A GROWERS GUIDE: Produce Safety Research

A Practical Examination of the Research Presented at

Center for Produce Safety 2010 Research Symposium

Prepared by:

PMA

WESTERN GROWERS

Fresh produce from our families to yours

March 8, 2011

ACKNOWLEDGEMENTS

This report was made possible by the dedicated contribution of the following individuals:

Robert Whitaker, Ph.D., Chief Science & Technology Officer, *Produce Marketing Association*
Hank Giclas, Sr. Vice President Strategic Planning, Science & Technology, *Western Growers*
Sonia Salas Gutierrez, Science & Technology Manager, *Western Growers*

Table of Contents

Center for Produce Safety 2010 Research Symposium

BACKGROUND

The Center for Produce Safety (CPS) was established by public and private partnership at the University of California, Davis in 2007 and is aimed at identifying and prioritizing critical produce food safety research needs, funding original research, offering a searchable produce safety database and providing industry outreach programs, all focused on enhancing produce safety.

The inaugural CPS Research Symposium was held on June 23, 2010 in Davis, Calif. It featured three research reporting sessions focused on specific findings from the first eleven CPS-funded research projects and ended with a food industry/ government discussion on the critical issues facing our industry. The symposium was attended by almost 300 people. During the sessions, short presentations were given by each project's principal investigator highlighting key research results followed by a panel discussion. The panels were composed of industry executives, industry scientists, regulatory experts and academic researchers. Their purpose was to lend perspective to the new research findings and provide commentary on how these findings might be incorporated into everyday growing, harvesting or processing operations.

The final reports for the first eleven projects funded by the Center for Produce Safety are posted on the CPS website. They have not been subject to peer-review at this time. When the principal investigators' articles based on their research are accepted for publication in a peer-reviewed professional journal, access to articles should be provided. The eleven projects featured in the Symposium resulted from the initial CPS request for proposals (RFP) in mid-2008 and a second RFP issued jointly by CPS and the California Leafy Greens Research Board in late 2008. CPS announced the recipients of its 2010 awards inSeptember 2010. To date, CPS has awarded nearly $7 million dollars to advance 41 research projects across a broad spectrum of food safety research areas and commodities. The 2011CPS RFP was announced on February 1, 2011 and can be found at http://cps.ucdavis.edu.

A Practical Examination of the Research Presented at Center for Produce Safety 2010 Research Symposium

DISCLAIMER

This report has been prepared to provide guidance and a meaningful summary of the 11 research reports discussed during the CPS Research Symposium at UC Davis and does not constitute legal advice nor does it supersede any regulations. The sections headed "What does this mean for you?" are based on author interpretation. Please also note that research findings from these 11 projects have not been subject to peer-review at this time.

SESSION I: *Survivability of* E. coli *Under Field Conditions*

PROJECT #1: *Survival of attenuated* Escherichia coli *0157:H7 ATCC 700728 in field-inoculated lettuce*

Principal Investigator: Linda Harris, Ph.D., University of California, Davis

Layman's Summary (Source: CPS Program Notebook- June 23, 2010)
From 1995 through 2006, 22 outbreaks of *E. coli* O157:H7 were associated with consumption of leafy green vegetables. This project was aimed at having a better understanding of how *E. coli* O157:H7 7 survives in field of growing lettuce. A non-pathogenic strain of *E. coli* O157:H7, with characteristics similar to the pathogen, was used to inoculate lettuce plants in the field. Drip and sprinkler irrigation systems were compared for their influence on *E. coli* survival on lettuce because moisture is known to impact pathogen survival. Lettuce plants were inoculated (mock contamination event) a single time either immediately or four weeks after seeding. After inoculation rapid decreases in numbers of *E. coli* were observed in the soil and on each lettuce plant. When the soil was inoculated prior to germination, *E. coli* O157:H7 could not be detected in the growing plants. However, when young plants were inoculated, a small number of *E. coli* did survive on the lettuce plants (primarily outer leaves or the inoculation site) for much longer periods up to and including the time of harvest (about eight to nine weeks after planting and four to five weeks after introduction of *E. coli*). No significant difference in counts was observed between plants that were irrigated by drip or overhead sprinkler. Neither drip nor overhead sprinkler irrigation consistently influenced the survival of *E. coli* O157:H7 on lettuce. The time of the mock contamination event had the greatest impact on the probability of isolating *E. coli* form harvested lettuce.

Technical Findings and What it Means for You:
The overall objective of this research project was to evaluate the survival of attenuated (non-pathogenic) *E. coli* O157:H7 inoculated onto Salinas Valley field-grown romaine lettuce under two irrigation methods (drip and sprinkler irrigation) and two seasons (spring and fall). The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Attenuated* E. coli *O157:H7 did not appear to transfer from the soil to the plant under these experimental conditions.* When soil was inoculated prior to plant emergence, attenuated *E. coli* O157:H7 could be isolated from the soil for up to 15 days post inoculation. However, pathogen transfer from the inoculated soil to the plant was not observed, i.e. *E. coli* O157:H7 ATCC700728 could not be recovered from plants when sampled 7 to 15 days post-inoculation.

   What does this mean for you? This result tells us that plants that emerge from seed through soil that is contaminated with this attenuated strain of *E. coli* O157:H7 are not themselves contaminated with the pathogen. It also tells us that the pathogen is detectable in the soil for up to 15 days after it was placed there. We know from research reports by other scientists that *E. coli* can persist in soils for varying periods of time depending on the type of soil, presence of organic materials, temperatures, moisture and other environmental factors. While the data from this study show that emerging plants do not become contaminated, the data also show that the pathogen may persist in

the soil and as such, represents a potential contamination risk for transfer to plants by humans, animals, water or other transfer vehicles. This result reaffirms the importance of Good Agricultural Practices (GAP) programs which are focused on preventing pathogen contamination of soils.

2. **Finding:** *Attenuated* E. coli *O157:H7 does not survive well on romaine leaves in field conditions.* When plant leaves were inoculated directly, the levels of attenuated *E. coli* O157:H7 rapidly declined in the first two hours (a 3-log reduction for the spring and fall field trials). After two days, attenuated *E. coli* O157:H7 could only be recovered from inoculated plants if enrichment techniques (i.e. a method employed to help the bacteria recover and grow when they cannot be detected by conventional methods) were employed. Indeed, after two days, 85% of the plants during spring and 74% during the fall field trial fell below the assay detection limit for *E. coli*. By 7 days, more than 90% of the Romaine lettuce plants for both trials were below the limit of detection for this pathogen. However, very low levels of pathogen could be detected on leaf material employing enrichment techniques out to 28 days (spring) and 35 days (fall) post-inoculation.

   What does this mean for you? The production environment of a romaine leaf is not particularly conducive to attenuated *E. coli* O157:H7 survival. It dies off significantly after only a few hours and by 2 days after the inoculation, it can only be detected using enrichment treatments. These results were consistent in both the spring and fall field trials. This observation might tell us that contamination events that occur close to harvest are likely more concerning that those that might occur early in production, i.e. the pathogen dies off and apparently continues to do so over time. This is precisely the reason why risk assessments conducted a few days prior to harvest are an important tool in any field-level food safety program, i.e. recent contamination or evidence of risk events (e.g. animal intrusion, flooding, etc.) can be detected and appropriate management procedures implemented. While this result is encouraging and supports the value of pre-harvest risk assessments, the data also tells us that even though the attenuated pathogen does not survive well on romaine leaves, it did not die off completely and could still be found 4 to 5 weeks post inoculation. Since *E. coli* O157:H7 can reportedly cause illness at levels as low as a few live cells, this observation is important. Even though the pathogen may not survive well on the leaves, any survival at all may represent a potential public health risk. Once again, we see the importance of having preventive risk-based GAP programs that provide multiple risk management hurdles or protective firewalls that reduce the risk of contamination from ever occurring.

3. **Finding:** E. coli *O157:H7 population sizes were not significantly different for plants irrigated by drip versus overhead sprinklers.* Romaine lettuce was inoculated with attenuated *E. coli* O157:H7 and then irrigated by overhead sprinklers or drip irrigation. Greater numbers of positive plants were uncovered in the spring field trial irrigated by overhead sprinklers in the first 2 weeks after inoculation than in the fall field trial. Conversely, in the fall trial a higher percentage of positive plants were found on those plants that were drip irrigated after a similar two week period. However, during the fall trial, 35 mm of rain fell in the first 7 days following inoculation whereas rainfall was not recorded during the spring trial. This may very well have confounded the data and affected the outcome. Irrespective of

irrigation method, by the time of harvest, 7%, of the plants in the spring and 1% of the plants in fall were contaminated with *E. coli* O157:H7.

What does this mean for you? There has been a lot of speculation that overhead sprinkler irrigation poses a greater contamination risk than drip irrigation owing to the direct exposure of the leaf surface to water or perhaps from potential "splash" effects from the water hitting the soil and splashing up onto the plant. We have to be careful about over-reaching based on a single spring and fall field trial, but these data demonstrate that while pathogen levels seem to fluctuate on purposely inoculated plants, even though greater number of positive plants were found after sprinkler irrigation and rain events, by the time the romaine lettuce was ready for harvest, the levels of pathogen that were recoverable were statistically the same. Therefore, it appears from this single study that irrigation method may not have a significant effect on pathogen survival on the leaf surface.

4. **Finding:** *Although survival of attenuated* E. coli *O157:H7 was very low in these studies, when the attenuated pathogen was detected it was always only found in the outer leaves of the romaine lettuce.* When evaluating previously inoculated plants, those that did have detectible attenuated *E. coli* O157:H7 were always found to have that contamination on the outer, more mature leaves.

   What does this mean for you? This result is important because when romaine lettuce is harvested, typically the outer leaves are trimmed away, i.e. those leaves that are most likely to harbor a contaminating pathogen (if a contamination event were to occur in the first place) are discarded in the field. The leaves are trimmed off the plant principally for quality reasons as these are the most mature and have been subjected to wind and other damaging influences that reduce their overall quality. Therefore, it turns out that a traditional harvest practice may indeed be a food safety risk management practice as well. This is yet another example of a preventive practice within the multi-level firewall approach employed by growers to reduce the risk of a pathogen contamination that could result in a public health event.

PROJECT #2: *Contribution of phyllosphere microbiata to the persistence of* Escherichia coli *0157:H7 ATTC 700728 on field-grown lettuce*

Principal Investigator: Maria L. Marco, Ph.D., University of California, Davis.

Layman's Summary (Source: CPS Program Notebook - June 23, 2010)
E. coli O157:H7 is a poor colonist of lettuce and other leafy greens, although certain environmental (abiotic and biotic) conditions in the field might promote the ability of this organism to establish persistent populations on lettuce and enter into food supply chains. This project is investigating the contribution of the indigenous plant-surface-associated (phyllosphere) microorganisms (microbiota) to the persistence of *E. coli* O157:H7 on Romaine lettuce in the Salinas Valley, CA. Lettuce plant microbiota was examined in collaboration with a concurrent CPS project (Harris et al.) aimed at determining the survival of a non-pathogenic (attenuated) *E. coli* O157:H7 strain after application onto field-gown Romaine lettuce exposed to different irrigation regimes (overhead and drip) and planted in different seasons (spring and fall). By applying culture-dependent and independent bacterial detection methods, we found significant differences in the populations of bacteria on Romaine lettuce over a four-week period depending on the season of planting, irrigation regime, and prior application of the attenuated *E. coli* O157:H7 strain. We also identified bacterial isolates from the Romaine lettuce plants with the capacity to inhibit the growth of *E. coli* O157:H7. These results indicate the total bacterial cell amounts and certain indigenous microbes present on Romaine lettuce plants might impair or improve ability of pathogenic *E. coli* O157:H7 to persist on plants.

Technical Findings and What it Means for You:
This project is focused on identifying and quantifying the bacterial populations on field grown lettuce. Dr. Marco's project goals were: 1) quantify differences in culturable and total phyllosphere microbiota (bacterial population) on drip and overhead irrigated field-grown romaine lettuce plants for time points before and after exposure to attenuated (non-pathogenic) *E. coli* O157:H7, and 2) identify strains of bacteria isolated from the phyllosphere of overhead and drip-irrigated plants which inhibit the growth of *E. coli* O157:H7. This study was complementary to the field trial designed by Dr. Harris et al. (see above) on field-grown romaine lettuce plants that were inoculated with same attenuated *E. coli* O157:H7. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *The total bacterial population (phyllosphere) on romaine lettuce changes over time both during the growing season and between different growing seasons (spring versus fall). In these experiments the phyllosphere differed significantly based on season, presence of inoculated attenuated* E. coli *O157:H7 and method of irrigation (overhead versus drip).* During spring 2009, bacterial populations were typically 2 to 5-fold larger for overhead or sprinkler irrigated plants than for drip irrigated plants. These observations were consistent whether the plants had been inoculated with attenuated *E. coli* O157:H7 or not (controls). When looking only at culturable bacteria, perhaps surprisingly, the plants inoculated with the attenuated pathogen harbored significantly higher populations. During this spring 2009 field trial, when attenuated *E. coli* O157:H7 was inoculated on the plants there was an inverse relationship between the amount of attenuated pathogen

present and the total number of bacteria present, i.e. the more attenuated pathogen detected, the lower the total bacterial (culturable plus non-culturable) population in the phyllosphere.

This was an interesting result so Dr. Marco's group looked to see if the same relationship held true during the fall 2009 field trial. Once again the same type of analysis was performed in the fall and both culturable and non-culturable bacterial populations were enumerated in plants that were inoculated with attenuated *E. coli* O157:H7 and uninoculated controls. However, the results in this trial were opposite of those observed in the spring, i.e. the higher the population of recovered attenuated pathogen the higher the total bacterial population. This fall 2009 field trial was impacted by a significant rainfall and resulted in the bacterial population sizes on all plants to increase to similarly high levels. This rainfall event makes specific conclusions from this study difficult regarding pathogen and total bacterial population relationships. Clearly more work needs to be performed and is, indeed planned for 2010.

What does this mean for you? The data from these experiments are not final but they do illustrate the complex environment that exists on the surface of growing plants. Sometimes we fail to recognize that a contaminating pathogen does not exist by itself on the leaf surface of a Romaine plant or another leafy green. There is a vibrant population of microorganisms present on those leaves and these results indicate that the relationships between these microorganisms can be variable based on growing conditions and perhaps even the size of the population of the contaminating pathogen. This project is focused on helping to define what types of interactions the phyllosphere may have on unwelcome human pathogens and we await those results. These data also show that a rainfall event during production can impact the phyllosphere and perhaps promote general bacterial growth.

2. **Finding:** *Using an array of advanced, DNA-based molecular methodologies, the researchers found that highly diverse microbial communities are present on romaine lettuce leaves and that many of these types of bacteria are not easily cultivated on standard laboratory media.* A limited culture-independent analysis of the phyllosphere microbiota indicates that some plants contain only a few bacterial species while others harbor remarkably diverse microbial communities. Ongoing analyses are aimed at identifying potential correlations between microbial diversity patterns or specific organisms which are associated with *E. coli* persistence on romaine lettuce in the field.

   What does this mean for you? This finding confirms the discussion above on the vibrant microbial ecology that exists on the surface of lettuce leaves and the importance of using new technologies to help uncover their presence. Using traditional microbial methods we have been historically limited to identifying only those bacterial strains that respond to our culture techniques. Using DNA-based methodologies we can now get a more dynamic picture of the total bacterial population that exists on plant surfaces and we can begin to develop a better and more complete understanding of how these bacteria interact and how they may assist human pathogens in surviving on romaine leaves or perhaps inhibit their growth and survival.

3. **Finding:** *Field-grown romaine lettuce harbors indigenous bacteria which are antagonistic towards the growth of virulent* E. coli *O157:H7.* A total of 98 isolates were collected from both the spring and fall 2009 field trials and tested for their ability to inhibit the attenuated *E. coli* O157:H7 strain and 5 additional *E. coli* O157:H7 strains previously associated with human illnesses. These "isolates" are simply different strains of bacteria that the researchers were able to recover using standard microbiology culture methods from romaine grown in the Salinas field trials. Twenty-eight of these bacterial isolates were found to inhibit one or more of the *E. coli* O157:H7 strains used in the experiments. Interestingly, isolates specifically recovered during the fall 2009 trial were more effective in inhibiting *E. coli* O157:H7 than those developed from the spring 2009 field trial.

   What does this mean for you? It is significant that there may be naturally occurring bacteria that can inhibit or are antagonistic toward *E. coli* O157:H7. At the very least, it is another element that needs to be considered when we think about the phyllosphere and the complexities of the biological system that exist on the surface of plants. It is encouraging that Dr. Marco and her team are continuing to explore this area as we can only understand why pathogens survive or decline in production environments when we develop a more complete picture of these complex microbial interactions. The significance of the role of naturally occurring antagonistic bacterial species remains to be determined, but the possibilities are intriguing. For example, could the presence or absence of these antagonistic species be used as indicators that pathogens may or may not be present? Perhaps our knowledge might advance to the point where we understand why these antagonists are present and we could fine tune our production practices to favor their presence? Lastly, perhaps in the future antagonistic species could be used as a biocontrol treatment to help manage the risk of pathogen contamination? Again, there is considerable work to be done in this area in the future but it is important to monitor progress going forward.

PROJECT #3: *Comparison of surrogate* E. coli *survival and epidemiology in the phyllosphere of diverse leafy green crops*

Principal Investigator: Trevor Suslow, Ph.D., University of California, Davis

Layman's Summary (Source: CPS Program Notebook- June 23, 2010)
Validated surrogates, safe substitutes for pathogens, for research on diverse issues in food safety are essential enabling tools to fill in critical data gaps on survival, spread, and control in open field environments, including most public experimental farm facilities. Our primary objective was to complete a second year of on-farm testing comparing a mixture of three regionally selected nonpathogenic *E. coli* (gEcoli) to two attenuated (lacking the ability to produce illness-associated toxins) *E. coli* 0157:H7 strains (att0157). To date, an experimental planting of fourteen different types of leafy greens used in Spring Mix salads was inoculated twice during growth with log 4.5 CFU>ml of these surrogate *E. coli* and analyzed for relative survival. As with all other tests thus far, the gEcoli survived longer and at higher numbers than att0157 treatments. Only gEcoli remained detectable in some but not all replicated plots and on some but not all leafy greens by 19 days after the initial simulated contamination event and 14 days after the second treatment. In similar field trials with romaine lettuce, the dose of applied nitrogen fertilizer had no effect on the survival of att0157 applied to leaves at three stages of plant growth, the final "contamination" within 14 days of harvest. Survival of applied att0157 was essentially undetectable at harvest. We feel that these test outcomes significantly expand the database that supports industry standards for microbiological quality. Once published in peer-reviewed journals, the gEcoli will be made available to researchers to facilitate broad controlled environment and field-based experiments.

Technical Findings and What it Means for You:
Dr. Suslow's team has been working for several years on various aspects of *E. coli* and *Salmonella* contamination and survival in production environments. The project reported on at the CPS Food Safety Symposium had three principal objectives: 1) compare the foliar survival and growth of specific isolates of nonpathogenic *E. coli* to non-toxigenic (attenuated) *E. coli* 0157:H7 on lettuce and leafy greens during late spring and late summer conditions, 2) conduct rapid response field surveys on fields identified as positive for Enterohaemorrhagic *Escherichia coli* (commonly called EHEC) to localize natural contamination events; and, 3) analyze the survival and persistence of attenuated *E. coli* O157:H7 on field romaine lettuce at different nitrogen fertilizer levels. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Experiments designed to characterize the survival and utility of surrogate* E. coli *O157:H7 strains for future research projects were inconclusive.* The survival of all applied *E. coli* surrogates (non-pathogenic and/or attenuated strains) was very low in this first trial attempt within a commercial operation rather than a designated experimental field. By definition, field-level experiments are like commercial production in that results can be severely impacted by environmental conditions. In this first trial in a commercial spring mix field, irrigation problems, excessive weeds and warm weather served to cause less than optimal plant growth. Although differences in survival were observed between various surrogate *E. coli* strains and attenuated *E. coli*, it is premature to ascribe any significance to the reliability of these data due to the quality of the field. The researchers

hope to repeat this trial in the near future. They feel that the usefulness of this data resides in the observation that even under the stress-inducing conditions of this field trial, survival of the applied attenuated at) *E. coli* O157:H7 was confirmed by enrichment-based detection at 8 and 14 days post-inoculation indicating potential usefulness of these strains in future field-level experiments.

What does this mean for you? It is premature to draw conclusions from the work reported on this objective. However, this work bears watching. The development and evaluation of attenuated and surrogate strains (safe substitutes for pathogens) of *E. coli* O157:H7 and other Shiga-toxigenic *E. coli* (STEC) pathogens are important because they enable field level studies of *E. coli* O157:H7 and their survival in a natural environment. Field-level experiments with pathogenic strains of live *E. coli* O157:H7 would generally not be permissible owing to the potential for inadvertent contamination and threats to public health. The surrogate and attenuated strains Dr. Suslow and his team are developing are not capable of causing disease and thus permit field experiments. Understanding their biological characteristics will help researchers understand their behavior relative to the authentic pathogen.

2. **Finding:** *Although opportunities for involvement in Rapid Response event analyses were less frequent than anticipated, significant learnings were derived from working with growers that had positive* E. coli *O157:H7 or* Salmonella *test results.* The rapid response aspects of this project derived from requests by growers for assistance to Dr. Suslow's group when they received presumptive or confirmed pathogen test results. These opportunistic events engaged the Suslow team as investigators in a real time contamination event where they conducted detailed risk assessments and performed additional pathogen testing in an attempt to determine the cause of the pathogen contamination. The key learnings and/or results from five specific events are:

   - **Event 1: Pig Intrusion before harvest.** Pre-harvest scouting determined feral pig intrusion on the edge of two lettuce blocks resulting in notification of the Suslow group to assist in the investigation. Samples of soil and lettuce from damaged lettuce plants in the area encompassing the pig intrusion as well fecal droppings resulted in no positive tests for EHEC or *Salmonella* within and beyond the 30-foot "no harvest zone". Interestingly, testing did reveal that generic *E. coli* was present on lettuce plants closest to the pig fecal droppings but at reduced levels inside of the buffer zone.

   What does this mean for you? First off, this incident shows the value of pre-harvest inspections, i.e. pig damage was observed, the damaged area was marked and a buffer zone established. The subsequent testing by the Suslow group showed that while no pathogens were detected, elevated levels of generic *E. coli* were found but not outside the buffer zone. This indicates that intruding pigs can indeed transfer bacteria to plants and soil but that the concept of a buffer zone can be effective in managing that risk. Therefore, the buffer zones prescribed by the Leafy Green Products Handler Marketing Agreement (LGMA) guidelines appear both appropriate and adequate.

- **Event 2: Deer Intrusion and Irrigation Source.** The Suslow team was notified of an animal intrusion near a surface source of irrigation water that demonstrated elevated levels of generic *E. coli*. Additionally, deer pellets were collected from the immediate area and made available to the Suslow group for testing. The microbiology testing revealed high levels of indicator *E. coli* present in deer pellets but no EHEC was detected. The water testing showed that the *E. coli* levels had fallen below the LGMA actionable levels (<235 MPN/100 ml) on consecutive days of testing. Additional testing using filter concentration of a larger volume of water followed by an enrichment step yielded a PCR-positive result for *E. coli* O157:H7. This result was confirmed by plating on selective media. These positive test results were repeated 72-hours later. The grower immediately destroyed the crop in question and discontinued used of the contaminated reservoir.

What does this mean for you? Again, this event demonstrates the value of pre-harvest inspection. Deer droppings around a water reservoir triggered a follow up investigation and a proactive decision to forego harvest was made in an exercise of reasonable caution. This event also shows the value and the limitations of "indicator" based tests. Elevated generic *E. coli* levels in deer pellets and reservoir water indicated a potential problem but follow up testing of the water showed levels had declined below the actionable levels prescribed by the LGMA guidelines. Sampling larger volumes of water and employing a concentration and enrichment step revealed that while generic *E. coli* levels may have declined, measurable levels of *E. coli* O157:H7 were present. It is important to note that the data do not lead us to any conclusions about whether the crop was contaminated with *E. coli* O157:H7 or that the presence of this pathogen in the irrigation water could have led to human disease. However, the data do show that it is important to understand what specific types of microbial tests mean. If the grower had only done generic *E. coli* testing and had seen the levels fall below actionable levels, they may have concluded that the reservoir and perhaps the field were "safe". By following up with specific pathogen testing and using larger sample volumes and enrichment steps, pathogenic *E. coli* was found and reasonable caution dictated destroying the crop and discontinuing use of the reservoir. It is always important to know what the results of specific tests may mean to understand the actions that will need to be taken if one gets a "negative" or "positive" result.

- **Event 3: Presumptive *E. coli* O157:H7 Positive on Bagged Spinach.** A commercial lab testing finished products for a processor found a positive sample for *E. coli* O157:H7. The processor contacted the Suslow group for a follow up risk assessment and investigative testing to see if the cause of the positive test could be identified. The team did not identify any obvious site risk factors. However, as the field was nearing harvest, an unusual early season rainfall followed by warm weather and then additional rains (note: this is the same weather discussed previously in the Harris and Marco projects) may have contributed to the positive test results. Additional sampling of finished product uncovered several PCR-positives for *E. coli* O157:H7 and EHEC. Indeed, 16% of the leaves in a 150-gram sample were positive for *E. coli* O157:H7. In the process of performing the microbiology analysis and doing PCR testing, the researchers also noted unusual PCR results and then atypical colony morphologies

compared to *E. coli* O157:H7. This may be indicative of the presence of more than one strain or genotype of *E. coli* O157:H7.

What does this mean for you? This event brings out at least three interesting points. The first is that there were no obvious risks identified with the growing environment for this crop. There was no evidence of animal intrusion or issues with irrigation water or any other of the potential risk factors associated with production and harvesting yet positive samples were detected in finished products. Once again, this speaks to the need to have multiple hurdles in food safety programs so that if an event is not detected, controlled or addressed at one hurdle, it may be at the next one. Secondly, the unusual PCR and atypical colonies associated with this event suggest that more than one *E. coli* strain may have been associated with this contamination event. Actually, the industry saw evidence of the same occurrence earlier in 2010 when an illness outbreak of *E. coli* O145 associated with romaine lettuce showed different phenotypic strains were involved. It is not clear yet what this observation tells us about product contaminations, but this report and the outbreak of earlier this year (with a completely different pathogen and location) may underscore the need to consider other phenotypic strains as we move forward in our efforts to understand the biology of contamination. Thirdly, typical commercial tests employ 25-gram samples of leaves. In these studies, 150-gram samples were used. If standard sampling had been used in these studies, it is possible that the contamination would have gone undetected. Indeed, only 16% of 190 leaves (150-grams) were found to be contaminated. One can easily imagine a scenario where if only a sixth of these leaves or 25-grams were selected for testing, only uncontaminated leaves might be included and the sample determined to be negative. The most important learning here is that sample size is critical in testing and obviously larger sample sizes are more desirable.

- **Event 4: Presumptive Salmonella and EHEC Positive in pre-harvest and harvested Spinach.** The Suslow team was contacted regarding PCR positive samples for EHEC and *Salmonella* in pre-harvest samples and harvested products in adjacent spinach blocks. Subsequent sampling by the UC-Davis team did not confirm *Salmonella* positives for the adjacent blocks but did find multiple EHEC positives for retained product samples for the original block. *Salmonella* was found on environmental samples by the team. Interestingly, the *Salmonella* strain detected by the rapid response team was different than the strain detected in the original samples. The key risk factor appeared to be the irrigation water source. However, the actual source of contamination could not be determined.

What does this mean for you? This event reinforces the observations from Event 3 in that apparently multiple strains of *Salmonella* were involved in this contamination event. Multiple potential pathogens may be present in environmental settings and/or crop inputs but persistence may be highly ephemeral under typical growing conditions. In this event the irrigation water source was suspected, but the team could not find EHEC or *Salmonella* on adjacent blocks irrigated from the same source as the block in question. This suggests that detecting pathogens in irrigation sources may not be readily correlated with detectable contamination of crop or product. This area deserves more research.

- **Event 5: Confirmed *Salmonella* Positive on Arugula.** The Suslow team was contacted regarding positive samples for *Salmonella* on Arugula. Although there was an unavoidable delay in conducting site evaluations and sampling, subsequent sampling by the UC-Davis team did not confirm *Salmonella* positives for the production block but did find *Salmonella* positives in environmental samples from an irrigation source. Interestingly, *Salmonella* could not be confirmed after 10 days under ambient conditions.

What does this mean for you? This event reinforces the observations from Event 4 in that apparently the persistence of pathogens may be highly ephemeral under typical growing conditions. Based on the site evaluations and sampling conducted, the survival of *Salmonella* on Arugula under field conditions seems to be limited.

3. **Finding:** *Nitrogen levels applied to the soil did not impact survivability of pathogens.* The survival and growth of pathogenic *E. coli* O157:H7 under different levels of nitrogen was evaluated; the results showed that nitrogen dose had no demonstrable or practical effect on detectable survival of attenuated *E. coli* O157:H7 applied as a foliar spray. Survival detection by enrichment revealed only one positive plant in a population of 360 plants at 14 days but none beyond that time.

   What does this means for you? The results from this objective indicate that nitrogen level does not impact survivability of attenuated *E. coli* O157:H7. Earlier research by another group had indicated the opposite. This area bears watching. There is still a lot to be learned about the nutritional environment on the surface of leafy greens and other crops and survivability of human pathogens. This experiment also seems supportive to the work previously reported by Harris and Marco at the Symposium as the Inoculated attenuated *E. coli* O157:H7 did not survive well in the production environment.

PROJECT #4: *Examination of the survival and internalization of* E. coli *on spinach under field production environments*

Principal Investigator: Steven Koike, UC Cooperative Extension, Monterey County

Layman's Summary (Source: CPS Program Notebook- June 23, 2010
Field trials in the Salinas Valley, a major leafy greens region, confirmed an earlier research that simulated *E. coli* contamination of soil and plants is short-lived. We applied *E. coli* strains (generic and attenuated 0157:H7 stx-neg) using different inoculation methods to field plots planted with spinach seed. Attenuated strains are *E. coli* 0157:H7 bacteria that lack genes for toxin production. The spinach was grown commercially using overhead sprinklers. Whether applied to the seedbed before seedlings emerge or at different stages of leaf development, the *E. coli* strains decreased 10,000-fold or greater in the first four days post-inoculation. When *E. coli* was introduced to the seedbed surface as solid inoculum in a porous sachet, survival was likewise brief; these bacteria were generally not recovered at sites distant from the sachet nor transferred to surrounding plants. When *E. coli* was delivered to spinach roots via sub-surface drip irrigation, we did not recover bacteria from spinach foliage; inoculated bacteria were readily recovered from soil adjacent to the drip lines, demonstrating that viable *E. coli* was introduced to the roots. When mature plants were inoculated and immediately disked into the soil, recovery on the inoculated strains from soil and spinach residue was possible for over 100 days post inoculation. Based on these experiments within a commercial production environment, it appears that the greater risk of persistent contamination may be the period close to harvest. These outcomes further suggest that current LGMA metrics of five-foot no-harvest zone around an in-field contamination point are adequate.

Technical Findings and What it Means for You:
This project objectives were 1) monitor survival of generic and attenuated *E. coli* O157:H7 strains in soil and on foliage in a field spinach production environment and 2) determine whether *E. coli* can be internalized by roots of field grown spinach and be systemically transported to foliage (internalization). The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Attenuated* E. coli *O157:H7 and generic* E. coli *sprayed on soil or placed on soil did not survive well under commercial growing conditions.* However, the same bacteria sprayed on whole plants that were then turned under the soil survived up to 85 days. The survival of various *E. coli* strains (mixtures of generic *E. coli* and attenuated *E. coli* 0157:H7rifampicin-resistant strains) applied as water-based sprays or mixed with sand and placed in mesh bags to simulate point sources of contamination did not survive in soil for long periods of time under commercial growing conditions in the Salinas Valley. After four days the level of pathogen was decreased by at least ten thousand-fold and by fifteen days after inoculation, recovery was below the detection limit for both strains; only generic strains were detectable by using enrichment methods. However, when mature spinach plants were spray inoculated and immediately disked into the soil; inoculated bacteria were recovered from field plots for over 85 days.

   What does this mean for you? These data indicate that these particular attenuated and generic *E. coli* strains do not survive long in soils under commercial production

conditions. However, generic *E. coli* strains were detectible using enrichment methods indicating that these bacteria were not totally eliminated. These data reinforce results from studies previously reported in the Harris and Suslow programs. It is important to note that other research reports have shown survival of inoculated pathogens in soil for extended periods of time. Indeed in these studies, the Koike team sprayed mature spinach plants with attenuated *E. coli* O157:H7 and generic *E. coli* and immediately disked the plants into the soil. Inoculated bacteria were recovered from disked field plots for over 85 days. So why did the bacteria survive when disked in on spinach plants and not when just applied directly to the soil? Up front it is important to recognize that there remains a great deal of work to do to better understand all of the factors that either increase or diminish pathogen survival in soils.

Additionally, the experimental protocol employed in this very preliminary study does not reflect current production practices for spinach. Typically, spinach is harvested by an automated harvest machine which clips the spinach an inch or less from the bed surface. Post-harvest, the field may sit un-touched for a few days and then the ground is disked and prepared for future plantings. Clearly, reality is different from the experiment and we need to bear that in mind when considering the preliminary data. That said, when the attenuated pathogen or generic *E. coli* was sprayed on spinach leaves and immediately disked under the soil, the bacterial survival may have been enhanced by the fact that the turned plant material could serve as a nutritional source for the bacteria. Indeed, it seems reasonable to assume that organic matter and other nutritional factors, degree of aeration and moisture levels in the soil as well as native microflora, temperature and exposure to sunlight can impact pathogen survival. This is clearly an area of produce safety research that bears continued monitoring.

2. **Finding:** *Spinach plants growing in soil inoculated with attenuated pathogen were not subsequently contaminated with those pathogens and the pathogens did not appear to move away from the inoculum source.* Attenuated pathogen or generic *E. coli* was either sprayed on the soil or placed in mesh bags to serve s a point source of contamination in a production field. Spinach was planted and then leaves were harvested and tested for the presence of attenuated *E. coli* O157:H7 and generic *E. coli*. In no instance was either organism recovered, via direct plating from the spinach plants growing through inoculated soil or next to bag inoculum. The researchers also used the bagged inoculum to see whether the attenuated pathogen or generic *E. coli* could move through the soil as some have previously postulated. For soil samples taken further away from the mesh bags, low populations of the generic strain were found on day one at both 25 and 50 cm distances. After day one, no generic strains were recovered at any time up until the experiment was ended after 15 days. From the 25 and 50 cm distances, no attenuated strains were recovered at any time during the experiment.

   What does this mean for you? This is a reassuring data point that tells us even if the soil might harbor low levels of pathogenic bacteria, plants emerging through that soil may not necessarily become contaminated with those pathogens. While this is reassuring, we must be careful not to over-estimate what this might mean in production. This is a single

data point and we must be careful not to interpret this too broadly so that we discount the importance of preventing any soil contamination that is within our capabilities.

The second point here is that it does not appear that pathogens can move freely through the soil in their own. This is an important observation as it relates to buffer zones and animal intrusion events. For example, it is not uncommon to observe animal intrusion events in a production field for any commodity. Sometimes these intrusions result in animal droppings being left behind in the field. A way of looking at this might be that these droppings could be considered a point source contamination event. The data presented by the Koike team suggests that if these droppings were indeed contaminated with a pathogen, it will likely remain localized to that site and not just move on its own through the field. Therefore, the approach of creating a buffer zone around the dropping site and preventing harvest within that zone is a reasonable mitigation step to manage the risk. However, if physical evidence exists that the droppings and perhaps any pathogens the droppings might contain were somehow spread, i.e. tracked by workers or washed by irrigation water to other parts of the field, then obviously one must reassess the risk and perhaps adjust risk management activities.

3. **Finding:** *Attenuated* E. coli *O157:H7 and generic* E. coli *does not appear to attach to spinach roots are be transported inside the plant through the root.* When attenuated *E. coli* O157:H7 and generic *E. coli* strains were inoculated onto spinach roots by using a subsurface drip irrigation system, the above ground foliage did not test positive for either *E. coli* strain using direct plating methods. Surface sterilizing plants with mercuric chloride followed by enrichment culture resulted in only one of 80 whole plants being positive for the rifampicin-resistant generic *E. coli*. Soil collected adjacent to the subsurface drip tapes tested positive, by direct plating, for both generic and attenuated strains, confirming that viable inoculum was delivered to the subsurface soil area. Populations of both generic and attenuated strains declined rapidly over time.

   What does this mean for you? There have been several reports indicating that pathogenic bacteria can be shown to adhere to root tissues or even become internalized within the root tissues. Generally, these experiments have been designed to expose plant root systems to very high concentrations of pathogen and under a specific set of lab conditions, pathogens have indeed been induced to associate with root tissues. This experiment by the Koike group addresses the same question but replaces the lab conditions with actual production conditions. In order to ensure the pathogen was delivered to the root area, a sub-soil drip irrigation system was used and confirmation testing verified that indeed the bacteria were delivered to the roots. Under commercial growing conditions, neither the attenuated pathogen nor the generic *E. coli* strains were shown to colonize the root or migrate to the aerial or edible parts of the plant. This is a valuable experiment as it serves as a counterbalance to lab-based experiments. However, again caution must be observed as this is a single experiment conducted over one growing season in a single environment. Clearly, more work is needed in this area, but the data dispute several lab-based studies and suggest that this mode of contamination may not be a principal route in commercial production.

SESSION II: *Enhanced Testing Methods for Pathogens in Produce*

PROJECT #5: *A high-throughput, culture-independent approach to identify index and indicator species for* E. coli *0157:H7 contamination*

Principal Investigator: Gitta Coaker, Department of Plant Pathology, University of California, Davis

NON-TECHNICAL SUMMARY (Source: CPS Program Notebook- June 23, 2010)
Recurrent outbreaks of *E. coli* 0157:H7 linked to lettuce and leafy greens produced in the California Central Coast region are of high concern to both the industry and public health regulators. The absence of recognized sporadic illnesses or outbreaks from product originating from winter-production regions in southern deserts has engendered broad speculation as to causes. One commonly held hypothesis is that although similar risk factors for pathogen contamination exist in both regions, climatic conditions and other factors result in an exclusionary leaf surface microflora in the desert regions. This proposal was aimed at identifying bacterial species that have potential as indicators of contamination for *E. coli* 0157:H7 on lettuce. Non-pathogenic microorganisms on the surface of fresh fruits and vegetables, including lettuce, can behave in a manner highly similar to those causing food-borne illness. Alternatively, some harmless bacteria are most abundant when conditions are not favorable for foodborne pathogens. The study will use a high-throughput DNA sequencing approach to identify bacterial species present on Romaine lettuce leaves grown in the Salinas, Imperial and Yuma districts. Indicator bacteria will be selected for positive or negative association with periods of *E. coli* 0157:H7 detection or growth. The verification of both positive and negative risk indicators will result in optimized testing strategies and lower overall costs for monitoring of product for *E. coli* 0157:H7. The development of novel rapid, DNA-based tests will help focus economic resources for pathogen testing on fields and lots with significant risk.

Technical Findings and What it Means for You:
The project objective was to use high-throughput DNA sequencing technologies to compare microbial communities associated with lettuce produced in the Salinas, Imperial, and Yuma districts during the seasonal cycle. By identifying both culturable and non-culturable bacterial species associated with romaine lettuce across multiple growing regions and seasons, we may gain a better understanding of potential interactions between bacterial genera and pathogenic *E. coli* species. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *The microbial population on lettuce leaves is extremely diverse and 90 to 99.9% of the population is not culturable in the laboratory.* The pyrosequencing approach used by the research group was highly successful in identifying and quantifying bacteria associated with lettuce. The DNA sequence information obtained from pyrosequencing can be used to identify bacteria at the species level. Five of the most abundant genera make up over half of the populations (Bacillus, Pseudomonas, Pantoea, Alkanindiges, and Xanthomonas).

   What does this mean for you? These data are really an affirmation that the microbial flora on the surface of lettuce leaves is abundant and that pyrosequencing is a valuable tool that permits researchers to detect unique DNA sequences that can be used to identify

specific bacterial genera. The data remind us that when pathogens like *E. coli* O157:H7 or *Salmonella* are present on a leaf surface they are not likely there by themselves. There are other species of bacteria also present. Some undoubtedly co-exist well with human pathogens and may be useful as indexing or indicator species and others perhaps are inhibitory to human pathogens or are antagonists to their survival.

2. **Finding:** *There are seasonal and geographic differences in the levels of culturable bacteria in romaine lettuce grown in Salina versus Yuma and Imperial and it seems that coliforms may hold promise as an indexing organism.* There are lower levels of culturable bacterial populations present in Imperial and Yuma districts in the winter season than in Salinas during the spring and summer season. Further, there are much higher levels of coliform bacteria present in Salinas than in Yuma or the Imperial district where coliform levels were often below the detection levels of the methods used by the researchers. From the initial data, it seems that coliforms may hold some promise as an indexing group of bacteria because their presence and abundance more closely follows the seasonality of historical detections of *E. coli* O157:H7 in the Salinas Valley. Additionally, coliform bacteria were much more sensitive to environmental changes (humidity, temperatures, etc.) and their population size changed significantly across the summer season in Salinas. Based on these initial observations, it is possible that select coliform species may hold significant promise as index/indicator organisms for *E. coli* O157:H7 contamination.

   What does this mean for you? The data show that bacterial species and population sizes on the surface of romaine lettuce leaves can be different depending on which region they are grown in, can change from season to season and can also be affected by environmental conditions like temperature and humidity. These data compare favorably with the work of Dr. Marco and her group discussed earlier in this paper. The diversity and responsiveness to environmental factors opens up the promise that indexing or antagonistic species may eventually be identified. However, as with all research that seeks to identify factors that change by season or environment, it takes multiple seasons of data collection to make positive or negative correlations. The research team will continue to evaluate leaf samples and characterize the resident microflora using pyrosequencing and culture methods to reinforce the data from the first year of this project. The research team also indicates that the future direction of this project will focus on collecting samples from fields that have either identifiable contamination risk factors or have actually tested positive for *E. coli* O157:H7 or related EHEC. This information will be used to identify bacterial species with promise as index/indicator organisms as well as antagonistic species that might be used as biocontrol agents (i.e. bacteria that inhibit pathogen growth or survival that could be used as a field treatment to protect crops from contamination). It will be important to follow these lines of research as they offer growers potential tools for the future. If one could identify a set of indexing bacteria, they could be used as detection tools in a multi-part screen to test production fields prior to harvest. If simple detection tests for indexing species could be combined with other measurable risk factors (e.g. temperature, humidity, observational risk assessment, etc.) one might be able to prescreen production fields prior to harvest and determine the level of risk that a pathogen might be present or even forecast potential "high risk" periods for pathogen growth in fields. If the risk analysis indicated a "high" risk then further pathogen testing would be warranted. If the

risk analysis indicated a very low likelihood of pathogen presence then preharvest testing could be minimized and/or the field could be cleared for harvest. On the other end, while bacterial biocontrol agents represent challenges, if antagonistic bacterial species that inhibit pathogens could be identified, one could easily envision crop applications when index testing and risk assessment indicated an increased risk of pathogen presence.

PROJECT #6: *Enhancing the effectiveness of human pathogen testing systems for the advancement of practical produce safety research and commercial management*

Principal Investigator: Carol D'lima, University of California, Davis

NON-TECHNICAL SUMMARY (Source: CPS Program Notebook- June 23, 2010)
This project accomplished its three primary objectives: 1) refine and validate a novel, rapid method for screening diverse environmental and product samples for a broad class of pathogenic *E. coli*; 2) verify the function of a simple sample "banking" method to allow delayed pathogen testing; and 3) application of the developed tools within a standardized scheme for investigation of natural contamination events of leafy greens. To accomplish the first objective, diverse samples were inoculated with very low concentrations of human pathogenic *E. coli*. The highly specific DNA probe detected all *E. coli* 0157:H7 and over 40 non-0157 pathogenic *E. coli*, including the six priority non-0157 types most associated with human illness. Importantly, the probe excluded a wide range of related coliform and non-pathogenic leaf, water and soil bacteria commonly found on produce. Sample DNA "banking" using a commercial system not requiring expensive freezing equipment, called FTA Cards, was effective for over two months storage with limited loss of detection efficiency. This technology will be used to more broadly secure growers permissions for farm access to conduct detailed grid-analysis of a crop separated in time from marketing. Putting the pieces together, naturally contaminated samples, including irrigation water, animal fecal matter, manure –laden soil, compost, and leafy greens, from natural field contamination events were tested. The probe for pathogenic *E. coli* proved useful for rapid molecular screening, confirmation test, and mass screening of colonies for inclusion or exclusion to identify the source of the initial pathogen test reactions that suggested a commercially contaminated crop.

Technical Findings and What they Mean for You:
This research project was directed primarily at developing pathogen testing tools for both researchers and growers. The research team has had a long history of working with the produce industry on food safety issues and in particular has worked to develop more selective pathogen tests for produce. The project objectives were: 1) Conduct a comprehensive analysis of available protocols and emerging technologies to stabilize and preserve enrichment samples and to develop a standard protocol for sample preparation and extraction; and, 2) design a probe based version of a specifically designed primer set that will detect the presence of pathogenic, shigatoxin-producing *Escherichia coli* (STEC) and Enterohaemorrhagic *Escherichia coli* (EHEC) and validate its use in produce plant tissue as well as other environmental samples. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Using FTA cards to store sample extractions for future PCR testing was shown to be feasible.* FTA card extraction and evaluation by PCR was done on a monthly basis over six months to determine their capacity to store DNA at room temperature without degradation. In some of the samples, detection of the DNA target from newly extracted FTA card DNA was lost over time indicating possible degradation of DNA. However, when considering factors such as space, expense, and ease of shipping, FTA cards do appear to have a clear advantage over tubes of frozen samples.

What does this mean for you? One of the limiting factors in on-farm investigations has always been laboratory capacity to process samples for DNA and then perform PCR. By being able to obtain samples, extract their DNA and then hold or store them without DNA breakdown, the researcher can take more samples to better help determine where a contamination might have originated without worrying about capacity to perform PCR and analyze the data all at once. This could be a valuable tool going forward. For example, if a pre-harvest sample for *E. coli* O157:H7 tests positive and the grower wants to determine where the contamination might have come from and how widespread it might be, a researcher could effectively lay out a sampling grid and take hundreds or thousands of samples in a precise pattern, extract the DNA according to the protocol developed by this research team and store the sample (product, soil or water) DNA on the FTA cards and then perform PCR to detect pathogen DNA as PCR capacity and time permits. The more extensive the sampling, the more likely the researcher is to find the root cause of the contamination. The time delay element is also crucial for more practical reasons. Many times growers have been hesitant to permit researchers to follow up on positive product tests during an active production season thereby missing an opportunity to learn from the event. Often it is much easier to simply plow under lots that test positive (thereby protecting public health) and not jeopardize the next crop available for harvest. Using these FTA cards, a researcher can take samples when a contamination event occurs and then hold those samples until after the growing season is over for PCR testing and analysis. This helps mitigate grower concerns and may afford the industry better opportunities to understand causative risks.

2. **Finding:** *An improved PCR assay was developed for detection of Total Pathogenic* E. coli *or TPEC (Total Pathogenic* E. coli *).* The research team examined collections of pathogenic *E. coli* and nonpathogenic bacteria using PCR tests based on known pathogenic *E. coli* genes, *rfb*E, *uid*A, *eae*A and TPEC. They found that these standard primer DNAs did not always detect non *E. coli* O157:H7 strains. However, they were able to fine tune the molecular structure of the *eae*A primer and when used in combination with the TPEC probe in a PCR assay, the researchers detected 32 out of 40 pathogenic *E. coli* strains (including the O-types O26, O145, O111, O121, and O103). The eight strains that were not detected originated from older isolates that have likely undergone genetic change through several years of subculturing. The research team also used the TPEC/*eae*A PCR test to test in two risk-based field evaluations conducted in fields with evidence of animal intrusion and also in finished spinach product provided for the evaluations. The redesigned PCR test proved to be valuable as a detection tool for pathogenic *E. coli*.

   What does this mean for you? This project employed a number of approaches to comparing existing PCR testing systems. The results highlight one of the key issues the produce industry has been dealing with for the last five years; many of the PCR primer sets used in testing for pathogens in produce samples simply do not detect all strains of pathogenic *E. coli* equally well. This project shows that primer DNA sequences can be modified to increase their selectivity to give the tests greater value for detecting broader collections of pathogenic *E. coli* strains. This work confirms how important it is that growers and processors (as well as buyers who request product testing) work with testing laboratories to understand the selectivity and sensitivity of the tests they are

employing and the apply them appropriately based on clear understanding of what the test results mean since these tests are used to make harvest, shipping, sanitation or other operational decisions.

PROJECT # 7: *A sensitive and specific molecular testing method for live* Salmonella *in produce*

Principal Investigator: Beilei Ge, Ph.D., Louisiana State University

NON-TECHNICAL SUMMARY

Current detection methods for *Salmonella* include: culture-based (it takes too long), immunological-based (used for confirmation only) and molecular-based (PCR is successful but may be costly). This project seeks to address the challenge associated with *Salmonella* detection in a variety of produce items. A novel molecular testing method, loop-mediated isothermal amplification (LAMP) was examined. Compared to the commonly used PCR methods, LAMP offers several attractive features such a high specificity, no requirement of a PCR machine, easy to read results, high sample through-put, and cost-effectiveness. Additionally, a novel reagent, propidium monoazide (PMA) was incorporated to differentiate live/dead cells during the testing. Specific objectives included 1) To design and optimize a LAMP assay that targets *Salmonella* strains; 2) To evaluate the sensitivity and specificity of the LAMP assay in detecting live *Salmonella*; and 3) To apply the assay in the detection of live *Salmonella* in experimentally contaminated produce items (baby spinach, sliced tomato, and cantaloupe cubes). Under the optimized conditions, the PMA-LAMP assay gave consistent negative results for heat-killed *Salmonella* cells with concentrations ranging from 101 CFU/ml to 107 CFU/ml and could detect 34 live *Salmonella* cells in the presence of 105 CFU/ml of heat-killed *Salmonella* cells, without false positive results. The complete assay took about 3 hours to complete when testing produce samples. This rapid, sensitive, and accurate detection assay for live *Salmonella* in fresh produce may provide the produce industry an increased ability to control potential microbial hazards that may contaminate the fresh produce.

Technical Findings and What They Mean for You:

The project objectives were: 1) design and optimize a LAMP assay that targets *Salmonella* strains, 2) evaluate the sensitivity and specificity of the LAMP assay in detecting live *Salmonella*; and, 3) apply the assay in the detection of live *Salmonella* in experimentally contaminated produce items (shredded lettuce, baby spinach, sliced tomato, sprouts, and cantaloupe cubes) of various stages of maturity. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Technical Finding: Five sets of LAMP primers were developed based on the *Salmonella inv*A gene.** Four of the five primer sets were shown to detect *Salmonella* effectively. The researchers chose the use of a real-time turbidimeter as a way to detect LAMP products instead of using color change as originally proposed. The assay sensitivity/assay specificity was tested and this LAMP test was found to be highly specific, i.e., no false positive or false negative results were observed when tested against a panel of 25 *Salmonella* and 25 non-*Salmonella* strains. The assay took 30 minutes and the assay was shown to detect as little as 13 cells using serial dilutions.

   What does this mean for you? In those commodities where product testing has become more routine, e.g. leafy greens, the industry has seen frequent instances where samples are originally "positive" by PCR testing but do not confirm when tested by further PCR

methods or standard BAM plating methods. This has been particularly acute when testing for *Salmonella* where initial positives plate out to be *Klebsiella* or *Acetobacter*. The development of this LAMP *Salmonella* test holds promise in that the test is fast and the use of the turbidity meter makes the test quantitative, i.e. PCR is basically a plus/minus test; it tells you if pathogen DNA sequences are present, but it doesn't tell you how much contamination is present. Using the measure of turbidity as an indicator, the LAMP test can be made to be quantitative. A standard curve can be generated and one can determine the initial level of contamination in the sample.

These initial results look promising in that LAMP in combination with turbidity monitors show potential as an effective test for *Salmonella*. Therefore, it is important to follow this line of research to see if this initial promise can be shown to have practical importance in produce systems.

2. **Finding:** *The use of PMA (propidium monoazide) can be useful for determining live versus dead cells.* One of the questions frequently asked when using DNA-based testing is whether the cells the DNA is extracted from were actually alive and capable of causing illness. Dead cells would be expected to have openings in cell membranes that would allow PMA to get inside the cell and bind to DNA thereby preventing amplification. Conversely, live cells would have structural integrity in their membranes thereby preventing infiltration by PMA so that DNA from live cells can be amplified. Assay design and optimization were tested using PMA treatments on live and dead (heat treated) *Salmonella* cells. PMA was shown to be effective in eliminating dead cell DNA from LAMP assay detection, i.e. PMA-LAMP on dead cells consistently gave negative results using a range of dead cell populations.

   What does this mean for you? The use of PMA is an interesting laboratory procedure to ensure the LAMP test only detects DNA from live cells. Today's testing methods generally include enrichment steps that permit pathogen cells that are in stationary physiological states to recover and grow reaching populations sufficient for PCR testing. Without further studies to test LAMP and LMAP/PMA versus standard PCR testing in naturally contaminated produce samples, it is difficult to determine the value of this procedure. Again, this is an area of research that warrants additional study. Some preliminary studies were reported in this study using PMA/LAMP and homogenized extracts of melons, tomatoes or spinach. These lab-based studies show the PMA/LAMP test was clearly able to eliminate dead cells from detection and demonstrated impressive sensitivity for detection of live *Salmonella* cells.

SESSION III: *Potential Vectors for Pathogen Transfer During Field Production*

PROJECT #8: *Fly reservoirs of* E. coli *0157:H7 and their role in contamination of leafy greens*

Principal Investigator: Astri Wayadande, Ph.D., Oklahoma State University

Layman's Summary

The proposal objectives were to 1) capture flies in Salinas Valley leafy green production areas within a ten-mile radius of a confined cattle operation or rangeland, and test them for presence of *E. coli* 0157:H7; and 2) determine if *E. coli* 0157:H7 colonization of the leaf phyllosphere occurs after fly regurgitation on the plant surface. Flies were sampled four times in 2009 at six Salinas Valley locations and tested singly or in groups of ten for *E. coli* 0157:H7 by plate isolation and serology followed by PCR. Using this strategy, only four of the 1250 flies' samples were positive for *E. coli*. The unexpected low numbers of positives using standard microbiological methods prompted a change to an assay that split the sample between plating for recovery and immediate PCR analysis using *flic*C, *rfb*E, *stx*1 and *stx*2 specific primers. Flies were then sampled twice during the winter months at one location in the Imperial Valley and tested in groups of ten for *E. coli* 0157:H7 by microbiological methods and multiplex PCR. To date, 17 of the 98 pooled samples were positive by PCR. Scanning electron micrographs of regurgitation spots from flies that had fed on inoculated manure or bacterial lawns revealed that regurgitated bacteria attach to the surface of spinach leaves and persist for at least one week. Regurgitation spots from flies exposed to control manure or uninoculated plates had a few isolated bacteria on the leaf surface. The SEM results suggest that regurgitated bacteria may survive and colonize spinach surfaces.

Technical Findings and What it Means for You:

Flies are common vectors of pathogens, with a lifespan of 7-14 days. Earlier research reported in 2008 suggested flies could be a potential vector for transfer of pathogenic *E. coli* O157:H7 from potentially contaminated areas (feedlots) to leafy greens production fields. This study was formulated to evaluate that possibility. Six sites were evaluated near leafy greens facilities. The project objectives were: 1) test flies in California and Oklahoma feedlots/rangeland for *E. coli* O157:H7 to determine what proportion of tested flies are *E. coli* O157:H7 positive, what time of the year they are more prevalent, what proportion of the samples are positive and if they transmit *E. coli* O157:H7 to plants 2) test flies captured in leafy green production areas to determine what times of year are *E. coli* O157:H7 positive flies more prevalent, what portion are positive and also what times of the year they are more prevalent; and, 3) examine flyspecks on spinach for evidence of bacterial colonization over time to determine if excreted bacteria form biofilms on the leaf surface and, if they do, how long do excreted bacteria remain viable on the leaf surface. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *A very low percentage of flies captured in the Salinas Valley were* E. coli *O157:H7, in contrast most flies from El Centro site were positive for multiple strains of* E. coli *O157.* Flies were collected from six locations in Monterey and San Benito counties from April through September 2009. Most flies captured in Salinas throughout the summer were houseflies. A very low proportion of flies in the Salinas area tested positive for *E. coli* O157:H7 using a standard microbiological plating approach. Flies were also captured

in southern California near El Centro in December 2009 and January 2010. Over 1500 flies were tested (and only 4 were positive), but this was far short of what the researchers expected to capture and test. The researchers believe that their findings underestimate the number of filth flies carrying *E. coli* O157:H7 and perhaps other STECs and EHECs. A much higher proportion of flies captured in southern California and tested for the presence of *E. coli* O157:H7-specific genes by PCR were positive. Indeed, even non-O157 strains were identified. The greater sensitivity and accuracy of PCR-based detection makes this diagnostic preferable to culture-based detection for the presence of bacterial DNA, but the test did not distinguish between live and dead bacteria.

What does this mean for you? Although a low percentage of flies tested positive in Salinas compared to the ones tested near El Centro, the sample numbers are still pretty low so this does not necessarily translate into a conclusion that there are more potential issues for one area versus another. The scientists on this team believe that there was an underestimation of the true number of *E. coli* positive flies when using plating techniques and they are switching over to PCR methods for future work. These results tell us that flies can carry *E. coli* O157:H7 and that apparently multiple strains of pathogenic *E. coli* can be found in flies. These studies do not definitively identify flies as a significant risk factor and more sampling is needed to define their actual risk potential as a vector.

2. **Finding:** *Preliminary data suggest that bacteria acquired from inoculated manure survive the ingestion and regurgitation process (part of the digestion process of a number of flies).* This is critical because flies place their saliva onto potential food sources and can transfer bacteria. To understand the potential for pathogenic bacteria to transfer from flies and survive on leaf surfaces, house flies were exposed to different laboratory-prepared sources of pathogens, released onto spinach plants and then regurgitation spots were analyzed. The electron micrographs clearly show transfer of *E. coli* to the surface of the spinach leaf and increased numbers of cells after seven days indicating the regurgitated bacteria may survive and grow in lab conditions. Indeed the researchers suggest that their observations are consistent with the formation of a biofilm on the leaf surface.

   What does this mean for you? These results are preliminary and should be validated with additional samples in order to confirm if fly regurgitation spots suggest the possibility of flies transmitting viable *E. coli* O157:H7 or related strains under optimal conditions. At this point, these results tell us that flies can carry multiple strains of *E. coli* O157 and that they may be able to transfer viable cells to spinach leaf surfaces. These data point clearly to the importance of having risk-based GAP programs that provide multiple risk management hurdles or protective firewalls that reduce the risk of contamination from flies ever occurring.

PROJECT #9: *Food safety risks associated with sheep grazing in vegetable stubble fields*

Principal Investigator: Bruce R Hoar, D.V.M., Ph.D., University of California, Davis

Layman's Summary

Sustainable crop and livestock production, particularly in organic and reduced- till settings, would benefit from utilization of sheep as aids in controlling unwanted or excess vegetation growth. However, recent outbreaks of *E. coli* 0157:H7 linked to consumption of California produce have dramatically raised concern that sheep and other ruminants may elevate levels of pathogens in the soil and in subsequent crops grown on the grazed location. In order to assess the validity of these concerns and to develop science-based recommendations regarding sheep grazing and food safety, several key questions must be addressed. First, what is the prevalence of potential human pathogens in the feces of California sheep that graze vegetable crop residue? Second, what is the range of concentration or intensity of these bacteria in sheep fecal material? Third, does this prevalence or intensity of fecal shedding shift upward or downward as sheep are rotated through different crop systems? Finally, what is the rate of inactivation of bacteria once it is deposited onto the soil surface and subsequently exposed to solar radiation or tilled into the soil in preparation for the next crop?

Technical Findings and What it Means for You:

In the San Joaquin and Imperial Valleys of California, vegetable growers have often cooperated with sheep producers; i.e. sheep producers graze sheep in vegetable fields prior to planting to remove unwanted weeds. This project was designed to evaluate the risk of contamination from pre-plant sheep grazing. The project objectives were:1) estimate the prevalence of fecal shedding of *E. coli* O157:H7 and *Salmonella spp.* by sheep grazing in different crop systems as well as to measure the intensity of fecal shedding of commensal *E. coli*, 2) determine if rotational grazing between crop systems of differing forage quality and energy content alters the prevalence of fecal shedding of *E. coli* O157:H7 and *Salmonella spp.* by sheep; and, 3) measure the rate of inactivation of *E. coli* O157:H7 and *Salmonella spp.* as a function of such parameters as time, tillage practice, irrigation, ambient temperature, etc., and compare these estimates to the fate of commensal *E. coli*. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Commensal* E. coli *was present at varying levels, but no fecal samples were positive for* E. coli *O157:H7.* However, *Salmonella* was detected in nine bands of sheep. The researchers estimated that approximately 28,000 sheep distributed in 19 bands were in the population from which the samples were obtained. Nine bands had at least one positive culture for *Salmonella spp.* At the time of this report, the serotyping on these *Salmonella* isolates was not complete. No fecal samples were positive for *E. coli* O157:H7.

   Commensal *E. coli* isolated from sheep feces was present at varying levels (5x105 CFU/ gram to greater than 1 x1010 CFU/gram. The research program was complicated in the first year owing to weather and rotational issues. As a result, the sheep in this study were grazed almost exclusively on alfalfa thereby making it impossible for the research team to look at Objective 2 on the impact of rotational grazing impacts pathogen shedding.

What does this mean for you? Prevalence and intensity of shedding of *E. coli* O157:H7 and *Salmonella spp.* by sheep in this management system is apparently low during the time period covered by this investigation. The data presented at the June 2010 Symposium covered five months of work (November 2009 to March 2010). Based on the data, it is difficult to read too much into the results. While the failure to detect *E. coli* O157:H7 is promising, we know from work in other systems that sheep can carry this pathogen. Indeed, the research team was concerned with this preliminary result and went back to be sure that their sampling and sample handling procedures were not somehow faulty (they were not). However, the absence of *E. coli* O157:H7 in this specific experiment is not sufficient to conclude that sheep represent no risk for cross contamination and additional data is clearly needed so proper context can be provided. Similarly, the finding of *Salmonella* in nine bands of sheep is, at first glance, concerning, but again, the data are limited and it would be unwise to place too much emphasis on these limited data.

This is another project which bears further attention as it moves toward completion. It is clear from the preliminary data that sheep can be a source of pathogens in the production environment. The question still remains as to whether sheep are a significant risk factor. It is not at all clear that pathogens potentially left behind following grazing can survive under field conditions (remember the San Joaquin and Imperial Valley's are subject to exceedingly high temperatures and very low rainfall) and then transfer to vegetable crops. This question embodies Objective 3 of this program and is yet to be addressed. This research team has been funded for a second project by CPS to answer some of these open questions.

PROJECT # 10: *Environmental effects on the growth or survival of stress-adapted* Escherichia coli *0157: H7 and* Salmonella spp. *in compost*

Principal Investigator: Xiuping Jiang, D.V.M., Ph.D., Clemson University

Layman's Summary (Source: CPS Program Notebook- June 23, 2010)
Raw or inadequately composted animal manure has been considered as a potential source of pre-harvest contamination of fresh produce. Composting, as a practical way for waste management on farm, can inactivate human pathogens, but many environmental factors can affect the outcome. Therefore, there is a need for developing composting guidelines and standards that apply to a wide range of conditions. In this proposed study, we hypothesize that the extended mesophilic phase due to suboptimal composting conditions may induce heat-shock response in human pathogens, which become resistant to subsequent lethal temperatures during thermophilic phase of composting. By simulating early-stage, on-farm composting in a humidity-controlled environmental chamber, we found out that composting with long come-up time (five days) can extend the survival of *Escherichia coli* 0157 significantly as compared with short come-up time (two days), suggesting the heat adaptation of microorganism. Other sub-optimal conditions (such as low moisture or C:N ratios of initial compost mixture) also extended the survival of *E. coli* 0157:H7 and *Salmonella spp.* in fresh dairy and poultry compost, respectively. To prevent possible re-growth of a few surviving pathogenic cells in cured compost, we evaluated environmental parameters conducive to pathogen growth, and applied the indigenous microorganism as a secondary treatment to the finished compost. Our study identified the critical levels of indigenous microorganisms and moisture content which can inhibit pathogen growth in compost.

Technical Findings and What it Means for You:
Raw organic fertilizers and soil amendments derived from animal sources are the only crop inputs where we are fairly certain that human pathogens have a reasonable chance of being present prior to treatment or composting that serve to inactivate these pathogens. Therefore it is critically important that the composting process be conducted and monitored closely to insure complete elimination of pathogens. This project examined three critical elements of composting to: 1) determine the thermal resistance of stress-adapted *E. coli* O157:H7 and *Salmonella spp.* in various types of compost at elevated composting temperatures in a humidity chamber by simulating early stages of on-farm composting, 2) apply competitive exclusion microorganisms as a secondary treatment to eliminate the re-growth of stress-adapted pathogens in cured compost; and, 3) improve the sensitivity of pathogen detection from compost by combing phage enrichment and the Pathatrix® detection system to improve sample testing methods. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Low moisture compost and long come-up time extends the survival of pathogens during composting.* The results consistently demonstrated that fresh compost with 40% moisture supported better survival of enteric pathogens than the compost with 50% moisture during composting. Compost with lower C:N (16:1) allowed longer survival of *E. coli* O157:H7 than the compost with optimal C:N ratio (25:1). Come-up time was one of the most critical factors during the composting trials with longer survival being observed for the compost which simulated slow heating process (5 days come-up time)

than the one with normal temperature rise (2 days of come-up time) regardless of the moisture level and C:N ratio. The thermal inactivation data for 16 out of 20 experimental series fit well into the mixed Weibull model, which is a model used to predict inactivation of *E. coli* O157:H7 during early stages of on-farm composting trials. Both plate count and modeling results suggest that microbial populations become adapted to the composting temperatures when the temperature rise during come-up time is slow or the composting was conducted under suboptimal conditions.

What does this mean for you? This finding suggests that composting processes need to be closely monitored to make sure a rapid temperature rise during early phase of composting occurs. It is advisable to keep the mesophilic phase (moderate temperature phase) of composting short and initial composting moisture as near optimal as possible. Maintaining a short come-up time (2 days) and optimal moisture (50%) during composting will help to reduce the survival of pathogens and preclude them from adapting to elevated temperatures and improving their chances of survival in "finished" compost. Growers need to understand the importance of using compost that has undergone a proper composting process that can be verified by the supplier. If preparation of compost is done on the farm, then the grower should be aware of the importance of properly treating compost materials and monitor critical elements of the process, e.g. moisture levels, temperatures, come up times and C:N ratios. Compost providers should be prepared to make process verification records and testing data available to growers as evidence of a well-controlled process.

Finding: *Competitive exclusion (CE) microorganisms can suppress the growth of* E. coli *O157:H7 and* Salmonella spp. The research team explored several environmental factors to see if the growth of *E. coli* O157:H7 or *Salmonella* could be inhibited. Competitive exclusion microorganisms were isolated from compost samples and then added back to compost as a secondary treatment to inhibit pathogen growth. These laboratory-based tests indicate that a mixture of naturally occurring microorganisms largely composed of *Bacillus*, *Brevibacillus* and Pseudomonad species can indeed inhibit *E. coli* O157:H7 or *Salmonella spp.* when present at concentrations of 6.5 log CFU/g or higher. When evaluating the effect of CE microorganisms, both the levels and types of indigenous microorganisms play an important role for controlling the pathogen growth in the compost. In the dairy compost used in these experiments, application of the minimal level of 6.5 log CFU CE microorganisms/g suppressed the growth of *E. coli* O157: H7 and *Salmonella spp.* By applying the competitive exclusion microorganisms into the compost, the growth potential of *E. coli* O157 and *Salmonella spp.* was reduced anywhere from 10- to 2,000-fold compared with the controls.The researchers also looked at temperature effects on the growth of *E. coli* O157:H7 in the presence of CE microorganisms. The results indicated that *E. coli* O157: H7 can grow in compost in the presence of CE microorganisms when outdoor temperatures are warm; such as in late spring, summer or early fall months. Experiments also showed that as moisture increases in finished compost from 20 to 40 %, the ability of *E. coli* O157:H7 to grow, even in the presence of CE microorganisms, also increases. It is clear that finished compost contains sufficient nutrients for a few pathogenic cells to grow under warm temperatures (≥22°C), pH in a range of 7.9 to 9 and water activity levels of 0.97 to 0.99.

What does this mean for you? The results suggest that finished compost is a very complex biological system. The data indicate that if composting is not completed, i.e. some small concentration of pathogens remain after composting or if finished compost is not stored properly and it becomes contaminated from another source, it contains sufficient nutrients to support pathogen growth under warm, moist conditions. The researchers point out the importance of keeping compost as dry as possible and to be especially vigilant when temperatures are warm. It is clear that compost storage conditions should be part of any risk analysis as growers put together GAP programs and should work with their suppliers to develop risk management practices to address these issues.

The data on CE microorganisms provides a window into another aspect of the exceedingly complex biological system of compost. The basic concept of looking for bacterial species that inhibit or are antagonistic to human pathogens like *E. coli* O157:H7 or *Salmonella* has been previously discussed in this paper as it shows itself as a key finding or strategy in the work of Marco (project 2) and Coaker (project 5). The data presented by the Jiang team indicate that certain types of microorganisms can indeed inhibit *E. coli* O157:H7 or *Salmonella* growth. These observations raise questions about the future potential for testing for these CE microorganisms in final compost as a way to determine the risk for pathogen growth in finished compost. These results also raise the possibility of adding back a cocktail of CE microorganisms to compost analogous to using a biocontrol agent to control pathogen levels in finished compost or to diminish recontamination risks. This is an important area of research to watch in the next few years.

3. **Finding:** *Pathogen detection sensitivity may be improved by combing phage enrichment and Pathatrix® detection system.* The research team sought to develop a more effective or sensitive pathogen detection system when testing compost. The basic concept was to isolate bacteriophages (essentially viruses that infect and kill specific bacteria) and use cocktails to eliminate non-pathogenic background bacteria in compost samples. If these bacteriophages cocktails could be used to eliminate other types of bacteria, the thought was that detection of the target pathogen bacteria might be made easier using PCR testing. The research team used a wide array of compost types and isolated 18 different bacteriophage stocks that were shown to be effective against 27 different bacterial species commonly found in composts or manures. Several different approaches to increase pathogen detection in compost using phage mixtures were attempted. The two-step application of bacteriophages to the enrichment culture resulted in the increased detection of *E. coli* O157:H7 and the reduction of interfering background microorganisms on the selective agar. Although the phage cocktail does not greatly reduce background populations of target bacteria during enrichment, there seems to be some inhibitory effects that are allowing *E. coli* O157:H7 to grow better during 4 ~ 6 h enrichment. Considering that the Pathatrix® procedure is designed to be followed by PCR, a four-fold increase in cell numbers during enrichment can enhance the ability to detect pathogenic bacteria like *E. coli* O157:H7. Since the microbial isolates can be obtained easily, those new isolates can be used to propagate better phage cocktails to target those indigenous microorganisms unique to compost and manure. Therefore, the use of bacteriophage in enrichments to inhibit background interference thus allowing the target pathogen to grow is a method with a great potential.

What does this mean for you? This finding suggests that bacteriophages specific for the compost microbial community can enhance current pathogen detection methods. This may benefit composters and the produce industry by providing the industry with improved analytical tools to evaluate the efficacy and safety of their processes.

PROJECT # 11: *Minimizing pathogen transference during lettuce harvesting by optimizing the desing of the harvesting device and operation practices*

Principal Investigator: Yaguang Luo, Ph.D., U.S. Department of Agriculture – Agricultural Research Service.

Layman's Summary (Source: CPS Program Notebook- June 23, 2010)
Laboratory studies have shown that *Escherichia coli* 0157:H7 can be transferred to lettuce by a harvesting-coring knife. However, specific scientific data that reflect realistic contamination conditions needed for risk assessment are not available. Also needed are methods to prevent/reduce pathogen contamination. The main objectives of this project were to: 1) determine soil pathogen levels required for pathogen transfer to the edible portions of lettuce via a contaminated coring knife; and 2) reduce the risk of pathogen transfer by improving coring knife design and disinfection. A series of experiments were performed with typical Salinas sandy-loam soil and *E. coli* 0157:H7 contamination levels ranging from 1 CFU/g to 100,000 CFU/g soil. Worst case scenario and real life fresh-cut processing conditions were also modeled. Results indicate that detection of *E. coli* 0157:H7 on lettuce transferred from soil via a contaminated knife is highly dependent on soil pathogen concentration, soil type and moisture level, and the portion of the lettuce head sampled. Results also show that when coring knives are washed in chlorine alone, at least 50 ppm free residual chlorine is needed in the wash solution to inactivate *E. coli* 0157:H7 on welded knife parts. However, when ultrasound was combined with chlorine, pathogen inactivation to below detection levels on all knife parts was achieved with a much lower free chlorine level. Additionally, two prototype lettuce coring-knives were designed and tested; and results showed that they improved sanitation efficacy and reduced the potential for pathogen adherence.

Technical Findings and What it Means for You:
The potential for coring knives to be a vector for pathogen transfer from the soil to the cut surface of harvested iceberg or romaine lettuce has been reported and debated for several years. Indeed, many harvesters have developed risk management practices that include dipping harvest knives into liquid sanitizers and various training practices for workers to minimize the chances for workers to contact soil with knives. This project was designed to look at this question of harvest knife cross contamination under conditions that closely resemble those found in the Salinas Valley where much of the iceberg lettuce is produced during the spring and summer months. Specifically, the project objectives were: 1) determine the pathogen levels in the soil required to permit pathogen transference to the edible portions of cored lettuce via contaminated coring knives, 2) reduce the risk of coring knife pathogen transference by developing improved coring knife design and sanitation procedures, 3) eliminate the potential for coring knife contamination via soil contact by separating the cutting and coring process and 4) identify post-harvest handling practices that can be used to effectively manage the potential food safety risks during coring-in-field (CIF) harvesting. The following are key findings (extracted from the presentation at the June Symposium and final research report) and potential implications (based on our interpretation):

1. **Finding:** *Optimizing harvesting practices can significantly minimize the potential for contamination.* Pathogens transferred from soil to harvested lettuce are a function of

pathogen concentration in soil, and the amount of soil present on the harvested lettuce, which are then impacted by a number of factors including lettuce growing and harvesting conditions, CIF lettuce harvesting methods, and knife design and disinfection. Major factors impacting pathogen transference from contaminated soil to lettuce via a harvesting knife were identified as: a) sufficiently contaminated soil; b) contact between a coring knife (blade or ring) and the contaminated soil; and c) contact between contaminated coring knife and harvested lettuce. Cutting knives are much more prone to soil contact when CIF harvest is carried out in wet soil (rain events, irrigation near harvest time, etc.) versus dry soil. Extreme care must be exercised to avoid harvest knife contact between soil, and lettuce, if CIF lettuce has to be harvested under wet conditions.

The research team looked at various soil types obtained from Salinas, CA and Yuma, AZ purposely contaminated with attenuated *E. coli* O157:H7. They examined a range of contamination levels from essentially 0 MPN/gram of soil (control) to 100,000 MPN/gram of soil. The data show that detection of *E. coli* O157:H7 on three successively cored heads is not possible at very low inoculation levels (essentially 102 MPN/gram and lower) whereas in the 102 or 103 MPN/gram range, *E. coli* O157:H7 could be found in the first head cored and not in the second and third head cored. Above an initial inoculum of 103 MPN/gram of soil, *E. coli* O157:H7 could be detected in all three heads cored.

What does this mean for you? These findings suggest that a cutting blade and coring ring have the potential to play a role in pathogen transfer. However, it is important to note that if coring is to be considered a significant contamination risk, then the blade or ring must first come into contact with the soil, in which there must be a reasonable high concentration of pathogens. We know from various environmental testing programs that we do not find high concentrations of *E. coli* O157:H7 in the production environment and we know from research reported at this same CPS Symposium that even when *E. coli* O157:H7 is purposely inoculated onto soil, it does not survive well in the production environment. We also know that equipment care and sanitation in the field is critical and most operations have SOPs requiring cleaning and disinfection of harvest knives that come into direct contact with soil. Therefore, the data presented here would indicate that unless there is a major failure in GAP risk management practices, pathogen transfer from soils (which should not normally) harbor *E. coli* O157:H7 should not be a critical risk factor. However, common sense also tells us that anything we can do to minimize soil contact with cutting blades or the product itself is important to do as we seek to diminish even small risk factors from impacting the product.

2. **Finding:** *Improving harvest knife design and disinfection can significantly minimize contamination risks.* The cutting blade and coring ring of a CIF (core in field) harvest knife can potentially play significantly different roles in pathogen cross-contamination and transference from soil to lettuce. Analysis of the current CIF harvesting practice observed in California reveals that the cutting blade has higher potential to be contaminated by the soil, but less opportunity to transfer pathogens onto to harvested lettuce. On the contrary, the coring ring has less potential to be contaminated by soil; but much higher potential to transfer pathogens onto the cut surfaces of harvested lettuce. The harvest knives currently used in the industry are designed so that the cutting blade and the coring ring are in

close proximity to each other. The research team showed that by extending the distance between the cutting blade and the coring ring, the opportunity for soil and other debris to contaminate the coring ring is diminished. Further, the research team showed that many current cutting and coring knives are constructed with less than optimal weld joints making them difficult to sanitize properly. The research team showed that by improving the weld quality (i.e. making them smoother) and extending the separation between the knife and the coring ring, cross contamination risks can be further diminished. The research team also looked at sanitation of coring knives and found that the ability of ultrasound to enhance chlorine disinfection was superior to chlorine alone.

What does this mean for you? This is one of those instances where a simple research project gives clear data and a relatively simple solution to managing a potential risk. As discussed in the previous section, transfer of pathogens via the cutting blade/coring ring is likely not a high frequency problem, but we can further manage that risk by a simple extension on the cutting tool separating the cutting knife from the coring ring. In addition, simply polishing the welds when the knives are constructed makes them much easier to sanitize providing another layer of risk management. These steps should be relatively easy to implement in any risk management program. The work on combining ultrasound with chlorine as a disinfecting method seems to hold promise. It remains to be seen whether this is feasible in field conditions, but certainly the concept may have broad applicability in the industry.

SESSION IV: *Conclusions and "Take Aways"*

This report has been prepared to provide guidance and a meaningful summary of the 11 research reports discussed during the CPS Research Symposium at UC Davis in June 2010 and does not constitute legal advice nor does it supersede any regulations. The research findings have not been peer reviewed and in many instances are single studies that need to be repeated and expanded upon in other settings to effectively identify trends and provide growers and processors greater confidence in data and findings for industry use. That said, the sections headed "What does this mean for you?" are based on author interpretation and some of the key take-aways from the interpretations above are listed below as opinions only.

1. The removal of outer leaves in Romaine and iceberg lettuce harvest is a common quality-related practice that may also promote food safety.

2. Greater understanding of the microbiota associated with plants and their interactions with contaminating human pathogens is an important area of research to be monitored.

3. Real-time rapid response investigations into food safety events should be encouraged in the produce industry as a mechanism to identify critical risk factors. Perhaps these investigations can be facilitated by the use of DNA storage techniques that permit increased sampling and subsequent analysis.

4. It is critical for operators to understand pathogen testing methods, their limitations and the meaning of results for samples collected in association with their operations or inputs.

5. Sample size is important when executing testing programs and larger samples are more desirable.

6. Buffer zones associated with the "Food Safety Guidelines for the Production and Harvest of Leafy Greens" appear to be adequate.

7. Uptake of pathogens through plant root systems does not appear to be a principal route of contamination in some commercial production settings.

8. Identification of indicator species for common pathogens is an important area of research that could lead to better risk assessment.

9. Technology innovations such as molecular testing methodologies with improved selectivity and sensitivity for target pathogens are an important area of research and development.

10. Optimized composting (rapid come-up times, adequate moisture content and C:N ratios) is important for effective pathogen kills.

11. Compost providers should provide evidence of well-controlled, verified processes to growers. It is also important to document compost storage protocols.

12. CIF knives and sanitation programs should be designed to minimize the potential for contamination.

Resources

This is a list of the 11 final project reports discussed in this document; they are listed by principal investigator's name. They can be found at the CPS website. (http://cps.ucdavis.edu/funding_opportunities_awards.php)

D'lima, Carol, Ph.D., University of California, Davis. *Enhancing the effectiveness of human pathogen testing systems for the advancement of practical produce safety research and commercial management.*

Ge, Beilei, Ph.D., Louisiana State University. A *sensitive and specific molecular testing method for live* Salmonella *in produce.*

Gitta Coaker, Ph.D., University of California, Davis. *A high-throughput, culture-independent approach to identify index and indicator species for* E.coli *O157:H7 contamination.*

Harris, Linda J, Ph.D., University of California, Davis. *Survival of attenuated* Escherichia coli *O157:H7 ATCC 700728 in field-inoculated lettuce.*

Hoar, Bruce, DVM, Ph.D., University of California, Davis. *Food safety risks associated with sheep grazing in vegetable stubble fields.*

Jiang, Xiuping, Ph.D., Clemson University. *Environmental effects on the growth or survival of stress-adapted* Escherichia coli *015:H7 and* Salmonella spp. *in compost.*

Koike, Steven, University of California Cooperative Extension, Monterey County. *Examination of the survival and internalization of* E. coli *on spinach under field production environments.*

Luo, Yaguang, Ph.D., USDA, ARS. *Minimizing pathogen transference during lettuce harvesting by optimizing the design of the harvesting device and operation practices.*

Marco, Maria, Ph.D., University of California, Davis. *Contribution of phyllosphere microbiota to the persistence of* Escherichia coli *O157:H7 ATCC 700728 on field-grown lettuce.*

Suslow, Trevor, Ph.D., University of California, Davis. *Comparison of surrogate* E. coli *survival and epidemiology in the phyllosphere of diverse leafy green crops.*

Wayadande, Astri, Ph.D., Oklahoma State University. *Fly reservoirs of* E. coli *O157:H7 and their role in contamination of leafy greens.*