PRODUCE

SURFACE-STERILIZATION METHODS FOR *ESCHERICHIA COLI* O157:H7 ON LETTUCE (*LACTUCA SATIVA* L.)

(G. Zhang, L. Ma, L.R. Beuchat, M.C. Erickson, V.H. Phelan, and M.P. Doyle)

Many outbreaks of *Salmonella* and *Escherichia coli* O157:H7 infections have been associated with consumption of fresh-cut leafy greens in the past decade. Questions remain regarding the ability of these pathogens to become internalized within lettuce and spinach. To differentiate internalized populations from surface contamination, an effective surface-sterilization method for lettuce is needed and was the focus of this study.

Iceberg lettuce (*Lactuca sativa* L.) was purchased from a local grocery store and cut into 3 x 3 cm pieces. Lettuce roots were purchased from a local farmer. Leaf pieces and roots were inoculated by immersing in 10^8 CFU of a five-strain mixture of GFP-labeled *E. coli* O157:H7/ml for 10 min at room temperature. Inoculated samples were put in a laminar flow biosafety cabinet for 30 min before further treatment. Thirteen surface-sterilization methods (including sodium hypochlorite, ethanol, $HgCl_2$, and hydrogen peroxide) were compared for their efficacy in killing/removing *E. coli* O157:H7 on lettuce leaf and root surfaces. Treated samples were washed 5 times with sterile water, and then assayed for *E. coli* O157:H7.

Among the 13 surface-sterilization methods evaluated, *E. coli* O157:H7 was not detected by enumeration with a direct plating procedure on treated samples for 3 treatments, including 20 min with 10,000 ppm sodium hypochlorite and 2 treatments containing ethanol and $HgCl_2$. There were 2.8 to 4.4 CFU *E. coli* O157:H7/leaf piece or root after surface-sterilization for the other methods. Plant tissue prints on agar and enrichment culture results were consistent with enumeration results. Our overall data revealed that the best surface-sterilization method for lettuce leaves and roots was dipping in 80% ethanol for 10 s, followed by immersion in 0.1% $HgCl_2$ for 10 min.

ENUMERATION OF *E. COLI* O157:H7 IN SPINACH OUTBREAK SAMPLES

(L. Ma, G. Zhang, P. Garner-Schmidt, and M. P. Doyle)

During August and September of 2006, more than 200 persons from 26 states were infected by a single strain (isolates indistinguishable by pulsed-field gel electrophoresis [PFGE]) of *E. coli* O157:H7. Fresh spinach was identified as the vehicle of the outbreak and *E. coli* O157:H7 with a PFGE pattern matching the outbreak strain was isolated from many open packages of fresh spinach consumed by patients. The objective of this study was to enumerate *E. coli* O157:H7 in outbreak-associated spinach samples obtained from patient refrigerators. A 3-tube Most Probable Number (MPN) method in combination with selective plating, multiplex PCR (targeting genes: *gad*, *eae*, *bfp*, *stx*1, and *stx*2) screening, confirmation by *E. coli* O157 latex agglutination assay, and PFGE pattern matching was used for the isolation and enumeration of the outbreak strain in the spinach samples. The estimated cell numbers in positive samples ranged from 0.61 MPN/g to 21 MPN/g. Multiplex PCR analysis revealed absence of the *stx* 1 amplicon in the outbreak isolate.

ISOLATION AND ENUMERATION OF *E. COLI* O157:H7 FROM POSSIBLE CONTAMINATED GROUND BEEF SAMPLES

(L. Ma, G. Zhang, P. Gerner-Smidt, and M. P. Doyle)

Direct plating on selective media (TC-CHROMagar O157) and a 3-tube Most Probable Number (MPN) method were used for the isolation and enumeration of *E. coli* O157:H7 from ground beef associated with an outbreak. Presumptive-positive isolates of *E. coli* O157:H7 were confirmed molecularly (multiplex PCR targeting five genes: *gad*, *eae*, *bfp*, *stx*1, and *stx*2) and immunologically (*E. coli* O157 latex agglutination assay). Subtyping by MLVA and PFGE confirmed the isolates were indistinguishable to the outbreak strain. Three out of eight of the original samples were positive for *E. coli* O157:H7, with cell numbers of the pathogen in the positive samples being generally low, ranging from <0.3 MPN/g to 24 MPN/g by the MPN method, or from <50 CFU/g to ca. 50 CFU/g by direct enumeration. Also, multiplex PCR revealed that all of the *E. coli* O157:H7 isolates contained *eae*, *stx1* and *stx2* genes. One sample contained a large number of colonies that had the same morphology (mauve) as *E. coli* O157 on TC-CHROMager O157 and reacted positively by the *E. coli* O157 latex agglutination assay; however, these isolates were later identified as *Serratia liquefaciens* by the API 20E test.

ATTACHMENT AND RECOVERY OF *ESCHERICHIA COLI* O157:H7 AND A NON-PATHOGENIC SURROGATE FROM ROMAINE LETTUCE AFTER CONTACT WITH CONTAMINATED ICE

(J. Kim and M. Harrison)

Ice, possibly contaminated with *E. coli* O157:H7, can be used to chill romaine lettuce and maintain relative humidity during transportation. Contamination of lettuce is of concern since it is usually consumed raw or minimally processed. The potential for *E. coli* O157:H7 contamination of romaine lettuce with either ice contaminated with the pathogen or by transfer from lettuce surfaces via melting ice was determined. In order to evaluate pathogen transfer by these means in actual commercial facilities, the use of non-pathogenic surrogates is needed. A non-pathogenic *E. coli* strain was selected and compared with *E. coli* O157:H7 to determine differences and similarities in attachment to and recovery from romaine lettuce in contact with contaminated ice. *E. coli* O157:H7 distributes onto other produce layers in shipping containers due to melted ice made of contaminated water and transfers from contaminated to uncontaminated surfaces. Based on cryotolerance and cell surface characteristics, *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for studies involving attachment and recovery from chilled produce.

CONTAMINATION AND POTENTIAL INTERNALIZATION OF *ESCHERICHIA COLI* O157:H7 IN PRE-HARVEST ICEBERG LETTUCE (*LACTUCA SATIVA L.*)

(G. Zhang, L. Ma, L.R. Beuchat, M.C. Erickson, V.H. Phelan, and M.P. Doyle)

The ability of foodborne pathogens to internalize within lettuce, especially under growing conditions, is an important unanswered question in need of elucidation for risk analysis and intervention purposes. The objectives of this study were (1) to determine the effect of inoculation sites (abaxial vs adaxial leaf surfaces) on survival and internalization of *E. coli* O157:H7 in lettuce; and (2) to evaluate the vulnerability of lettuce at different ages to *E. coli* O157:H7.

Iceberg lettuce (*Lactuca sativa L.*) was grown in sandy soil in an envirotron at 23°C during the day and 7°C at night. A 5-strain mixture of GFP-labeled *E. coli* O157:H7 at 10^6 CFU/ml in water and cow manure extract was used as inoculum. Plants were inoculated on abaxial and adaxial sides of leaf surfaces at 3, 30, and 60 days after transplantation and sampled 2 to 3 times for each inoculation treatment. At each sampling time, *E. coli* O157:H7 in soil and in/on shoots and roots were analyzed. For surface-sterilization, leaves and roots were dipped in 80% ethanol for 10 s, followed by immersion in 0.1% $HgCl_2$ for 10 min.

Twenty-five days after inoculation, 2 of 12 samples were *E. coli* O157:H7-positive on inoculated leaves. No *E. coli* O157:H7 was detected on inoculated leaves at 54 days. All surface-sterilized root and leaf samples were negative for *E. coli* O157:H7 regardless of plant age at inoculation, sampling time, or abaxial- or adaxial-side inoculation. Substantially more lettuce leaves inoculated on the abaxial side were *E. coli* O157:H7-positive after 3 to 25 days than those leaves inoculated on the adaxial side.

Internalization of the *E. coli* O157:H7 in iceberg lettuce by leaf inoculation did not occur. Age of lettuce plants did not affect internalization of *E. coli* O157:H7 in lettuce. Inoculated *E. coli* O157:H7 survived longer on the abaxial side of the leaves than on the adaxial side.

CONTAMINATION AND POTENTIAL INTERNALIZATION OF *ESCHERICHIA COLI* O157:H7 IN LETTUCE (*LACTUCA SATIVA* L.) BY SOIL INOCULATION

(G. Zhang, L. Ma, L.R. Beuchat, M.C. Erickson, V.H. Phelan, and M.P. Doyle)

Understanding whether internalization of foodborne pathogens occurs through plant roots will be helpful in conducting risk assessments and developing effective interventions to reduce pathogen contamination in produce. The objectives of this work were (1) to determine if internalization of *E. coli* O157:H7 through lettuce roots occurs; and (2) to determine if differences exist among *E. coli* O157:H7 isolates and lettuce types regarding *E. coli* O157:H7 internalization, survival and growth in and on lettuce plants.

Iceberg, Romaine and leaf lettuces were grown in sandy soil in an envirotron using two temperature regimes. Soil was inoculated with 5 GFP-labeled *E. coli* O157:H7 isolates individually at 10^6 or 10^3 CFU/g of soil when lettuce seedlings were transplanted. Lettuce plants were sampled 2 to 3 times after transplantation and assayed for

E. coli O157:H7 in soil and in/on shoots and roots. For surface-sterilization, leaves and roots were dipped in 80% ethanol for 10 s, followed by immersion in 0.1% $HgCl_2$ for 10 min.

Results revealed that surface-sterilized leaf and root samples were negative (except for 2 root samples) for *E. coli* O157:H7. Seventeen days after transplantation and inoculation, most leaf surfaces were positive for *E. coli* O157:H7 which was likely due to cross-contamination from the inoculated soil. The 26-, 45- and 60-day samplings revealed no *E. coli* O157:H7 on leaf surfaces. Some soil and rhizosphere samples were positive for *E. coli* O157:H7 at 60 days when the trials were terminated.

In conclusion, internalization of *E. coli* O157:H7 in lettuce did not occur through the roots; however, the pathogen could survive in soil for at least 60 days. There were no differences among *E. coli* O157:H7 isolates or lettuce types with regard to *E. coli* O157:H7 internalization in lettuce.

PRE-HARVEST FACTORS AFFECTING INTERNALIZATION OF ZOONOTIC PATHOGENS INTO LETTUCE

(M.C. Erickson, J. Liao, A. Payton, C. Webb, L. Ma, G. Zhang, M. Doyle, and L.R. Beuchat)

In the past two decades, the fresh fruit and vegetable industry has rapidly evolved and contributed to increased retail and food-service sales. Accompanying this growth has been an increasing number of outbreaks associated with fresh produce consumption that has often been traced back to the farm. Potential pre-harvest vehicles for contamination of vegetables include soil amendments (manure or improperly-composted manure) or contaminated irrigation or runoff water. Based on laboratory studies, however, both surface and internalized contamination occurred when seeds or seedlings were exposed to contaminated soil or water solutions. Whether internalization occurred in older plants and the fate of any internalized populations was one of the objectives of this study.

Differences in the robustness of plant defense mechanisms that target bacterial extracellular components for subsequent subcellular compartmentalization and degradation have been suggested as one factor affecting internalized pathogen populations. Since plant stress associated with drought conditions could affect plant defensive activities, the level of internalization of zoonotic pathogens could, in turn, also be affected. Another factor that is likely to affect internalization of zoonotic pathogens is the level of indigenous microorganisms in the soil environment. Since the abundance of an indigenous population is dependent on the relative availability of nutrients, internalization of zoonotic pathogens by plants could, in turn, be affected by the level of fertility in the soil. A second objective of this study therefore addressed both the influence of plant stress and soil fertility levels on internalization of zoonotic pathogens by lettuce plants.

Green leaf lettuce (variety Two star) was grown in pots using either 0:5, 1:5 or 2:5 manure compost:top soil mixtures. Pots were held in an envirotron at 20°C during the day and 7°C at night. An inoculum mixture of green-fluorescent protein (gfp)-labeled *Escherichia coli* O157:H7 isolates or an inoculum mixture of gfp-labeled *Salmonella* spp. was prepared and added to water to give concentrations of 10^3 or 10^6 CFU/ml. Contaminated water was applied to the soil of 3- or 33-day post-transplanted plants (30-50 ml/plant) and a portion of those plants were sampled 3 days later and at 60-days post-transplantation. For a sub-group of plants exposed at 33-days post-transplantation, a reduced watering rate was applied for 2-3 weeks prior to the contamination event. With all plants, a physical barrier separated leaves and soil to prevent direct transfer of pathogens from soil to leaves. Leaves were analyzed separately from washed roots and both surface and internalized populations were enumerated for these samples. Using an ethanol and mercury chloride wash, surface sterilization of samples preceded enumeration of internalized populations.

Pre-harvest internalization of *Escherichia coli* O157:H7 or *Salmonella* spp. into roots or leaves of green leafy lettuce cultivated in a growth chamber did not occur when plants were watered with a contaminated water source. Pathogen internalization was not affected by the level of soil fertility. A 2-week period of reduced watering prior to the contamination event also did not induce internalization of pathogens. The absence of internalized populations is of merit as post-harvest interventions need only target surface contamination.

SURVIVAL, GROWTH, AND TOXIGENESIS OF *CLOSTRIDIUM BOTULINUM* IN FRESH CARROT JUICE

(L. Ma, G. Zhang, P. Gerner-Smidt, and M. P. Doyle)

During September and October of 2006, 5 cases of botulism associated with commercial fresh carrot juice were reported. These cases have raised questions regarding the safety of fresh carrot juice. The objectives of this project were to evaluate the survival, growth, and toxigenesis of *Clostridium botulinum* in fresh carrot juice as affected by a *C. botulinum* inoculum of 1 to 1000 spores/ml, storage temperature of 4, 10, 15, 25, and 31°C, storage time of up to 8 days at 25 and 31°C and 6 weeks at lower temperatures, type of carrot juice (baby vs. mature carrots), food preservatives (potassium sorbate and nisin) and biological control agents (lactic acid bacteria) by using factorial design experiments. To date, lactic acid bacteria have been isolated from naturally fermented vegetables and six were selected for further study based on antagonistic activity against *C. botulinum* in vitro. Studies conducted at 15 and 25°C indicated that *C. botulinum* grew well in fresh carrot juice when stored at either temperature. Even an inoculum of 1 spore/ml of fresh carrot juice became toxic at day 5 or 6 when stored at 25°C and week 4 or 5 at 15°C. In general, fresh carrot juice made from baby carrots was less prone to the growth and toxigenesis of *C. botulinum* than carrot juice made from mature carrots. The addition of nisin had no effect on the growth and toxigenesis of *C. botulinum*, whereas potassium sorbate delayed the time to toxin production. Lactic acid bacteria with an inoculum of 1 CFU/ml prevented the germination, growth and toxigenesis of *C. botulinum* at both temperatures. The project is in progress.

SURVIVAL AND GROWTH OF ACID-ADAPTED AND UNADAPTED *SALMONELLA* IN AND ON RAW TOMATOES AS AFFECTED BY STAGE OF RIPENESS AND STORAGE TEMPERATURE

(L. R. Beuchat and D. A. Mann)

Several outbreaks of salmonellosis have been associated with the consumption of raw tomatoes. Once *Salmonella* attaches to the surface of tomatoes or infiltrates tissues, it can persist and may grow. Temperature and relative humidity affect the extent to which cells attach to ripe tomatoes. Populations of *Salmonella* Montevideo on the surface of mature green tomatoes stored at 10ºC for 18 days have been observed to not change significantly. Populations of the same serotype inoculated on the surface of green tomatoes did not change significantly when tomatoes were treated with 100 ppm ethylene at 100% relative humidity and 20ºC for 6 days. Several other *Salmonella* serotypes have been reported to persist on the surface of green as well as ripe (red) tomato fruits, leaves, and stems. Depending on temperature, relative humidity, and other factors, *Salmonella* may grow on the surface of tomatoes. *Salmonella* can also grow in diced red tomatoes at 22ºC to populations exceeding 10^8 CFU/g. Salmonellae are known to be able to grow on sliced tomatoes.

Survival and growth characteristics of *Salmonella*, as affected by variety of tomato and stage of ripeness, has received little research attention. While Roma tomatoes have been reported to have a significantly higher pH than round tomatoes, survival of salmonellae in wounds and on the surface has been observed to be unaffected by variety. The behavior of acid-adapted *Salmonella* in and on tomatoes has likewise been given only meager research attention. Tolerance of *Salmonella* Baildon upon exposure to an agar medium at pH 4.5 is not influenced by the pH of tomato juice (4.8 or 5.8) or broth (pH 7.2) in which it had been grown. However, acid-adapted cells of *S.* Montevideo inoculated into homogenized Roma tomatoes are more resistant than unadapted cells to electron beam irradiation.

A study was done with the objective to determine if survival and growth of *Salmonella* in and on tomatoes is affected by the variety of tomato (round, Roma, and grape), stage of ripeness, and storage temperature. The influence of acid adaptation of cells and site of inoculation on survival and growth was studied. The influence of acid adaptation of cells and site of inoculation on survival and growth was studied. *Salmonella* grew in stem scar and pulp tissues of round, Roma, and grape tomatoes stored at 12 and 21ºC but not at 4ºC. Survival and growth was largely unaffected by variety and stage of ripeness at the time of inoculation. The pathogen did not grow on the skin of grape tomatoes stored at 4, 12, and 21ºC. Survival and growth of *Salmonella* inoculated into stem scar and pulp tissues of round and Roma tomatoes were unaffected by prior exposure of cells to an acidic pH environment before inoculation. Results emphasize the importance of preventing contamination of tomatoes with *Salmonella* at all stages of ripeness, regardless of variety or previous exposure of cells to an acidic environment.

EFFICACY OF GASEOUS CHLORINE DIOXIDE AS A SANITIZER AGAINST *CRYPTOSPORIDIUM PARVUM*, *CYCLOSPORA CAYETANENSIS*, AND *ENCEPHALITOZOON INTESTINALIS* ON PRODUCE

(Y.R. Ortega, A. Mann, M.P. Torres, and V. Cama)

Parasites have frequently been identified in fresh produce or vegetables and have caused several foodborne outbreaks. *Cryptosporidium parvum*, a parasite often linked to waterborne transmission, has also been reported causing disease through the consumption of unpasteurized milk or apple cider and by eating unwashed contaminated vegetables and fruits. Most cases of cyclosporiasis have been almost exclusively associated with the consumption of contaminated fresh vegetables, such as raspberries, lettuce, basil, mixed greens, and snow peas.

Recently, chlorine dioxide gas has been evaluated for the killing of *Salmonella*, yeast and molds on berries as an alternative to rinse sanitizers for fruits and vegetables eaten raw.

The efficacy of gaseous chlorine dioxide to reduce parasite and bacterial burden in produce was investigated in this study. Basil and lettuce leaves were inoculated with *C. parvum* and *Cyclospora cayetanensis* oocysts, *Encephalitozoon intestinalis* spores, and a cocktail of two isolates of nalidixic acid-resistant *Escherichia coli* O157:H7 and subsequently treated for 20 minutes with gaseous chlorine dioxide at 4.1 mg/l. *Cryptosporidium*, *Encephalitozoon*, and *E. coli* loads were significantly reduced (2-4 log), although *Cyclospora* was resistant to the treatment. Our findings demonstrate that *Cyclospora* oocysts are resistant to gaseous chlorine dioxide treatment but other pathogens such as *Cryptosporidium, Encephalitozoon* and *E. coli* can be inactivated using gaseous chlorine dioxide, therefore providing an alternative treatment for safer vegetables.