

ESCHERICHIA COLI O157:H7

SURVIVAL AND CONTROL OF *E. COLI* O157:H7 IN DRINKING WATER FOR CATTLE (P. Zhao, M. P. Doyle, and T. Zhao)

A study published in 1999 by the Centers for Disease Control and Prevention estimated that *E. coli* O157:H7 accounts for more than 73,000 cases of foodborne illness each year in the United States. Although a variety of different foods have been implicated as vehicles of infection, most *E. coli* O157:H7 foodborne outbreaks are associated with the consumption of undercooked ground beef. In 2002 alone, approximately 20 million pounds of ground beef contaminated with *E. coli* O157:H7 were recalled by the United States Department of Agriculture. Researchers have determined previously that *E. coli* O157:H7 can survive in bovine feces and water contaminated with cattle feces for long periods of time and retain their ability to produce verotoxins. Cattle are a principal reservoir of *E. coli* O157:H7 and their drinking water at the farm is an important source for their transmission. The objectives of this project were to evaluate the survival characteristics of *E. coli* O157:H7 in water after it is contaminated by rumen content, a site in cattle where *E. coli* O157:H7 resides, and to develop methods to control *E. coli* O157:H7 in contaminated water containing rumen fluid.

Survival of *E. coli* O157:H7 (a 5-strain mixture, including E0018, E009, 932, E0122, and E0139) in water contaminated with rumen content at different ratios, cell numbers and temperatures were determined. At 8°C, results revealed that *E. coli* O157:H7 at a high level of inoculum (10^5 cfu/ml) survived for 16, 6, 8, 3, and 5 weeks at ratios (tap water to rumen content) of 5:1, 10:1, 25:1, 50:1 and 100:1, respectively, and at low level of inoculum (10^2 cfu/ml) for 2, 3, 1, 1, and 1 weeks at ratios of 5:1, 10:1, 25:1, 50:1 and 100:1, respectively. However, at 21°C, results revealed that *E. coli* O157:H7 at a high level of inoculum survived 8, 15, 23, >56 and 24 weeks at contamination ratios of 5:1, 10:1, 25:1, 50:1 and 100:1, respectively, and at a low level of inoculum survived 8, 11, 10, 11, and 10 weeks at ratio of 5:1, 10:1, 25:1, 50:1 and 100:1, respectively. Results of studies at 8°C indicate the more concentrated the rumen content, the greater the survival of *E. coli* O157:H7. On the contrary, survival trends at 21°C were greater when the rumen content was more dilute. With equivalent cell numbers of *E. coli* O157:H7 inoculated, the survival of the pathogen was much greater at 21°C than at 8°C. DNA profile analysis of the isolates obtained at 56 weeks when held at 21°C revealed that E0122 (cattle isolate), 932 (meat isolate) and E0139 (deer isolate) were the dominant surviving strains.

Treatment of water with chlorine revealed that free chlorine at 1 ppm (the concentration present in tap water) is sufficient to kill up to 10^7 cfu *E. coli* O157:H7/ml within minutes. However, results from studies with tap water contaminated with different levels of rumen content, which included 10:1, 25:1, 50:1 and 100:1, revealed that the concentration of free chlorine was eliminated within minutes. Free chlorine (5 ppm) was added to tap water contaminated with rumen content at a ratio of 10:1, 25:1, 50:1 and 100:1. *E. coli* O157:H7 cell numbers ranging from 10^5 to 10^7 cfu/ml were added at 21°C and sampled at 0, 1, 2, 5, 10 and 20 minutes for enumeration of *E. coli* O157:H7. Results revealed there was no change in *E. coli* O157:H7 counts within 2 min for any of the contamination ratios evaluated. For samples with a contamination ratio of 100:1, *E. coli* O157:H7 counts at 5, 10 and 20 min were reduced by 0.3, 1.1 and 3.8 \log_{10} cfu/ml, respectively; at 25:1, *E. coli* O157:H7 counts were reduced by 0, 0.2 and 1.1 cfu/ml, respectively; and at 10:1, *E. coli* O157:H7 counts were not reduced within 20 min.

The effect of ozonated water on *E. coli* O157:H7 was also evaluated. The concentration of ozone in water for all determinations ranged from 22-24 ppm, and the temperature was 5°C. The *E. coli* O157:H7 inoculum ranged from $10^{5.2}$ to $10^{6.7}$ cfu/ml. Four to 6 trials have been completed for studies with water to rumen content at ratios of 50:1 and 100:1. Results revealed that 22-24 ppm of ozone in water with no rumen content killed up to 10^5 *E. coli* O157:H7/ml within 1 min. However, the influence of ozone in killing *E. coli* O157:H7 was related to the amount of rumen content present in water. At a water to rumen content ratio of 20:1 and 50:1, a contact time of up to 20 min reduced *E. coli* O157:H7 by only 0.4 \log_{10} cfu/ml. Inconsistent results were obtained for studies done with ozone in water containing rumen content at a ratio of 100:1. For three of the 6 trials, *E. coli* O157:H7 populations were reduced by greater than 4 \log_{10} cfu/ml within 1 min of exposure to ozone. For one trial, a greater than 4 \log_{10} cfu/ml reduction occurred after 5 min or more exposure time. Interestingly, there was no substantial reduction of *E. coli* O157:H7 for two trials, i.e., ca. 0.7 \log_{10} cfu/ml after 10 min or exposure for one trial and no inactivation of *E. coli* O157:H7 after 20 min of exposure for the other trial. These results indicate that the effect of ozone on *E. coli*

O157:H7 is related to the amount of rumen content in water. Contamination of water with rumen content at a ratio more concentrated than 50:1 neutralizes the killing effect of 22-24 ppm ozone.

Treatment of water with probiotic *E. coli* (a mixture of 3 strains, including #271, #786 and #797, all of which produce metabolites antagonistic to *E. coli* O157:H7) to inactivate *E. coli* O157:H7 was studied at 21°C for a period of 15 days. Experimental conditions included water to rumen content ratios of 50:1 and 100:1, a 5-strain mixture of *E. coli* O157:H7 at 10³ cfu/ml and probiotic *E. coli* at 10⁶ cfu/ml. The studies were repeated five times. Within 15 days, the population of *E. coli* O157:H7 in rumen content-contaminated water increased (up to 2.5 log₁₀ cfu/ml), whereas the population of *E. coli* O157:H7 treated with probiotic *E. coli* decreased from 0.2 to 2.5 log₁₀ cfu/ml. Greater *E. coli* O157:H7 inactivation occurred in water to rumen content at a ratio of 100:1 than at 50:1.

A group of chemicals, including lactic acid, acidic calcium sulfate, chlorine, chlorine dioxide, sodium hydroxide, caprylic acid, propionic acid, and butyric acid were tested for their effect on the killing of *E. coli* O157:H7 individually or as a combination. Of these, a combination of lactic acid (0.1-0.5%), acidic calcium sulfate (0.5.0.9%) and chlorine dioxide (50-100 ppm) or lactic acid (0.1-0.5%), acidic calcium sulfate (0.9%) and caprylic acid (0.1-0.5%) at 21°C effectively killed > 5.0 log₁₀ *E. coli* O157:H7 within 2 min in water heavily contaminated with rumen content at a ratio of 10:1. Additional experiments are underway to identify the most cost effective combinations for on-farm use.

RADIO-FREQUENCY HEATING OF ALFALFA SEED FOR REDUCING HUMAN PATHOGENS (S. O. Nelson, C.-Y. Lu, L. R. Beuchat, and M. A. Harrison)

The production of sprouts from alfalfa and other seeds for human consumption is a substantial industry; however, there have been several outbreaks of illness associated with sprouts, and contamination by *Salmonella* and *Escherichia coli* O157:H7 has been identified as the cause. Contaminated seed used for sprouting is considered the most likely source of these human pathogens. No sprout-related illness attributable to *Listeria monocytogenes* has yet been documented, but this pathogen also poses a potential threat. Because most of the outbreaks of infections have been attributed to contaminated sprouting seed, several methods have been studied for decontaminating seed. Treatment of alfalfa seed in hot water at 54°C significantly reduced seed viability. Several aqueous solutions of chemicals, including chlorine, chlorine dioxide, hydrogen peroxide, trisodium phosphate, ethanol, peracetic acid, and some commercial fruit and vegetable produce wash solutions have been studied for decontaminating alfalfa seed. None of these treatments eliminated *E. coli* O157:H7 or *Salmonella* from alfalfa seed intended for sprouting.

Earlier research has shown that radio-frequency (RF) and microwave dielectric heating treatments are effective for increasing the germination percentage of alfalfa seed lots containing high percentages of hard seed. Hard seeds occur naturally and are viable seeds with seed coats that are impermeable to water. Therefore, they will not germinate promptly when planted, but they may germinate several weeks, months, or years later when the seed coat becomes permeable through natural processes. Mechanical scarification of such seed lots to increase germination is common practice for alfalfa, but the abrasive process scratches the seed coat thus providing a favorable environment for bacterial attachment, which may make sanitization with liquids more difficult. Thus, it appeared reasonable to explore the possible use of dielectric heating for reduction of bacterial populations on alfalfa seed, especially since the improvement of germination and subsequent sprout yield can be achieved without mechanical abrasion of the seed coat. Similar consistent increases in alfalfa seed germination through hard seed reduction have been achieved by dielectric heating at frequencies of 5, 10, 39, or 2,450 MHz. Treatment at 39 MHz was selected for this study because of equipment availability and because it provides a more uniform electric field for exposure of the samples than is commonly available in microwave ovens.

The potential for controlling human bacterial pathogens on alfalfa seed used in the production of sprouts by dielectric heating was studied by experimental exposure of alfalfa seed artificially contaminated with *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* to RF dielectric heating treatments at 39 MHz and different electric field

FATE OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, AND *SALMONELLA* SPP. IN REDUCED SODIUM HOME-STYLE BEEF JERKY (M. A. Harrison)

Interest in low-sodium food products necessitates re-examination of home preservation processes relying in part on salt for antimicrobial effects. The fates of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. in reduced sodium home-style beef jerky was determined using different salt levels in ground or

whole beef strips. Samples were either dried in a 60°C dehydrator or heated to an internal temperature of 71.1°C prior to drying in a 60°C dehydrator. Populations were determined at time 0 and 2 h intervals until dry. Population reductions were greater in ground beef with non-reduced salt levels compared to that with reduced salt levels, and in most cases, greater reduction (1.0 – 1.5 log₁₀) was observed for ground beef strips heated prior to drying. For dried whole jerky strips, there generally were no significant differences (p > 0.05) in pathogen populations between the non-reduced and reduced salt marinade in the end product. The results from this study support the importance of the antimicrobial effect of sodium chloride in particular products on the pathogens used in this experiment.

**VIABILITY OF ACID-ADAPTED *ESCHERICHIA COLI* O157:H7 IN GROUND BEEF
TREATED WITH ACIDIC CALCIUM SULFATE
(L. R. Beuchat and A. J. Scouten)**

Exposure of microorganisms to sublethal stress can result in greater resistance of cells upon subsequent exposure to the same or unrelated stress. Acid adapted or acid shocked *Escherichia coli* O157:H7 has been shown to have increased resistance to heat, salt, and acidic pH. The type of acidulant used to achieve a given pH can influence the rate of inactivation of *E. coli* O157:H7 as well as its sensitivity upon subsequent exposure to acid or other stress environments. The effectiveness of treatment of beef carcasses with organic acids for the purpose of reducing or eliminating surface microflora, including *E. coli* O157:H7 and other pathogens, varies with type, concentration and temperature of acid, the presence of surfactants, contact time, application pressure, tissue type, and sensitivity of the microorganism. The potential for *E. coli* O157:H7 surviving acid rinse treatment of beef carcasses to exhibit increased tolerance to acidic environments or refrigeration temperatures to which processed beef may be exposed raises a concern about the impact of organic acid sanitizers on safety risks. Supplementation of ground beef with acidic calcium sulfate (ACS), the basis for a commercial food additive called Safe₂O™, which contains calcium hydroxide, sulfuric acid, and calcium sulfate, has been suggested as an intervention to control the growth of spoilage and pathogenic bacteria. Claims are that ACS kills bacteria at levels nearly equal to irradiation and continues to inhibit microbial growth and recontamination long after treatment. Bacterial kill in excess of 5 log₁₀ is claimed. The effects of ACS on survival of acid-adapted *E. coli* O157:H7 in refrigerated ground beef have not been reported.

The objectives of this study were to determine if treatment of three strains of *E. coli* O157:H7 in broth acidified with lactic acid, acetic acid, or ACS results in a change in tolerance of cells upon exposure to a second acidic environment and to determine if *E. coli* O157:H7 grown on an agar medium at pH 4.5 achieved using ACS as an acidulant changes in its ability to survive in ACS-treated ground beef. Differences in tolerance to acidic environments were observed among strains but the level of tolerance was not affected by the acidulant to which cells had been exposed. Cells of *E. coli* O157:H7 adapted to grow on tryptic soy agar acidified to pH 4.5 with ACS were compared to cells grown at pH 7.2 in the absence of ACS for their ability to survive after inoculation into ground beef treated with ACS, as well as untreated beef. The number of ACS-adapted cells recovered from ACS-treated beef was significantly ($\alpha = 0.05$) higher than the number of control cells recovered from ACS-treated beef during the first 3 days of a 10-day storage period at 4°C, suggesting that ACS-adapted cells are initially more tolerant than unadapted cells to reduced pH in ACS-treated beef. Regardless of treatment of ground beef with ACS or adaptation of *E. coli* O157:H7 to ACS before inoculating ground beef, the pathogen survived in high numbers.

**SENSITIVITY OF *ESCHERICHIA COLI* O157:H7 TO COMMERCIAL ALKALINE CLEANERS
AND SUBSEQUENT RESISTANCE TO HEAT AND SANITIZERS
(M. Sharma and L. R. Beuchat)**

Exposure to bacterial cells to extreme pH may make bacterial cells more resistant to subsequent extreme pH environments that would otherwise be lethal. Acid-adapted stationary phase cells of *Escherichia coli* O157:H7 are more resistant than unadapted cells to heat. Increased heat tolerance of acid-adapted cells correlates well with the synthesis of heat-shock proteins by acid-adapted non-pathogenic *E. coli* but little is known about the survival and potential for induction of cross protection of *E. coli* O157:H7 upon exposure to alkaline environment. The pathogen may, however, be exposed to alkaline conditions in a variety of pre- and post-processing and handling environments resulting from the use of alkaline cleaners and sanitizers in food processing plants and the food service industry.

The *rpoS* gene has been reported to play an important role in the survival of *E. coli* and *Salmonella* cells exposed to chemical and physical stresses. *E. coli* O157:H7 cells deficient in the expression of the *rpoS* gene were more susceptible to acidic, osmotic, and heat stresses than were wild-type cells. The *rpoS* gene may also aid in survival of *E. coli* O157:H7 in high pH environments, providing cells with a simple mechanism to tolerate extreme alkaline conditions they may encounter in the gastrointestinal system of a host. However, studies evaluating the role of *rpoS* in *E. coli* O157:H7 upon exposure to alkaline cleaners and sanitizers commonly used in food processing environments have not been reported. The objective of this study was to determine the survival characteristics of *E. coli* O157:H7 upon exposure to alkaline cleaners commonly used in food processing plants. Cells surviving exposure to alkaline cleaners were evaluated for changes in thermotolerance and resistance to sanitizers. The *rpoS* was examined for its role in protecting cells treated with alkaline cleaners and potential cross protection of treated cells against subsequent exposure to heat and sanitizers.

The effects of seven commercial alkaline cleaners used in the food processing industry, 0.025 M NaOH, and 0.025 M KOH on viability of wild-type (EDL 933) and *rpoS*-deficient (FRIK 816-3) strains of *E. coli* O157:H7 in logarithmic and stationary phases of growth were determined. Cells were treated at 4 or 23°C for 2, 10, or 30 min. Cleaners 2, 4, 6, and 7, which contained hypochlorite and < 11% NaOH and/or KOH (pH 11.2 – 11.7), killed significantly ($P \leq 0.05$) higher numbers of cells compared to treatment with cleaner 3, containing sodium metasilicate (pH 11.4) and < 10% KOH, and cleaner 5, containing ethylene glycol monobutyl ether (pH 10.4). Treatment with KOH or NaOH (pH 11.2) was not as effective as four out of seven commercial cleaners in killing *E. coli* O157:H7, indicating that chlorine and other cleaner components have bactericidal activity at high pH. Stationary phase cells of strain EDL 933 that had been exposed to cleaner 7 at 4 or 23°C and strain FRIK 816-3 exposed to cleaner 7 at 23°C had significantly ($P \leq 0.05$) higher $D_{55^\circ\text{C}}$ values than cells of FRIK 816-3, indicating that exposure to cleaner 7 confers cross protection to heat. Cells of EDL 933 treated with cleaner 7 at 12°C showed significantly higher $D_{55^\circ\text{C}}$ values than cells of FRIK 816-3, indicating that *rpoS* may play a role in cross protection. Stationary phase cells treated with cleaner 5 or cleaner 7 at 4 or 12°C were not cross protected against subsequent exposure to sanitizers containing quarternary ammonium compounds or sodium hypochlorite, or to cetylpyridinium chloride and benzalkonium chloride.

**INTERACTION OF A FREE-LIVING SOIL NEMATODE, *CAENORHABDITIS ELEGANS*,
WITH SURROGATES OF FOODBORNE PATHOGENIC BACTERIA
(G. L. Anderson, K. N. Caldwell, L. R. Beuchat, and P. L. Williams)**

The agricultural impacts of plant and animal parasitic nematodes have long been recognized and, by virtue of their effects on fruit and vegetable production, have been extensively studied. Comparatively little is known regarding the impact of free-living microbiovorous nematodes on produce production and safety, although they are the most abundant and wide spread soil mesofauna. The association of free-living nematodes and various genera of bacteria has been studied. While it is recognized that free-living nematodes avoid certain bacteria, it is clear that they do not uniformly avoid foodborne pathogens. Two human enteric pathogens, *Salmonella* and *Shigella*, are reportedly ingested and defecated by free-living saprozoic nematodes and *Salmonella* Typhimurium is known to infect the free-living soil nematode *Caenorhabditis elegans*. From these reports, it appears that free-living nematodes may be important as vectors of pathogenic bacteria, including some forms capable of causing human disease.

Soil is a source of microbial contamination of fruit and vegetables, as evidenced by the isolation of soil-residing pathogenic bacteria from produce. In a survey of vegetables for the presence of amoebae and *Salmonella*, nematode eggs and larvae have been recovered using a naccional-ether method. The recovery of nematodes from uncooked vegetables indicates that agronomic conditions and marketing practices may be conducive to the survival of nematodes on fresh produce. This also indicates that if free-living nematodes are present on raw produce, they may serve as vehicles for contamination with pathogenic bacteria, either by contact with their surface or via eggs or voided material from their gastrointestinal tract.

We undertook a study to evaluate the interaction of *C. elegans* with bacterial surrogates for foodborne pathogens occasionally occurring or persisting in soil. Nematode/bacterial interactions were characterized to determine the propensity of young adult worms to be attracted to bacterial colonies, to compare the feeding and development of young adult worms cultured on this diverse group of bacteria, and to examine the dispersal of bacteria by *C. elegans* following feeding on monoxenic cultures. We evaluated the association between a free-living soil nematode, *C. elegans*, with *Escherichia coli*, an avirulent strain of *S. Typhimurium*, *Listeria welshimeri*,

and *Bacillus cereus*. On an agar medium, young adult worms quickly moved toward colonies of all four bacteria; over 90% of 3-day adults entered colonies within 16 min after inoculation. After 48 h, worms moved in and out of colonies of *L. welshimeri* and *B. cereus*, but remained associated with *E. coli* and *S. Typhimurium* colonies for at least 96 h. Young adult worms fed on cells of the four bacteria suspended in K medium. Worms survived and reproduced using nutrients derived from all test bacteria, as evidenced by eggs laid by second generation worms after culturing for 96 h. Development was slightly slower in worms fed on Gram-positive bacteria compared to Gram-negative bacteria. Worms fed for 24 h on bacterial lawns formed on tryptic soy agar dispersed bacteria over a 3-h period when transferred to a bacteria-free agar surface. Results suggest that *C. elegans* and, perhaps, other free-living nematodes are potential vectors for both Gram-positive and Gram-negative bacteria, including foodborne pathogens in soil.

**ATTACHMENT OF *ESCHERICHIA COLI* O157:H7 GROWN IN TRYPTIC SOY BROTH
AND NUTRIENT BROTH TO APPLE AND LETTUCE AS RELATED
TO CELL HYDROPHOBICITY, SURFACE CHARGE, AND CAPSULE PRODUCTION
(A. N. Hassan and J. F. Frank)**

This study investigated the effect of growth in tryptic soy broth (TSB) and nutrient broth (NB) on the ability of *E. coli* O157:H7 to attach to lettuce and apple. In addition, surface hydrophobicity, charge and capsule production were determined by cells grown in these media. Cells grown in NB attached less to lettuce and apple surfaces than did those grown in TSB. TSB, but not NB, supported capsule production by *E. coli* O157:H7. Cells grown in TSB were more hydrophilic than those grown in NB. No difference was found in the electrokinetic properties of cells grown in these media. Electrostatic and hydrophobic interactions and surface proteins did not appear to play an important role in the attachment of *E. coli* O157:H7 to these surfaces. Of the factors studied, only capsule production was associated with attachment ability.

**ETHANOL-MEDIATED VARIATIONS IN CELLULAR FATTY ACID COMPOSITION AND PROTEIN PROFILES
OF TWO GENOTYPICALLY DIFFERENT STRAINS OF *ESCHERICHIA COLI* O157:H7
(R. Y.-Y. Chiou, R. D. Phillips, P. Zhao, M. P. Doyle, and L. R. Beuchat)**

Ethanol can be found in foods, particularly in fruits and fruit products and in fermented foods and beverages, and in food processing environments as a result of fermentation of sugars by naturally occurring microorganisms. Ethanol and cleaners containing ethanol and other alcohols are also used in some areas of food processing plants to reduce or remove microorganisms on equipment and for promoting good worker hygiene. Occasionally, low concentrations of residual ethanol may be present on treated surfaces of equipment and in environmental niches not properly cleaned and sanitized. This provides an opportunity for pathogens to adapt and grow in environments with sublethal concentrations of ethanol.

The use of ethanol in the food processing industry is largely for the purpose of killing microorganisms rather than as a preservative, although low concentrations of ethanol have been examined for controlling the growth of spoilage and pathogenic species. The efficacy of ethanol as a preservative in a wide range of foods was studied. Growth inhibition of *Staphylococcus aureus* by ethanol has been shown to be caused by factors other than reduced a_w . Treatment of a high-moisture bakery product with ethanol vapor delays the growth of and toxin production by *Clostridium botulinum*. Little is known about physiological changes in *Escherichia coli* O157:H7 and other foodborne pathogens exposed to sublethal concentrations of ethanol. However, the prospect of using ethanol as a preservative in foods raises the need for more information on the behavior of spoilage and pathogenic microorganisms that may adapt to otherwise lethal concentrations of ethanol and subsequently exhibit altered survival or growth behavior and increased resistance to other environmental stresses imposed by traditional preservation technologies.

It is important to gain a better understanding of the mechanisms *E. coli* O157:H7 may possess to adapt and grow in ethanol-containing substrates in order to more accurately assess the level of safety hazard they may represent. In this study, two strains of *E. coli* O157:H7 isolated from unpasteurized apple juice and salami were grown in ethanol-supplemented tryptic soy broth (TSB) as a model food system. Characterization of growth patterns and changes in pH and glucose content in TSB as affected by ethanol content were determined. Early stationary-growth phase cells grown in TSB supplemented with 5.0% ethanol were analyzed for fatty acid

composition, protein content, and SDS-PAGE protein patterns. Cells grown in TSB and in TSB supplemented with ethanol were subjected to pulsed-field gel electrophoresis (PFGE) analysis to compare genomic DNA fingerprints.

Two strains of *E. coli* O157:H7 were grown in tryptic soy broth (TSB, pH 7.1) supplemented with 0, 2.5, 5.0, 7.5, and 10% ethanol at 30°C for up to 54 h. Growth rates in TSB supplemented with 0, 2.5, and 5.0% ethanol decreased with an increase of ethanol concentration. Growth was not observed in TSB supplemented with 7.5 or 10% ethanol. The pH of TSB containing 5.0% ethanol decreased to 5.8 within 12 h, then increased to 7.0 at 54 h. The ethanol content in TSB supplemented with 2.5 or 5.0% ethanol did not change substantially during the first 36 h of incubation but decreased slightly thereafter, indicating utilization or degradation of ethanol by both strains. Glucose was depleted in TSB supplemented with 0, 2.5, or 5.0% ethanol within 12 h. Cells grown under ethanol stress contained a higher amount of fatty acids. With the exceptions of *cis*-oleic acid and nonadecanoic acid, higher amounts of fatty acid were present in stationary-phase cells of the two strains grown in TSB supplemented with 5.0% ethanol for 30 h compared to cells grown in TSB without ethanol for 22 h. The *trans*-oleic acid content was 10-fold higher in the cells grown in TSB with 5.0% ethanol than in TSB without ethanol. In contrast, *cis*-oleic acid was not detected in ethanol-stressed cells but was present at concentrations of 0.32 and 0.36 mg/g of cells of the two strains grown in TSB without ethanol. Protein content was higher in ethanol-stressed cells than in non-stressed cells. SDS-PAGE protein profiles varied qualitatively as affected by strain and the presence of ethanol in TSB. An ethanol-mediated protein (MW 28 kDa) was observed in the ethanol-stressed cells but not in control cells. It is concluded that the two test strains of *E. coli* O157:H7 underwent phenotypic modifications in cellular fatty acid composition and protein profiles in response to ethanol stress. The potential for cross protection against subsequent stresses applied in food preservation technologies as a result of these changes is under investigation.

**FACTORS AFFECTING PRODUCTION OF EXTRACELLULAR CARBOHYDRATE COMPLEXES
BY *ESCHERICHIA COLI* O157:H7
(J.-H. Ryu and L. R. Beuchat)**

Numerous environmental factors have been reported to promote exopolysaccharide (EPS) production. These include high levels of oxygen, limited availability of nitrogen, desiccation, low temperature, and nutrient deprivation. The influence of waxes on colonization of microorganisms on plants and implication of biofilms in the ecology and management of epiphytic bacteria on plant leaves have been described. However, environmental factors specifically related to EPS production and biofilm formation by foodborne pathogens on the surface of raw fruits and vegetables have been given only meager research attention. The rate and amount of EPS produced by *Escherichia coli* O157:H7 and other pathogens, as affected by environmental conditions to which whole and fresh-cut produce is commonly exposed, are unknown.

In this study, we studied the effects of atmospheric gas composition, pH, nutrient source, and temperature on production of extracellular carbohydrates complexes (ECC) by *E. coli* O157:H7 and investigated the production of ECC on media formulated from juice of raw fruits and vegetables. ECC is defined in this study as those carbohydrates secreted from cells or loosely attached to the cell surface which can be detached by heat treatment. The reason for measuring the amount of ECC rather than the amount of purified EPS produced was that it is difficult to achieve a complete physical separation of EPS from other polysaccharides such as lipopolysaccharides. EPS is probably present with other carbohydrates, including mono- or oligosaccharides secreted by cells or cleaved from the EPS physically or enzymatically, and polysaccharides derived from cell surface components in the form of carbohydrate complexes.

The influence of environmental conditions on cell growth, the total amount of ECC produced, and the amount of ECC produced on a per cell basis by *E. coli* O157:H7 strains ATCC 43895 (wild type) and 43895-EPS (natural mutant, extensive EPS producer) was studied. To determine the effects of pH on the production of ECC on a per cell basis, *E. coli* O157:H7 was grown aerobically at 12°C and 22°C on tryptic soy agar (TSA) acidified at pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, and 4.0. Lettuce, alfalfa sprout, cantaloupe, tomato, and apple juice agars (pH 4.46 to 6.50) were also evaluated for their support of the ECC production. Conditions generally favorable for growth of *E. coli* O157:H7 were rich nutrient medium (TSA) versus heated lettuce juice agar (HLJA) or minimal salts medium (MSM), 22°C and an aerobic atmosphere compared to modified atmosphere (1% O₂, 10% CO₂, and 89% N₂). Conditions favorable for production of ECC on a per cell basis were HLJA, 12°C, and an aerobic atmosphere. There was a negative relationship between cell growth and production of ECC on a per cell basis, and environmental conditions that affected the total amount of ECC produced based on initial population reflected a combination of environmental conditions influencing both cell growth and ECC production on a per cell basis. A

relative growth index factor (RGIF) was calculated to better understand ECC production as affected by various environmental conditions simultaneously. The production of ECC on a per cell basis by strain 43895-EPS showed a negative linear relationship with pH of TSA at both 12°C and 22°C. This strain generally produced a greater amount of ECC on fresh juice agar than on TSA at the same pH but production of ECC on alfalfa sprout juice agar (FJA, pH 6.45) at 22°C was significantly less than on TSA (pH 6.50). This indicates that nutrient limitation is not based only on nutrient availability. There may be other factors that repress the production of ECC on FJA and the effects of those factors may be temperature dependent. Further studies will be required to better understand the relationship between nutrient availability and other factors on the production of ECC by *E. coli* O157:H7 on raw produce.

**EVALUATION OF INOCULATION METHOD AND INOCULUM DRYING TIME FOR THEIR EFFECTS ON SURVIVAL AND EFFICIENCY OF RECOVERY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* INOCULATED ON THE SURFACE OF TOMATOES
(M. M. Lang, L. J. Harris, and L. R. Beuchat)**

Contamination of raw produce with pathogenic microorganisms can occur at any of several points from the field through the time of consumption. Given sufficient time and appropriate environmental conditions, pathogens can grow to populations exceeding 10^7 cfu/g of tomato. Work has been done to define conditions that result in contamination of produce and subsequent growth of pathogens during storage. Researchers have also evaluated the effectiveness of a wide range of chemical sanitizers and physical treatments to decontaminate fresh produce. Results of studies done in different laboratories are difficult to compare, however, because of numerous variations in methodologies employed and incompleteness in describing results. A study was undertaken with the objective to evaluate procedures for inoculating *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* onto the surface of tomatoes with the goal of selecting an inoculation procedure to be used in a standard method. Dip, spot, and spray inoculation were evaluated. A second objective was to examine the effect of time between application of inoculum and analysis of tomatoes on the viability and retrievability of pathogens. Inocula applied to tomatoes were subjected to two drying times followed by either no treatment or treatment with water (control) or chlorine (200 µg/ml), then analyzed for the presence of surviving cells.

Five-strain mixtures of *Escherichia coli* O157:H7, *Salmonella*, or *Listeria monocytogenes* were applied to tomatoes by dip, spot, or spray inoculation methods. Inocula were dried for 1 or 24 h at 22°C before tomatoes were treated with water (control) or chlorine (200 µg/ml). Significantly ($\alpha = 0.05$) higher populations (cfu/tomato) of *E. coli* O157:H7 and *Salmonella* were recovered from dip-inoculated tomatoes compared to spot- or spray-inoculated tomatoes. This is attributed to larger numbers of cells adhering to tomatoes subjected to dip inoculation. Populations of *E. coli* O157:H7 and *Salmonella* recovered from spot- and spray-inoculated tomatoes containing the same initial number of cells were not significantly different. Significantly different populations of *L. monocytogenes* were recovered from inoculated tomatoes (dip > spot > spray). Populations of pathogens recovered from tomatoes were significantly higher when inocula were dried for 1 h compared to 24 h. Significant differences (water > chlorine) were observed in populations of all pathogens recovered from tomatoes treated with chlorine, regardless of inoculation method or drying time. Results indicate that inoculation method, drying time, and treatment affect survival and/or recovery of foodborne pathogens inoculated onto the surface of tomatoes. It is recommended that spot inoculation with a drying time of 24 h at 22°C be used in a standard method to determine the efficacy of chlorine and other sanitizers in killing foodborne pathogens on tomatoes.

**SURVIVAL AND RECOVERY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* ON LETTUCE AND PARSLEY AS AFFECTED BY METHOD OF INOCULATION, TIME BETWEEN INOCULATION AND ANALYSIS, AND TREATMENT WITH CHLORINATED WATER
(M. M. Lang, L. J. Harris, and L. R. Beuchat)**

Given sufficient time and appropriate environmental conditions after contamination, pathogens can grow to populations exceeding 10^7 cfu/g of lettuce and 10^6 cfu/g of parsley. Conditions that result in contamination of produce with pathogens and subsequent growth during storage have been described. A wide range of chemical sanitizers and physical treatments for decontamination of fresh produce has been evaluated. Results are difficult to compare, however, because of the numerous variations in methodologies. The lack of uniformity of methods used to treat produce with sanitizers and enumerate microorganisms surviving treatments makes it difficult to assess their

effectiveness and establish industry recommendations and guidelines for their use. The development of a standard method would minimize or eliminate variations in methodologies used in various laboratories, thereby enabling a comparison of pathogen reductions resulting from treatment with various sanitizers. A single method may not be applicable for all fruits and vegetables but a basic test method that could be modified as necessary to accommodate natural variations in fresh and fresh-cut produce would be the goal.

One of the objectives of the study reported here was to evaluate three methods (dip, spot, and spray) for inoculating *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* onto the surface of two types of leafy salad vegetables, lettuce and parsley, with the goal of establishing a procedure for use in a standard method to test the efficacy of sanitizers. A second objective was to examine the effect of time between application of inoculum and analysis of lettuce and parsley on the viability and recovery of pathogens. Inocula applied to lettuce and parsley were subjected to two drying times, followed by either no treatment or treatment with water or chlorine then analyzed for the number of surviving cells. Inocula were dried for 2 h at 22°C or for 2 h at 22°C followed by 22 h at 4°C before treating with water (control) or chlorine (200 µg/ml). Significantly ($\alpha = 0.05$) higher populations (cfu/lettuce or parsley sample) of *E. coli* O157:H7 and *Salmonella* were recovered from dip-inoculated produce compared to spot- or spray-inoculated produce. This is attributed to larger numbers of cells adhering to lettuce and parsley subjected to dip inoculation. Populations of *E. coli* O157:H7 and *Salmonella* recovered from lettuce inoculated by spot and spray methods were not significantly different but populations recovered from spot-inoculated parsley were significantly higher than those recovered from spray-inoculated parsley, even though the number of cells applied was the same. Significantly different populations of *L. monocytogenes* were recovered from inoculated lettuce (dip > spray > spot); populations recovered from dip-inoculated parsley were significantly higher than those recovered from spot- or spray-inoculated parsley, which were not significantly different from each other. Populations of pathogens recovered from lettuce and parsley after drying inoculum for 2 h at 22°C were significantly higher than or equal to populations recovered after drying for 2 h at 22°C followed by 22 h at 4°C. Significant differences (water > chlorine) were observed in populations of all pathogens recovered from treated lettuce and parsley, regardless of inoculation method and drying time. It is recommended that spot inoculation with a drying time of 2 h at 22°C followed by 22 h at 4°C be used to determine the efficacy of chlorine and other sanitizers in killing foodborne pathogens on lettuce and parsley.

**DEVELOPMENT OF A METHOD TO QUANTIFY EXTRACELLULAR CARBOHYDRATE COMPLEXES
PRODUCED BY *ESCHERICHIA COLI* O157:H7
(J.-H. Ryu and L. R. Beuchat)**

Escherichia coli O157:H7 is known to produce exopolysaccharides (EPS). Production on the surface of foods and food processing equipment may result in enhanced protection against various environmental stresses. EPS may also be involved in cell/cell recognition and interaction, biofilm formation, and adhesion. Biofilm formation and adhesion are extremely important because cells become more resistant to removal from foods and food-contact surfaces in processing plants and to inactivation by sanitizers. There is evidence that EPS is involved in the development of surface film, adhesion of cells, and the formation of a complex three-dimensional biofilm structure. Since EPS may be involved in many steps of biofilm formation and, in fact, be a component of biofilm, it is important to use a standard method to quantify the amount of EPS produced under various environmental conditions in order to more easily develop intervention strategies to prevent or eliminate biofilm development.

Bacterial surface polysaccharides can be categorized in two general groups, those bound to the cell surface by attachment to lipid A, i.e., lipopolysaccharides (LPS), and those associated with the cell surface as a capsule and extracellular slime, EPS. It is difficult to separate LPS from EPS according to their presence, functions, and roles in biofilm formation. It may be difficult to make a complete physical separation of the two polymers because components of the microbial cell wall undergo constant turnover and may be excreted and lost from the cell surface as the cell ages or is subjected in natural or laboratory environments to high shear forces. Exopolysaccharides are probably present with other carbohydrates as carbohydrate complexes. Other carbohydrates may include mono- or oligosaccharides secreted by the cell or cleaved from the EPS physically or enzymatically, and polysaccharides derived from cell surface components. Consequently, the effects of EPS on cell physiology and behavior in response to external factors should be considered in broader terms of extracellular carbohydrate complexes (ECC). We defined ECC as those carbohydrates secreted from cells or loosely attached to the cell surface which can be detached by heat treatment. ECC might include EPS (slime and capsule) and other carbohydrates such as components of LPS, cell walls, and degraded EPS.

We undertook a study with the objective of optimizing a procedure to separate ECC fractions I and II present in EPS formed by *E. coli* O17:H7 and to standardize a method to quantify these complexes on a per cell basis. The ultimate goal is to develop a standard procedure to examine the production of ECC by *E. coli* O157:H7 in biofilms formed on food contact surfaces in processing plants and on foods as affected by conditions to which they are exposed. ECC fraction I was removed from *E. coli* O157:H7 cells produced on tryptic soy agar and lettuce juice agar by centrifugation. To remove ECC fraction II, cells were heated at 100°C for 10 min, then centrifuged. The sum of ECC fraction I and II was considered as the total ECC produced by *E. coli* O157:H7. A correlation between cell mass and turbidity (OD_{750nm}) of cell suspensions was determined. Cell mass has a linear relationship ($R^2 = 0.93$) with turbidity of cell suspensions from which ECC is removed. The amount of ECC produced on a per cell basis was calculated by dividing total amount of ECC ($\mu\text{g/ml}$) produced by the turbidity (OD_{750nm}) of heated cell suspension after removal ECC fractions I and II. We succeeded in developing a method for separating ECC from cells of *E. coli* O17:H7 and conditions have been optimized. A standard method to estimate the amount of ECC produced on a per cell basis was also developed. Using these procedures to prepare extract of ECC from *E. coli* O157:H7 and standardize values, production of ECC on a per cell basis can be estimated and a comparison of the amount of ECC produced by the pathogen grown under different environmental conditions can be accurately measured.

