CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Sources and mechanisms of transfer of *Salmonella* in the production and post-harvest tree nut environment

Project Period
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Objectives
1. *Objective 1a. Evaluate the microbial composition of bioaerosols and dust originating from livestock operations located in close proximity to almond and pistachio production areas in the California Central Valley.*

2. *Objective 1b. Evaluate the movement of microorganisms from livestock areas to nearby almond and pistachio orchards compared with control orchards not in proximity to livestock operations. Standardized and validated bioaerosol collection and analytical techniques will be used to measure the occurrence, dispersion and transport of Salmonella and non-pathogenic indicator E. coli from livestock sources (solid stacks, lagoon, pen floors/bedding) to nearby almond and pistachio crops. Molecular subtyping approaches will be used to compare genetic relatedness and source track movement of strains from livestock operations to tree nut study sites. Pyrosequencing will be used on a subset of samples to evaluate the potential for this technique.*

3. *Objective 2: Evaluate microbial composition of bioaerosols and dusts at a) almond hullers/shellers and b) pistachio hulling/processing facilities.*

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FINAL REPORT

Abstract
The primary vectors and transport pathways leading to the contamination of almonds and pistachios by *Salmonella* and other zoonotic enteric pathogens remain unclear and may be impacted by production and harvest practices. The California Central Valley is the largest producer of almonds, pistachios, and milk in the United States; an unintended consequence is that orchards and animal operations are often in very close proximity. To further understand how bacterial populations may move among animal operations and orchards, various samples (e.g., air, soil, leaves, manure) were collected from two pistachio orchards and adjacent livestock operations. Additionally, samples were collected from two almond orchards; one orchard was in close proximity to a poultry operation and the other served as a control that was at a distance from animal operations. Before harvest amounts of dust were significantly greater on leaves collected from trees that were closest to the animal operation than on leaves from trees further into the orchard. Differences disappeared during and after harvest. In contrast, microbial populations determined by plate counts in air samples and from leaves were consistent throughout an orchard but differed by sample date. *E. coli* was never isolated from air samples from the control orchard, whereas the organism was detected frequently in the orchards adjacent to animal operations. *Salmonella* was detected in 7 of 89 (7.9%) manure and wastewater samples at dairy operations adjacent to pistachio orchards but not from any of 90 air samples analyzed. None of the over 1,000 air soil or leaf samples collected in any of the four orchards was positive for *Salmonella*. *Salmonella* was found on a single occasion at each of the orchards adjacent to the animal operations in pooled drag swabs (of 115 total pooled swabs) None of the 15 pooled swabs from the control orchard were positive for this organism. The same *Salmonella* serovar (Give) found in calf manure and lagoon samples in February and April 2013 was also found in the corresponding pistachio orchard in June 2013. Microbial community sequencing of air, soil, and leaf rinsate samples showed distinct bacterial populations among the sample types. Analysis of the bacterial taxa associated with almond orchards revealed that members of the family *Bacillaceae* were significantly more abundant in the air and leaf rinsate samples in the orchard near the poultry operation. This study provides preliminary evidence that microbial populations in tree nut orchards may be altered by proximity to large scale animal operations. However, further data from paired orchards (next to and at a distance from animal operations) are needed to characterize the significance of these altered microbial communities to the safety of tree nuts.

Background
Foodborne disease outbreaks have been linked to consumption of raw almonds (*Salmonella*; CDC, 2004; Isaacs et al., 2005) and inshell hazelnuts (*E. coli* O157:H7; CDC, 2011; US FDA, 2011), and have been epidemiologically linked to consumption of pistachios (*Salmonella*; US FDA, 2014) and walnut kernels (*E. coli* O157:H7; CFIA, 2011). Between 2009 and 2013, type I recalls due to *Salmonella* contamination occurred in California inshell pistachios (US FDA, 2009a, 2013a, 2013b), Oregon hazelnuts (US FDA, 2009b, 2010a), Turkish pine nuts (US FDA, 2010b), and California walnuts (US FDA 2010c).

The primary vectors and transport pathways leading to the contamination of almonds and pistachios by *Salmonella* and other zoonotic enteric pathogens remain unclear and may be impacted by production practices and harvest and postharvest handling. However, for both almonds and pistachios, dust and bioaerosols have the potential to be important routes of transmission during production, harvest and, in some cases, post-process handling. Bioaerosols are defined as biological particulates, such as viruses and bacteria suspended in air, whereas dust comprises mineral particulates that may serve as carriers. It is well documented that

concentrations of livestock may generate bioaerosols and airborne dust that can be transported off-farm to surrounding areas (Millner, 2009).

In the U.S., California's Central Valley is the sole producer of almonds and the major producer of pistachios. This region also is home to the largest dairy industry in the nation. An unintended consequence of the growth of the dairy and tree nut industries over the past 20 years is the large concentration of cattle often found in the vicinity of many other agricultural crops, including tree nuts, citrus, and vegetables (Figure 1). The risk of pathogen transport from large dairies and feedlots to surrounding food crops is not well-characterized, although dairy waste was implicated in an *E. coli* O157:H7 outbreak linked to iceberg lettuce grown in the Central Valley as a result of cross contamination of irrigation water (CDPH, 2008).

Results from *Salmonella* prevalence surveys for California almonds suggested that cattle environments, especially dairies, may serve as a source of *Salmonella* contamination of orchards in the Central Valley (Bansal et al., 2010; Danyluk et al., 2006; Harris, unpublished). From 2001 to 2007, our laboratory isolated 137 *Salmonella* strains from almonds, representing 43 different serovars. The range of serovars identified was similar to other environmental surveys in California (Gorski et al., 2011; Kinde et al., 1997). Some isolates, however, stood out because of known association with cattle (Cobbold et al., 2006; Hoelzer et al., 2010). In particular, several isolates identified across the 7-year survey were resistant to multiple (4 to 12) antibiotics: *Salmonella* Agona (two isolates: 2006), *Salmonella* Anatum (four isolates: 2002, 2004, 2004, 2006), *Salmonella* Newport (four isolates: 2001, 2002, 2006, 2007) and *Salmonella* Typhimurium DT104 (1 isolate: 2004).

Research Methods and Results

Our goal for objective 1 was to identify almond and pistachio orchards that were next to a collaborating dairy or feedlot ("livestock operation-orchard unit"), and control orchards that were approximately 0.5 miles and upwind from the nearest livestock operation (preferably surrounded by other orchards). We proposed to investigate two livestock operation orchard units and two control orchards that would be sampled for two growing seasons from July through November, and with greater sampling frequency as those orchards were harvested (objective 1).

Unfortunately, we were not able to identify collaborating growers until very late in the first year of the project. When we did, we were limited in choice. We were ultimately able to confirm two pistachio orchards; one next to a dairy feedlot (identified mid 2012) and the other next to a dairy calf operation (late 2012). Access was permitted to both the dairy and calf operations. However, access to control orchards was not given. The orchard next to the dairy feedlot was very small and for this reason was ultimately sampled only in 2012. The only collaborating almond orchard we identified was next to a poultry layer operation rather than a dairy. The manager of the poultry farm acknowledged and did not object to our sampling of the almond orchard but was uncomfortable with us on his property, in part because of his biosecurity program. Therefore, we were not able to collect samples within the layer operation. We did, however, have access to a corresponding control almond orchard that was not near domestic animal production.

For objective 2 we had proposed to follow the harvested almonds and pistachios from the test orchards to the hulling facilities where they are processed, and sample dusts in and around these facilities as corresponding almonds are hulled and shelled or pistachios are hulled and dried. However, despite our best efforts we could not identify either almond or pistachio hulling facilities that were willing to allow us to collect samples. Thus we were not able to accomplish objective 2.

Sampling Locations (Figure 1)

Samples were collected from two pistachio orchards in close proximity to livestock operations: one orchard was next to a dairy (denoted "Pistachio Dairy") and the other was next to a calf operation ("Pistachio Calf"). In 2012, Pistachio Dairy was sampled twice – before and after the harvest. In 2013, samples were collected three times from Pistachio Dairy and seven times from Pistachio Calf. Samples were also collected from the adjacent livestock operations upon each visit. In addition, samples were collected from two almond orchards: one orchard was in close proximity to a poultry operation ("Almond Poultry") and the other served as a control orchard ("Almond Control") and was not in proximity to any animal operation. In 2012, Almond Poultry was sampled three times (before, during, and after harvest), and eight times in 2013. Samples were collected on five occasions from Almond Control in 2013. Because sampling within the poultry operation was not permitted, leaves were collected from bushes that were adjacent to neighboring poultry operation and the single lane road that adjacent to the orchard. For Pistachio Calf, Almond Poultry, and Almond Control the final sampling in 2013 occurred after the trees had been shaken and the nuts harvested; all other sampling in 2013 occurred before harvest activities.

Sampling Procedures for 2012

Collection locations. Leaf and air samples were collected from 15 locations within Pistachio Calf and Almond Poultry during the harvest period. Orchards are planted in grids with enough room between rows to allow for farm vehicles to travel. For the collaborating orchards, five rows of trees were selected for sampling: row 1 was at the edge of the orchard, closest to the adjacent animal operation with typically a dirt road used to access the property; row 2 was two rows of trees into the orchard, and rows 3 to 5 were evenly spaced over the remaining orchard, with row 5 approximately 60 and 150 m into the orchard from row 1 for Pistachio Dairy and Almond Poultry, respectively. Three sampling locations were identified along the rows (columns A, B, C). Drag swabs were collected along each of the sample rows in between the trees. Manure and wastewater (lagoon) samples were collected from the livestock operation adjacent to Pistachio Calf. Dairy wastewater (flush alley, lagoon) and stacked manure were collected at Pistachio Dairy. Additionally, atmospheric data was collected at each location within an orchard (wind speed and direction, temperature, and relative humidity).

Manure. For Pistachio Dairy, stacked manure (approximately 100 g) and wastewater (1 L) were collected randomly from using sterile scoops and bottles. Fresh manure samples were collected from the pen floors at Pistachio Calf and placed in sterile Whirlpak bags. The samples were put on ice and transported back to the laboratory. The samples were mixed thoroughly and a 10-g subsample of the manure or 100 ml of wastewater samples were enriched for *Salmonella* by adding to 90 ml buffered peptone water (BPW; Becton, Dickinson, and Company (BD), East Rutherford, N.J., U.S.A) and incubating for 20 ± 2 h at 37 ± 2°C. The enrichment (10 µl) was then transferred into 1 ml Rapport-Vassiliadis R10 broth (RV; Neogen, Lansing, Mich., U.S.A.) and incubated for 48 ± 2 h at 42 ± 2°C. The enriched RV was streaked onto XLT4 for detection of *Salmonella.* These cultures were incubated for 35 ± 2°C, 48 ± 2 h and then evaluated for the presence of typical *Salmonella* colonies.

Air. Air samples were collected using Mas-100 Eco samplers (Millipore Billerica, Mass., U.S.A). Air is pulled through the sampler resulting in the impaction of airborne microorganisms onto agar plates. Air samples were collected for 1 min (100 liters of air) in triplicate onto tryptic soy agar (TSA; BD) supplemented with cycloheximide (50 mg/liter) in an attempt to minimize the growth of molds. Additionally, air samples were collected for 10 min (1,000 liters of air) in triplicate onto MacConkey agar (Mac; BD) supplemented with cycloheximide (50 mg/liter) and onto semi-solid TSA (semiTSA) made as for standard TSA, but with half the standard amount of

agar (7.5 g agar/liter). TSA and Mac were incubated at 35 ± 2°C for 18–24 h and colonies enumerated. After enumeration, Mac plates were replica plated using sterile velvet squares onto CHROMagar ECC (ChromECC; CHROMagar, Paris, France) and XLT4 agar (XLT4; BD) for enumeration of generic *E.coli,* other coliforms, and *Salmonella*. The entire semiTSA plate was added to 90 ml BPW and enriched for *Salmonella* using the technique described above for manure samples.

Leaves. Leaf samples were collected from orchard trees that were located near each air sampling location. The leaves were collected from the trees at approximately eye level. A sterile bag (710-ml Whirl-pak bags (Nasco, Modesto, Calif., U.S.A.). was placed over the branch and the leaves were stripped from the branch and into the bag. At the laboratory, any twigs, branches, and almonds that had been inadvertently collected were removed aseptically and 0.1% peptone (0.1peptone, BD) was added to the leaves in a ratio of 10 ml 0.1peptone:1 g leaf. Samples were shaken 20 times in a 30° arc, rubbed by hand for 30 s, and shaken 20 more times in a 30° arc. Samples were serially diluted and spread plated onto TSA and Mac, and cultures were incubated as described previously and then enumerated. Colonies on Mac agar were stabbed or transferred by replica plating onto ChromECC and XLT4 for enumeration of generic *E. coli,* other coliforms, and *Salmonella*.

Drag swabs. The drag swabs were prepared by tying approximately 1 m of butcher's string tightly around the center of a 12-ply 4 x 4 inch (10 x 10 cm) sterile gauze pad (CVS, Woonsocket, R. I., U.S.A.). Swabs were autoclaved and aseptically added to 1623-ml Whirl-pak bags, leaving approximately 10 cm of the string outside the bag; the string piece aids in aseptic removal of the swab in the field. Sterile nonfat condensed milk (12 ml) was added to each swab before the swab was frozen for storage. Swabs were thawed, but kept refrigerated (4 ± 2°C) for up to 1 day before use. Prepared swabs were dragged along each of the sampling rows before any other orchard sampling took place. Four swabs were collected for each row and pooled to make a single sample. Pooled samples were submitted to the California Animal Health and Food Safety Laboratory (CAHFS) at UC Davis for *Salmonella* enrichment.

<u>Sampling Procedures for 2013</u>
Collection locations. Sampling procedures for 2013 were modified in order to address some of the issues we identified in 2012 (see results). Leaf, soil, and air samples from within three of the orchards were taken from nine locations. For the Pistachio Calf, Almond Poultry, and Almond Control orchards, three "rows" were selected. Row 1 was at the very front of the orchard, closest to the adjacent animal operation or access road, row 2 was 60 m from the front row into the orchard, and row 3 was an additional 60 m from row 2. Three evenly separated points within each row served as the 3 "columns" for a total of nine sampling locations. Due to the small size of Pistachio Dairy (50 x 50 m), soil was collected from five locations (each corner and the middle) and air samples taken from four locations (four corners). Rows within each orchard were sampled using drag swabs.

Air and manure samples were collected within the livestock operations. Air samples were collected in three locations in each livestock operation. For Pistachio Dairy, air samplers were set up next to the lagoon, manure stacks, and stalls (loafing barn). For Pistachio Calf, air samplers were set up next to the lagoon, pens, and calf hutches. Manure samples were taken from each location, with the exception of the lagoon which was only sampled when the lagoon was full enough to be sampled safely.

Manure. Manure samples were processed in a similar manner as in 2012. In addition, 10 ml of the BPW enrichment was transferred to 90 ml of double strength Universal Pre-enrichment

Broth (UPB; BD) and incubated for 15 ± 2 h at 37 ± 2°C. The enriched UPB was streaked onto ChromECC for detection of generic *E. coli* and other coliforms and a portion was reserved for DNA extraction. For *Salmonella*, 10-ml aliquots of the enriched UPB were subsequently transferred to 90 ml of Tetrathionate Broth Base (TTB; BD) supplemented with iodine (20 ml/liter) and were incubated for 6 ± 1 h at 42 ± 2°C. Ten ml of the enriched TTB was transferred to 90 ml of mBroth broth (mBroth; Biocontrol Systems, Inc., Bellevue, Wash., U.S.A.) for 12 ± 1 h at 37 ± 2°C. The enrichment was streaked onto XLT4 agar (XLT4; Neogen) for *Salmonella* detection. When present, three colonies exhibiting characteristics of *Salmonella* from each plate were purified and confirmed to be positive for *Salmonella* using a standard PCR method. PCR-positive isolates were sent to the CAHFS for serotyping.

Air. Air samples were collected in triplicate using Mas-100 Eco samplers as in 2012. Samplers were run for 10 min (1000 liters of air) with agar plates of 20% Reasoner's 2A agar (Oxoid Limited, Basingstoke, U.K.) with pyruvic acid supplemented at (1 g/L; R2A). Samples were incubated for 18 ± 3 h during transport to the laboratory and in incubators at the laboratory (25–35°C). Colonies appearing at this time were enumerated and then all of the agar in the petri dish was dissolved in 50 ml BPW (BD) and incubated for 6 ± 1 h at 35 ± 2°C. Procedure for determining generic *E. coli*, coliforms and *Salmonella* proceeded as described above for manure samples.

Leaves. Leaf samples were collected by breaking small branches off of trees near each air sampling location. Dust was rinsed off the leaves using two different methods to extract DNA for sequencing and to quantify the amount of adhering dust (total solids). For DNA extraction, leaves and branches were packed into 1623-ml filtering Whirl-pak bags and 100 ml of 0.01 M K_2PO_4 buffer supplemented with Tween 20 (0.5 ml/L) was added. The contents were vigorously shaken for 30 s and the bags were then sonicated for 15 min. The mixture of buffer and dust was evenly pipetted into two 50-ml falcon tubes (BD) and centrifuged at 8000 rpm for 10 min. The supernatant was poured off and DNA was extracted from the pelleted dust. For total solids analysis, leaves were broken off at the stem and 25.0 ± 0.2 g of leaves were shaken with 75.0 ± 0.1 ml of MilliQ water. The leaf rinse water (rinsate) (10.0 ± 0.1 ml) was pipetted into pre-weighed aluminum dishes and all the moisture in the sample was evaporated in an oven.

Leaf samples were also collected from the trees and brush located between Almond Poultry and the adjacent poultry houses. Leaves were sampled in duplicate from three locations; 100 g of leaves were vigorously shaken for 30 s with 100 ml of 0.01 M K_2PO_4 buffer supplemented with Tween 20 (0.5 ml/L). The rinsate (100 ml) was added to 100 ml of UPB and incubated for 18 ± 2 h at 37 ± 2°C. Detection of *Salmonella*, generic *E. coli*, and other coliforms proceeded in the same manner as with the manure samples.

Drag swabs. Drag swabs were constructed and collected using the same procedure as 2012 with the exception of sampling in Pistachio 2. Two pools of four swabs instead of one pool were collected for each row due to the orchard's greater width, approximately double that of the other orchards.

Soil. Soil samples were collected using four scoops of top soil (<3 cm deep) and pooled from each orchard location with a portion being reserved for DNA extraction. Pooled soil (100 g) was mixed with 200 ml of 0.02 M Na_2PO_4 buffer supplemented with Tween 20 (0.5 ml/L; Sigma-Aldrich Corp., St. Louis, Mo.) in filtered 532-ml Whirl-pak bags and vigorously shaken for 30 s. Filtrate (100 ml) was added to 100 ml of UPB and incubated for 18 ± 2 h at 37 ± 2°C. Detection of *Salmonella*, STEC, generic *E. coli*, and other coliforms proceeded in the same manner as described above for manure samples.

Culture-independent bacterial community analysis. Leaf rinsate, soil, and enriched air from Pistachio Calf, Almond Poultry, and Almond Control from July and August samplings were used for characterization of the orchard microbiome. DNA from the air samples was extracted from the air UPB enrichments using the PowerFood kit from Mo-Bio (Carlsbad, Calif., U.S.A.). DNA from soil and leaf rinsate samples was extracted using the PowerSoil kit, also from Mo-Bio. The V4 domain of bacterial 16S rRNA gene was amplified using PCR with forward primer F515 with an 8 nucleotide (nt) barcode sequence on the 5' end and reverse primer R806 in a similar manner to that described by Bokulich et al. (2013).

The barcoded amplicons were multiplexed into an equi-molar single sample and submitted for library construction and pared-end DNA sequencing using the Illumina MiSeq platform at the UC Davis Genome Center (Davis, Calif. U.S.A.). The 16S rRNA gene sequences were quality-filtered and processed in the software package Qiime (www.qiime.org). First, the sequences were de-multiplexed according to the unique 8 nt barcode sequence associated with each sample. During this de-multiplexing step, the sequences were also analyzed filtered to remove low quality reads and the resulting dataset was used for the downstream comparisons. Secondly, the 16S rRNA gene sequences were assigned to operational taxonomic units (OTU) at the 97% similarity level and then a representative sequence from each OTU was assigned a taxonomic identity. Then, the alpha-diversity metric phylogenetic diversity (PD), which represents the total diversity in a single sample, was used to compare the diversity of bacteria in the orchard microbiome. For Beta-diversity, or between sample analyses, principal coordinate analysis (PCA) was used to compare the total diversity differences among samples using the weighted UniFrac metric. Significance tests on the taxonomic abundances in the individual samples were performed using Student's t-test with a $P < 0.05$ limit. Alpha-diversity and Beta-diversity comparisons were also performed in QIIME.

Results

2012

Sampling within the livestock operation:

Manure. All 10 stacked manure and 6 dairy wastewater samples from the livestock operation adjacent to Pistachio Dairy were negative for *Salmonella* upon enrichment.

Sampling within the orchards:

Air. A total of 265 air samples were collected on five sampling dates (one trip to each orchard before and after harvest and an additional trip to Almond Poultry mid-harvest). Samples that were collected onto TSA were often difficult to impossible to accurately enumerate due to a high density of colonies and the presence of large spreading colonies. Upon replica plating of these plates onto MacConkey agar, samples were generally below the limit of detection (<1 CFU/100 liter of air) for both presumptive generic *E. coli* and other coliforms. No *Salmonella* was detected from either direct replica plating onto selective agar or by enrichment of the entire agar sample for any of the air samples.

Leaves. A total of 60 leaf samples were collected from Almond Poultry (before and mid harvest) and Pistachio Dairy (before and after harvest). Aerobic plate counts on TSA ranged between 3.7 and 5.2 log CFU/g of leaf (Table 1), counts for generic *E. coli* ranged between <1.0 (the lower limit of detection) to 4.0 log CFU/g of leaf and counts for other coliforms ranged from 1.1 to 3.1 log CFU/g leaf. The counts were relatively uniform across the orchard. *Salmonella* was not isolated from any of the leaf samples.

Drag swabs. Twenty-five pooled drag swabs were enriched for *Salmonella*. Positive samples were identified in the first row of both Pistachio Dairy and Almond Poultry post-harvest. The

isolate found in Pistachio Dairy was serotyped as *Salmonella* Mbandaka, while the isolate in Almond Poultry was untypeable although it was confirmed to be a *Salmonella* (Table 2).

2013

Sampling within the livestock operations:

Air. A total of 90 air samples were collected from within livestock operations adjacent to the pistachio orchards. Counts on R2A ranged from 0.8 to >3.4 log CFU/1000 liters of air (upper limit of detection; Figure 2). None of the samples collected were positive for *Salmonella* upon enrichment. Due to the low prevalence of *Salmonella* in orchard samples in 2012, orchard air and soil samples were enriched for generic *E. coli*, and coliforms in 2013. Twenty seven and 41 samples were positive for generic *E. coli* and coliforms, respectively, of the 72 samples tested for these organisms (Table 3). The method for enumerating air samples was greatly improved in 2013 by switching to R2Agar. The low nutrient content helped prevent the growth of the spreading organisms that often overtook the TSA plates in 2012.

Manure. A total of 73 manure samples were collected from within the livestock operations. Seven were positive for *Salmonella* upon enrichment (two from Pistachio Dairy and five from Pistachio Calf); isolates from one sample were *Salmonella* Newport, four samples had *Salmonella* Give, and one had both *Salmonella* Give and a strain that was untypeable although it was confirmed as a *Salmonella* (Table 2). Forty five samples were enriched for generic *E. coli* and coliforms; all 45 were positive (Table 3).

Sampling within the orchards:

Air. A total of 549 air samples were collected and processed from within almond and pistachio orchards. Counts on R2A ranged from 0.7 to 3.4 log CFU/1000L of air (Figures 2 and 3). Of the 549 samples collected and enriched, none were positive for *Salmonella*. Four hundred and ninety two samples were tested for generic *E. coli* and coliforms; 113 tested positive for generic *E. coli* and 327 were positive for coliforms (Tables 4 and 5). In general counts were higher closer to or during harvest. Counts for both poultry and almond orchards exceeded the limit of detection for APC in all orchard locations in June and after/during harvest. Counts were usually consistent across an orchard; greater differences were seen among sampling dates. Generic E. coli was isolated from air samples on five of six sampling dates for Almond Poultry and from none of six sampling dates for Almond Control. For samples collected in mid-harvest (after one variety of tree had been shaken and almonds collected from the ground) 0 and 27 of 27 air samples for Almond Poultry and Almond Control, respectively, were positive for *E. coli*.

Soil. A total of 211 soil samples were collected and processed from within the orchards. None were positive for *Salmonella*, 57 were positive for generic *E. coli* (87 were tested for generic *E. coli*), and 87 were positive for coliforms (87 were tested for coliforms) upon enrichment (Tables 4 and 5).

Drag swabs. A total of 90 pools of drag swabs were tested for *Salmonella*. Pooled samples from all three rows in Pistachio Calf in June were positive for *Salmonella* and were all serotyped as *Salmonella* Give (Table 2). There were no other pooled drag swabs positive for *Salmonella* in 2013.

Leaves. Leaves from trees and brush between Almond Poultry and the adjacent poultry operation were sampled on eight occasions. Of the 51 samples of “border leaves” collected during this time period, none tested positive for *Salmonella*.

It was visually apparent that the leaves in the front part of all orchards were covered with greater amounts of dust. In all cases before harvest, leaf rinsates from locations in the first row of each orchard had significantly ($P < 0.05$) higher amounts of dry solids than leaves collected from a distance of 60 or 120 m. Dry solids on leaves in the first row of the Almond Control were, however, significantly lower than either Pistachio Calf or Almond Poultry. However, leaf rinsate samples collected after tree shaking and tree nut pick-up showed no significant differences in amounts of dry solids (Figure 4). The impact of the dirt road that separated the both orchards and animal operations could not be determined. Although there was no adjacent animal operation, row 1 in Almond Control was next to the access road for the orchard.

Culture-independent bacterial community analysis. In 2013 the microbiota associated with leaves, soil, and air in proximity to the orchards was analyzed by sequencing the 16S rRNA genes of the bacteria in these communities. The microbial taxa associated with the three different sample types were distinct from one another. At the phylum level, the air samples from all locations were dominated by *Proteobacteria* and *Firmicutes*; the most abundant bacterial families were *Enterobacteriaceae*, *Bacillaceae*, and *Planococcaceae* (Tables 5 and 6). *Bacillaceae* and *Enterococcaceae* were significantly more abundant in the air samples collected in Almond Poultry than in Almond Control, whereas *Pseudomonadaceae* was plentiful in the air samples collected from Almond Control. This finding suggests that the proximity to the poultry operations may impact the microbial communities in nearby orchards.

In the leaf rinsate samples, the most abundant bacterial phyla were *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Table 5) and these samples were generally high in sequences from bacterial families of *Enterobacteriaceae*, *Sphingomonadaceae*, and *Cyptophagaceae* (Table 6). Similar to the air samples, some of the identified bacterial families were significantly plentiful dependent on the proximity to poultry operation. For example, the leaf rinsate samples collected from Almond Poultry were, like the air samples, significantly higher in *Bacillaceae* and were also abundant in *Micrococcaceae* and *Corynebacteriaceae*. The leaf rinsate samples from Almond Control were significantly plentiful in *Methylobacteriaceae* and *Sphingomonadaceae* which are commonly identified as typical phyllosphere colonists. Moreover, the bacterial diversity in the leaf rinsate samples from Almond Poultry was significantly higher than the leaf rinsate samples collected from Almond Control (Figure 5B).

In the soil collected in these locations, the identified microbial communities were significantly more diverse than the microbial communities in the leaf rinsate and air samples (Figure 5A). The dominant bacterial phyla were *Actinobacteria*, *Chloroflexi*, and *Proteobacteria* and these samples were enriched in populations of the bacterial families *Micrococcaceae*, *Bacillaceae*, and *Geodermatophilaceae* (Table 5 and 6). Similar to the leaf rinsate samples, *Micrococcaceae* was significantly more abundant in Almond Poultry.

With this 16S rRNA dataset, we were able to compare the total bacterial communities using PCA on the weighted UniFrac community distance metric. This analysis revealed that the microbial communities in the air, leaf rinsate, and soil were uniquely clustered and each sample type was distinct (Figure 6). This result was not surprising based on the current knowledge of microbial communities in these kinds of samples; however, with this analysis we were able to identify distinct trends in the total microbial communities in the samples collected from Almond Control and Almond Poultry (Figure 7). This finding will assist in future studies of the microbial communities associated with these samples and the possible correlations between the physical location within the orchard and specific bacterial taxa.

Outcomes and Accomplishments

A total of 1,477 samples were collected in almond and pistachio orchards or adjacent animal operations to evaluate the microbial composition of bioaerosols and dusts near and within almond and pistachio orchards and to evaluate to what extent microorganisms were able to move from livestock areas to neighboring orchards.

A significant amount of effort was devoted to evaluating the methodology used to collect data on microbiological populations. Our initially-proposed methods for air sampling did not work well (counts were virtually impossible for a vast majority of samples) and they were thus revised in the second year of the study. While there was a visible gradient in the amount of dust present on leaves at the edge of the orchard, we could not demonstrate differences in microbial counts. However, in year 2 we also measured weights of adhering dry solid matter on the leaves and this method did support our observations. We had originally proposed to evaluate the potential of pyrosequencing (on a subset of samples) as a means to evaluate the potential for this culture-independent technique to characterize microbial communities. However, from the time the proposal was written to the time we were ready to evaluate samples methods in this area had advanced significantly and we chose instead to evaluate Illumina sequencing. This method showed definite promise as a tool for future research in this area.

Summary of Findings and Recommendations

We made the following general observations:

1. Microbial populations in air samples as measured by collection on agar were consistent throughout an orchard but differed with sample date.

2. Before harvest amounts of dust were significantly greater on leaves collected from trees that were at the edge of the orchard than on leaves from trees further into the orchard. Differences in dust levels on leaves collected throughout the orchards were insignificant after harvest. This observation is likely due to shaking of the trees and subsequent sweeping (in the case of almonds) that takes place during harvest.

3. Generic *E. coli* was detected in air from within the almond and pistachio orchards that were next to animal operations but not from the almond control orchard.

4. *Salmonella* was isolated from some but not all samples of manure collected from the dairy or calf operation. *Salmonella* prevalence in manure and wastewater was consistent with other surveys of dairy operations. *Salmonella* was isolated at a single time point from each of the three orchards from pooled drag swabs and never from any of the other samples. For Almond Poultry and Pistachio Dairy *Salmonella* was found during harvest in the row closest to the animal operation. *Salmonella* was isolated in June from Pistachio Calf in all three of the rows examined. The *Salmonella* identified in the calf manure and lagoon samples (serovar Give) in February and April was the same as that identified in the corresponding pistachio orchard in June.

5. The microbial communities identified by Illumina sequencing revealed that there were distinct bacterial populations associated with the air, leaf rinsate, and soil collected in the orchards. Bacterial populations in the leaf rinsate and air samples from Almond Poultry were significantly more diverse compared to the leaf rinsate samples from Almond Control. Almond Poultry leaf rinsate samples form unique clusters in PCA analysis and different bacterial families were associated with Almond Poultry.

Collectively these data provide preliminary evidence that microbial populations in tree nut orchards may be altered by proximity to large-scale animal operations. However, further data from paired orchards (next to and at a distance from animal operations) are needed to characterize the significance of these altered microbial communities to the safety of tree nuts.

Of the measurements taken the following would be recommended for further study: amounts of dust on leaves; pooled drag swabs in the orchards for *Salmonella*; *E. coli* in air samples; and microbial communities on leaf rinsates and soil.

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APPENDICES

Publications and Presentations (required)

No publications to report.

Presentation

Harris LJ, Jay-Russell MT, Gutierrez-Rodriguez E. 2013. Sources and mechanisms of transfer of *Salmonella* in the production and postharvest tree nut environment. Center for Produce Safety Research Symposium, June 25-26.

Theofel C, Gutierrez-Rodriguez E, Davidson G, Jay-Russell M, Harris L. 2013. Sources and mechanisms of transfer of *Salmonella* in the production almond environment. The Almond Conference, December 3-5.

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.

Suggestions to CPS (optional)

None.

Appendix 3. Figures and Tables

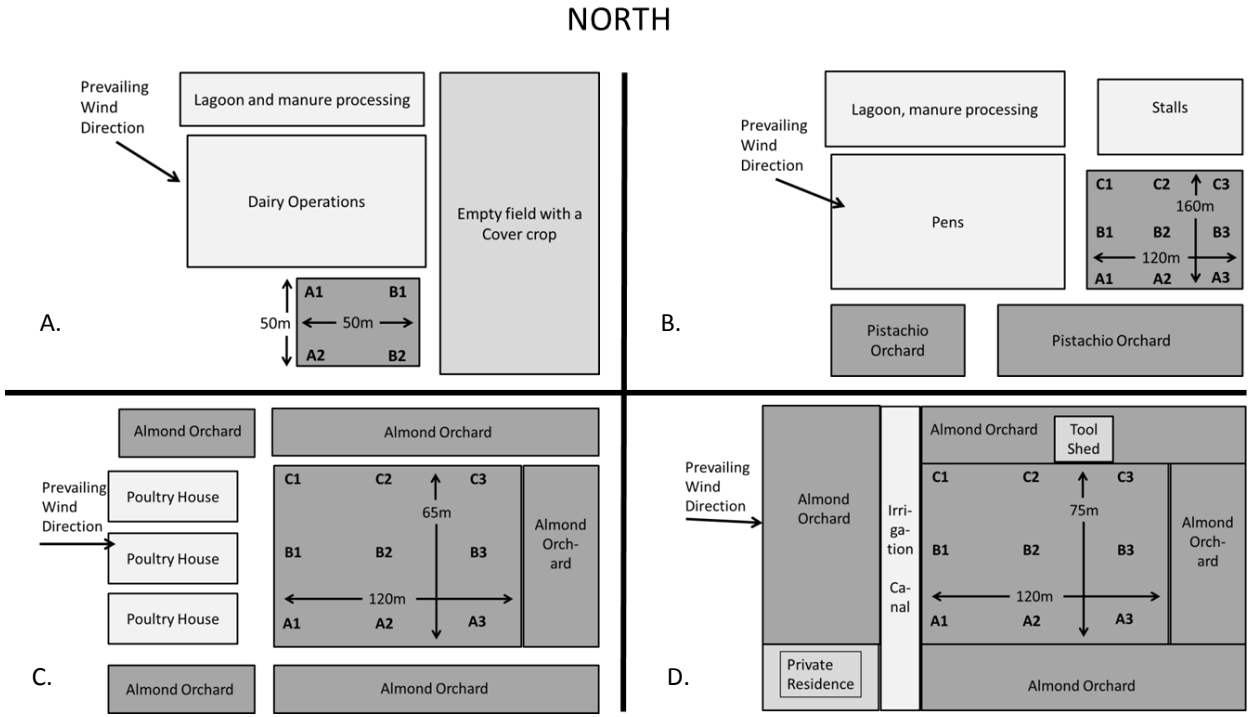

Figure 1. Schematics of collaborating orchards and neighboring agricultural activities; A. Pistachio Dairy, B. Pistachio Calf, C. Almond Poultry, D. Almond Control.

Sampling month		Pistachio Dairy					Pistachio Calf				
		A	B	Livestock			A	B	C	Livestock	
February	1	1.7	1.6	Lagoon	1.7		2.4	2.6	1.8	Lagoon	2.8
	2	1.9	1.9	Stall	>3.4		1.9	1.9	1.1	Stall	>3.4
	3	NP[a]	NP	Stack	1.8		2.0	1.8	1.0	Stack	1.8
March	1	1.3	0.7	Lagoon	0.9		2.1	2.1	1.8	Lagoon	1.7
	2	1.3	0.8	Stall	3.0		2.1	1.3	1.6	Stall	0.8
	3	NP	NP	Stack	2.1		1.6	1.3	1.6	Stack	1.8
April	1	3.2	>>3.4	Lagoon	2.3		2.4	1.9	2.7	Lagoon	2.0
	2	2.8	2.4	Stall	3.0		1.9	1.9	2.2	Stall	2.0
	3	NP	NP	Stack	2.4		2.0	1.8	2.1	Stack	2.3
June	1	NS[b]	NS	Lagoon	NS		>3.4	>3.4	>3.4	Lagoon	>3.4
	2	NS	NS	Stall	NS		>3.4	>3.4	>3.4	Stall	>3.4
	3	NP	NP	Stack	NS		>3.4	>3.4	>3.4	Stack	>3.4
July	1	NS	NS	Lagoon	NS		2.5	2.6	2.3	Lagoon	2.2
	2	NS	NS	Stall	NS		2.2	2.2	2.3	Stall	2.5
	3	NP	NP	Stack	NS		2.3	2.0	2.2	Stack	2.2
August	1	NS	NS	Lagoon	NS		3.2	3.1	3.1	Lagoon	3.0
	2	NS	NS	Stall	NS		3.0	3.2	2.9	Stall	>3.4
	3	NP	NP	Stack	NS		2.9	3.1	2.8	Stack	3.3
Mid-Harvest	1	NS	NS	Lagoon	NS		>3.4	>3.4	>3.4	Lagoon	>3.4
	2	NS	NS	Stall	NS		>3.4	>3.4	>3.4	Stall	>3.4
	3	NP	NP	Stack	NS		>3.4	>3.4	>3.4	Stack	>3.4

[a] NP, sample not present in orchard.
[b] NS, orchard not sampled

Microbial population key (log CFU/1000 L air)	<2.5	2.5 - 3.4	>3.4

Figure 2. Distribution of microbial populations in air (APC log CFU/ 1000 liter of air) in pistachio orchards by row (1, 2, 3) and column (A, B, C) in 2013, *n* = 3.

Sampling month		Almond Poultry			Almond Control		
		A	B	C	A	B	C
January	1	2.4	2.3	2.2	NS[a]	NS	NS
	2	1.7	1.3	1.6	NS	NS	NS
	3	1.8	1.4	1.5	NS	NS	NS
March	1	2.3	2.4	2.1	NS	NS	NS
	2	2.3	2.7	2.0	NS	NS	NS
	3	2.3	2.3	2.0	NS	NS	NS
April	1	1.3	1.2	1.5	NS	NS	NS
	2	1.1	1.2	1.5	NS	NS	NS
	3	1.2	1.0	1.6	NS	NS	NS
May	1	2.4	2.4	2.3	2.1	2.2	2.3
	2	2.2	2.3	2.4	2.2	2.2	2.4
	3	2.2	2.2	2.2	2.1	2.2	2.1
June	1	2.7	2.4	2.4	>3.4	>3.4	>3.4
	2	2.5	2.3	2.4	>3.4	>3.4	>3.4
	3	2.9	2.3	2.4	>3.4	>3.4	>3.4
July	1	3.0	>3.4	3.3	2.5	2.5	2.5
	2	>3.4	3.1	3.0	2.6	2.5	2.6
	3	2.7	2.5	2.4	2.4	2.6	2.6
August	1	3.0	2.6	NC[b]	2.3	2.5	2.3
	2	2.9	2.9	NC	2.2	2.2	2.2
	3	2.7	2.4	NC	2.4	2.3	2.4
Mid-Harvest	1	>3.4	>3.4	>3.4	>3.4	>3.4	>3.4
	2	>3.4	>3.4	>3.4	>3.4	>3.4	>3.4
	3	>3.4	>3.4	>3.4	>3.4	>3.4	3.2

[a] NS, orchard not sampled.
[b] NC, sample not collected.

Microbial population key (log CFU/1000 L air)	<2.5	2.5 - 3.4	>3.4

Figure 3. Distribution of microbial populations in air (APC log CFU/ 1000 L air) in almond orchards by row (1, 2, 3) and column (A, B, C) in 2013, *n* = 3.

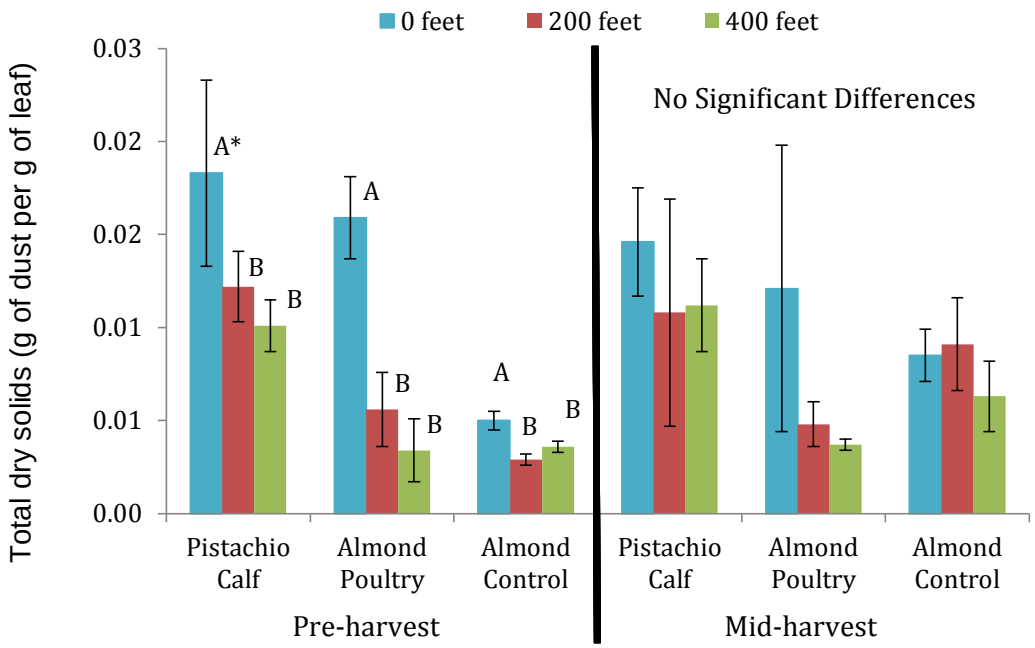

Figure 4. Average dry weight of dust rinsed off of orchard leaves (25.0 ± 0.2 g) collected from 0, 200, or 400 feet from the edge of the orchard closest to the poultry operation or entry road (n ≥ 9). * Different letters for samples within an orchard indicate significant differences ($P < 0.05$) in dust levels were observed.

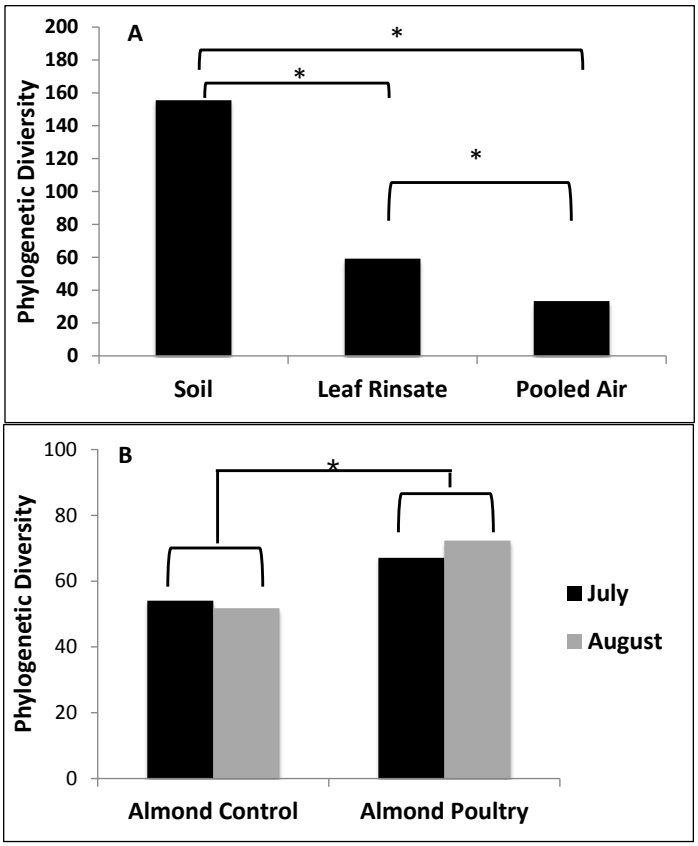

Figure 5. The alpha-diversity of the microbial communities identified by Illumina sequencing. The phylogenetic diversity (PD), which quantifies the bacterial diversity in a single sample, is shown here. (A) The average PD is given for each sample type. (B) The average PD is given for Almond Control and Almond Poultry leaf rinsate samples collected in two different months. Asterisk (*) indicates significant difference ($P < 0.01$) between samples as determined by Student's *t*-test.

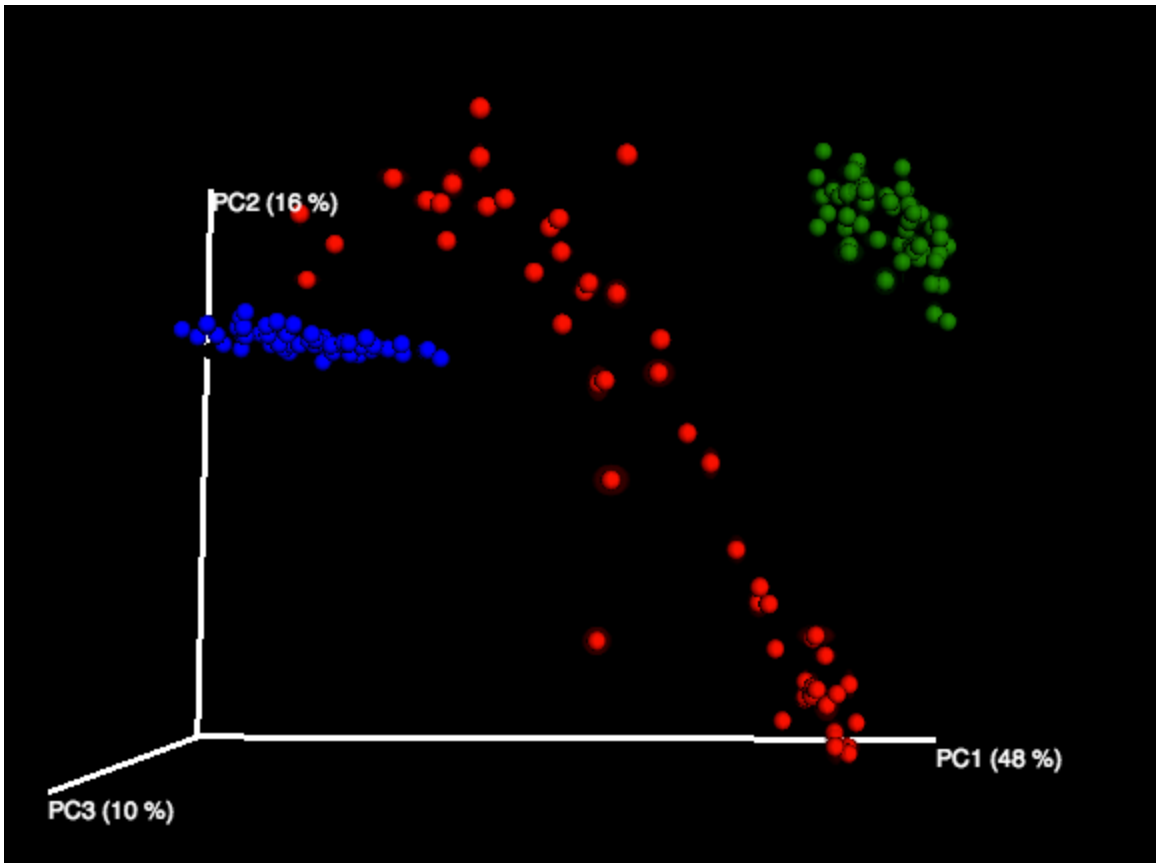

Figure 6. Principal coordinate analysis using the weighted UniFrac community distance metric. Each point represents the entire bacterial community identified by Illumina sequencing of the 16S rRNA genes in a single sample. The blue points represent air samples, the red points represent the leaf rinsate, and the green points represent the soil samples.

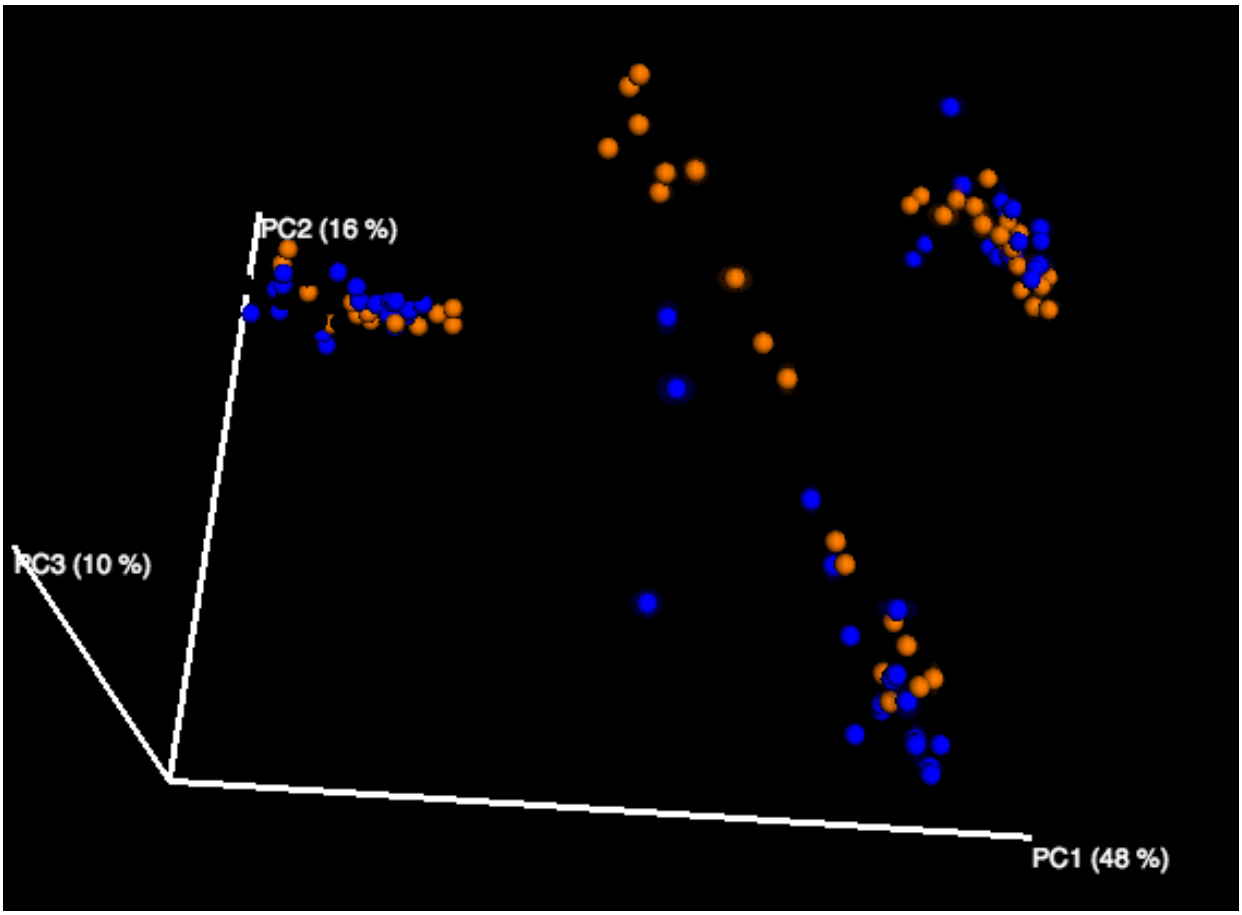

Figure 7. Principal coordinate analysis using the weighted UniFrac community distance metric. Each point represents the entire bacterial community identified by Illumina sequencing of the 16S rRNA genes in a single sample. The orientation is the same as in Figure 6, where the cluster on the left represents the air samples, the middle cluster represents leaf rinsate samples, and the right cluster represent soil samples. In this figure, the Pistachio Calf orchard samples have been removed for simplicity. The orange points represent the bacterial communities from Almond Poultry and the blue points represent those from Almond Control.

Table 1. Bacterial populations on orchard leaves (log CFU/g leaf) by orchard row in 2012 samplings, n = 3.

Orchard and row	Pre-harvest populations			Mid- or post-harvest populations[1]		
	APC[2]	*E. coli*[3]	Other coliforms[4]	APC	*E. coli*[4]	Other coliforms
Almond Poultry						
1	4.0 ± 0.3	<1.7	2.4 ± 0.6	4.2 ± 0.4	<1.0	1.4 ± 0.4
2	4.2 ± 0.4	1.7 ± 0.0	2.2 ± 0.8	4.0 ± 0.1	<1.0	1.6 ± 0.5
3	3.8 ± 0.1	<1.7	1.8 ± 0.2	4.0 ± 0.5	<1.0	1.8 ± 0.5
4	4.0 ± 0.4	<1.7	<1.7	3.7 ± 0.1	<1.0	1.1 ± 0.2
5	3.8 ± 0.2	<1.7	<1.7	3.8 ± 0.1	<1.0	1.5 ± 0.2
Pistachio Dairy						
1	5.2 ± 0.0 A[5]	3.3 ± 0.3	3.1 ± 0.8	ND[6]	1.7 ± 0.6 BC	1.2 ± 0.3
2	5.2 ± 0.1 A	2.9 ± 1.3	2.0 ± 0.5	ND	1.5 ± 0.6 C	1.1 ± 0.2
3	5.0 ± 0.1 AB	3.2 ± 0.9	2.9 ± 0.7	ND	3.2 ± 1.6 AB	1.6 ± 1.0
4	4.8 ± 0.2 B	2.2 ± 0.5	2.4 ± 1.2	ND	2.5 ± 1.0 AB	1.3 ± 0.6
5	5.0 ± 0.1 AB	2.9 ± 0.5	2.0 ± 0.1	ND	4.0 ± 0.3 A	1.7 ± 0.6

[1] Almond Poultry was sampled mid-harvest, Pistachio Dairy was sampled post-harvest.
[2] APC, aerobic plate count.
[3] Limit of detection = 1.7 log CFU/g.
[4] Limit of detection = 1.0 log CFU/g.
[5] Within columns for each orchard, means with different letters are significantly difference ($P < 0.05$).
[6] ND, not determined; plates were uncountable.

Table 2. Serotypes of *Salmonella* isolates from orchard and livestock operation sampling (in 2012 and 2013).

Sampling site	Sampling time	Sample type	Location	*Salmonella* serotype
2012				
Almond Poultry – orchard	October	Drag swab	Row 1	Untypeable: Monophasic
Pistachio Dairy – orchard	September	Drag swab	Row 1	Mbandaka
2013				
Pistachio Dairy – livestock	February	Wastewater	Lagoon	Give
	February	Wastewater	Lagoon	Give
Pistachio Calf – livestock	February	Manure	Stalls	Give
	April	Manure	Stalls	Give, Untypeable: Monophasic
	April	Manure	Stalls	Newport
	April	Manure	Stalls	Give
	April	Wastewater	Lagoon	Give
Pistachio Calf – orchard	June	Drag swab	Row 1	Give
	June	Drag swab	Row 2	Give
	June	Drag swab	Row 3	Give

Table 3. Number of samples that were positive by enrichment for *Salmonella,* generic *E. coli,* and other coliforms from livestock operations adjacent to pistachio orchards (2013 sampling).

Orchard	Month	*Salmonella*		Generic *E. coli*		Other Coliforms	
		Air	Manure[a]	Air	Manure	Air	Manure
Pistachio Dairy[b]	February	0/9	2/8	ND[c]	ND	ND	ND
	March	0/9	0/7	1/9	7/7	3/9	7/7
	April	0/9	0/8	3/9	8/8	4/9	8/8
Pistachio Calf	February	0/9	1/8	ND	ND	ND	ND
	March	0/9	0/6	2/9	6/6	3/9	6/6
	April	0/9	4/6	3/9	6/6	5/9	6/6
	June	0/9	0/9	6/9	9/9	7/9	9/9
	July	0/9	0/9	1/9	9/9	3/9	9/9
	August	0/9	0/6	7/9	ND	9/9	ND
	Mid-harvest	0/9	0/6	4/9	ND	7/9	ND
Total: 10 samplings		0/90	7/73	27/72	45/45	41/72	45/45

[a] Includes fresh feces, stacked manure, and lagoon wastewater.
[b] Sampling discontinued after April in the adjacent pistachio orchard.
[c] ND, sample not done.

Table 4. Number of samples that were positive by enrichment over total sampled for *Salmonella,* generic *E. coli,* and other coliforms in pistachio orchard samples (2013 sampling).

Orchard	Month	*Salmonella*			Generic *E. coli*		Other Coliforms	
		Air	Soil	Drag swabs	Air	Soil	Air	Soil
Pistachio Dairy[b]	February	0/12	0/5	0/3	ND[a]	ND	ND	ND
	March	0/12	0/5	0/3	0/12	ND	4/12	ND
	April	0/12	0/5	0/3	6/12	ND	6/12	ND
Pistachio Calf	February	0/27	0/9	0/6	ND	ND	ND	ND
	March	0/27	0/9	0/6	1/27	ND	6/27	ND
	April	0/27	0/9	0/6	0/27	ND	4/27	ND
	June	0/27	0/9	3/6	11/27	5/9	18/27	9/9
	July	0/27	0/9	0/6	2/27	7/9	14/27	9/9
	August	0/27	0/9	0/6	7/27	ND	26/27	ND
	Mid-harvest	0/27	0/9	0/6	4/27	4/9	14/27	9/9
Total: 10 samplings		0/225	0/78	3/51	31/186	16/27	92/186	27/27

[a] ND, sample not done.
[b] Sampling discontinued after April.

Table 5. Number of samples that were positive by enrichment over total sampled for *Salmonella,* generic *E. coli,* and other coliforms in almond orchard sampling (2013 sampling).

Orchard	Month	*Salmonella*				Generic *E. coli*		Other Coliforms	
		Air	Soil	Drag swabs	Border leaves	Air	Soil	Air	Soil
Almond	January	0/18	0/11	ND[a]	0/9	ND	ND	ND	ND
Poultry	March	0/27	0/11	0/3	0/6	2/27	ND	9/27	ND
	April	0/27	0/11	0/6	0/6	9/27	ND	13/27	ND
	May	0/27	0/11	0/3	0/6	0/27	ND	23/27	ND
	June	0/27	0/11	0/3	0/6	8/27	3/11	25/27	11/11
	July	0/18	0/11	0/3	0/6	1/18	2/11	12/18	11/11
	August	0/18	0/11	0/3	0/6	10/18	ND	10/18	ND
	Mid-harvest	0/27	0/11	0/3	0/6	27/27	11/11	27/27	11/11
Total	8 dates	0/189	0/88	0/24	0/54	57/151	16/33	119/151	33/33
Almond	May	0/27	0/9	0/3	NA[b]	0/27	ND	23/27	ND
Control	June	0/27	0/9	0/3	NA	0/27	0/9	26/27	9/9
	July	0/27	0/9	0/3	NA	0/27	2/9	23/27	9/9
	August	0/27	0/9	0/3	NA	0/27	ND	18/27	ND
	Mid-harvest	0/27	0/9	0/3	NA	0/27	5/9	26/27	9/9
Total: 13 samplings		0/135	0/45	0/15	NA	0/135	7/27	116/135	27/27

[a] ND, sample not done.
[b] NA, sample not available in orchard.

Table 6. Percentages of dominant bacterial phyla in the orchard microbial community[a] as determined by sequencing.

Sample	Bacterial phylum	Pistachio Calf		Almond Control		Almond Poultry	
		July	August	July	August	July	August
Soil	*Actinobacteria*	21.70%	26.20%	30.60%	21.58%	28.68%	24.78%
	Chloroflexi	13.47%	19.00%	5.62%	15.34%	9.88%	13.01%
	Proteobacteria	28.46%	16.40%	30.43%	21.27%	31.72%	21.75%
Leaf rinsate	*Actinobacteria*	33.53%	30.98%	8.97%	11.27%	20.29%	28.52%
	Firmicutes	39.83%	35.84%	5.52%	19.38%	22.63%	40.08%
	Proteobacteria	8.52%	30.28%	47.47%	39.63%	33.45%	19.73%
Air	*Actinobacteria*	0.34%	0.30%	0.46%	0.40%	0.48%	1.00%
	Firmicutes	59.50%	40.30%	49.17%	63.20%	60.99%	65.60%
	Proteobacteria	39.90%	58.90%	50.02%	36.00%	38.25%	33.00%

[a] Microbial communities of three different orchards on the basis of July and August sampling. Within each orchard and for each month, the percentage of three bacterial phyla in the total bacterial community is shown among each sample type. In general, the top three most abundant phyla are given.

Table 7. Percentages of dominant bacterial families in the orchard microbial community[a] as determined by sequencing.

Sample	Bacterial family	Pistachio Calf		Almond Control		Almond Poultry	
		July	August	July	August	July	August
Soil	*Micrococcaceae*	6.9%	5.0%	4.0%	4.1%	9.7%	5.0%
	Bacillaceae	4.7%	4.9%	1.7%	3.2%	2.1%	1.8%
	Geodermatophilaceae	3.4%	3.2%	3.2%	2.1%	3.7%	2.7%
Leaf rinsate	*Sphingomonadaceae*	19.7%	0.14%	13.2%	13.4%	2.1%	6.4%
	Cytophagaceae	14.0%	0.05%	11.4%	10.1%	2.0%	4.5%
	Enterobacteriaceae	0.96%	28.8%	1.1%	5.3%	5.8%	2.7%
Air	*Enterobacteriaceae*	25.65%	51.6%	40.5%	27.9%	31.3%	21.1%
	Bacillaceae	40.83%	15.8%	39.6%	15.8%	42.0%	17.6%
	Planococcaceae	10.17%	9.6%	8.2%	4.8%	9.2%	7.9%

[a] Microbial communities of three different orchards on the basis of July and August sampling. Within each orchard and for each month, the percentage of three bacterial families in the total bacterial community is shown among each sample type. In general, the top three most abundant families are given.