Fresh Express Produce Safety Research Initiative e

In April, 2007, Fresh Express awarded $2 million dollars to fund nine separate research projects designed to further the understanding of contamination by the pathogen *Escherichia coli* O157:H7 in lettuce and leafy greens.

The projects were chosen by an independent and voluntary panel of scientific advisors from a total field of 65 proposals. All proposals were required to address one or more areas of needed research identified by the panel. All research projects are now underway.

Five Areas of Needed Research

1. The potential for the internalization of *E. coli* O157:H7 into lettuce tissue
2. Mitigation strategies and technologies
3. Environmental sources and vectors for contamination
4. Ability of *E. coli* O157:H7 to multiply in the presence of normal background flora
5. Ability of *E. coli* O157:H7 and other enteric pathogens to survive composting processes

Voluntary Advisory Panel of Independent Scientific Experts

Panel Chair:
Dr. Michael T. Osterholm, Executive Director, University of Minnesota Center for Infectious Disease Research and Policy
Members:
Dr. Jeff Farrar, California Department of Health Services
Dr. Bob Buchanan, U.S. Food and Drug Administration
Dr. Robert Tauxe, U.S. Centers for Disease Control and Prevention
Dr. Bob Gravani, Cornell University
Dr. Craig Hedberg, University of Minnesota

Current Research Summaries

Subsurface contamination and internalization of *Escherichia coli* O157:H7 in pre-harvest lettuce

PRINCIPAL INVESTIGATOR: MICHAEL DOYLE, UNIVERSITY OF GEORGIA
Co-investigators: Larry Beuchat, Marilyn Erickson, David Riley, Guodong Zhang, and Li Ma, University of Georgia

This project examines factors affecting the uptake and internalization of *E. coli* O157:H7 in lettuce plants. Growth and survival of the pathogen will be evaluated according to several key variables, including pathogen strain, produce type, growth cycle phase, environmental factors, and mechanical processing. The results will improve our understanding of the factors that influence *E. coli* O157:H7 growth and survival in and on produce, enabling the development of enhanced risk mitigation strategies.

It is anticipated that *E. coli* O157:H7 is internalized through the roots of seedlings or older plants when the plants are subjected to stressful conditions (such as excessive heat or restricted water/nutrients), although this route of internalization may result in the pathogens either not surviving or failing to be transported up through the plants' vascular system to their edible tissues. Internalization may occur more readily through the leaf surface, depending on the physical location of *E. coli* O157:H7 on the leaves, availability of nutrients, and insect-related mechanical damage to the leaf. Contaminated cutting blades used to harvest the lettuce could also serve as a tool to expose internal tissues to *E. coli* O157:H7, although treatment with a chlorinated water rinse soon after the contamination may reduce pathogen levels.

In this study, the following factors will be evaluated systematically for their impact on the extent of *E. coli* O157:H7 internalization, colonization, survival, and growth in and on lettuce plants:

- Strain differences among *E. coli* O157:H7 isolates;
- Types of lettuce and phases of the plant's growth cycle;
- Environmental stress (heat or water) and soil fertility;
- Level of contamination;
- Insect damage to lettuce leaf tissue;
- Physical location of contamination (abaxial or lower leaf surface versus adaxial or upper leaf surface);
- Contribution of soluble organic matter (simulating materials in which *E. coli* O157 can be found); and
- Coring by an *E. coli* O157:H7-contaminated knife.

All experiments will be conducted in environmentally controlled growth chambers at the Georgia Envirotron facility at the University of Georgia. Lettuce seeds will first be germinated in trays containing horticultural vermiculite. One week after germination, healthy seedlings will be transplanted into pots containing either 1:5 dairy manure:soil (leaf surface contamination studies) or sifted sandy loam soil (root contamination studies) obtained from horticultural fields on the Tifton Campus at the University of Georgia. The pots will be illuminated (14 hours per day) in growth chambers held at 23°C during the day and 7°C at night.

All *E. coli* O157:H7 strains used in the project will be labeled with the green-fluorescent protein (gfp) and will either be suspended in a carrier solution or manure filtrate (approximately 10^4 or 10^6 CFU/ml) for leaf surface contamination studies or incorporated into the soil (approximately 10^3 or 10^6 CFU/g) for root contamination studies. Exposure of lettuce plants to contaminated soil will occur either initially at seedling transplantation or 30 days after seedling transplantation. In all leaf surface contamination studies, the pathogen mixture will be applied as drops to either the abaxial or adaxial leaf surfaces at one of three different times (7, 30 and 60 days) following seedling transplantation.

Studies addressing the potential for insects to act as contributing factors in the contamination of lettuce tissues with *E. coli* O157:H7 will involve two scenarios: pathogen contamination occurring 24 hours before insect infestation and immediately following insect infestation.

For all studies, triplicate samples will be obtained following contamination at selected intervals up to and including harvest. Plants will be carefully removed and dissected into root and aerial tissue portions. Enumeration will be conducted for both surface and internalized *E. coli* O157:H7 in lettuce root and aerial tissue samples. In addition, soil samples beneath and in contact with plant roots will be obtained for enumeration of *E. coli* O157:H7 in root contamination studies. When pathogens cannot be detected by direct plating, a selective enrichment method will be used and representative colonies will be confirmed by the *E. coli* O157:H7 latex agglutination test. At selected sampling times, plant tissues will also be obtained for confocal and epifluorescent microscopic analyses.

To simulate contamination of iceberg head lettuce through mechanical damage incurred during harvest, uncored head lettuce will be cored with a field corer that has been exposed to *E. coli* O157:H7-contaminated soil (10^2 or 10^5 CFU/g). Following coring, the lettuce heads will be rinsed with chlorinated water (0, 100, or 200 ppm) and examined for *E. coli* O157:H7 through enumeration and enrichment culture.

►Addresses Fresh Express Produce Safety Initiative research priority area 1.

Movement of *Escherichia coli* O157:H7 in spinach and dissemination to leafy greens by insects

PRINCIPAL INVESTIGATOR: JACQUELINE FLETCHER, OKLAHOMA STATE UNIVERSITY
Co-investigators: Astri Wayadande, Justin Talley, and Stanley Gilliland, Oklahoma State University

The role of insects and other arthropods as potential vectors for *E. coli* O157:H7 dissemination resulting in contamination of produce is examined in this study. The likelihood of insect transmission of *E. coli* O157:H7 from animal areas to leafy green produce is not known, although flies have been previously implicated in the transmission of *E. coli* O157:H7 to cattle, feed, and water. This project will evaluate *E. coli* O157:H7 internalization patterns and titers in spinach plants, based on the route of internalization, the type of cultivar, and the age of the plant. It will also determine whether key insect species are capable of transmitting or disseminating *E. coli* to leafy greens. The results will inform the development and prioritization (based on bacterial internalization sites, titer and translocation propensity) of strategies used to prevent or interrupt bacterial dissemination and/or invasion of plant tissues, including choice of cultivar, pest management practices, and bacterial removal or inactivation treatments.

Based on the following hypotheses, the study includes five objectives:

E. coli 0157:H7 colonizes different tissues of the spinach plant, and achieves different titers, depending on the site and method of entry. Phytopathogenic bacteria enter plants through natural openings, wounds, or deposition by insects. Depending on the mode of entry, bacteria encounter different plant micro-environments and develop different interactions with the host.

- Objective 1: Assess whether a green fluorescent protein-tagged *E. coli* 0157:H7, introduced into different plant tissues, colonizes and moves through intercellular spaces, the vascular system, or both, using confocal and fluorescence microscopy of roots, stems and leaves. Spinach cultivars will be inoculated by root drench, leaf surface contamination, needle injection, vacuum infiltration, and an insect vector. Plate counts and quantitative (Q-PCR) will be used to assess bacterial titers reached after internalization.

Spinach cultivar and plant age at the time of inoculation affect susceptibility to *E. coli* O157:H7 internalization, colonization, and titer.

Different plant cultivars often differ in susceptibility to bacterial invasion.

- Objective 2: Test susceptibility to *E. coli* 0157:H7 for a variety of spinach cultivars. Plant tissues (mature and developing leaves and roots) will be assessed by quantitative polymerase chain reaction (Q-PCR) and culturing. The effect of plant age on colonization will be tested using 1, 2, 3, and 4 week-old plants.

Insect species that are known phytopathogen vectors inhabit livestock pasture areas in close proximity to leafy greens production areas.

- Objective 3: Survey cattle pastures and nearby lettuce/spinach growing areas for insects. Insect sticky traps will be placed in fencerows bordering pastured cattle at three to four locations in Southern California and the Salinas Valley. Sweep samples will be collected from each pasture and insects identified. Pasture grasses will be sampled and tested for *E. coli* O157:H7.

Insects common to livestock and field production areas (muscid flies, cabbage loopers, leafhoppers, and aphids) are capable of acquiring *E. coli* from contaminated livestock sources.

- Objective 4a: Confirm that houseflies acquire bacteria. Flies will be exposed to *E. coli* by caging with open agar cultures, dishes of *E. coli* suspension, fecal material containing *E. coli*, or potted grasses sprayed with 10^5 cells/ml bacteria, and flies will be held in mesh cages and tested for *E. coli* by polymerase chain reaction (PCR) methodology;
- Objective 4b: Test a butterfly model for its capacity to drink and acquire from above sources. Cabbage looper adults will be enclosed with *E. coli*-contaminated water or fresh fecal material and then tested along with liquid inoculation sources by PCR and cell plating;
- Objective 4c: Test phytopathogen vectors for their capacity to acquire pathogens from the above sources and from traditional artificial feeding sachets. *Circulifer tenellus* (a vector of spinach virus), and *Acyrthosiphon pisum* or *Myzus persicae* (aphid vectors of plant viruses) will be caged with *E. coli* contaminated water, fecal material, and plant material, or placed in feeding sachets containing *E. coli* and insects will be tested after 1, 2 or 3 weeks by PCR and culturing.

Flies, butterflies, leafhoppers, and aphids are competent vectors of *E. coli* transmitted to leafy greens*.*

- Objective 5a: Determine dispersal flight distances of flies and cabbage looper butterflies marked with fluorescent powder, released in cattle production area, and recaptured at distances of 0.8 km, 1.6 km, and 2.4 km using fly-bait traps and sugar-water traps set in concentric circles around the release point;

- Objective 5b: Assess propensity of contaminated flies, butterflies to alight on and contaminate greens. Houseflies or butterflies exposed to *E. coli* will be released into a chamber containing lettuce or spinach plants, and plants will be tested for *E. coli* by PCR and cell plating, at 1, 3 and 5 days;
- Objective 5c: Evaluate *E. coli* vector competence of leafhoppers and aphids; *C. tenellus, A. pisum*, *M. persicae* and *Bemesia tabaci* will acquire *E. coli* from feeding sachets and caged with lettuce or spinach and plants will be tested for *E. coli* by PCR or culturing. If any experimental transmissions are successful, transmission of *E. coli* O157:H7 by those insect species will be tested.

►Addresses Fresh Express Produce Safety Initiative research priority areas 1 and 3.

Interaction of *Escherichia coli* O157:H7 with fresh leafy green produce

PRINCIPAL INVESTIGATOR: JORGE GIRÓN, UNIVERSITY OF ARIZONA
Co-investigators: TBD

The aim of this project is to elucidate the mechanism by which *E. coli* O157:H7 colonizes fresh produce and survives industrial decontamination. The results will be applicable to the development of an effective method of reducing the survival of this pathogen in fresh produce.

Environmental, ultrastructural, molecular, and bactericidal testing approaches will be applied in this project. Environmental studies will assess the role of temperature, pH, incubation time, gas requirements, plant age, and bacterial determinants on the colonization of leafy greens. Ultrastructural research will examine the structures produced by the bacteria that may be involved in bacterial adherence and colonization, such as pili and flagella. Molecular testing will focus on the introduction of specific bacterial effectors into the leaf to determine if they have a role in this phenomenon. Bactericidal testing will be used to assess various bactericidal agents that are non-toxic to humans to determine a more effective method of eliminating this organism from fresh produce.

Preliminary results indicate that *E. coli* O157:H7 can colonize the surface of spinach leaves using structures resembling flagella and can gain access to the internal leaf structure using the stomata. Colonization of the stomata may be the mechanism by which the *E. coli* can survive the industrial decontamination process. Further testing will focus on (1) identifying the pathogen's mechanism for manipulating the stomata, which aids internal colonization; (2) identifying the specific structures that play a role in leaf surface adherence; and (3) developing a method of sterilization that can be applied at the industrial level to eliminate *E. coli* O157:H7 from produce.

►Addresses Fresh Express Produce Safety Initiative research priority areas 1 and 2.

Factors that influence the ability of *E. coli* O157:H7 to multiply on lettuce and leafy greens

PRINCIPAL INVESTIGATOR: LINDA HARRIS, UNIVERSITY OF CALIFORNIA–DAVIS

Co-investigators: Mysore Sudarshana, Hai-Ping Li, and Trevor Suslow, University of California–Davis

This project investigates the factors that influence the ability of *E. coli* O157:H7 to multiply on lettuce and leafy greens in the presence of normal background flora. Comparative growth kinetics of *E. coli* O157:H7 on bagged romaine lettuce and spinach in simulated standard and sub-optimal post-harvest temperatures, and the various factors that influence growth rates, will be evaluated. It is hypothesized that sub-optimal temperature management may play a significant role in the growth of *E. coli* O157:H7 on produce during harvesting, processing, distribution, and pre-consumption handling. How well *E. coli* O157:H7 is able to grow under these conditions may depend also on conditions of the plant (variety, age, growing conditions, day length, method of harvest, moisture at harvest, and soluble nutrients), the bacteria (pre-inoculation stress and growth conditions) and the post-inoculation environment. Results of this project will support the development of optimal pre-harvest, harvest, and post-harvest preventive programs and improved predictive models for host-pathogen and environment-pathogen interactions.

Growth of *E. coli* O157:H7 inoculated onto lettuce or spinach will be evaluated according to these three objectives:

- For inoculated *E. coli* O157:H7 on spinach, characterize growth kinetics and evaluate the effects of variety, age of plant, growing conditions, fertility management, growth rate, and day length.
- For inoculated *E. coli* O157:H7 on washed and bagged romaine lettuce, characterize growth kinetics under different pre-inoculation bacterial growth conditions and stresses.
- For inoculated *E. coli* O157:H7 on washed and bagged romaine lettuce and spinach, characterize growth kinetics at different holding temperatures, both static and fluctuating. Romaine lettuce and spinach will be inoculated with different levels of *E. coli* O157:H7 and held at static temperatures or under conditions where the temperature is shifted for various periods of time from refrigerated to mildly abusive temperatures that permit growth of *E. coli* O157:H7.

Three varieties of spinach will be grown to three different stages of maturity, at different nitrogen fertilization rates, and using different amounts of supplemental light.

E. coli O157:H7 inoculum will be prepared by several approaches: on solid or liquid medium; in minimal medium or tryptic soy broth; at minimal, moderate, and optimum temperatures; in different inoculum carriers (irrigation water, MilliQ water, 0.1% peptone, and dilute compost); to early or late stationary phase; and held for different times between preparation of inoculum and inoculation.

Results of this study will include the identification of factors under controlled greenhouse conditions that affect leaf structure, leaf breakage, cotyledon dehiscence or senescence, and other aspects of plant morphology relevant to food safety. Data on culture conditions determined to have the greatest impact on the fate of *E. coli* O157:H7 inoculated onto lettuce will be applicable to methods development and inoculation studies. Data on *E. coli* O157:H7 survival and growth at constant and fluctuating storage temperatures generated by these experiments will greatly expand the available information relevant to leafy greens.

►Addresses Fresh Express Produce Safety Initiative research priority area 4.

Fate of *Escherichia coli* O157:H7 on fresh and fresh-cut iceberg lettuce and spinach in the presence of normal background microflora

PRINCIPAL INVESTIGATOR: MARK HARRISON, UNIVERSITY OF GEORGIA
Co-investigators: William Hurst and William Kerr, University of Georgia

The objective of this study is to determine the ability of *E. coli* O157:H7 to multiply in the presence of normal background microorganisms on iceberg lettuce and spinach under conditions that mimic actual practices between production and retail sale, including:

- Transportation from harvest field to cooler
- Refrigerated storage
- Transportation and distribution as (1) cored product in a nitrogen atmosphere (lettuce), (2) open 20-pound returnable plastic totes (spinach), or (3) finished packaged salads in a low oxygen/high carbon dioxide atmosphere in both common and abusive temperatures.

By simulating conditions and practices of the fresh-cut industry, this study allows for the evaluation of the fate of *E. coli* O157:H7 on produce during typical handling practices. The results will provide insight into how *E. coli* O157:H7 on iceberg lettuce and spinach interacts with naturally-occurring bacteria, which may have competitive or antagonistic influences on the growth of *E. coli* O157:H7 in these plants. This knowledge can be used to identify handling and packaging routines that reduce the potential for contamination *E. coli* O157:H7 in fresh produce.

Variations on the way iceberg lettuce and spinach are handled in commercial operations and packaged will be reflected in the sampling plans. Green fluorescent pigment-expressing (GFP) *E. coli* O157:H7 will be used to trace the organism on inoculated product during handling and storage treatments.

Fresh iceberg lettuce and spinach, harvested from fields in central California, will be forced-air cooled and shipped overnight to the University of Georgia's Department of Food Science and Technology. Field location, proximity of harvest location to cattle operations, rivers, and other wildlife habitats, and temperature during harvest will be recorded. Prior to inoculation, the greens will be removed from the cooler and equilibrated to 25^{o} or 32^{o}C to allow for a comparison of two harvest conditions (one considered desirable and the other considered abusive). For spinach, individual leaves will be sampled. For iceberg lettuce, outer leaves will be removed and the heads cored by hand and washed in chlorinated water as typically done in the field. Exterior leaves and those from the next layer underneath will be sampled to determine the background microflora (aerobic mesophilic and psychrotrophic bacteria, coliforms, yeasts and molds, and lactic acid bacteria).

Multiple areas on the exterior leaves of the iceberg lettuce and leaves in the next layer will be surface-inoculated with 1,000-10,000 GFP-expressing *E. coli* O157:H7 per cm^2. For spinach, individual leaves will be inoculated. After the inoculum dries, a section (approximately 25 cm^2) will be removed from the inoculated leaves and the *E. coli* O157:H7 will be enumerated.

Heads of lettuce inoculated with the GFP-expressing *E. coli* O157:H7 will be placed in returnable plastic totes, held at 25^{o}C and 32^{o}C, and sampled for GFP-expressing *E. coli* O157:H7 and background microflora at 0, 5 and 10 hours. These temperatures and times correspond to reasonable conditions and abusive conditions for transporting greens from the field to the cooling facility. After sampling, totes will be placed in the vacuum cooler and chilled to approximately 40^{o}F within 20-40 min. Totes will be held at either 4^{o}C or 12^{o}C and the inoculated heads will be sampled for GFP-expressing *E. coli* O157:H7 and background microflora at 0, 10, and 72 hours. Sampling after 10 hours represents transit to a fresh-cut operation within the region where the lettuce was harvested, while sampling after 72 hours represents the typical transit from central California to eastern areas like Atlanta.

Lettuce will be chop-cut and held in chilled water with or without chlorine (pH adjusted), with agitation to simulate contact with water in fresh-cut operations. After dewatering and bagging, lettuce samples will be held at storage temperatures (desired and abusive temperatures that could occur during distribution of the retail product) and sampled over several days. Baby spinach will be forced air cooled to 4^{o}C and bagged in macro-perforated bags, not chopped or shredded, simulating industry practice.

Naturally-occurring bacteria on the product will be screened for their ability to inhibit the pathogen. Randomly selected isolates from the plates used to enumerate the various types of bacteria, yeasts, and molds present on the lettuce and spinach at the different stages of handling and processing will be tested for any notable competitive or antagonistic effect on *E. coli* O157:H7.

►**Addresses Fresh Express Produce Safety Initiative research priority area 4.**

Determining the environmental factors contributing to the extended survival or regrowth of foodborne pathogens in composting systems

PRINCIPAL INVESTIGATOR: XIUPING JIANG, CLEMSON UNIVERSITY
Co-investigators: Geoff Zehnder and Feng Luo, Clemson University

This study examines the effectiveness of composting for inactivating pathogens in manure, given that raw or inadequately composted animal waste applied to growing fields is a potential pre-harvest source of produce contamination. The primary mechanism for pathogen inactivation during outdoor composting is microorganism-related heat generation. In practice, the effectiveness of pathogen inactivation varies with environmental factors, including temperature, rainfall, nutrient sources, compost ingredients, and pathogens' induced heat resistance.

Key environmental factors related to the regrowth and survival of three foodborne pathogens in compost are evaluated in this study. Data from this project can be applied to the design of composting practices in California, the identification of environmental factors conducive to pathogen regrowth, and to the prediction of pathogen inactivation during on-farm composting.

The specific objectives are to:

- Identify the optimal conditions for *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* regrowth in composts under field application; The most desirable environmental conditions for inactivation, such as heavy rain/overhead sprinkling/flooding, nutrient sources (types of compost), and warm temperatures, will be evaluated for pathogen regrowth in compost. A cocktail of three strains for each pathogen will be heat-shocked and then inoculated into the compost with low initial populations. The inoculated compost will be maintained inside a greenhouse for four weeks and sampled periodically for pathogen analysis. Different varieties of compost produced in California will be used for studying pathogen regrowth.

- Determine the fate of heat-adapted avirulent pathogens during composting on the farm; Composting practices in California will be followed and simulated, using on-farm composting of vegetables with dairy manure and ingredients used in California (which can also be found in South Carolina.) The green wastes from either summer or fall organic crops will be collected from Clemson University's Calhoun Field Laboratory farm. Duplicate compost heaps will be set up on site. The survival of compost-adapted and heat-adapted avirulent strains of *E. coli* O157:H7, *Salmonella* spp., and *L. innocua* will be determined at three locations (surface, center, and bottom) of composting heaps. Field studies will be conducted during summer months (25-35°C) and winter months (5-15°C) to determine the influence of outdoor temperatures and precipitation on the fate of pathogens.

- Study the impact of initial heat resistance of pathogens on their survival during composting. Three strains for each pathogen will be heat-shocked at 45°C prior to the inoculation of compost mixture separately. Cultures without heat-shock treatment will be used as control. The inoculated compost held in a Tyvek® pouch will be kept in an incubator at a range of temperatures (45, 50, 55 and 60°C) to simulate the conditions inside compost heaps during thermophilic composting. At certain intervals, samples will be taken out and analyzed for surviving bacterial counts. Mathematic models based on these data will be developed to predict the thermal inactivation of pathogens during composting.

►Addresses Fresh Express Produce Safety Initiative research priority area 5.

Quantifying the risk of transfer and internalization of *Escherichia coli* O157:H7 during processing of leafy greens

PRINCIPAL INVESTIGATOR: ELLIOT RYSER, MICHIGAN STATE UNIVERSITY
Co-investigators: Bradley Marks and Ewen Todd, Michigan State University

This project evaluates the role of three key produce processing steps—conveying, dewatering, and shredding—in the potential transfer of bacterial pathogens immediately after leafy greens exit the flume tank. The resulting data will help to quantify the extent and likelihood of bacterial transfer during the processing of leafy greens. The project will also develop a set of recommendations on cleanability and sanitary design of processing equipment for leafy greens. The goal is to develop a scientific basis for minimizing the risk of contamination of fresh-cut leafy greens through pathogen dissemination in commercial processing equipment.

The project's specific objectives are to:

- Determine the transfer coefficients for the numbers of *E. coli* O157:H7 transferred from dip-inoculated, wet head lettuce and wet spinach to equipment surfaces during conveyance on a conveyor belt, dewatering by shaking/centrifugation, and mechanical shredding using commercial processing equipment.
- Determine the extent of *E. coli* internalization during processing of head lettuce and spinach; and
- Develop a mathematical risk model for *E. coli* O157:H7 cross-contamination of head lettuce and spinach during processing, using the model to identify candidate risk mitigation strategies.

Fresh-cut, pre-washed baby spinach and intact heads of California head lettuce (Iceberg variety) will be surface-inoculated with an avirulent strain of *E. coli* O157:H7 containing the green fluorescent protein (gfp) plasmid by dipping so as to contain approximately 105 CFU/g or cm^2. After brief draining to simulate product exiting the flume tank, numbers of *E. coli* transferred to equipment surfaces will be assessed during conveying, dewatering and shredding. Selected lettuce and spinach samples containing the highest numbers of gfp-labeled *E. coli* O157:H7 will be examined for both presence and numbers of internalized cells. For quantification, samples will be surface-sterilized by immersion in 1% silver nitrate, rinsed, ground, and then plated on an appropriate medium. Selected samples will also be examined for internalized cells of gfp-labeled *E. coli* O157:H7 using Confocal Scanning Laser Microscopy. The results will be compared to those from Objective 1 to determine percent and extent of internalization. After determining the transfer coefficients for the different transfer scenarios, the data will be subjected to our existing bacterial transfer model. As the *E. coli* transfer coefficients are collected, the information will be plotted to determine the relationship between type of contact surface and the rate of bacterial transfer. By examining the plotted experimental results, several mathematical models will be proposed. Software dedicated to model development, such as TableCurve® 2D and 3D, will be used to identify alternative mathematical algorithms. In all objectives, transfer of *E. coli* from an inoculated to an uninoculated surface and vice versa will be assessed using statistically-based models.

A quantitative risk assessment model will be developed for assessing the risk of California-grown leafy greens for consumers, and include mitigation strategies, in accordance with the Codex Alimentarius Commission's scheme: exposure assessment, hazard characterization, risk characterization and sensitivity analysis.

It is anticipated that conveyance on a conveyor belt, dewatering by shaking or centrifugation, and mechanical shredding will prove to be important multi-directional transfer points for *E. coli* O157:H7 dissemination, due to incoming contamination on the leafy greens, low cleanability of some areas of the equipment surface, and potential for *E. coli* O157:H7 to penetrate into cut surfaces of leafy greens during shredding. Transfer coefficients generated from these data can be modeled and incorporated into risk assessments to enhance the safety of leafy greens for consumers.

►Addresses Fresh Express Produce Safety Initiative research priority areas 1, 2, and 3.

A novel approach to investigate internalization of *Escherichia coli* O157:H7 in lettuce and spinach

PRINCIPAL INVESTIGATOR: MANAN SHARMA, AGRICULTURAL RESEARCH SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE
Co-investigator: Michael Donnenberg, University of Maryland–Baltimore

A molecular method based on chromosomal integration of the green fluorescent protein (*gfp)* gene into *E. coli* O157:H7 is used in this project to provide an alternative, and potentially more accurate, approach to the measurement of *E. coli* O157:H7 internalization in fresh produce. Use of the *gfp* marker in previous studies typically involved integration of the *gfp* gene into plasmid DNA along with an antibiotic resistance gene marker, which may lead to detection of fewer *E. coli* O157:H7 cells than actually present under conditions of physiological, nutritional, or antimicrobial stress. The chromosomal integration method used in this study will help to determine whether and to what extent internalization of *E. coli* O157:H7 occurs in fresh produce.

The project will apply this novel approach to evaluate the following hypotheses: (1) that *E. coli* O157:H7 strains isolated from produce-associated outbreaks are better able to be internalized into plants than are strains isolated from beef-associated outbreaks or non-pathogenic *E. coli* strains; (2) that *E. coli* O157:H7 requires an intact stress response to survive in plants; and (3) that established *E. coli* O157:H7 virulence factors are heightened, or up-regulated, during growth on leafy greens compared with growth in ground beef.

Specific objectives of the project include:

- Development of strains of *E. coli* O157:H7 and non-pathogenic *E. coli* that contain the *gfp* gene inserted into the bacterial chromosome, using newly developed transformation methods based on transposons as vectors. These strains will have improved bacterial fitness over plasmid *gfp* strains and will not require a selective agent for detection, enabling accurate assessment of *E. coli* O157:H7 survival and growth.
- Determination of the survival and growth of *gfp*-labeled wild type versus stress response gene (*rpoS)*-deficient *E. coli* O157:H7 populations in internalized plant tissues in different spinach and lettuce varieties. Enumeration and microscopic analysis of *E. coli* O157:H7 from internalized tissues will reveal the possibility and extent of internalization in plant tissues at specific points during the growing process.

- Determination of differences in the expression of virulence factors in *E. coli* O157:H7 grown on leafy greens compared with *E. coli* O157:H7 grown in ground beef. Virulence factors such as *ler* (positive regulator of type III secretion), *eae* (outer membrane adhesin intimin), *espA* (type III secretion filament), *ehxA* (enterohemolysin), *ihaA* (adherence factor), *rfbE* (O157 antigen), and *stxII* (shiga toxin 2) will be evaluated by real time reverse transcriptase (RT) polymerase chain reaction (PCR) methods. A ratio between virulence factors and housekeeping genes will be determined, and the ratio for *E. coli* O157:H7 grown on lettuce will be compared to the ratio for *E. coli* O157:H7 grown on ground beef. This comparison will yield insights into the potential of *E. coli* O157:H7 to cause human illness through the consumption of contaminated produce.

►Addresses Fresh Express Produce Safety Initiative research priority area 1.

Sanitization of leafy vegetables by integrating gaseous ozone treatment into produce processes

PRINCIPAL INVESTIGATOR: AHMED YOUSEF, OHIO STATE UNIVERSITY
Co-investigator: Sudhir Sastry, Ohio State University

This study investigates the feasibility of sequentially applying sanitation technologies—vacuum, gaseous ozone, and pressurized gases—to leafy green produce to overcome external and internal contamination with *E. coli* O157:H7. The development of this approach involves: (1) adapting existing vacuum-cooling technology for freshly harvested produce to include a sanitization step involving repressurization with gaseous ozone; (2) determining the kinetics of *E. coli* O157:H7 inactivation in leafy green produce in relation to the treatment variables; and (3) monitoring the viability of *E. coli* O157:H7 during ozone treatment and refrigerated storage of fresh produce.

Ozone is a potent sanitizer that is well-suited for use with fresh produce. Results of this project may provide the fresh produce industry with a critical decontamination step that can be incorporated into exiting production and processing lines, potentially reducing the health risks associated with sporadic contamination of fresh produce with *E. coli* O157:H7.

The preliminary experiments will involve the use of an elaborate ozone treatment vessel located in the investigators' biosafety level-2 facility. Processing variables to be tested include: vacuum level; vessel pressure after ozone introduction; ozone concentration; treatment time; average temperature; and product loading. Preliminary studies will be conducted on diffusion of ozone into various locations within the treatment chamber. A sampling manifold will be installed to draw gas samples from multiple locations when different product loads are tested. Product/vessel volume ratio will be optimized to make sure all leaves are exposed to the sanitizer. If needed, a slow tumbling of the product within the vessel during the treatment will be applied. In all cases, *E. coli* O157:H7 will be counted (or detected, if the bacterial population is uncountable) in produce before and after treatment, as well as during storage.

This study will identify treatment conditions sufficient to eliminate *E. coli* O157:H7 in fresh produce, at realistic contamination levels, while maintaining product quality and integrity. It will also optimize an ozonation process for scale-up and incorporation into pre-packaging treatments of fresh produce.

►**Addresses Fresh Express Produce Safety Initiative research priority area 2.**