ECOLOGY OF PATHOGENS

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.

CULTURE AND DETECTION OF *CAMPYLOBACTER JEJUNI* WITHIN MIXED MICROBIAL POPULATIONS OF BIOFILMS ON STAINLESS STEEL

(S.Q. Sanders, D.H. Booth, J.F. Frank, and J.W. Arnold)

The objective of this research was to observe the formation and composition of biofilms that contain *Campylobacter jejuni*. Biofilms containing natural populations of bacteria from the poultry processing environment and the target pathogen, *C. jejuni*1221gfp, were produced. Growth kinetics were observed at four cell densities to determine temporal compatibility with biofilm mixtures. Thus, a protocol was developed for growing *C. jejuni* within biofilms on stainless steel coupons. Analysis of the biofilms by confocal laser scanning microscopy showed that *C. jejuni*1221gfp formed a biofilm on stainless steel when incubated without other bacteria. The average surface area of steel covered by *C. jejuni*1221gfp increased from 24 h to approximately equivalent levels at 48 and 96 h. *C. jejuni*1221gfp and natural bacterial populations formed biofilms on stainless steel. This mixture was characterized by an initial increase and subsequent decrease of the surface area coverage of stainless steel by *C. jejuni*1221gfp for each time period. Data on the surface area of stainless steel associated with *C. jejuni*1221gfp when incubated with either of two different initial inoculum densities of other bacteria suggested that the presence of natural populations of bacteria enhanced the numbers of *C. jejuni* on stainless steel. This work provides the basis to study interactions of *C. jejuni* with other bacteria.

PROTEOMIC ANALYSIS OF A HYPOCHLOROUS ACID TOLERANT *LISTERIA MONOCYTOGENES* CULTURAL VARIANT EXHIBITING ENHANCED BIOFILM PRODUCTION

(J.P. Folsom and J.F. Frank)

Following exposure of *Listeria monocytogenes* ScottA (SA) to hypochlorous acid, rough colony variants were identified that were tolerant of hypochlorous acid and produced increased amounts of biofilm. A derivative of one of these variants was smooth, produced even more biofilm and exhibited greater biofilm chlorine resistance. The objective of this research was to compare the protein expression of a cultural variant to SA, and identify proteins that may be associated with biofilm production and chlorine tolerance. Suspension chlorine tolerance for several cultural variants (SAR, SAR5, and SBS) was determined by exposure to 60-120 ppm hypochlorous acid for five minutes. Hypochlorous acid tolerance of biofilms was determined after growing biofilms on stainless steel followed by exposure to 200 ppm hypochlorous acid for 5 min. All cultural variants were able to survive 120 ppm of hypochlorous acid in suspension. There was little difference in the hypochlorous acid tolerance of the cultural variant planktonic cells. The cultural variants produced greater amounts of biofilm than the SA form, and were more hypochlorous acid tolerant. The SBS variant was selected for proteomic comparison because it was the variant that produced the most biofilm and was most tolerant of hypochlorous acid when grown as a biofilm. Protein expression of planktonic and biofilm cells of SBS was compared to SA by using two dimensional difference gel electrophoresis. The 50s ribosomal protein, L10 was down regulated in biofilm SBS. Other proteins down regulated in planktonic SBS were the peroxide resistance protein (Dpr) and a sugar binding protein (LMO0181). This sugar binding protein was also up regulated in biofilm SBS. One protein spot down regulated in planktonic SBS contained both 50s ribosomal protein L7/L12 and an unknown protein (LMO1888).

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^{o}C, storage time of up to 8 days at 25 and 31^{o}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.

FATE OF *ENTEROBACTER SAKAZAKII* ATTACHED TO OR IN BIOFILMS ON STAINLESS STEEL UPON EXPOSURE TO VARIOUS TEMPERATURES OR RELATIVE HUMIDITIES

(H. Kim, J. Bang, L. R. Beuchat, and J.-H. Ryu)

Concerns about the occasional presence of *Enterobacter sakazakii* in powdered infant formula have surfaced as a result of reports of outbreaks of infections associated with consumption of reconstituted products. The bacterium may enter formulas via contaminated ingredients after spray drying of milk or soy components or by cross-contamination from the environment before packaging or during reconstitution in preparation areas. *E. sakazakii* has been observed to attach to or form biofilms on the surface of silicon, latex, polycarbonate, glass, polyvinyl chloride, and stainless steel. Cells that have attached to stainless steel and formed biofilms have enhanced resistance to disinfectants. *E. sakazakii* is reported to produce extracellular polysaccharides which may enhance the resistance of cells to environmental stresses such as in low a_w environments. Meager research attention has been given to characterizing the survival of cells of *E. sakazakii* attached to abiotic surfaces or in biofilm upon exposure to dry environments.

To develop effective strategies and practices for eliminating *E. sakazakii* in processing or preparation kitchen environments, factors affecting the survival of attached cells and cells in biofilm need to be better understood. We undertook a study to determine the survival characteristics of *E. sakazakii* cells suspended in water and reconstituted infant formula and dried on the surface of stainless steel as affected by subsequent incubation temperature at 43% relative humidity (RH) for up to 60 days. Maturation curves of biofilms formed in M9 medium and reconstituted infant formula, and survival of cells in biofilms formed in these media upon exposure to RH of 23 – 100% for up to 42 days were determined.

Initial populations of 7.4 - 8.6 log CFU/coupon decreased significantly ($p \leq 0.05$) at 4, 25, and 37°C within 10, 3, and 1 day(s), respectively, but the pathogen remained viable for up to 60 days. At a given storage temperature and time, reductions were significantly greater when cells had been suspended in water rather than infant formula before drying. Formation of biofilm by *E. sakazakii* on stainless steel immersed in M9 medium, which contains minimal concentrations of nutrients, and infant formula at 25°C and subsequent survival of cells at 25°C as affected by exposure to 23, 43, 68, 85, and 100% RH were investigated. Some of the cells in these biofilms survived under all test RHs for up to 42 days. The overall order of survival as affected by RH was 100 > 23 = 43 = 68 > 85% RH, regardless of the medium in which the biofilm was formed. Reduction in viability of cells was significantly greater in biofilm that had formed in M9 medium than in biofilm formed in infant formula. Results indicate that infant formula provides protection for attached cells, as well as cells in biofilm, against lethality upon exposure to desiccation. These results are useful when predicting the survival characteristics of *E. sakazakii* on stainless steel, thereby providing insights to developing and applying effective strategies and practices for elimination of the pathogen in processing and preparation kitchen environments.

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.

DETECTION OF *CRYPTOSPORIDIUM, GIARDIA,* AND *CYCLOSPORA* IN WATERS FROM GEORGIA

(Y.R. Ortega)

Protozoan parasites have been associated with gastrointestinal infections in animals and humans. In the U.S., food and waterborne infections of cryptosporidiosis, cyclosporiasis and giardiasis have been well recognized. *Cryptosporidium, Giardia* and *Toxoplasma* are zoonotic parasites that infect domestic and farm animals. Surface water may become contaminated via the entry of infectious oocysts by agricultural run-off from adjacent farm animals or by accidental contamination from human sewage. The ubiquitous nature of these parasites, resistance to environmental conditions, small size, and low sedimentation rate make water or moist environments an optimal matrix where the oocysts can remain viable and infectious for long periods of time. These parasites can cause zoonotic infections and have a low infectious dose. They are resistant to sanitizers and disinfectants commonly used in the water and produce. Because of these factors, parasites and water quality are a priority for food production and processing.

The role man and domestic/farm animals may play in contaminating recreational waters (lakes and rivers) and the subsequent contamination of our food supply in Georgia is unknown. In 2007, rivers and lakes of the central regions of Georgia were sampled and examined for the presence of parasites. Of those 18 environmental water samples, 4 had *Cryptosporidium*, 9 *Giardia* and 1 *Eimeria*. The locations with more parasites were the Flint and Appalachian Rivers.

Parasites identified in waters suggest that human and animal waste are present in these rivers and that they may play a role in contaminating animals and crops that use this water for irrigation. More studies are needed to evaluate the impact of these parasites in irrigation waters.

INACTIVATION OF PATHOGENS IN COMPOST MIXTURES AS INFLUENCED BY TYPE OF MANURE

(M.C. Erickson, C. Smith, X. Jiang, and M.P. Doyle)

During aerobic composting, heat is generated from the metabolic activity of thermophilic microorganisms and may contribute to inactivation of contaminant pathogens at internal sites of static piles. At the surface of compost piles, however, heat dissipation contributes to reduced temperatures and in turn reduced pathogen inactivation. It was the objective of this study to investigate whether pathogen inactivation at the surface would be affected by the compost composition and in particular the type of manure.

Chicken, cow, and hog manures served as the source of nitrogen in compost mixtures while straw and cottonseed meal were used as carbon amendments. Mixtures varied in the C:N ratio, having initial values of 20:1,

30:1, or 40:1 and were inoculated with both gfp-labeled *Salmonella* spp. and gfp-labeled *Listeria monocytogenes*. Mixtures were placed in trays (simulating surface sites of static compost piles) and held in environmental controlled chambers at 20° or 30°C and under different levels of light exposure. On a weekly basis, moisture levels in samples were adjusted to initial values (30% or 60%). Samples were periodically taken for enumeration of pathogens and measurement of moisture and pH.

At both 20° and 30°C, pathogen survival was greatest in compost mixtures formulated with cow manure followed by mixtures formulated with chicken manure and then hog manure. Regardless of the manure used in the compost mixture formulation, however, *L. monocytogenes* populations decreased faster than *Salmonella* spp. populations. Exposure to conditions simulating bright sunlight accelerated pathogen inactivation.