LISTERIA MONOCYTOGENES

GREEN FLUORESCENT PROTEIN LABELING OF *LISTERIA* AND *SALMONELLA* FOR FOOD SAFETY-RELATED STUDIES

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

Many studies with foodborne bacterial pathogens require tracking of the introduced bacterial strain in order to monitor its fate in complex environments. With an easily detectable phenotype, the green fluorescent protein (GFP) gene (*gfp)*, has been used to label many microorganisms for localization and gene expression studies. The objectives of this study were to label *Listeria monocytogenes, Listeria innocua* and *Salmonella* strains with GFP and characterize the *gfp*-labeled strains in terms of stability of label and the effect of the label on bacterial growth, two important characteristics pertinent to their intended application.

Plasmids containing the *gfp* gene were introduced into *Salmonella* and *Listeria* strains by conjugation and electroporation. Expression of GFP in labeled strains was determined by epifluorescence microscopy of colonies. Stability of the label was investigated through sequential propagations of labeled strains in the absence of antibiotic selection, and rates of plasmid-loss were calculated. Growth curves were determined comparing the parent strain with its corresponding labeled derivatives to determine the effect of *gfp*-labeling on bacterial growth. *Salmonella* strains were labeled by the calcium chloride method. *gfp*-labeling of *Listeria* by conjugation was easily performed but not universally effective for all strains, whereas electroporation was an effective method for labeling all *Listeria* strains. Plasmid stability varied among the labeled strains. When grown in non-selective media for two consecutive subcultures (ca. 40 generations), the rates of plasmid-loss among labeled *Salmonella* and *Listeria* strains ranged from 15.8%-99.9% and 8.1% -93.4%, respectively. Complete loss (>99.9%) of the plasmid was observed in some labeled strains when grown for five consecutive subcultures in the absence of selective pressure, whereas it remained stable in other cultures. Maintaining the *gfp*-plasmid had an insignificant effect on growth of most labeled strains. In conclusion, *Salmonella* and *Listeria* strains can be effectively labelled with the plasmid-borne *gfp* gene and in several isolates will be stable for many generations without adversely affecting growth rates.

MECHANISMS OF *LISTERIA MONOCYTOGENES* INDUCED-STILLBIRTHS IN PREGNANT GUINEA PIGS

(E. A. Irvin, D. Williams, S. Lambert, A. Richardson, and M. A. Smith)

Listeria monocytogenes has been identified as the causative agent in spontaneous abortions and stillbirths in humans after consumption of contaminated foods. Approximately 2500 cases occur each year in the U.S., and pregnant women are 20 times more likely to develop listeriosis than the general population. Previous studies indicate that pregnant guinea pigs are an appropriate model for human listeriosis. We hypothesize that pregnant guinea pigs orally exposed to *L. monocytogenes* have increased apoptosis and changes in cytokines in the placenta and that these are correlated with placental infection and stillbirth. Pregnant guinea pigs were treated orally on gestation day (gd) 35 with 10^5, 10^6, and 10^7 colony forming units (CFU) *L. monocytogenes* in whipping cream. Pregnancies were allowed to proceed normally until sacrifice on gd 56. Tissues were collected from mothers and fetuses for cytokine and apoptosis analyses. Tumor Growth Factor-β1 (TGF-β1) was analyzed in maternal serum using a human TGF-β1 ELISA kit from R & D Systems. The presence of apoptosis in placentas was analyzed by PCR with an apoptosis-specific kit (PCR Kit For DNA Ladder Assay) from Maxim Biotech, Inc. Fetal lengths and weights were not affected by *L. monocytogenes* infection. Stillbirths occurred after treatment with *L. monocytogenes* at 10^6 and 10^7 CFU. Average fetal lengths were 8.2, 7.4, 7.9, 7.6 cm and average fetal weights were 55.8, 59.9, 58.6, 48.2 g for control, 10^5, 10^6, and 10^7 CFU, respectively. TGF-β1 concentrations were not significantly different in maternal serum regardless of *L. monocytogenes* infection with 10.1 ng/mL, 11.3 ng/mL, 9.86 ng/mL, and 11.3 ng/mL for control, 10^5, 10^6, 10^7 CFU,

respectively. Placentas positive for apoptosis showed a dose-dependent trend with 51% of placentas from control animals showing evidence of apoptosis based on PCR. Treated animals had 57%, 73%, and 87% of placentas positive for apoptosis after treatment with 10^5, 10^6, and 10^7 CFU *L. monocytogenes*. TGF-β1 was not changed with infection of *L. monocytogenes*. Increases in apoptosis in placentas after treatment with *L. monocytogenes* suggests infection-related changes occur in the placenta and this may impact fetal viability.

EVALUATION OF *LISTERIA INNOCUA* AS A SURROGATE FOR *LISTERIA MONOCYTOGENES* IN AEROSOL STUDIES (G. Zhang, L. Ma, and M. P. Doyle)

Airborne contamination of *Listeria monocytogenes* may be a contributing factor in disseminating *L. monocytogenes* in food processing plants. However, aerosol studies in such facilities have been limited by lack of a suitable surrogate microorganism for *L. monocytogenes*. The objective of the study was to investigate the potential of using *Listeria innocua* as a surrogate for *L. monocytogenes* in an aerosol study.

Five strains of *L. innocua* and 5 strains of *L. monocytogenes* labeled with jellyfish green florescent protein genes were used. The study was carried out at room temperature in a bioaerosol chamber (124 x 51 x 51 cm). *Listeria* cells were released into the chamber by a nebulizer at 10^5 and 10^3 CFU/L air. Two air flow conditions were used: no fan blowing after plates were opened, and continuous fan blowing throughout the entire 3-h experiment. Trypticase™ soy agar (TSA) plates, Oven Roasted Breast of Chicken, and Oven Roasted Breast of Turkey in Petri plates were placed in the chamber to monitor *Listeria* cell number changes in the aerosol. Plates with TSA, turkey meat or chicken meat were exposed for 30 minutes to aerosol. Every 30 minutes new plates were exposed. A single trial lasted 3 hours. TSA plates were incubated at 37°C for 24 h. Turkey and chicken breast meat were stomached and enriched overnight in University of Vermont broth at 30°C, and then streaked onto modified Oxford plates and incubated at 35°C for 24 h. GFP-labeled *Listeria* colonies on all plates were counted with Leica X-Cite® 120 Fluorescence Illumination System. Experiments were repeated in triplicate.

Results revealed that *L. monocytogenes and L. innocua* survived as well on chicken and turkey breast meat as on TSA plates. When only one *Listeria* colony was detected on TSA plates, turkey and chicken breast meat plates were usually *Listeria*-positive also. When there was no detectable *Listeria* on TSA plates, turkey and chicken breast meat plates were usually *Listeria*-negative. In two cases, the chicken breast meat purchased for this study was contaminated with *Listeria;* however, by counting the fluorescent colonies only, we were still able to obtain reliable data. Therefore, GFP-labeled bacteria enabled the enumeration of *Listeria* in the presence of environmental contaminants. During the three hours of experiments, air flow with or without fan activity did not have a significant effect on settling rates of aerosolized *L. monocytogenes* or *L. innocua*. Settling rates of aerosolized *L. monocytogenes and L. innocua* were similar under both air flow conditions and as detected by all three media used. These results indicate that *L. innocua* could be used as a surrogate for *L. monocytogenes* in an aerosol study.

EFFECTS OF BILE SALTS ON GROWTH OF *LISTERIA MONOCYTOGENES* (G. Anderson, S. Lambert, and M.A. Smith)

Pregnant women are susceptible to *Listeria* infection, and stillbirths can occur especially upon exposure during the third trimester. Studies in the Rhesus monkey suggest that persistence in the host GI tract may be an important determinant of subsequent systemic infection and stillbirths. The current study characterizes the effects of bile salts on growth of strains of *Listeria monocytogenes* and compares bile salt tolerance in these strains to that of *Listeria innocua*. All *Listeria spp.* grew vigorously in the presence of bile salts. Specific growth rates in the presence of bile salts, either 2 or 4%, were not different ($p<.05$) from control growth rates which ranged between 0.95/h and 1.06/h. There were no significant differences among strains in specific growth rates in the absence or presence of bile salt. In contrast, lag times

exhibited both dose and strain dependence. In 2% bile salt, lag time for *L. monocytogenes* strains 12443 and H9666 were 0.29 ± 0.12 h and 0.39 ± 0.18 h, respectively. In contrast, the lag time for *L. innocua* was significantly longer (0.80 ± 0.13 h). The same pattern of responses occurred for growth in 4% bile salt. Lag differences resulted in strain dependent differences in net growth during 3.5 h culture in the presence of bile salts. Populations of *L. innocua* increased by 12.1 fold in 2% bile salts compared to 17.6 and 16.1 fold for *L. monocytogenes* 12443 and H9666, respectively. We conclude that pathogenic *Listeria* was less sensitive to bile salts than non-pathogenic *Listeria*.

DEATH OF *SALMONELLA, ESCHERICHIA COLI* O157:H7, AND *LISTERIA MONOCYTOGENES* IN SHELF-STABLE, DAIRY-BASED, POURABLE SALAD DRESSINGS

(L. R. Beuchat, J.-H. Ryu, B. B. Adler, and M. D. Harrison)

Commercial sterilization of salad dressings by treatment at high temperatures is not an option for eliminating microorganisms because it would destroy the physical integrity and result in products with substantially different sensory qualities. Commercial processing and preservation of salad dressings instead depends on a combination of intrinsic factors, and possibly mild heat treatments, to reduce, control, or eliminate microorganisms. Commercial salad dressings are also manufactured under strict quality controls, as manufacturers adhere to good manufacturing practices. Storage temperature can affect the physical stability and sensory quality of salad dressings, as well as the rate of growth of spoilage microorganisms. The lethality of the harsh environment imposed by intrinsic factors characteristic of salad dressings to foodborne pathogens that may become contaminants during postprocess handling would be anticipated to act synergistically or additively with non-refrigerated temperatures to cause death of these pathogens at a more rapid rate. The amounts and types of pourable salad dressings available for purchase in large containers for use in food service and home settings have increased in recent years. This presents an increased possibility of postprocess contamination, e.g., at salad bars where portions are removed from the same container by several different people over an extended period of time. The behavior of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* that may contaminate salad dressings at some point after opening containers in foodservice or home settings has not been critically evaluated.

The objectives of this study were to determine the death rates of *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* in three commercially manufactured full-fat ranch salad dressings, three reduced-fat ranch salad dressings, two full-fat blue cheese salad dressings, and two reduced-fat blue cheese salad dressings and to affirm the expectation that these dressings do not support the growth of these pathogens. The respective initial pH values of the four types of shelf-stable, dairy-based, pourable dressings were 2.87 - 3.72, 2.82 - 3.19, 3.08 - 3.87, and 2.83 - 3.49. Dressings were inoculated with low (2.4 - 2.5 $\log_{10}$ CFU/g) and high (5.3 - 5.9 $\log_{10}$ CFU/g) populations of separate five-strain mixtures of each pathogen and stored at 25°C for up to 15 days. Regardless of the initial inoculum population, all test pathogens rapidly died in all salad dressings. *Salmonella* was undetectable by enrichment (<1 CFU/25-ml sample in three replicate trials) in all salad dressings within 1 day, and *E. coli* O157:H7 and *L. monocytogenes* were reduced to undetectable levels by enrichment between 1 and 8 days and 2 and 8 days, respectively. *E. coli* O157:H7 was not detected in four of the ten salad dressings stored for 2 or more days and nine of the ten dressings stored for 6 or more days after inoculation. *L. monocytogenes* was detected in nine of the ten salad dressings stored for 3 days but in only one dressing, by enrichment, at 6 days, indicating that it had the highest tolerance among the three pathogens to the acidic environment imposed by the dressings. Overall, the type of dressing (i.e., ranch vs. blue cheese) and level of fat in the dressings did not have a marked affect on the rate of inactivation of pathogens. Total counts and populations of lactic acid bacteria and yeasts and molds remained low or undetectable (< 1.0 $\log_{10}$ CFU/ml) throughout the 15-day storage period. Based on these observations, shelf-stable, dairy-based, pourable ranch and blue cheese salad dressings manufactured by three companies and stored at 25°C do not support the growth of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* and should not be

considered as potentially hazardous foods (time/temperature control for safety foods) as defined by the U.S. Food and Drug Administration Food Code.

Application of Competitive Exclusion Bacteria for Control of *Listeria* in Floor Drains in a Ready-to-Eat Poultry Processing Plant

(T. Zhao, M. P. Doyle, T. C. Podtburg, P. Zhao, D. A. Baker, and B. Cords)

Controlling the widely occurring *Listeria monocytogenes* in food processing facilities has been a formidable challenge for the entire food industry. Floor drains in particular are an important harborage for *Listeria*. Drains are difficult to clean because listeriae can become entrapped on drain surfaces in a slimy, protective covering known as biofilms.

We have obtained from floor drains some bacteria, including *Lactococcus lactis* subsp. *lactis* (#C-1-92) and *Enterococcus durans* (#152) (competitive exclusion bacteria; CE), that are inhibitory to the growth of *L. monocytogenes* in biofilms at 4 to 37°C. In a previous fresh poultry plant trial, we combined these two isolates as a treatment in floor drains to determine their effect in reducing *Listeria* in drains that were located in rooms at different temperatures. Results indicated that these two CE can greatly reduce *Listeria* numbers in floor drains at 2 to 30°C.

With the collaboration of two industry partners (Gold Kist and Ecolab), a ready-to-eat processing plant was selected for further study to verify the usefulness of this CE treatment to reduce/eliminate *Listeria* in floor drains. Seventeen floor drains in four different locations within the plant were selected for initial screening, and four sites were sampled in each drain. These included: #1, inside surface of the drain's cover; #2, outer surface of the drain basket; #3, sides at top of the drain; and #4, sides at ca. 5 inches within the drain.

Each floor drain was sampled three times before CE treatment to determine which drains were consistently *Listeria*-positive. Results revealed that seven were positive at all three samplings and two were positive at two samplings. *Listeria* counts in all positive floor drains were low, with a maximum of 100 *Listeria*/cm^2 and most were *Listeria*-positive only by selective enrichment culture (<50 *Listeria*/cm^2).

Six of seven floor drains (one in a construction area) that were consistently *Listeria*-positive were selected for CE treatment and the two drains that were *Listeria*-positive 2 out of the 3 samplings were used as the controls (no CE treatment). The CE preparation included 25 ml of two bacteria, *L. lactis* subsp. *lactis* (#C-1-92) and *E. durans* (#152), at ca. 10^9 CFU/ml, 20 ml of Dy-gest , 20 ml of Dy-gest II, and 1 gallon of water. CE treatment was applied as a foam a total of 10 times, with the first, second, third and fourth treatments introduced daily during the first week, then twice a week during the following three weeks.

Results revealed that the CE treatment substantially reduced or eliminated *Listeria* from all of the CE-treated floor drains, but not the untreated control drains (Table 1). The CE treatment appears to effectively control *Listeria* in most drains (5 of 6) for up to 8 weeks following the last CE application. Results suggest the CE treatment should be applied to drains every 2 months for optimal *Listeria* control.

Surrogate Bacteria for In-Plant Critical Control Point Validation of Thermal Inactivation of *Listeria monocytogenes*

(L. Ma, J. L. Kornacki, and M. P. Doyle)

Heat treatment of meat and poultry is among the most common of processing techniques to assure their microbiological safety and is considered a critical control point in the Hazard Analysis Critical Control Point (HACCP) system. However, it is not feasible in processing plants to validate thermal processes on a periodic basis using pathogenic bacteria. Hence, a suitable non-pathogenic (surrogate) microorganism is needed for process validation and verification. The goal of this research was to validate the relationship of thermal destruction of the surrogate non-pathogenic *Enterococcus* sp. B2354 (formerly

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known as *Pediococcus* sp. NRRL B-2354 and *Micrococcus freudenteihii*) to that of pathogens of concern in meat products (*Listeria monocytogenes* and *Salmonella*).

Several trials were done to determine the heat resistance of *Enterococcus* sp. B2354, *L. monocytogenes* 101M, and *S.* Senftenberg 775W at four temperatures (58, 62, 65, and 68°C) in ground beef of 4% (lean) and 12% (normal) fat content. In lean ground beef, *L. monocytogenes* was more sensitive to thermal inactivation at 58 and 62°C than *S.* Senftenberg, but slightly more resistant at temperature above 62°C. However, in normal ground beef, *L. monocytogenes* was consistently more heat sensitive than *S.* Senftenberg at all four temperatures tested. Higher fat content protects bacteria from thermal inactivation, especially at temperatures lower than 68°C. D-values for *Enterococcus* sp. B2354 in lean and normal ground beef were 4.5 to 18 and 3.6 to 15 times greater, respectively, than those for the most resistant pathogenic microorganisms (*L. monocytogenes* or *S.* Senftenberg 775W) at all temperatures tested, with the greatest difference in D-values occurring at 58° and 62°C. These results indicate that thermal treatments of ground beef at 58° to 68°C that kill *Enterococcus* sp. B2354 will also kill *Salmonella* and *L. monocytogenes*. Hence, depending on the margin of safety desired, processors could use this strain of *Enterococcus* sp. B2354 as a surrogate for validation studies of thermal processes in lean and normal ground beef at 58° to 68°C.

In search for a less heat resistant surrogate than *Enterococcus* sp. B2354, the heat resistance of two other potential surrogate microorganisms, *Pediococcus parvulus* HP and *Pediococcus acidilactici* LP, isolated from a commercial meat starter culture, was compared with the three strains under study (*L. monocytogenes* 101M, *S.* Senftenberg 775W, and *Enterococcus* sp. B2354) in broth at 62°C. D-values of *P. parvulus* HP and *P. acidilactici* LP were lower than those of *Enterococcus* sp. B2354 but 4.1 and 2.5 times greater, respectively, than those of the most resistant pathogen (*S.* Senftenberg 775W). Therefore, these two *Pediococcus* strains may serve as alternate surrogates for validation studies when a less heat resistant surrogate is desired; however, studies at additional temperatures are needed with these strains for validation of the entire range of 58° to 68°C.

EVALUATION OF GASEOUS CHLORINE DIOXIDE AS A SANITIZER FOR KILLING *SALMONELLA, ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES,* AND YEASTS AND MOLDS ON FRESH AND FRESH-CUT PRODUCE

(K. V. Sy, M. B. Murray, M. D. Harrison, and L. R. Beuchat)

Treatment of fruits and vegetables with sanitizers often results in reductions in populations of pathogens not exceeding 2 to 3 log_{10} CFU/g and cannot be relied upon to eliminate safety risks. The lack of effectiveness of sanitizers for killing high numbers of pathogens on produce can be attributed in part to difficulties in delivering aqueous chemical sanitizers to surface or subsurface areas where pathogens may be lodged. Treatment with aqueous chemical solutions can result in residual moisture on the surface of fruits and vegetables, which can promote the growth of yeasts and molds, thus reducing fresh-market shelf life. Growth of molds can in turn increase the pH of produce tissues and enhance the growth of infectious toxigenic foodborne pathogens thereby increasing safety risks.

We undertook a study to evaluate ClO_2 gas for its effectiveness in killing *Salmonella enterica, E. coli* O157:H7 and *L. monocytogenes* inoculated onto the surfaces of fresh-cut cabbage, carrot, and lettuce and its effectiveness in killing *Salmonella*, yeasts, and molds on the surfaces of fresh apples, tomatoes, onions, and peaches. Inoculum (100 µl, ca. 6.8 log_{10} CFU) containing five serotypes of *Salmonella enterica*, five strains of *E. coli* O157:H7, or five strains of *L. monocytogenes* was deposited on the skin and cut surfaces of fresh-cut vegetables, dried for 30 min at 22°C, held for 20 h at 4°C, and then incubated for 30 min at 22°C before treatment. The skin surfaces of apples, peaches, tomatoes, and onions were inoculated with 100 µl of a cell suspension (ca. 8.0 log_{10} CFU) containing five serotypes of *Salmonella*, and inoculated produce was allowed to dry for 20 to 22 h at 22°C before treatment. Treatment with ClO_2 at 4.1 mg/liter significantly ($\alpha = 0.05$) reduced the population of foodborne pathogens on all produce. Reductions resulting from this treatment were 3.13 to 4.42 log_{10} CFU/g for fresh-cut cabbage, 5.15 to 5.88 log_{10} CFU/g for fresh-cut carrots, 1.53 to 1.58 log_{10} CFU/g for fresh-cut lettuce, 4.21 log_{10} CFU per apple, 4.33 log_{10} CFU per tomato, 1.94 log_{10} CFU per onion, and 3.23 log_{10} CFU per peach. The highest reductions in yeast and mold populations resulting from the same treatment were 1.68 log_{10} CFU per apple and 2.65 log_{10} CFU per peach. Populations of yeasts and molds on tomatoes and onions were not significantly reduced by treatment with

4.1 mg/liter ClO_2. Substantial reductions in populations of pathogens on apples, tomatoes, and onions but not peaches or fresh-cut cabbage, carrot, and lettuce were achieved by treatment with gaseous ClO_2 without markedly adverse effects on sensory qualities.

THERMAL TOLERANCE OF ACID-ADAPTED AND UNADAPTED *SALMONELLA, ESCHERICHIA COLI* O157:H7, AND *LISTERIA MONOCYTOGENES* IN CANTALOUPE JUICE AND WATERMELON JUICE

(M. Sharma, B. B. Adler, M. D. Harrison, and L. R. Beuchat)

Outbreaks of foodborne infections associated with the consumption of fresh fruits and vegetables as well as unpasteurized juices contaminated with pathogenic bacteria have been documented. Outbreaks of salmonellosis and *Escherichia coli* O157:H7 infections have been linked to the consumption of cantaloupes. Watermelons have been implicated in outbreaks of salmonellosis and shigellosis. Pathogens known to be contaminants on the surface of melon rinds can be translocated to the edible tissues and juices when melons are cut to prepare for consumption. *Salmonella* can rapidly grow on sliced cantaloupe, watermelon, and honeydew melon, and in cantaloupe juice and watermelon juice. *Escherichia coli* O157:H7 has been reported to grow on cantaloupe and watermelon cubes and *Listeria monocytogenes* can grow in cantaloupe and watermelon pulp. The U.S. Food and Drug Administration has implemented a HACCP program that focuses on minimizing microbiological safety risks that may be associated with fruit and vegetable juices. One of the interventions to eliminate foodborne pathogens is heat treatment. The use of melon juice in blends of non-pasteurized and pasteurized fruit juices offered for sale to the consumer has increased in recent years. To date, research efforts on the microbiological safety of pasteurization processes for fruit juices have concentrated largely on determining *D* values (decimal reduction times) for *Salmonella, E. coli* O157:H7, and *L. monocytogenes* in apple juice. We undertook a study to determine the *D* values of these pathogens in cantaloupe juice and watermelon juice as affected by acid adaptation preceding exposure to heat.

Salmonella enterica serotype Poona, *Salmonella enterica* serotype Saphra, two strains of *E. coli* O157:H7, and two strains of *L. monocytogenes* were grown in tryptic soy broth (TSB) and TSB supplemented with 1% glucose for 24 h at 37°C. Decimal reduction times (*D* values) of cells suspended in unpasteurized cantaloupe juice and watermelon juice were determined. Acid-adapted cells of *Salmonella* and *E. coli* O157:H7, but not *L. monocytogenes*, had increased thermal tolerance compared to cells that were not acid-adapted. There was no correlation between soluble solids content of the two types of juice and thermal resistance. Growth of *Salmonella* and *E. coli* O157:H7 in cantaloupe juice, watermelon juice, or other acidic milieu, either in preharvest or postharvest environments, may result in cross protection to heat. The pasteurization conditions necessary to achieve elimination of pathogens from these juices would consequently have to be more severe if cells are habituated to acidic environments. Insights from this study provide guidance to developing pasteurization processes to eliminate *Salmonella, E. coli* O157:H7, and *L. monocytogenes* in cantaloupe juice and watermelon juice.