

LISTERIA MONOCYTOGENES

DEVELOPMENT OF A DNA MICROARRAY CHIP FOR IDENTIFICATION OF *LISTERIA* SPECIES, PARTIAL SEROTYPING AND ASSESSMENT OF THE VIRULENCE POTENTIAL OF *LISTERIA MONOCYTOGENES* ISOLATES

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

Listeria monocytogenes is a leading cause of death attributed to foodborne bacterial pathogens. Lack of sufficient scientific information has led regulatory agencies to consider any strain of *L. monocytogenes* to be potentially pathogenic for humans although several lines of evidence indicate there are differences in virulence potential among *L. monocytogenes* isolates. Our ultimate goal is to develop a DNA microarray chip that can differentiate and identify *Listeria* species, including *L. monocytogenes*, as well as subtype and assess the virulence potential of *L. monocytogenes* isolates. Our initial approach to developing this chip was to design a set of 128 probes targeting 168 rRNA and *iap* genes of *Listeria* spp. (for differentiation and identification of *Listeria* spp.), and the *iap*, *gltA-gltB*, and *inlB* genes of *L. monocytogenes* (for partially serotyping and assessing the virulence potential of *L. monocytogenes* isolates). The probes (17- to 37-mer) were designed through the following steps: retrieving all available related gene sequences from GenBank; alignment of retrieved sequences using Clustal-X version 1.80; manual editing of alignment by SeaView; identification of regions for probe design; and design of specific probes using probe design software Sarani. For every probe, a control probe containing one central mismatch was included for accurate discrimination between true signal and random hybridization. The probes were synthesized with 5' or 3' amine modification for covalent attachment to substrate slides and several probes were synthesized with additional 12-mer spacers for studying the effect of general steric hindrance. Construction of the microarray chip is underway using an OmniGrid Accent Microarrayer and the functionality of the chip will be validated through serial hybridization with genomic DNAs from a collection of *Listeria* strains. The developed DNA microarray chip will enable rapid and accurate discrimination among six *Listeria* species and partial serotyping, and assess the virulence potential of *L. monocytogenes* isolates, such as those of food origin. With addition of more probes that specifically target virulence-associated genetic markers, the chip should be useful for rapid and more accurate global assessment of the virulence potential of any *L. monocytogenes* isolate. This DNA chip can be used for large-scale population genetic analysis of *L. monocytogenes* isolates and information gained through such studies would be the scientific basis for differentiating highly virulent from less virulent or avirulent strains of *Listeria*. This would provide an assay to enable food processors, regulatory agencies, and public health organizations to differentiate significant public health concerns such as food contaminated with a highly virulent strain of *L. monocytogenes* in contrast to an unimportant avirulent *L. monocytogenes* food contaminant. In addition, with resolution beyond the species level, the chip may be a useful subtyping tool for tracking contamination sources in the food-processing environment.

REMOVAL AND DISINFECTION OF *LISTERIA MONOCYTOGENES* AND POULTRY SOIL-CONTAINING BIOFILMS USING CHEMICAL CLEANING AND SANITIZING AGENTS UNDER STATIC CONDITIONS

(J. F. Frank, J. Ehlers, and L. Wicker)

Cleaning and sanitizing the food processing environment often involves the application of chemical agents in the form of foam or gel (viscous liquid or thin film) to avoid the use of high pressure sprays and hand scrubbing that can facilitate the spread of pathogenic bacteria. In addition, these chemical agents are often applied without application of heat to ambient or cold surfaces. The objective of this research was to evaluate the effectiveness of cleaning and sanitizing chemicals applied under static conditions without application of heat for the removal of *Listeria monocytogenes* biofilms coated with soil of poultry origin. Chemicals evaluated were alkali and neutral cleaning compounds, sodium hypochlorite, acidified sodium chlorite, peroxyacetic acid, peroxyacetic acid/octanoic acid mixture, and quaternary ammonium compound sanitizing agents. Biofilms were prepared by growing *L. monocytogenes* on stainless steel for 24 h at 25°C. The resulting biofilms were then coated with chicken serum albumin and rendered chicken fat. Chemical treatments were at 4°C or 25°C for 1 to 30 min. At 25°C, the alkali cleaning agent removed 99% of fat and 93% of protein after 30 min exposure. The neutral cleaning agent was equally effective at removing fat, but removed only 77% of protein. The alkali cleaning agent also effectively

removed *L. monocytogenes* biofilm coated with protein, decreasing cell numbers on the surface by over 7 log₁₀ after 10 min exposure. Acidified sodium chlorite and peracetic acid/octanoic acid mixture were the most effective sanitizers at killing *L. monocytogenes* biofilm coated with fat and protein, both achieving > 5 log₁₀ reduction after 1 min exposure at 25°C. A combination of 10 min cleaning with alkali and 30 min sanitizing with acidified sodium chlorite achieved > 7 log₁₀ reduction of *L. monocytogenes* to nearly undetectable levels (> 0.2 cfu/50 cm²) at 25°C. The combination of alkali cleaning (10 min) and use of either acidified sodium chlorite or peracetic acid/octanoic acid (10 min) were effective at inactivating the *L. monocytogenes* biofilm at 4°C, achieving > 6.0 and 5.3 log₁₀ reductions, respectively. This research has demonstrated that processing plant environmental surfaces can be effectively cleaned and sanitized using static application of chemicals on surfaces and ambient and cold temperatures.

HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES*-CONTAINING BIOFILMS (R. Chmielewski and J. F. Frank)

Recent outbreaks of *Listeria monocytogenes* have been associated with the consumption of contaminated processed meat products and refrigerated products and have raised concern that recontamination is occurring during or after processing. Possible sources of recontamination in food processing plants could be due to ineffective cleaning and redeposition of soil especially in stagnant areas such as joints and dead ends. The objective of this research was to develop a predictive model to determine the importance of time and temperature for predicting survival of *Listeria monocytogenes*, *Pseudomonas*, and *Listeria* -*Pseudomonas* mixed culture biofilms formed on stainless steel and buna-N rubber coupon surfaces.

Coupons were added to 10% TSB inoculated with 0.1% *Pseudomonas* spp M21, *L. monocytogenes*, or 1:4 *Pseudomonas* spp M21- *L. monocytogenes* mixed culture and incubated for 4 h at 25°C. After attachment, coupon surfaces were rinsed with phosphate buffer and transferred to 10% TSB and incubated for 48 h at 25°C. Duplicate coupons were tested for each heating time (1, 3, 5, or 15 min) and temperature (70, 72, 75, or 77°C). Heat treated samples were enumerated using the fraction negative enumeration method. Positive controls were vortexed with glass beads and enumerated using PCA and *Listeria* selective agar. The experiment was repeated six times.

Time was the predominant predictive factor for biofilm survival on stainless steel while both temperature and time contributed equally to predicting the survival of biofilm on buna-N rubber. Overall, *Pseudomonas* was more heat resistant than *Listeria* on stainless steel, probably due to its higher initial load. On rubber, *Listeria* in the mixed culture biofilm had the greatest probability of survival. *Pseudomonas* in biofilms on stainless steel has a 16% probability of survival after heat treatment of 77°C for 15 min and 0.04% on buna-N rubber. For *Listeria* in biofilms, the probability of survival is 7% on stainless steel and 0.094% on buna-N while in mixed culture biofilms, the probability of survival of *Listeria* was 0.3% on stainless steel and 0.4% on buna-N rubber.

EFFICACY OF CHLORINE AND A PEROXYACETIC ACID SANITIZER IN KILLING *LISTERIA MONOCYTOGENES* ON ICEBERG AND ROMAINE LETTUCE USING CONDITIONS SIMULATING THOSE USED IN A COMMERCIAL PROCESS (L. R. Beuchat, B. B. Adler, and M. M. Lang)

Several studies have described the efficacy of chlorine and other sanitizers in killing *Listeria monocytogenes* on inoculated, cut lettuce. Various ratios of weight of lettuce and volume of treatment solution, as well as different treatment times and temperatures, have been used in these studies, making comparisons across laboratories difficult. In some instances, treatment conditions did not mimic those used in commercial fresh-cut lettuce operations. A recent study, done in collaboration with a commercial fresh-cut iceberg lettuce processor in Australia, evaluated the effectiveness of chlorine and a mixture of hydrogen peroxide and peroxyacetic acid in killing *L. monocytogenes* on shredded lettuce. The ratio of lettuce weight to treatment solution volume was 1:19. Results of the experiment were used to demonstrate how the manufacturer could meet a food safety objective of < 100 cfu of *L. monocytogenes*/g of lettuce.

We undertook a study to determine the efficacy of chlorine (100 µg/ml) and a peroxyacetic acid sanitizer (Tsunami 100) (80 µg/ml) in killing *L. monocytogenes* inoculated at populations of 1 – 2, 2 – 3, and 4 – 5 log₁₀ cfu/g of iceberg lettuce pieces, shredded iceberg lettuce, and Romaine lettuce pieces. Treatment conditions simulated those used by a commercial fresh-cut lettuce processor. The ratio of lettuce:treatment solution was 1:100 (wt:vol), treatment temperature was 4°C, and total treatment time was 30 sec. Compared to washing with water,

treatment of iceberg lettuce pieces containing all levels of inoculum and shredded iceberg lettuce containing 2 – 3 or 4 – 5 log₁₀ cfu/g with chlorine or Tsunami resulted in significant reductions ($P \leq 0.05$) in populations of the pathogen. Populations recovered from Romaine lettuce pieces treated with chlorine or Tsunami were not significantly different than populations recovered from pieces washed with water, regardless of the inoculum level. Within lettuce type and inoculum level, in no instance was the number of *L. monocytogenes* recovered from lettuce treated with chlorine or Tsunami significantly different. The rate of decrease in free chlorine concentration in treatment solution as affected by the wt:vol ratio (1:100, 1:10, 2:10, and 4:10) of lettuce:solution was determined. The rate of reduction increased as the ratio decreased, with an overall order of magnitude of reduction being shredded iceberg lettuce > iceberg pieces > Romaine pieces. Highest reductions in free chlorine concentration in solutions used to treat shredded lettuce are attributed to the release of tissue juices, which increases the concentration of soluble organic materials available for reaction with chlorine.

FATE OF AEROSOLIZED *LISTERIA MONOCYTOGENES* IN A CLOSED BIOAEROSOL CHAMBER (Z. Yan, C. M. Lin, J. Kornacki, and M. Doyle)

The role of aerosols in transmission of *Listeria monocytogenes* in a ready-to-eat meat processing facility environment is uncertain. A 315-liter-enclosed Plexiglas bioaerosol chamber was designed to study the fate of aerosolized *L. monocytogenes* at various levels under selected moisture conditions. *L. monocytogenes* cells were grown in TSB broth, suspended in Butterfield's phosphate buffer (pH 7.2) to selected levels and released into the chamber through a commercial nebulizer. Saturated magnesium chloride and sodium chloride were used to adjust the chamber to 40 – 45% and 75 – 80% relative humidity, respectively. Aerosol particle sizes released from the nebulizer at both moisture conditions ranged from 0.2 μm – 1 μm . Aerosols and air were mixed thoroughly through an air pump.

The settling rate of aerosol-borne *L. monocytogenes* released into the chamber at 10⁸ cfu/liter air was monitored. TSA YE plates at the bottom of the chamber were opened for 15 min each at 0, 0.5, 1, 1.5, 2, 2.5, and 3 h after releasing *L. monocytogenes*. *L. monocytogenes* cells settled on TSA YE plates were undetected 3 h after releasing.

Settling of *L. monocytogenes* onto food at different concentrations under both RH conditions was studied. Ham slices were heat-treated for 5 min at 71°C, aseptically cut to fit into Petri dishes (100 mm x 15 mm) and placed into the chamber and the ham was exposed. Bacteria were released into the chamber at 30 – 40, 10² – 10³, 10⁵, and 10⁷ cfu/liter air at both RH conditions. Plates were covered at 5, 30, 60, 120, 180, and 240 min after release of aerosolized *L. monocytogenes*. Ham slices in plates were enriched in UVM broth, transferred to Fraser broth incubated appropriately, streaked onto MOX agar, and confirmed by an enzyme-linked fluorescent antibody assay. The incidence of positive ham slices/total ham slices was recorded. Similar patterns were obtained for both RH conditions. No *L. monocytogenes* contaminated ham slices were detected at 30 – 40 cfu/liter of air at both RH conditions.

Cell injury in the air was determined by recovering *L. monocytogenes* cells on TSA YE, MOX and TSA YE/MOX overlay technique. Nearly all cells were injured as evidenced by an absence of colonies on MOX but very high numbers of cells were recovered on the TSA YE and TSA YE/MOX overlay.

Preliminary results from this research revealed that *L. monocytogenes* may not be recovered 4 h after releasing into the bioaerosol chamber. Ham was not contaminated when exposed to levels close to what may occur under industrial conditions, although direct fallout of *L. monocytogenes* onto ham was observed at higher cell numbers of *L. monocytogenes*. Air and aerosols appear to be unlikely sources of direct measurable *L. monocytogenes* of product in meat processing facilities.

ISOLATION OF *LISTERIA MONOCYTOGENES* FROM LATINA-STYLE SOFT CHEESE (C.-M. Lin, L. Zhang, B. Swaminathan, and M. P. Doyle)

Continuous outbreaks of listeriosis occurred for several months in 2003 in Hispanic communities in Texas. The Texas Department of Health determined that most cases of listeriosis were associated with Latina-style soft cheese such as queso fresco. The contaminated cheese was either imported from Mexico or home-made. The Texas Department of Health collected and shipped two sets of cheese samples (total of 34 samples) to the UGA Center for Food Safety in July and August. The cultural methods described in the FDA Bacteriological Analytical Manual (BAM, 9th ed. 2001) and a PCR-based assay (BAX[®] system, DuPont) were used simultaneously to detect

L. monocytogenes in the cheese samples, and a three-tube MPN procedure was used to enumerate *L. monocytogenes* in the positive samples. Four samples were confirmed *L. monocytogenes*-positive. Isolates of *L. monocytogenes* from the positive samples were sent to the CDC for serotyping and PFGE analysis. One isolate had the same PFGE profile as patient isolates collected in Texas and Georgia and another isolate had the same pattern as an isolate from a patient associated with an outbreak in Los Angeles County, California. Distribution of *L. monocytogenes* in the cheese samples was not homogenous. All samples were analyzed twice. When a sample was confirmed as positive by one of the two analyses, the sample was analyzed again. Of the four positive samples, two were positive by both analyses, one was positive in only one of three analyses, and one was positive with the second and third analyses. *L. monocytogenes* counts ranged from < 0.3 to 149 MPN/g in the positive cheese samples.

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.

**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.

**SURFACE MATERIAL, TEMPERATURE AND SOIL EFFECTS ON THE SURVIVAL
OF SELECTED FOODBORNE PATHOGENS IN THE PRESENCE OF CONDENSATE
(J. Allan, J. L. Kornacki, and Z. Yan)**

Survival of foodborne pathogens in food processing facilities is affected by factors including surface materials, nutrients, moisture and temperatures. The effects of surface-type [stainless steel, Delrin[®] (DuPont) acetal resin, and fiberglass reinforced plastic wall paneling (FRP), and mortar surfaces], soil, and temperature on the survival of *Listeria monocytogenes*, *Salmonella* spp., and *Yersinia enterocolitica* were evaluated in the presence of condensation. Surface coupons soiled and non-soiled with sterile porcine serum were exposed to cell suspensions made from five-strain cocktails of each organism (10^7 cfu/ml) in Butterfield's phosphate buffer (BPB) and incubated for 2 h at 25°C allowing attachment of cells. Three surfaces, stainless steel, Delrin and FRP, were included in the same experiment. The coupons were rinsed to remove unattached cells, incubated at either 4°C or 10°C under condensate-forming conditions, and sampled at six time intervals over a 15-day period. For

enumeration, cells were removed from the coupons by vigorous shaking in 100 ml BPB with 3 g of glass beads and the BPB was plated on tryptic soy agar with 0.6 % yeast extract. The results showed that stainless steel did not support the survival of *Listeria* as well as acetal resin or FRP. Acetal resin and stainless steel were less supportive of *Salmonella* than FRP. All three surfaces supported the survival of *Yersinia* over the 15-day trial equally at 10°C. Temperature had little effect on survival of *Listeria* or *Salmonella* across all three surfaces. However, *Yersinia* displayed growth on FRP at 10°C, but death at 4°C. Serum had a protective effect on *L. monocytogenes* on all surfaces, but did not affect survival of *Salmonella* or *Yersinia* on stainless steel, acetal resin, or FRP.

Since mortar surface is very different from the three surfaces described above, it was tested separately. The method to enumerate bacterial cells on the mortar surface involved applying sonication to remove bacterial cells and determining the cfu/coupon at 9 to 10 sampling periods over a total of 120 h. In general, the mortar surface had a significant inhibitory effect against all the bacteria tested compared to the three surfaces described above because of alkaline pH (increased to pH 11 within 6 h) when submerged in BBP. *Listeria* and *Salmonella* survived better on mortar than *Yersinia* throughout the 120-h incubation period, partially due to the alkaline resistance of *L. monocytogenes* and *Salmonella* spp. Serum had a protective effect on the survival of all three organisms. Differences in temperature did not affect the survival of *Salmonella* or *Yersinia*, whereas populations of *L. monocytogenes* declined more rapidly at 10°C than at 4°C after 24 h.

VIRULENCE OF *LISTERIA MONOCYTOGENES* IN IMMUNOCOMPROMISED AND NORMAL MICE (N. Mytle, K. Takeuchi, M. P. Doyle, G. L. Anderson, and M. A. Smith)

Listeriosis is a significant foodborne pathogen with high rates of mortality principally in immunocompromised populations. Differences in the ability of *L. monocytogenes* strains to cause illness may in part be attributed to differences in virulence factors associated with different strains.

The objective of this study was to determine the virulence based on LD₅₀ and infectivity, of ten *L. monocytogenes* strains in immunocompromised mice and three *L. monocytogenes* strains in normal mice. Infectivity was determined by quantitating the colony-forming units (cfu's) of *L. monocytogenes* in the mouse liver and spleen.

For LD₅₀ studies, 10 pathogenic isolates (H9666, ScottA, H7550, G3982, 12375, 12443, Vacherin, F6854, H7776, and 101M) were used in immunocompromised mice and three pathogenic isolates (12443, G3982, and H9666) were used in normal mice. Normal and immunocompromised female ICR mice were intraperitoneally (i.p.) injected with test strains in 0.1 ml PBS at 10⁰-10⁹ cfu. For the determination of LD₅₀, five mice were injected with each *Listeria* strain and observed for 5 days. *Listeria* isolates that caused at least one death in 5 days were considered pathogenic. In addition pulsed-field gel electrophoresis (PFGE) patterns of isolates from tissues were compared with the original treatment strains to verify their stability during passage through the mouse. Data were analyzed statistically using analysis of variance in SAS statistical software.

As expected, the dose for the LD₅₀ at 3-days post treatment was higher than the dose for the LD₅₀ at 5-days for all strains. In immunocompromised mice, the most virulent strain of *L. monocytogenes* as determined by the LD₅₀ at both 3-days and 5-days post-exposure was G3982. In normal (not immunocompromised) mice, strain 12443 was the most virulent based on the 5-day LD₅₀ determinations. However, there was statistically no significant difference (P < 0.05) between 12443, G3982 and H9666 strains for the 3-day LD₅₀ dose in normal mice, indicating that they are all about equally virulent. When comparing normal and immunocompromised mice, there was no significant difference (P < 0.05) in strain 12443 for the 3- or 5-day LD₅₀'s. However, there was a significant difference (P < 0.05) for immunocompromised and normal mice for strains G3982 and H9666 for LD₅₀'s at both 3-days and 5-days. The 5-day LD₅₀ of G3982 for immunocompromised mice was 3.00 x10² cfu and 1.45 x10⁶ cfu for normal mice. Similarly, for H9666 strain, the LD₅₀ at 5-days was 5.37 x10² for immunocompromised mice and 2.06 x10⁶ cfu for normal mice.

With all three strains of *L. monocytogenes* the viable counts recovered from liver of infected normal mice were about the same as those, obtained from the spleen. Selected colonies from each treated group of mice were tested by PFGE analysis. PFGE patterns were indistinguishable from the treatment strains in all cases.

Conclusions: For ten *L. monocytogenes* strains tested in immunocompromised mice, G3982 was the most virulent based on a 5-day LD₅₀ assay. Three strains (G3982, H9666, and 12443) were tested in both immunocompromised and normal mice. When comparing results from normal and immunocompromised mice, there was no significant difference (P < 0.05) in 3- day or 5-day LD₅₀ determinations, for strain 12443, but there was a significant difference (P < 0.05) for strains G3982 and H9666. In this study we identified three strains that

appear to be more virulent than the others tested. Further characterization of genetic differences and virulence factors of strains will aid in understanding the factors affecting the variation in virulence among strains.

**INFLUENCE OF VARIATIONS IN METHODOLOGY ON POPULATIONS OF *LISTERIA MONOCYTOGENES*
RECOVERED FROM LETTUCE TREATED WITH SANITIZERS**

(A. B. Burnett, M. H. Iturriaga, E. F. Escartin, C. A. Pettigrew, and L. R. Beuchat)

The lack of a standard method(s) to quantitate pathogens on raw fruits and vegetables has resulted in great variations in methodology used by researchers and in commercial testing laboratories. While some of these variations may not affect the efficiency of enumerating pathogens on a given produce item, most have not been properly evaluated or validated. The importance of optimum procedures for recovering pathogens that may be stressed or injured as a result of desiccation or exposure to chemical sanitizers, for example, is increased when the efficacy of decontamination treatments is being assessed. If the enumeration method does not recover all viable cells from treated produce, an underestimation of populations will result.

Progress is being made in developing and validating a standard method to evaluate the effectiveness of produce sanitizers, although modifications of a basic method will likely be necessary to achieve maximum recovery of pathogens surviving treatment. Major variations in sanitizer efficacy methodology currently used by researchers include inoculation of produce by dipping in a cell suspension versus spot inoculation of the test pathogen, drying inoculum for times ranging from a few seconds to 24 h, failure to use a neutralizing agent to terminate the activity of the lethal component, and rinsing, stomaching, or blending to process treated samples for enrichment or direct plating. In only a few studies have comparisons of variations in specific steps in produce sanitizer efficacy methods been made. Survival of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on tomatoes, as affected by drying time after application of inoculum, and subsequent effectiveness of point-of-use sanitizers in reducing populations have been investigated. We have examined sample processing methods for their influence on populations of *Salmonella* recovered from twenty-six types of raw fruits, vegetables, and herbs. Overall, processing samples by washing in 0.1% peptone, stomaching, or homogenizing did not substantially affect the number of *Salmonella* recovered.

The study reported here was done to determine if variations in methodology influence the efficacy of chlorinated (200 µg/ml free chlorine) water and FIT® Professional Line Produce Cleaner (FIT®) in killing *L. monocytogenes* spot inoculated onto iceberg lettuce. Variations in methodology included composition of broth media used to grow cells to prepare inocula, number of strains present in the inoculum, time and temperature between inoculation and treatment with sanitizers, sample processing method, and composition of direct plating media used to enumerate the pathogen. The efficacy of the two sanitizers was not influenced by the composition of the medium used to culture *L. monocytogenes* used in inocula, the number of strains in the inoculum, or the recovery medium used to enumerate the pathogen on lettuce after treatment. Drying inoculum on lettuce for 45 min at 37°C caused more cells to die or not be retrieved compared to drying inoculum for 30 min at 25°C. However, the percentage of cells in the inoculum recovered from lettuce treated with chlorine or FIT® was not significantly different, regardless of the drying method. Stomaching, homogenizing, or stomaching followed by homogenizing lettuce treated with sanitizers resulted in recovery of similar numbers of *L. monocytogenes*, indicating that stomaching and homogenizing are equivalent in extracting cells; the sequential use of both processing methods did not substantially increase the efficiency of recovery. Washing lettuce with water or treating lettuce with 200 µg/ml chlorine or FIT® resulted in decreases in populations of 0.60, 1.76, and 1.51 log₁₀ cfu/lettuce, respectively, regardless of variations in test parameters. Reductions caused by sanitizers were significantly greater ($\alpha = 0.05$) than that observed for water but not significantly different from each other. It is concluded that evaluation of sanitizers for their efficacy in killing *L. monocytogenes* on lettuce can be determined by spot inoculating 50 µl of a five-strain mixture of cells from 24-h cultures suspended in 5% horse serum albumen, followed by drying the inoculum for 45 min at 37°C, treatment by submerging in 50 ml of sanitizer for 5 min, stomaching samples in 50 ml of Dey-Engley neutralizing broth for 2 min, and enumerating survivors on modified Oxford medium.

**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.

**COMPARISON OF USDA CULTURE PROCEDURE AND POLYMERASE CHAIN REACTION (PCR)
DETECTION OF *LISTERIA MONOCYTOGENES* ON DELI MEATS
(C.-M. Lin, L. Zhang, and M. P. Doyle)**

A polymerase chain reaction (PCR) assay (BAX[®], DuPont, Wilmington, DE) for detecting *Listeria monocytogenes* in meat products has been adopted by the US Department of Agriculture (USDA). In addition, USDA-FSIS has promoted using a large sampling size such as an entire package for detecting *L. monocytogenes* in meat products. This study was conducted to compare the BAX-PCR assay and the USDA culture enrichment method to detect *L. monocytogenes* on deli meats contaminated during slicing by a slicer blade inoculated with listeriae. Two types of deli meat, salami and bologna, were tested. The salami had a low pH and moisture content and the bologna contained antimicrobials to *L. monocytogenes*, hence neither meat would support the growth of listeriae. A five-strain (Bil Mar, Coleslaw, Plantation, Jalisco, OM) mixture of *L. monocytogenes* at 10² cfu was inoculated onto the surface of the blade of a commercial slicer. A commercial log of meat product was sliced in its entirety and five slices per package were packed, vacuum-sealed, stored at 4°C, then sampled at 1, 30, 60, and 90 days post-slicing. Meat sliced before the blade was inoculated with *L. monocytogenes* was used as the negative control. Meat from eight negative-control packages was inoculated with 10¹ or 10² cfu of *L. monocytogenes* to serve as positive controls. Studies with each type of meat were done in duplicate. A total of 132 samples were analyzed at each sampling date. The entire package of deli meat was stomached with UVM broth and incubated at 30°C for 24 h. For the USDA culture enrichment procedure, 0.1 ml of UVM enrichment culture was transferred into Fraser broth then incubated and streaked onto modified Oxford agar (MOX) plates, and a loopful of the enrichment culture was streaked directly onto MOX plates. Presumptive isolates of *L. monocytogenes* obtained from either culturing in Fraser broth or by direct plating onto MOX were confirmed by hemolysis on horse blood agar plates, then biochemical assay with API strips (bioMérieux Inc., Hazelwood, MO) and BAX-PCR. For the BAX-PCR assay, 0.1 ml of the UVM-enrichment culture was transferred into BAX-*Listeria* enrichment broth. PCR-positive samples were further assayed by streaking the BAX-*Listeria* broth onto MOX and horse blood agar plates, then isolates were confirmed with API strips. A three-tube most probable number (MPN) method was used to enumerate *L. monocytogenes* populations in positive samples. There were very few *Listeria*-positive samples. For the two trials for each type of meat, at most two meat packages were confirmed *L. monocytogenes*-positive on the first sampling day. The number of *Listeria*-positive samples declined during storage. No sample was confirmed *Listeria*-positive at 30 and 60 days for either salami or bologna; however, one 90-day sample of salami in the second trial was confirmed *L. monocytogenes*-positive. None of the negative controls was positive and *L. monocytogenes* in the positive controls inoculated at the 10¹ cfu level could not be recovered at 30 and 60 days on either salami or bologna. Based on MPN, *L. monocytogenes* populations were very low (from < 0.06 to 0.75 MPN/g) and decreased gradually during storage, even in positive control samples. The number of *L. monocytogenes*-positive samples determined by PCR or culture enrichment was similar. Most of the *Listeria*-positive samples were detected by both PCR and culture enrichment; however, some samples were determined *Listeria*-positive by PCR but not by culture enrichment and vice versa. Our results indicate that the ability of the BAX-PCR assay and the USDA culture enrichment to detect small cell numbers of *L. monocytogenes* on salami and bologna was similar, and that neither bologna with antimicrobial agents nor salami supported the growth of *L. monocytogenes* throughout 90 days of storage at 4°C.

A SOLID AGAR OVERLAY METHOD FOR RECOVERY OF HEAT-INJURED *Listeria monocytogenes*
(Z. Yan, J. Gurtler, and J. L. Kornacki)

A solid agar overlay method was developed for recovery of heat-injured *Listeria monocytogenes*. A pre-solidified non-selective medium, tryptic soy agar with 0.6% yeast extract (TSAYE, 2% agar), was aseptically overlaid onto the top of a solidified selective medium; modified oxford agar (MOX). In principle, injured bacterial cells are resuscitated on the TSAYE overlay before diffusion of selective agents from MOX can inhibit their recovery. A five-strain cocktail of *L. monocytogenes* was heat injured by subjecting the cells to 58°C for 6 min in a water-jacketed flask filled with TSB broth. Both freshly grown and heat-treated cells of *L. monocytogenes* were plated onto TSAYE, MOX and TSAYE/MOX overlaid plates. No significant differences ($P < 0.05$) were found among the three media for recovery of freshly grown (e.g., uninjured) bacterial cells. Selective medium MOX recovered significantly ($P < 0.05$) less *L. monocytogenes* cells than on non-selective medium TSAYE and the TSAYE/MOX overlaid plates. In contrast, there were no significant differences among the TSAYE and TSAYE/MOX overlaid agar plates prepared 0, 2, 4, 6, 8, 16, and 24 h prior to plating heat-injured bacterial cells. TSAYE/MOX overlaid agar was able to differentiate *L. monocytogenes* from a mixture of three additional foodborne pathogens; *Salmonella* spp, *E. coli* O157:H7 and *Yersinia enterocolitica*. This solid agar overlay method for recovery of heat-injured *L. monocytogenes* cells is less time-consuming and less complicated than the conventional overlay/underlay technique and reported thin agar layer overlay methods.

