductions observed at higher moisture levels. Moisture levels should be routinely monitored in thermal validation studies after inoculation and drying of almonds. The current standard methods for preparation of inoculated almonds should be modified to include longer drying periods after inoculation and evaluation of moisture and water activity prior to initiating the study. A target moisture level should be specified in the protocol. At 121°C the heat sensitivity of four *Salmonella* isolates was similar in oil roasting. The sensitivity of these isolates to 127°C should also be compared.

Salmonella Enteritidis PT30 is more desiccation tolerant than several other *Salmonella* isolates compared. Survival of a six-strain cocktail of *Salmonella* on almonds stored at room temperature was similar to survival observed for *Salmonella* Enteritidis PT30. No reductions in population of *Salmonella* were observed at 4 or -20°C. These data indicate that risk assessments for almonds that are based on data derived for *Salmonella* Enteritidis PT30 are more broadly applicable to *Salmonella* spp.

APPENDICES

Publications and Presentations (required)

Kaur, H., and L.J. Harris. 2010. Impact of almond moisture on the survival of *Salmonella* Enteritidis PT30 after exposure to hot oil. International Association for Food Protection, Anaheim, CA, August 1-4. (Abstract P3-47).

Budget Summary (required)

The funds requested were adequate to support the research. Salary and benefits of $57,801 were granted and $61,058 was expended; $21,000 in materials and supplies were granted, $20,145 was expended; $5,300 in travel were requested, $1,654 were expended. A total of $1,244 remains to support travel for the PI to the CPS research symposium in Orlando in June, 2011.

Tables and Figures (optional)

Table A-1. Moisture content and water activity of Mission and Nonpareil almonds either uninoculated or inoculated with *E. coli* K12 after storage in the presence of saturated K_2SO_4 (n=3).

	Uninoculated				Inoculated *E. coli* K12[a]	
Day	Moisture (%)	Water activity	Moisture (%)	Water activity
Mission				
0	4.8 ± 0.1	0.47 ± 0.0	4.9 ± 0.1	0.47 ± 0.0
1	6.1 ± 0.1	0.63 ± 0.0	6.2 ± 0.2	0.63 ± 0.0
2	7.2 ± 0.4	0.74 ± 0.0	7.3 ± 0.3	0.75 ± 0.0
3	8.5 ± 0.2	0.78 ± 0.1	8.6 ± 0.1	0.79 ± 0.0
Nonpareil				
0	4.5 ± 0.1	0.44 ± 0.0	4.5 ± 0.1	0.45 ± 0.0
1	5.4 ± 0.1	0.60 ± 0.0	5.5 ± 0.1	0.62 ± 0.0
2	6.7 ± 0.1	0.73 ± 0.0	6.9 ± 0.1	0.74 ± 0.0
3	8.5 ± 0.1	0.78 ± 0.0	8.6 ± 0.2	0.78 ± 0.0

[a] Almonds were inoculated with *E. coli* K12 and dried for 72 h under ambient conditions prior to exposure to salt solutions.

Suggestions to CPS (optional)

None.