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Influence of sodium pyrophosphate, sodium chloride, or combinations on the thermal inactivation of Listeria monocytogenes in pork slurry or ground pork

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Influence of sodium pyrophosphate, sodium chloride, or combinations on thermal inactivation of *Listeria monocytogenes* in pork slurry or ground pork

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To Noah

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ABSTRACT

The thermal inactivation (55-62.5° C) of *Listeria monocytogenes* (mixture of Scott A, V7, and ATCC 19116) in pork slurry and ground pork that contained 0, 0.25 or 0.5% sodium pyrophosphate (SPP) was evaluated. Also, a mathematical model describing the effects and interactions of heating temperature (57.5 to 62.5 ° C), sodium pyrophosphate (SPP; 0 to 0.5%), and salt (NaCl; 0 to 6%) on the thermal inactivation of starved *L. monocytogenes* ATCC 19116 in pork slurry was developed. The model was based on a split-split plot experimental design and means of decimal reduction times (D-values) were modeled as a function of heating temperature, SPP, and NaCl levels. Surviving cells were enumerated either on Modified Oxford Medium or on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE). In the first study, D-values in pork slurry control (0% SPP) were 8.15, 2.57, 0.99, and 0.18 min, at 55, 57.5, 60, and 62.5° C, respectively; D-values in ground pork ranged from 15.72 min at 55° C to 0.83 min at 62.5° C. D-values in pork slurry that contained 0.25% SPP (w/v) were 4.75, 1.72, and 0.4 min, at 55, 57.5, and 60° C respectively; the values in ground pork ranged from 16.97 at 55° C to 0.80 min at 62.5° C. Addition of 0.5% SPP further decreased ($P< 0.05$) the heat resistance of *L. monocytogenes* in pork slurry but not in ground pork. The z-values in slurry ranged from 4.63 to 5.47° C whereas higher z-values (5.25 to 5.77° C) were obtained in ground pork. Degradation of added SPP to orthophosphates in ground pork was two to three times greater than in pork slurry. The predictive model study indicated that increasing concentrations of SPP or NaCl in pork slurry protected starved *L. monocytogenes* from the destructive effect of heat. Combinations of 6.0% NaCl and SPP (0.25 or 0.5%) increased the thermal inactivation of the organism

compared to 6% NaCl alone. Possible reasons for the difference in the effect of SPP in the two studies could be that *L. monocytogenes* cells that have endured starvation are more tolerant to changes in heating temperature. This work showed the need to use starved *L. monocytogenes* to design thermal processes with an adequate margin of safety.

INTRODUCTION

Listeria monocytogenes is a psychotropic pathogen, which poses a major threat to the safety of refrigerated foods (68). This organism has a fatality rate of about 20% (77) and is the second leading cause of death due to bacterial food-borne disease in the U.S. (56). US regulatory agencies specify a zero tolerance for *L. monocytogenes* in cooked and ready-to-eat foods because of the relatively high fatality rate linked to food-borne listeriosis and the uncertainty of the infectious dose for immuno-compromised persons (15, 68).

Phosphates such as sodium pyrophosphate (SPP) and sodium tripolyphosphate (STPP) are used routinely in meat industry (100). These food additives are permitted for use at a maximum level of 0.5% in meat and poultry products (90). The main functions of phosphates in meat products are to increase water-holding capacity, bind structured meats, and protect flavor. In the past decades, there has been an interest in using phosphates as an antimicrobial to substitute for sodium nitrite in meat products. Although phosphates may be incorporated into processed meats to improve microbial safety, there is a controversy on the antimicrobial effectiveness of phosphates. Research has shown that the antimicrobial efficacy of phosphates vary depending on the type of phosphates used, the medium used (laboratory media, slurry, or real food system), and whether meat is fresh or cooked.

Some consumers are nutrition conscious and are looking for meats cooked at lower temperatures. New types of raw and ready-to-eat products including ground pork, pork sausage, vacuum-packaged pumped chops or roasts, and vacuum-packaged cooked and sliced roast pork depend mainly and often upon refrigeration for control of pathogenic and spoilage microorganisms. Processors rely on phosphates, salts, and some other spices to protect consumers from food borne illnesses if these foods are temperature abused or held for

prolonged periods at refrigeration temperatures. A major research objective is to accurately predict the death kinetics of *Listeria monocytogenes* under various environmental conditions including temperature, pH, and concentration of sodium pyrophosphates.

The composition of the heating menstruum can affect the thermal inactivation of *L. monocytogenes* (8). Since most predictive models for microbial inactivation in foods is done in model systems (e.g. broth, slurries) we investigated whether effects and interactions of multiple food barriers such as temperature, salt and phosphate result in the same inactivation of *L. monocytogenes* in slurry and in ground pork. There is a consensus that salt (NaCl) is protective against thermal inactivation of *L. monocytogenes*. Salt was protective against the thermal inactivation of *L. monocytogenes* at temperatures of 55 to 65°C but this protective effect was decreased at level of 6% salt in a predictive model that involved combinations of temperature, pH, salt, and sodium pyrophosphate (35). Tuncan and Martin (88) reported that the protective effect of salt was due to its lowering of water activity and increased osmotic pressure of foods.

In sausages where phosphates and salts are used in combination, mathematical models that describe the combined effects to these ingredients on the heat inactivation of pathogens are used. Researchers from the USDA and others have developed predictive models to explain the behavior of *L. monocytogenes*, grown in rich laboratory media, in sausages with different levels of sodium pyrophosphate and salt. In the environment, microorganisms are routinely exposed to stresses. Stressed bacteria may develop some mechanisms to repair stress-induced damages and prevent death by the synthesis of stress proteins (43). Jenkins et al. (31) reported that proteins induced by starvation cross-protected *Escherichia coli* not only against heat but also against oxidative challenge. Another study has

shown that bacteria (*Escherichia coli* K165 and Sc122) that survived starvation underwent physiological changes and became hard to kill by heat (34). Lou and Yousef (43) investigated the thermotolerance of *L. monocyotogenes* that was adapted to certain environmental stresses. *L. monocytogenes* Scott A starved in phosphate buffer for 156 h showed a 13-fold increase in D-value at 56°C.

There is a need to develop predictive modeling using starved *L. monocytogenes* to accurately predict the death kinetics for *L. monocytogenes* starved in water or on food contact surfaces in processing plants. This research will provide useful data on the effects and interactions of multiple barriers in foods on the heat resistance of starved and non-starved *L. monocytogenes* and thus extend the research efforts of USDA in its mission to generate sound processing guidelines to ensure the microbial safety of processed meats.

Thesis organization

The body of this thesis is divided into two papers. Each paper has its own abstract, introduction, materials and methods, results, discussion, and references cited. Prefacing the main body is a general introduction and literature review. References cited in the general introduction and literature review are placed at the end of the thesis.

LITERATURE REVIEW

Phosphates in meats

Molins (63) published an extensive review in the use of phosphates in meats. He reported that as many as 40 million pounds of phosphates (80% for brines in hams and bacon) were used in meat, poultry, and fish in the 1970s. That figure has likely increased considerably in recent years. Sodium acid pyrophosphate contains two phosphorus atoms, each of which is attached to four oxygens, one sodium, and one hydrogen. It is acidic in aqueous solution (48). Sodium acid pyrophosphate (SPP) and sodium tripolyphosphate (STPP) are routinely used in meat industry (100). These food additives are permitted for use at a maximum level of 0.5% in meat and poultry products (90). The main functions of phosphates in meat products are to increase water-holding capacity, bind structured meats, and protect flavor and to prevent lipid oxidation. In the early 1980s, searches to substitute sodium and nitrites in foods led to the reevaluation of phosphates as meat additives (65). This new interest spurred research in antimicrobial proprieties of phosphates. Currently, phosphates may be incorporated into processed meats to improve microbial safety (36).

Water-holding capacity of phosphates

The primary functional use of phosphates is to increase water-holding capacity (WHC) of meats, particularly in hams. Phosphates increase retention of native or added water and subsequently reduce cooking losses. Earlier theory (25, 97) attributed the WHC of phosphates to its capability to bind calcium and magnesium ions. Later research could not substantiate the importance of calcium chelation in enhancing WHC (5, 29, 30, 72, 73, 75). Sherman (74) used EDTA that binds calcium and magnesium, but could not reproduce WHC of phosphates. Hellendoorn (29) found that EDTA depressed rather than enhanced WHC in

meat. Berman and Swift (5) suggested that the binding by phosphates of zinc, not calcium and magnesium, was related to WHC.

WHC depends on the amount of phosphates used and temperature. Sherman (73) added phosphates to ground pork stored overnight at 0°C. He observed that, during cooking of ground pork with less than 2% of phosphates, WHC decreased initially and increased after 40°C to reach a maximum at 50°C. However, ground pork with more than 2% of phosphates absorbed all the added fluid before heating.

Hydrolysis of phosphates does not seem to affect the extent of WHC. Because the absorption of water was not affected by the storage of ground pork overnight (72), Molins suggested that the amount of water absorbed by meat after phosphate addition became permanent regardless of the nature of phosphates (pyrophosphates or orthophosphates). This confirmed report by Swift and Ellis (84) who did not find any differences in WHC between orthophosphates and pyrophosphates in rabbit muscle WHC.

The pH affects WHC by phosphates. Earlier work by Swift and Ellis (84) demonstrated that pH and ionic strength affected absorption of water by phosphates in rabbit muscle. They suggested that phosphates, not specifically pyrophosphates, dissolved proteins based on the pH and ionic strength. Sherman (72) attributed increased WHC in meat treated with TSPP and sodium polyphosphate glassy (SPG) to the degree of solubilization of actomyosin during storage of treated phosphate at 0°C. This phosphate-induced solubilization was positively related to pH greater than 6.25.

Sherman (72) reported that ionic strength (μ) was important only insofar as it controlled the rate of ion absorption by meat, so that more ions were absorbed as μ increased. Anions were preferably retained as compared to cations when ground pork was heated at

100°C. This suggested that the phosphate anion had some role to play in WHC increase. Sherman (74) further noticed that there was a synergism between phosphates and sodium chloride in affecting ionic strength. The combination of polyphosphates with NaCl increased the absorption of sodium and chloride to a level higher than the sum of the individual contributions.

Phosphates such as STPP, TSPP, SPP, SPG can affect the pH of raw or cooked meat. Alkaline phosphates raise the pH of meat away from the isoelectric point. Shults et al. (75) and Wierbicki et al. (96) showed that the rate at which alkaline phosphates increase pH was proportional to their rate of increasing WHC. Their increasing order was SPG<STPP<TSPP. This order was different from that reported by Tims and Watts (1958) (SPG<TSPP<ortho-P<STPP), or by Hamm (26) (ortho-P<TSPP<SPG<STPP). Unlike alkaline phosphates, sodium acid pyrophosphate reduces the pH of meat. This, in turn, has been shown to increase shrinkage (86), decrease cooking yields (Hargett et al, 1980), and reduce the swelling of raw meat (29).

Hydrolysis of added phosphates in meats

Muscle foods have acid and alkaline phosphatases that participate in the breaking down of added phosphates. Besides the enzyme-catalyzed hydrolysis, heat-induced hydrolysis was demonstrated in cooked pork sausage (73). The rate of enzymatic hydrolysis of phosphate depends on the concentration and type of phosphate (60). A summary of hydrolysis of phosphates in beef showed that the hydrolysis of phosphates in raw beef was rapid enough to degrade between 46 and 96% of added phosphates within 48 h depending on the type of phosphates; STPP hydrolyzed at 47% within 1 h at 4-5°C while TSPP was broken

down at 10% and SPG was degraded at 34% after 1 h (63). Mihalyi-Kengyel and Kormendy (58) investigated the effect of NaCl on the activity of some phosphatases in cured, noncomminuted meat. He noticed that NaCl seemed to activate pyrophosphatases although the rate of hydrolysis for pyrophosphates was lower. Also, NaCl decreased the rate of hydrolysis of tripolyphosphates as tripolyphosphatases were inhibited. In general enzyme activity is a function of temperature. Molins (62) was surprised to notice a breakdown of condensed phosphates in frozen ground beef patties at temperature below -20°C. This finding indicated activity of some phosphatases in frozen muscle foods. Awad (1) reported meat phosphatases were inactivated by heat during cooking at approximately 40°C and that inactivation was complete at 60°C.

Antimicrobial properties of phosphates

Effect of phosphates in media

Antimicrobial properties of phosphates have been demonstrated in laboratory media. Marcy (50). He reported that factors that affect the efficacy of phosphates as antimicrobials were the age of the microbial culture, and the formation of lethal superoxide radicals following exposure of phosphate or glucose in the media to atmospheric oxygen. Phosphates exert their antimicrobial effect by chelating the essential cations of calcium and magnesium. This chelation results in leakage of cell solutes, loss of viability and rapid lysis of cells. Also, chelation can lead to the impairment of the function of the cytochrome system with subsequent decrease in the synthesis of ATP. Another factor that explain the antimicrobial property of phosphates is the ability to change the water activity and pH of media. In their study with radiation-resistant strains of *Moraxella-Acinobacter*, Snyder and Maxcy (80) showed that NaCl, yeast extract, dehydrated m-plate count broth, or SPG decrease the water

activity of standard laboratory media to values below 0.99 resulting in inhibition of microorganisms. However, this inhibition effect disappeared as the water activity was returned to its original value. Phosphates affect the solubility of proteins because they alter the net charge of proteins, influence the pH of the solution, and increase the ionic strength of solutions (63). Much of the work on phosphates effect on microorganisms in media was focused on *Clostridium botulinum* as scientists were looking for way to substitute sodium nitrite with phosphates. It was found that the addition of SPP did not affect the outgrowth of spores at pH 5.85 to pH 5.55. On the contrary, vegetative cells of *C. botulinum* were inhibited by addition of SPP at pH 5.55 but not at pH 5.85 (92). The concentration of phosphate used is another factor that affect the effectiveness of phosphates as antimicrobial. At 0.4%, SPP as well as orthophosphates, were unable to affect the normal growth of *C. botulinum* in laboratory media (92, 94). On the other hand, 0.5% STPP added along with 1.5% sorbate resulted in greater inhibition of normal cell growth of *C. botulinum* type E when compared with 1.5% sorbate (76). The types of phosphates play a role in the efficacy of the antimicrobial solution. Even though SPP or orthophosphates at 0.4% did not inhibit vegetative cell growth of *C. botulinum*, SPP appeared to be more toxic than orthophosphates. SPP affected the morphology of the cells to make them longer and wider than normal (93, 94). Later, Wagner and Busta (95) added SPP labeled with ^{32}P in a medium inoculated with *C. botulinum* 52A vegetative cells. They noticed an increase in ^{32}P following growth when SPP was present. They believed that SPP bound anion sites of proteins and protein fragments and hypothesized that SPP affected the synthesis of proteases from RNA by associating itself with the RNA. Therefore, it was concluded that SPP prevented the synthesis of protease needed to activate the protoxin in *C. botulinum* cells.

Zaika and Kim (100) evaluated the effect of sodium polyphosphates (pyrophosphate, tripolyphosphate, sodaphos, hexaphos, and glass H with 2, 3, 6, 13, and 21 chains, respectively) on growth of *L. monocytogenes.* Only the long-chained polyphosphates inhibited growth of *L. monocytogenes* in brain heart infusion + 0.3% glucose at pH 6.0 and at 28, 19, 10, and 5°C; hexaphos and glass H were more inhibitory than sodaphos. Use of these compounds from 0.3% to 1.0% resulted in significant increase in lag time and no growth was observed after 40 days in the presence of 2.0%. The addition of 2.0% NaCl increased the inhibitory effect of sodium polyphosphates.

Effect of phosphates in meats

A distinction has to be made in the use of phosphates in fresh and cooked meats. Fresh meat contains hydrolytic enzymes which breakdown pyro-and polyphosphate to orthophosphate. Orthophosphate is known to have less antimicrobial activity than its precursors. Molins et al. (61) found that orthophosphate did not inhibit psychrotropic spoilage bacteria in fresh ground pork even at level as high as 1.0%. However, SPP extended the shelf life of the same ground pork by 50% when compared with treatment with orthophosphate. The activity of phosphatases is more pronounced in ground meat, beef, and pork as compared with poultry meat. In poultry, phosphatases are membrane-bound, and hence, not easily released. In ground or comminuted meat, grinding ruptures muscle cells resulting in additional release of phosphatases. Antimicrobial activity of phosphates has been demonstrated in cured and cooked meats. In processed meats the presence of ingredients such as salt, sodium lactate, and sodium nitrites makes it difficult to separate the effect of phosphates from that of other food additives.

Effects of phosphates in fresh meats

Most the work to improve the shelf life of fresh meats have been done in poultry. Chen et al. (9) used a combination of 75% sodium tripolyphosphate (STPP) and 25% tetrasodium pyrophosphate (TSPP) (Kena) and tested it against 17 cultures of microorganisms associated with chicken. When 3.0% Kena was applied as precooking or presoaking treatments, most of the surface organisms were eliminated. Gram-negative microorganisms, however, were more resistant to this treatment that were the Gram-positive species except *Streptococcus lactis*. Also, other researchers studied the antimicrobial action of Kena. Spencer and Smith (79) noticed that when iced water with 7% of Kena was used in the chilling of broiler carcasses, carcasses prolonged their refrigeration shelf life by 1 or 2 d. Steinbauer and Banwart (81) reported that when broiler carcasses were chilled by ice with 6% Kena, the bacterial counts were kept low in broilers for 20 days at storage at 5°C. A second experiment by the same authors did not show a similar effect of Kena. At a storage temperature of 2.2°C, the shelf life of poultry carcasses that were chilled in ice containing 3 and 8% Kena was extended by 17 and 65%, respectively (14).

Use of phosphate in fresh ground pork or beef has shown little antimicrobial effect. A study testing the antimicrobial action of different forms of phosphate in uncooked bratwurst, refrigerated at 5°C did not reveal any effect of 0.5% TSPP, STPP, or SPG on the number of endogenous spoilage bacteria or inoculated *Staphylococcus aureus* (59). The treatment with 0.5% SPP, however, lowered the total plate counts. In another study, 0.4% TSPP, STPP, or three phosphate blends were mixed with ground beef during preparation of beef patties (62). No effect was noticed in the total bacterial counts in patties held at -20°C. Also, none of the phosphates delayed spoilage when patties were thawed and kept at 24 to 25°C for 24 h. Only

TSPP and one blend of Brifisol 414 (SPP, TSPP, SPG) delayed the growth of lactic acid bacteria.

Phosphate has been used in combination with other preservatives to prolong shelf life of fresh meats (57). Bacteria from the *Enterobacteriaceae* group were significantly inhibited when pork chops were treated with a combination of preservatives, vacuum packaged and stored until 10 weeks at 2 to 4°C. The treatment solution used contained potassium sorbate, NaCl, sodium acetate and phosphates (SPP or Brifisol 414). Even though, potassium sorbate contributed most of the antibacterial effect, it could not be used alone because of its negative effect on the color of the product. A combination of 10% of potassium sorbate and Brifisol 414 produced microbial control similar to that of sorbate alone and preserved the natural color of the pork chops. By using a combination of 5% Brisifol, 10 % potassium sorbate, 5% NaCl, and 10% sodium acetate, Unda et al. (89) kept the mesophilic, psychotropic, anaerobic and facultative anaerobic bacteria and lactobacilli counts low in fresh rib eye steaks for 12 weeks at 2 to 4°C. In the same time, the growth of *Enterobacteriaceae* was totally inhibited and the color of the meat preserved.

Effects of phosphates in cured, cooked and processed meats

The effects of phosphates in combination with sodium nitrite, sorbic acid, and/or potassium sorbate against *clostridium spp.* have been investigated as alternatives to sodium nitrite in meats. Much of these studies have dealt with either clostridial growth or, in the case of *C. botulinum*, toxin production. Molins (63) published an extensive review of these studies. In this chapter, we report research on the use of phosphates in extending the shelf life of processed and cooked meats.

Nielsen and Zeuthen (66) used a blend of sodium tripolyphosphate (STP), tetrasodium pyrophosphate (TSPP), and SPP or STP alone to study their effectiveness against spoilage bacteria in bologna. They used cooked (75°C), sliced, vacuum-packaged bologna formulated with 55 ppm NaNO2, 168 ppm sodium ascorbate. None of the phosphates or mixtures had any effect on lactic acid bacteria. On the other hand, 0.3% of acidic blend reduced the population of *Brochothrix thermosphacta* by 1 log after 10 days of storage at 2°C. The acidic blend strongly inhibited *B. thermosphacta* by at least 3 logs after 30 days storage at 2°C.

Madril and Sofos (53) indicated that SPP was the only phosphate to improve the antimicrobial properties when formulated with 1.25% NaCl in comminuted meat products. The antimicrobial properties of SPP could not be explained whether it was due to a pH decline in the product and /or a specific phosphate effect. Also, Madril and Sofos (54) suggested that SPP might have more antimicrobial properties at pH 6.0 than at pH 5.7. They observed that concentrations of 0.5% SPP and 1.3% NaCl (2.3% brine) in comminuted meats at pH <6.3 inhibited microbial growth for up to 2 weeks at 27°C. Also, there was delay in initial formation of gas and reduced gas production. However, 0.4% SPP in combination with 1.3% NaCl was less effective compared with 2.5% NaCl without SPP in delaying gas production in the same product.

Marcy et al. (49) studied the combined effects of acid and alkaline pyrophosphates on the natural flora of a cooked meat system. They found that 0.4% SPP, tetrasodium pyrophosphate (TSPP), or combinations of these phosphates did not have any effect on total microbial counts in cooked pork sausage (2.0% salt, 0.5% pepper), vacuum-packaged and held refrigerated at 5°C for 21 days. Different from TSPP, SPP significantly affected counts

of mesophilic and facultative anaerobic organisms after to 48 h of temperature abuse (20-22°C). The organisms affected were streptococci or very similar coccobacilli.

Similar results of the effects of SPP were reported in an experiment with the same type of sausage (50). In that experiment, neutral trisodium pyrophosphate (PYRO-3) and SPP were used (0 and 0.4% or combinations). Both phosphates lowered the counts of mesophilic bacteria when the sausage was held at 7°C for 21 days. Also, both phosphates significantly affected counts of mesophilic and facultative anaerobic microorganisms after 48 h temperature abuse at 20-22°C.

Later, Marcy et al. (51) concluded that phosphate level, not phosphate type, was the determining factor in bacterial inhibition of the natural bacterial flora of a cooked meat system. The sausages were cooked as in previous work (49, 50). When four commercial phosphate blends and a neutral pyrophosphate were used at levels of 0.30-0.65%, higher counts of mesophilic and facultative anaerobic bacterial were always present in untreated sausage during refrigerated storage (5°C for 21 days) or temperature abuse (20-22°C for 48 h).

Recently, Flores et al. (20) found that a phosphate blend Bekaplus MSP (sodium polyphosphate, sodium metaphosphate, and sodium orthophosphate) at 0.5% had no effect in controlling *E. coli* 0157:H7 in ground beef; but it did have a significant effect on controlling *E. coli* 0157:H7 in fresh pork sausage when stored at 4°C over 7 days. The sausage was seasoned with 2.3% of a blend composed of 8.3% sodium chloride 8.5% sage and 8.5% white pepper. The authors believed that the difference in effectiveness of the phosphate in the sausage compared with the ground beef could be attributed to inherent differences in the meat or, more likely, to the antimicrobial properties of the spices in the sausage. There was

minimal or no effect of the phosphate blend on the growth of *L. monocytogenes* or *S. typhimurium* in boneless ham and smoked sausage.

Antimicrobial properties of Sodium chloride (NaCl)

The antimicrobial activity of NaCl may be direct or indirect. The inhibition of microbial growth depends on the amount of NaCl. For example, 16.54% NaCl can prevent microbial growth. This large amount of NaCl can be used in the processing of dried and smoked meats and fishes. In this case, the antimicrobial action of NaCl is direct. On the other hand, NaCl can be used as a flavoring and functional ingredient in some foods. Sodium chloride in these foods acts with other preservatives to prevent microbial growth and its antimicrobial action is called indirect. Also, the indirect action of NaCl involves decreasing the water activity to cause inhibition of microbial growth. Low water activity can lead to osmotic shock of the bacterial cell. During osmotic shock, the cell loses fluids and eventually dies. Sodium chloride may inhibit bacteria growth by decreasing the solubility of oxygen making it less available to the microbial cell, by lowering pH, by increasing the toxicity of sodium and chloride ions, by causing loss of magnesium ions (4), or by interfering with the cellular enzymes (71).

Various bacteria can be grouped based on their salt tolerance (2). First, there is the group of nonhalophile bacteria that can grow well at levels of 0 to 0.5% NaCl. Some bacteria belonging to the genera *Staphylococcus, Micrococcus* and *Clostridium* belong to this group. Second, there are bacteria that can tolerate and grow in the range of 1.5% to 5.0% NaCl. They are called slight halophiles and members of this group include *Achromobacter, Flavobacterium, Pseudomonas,* and *Vibrio*. Third, the group of moderate halophiles

comprises bacteria that can tolerate up to 5 to 20% NaCl. Some of the lactic acid bacteria and some spore formers are in this category. The last group is composed of extreme halophiles. These bacteria require at least 9% sodium chloride for growth, with the optimum concentration for growth ranging between 12 to 23% and the maximum being 32% (47). An example of extreme halophile is *Halobacterium salinarum* which has a cell wall that is stabilized by sodium ions. In low sodium environments the cell wall of this organism breaks down and the cell lyses (47). Obligate halophiles require 15% NaCl and can be found in saturated brines.

The antimicrobial mechanism of sodium chloride is explained by the lowering of water activity. Tuncan and Martin (88) showed that heat resistance of *S. aureus* in NaCl increased as the degree of salt-water association increased. They believed that the effect of NaCl on thermal inactivation of microorganisms was due to reduced water activity and increased osmotic pressure of the heating menstruum. Leistner and Russell (39) acknowledged that for a given solute there was an optimum concentration of the solute that gave a maximum heat protection to a microorganism. But outside of this concentration, the heat resistance of the microorganism decreased.

Many studies were conducted on the effects of sodium chloride on the thermal inactivation of *Listeria monocytogenes.* One of the studies investigated the effects of sodium chloride, temperature, and pH on the growth of *L. monocytogenes* (12) in cabbage juice. Concentration of 2% sodium chloride or higher were found to inhibit *L. monocytogenes.* Survival studies showed that there was 90% decline in *L. monocytogenes* Scott A in 5% sodium chloride over 70 days at 5°C. Another strain (coleslaw outbreak strain) survived in cabbage juice containing 3.5% or less sodium chloride over a 70 days period. Both strains

had extended lag times at 1.5 and 2.0% sodium chloride. Initial exposure to heat and ethanol increased the tolerance of *L. monocytogenes* to sodium chloride (43).

Studies in broth, pork, and beef reported a reduction in thermal inactivation of *L. monocytogenes* in various meat blends containing 3 to 4% NaCl (11, 18, 46, 99). Yen et al. (99) found NaCl protected *L. monocytogenes* against thermal destruction at concentrations up to 3%. They inoculated ground pork (15% fat) with a mixture of nine strains of *L. monocytogenes* (10^7–10^8 CFU/g) and cooked it at 60°C. They found that the destruction of these microorganisms was 3 log per gram less in ground pork with 3% NaCl than in ground pork without added sodium chloride. Juneja and Eblen (35) studied the interactive effects of temperature, pH, NaCl and sodium pyrophosphate on the heat inactivation of *L. monocytogenes* in beef gravy. In general, NaCl protected the organism against the heat. Although sodium pyrophosphate (0.3%) decreased the heat resistance of cells at pH 8.0 and 6% NaCl, there was a protective effect of NaCl against heat at lower pH.

Short-term exposure to salt can protect *L. monocytogenes* against heat. JΦrgensen et al. (33) reported an increase in heat resistance of *L. monocytogenes* grown in 0.09 mol/liter NaCl and exposed to test media containing 0.5, 1.0, or 1.5 mol/liter NaCl.

Heat inactivation kinetics predictive models

Mathematical modeling of microbial death is a technique used to determine the effects two or more food parameters on microbial heat resistance. Environmental food parameters include but are not limited to temperature, pH, water activity, salt, sodium nitrite, and gaseous atmosphere (aerobic or anaerobic). Models try to simplify complex food interactions on the survival of microorganisms in broths or food models. Hence, they cannot

be applicable to broad range of foods. It is crucial that any model be validated in the specific food of interest before it can be used for thermal processing or food safety decisions (98). There are numerous predictive models describing the behavior of *L. monocytogenes* in broths, food model systems, or foods with two or more factors.

Typical predictive models are linear regression analyses of D-values (41). D-values are calculated using the inverse of the slope of the best fit line when log of the number of survival cells is plotted against heating time in minutes. The log-linear thermal death time model assumes that the observed data are closely scattered along the slope and, that the cells have the similar heat resistance. This is typical of high processing temperatures in canning (42). However, studies (3, 41) have shown that non-linear inactivation of microorganisms occurred during heating at lower temperatures. In those experiments, thermal inactivation slopes display an initial shoulder or lag region followed by a linear exponential decline region and a final tailing region. Different approaches have been used to accurately estimate heat resistance for nonlinear data.

One of these approaches is the use of the Gompertz equation. The Gompertz equation was originally used to describe the asymmetrical sigmoid shape of microbial growth curves based on temperature, pH, and NaCl. Bhaduri et al. (3) adapted the Gompertz equation to account for thermal destruction of *L. monocytogenes* in liver sausage slurry. Those researchers found that the Gompertz model gave a good prediction of the sigmoidal survival curves in that study. Later, Linton et al. (41) adapted the Gompertz equation to model nonlinear survival curves for *L. monocytogenes* Scott A. They modified a model used the describe growth of *Clostridium botulinum* type A in pasteurized pork slurry by Gibson et al. (23). A log surviving fraction (LSF; LSF = log [CFU(t)/CFU(0)] instead of microbial growth

was modeled. They proceeded using a "by-cell" analysis to determine the accuracy of individual treatment to describe the survival as they heated *L. monocytogenes* Scott S in 0.1M KH_2PO_4 buffer at three temperatures (50,55, and 60°C), three pH levels (5,6, and 7), and three NaCl concentrations (0,2, and 4%). After, they used the data from all treatment cells to form a "full model" modified Gompertz equation. In that study, large shoulders and lag regions were common at 50°C and 55°C with linear curves at higher temperatures. The correlation of the full model modified Gompertz model as R^2 was 0.89. Linton et al. (42) applied the Gompertz equation to predict the thermal inactivation of *L. monocytogenes* in a real food system. *L. monocytogenes* Scott A was inoculated in infant formula and its heat resistance was observed for combinations of temperature (50,55, and 60°C), pH level (5,6,7) and NaCl (0,2,and 4%). This study confirmed that *L. monocytogenes* Scott A survival curves were more linear as temperature was increased from 50 to 60°C. The correlation of observed LSF versus predicted LSF was good at 0.92.

Recently, Chhabra et al. (10) used the same modified Gompertz model to determine the effects on formulated dairy milk of pH (5.0, 6.0, 7.0) milk fat (0,2.5,5.5%), and temperature on the thermal survival of *L. monocytogenes* Scott A. The full model at temperatures of 50,60, and 65°C, produced a poor LSF correlation R^2 of 0.72. At 55°C and pH 5.0, predictive values from the Gompertz equation were twofold the values of individual sets, 4D values for linear equation were closer to those of individual sets. At 60 and 65°C, 4D values obtained by the three methods were similar. In view of the fact that the modified Gompertz model overestimated survival compared to the observed data for temperatures below 61°C, the authors suggested that the use of Gompertz equation would be accurate for

processing temperatures above 61°C. Another limitation of the Gompertz equation is that it is reliable only if used for a maximum level of 4 to 5 log of cells.

A log-logistic function was applied successfully to quantify the effects of temperature on the thermal inactivation of *L. monocytogenes* (11) and the effect of heating rate on the inactivation of *L. monocytogenes* (82). In another experiment, a log-logistic function was used to develop 3-factor thermal inactivation models for *Salmonella enteriditis* and *Escherichia coli* 0157:H7 based on temperature (54.5 to 64.5°C), pH (4.2 to 9.6) and NaCl concentration (0.5 to 8.5% w/w) (6). Results indicated that 84 % and 83% of survival curves represented a linear logarithmic death for *S. enteritidis* and *E. coli* 0157:H7, respectively. Only the remaining curves showed shoulder and tailing regions.

A secondary model of quadratic response was used to study the effects and interactions of temperature (55 to 65°C), pH (4 to 8), NaCl (0 to 6%) and SPP (0 to 3%) levels in beef gravy on the inactivation of four-strain *L. monocytogenes* cocktail (35). After the D-values were transformed to the natural logarithm form, a quadratic response surface model was developed for curves fitting the linear function. At 55°C, lag periods were observed and correction was made in the calculation of D-values. The mathematical model generated gave a good prediction of the survival of the pathogen within the combinations of the four environmental factors studied ($R^2 = 0.95$).

No single predictive model can be applied to all temperatures. Depending on the temperature, a linear or non-linear model can be appropriate. One can argue that the Gompertz equation fit best the thermal inactivation of *L. monocytogenes*. But results from previously mentioned studies showed that this type of equation had a poor correlation between observed and predicted values at higher temperature (61°C and above). On the other

hand, linear regression model gives a better correlation at high canning processing temperatures but does not provide a good correlation at lower temperatures. Additional mathematical and statistical techniques may be used to adapt a linear or a non-linear equation to model a wide range of temperatures. Regardless of which equation is used to represent data, a predictive model should be used as an initial tool to estimate pathogenic inactivation and to provide an understanding of how various conditions affect the death of pathogens (10).

Thermal resistance of *Listeria monocytogenes*

Different strains of *L. monocytogenes* may behave differently when exposed to heat treatment. Other factors that affect heat resistance are age of the culture, growth conditions, physiological status of the vegetative or stationary cells, recovery media, and characteristics of foods such as salt content, a_w, acidity, and the presence of other inhibitors.

Research has shown that strains of *L. monocytogenes* under similar experimental conditions respond differently to heat. For example, Golden and al. (24) reported D-values of 16.0, 10.4, 7.4, and 5.7 min for *L. monocytogenes* strains Brie-1, LCDC 81-861, and DA-3 in tryptose phosphate broth at 56°C, respectively. In another study in tryptone soy broth, Mackey et al (46) found $D_{57°C}$ ranging from 6.5 to 26 min for 29 *L. monocytogenes* strains. *L. monocytogenes* Scott A is the strain that is routinely used for thermal inactivation studies. Its heat resistance appears to be intermediate among strains of *L. monocytogenes*. *Listeria innocua* has shown D-value higher than some *L. monocytogenes* strains, Scott A including (21).

Studies in foods have produced differences in heat resistance for *L. monocytogenes*. Investigators have reported various D-values for *L. monocytogenes* in ground meat. According to Farber (17), *L. monocytogenes* (strain not specified) had a D-value of 3.12 min in ground meat packaged in laminated pouches and heated to an internal temperature of 60°C. Boyle et al. (8) reported a D-value of 2.54 min for *L. monocytogenes* Scott A in ground beef slurry at 60°C. Schoeni et al. (70) found a D-value of 3.5 to 4.4 min for *L. monocytogenes* (5 strains) in ground roast beef at 60°C. At 63°C, D-values for *L. monocytogenes* Scott A ranged from 0.5 to 0.6 min and 1.1 to 1.2 min in lean (2% fat) ground beef and fatty (30.5%) ground beef, respectively (16). *L. monocytogenes* Scott A in ground pork had a D-value at 60°C of 1.14 min (67). In that study, the investigators reported a D-value of 1.70 min at 60°C for *L. monocytogenes* Scott A that contained soy hulls. Factors such as fat composition, water activity, pH of the ground meat, type of container, strains of *L. monocytogenes*, and type of plating medium could account for differences among D-values in different food systems.

The age of the microorganisms affects thermotolerance. Cells in the stationary phase are believed to be more heat resistant than exponential phase cells. Results from a study showed that at 60°C, cells of stationary phase *L. monocytogenes* strain 13-249 exhibited D-values four times higher than those of exponential cells (2.22 min as compared to 0.6 min) (32).

Growth condition is another factor that affect thermotolerance. Temperature affects the growth and synthesis of cellular constituents and subsequently determines the thermal tolerance of bacterial cells (13). Thermotolerance of bacteria increases as the growth

temperature of the culture increases prior to heating increases (37, 44, 45). *L. monocytogenes* cells grown at 10°C had a D-value at 60°C of 0.8 min when heated in sausage slurry, whereas cells grown at 37°C had a D-value at 60°C of 1.6 min (3). Heating at a lower temperature efficiently destroyed cells of *L. monocytogenes* grown on refrigerated meat (7). Cells grown at low temperatures show an increase in unsaturated fatty acids in the cytoplasmic membrane, increased membrane fluidity, reduced membrane viscosity, and increased heat sensitivity (22, 27, 52). Increased heat sensitivity of bacterial cells grown at low temperatures can be lost if cells are held for 1 to 5 hrs at 37°C prior to heating.

Environmental stress is another factor contributing to differences in heat resistance. Heating bacterial cells slowly prior to thermal inactivation increases their thermotolerance. Increased thermotolerance of *L. monocytogenes* that were heated slowly was linked to induction of the heat-shock response (82). The synthesis of a new set of proteins in response to heat challenge may be the basis for acquired thermotolerance in microorganisms (69). Although a direct cause and effect relationship between the synthesis of heat shock proteins and acquired thermotolerance is debatable (91), this relationship seems to be universal and has been investigated in *L. monocytogenes*, *Salmonella*, and *E. coli* 0157:H7 (18, 44, 64). Increased heat tolerance can be induced by short-term exposure to high salt. *L. monocytogenes* Scott A increased by 1.3 to 8 fold its heat resistance when they were grown in 0.09 mol/liter NaCl and exposed to 0.5, 1.0, or 1.5 mol/liter NaCl. On the other hand, a downshock from 1.5% NaCl to 0.09 % NaCl resulted in significant reduction in heat resistance(33). Lihono et al. (40) found that *L. monocytogenes* Scott A cells grown in 5% NaCl exhibited a 1.4 fold increase in heat tolerance compared with the control cells grown in

trypticase soy broth supplemented with 0.6% yeast extract. However, these osmotic adapted cells lost their increased thermotolerance within 8 min of osmotic downshift in pork slurry.

Starvation significantly increases the heat resistance of *L. monocytogenes*. This was proven by Lou and Yousef (43) who found that *L. monocytogenes* Scott A, starved in phosphate buffer for 156 h, exhibited a 13-fold increase in D-value at 56°C. Mazzotta and Gombas (55) starved an outbreak strain of *L. monocytogenes* in phosphate-buffered saline, pH 7, for 6h at 30°C. Those authors found that stationary phase cells starved for this short period had D-values twice higher than those of stationary phase cells (11.2 min versus 5.9 min at 58°C and 2.1 min versus 0.9 min at 62°C) in broth but not in hot dog batter.

Cells starved in the laboratory are comparable to cells surviving cleaning and sanitizing operations on raw meat processing equipment and being deprived of nutrients long enough to induce the stress response (55). Starvation is one of the most important stresses because it induces a general stress response in bacteria. Jenkins et al. (31) have demonstrated that *E. coli* cells starved in 0.3% glucose for 4 h produced about 30 stress proteins. Several of these proteins were heat shock proteins that protect against other homologous or heterologous stresses (acid, heat, oxidative) (31, 43). Other stresses that have been found to increase heat resistance of *L. monocytogenes* are acid stress (38), decrease in a_w (Sumner et al, 1991), and increase in solute concentrations (78).

INFLUENCE OF SODIUM PYROPHOSPHATE ON THERMAL INACTIVATION OF *LISTERIA MONOCYTOGENES* IN PORK SLURRY AND GROUND PORK

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Summary

The thermal inactivation (55-62.5° C) of *Listeria monocytogenes* in pork slurry and ground pork that contained 0, 0.25 or 0.5% sodium pyrophosphate (SPP) was evaluated. Surviving cells were enumerated on Modified Oxford Medium. Decimal reduction (D)- values in pork slurry control (0% SPP) were 8.15, 2.57, 0.99, and 0.18 min, at 55, 57.5, 60, and 62.5° C, respectively; D-values in ground pork ranged from 15.72 min at 55° C to 0.83 min at 62.5° C. D-values in pork slurry that contained 0.25% SPP (w/v) were 4.75, 1.72, and 0.4 min, at 55, 57.5, and 60° C respectively; the values in ground pork ranged from 16.97 at 55° C to 0.80 min at 62.5° C. At 62.5° C, *L. monocytogenes* in slurry that contained SPP were killed too rapidly to allow determination of the D-value. Addition of 0.5% SPP further decreased ($P < 0.05$) the heat resistance of *L. monocytogenes* in pork slurry but not in ground pork. The z-values in slurry ranged from 4.63 to 5.47° C whereas higher z- values (5.25 to 5.77° C) were obtained in ground pork. Degradation of SPP to orthophosphates in ground pork was 2 to 3 times greater than in pork slurry. Possible reasons for failure of SPP to reduce the thermal resistance of *L. monocytogenes* in ground pork are discussed.

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Introduction

Listeria monocytogenes is a psychrotrophic pathogen, which poses a major threat to the safety of refrigerated foods (Ryser and Marth, 1999). This organism has a fatality rate of about 20% (Slutsker and Schuchat, 1999) and is the second leading cause of death due to bacterial food-borne disease in the U.S. (Mead et al., 1999). US regulatory agencies specify a zero tolerance for *L. monocytogenes* in cooked and ready-to-eat foods because of the relatively high fatality rate linked to food-borne listeriosis and the uncertainty of the infectious dose for immuno-compromised persons (Engle et al., 1990; Ryser and Marth, 1999).

Phosphates such as sodium pyrophosphate (SPP) and sodium tripolyphosphate (STPP) are used routinely in meat industry (Zaika and Kim, 1993). These food additives are permitted for use at a maximum level of 0.5% phosphates in meat and poultry products (USDA, 1982). The main functions of phosphates in meat products are to increase water-holding capacity, bind structured meats, and protect flavor. However, phosphates may be incorporated into processed meats to improve microbial safety (Kijowski and Mast, 1988). Juneja and Marmer (1998) demonstrated a significant ($P<0.05$) decrease in heat resistance of *Clostridium perfringens* in precooked ground beef and turkey that contained 0.15 or 0.3% SPP compared to controls. In contrast, a mixture of sodium tripolyphosphate and sodium hexametaphosphate in combination with dextrose protected *L. monocytogenes* from thermal destruction in fresh ground pork (Yen et al., 1991).

The composition of the heating menstruum can affect the thermal inactivation of *L. monocytogenes* (Boyle et al., 1990). The addition of salts to comminuted meat can increase the heat resistance of *L. monocytogenes* (Schoeni et al., 1991; Yen et al., 1991). Previous

published information on thermal inactivation of *L. monocytogenes* is based on studies conducted in various nutrient media (Stephens et al., 1994; Patchett et al., 1996), on meat surfaces (Huang et al., 1993) in meat slurries or suspensions (Duffy and Sheridan, 1997), ground pork (Kim et al., 1994; Ollinger-Snyder et al., 1995) and beef (Hansen and Knochel, 1996). Boyle et al. (1990) investigated the thermal inactivation of *L. monocytogenes* in meat slurry and in ground beef. Murphy et al. (1999) evaluated the thermal inactivation of *Salmonella* spp. and *Listeria innocua* in ground chicken breast meat and in 0.1% peptone-agar solution. There is no published information that evaluates the effect of phosphates such as SPP on thermal inactivation of *L. monocytogenes* in ground pork and in pork slurry. Assessment of the thermal inactivation of pathogens in different media systems containing food additives can provide useful data for designing commercial thermal processes that ensure the microbiological safety of foods. The present study was carried out to quantitatively assess the influence of SPP in pork slurry and in ground pork on the thermal inactivation of *L. monocytogenes*.

Materials and Methods

Raw material

Fresh ground pork, purchased from a local supermarket, was tested using the Food and Drug Administration method (Hitchins, 1998) for naturally occurring *Listeria*. The meat was separated into appropriate portions and stored at -20° C for less than four weeks before use. The same batch of ground meat (2.3 kg) was used for all of the experiments in this study. Prior to each experiment, portions of frozen meat were thawed for 18 h at 4°C.

Cultures

The three strains of *L. monocytogenes* used in this study were Scott A (serotype 4b; clinical isolate), V7 (serotype 1/2a; milk isolate), and ATCC 19116 (chicken isolate). These strains, maintained as frozen (-70° C) stocks in brain heart infusion broth (BHI; Difco) supplemented with 10% glycerol, were obtained from the culture collection of the Department of Microbiology at Iowa State University. During the course of this study, individual cultures were maintained in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) at 4° C with monthly transfers to maintain viability of the cultures.

Preparation of inoculum

To prepare the inoculum, 0.1 ml of stock culture was transferred to 10 ml of TSBYE and incubated at 35° C for 18 h. After two consecutive transfers using 0.1 ml inoculum, cells were sedimented by centrifugation (9000 x g, 10 min, 4° C) and washed once in 0.1% peptone water (w/v). The cell pellets were re-suspended in 10 ml peptone water. The cell population in each inoculum suspension was determined by surface plating appropriate dilutions (in 0.1% peptone water), in duplicate, onto tryptic soy agar supplemented with 0.6% yeast extract (TSAYE) plates, which were then incubated at 35° C for 48 h. Thereafter, 5 ml suspensions of each strain were combined in a sterile test tube and mixed thoroughly by vortexing. An appropriate dilution of this mixture of strains was prepared in peptone water to obtain the desired cell concentration for inoculating the pork slurry or ground pork.

Sample preparation and inoculation

Pork slurry was prepared by mixing 40 g ground pork (80% lean) with 360 ml of 0.1% sterile peptone water in a sterile mesh-lined filter bag (Celsis Inc., Evanston, IL). The mixture was

pummeled using a Seward Stomacher 400 Lab-blender (Seward Ltd., London, England) for 1 minute at medium speed. Portions (100 ml) of the slurry were transferred into separate 250 ml Erlenmeyer flasks to which 0, 0.25 or 0.50 g SPP (Brifisol K, BK Landenburg, Simi Valley, CA) were added. One ml of the washed cells of L. monocytogenes was used to inoculate 100 ml of the pork slurry to give a final cell concentration of approximately 10^7 CFU ml^{-1}. The inoculated pork slurry was aseptically dispensed into sterile Pyrex thermal-death-time (TDT) tubes (2.5 ml per tube). The TDT tubes were heat-sealed by using a type 3A blowpipe (Veriflow Corp. Richmond, Calif.) and then held in a laboratory refrigerator (4° C) for at least 15 min to allow temperature equilibration before heating. Samples (100 g) of ground pork were weighed in sterile 1000 ml beakers. SPP was added to the ground pork and thoroughly mixed for 2 min using a sterilized spatula to give a final SPP concentration of 0.25% or 0.50% (w/v). Ground pork without SPP served as control. Pork samples (5 g) with or without SPP were placed into sterile stomacher bags. Each sample of ground pork was inoculated with 0.1 ml of the washed L. monocytogenes cell suspension to give a final cell concentration of approximately 10^7 CFU g^{-1}. Pork samples inoculated with 0.1 ml of sterile 0.1% (w/v) peptone water served as negative controls. All bags of inoculated ground pork were manually mixed to evenly distribute the inoculum in the meat samples then pressed against a flat surface to form a sample thickness of approximately 1-2 mm. The samples were vacuum-sealed using a Multivac vacuum packaging machine (Multivac, Wolfertschwenden, Germany) then held in a laboratory refrigerator (4° C) for at least 15 minutes prior to heating.

Determination of pH and soluble orthophosphate

Measurements of pH and soluble orthophosphates were performed on uninoculated samples. The pH of ground pork was determined using the method described by Sebranek (1978). A 5 g portion of ground pork was homogenized with 30 ml of distilled, deionized water for 2 min in a Stomacher. A combination electrode and Orion Model 525 pH meter (Orion Research Inc., Boston. MA) were used to measure the pH of the homogenate and the pork slurry. Analysis of soluble orthophosphates was conducted according to the method described by Molins et al. (1985) using inoculated samples of pork slurry or ground pork with or without added SPP. All samples were analyzed at approximately 1 h after preparation. A standard curve was prepared by determining the absorbance (690 nm) values of dilutions of a standard solution containing known amounts of 0, 5, 10, 15, 20, and 25 $\mu g\ ml^{-1}$ of orthophosphate. Orthophosphate concentrations ($\mu g\ ml^{-1}$) were then plotted against absorbance values to generate the standard curve. An extract of ground pork was prepared by blending 40 g meat with 360 ml sterile distilled water in a stomacher Lab-blender 400. The extract of ground pork as well as the pork slurry (1:10 dilution of pork in 0.1% peptone) was further diluted 1:10 with sterile deionized water to obtain orthophosphate concentrations within the measurable range (1-20 $\mu g\ ml^{-1}$) of the standard curve. Soluble orthophosphate content was determined by multiplying the value obtained from the standard curve by the reciprocal of the dilution factor and expressing the result as μg soluble orthophosphate $g^{-1}$of sample.

Thermal inactivation

Three replicate experiments were conducted. Each replicate experiment was conducted on separate days and the temperatures were randomly assigned. All samples were heated within

2 h of preparation. Sealed TDT tubes of pork slurry or vacuum-packaged pork samples were submerged in a thermostat-controlled Isotemp 1013S heater water bath (Fisher Scientific, Pittsburgh, PA) stabilized at 55, 57.5, 60, or 62.5° C. The internal temperature of the samples was continuously monitored by a copper-constantan thermocouple inserted, prior to sealing, at the center of an uninoculated sample in TDT tube or bag. Thermocouple readings were measured using a digital meter. The average number of viable *L. monocytogenes* in unheated samples represented the number of *L. monocytogenes* present at time zero. Thereafter, samples in TDT tubes or bags corresponding to the three treatments (0, 0.25, and 0.50% phosphates) were removed at set time intervals and submerged in 50/50 ice/water slush (0° C) for about 5 min. Samples were analyzed within 30 min for *L. monocytogenes* using procedures described below. The frequency of sampling was based on the heating temperature.

Microbiological analysis

TDT tubes (2 tubes per treatment) were opened and their contents were aseptically pooled in separate sterile test tubes. One ml from each pooled sample was removed and serially diluted in sterile 0.1% peptone. Packages of vacuum-packaged ground pork were aseptically cut open and 45 ml of sterile 0.1% peptone was aseptically added to each pork sample. The mixtures were pummeled for 1 min in a Stomacher Lab-blender and 1.0 ml samples were serially diluted in sterile 0.1% peptone solution. Samples (0.1 ml) of appropriate dilutions of the pork slurry or ground pork were surface-plated, in duplicate, onto plates of Modified Oxford (MOX) agar. In instances when increased sensitivity was required, 1.0 ml samples of undiluted slurry or meat mixture were plated directly onto MOX agar plates. Uninoculated

samples were plated as controls. All inoculated plates were incubated at 30° C. Bacterial colonies were counted at 96 h then checked for presumptive *L. monocytogenes* as described by McClain and Lee (1988).

Calculation of D- and Z-values

The D-values (time for 90% reduction in viable cells) expressed in minutes, were determined by plotting the $\log_{10}$ number of survivors versus time for each heating temperature using Microsoft Excel 98 Software (Microsoft Inc., Redmond, WA). Linear regression analysis (Ostle and Mensing, 1975) was used to determine the line of best fit for data on each survivor curve. The D-value for each phosphate/temperature treatment combination was determined by calculating the negative reciprocal of the slope of the survivor curve. The z-values were calculated by plotting the mean $\log_{10}$ D-values versus their corresponding heating temperature (° C) using Microsoft Excel 98 Software, and determining the change in temperature required to give a 1-log difference in D-value.

Statistical analysis

Means and standard deviations of D-values from three replicate experiments were determined. Each replicate experiment included samples heated at 55, 57.5, 60, and 62.5° C on the same day. The D-values and z-values were analyzed by t-test using SAS 96 (SAS, 1996). The z-values were compared by 2-way analysis of variance (ANOVA) (SAS, 1996).

Results and Discussion

L. monocytogenes cells heated at 55 to 62.5° C in pork slurry or ground pork demonstrated log-linear decline in number of survivors over time. No marked shoulders or tailing were observed in any of the survivor curves. The linearity of the survivor curves indicated homogeneity in thermal resistance of the *L. monocytogenes* cell population used in the present study.

The thermal resistance (D-values in min) of *L. monocytogenes* in pork slurry and ground pork that contained 0, 0.25, and 0.5% SPP is presented in Table 1. The addition of 0.25% or 0.5% SPP to pork slurry significantly decreased ($P<0.05$) the D-values of *L. monocytogenes* at all temperatures tested (Table 1). Rapid cell death occurred in pork slurry that contained 0.25 or 0.5% SPP and heated at 62.5° C; no viable *L. monocytogenes* cells were detected after 1 min of heating. In contrast, the addition of 0.25% or 0.5% SPP to ground pork did not significantly decrease ($P>0.05$) the D-values for this organism. The z-values for *L. monocytogenes* in pork slurry and ground pork that contained 0, 0.25, and 0.5 % SPP are shown in Figure 1. Significant decreases ($P<0.05$) in z-values were evident in pork slurry that contained 0.25 or 0.5% SPP. Only very small decreases in z values were evident in ground pork that contained 0.25 or 0.5% SPP; however, those decreases were not statistically significant ($P>0.05$).

Model food systems such as broth media and meat slurries are commonly used as heating menstruums in studies to develop predictive thermal inactivation models for *L. monocytogenes*. The compositional differences in those media can alter the influence of phosphates or other antimicrobials on thermal resistance of *L. monocytogenes* due to changes in intrinsic factors, including water activity, pH, and protein and/or fat content. In the

present study, the greater extent of thermal inactivation of *L. monocytogenes* in pork slurry compared to ground pork slurry could be partly attributed to differences in properties of each heating menstruum such as moisture content and pH. As expected, the D-values for *L. monocytogenes* were lower in pork slurry than in ground pork at all temperatures tested (Table 1). These results are consistent with those of previous studies, which reported greater rates of thermal destruction of *L. monocytogenes* when more free water was present in the heating menstruum (Boyle et al., 1990; Ollinger-Snyder et al., 1995).

The pH of the heating medium is considered as a major factor affecting the thermal resistance of bacteria. An assessment of the thermal inactivation of seven foodborne pathogens as a function of temperature, water activity, pH, and redox potential in synthetic media revealed that thermal inactivation increased with increasing water activity and decreasing pH (Reichart and Mohacsi-Farkas, 1994). In the present study, pork slurry and ground pork controls had pH values of 6.16 and 6.45, respectively. The addition of 0.25% or 0.5% SPP resulted in a greater drop in pH of pork slurry compared to that of ground pork (Table 1). Juneja and Eblen (1999) demonstrated that decreasing the pH of beef gravy from 8.0 to 4.0 resulted in a parallel decrease in the predicted D-value for *L. monocytogenes* at 55, 57.5, 60, 62.5, and 65° C. A theoretical interpretation of how pH affects thermal inactivation of bacteria was given by Reichart (1994), who described a linear relationship between pH and the logarithm of the D-values for *Escherichia coli*. The author explained that the D-value decreases linearly in the acid or alkaline range and has a maximum at the optimum pH for growth of the organism.

A wide variety of alkaline and acid phosphatases are present in the muscles of animals. Earlier studies have reported that native phosphatases in fresh meat and shrimp

were responsible for the observed breakdown of added tetrasodium pyrophosphate, sodium tripolyphosphate, and sodium polyphosphate glassy (Awad, 1968; Tenhet et al., 1981). Molins et al. (1985) demonstrated that the concentration of orthophosphates in fresh pork increased at the expense of added pyrophosphates. In the present study, the soluble orthophosphate content of ground pork or pork slurry increased with increasing concentration of added SPP. The concentration of soluble orthophosphates, derived from SPP added to ground pork, was two to three times greater than that of pork slurry with corresponding amounts of added SPP (Table 2). The lower accumulation of soluble orthophosphates in pork slurry could be attributed to lower concentrations of phosphatases that resulted from the ten-fold dilution of ground pork to prepare the pork slurry. Samples of pork slurry or ground pork with or without added SPP were analyzed for soluble orthophosphates within 60 min of preparation. This time frame is consistent with the time that elapses prior to heating of the inoculated samples of ground pork or pork slurry in heat inactivation experiments. Therefore, the residual SPP reported in this study represent a reasonably estimate of SPP concentration to which *L. monocytogenes* is exposed during heating of pork slurry or ground pork.

Some studies have demonstrated that sodium phosphates decrease the thermal resistance of Gram-positive pathogens, including *L. monocytogenes*, whereas others have reported that phosphates may protect *L. monocytogenes* from the destructive effect of heat. Juneja and Marmer (1998) demonstrated that SPP (0.1 or 0.3%) significantly ($P<0.05$) decreased the heat resistance of *Clostridium perfringens* in ground beef and turkey meat; both meats were sterilized by autoclaving at 121 $^{\circ}C$ for 15 min prior to adding SPP. Heat-induced hydrolysis may be involved in the loss of the antimicrobial properties of SPP in heat-

sterilized laboratory media (Molins and Kraft, 1984). Awad (1968) determined that inactivation of meat phosphatases by heat during cooking began at about 40° C and was complete at 60° C. Therefore, in the study conducted by Juneja and Marmer (1998), the heat sterilization of meats most likely inactivated the native phosphatases and spared the added SPP from degradation to orthophosphates. More recently, the sensitivity of *L. monocytogenes* to heating in a model food system (beef gravy) was shown to increase with increasing concentrations of SPP up to 0.2% (Juneja and Eblen, 1999). The results of those studies agree with results of the present study derived from the use of pork slurry but not ground pork, in which up to 0.5% SPP did not decrease the heat resistance of *L. monocytogenes* (Table 1). Yen et al. (1991) reported demonstrated that the addition of 0.4% sodium polyphosphates to ground pork failed to increase the thermal inactivation of *L. monocytogenes* compared to ground pork controls. In fact, the thermal inactivation of *L. monocytogenes* in ground pork that contained 0.4% sodium polyphosphates was 0.8 log CFU g^{-1} less than in ground pork alone. Those same authors concluded that the added polyphosphates significantly ($P<0.05$) protected *L. monocytogenes* from thermal destruction.

In conclusion, the D- and z-values reported in the present study indicate contrasting effects of SPP on the thermal inactivation of *L. monocytogenes* in pork slurry compared to ground pork. The intrinsic properties of each heating menstruum including differences in moisture content, pH, and phosphatase activity could account for variations in the thermal death time values reported in this study and in previous studies. The thermal death time values from this study further emphasize the importance of validating in real products, thermal inactivation data derived from use of model food systems. Thermal inactivation data from validation studies could be utilized to establish adequate heat processing regimes that

would minimize the potential danger of foodborne illness from ground meats contaminated with *L. monocytogenes*.

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Table 1. Heat resistance (D-values [a] in min) of a three-strain mixture *of Listeria monocytogenes* in pork slurry and ground pork that contained 0, 0.25, or 0.50% sodium pyrophosphate (SPP) at 55-62°C.

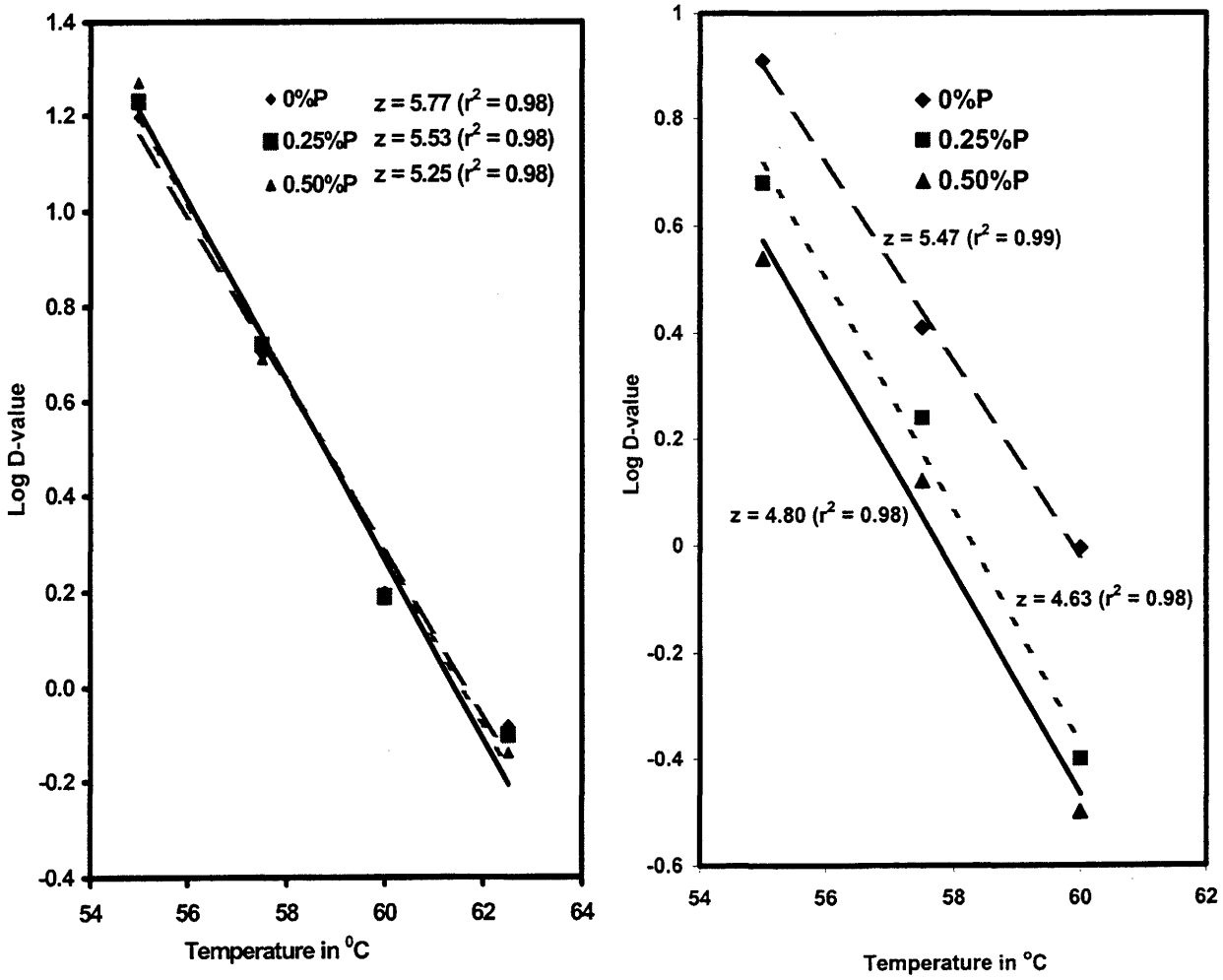
Heating Medium	pH	55°C	57.5°C	60°C	62.5°C
Pork Slurry w/o SPP	6.16 ± 0.22	8.15 ± 0.90^{b}	2.57 ± 0.13^{b}	0.99 ± 0.10^{b}	0.18 ± 0.04
Pork Slurry + 0.25% SPP	5.33 ± 0.04	4.75 ± 1.14^{c}	1.72 ± 0.48^{c}	0.40 ± 0.02^{c}	ND
Pork Slurry + 0.50% SPP	4.86 ± 0.04	3.51 ± 0.75^{c}	1.31 ± 0.31^{c}	0.31 ± 0.03^{c}	ND
Ground Pork w/o SPP	6.45 ± 0.12	15.72 ± 0.88^{d}	5.01 ± 0.21^{d}	1.60 ± 0.23^{d}	0.83 ± 0.20^{d}
Ground Pork + 0.25%SPP	6.15 ± 0.04	16.97 ± 2.60^{d}	5.28 ± 0.78^{d}	1.56 ± 0.15^{d}	0.80 ± 0.16^{d}
Ground Pork + 0.50% SPP	5.97 ± 0.04	18.61 ± 2.75^{d}	4.92 ± 0.42^{d}	1.55 ± 0.26^{d}	0.71 ± 0.24^{d}

[a] D-values are the means of three replications and expressed as mean ± standard deviation; means that have different superscripts within the same column are significantly different ($P<0.05$).
ND: No viable cells detected

Table 2. Amount[a] of soluble orthophosphates (μg g^{-1} of sample) derived from sodium pyrophosphate (SPP) added to samples of pork slurry and ground pork; samples analyzed 1hr after treatment.

	Soluble Orthophosphates (μg g^{-1})	Derived Orthophosphates (μg g^{-1})	% Loss[b]	% Residual[b]
Ground pork without SPP	1126 ± 51			
with 0.25% SPP	1374 ± 51	248 ± 28	0.04	0.21
with 0.50% SPP	1411 ± 28	285 ± 27	0.04	0.46
Slurry without SPP	116 ± 4			
with 0.25% SPP	193 ± 1	77 ± 5	0.01	0.24
with 0.50% SPP	271 ± 3	155 ± 5	0.02	0.48

[a]Values are the means of three replications and expressed as mean ± standard deviation
[b]Loss or residual SPP as % of sample weight; P_2O_5 content is 64.5% of SPP (manufacturer analysis)



A. B.

Fig.1. Thermal-death-time curves (z-values) for a three-strain mixture of *Listeria monocytogenes* in pork slurry (A) or in ground pork (B) which contained 0, 0.25, or 0.50% sodium pyrophosphate, at a temperature range of 55-60 °C(A) or at a temperature range of 55-62.5 °C(B). D-values used for determining the z-values, were the means of three replications.

A predictive model to determine the effects of temperature, sodium pyrophosphate, and sodium chloride on thermal inactivation of starved *Listeria monocytogenes* in pork slurry

A paper to be submitted in the Journal of Food Protection

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ABSTRACT

The effects and interactions of heating temperature (57.5 to 62.5 ° C), sodium pyrophosphate (SPP; 0 to 0.5%, wt/vol), and salt (NaCl; 0 to 6% wt/vol) on the thermal inactivation of starved *Listeria monocytogenes* ATCC 19116 in pork slurry were investigated. A split-split plot experimental design was used to compare 27 combinations of heating temperature, SPP, and NaCl levels. *L. monocytogenes* survivors were enumerated using tryptic soy agar supplemented with 0.6% yeast extract (TSAYE). Means of decimal reduction times (D-values) were modeled as a function of heating temperature, SPP, and NaCl levels. Increasing concentrations of SPP or NaCl in pork slurry protected starved *L. monocytogenes* from the destructive effect of heat. Combinations of 6.0% NaCl and SPP (0.25 or 0.5%) increased the thermal inactivation of the organism compared to 6% NaCl alone. All three variables interacted to affect thermal inactivation of *L. monocytogenes*. A mathematical model describing the combined effect of temperature, SPP, and NaCl levels on thermal inactivation of starved *L. monocytogenes* was developed. There was a high correlation ($R^2 = 0.97$) between D-values predicted by the model and those observed experimentally. The model can predict D-values for any combinations of temperature, SPP,

and NaCl that fall within the range of those tested. This predictive model can be used to assist food processors to design thermal processes that include an adequate margin of safety for controlling *L. monocytogenes* in processed meats.

INTRODUCTION

Listeria monocytogenes, is a foodborne of major concern to food processors as well as public health regulatory agencies because it is ubiquitous in the environment and can grow at refrigerator temperatures (27, 30). *L. monocytogenes* has been isolated from a variety of foods including raw and ready-to-eat meat, poultry, seafood, dairy products and vegetables (3, 8, 11, 13, 28, 37). Undercooked meat and poultry have been cited as sources of listeriosis, and investigations of outbreaks in many countries have linked this disease to consumption of contaminated turkey, chicken, and pork products (5, 30, 31). Listeriosis has a high human fatality rate (20 to 30%) (27, 33) and is the second leading cause of mortality from bacterial foodborne illness in the US (25). Based on the high fatality rate of foodborne listeriosis and the uncertainty of the infectious dose for immunocompromised individuals, U.S. regulatory agencies specify zero tolerance for *L. monocytogenes* in cooked and ready-to-eat foods (7, 30).

Thermal processing is one of the most common techniques used to control *L. monocytogenes* in foods. However, effective control of this organism is challenging to food processors due to its high heat tolerance compared to that of other non-spore forming foodborne pathogens (21) and the potential for post-processing contamination of foods in the

processing plant. Farber and Peterkin (10) identified post-processing contamination with *L. monocytogenes* as a major source of contamination of food products.

Previous studies involving *L. monocytogenes* demonstrated that the composition of the heating medium affected the thermal inactivation of this organism. For example, the heat resistance of *L. monocytogenes* was greater in ground pork than in pork slurry (18) and the heat resistance in meat slurry was greater than in phosphate buffer (2). In comminuted meats, the addition of phosphates, salt, or curing-salt mixes increased the thermotolerance of *L. monocytogenes* (22, 32, 39). Thermotolerance of *L. monocytogenes* is further increased when this organism has endured environmental stress such as sublethal heat shock, osmotic stress, acid or alkali, ethanol, hydrogen peroxide, and starvation (9, 15, 19, 20, 23, 34). Consequently, heat-processing procedures for foods should be designed to destroy the most heat resistant state of *L. monocytogenes* and thus provide an adequate margin of safety against this pathogen. Doyle et al. (6) suggested that there is need for additional data on D- and z-values using stressed *L. monocytogenes* in specific food products.

Predictive modeling of microbial inactivation in foods can be used to determine the effects of interactions between two or more food parameters on microbial heat resistance. In addition, predictive models permit estimation of the effect of various combinations of parameters that have not been tested definitively (38). Heat inactivation predictive models predict the survival of the target organism within a defined range of food formulation variables. These models, once validated in actual foods, can greatly assist processors in the formulation or reformulation of food products that are safe for the consumer (29).

Microbial starvation is important to the food industry because water used for cleaning and rinsing food contact surfaces generally provides a low nutrient environment for

microorganisms. Certainly, the stress from prolonged deprivation of nutrients can induce increased microbial resistance to subsequent chemical and physical challenges (14). There is however a scarcity of published information on thermal inactivation of starved *L. monocytogenes* within a range of food formulation variables, including phosphates and salt. Accordingly, the objective of this study was to assess the effects and interactions of heating temperature, sodium pyrophosphate (SPP), and sodium chloride (NaCl) on thermal inactivation of *L. monocytogenes* in a model food system (pork slurry) following nutrient starvation. The data were subsequently used to develop a quadratic linear response model that describes the combined effects and interactions of these parameters on the thermal inactivation of *L. monocytogenes*.

MATERIALS AND METHODS

Culture and culture conditions. *L. monocytogenes* ATCC 19116, a chicken isolate obtained from the culture collection of the Department of Microbiology at Iowa State University, was used throughout this experiment. The culture was maintained as frozen (-70° C) stock in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 10% glycerol until used. Prior to each experiment, the stock culture was transferred twice in 10 ml of TSBYE and incubated at 35° C for 18 h.

Nutrient starvation. A portion (1.0 ml) of *L. monocytogenes* stock culture was transferred to 100 ml of TSBYE in a screw-capped 250-ml Erlenmeyer flask. The inoculated medium was incubated at 35° C with shaking at 150 rpm in a shaker/incubator (New Brunswick Scientific Co. Inc., Edison, N.J.). Growth was monitored via optical density (OD) measurements using a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY).

When the OD_{600} of the culture was about 0.25, exponential phase cells were harvested by centrifugation (9000 x g, 10 min, 4° C) and washed once in 0.85% NaCl (w/v). The cell pellets were re-suspended in fresh 0.85% NaCl (w/v). Cells were starved by holding the cell suspension statically in a screw-capped 250-ml flask at 25° C. $D_{60}{}^{\circ}C$-values were determined at set time intervals to establish the time of starvation that induced maximal heat resistance of the cells.

Preparation and inoculation of pork slurry. A batch of fresh ground pork (80% lean) from the Meat Science Lab at Iowa State University was divided into 454-g portions, vacuum-packaged (Multivac, Wolfertschwenden, Germany) then frozen (- 20° C). The packages of frozen ground pork were sterilized by irradiation at 40 kGy then immediately stored in a laboratory freezer. The day before each heating experiment, one package of ground pork was thawed overnight in a refrigerator (4° C). Pork slurry was prepared by aseptically transferring 62.5 g thawed ground pork to 337.5 ml of 0.1% sterile peptone water in a sterile mesh-line filter bag (Celsis Inc., Evanston, IL, US). The mixture was pummeled using a Seward Stomacher 400 Lab-blender (Seward Ltd., London, England) for 1 minute at medium speed. Ground pork from a separate package of the same batch was used for each replicate experiment. Stock solutions of SPP and NaCl, prepared using 0.1% peptone water, were sterilized by filtration through 0.22 um pore size filters. Aliquots (20 ml) of sterile SPP or NaCl stock solutions were aseptically added to 80-ml portions of sterile pork slurry to obtain final concentrations (%w/v) of SPP (0%, 0.25%, or 0.5%), NaCl (0%, 3%, or 6%), or various combinations of SPP and NaCl. The pH of samples were measured using an Orion Model 525 pH meter (Orion Research Inc., Boston MA) fitted with a combination electrode.

Portions (25 ml) of sterile pork slurry, with or without SPP, NaCl, or combinations, were inoculated with 0.25 ml of exponential- or stationary-phase cells (control) or cells that were starved for 10 days in 0.85% saline to give a final cell concentration of approximately 10^6 CFU/ml. Exponential phase cells were used as controls at all three heating temperatures whereas, both exponential- and stationary-phase cells were used as controls at one heating temperature (60° C). The inoculated pork slurry solution was aseptically dispensed into sterile Pyrex thermal-death-time (TDT) tubes (2.5 ml/tube). The TDT tubes were heat-sealed by using a type 3A blowpipe (Veriflow Corp., Richmond, CA) and then held in a laboratory refrigerator (4° C) for at least 15 min to allow temperature equilibration before heating at 57.5, 60, or 62.5° C.

Thermal inactivation. Samples were heated within 3 h of inoculation. Sealed TDT tubes of pork slurry were submerged in a thermostat-controlled Isotemp 1013S heater water bath (Fisher Scientific, Pittsburgh, PA) stabilized at the appropriate heating temperature. The internal temperature of the samples was continuously monitored by a copper-constantan thermocouple inserted, prior to sealing, at the center of an uninoculated sample in a TDT tube. Thermocouple readings were measured using a digital meter. The average number of viable cells in unheated samples represented the number of *L. monocytogenes* present at time zero. TDT tubes were removed at set time intervals and immediately submerged in 50/50 ice/water slush (0° C) for about 5 min. Samples were analyzed within 30 min for *L. monocytogenes* survivors using procedures described below.

Microbiological analysis. TDT tubes (2 tubes per treatment) were opened and their contents were aseptically pooled in separate sterile test tubes. One ml from each pooled sample was removed and serially diluted in sterile 0.1% peptone. Samples (0.1 ml) of

appropriate dilutions were surface-plated, in duplicate, onto plates of TSAYE. In instances when increased sensitivity was required, 1.0 ml samples of undiluted slurry were plated directly onto TSAYE plates. All inoculated plates were incubated at 35° C. Bacterial colonies were counted at 72 h then checked for presumptive *L. monocytogenes* as described by McClain and Lee (24).

Calculation of D-values. The D-values (time for 90% reduction in viable cells) expressed in minutes, were determined by plotting the $\log_{10}$ number of survivors versus time for each heating temperature using Microsoft Excel 98 Software (Microsoft Inc., Redmond, WA). Linear regression analysis (26) was used to determine the line of best fit for data on each survivor curve. The D-value for each temperature/phosphate/salt treatment combination was determined by calculating the negative reciprocal of the slope of the survivor curve.

Experimental design and analysis. A split-split plot design was used to assess the effects and interactions of heating temperature (57.5° C, 60° C, 62.5° C), phosphates (0%, 0.25%, 0.50%), and salt (0%, 3.0%, 6.0%) on thermal inactivation of *L. monocytogenes*. Of all 27 variable combinations, 18 were replicated three times and 9 (62.5° C– treated samples) were replicated twice. For each replicate, temperature was randomly assigned to days, phosphate level was randomly assigned to heating run, and salt was randomly assigned to TDT tube. Samples of starved cells were heated along with those of washed exponential cells. At 57.5° C, samples were removed at 0, 8, 12, 16, 24, 36, and 48 min. At 60° C, samples were pulled out at 0, 2, 4, and 8 min. At 62.5°C, samples were removed at 0, 0.5, 0.75, 1.5, 3.0, and 4.5 min.

Statistical modeling. Means of D-values were modeled as a function of the heating temperature, SPP, and NaCl concentrations. D-values were transformed into the natural

logarithm form to stabilize the variance of the response parameter. The GLM procedure (SAS Institute Inc., Cary, N.C.) was used to analyze the D-values. A model with all quadratic terms was used. Effects of individual parameter and effects of interactions between parameters were analyzed by t-test. Significant effects were considered and a quadratic response model was developed. A correlation coefficient between the model and the observed data was computed. Predictions of D-values and 95% prediction interval were obtained within the range of experimental conditions.

RESULTS AND DISCUSSION

Preliminary studies in our laboratory indicated that six of seven test strains of *L. monocytogenes*, starved for 14 days in 0.85% NaCl (25° C), exhibited a dramatic increase in heat resistance at approximately10 days of starvation. Starved *L. monocytogenes* ATCC 19116 had the highest D-value at 60° C and consistently exhibited higher D-values than non-starved exponential-phase or stationary-phase cells when heated (57.5 to 62.5° C) in pork slurry with or without added SPP or NaCl (data not shown). Therefore, we selected this organism for further studies on thermal inactivation. During starvation, this strain of *L. monocytogenes* exhibited a steady increase in heat resistance from day 0 to day 8 (D_{60}-value of 0.45 at day 0 and 0.72 on day 8). Between days 8 and 10 the heat resistance further increased and reached a maximum D-value of 1.16 at day 10 (Fig. 1). *L. monocytogenes* ATCC 19116, starved for 10 days in 0.85% NaCl, was used in development of a mathematical model that predicts the effects and interactions of temperature, NaCl, and SPP, on the thermal inactivation of *L. monocytogenes*. Starvation of *L. monocytogenes* in the laboratory is analogous to this organism surviving without nutrients on equipment surfaces or

in the processing plant environment for an extended time period that is long enough to elicit the stress response.

A mathematical model that predicts the D-value of starved *L. monocytogenes* as a function of heating temperature, SPP, and NaCl was developed. Parameters and interactions between parameters were considered for their probability to significantly influence the model. A quadratic linear model was generated and the coefficients obtained for the model were as follows.

For starved cells, $\log_e$ D-value = 77. 0248 – 2.0737 (temp) + 0.0131 $(\text{temp})^2$

$$-16.5308\ (\text{SPP}) + 0.2599\ (\text{NaCl})$$

$$+ 0.2887\ (\text{temp})\ (\text{SPP})$$

$$- 0.3105\ (\text{SPP})\ (\text{NaCl})$$

The above multiple-regression equation for the $\log_e$ D-values yielded coefficients of correlation (R^2) of 0.97 and sum of squares error of 2.09. The equation is based on 27 unique combinations of temperature, SPP, and NaCl and can predict D-values for changes in parameter values (within the range tested) from any combination of these three environmental factors.

Table 1 shows the D-values of *L. monocytogenes*, based on survivor curves generated using the quadratic model for variables of temperature, NaCl and SPP levels. There was a very good fit ($R^2 = 0.9795$) between D-values predicted by the model and those of the observed experimentally (Fig. 2). Therefore, the model gives a valid description of the data that were used to generate it.

Survivor curves exhibited a linear decrease in numbers of *L. monocytogenes* as heating time increased; however, at 57.5° C the survivor curves initially showed a shoulder or

lag period prior to a linear decline. The "shoulder effect" has been attributed to poor heat transfer through the heating medium. In addition, this effect may involve an initial requirement for the bacterial cells to sustain a certain amount of injury before the onset of first order inactivation kinetics in the $\log_{10}$ numbers of surviving cells (12). Calculation of D-values from the linear portion of survivor curves, while disregarding "shoulders" or lag periods, could result in an underestimation of the time needed to attain a desired reduction in numbers of a microorganism. Therefore, in the present study "shoulders" in survivor curves for *L. monocytogenes* in pork slurry heated at 57.5° C were accounted for when calculating D-values.

Figure 3 shows the effects of combinations of SPP and heating temperature on thermal inactivation of starved *L. monocytogenes* in pork slurry. Inactivation of *L. monocytogenes* increased as heating temperature increased. However, within each heating temperature the heat resistance of the organism increased with increasing SPP concentrations. For example, the predicted D-values at 60° C increased (50.6%) from 0.79 min in pork slurry without SPP to 1.19 min in slurry with 0.5% SPP. At 62.5° C, predicted D-values increased (112%) from 0.24 min in pork slurry without SPP to 0.51 min in slurry with 0.5% SPP. These results agree with those of a previous study (39) in which *L. monocytogenes* exhibited greater heat resistance in ground pork containing 0.2% sodium tripolyphosphate and 0.2% sodium hexametaphosphate compared to pork without added phosphates. In that study, survival of *L. monocytogenes* was 0.8 $\log_{10}$ CFU/g greater in ground pork with added phosphates than in ground pork alone following heating at 60° C. Juneja and Eblen (16) also reported increased D-values at 55 to 65° C for *L. monocytogenes* Scott A in beef gravy (pH 4.0 or 8.0) containing 3% SPP. In contrast, Unda et al. (36)

indicated that a phosphate blend reduced the survival of *L. monocytogenes* in beef roasts that were pumped with brine and phosphates and cooked once or twice to 62.8° C. However, those authors did not state whether the phosphate blend reduced survival of *L. monocytogenes* during heating or whether it inhibited survivors during refrigerated storage of the beef roasts.

Figure 4 shows the effects of combinations of NaCl and heating temperature on thermal inactivation of starved *L. monocytogenes* in pork slurry. The addition of 3% and 6% NaCl to pork slurry increased the D-values for the organism by about 118% and 120% respectively, irrespective of the heating temperatures used in the present study. Juneja and Eblen (16) demonstrated that the addition of NaCl (1.5 to 6%) to beef gravy protected against thermal inactivation of *L. monocytogenes* at all temperatures (55 to 65° C) tested in their study. Similar findings were reported by Yen et al. (39) who demonstrated that the heat resistance of *L. monocytogenes* increased when the organism was heated at 60° C in ground pork with added NaCl. Generally, our results on thermal inactivation of starved *L. monocytogenes* in pork slurry with added NaCl are consistent with those of other studies on thermal inactivation of non-starved cells of this pathogen in broth, pork, and beef (4, 11, 22, 39). Those studies reported a decrease in thermal inactivation of *L. monocytogenes* in various meat blends containing 3 to 4 % NaCl. Therefore, the results of the present study can be used to predict the thermal inactivation of *L. monocytogenes* as affected by added NaCl in meat products. The influence of salts on thermal inactivation of microorganisms is largely due to reduced water activity and increased osmotic pressure of the heating medium (35).

Figures 5a, 5b, and 5c show the effects and interaction of 0 to 0.5% SPP and 0 to 6% NaCl on the predicted D-values of starved *L. monocytogenes* in pork slurry at all heating

temperatures tested. The addition of NaCl to pork slurry that contained SPP dramatically increased the extent of heat resistance irrespective of heating temperature. When heated at 60° C in pork slurry containing 0.25% SPP, starved *L. monocytogenes* had a predicted D-value of 0.94 min; however, the addition of 3% and 6% NaCl increased the D-value to1.62 min and 2.80 min, respectively. Previous studies have demonstrated the protective effect of NaCl in the presence of SPP (11, 22). Our findings are consistent with those previous studies (11, 22) and indicate that additives such as NaCl in processed meats may protect starved *L. monocytogenes* from the lethal effects of heat. Also, we observed that the protection of starved *L. monocytogenes* against heat inactivation occurred despite the decreases in pH of the pork slurry from the addition of SPP or NaCl.

Table 2 shows the changes in pH of the pork slurry from added SPP, NaCl, or combinations. The pH of pork slurry without added SPP or NaCl was 7.15. Addition of SPP, NaCl or combinations decreased the pH of the pork slurry. For example the pH of pork slurry that contained 6% NaCl, 0.5 % SPP, or 6% NaCl plus 0.5 % SPP decreased to 6.52, 5.38, or 4.81, respectively. Although pH was not one of the variables included in the model, the influence of these pH differences on thermal inactivation of *L. monocytogenes* in the pork slurry cannot be discounted. Juneja and Eblen (16) reported a parallel decrease in D-values for *L. monocytogenes* in beef gravy as the pH of the gravy decreased from 8.0 to 4.0. In the present study, the increased D-values observed for starved *L. monocytogenes* in pork slurry despite the relative decreases in pH were most likely due to the protective effect of added SPP, NaCl or combinations.

Figure 6 shows the predictive relative effect of various levels of SPP and NaCl on the heat sensitivity of starved *L. monocytogenes*. The z-value calculated from predicted D-

values for this organism in pork slurry with no SPP or NaCl was 4.0° C. The single or combined addition of SPP and NaCl to pork slurry resulted in higher z-values for starved *L. monocytogenes*. Pork slurry with 3% or 6% NaCl slightly increased the z-value to 4.6° C whereas increasing levels of SPP alone resulted in a corresponding increase in z-value. For example, z-values of starved *L. monocytogenes* in pork slurry with 0.25 and 0.5% SPP increased to 5.6 and 6.7° C, respectively. In the present study, the increase in z-values for starved *L. monocytogenes* in pork slurry with added SPP, NaCl, or combinations confirmed the findings of Schoeni et al. (32). Those authors reported that the z-value for a five-strain cocktail of *L. monocytogenes* increased from 7.9° C in ground beef roast to 10° C in a fermented beaker sausage that contained a cure premix (formulated for 3.3% NaCl, 1% sucrose, and 156 ppm sodium nitrite).

Our findings are not in agreement with those of Farber et al. (11) and Juneja and Eblen (16). Farber et al. (11) reported a decrease in z-values of *L. monocytogenes* from 4.92° C in ground meat to 3.5° C in ground meat with curing salts. Juneja and Eblen (16) reported that beef gravy with added NaCl (1.5 to 6.0%), SPP (0.1 to 0.3%) or combinations decreased the z-values for a four-strain cocktail of *L. monocytogenes*. The findings of the present study are also in disagreement with results of our previous work in which the presence of 0.25% or 0.5% SPP significantly ($P<0.05$) decreased the z-value for a three-strain mixture of non-starved *L. monocytogenes* (ATCC 19116, V7, and Scott A) in pork slurry (18). A plausible explanation for these contradictory findings could be that could be that *L. monocytogenes* cells that have endured certain environmental stresses, such as acidity or starvation, are more resistant to subsequent heat treatment and are therefore more tolerant to changes in heating temperature. For example, in the study conducted by Schoeni et al.

(32), a sausage mixture was inoculated with a five-strain cocktail of *L. monocytogenes* along with a *Pediococcus acidilactici* starter culture. Prior to heating, the inoculated sausage was held for about16 to 20 h at 32.2° C until the pH reached 4.8 to 5.0. The exposure of *L. monocytogenes* to decreasing pH in fermented beaker sausage for 16 to 20 h could have elicited the stress response that contributed to increased z-values reported for this organism. Based on the results of the present study, larger changes in heating temperature are necessary to obtain 90% reduction in D-value when starved *L. monocytogenes* ATCC 19116 is heated in pork slurry with increasing levels of SPP or NaCl. Lou and Yousef (19) demonstrated that adaptation *of L. monocytogenes* Scott A to certain environmental stresses including acid, starvation, ethanol, and hydrogen peroxide significantly increased the heat resistance of this pathogen.

The present study reports the development of a mathematical model that describes and predicts the effects and interactions of heating temperature, SPP, and NaCl levels on thermal inactivation of starved *L. monocytogenes*. Starvation causes stress on a bacterial population (17) that can induce cross-protection against subsequent unrelated stress (1). As non stress-adapted bacteria are commonly used in food safety studies, predictive models that use such data are likely to underestimate the actual ability of stress-adapted pathogens to survive food preservation methods. The predictive model developed in this study can assist food processors in designing thermal processes with an adequate margin of safety for effective control of *L. monocytogenes* in processed meats.

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TABLE 1. Observed and predicted D-values at 57.5 to 62.5°C of starved *L. monocytogenes* ATCC 19116 in pork slurry supplemented with sodium pyrophosphate (SPP) (0.0 to 0.5% w/v) and NaCl (0.0 to 6.0%, w/v)

Temperature (° C)	SPP (%)	NaCl (%)	D-value observed[a]	D-value predicted
57.5	0	0	2.79(0.05)	2.93
57.5	0	3	7.75(0.48)	6.40
57.5	0	6	14.59(0.99)	13.95
57.5	0.25	0	3.42(0.33)	2.98
57.5	0.25	3	4.48(0.28)	5.16
57.5	0.25	6	8.20(1.05)	8.91
57.5	0.5	0	2.78(0.07)	3.03
57.5	0.5	3	4.03(0.03)	4.16
57.5	0.5	6	6.05(0.25)	5.69
60	0	0	0.77(0.15)	0.79
60	0	3	1.56(0.32)	1.68
60	0	6	3.50(0.46)	3.66
60	0.25	0	0.97(0.14)	0.94
60	0.25	3	1.57(0.14)	1.62
60	0.25	6	2.58(0.03)	2.80
60	0.5	0	1.10(0.09)	1.19
60	0.5	3	1.44(0.23)	1.56
60	0.5	6	3.07(0.35)	2.14
62.5	0	0	0.21(0.08)	0.24
62.5	0	3	0.54(0.04)	0.52
62.5	0	6	1.28(0.05)	1.13
62.5	0.25	0	0.44(0.10)	0.35
62.5	0.25	3	0.76(0.21)	0.60
62.5	0.25	6	0.88(0.00)	1.04
62.5	0.5	0	0.59(0.22)	0.51
62.5	0.5	3	0.58(0.04)	0.69
62.5	0.5	6	0.76(0.06)	0.95

[a] Values represent means (standard deviations) of 27 variable combinations. Eighteen combinations were replicated three times; 9 combinations (62.5 ^{O}C) were replicated twice.

TABLE 2. pH values[a] for pork slurry with or without added sodium pyrophosphate (SPP), sodium chloride (NaCl), or combinations

	NaCl (% w/v)		
SPP (% w/v)	0.0	3.0	6.0
0.0	7.15 (±0.05)	6.67 (± 0.02)	6.52 (± 0.02)
0.25	5.85 (± 0.28)	5.35 (±0.32)	5.19 (± 0.30)
0.50	5.38 (±0.20)	4.98 (± 0.21)	4.81 (± 0.22)

[a] pH values are the means of three replications and expressed as mean ± standard deviation.

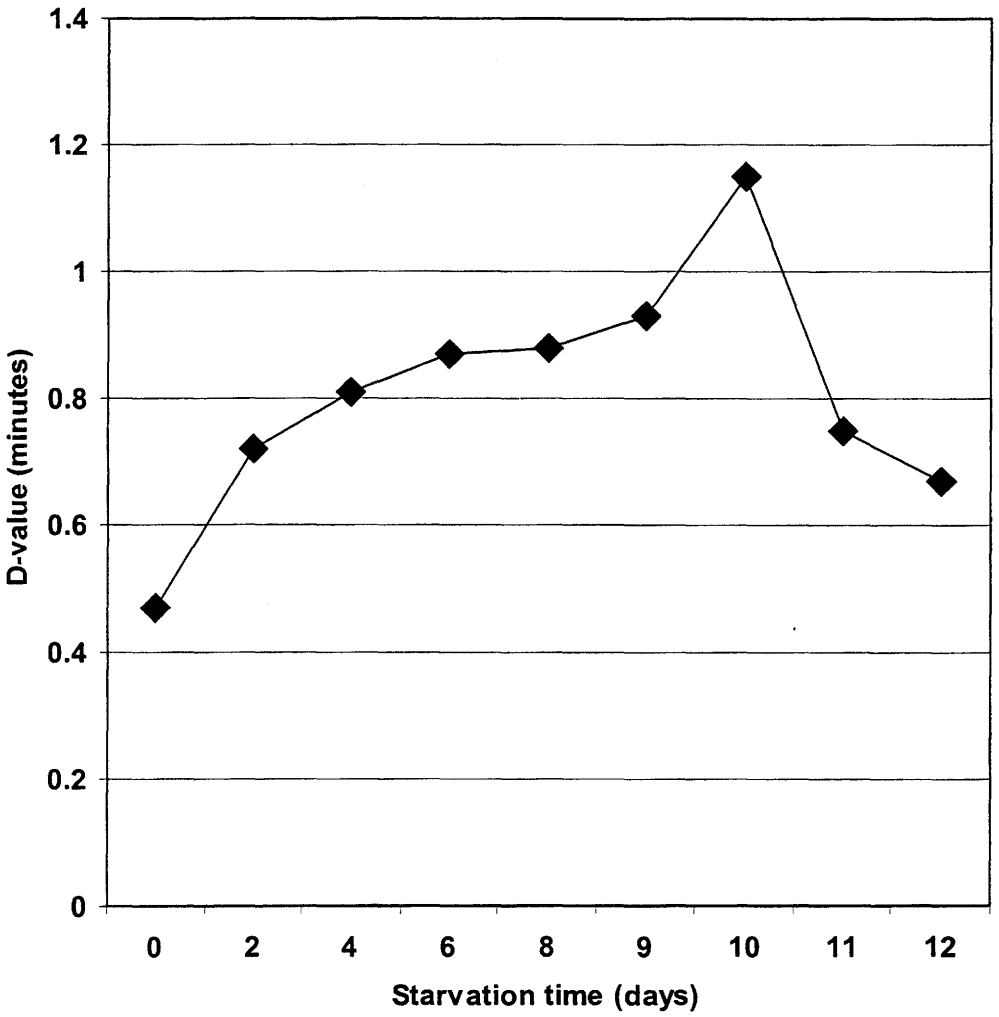

Figure 1. Heat resistance (D-values) of L. monocytogenes ATCC 19116 during starvation for 12 days in 0.85% (w/v) NaCl at 25° C. D-values were derived from the thermal death rate curves of survivors following heating in 0.85% (w/v) NaCl at 60° C.

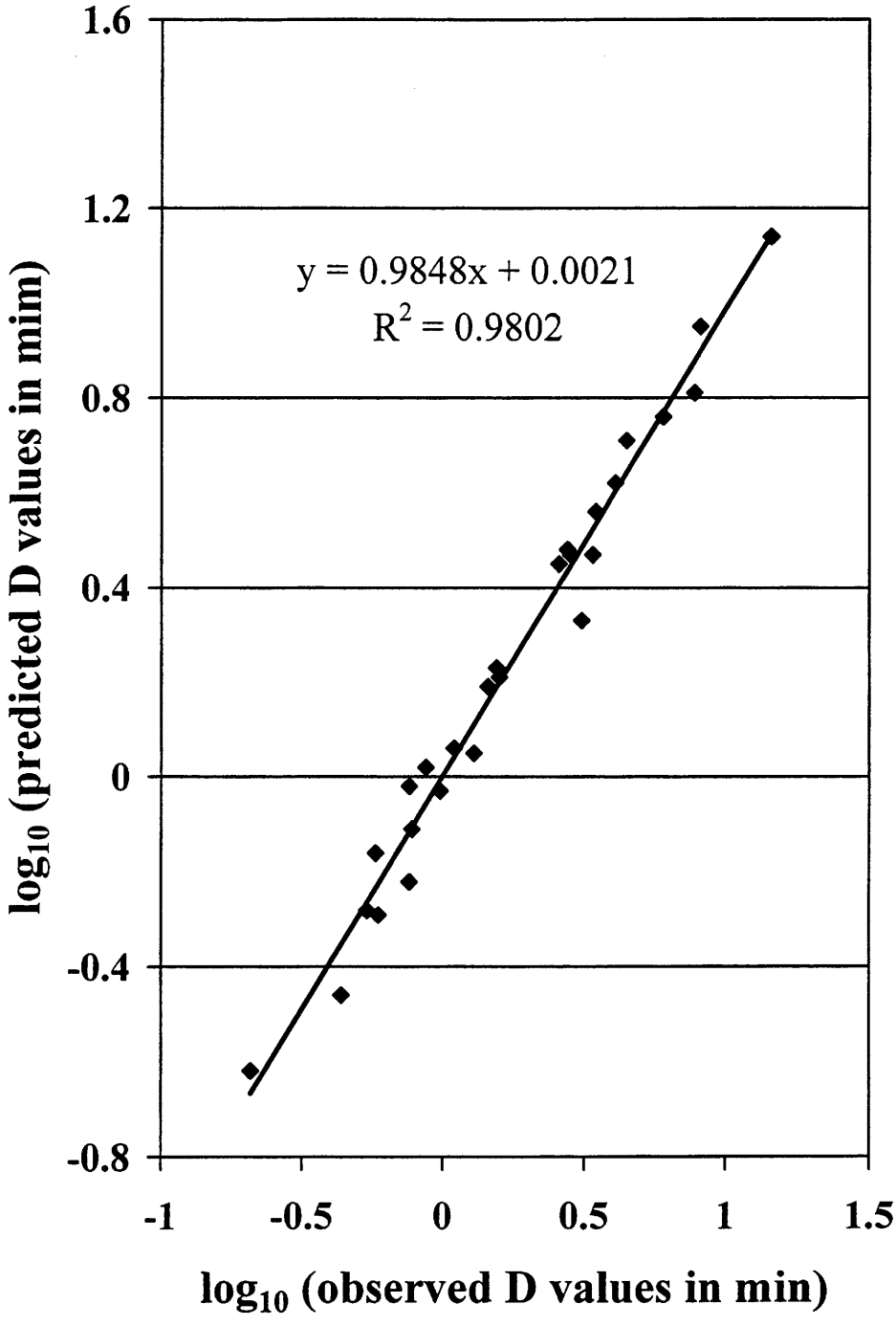

Figure 2. Agreement between predicted and observed D-values for starved *L. monocytogenes* ATCC 19116 in pork slurry.

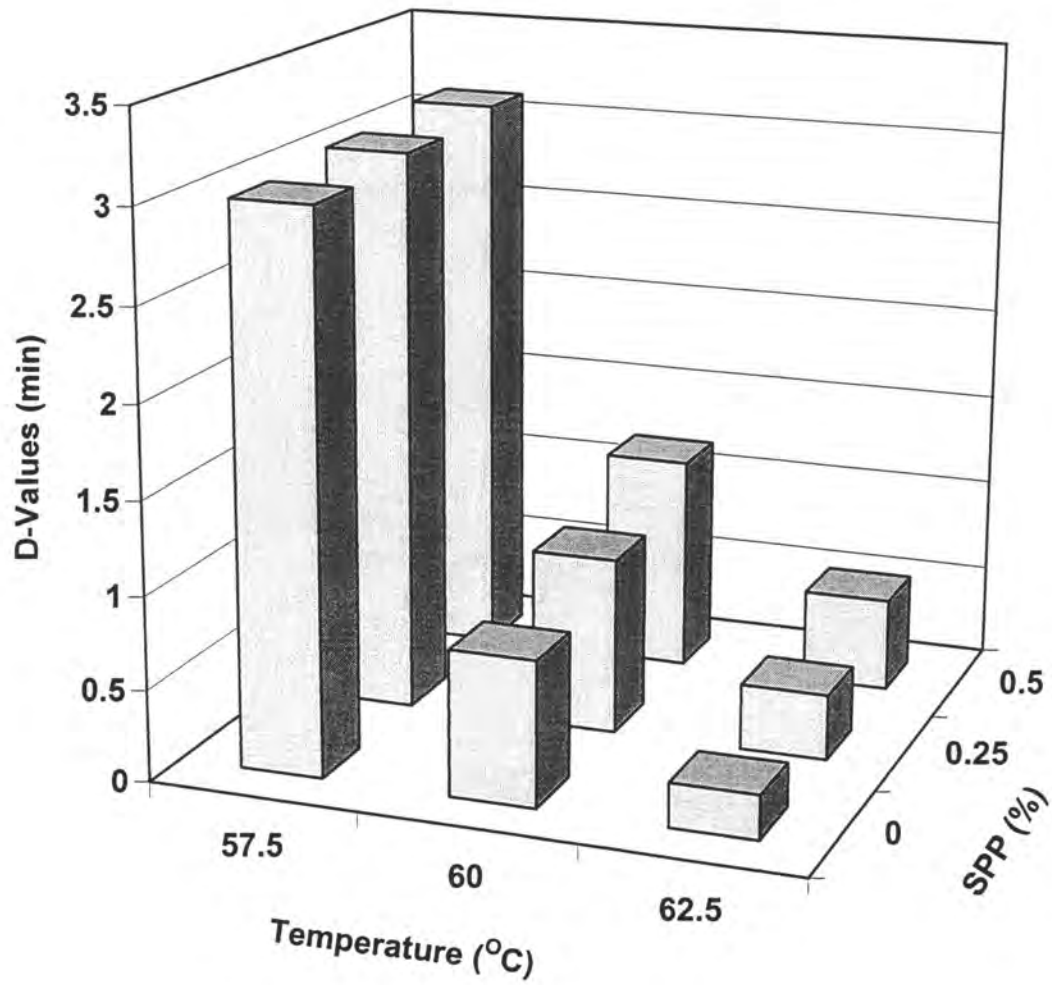

Figure 3. Combined effect of SPP and heating temperature on the predicted D-values of starved L. monocytogenes ATCC 19116 in pork slurry.

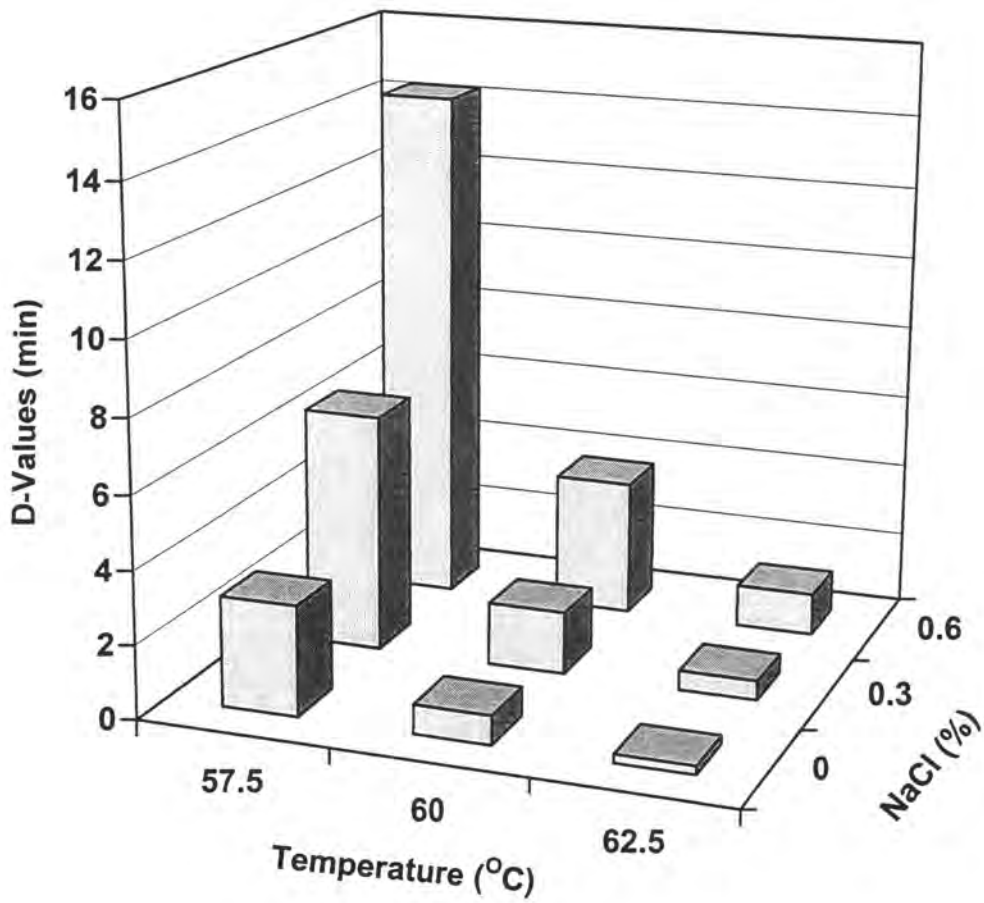

Figure 4. Combined effect of NaCl and heating temperature on the predicted D-values of starved L. monocytogenes ATCC 19116 in pork slurry.

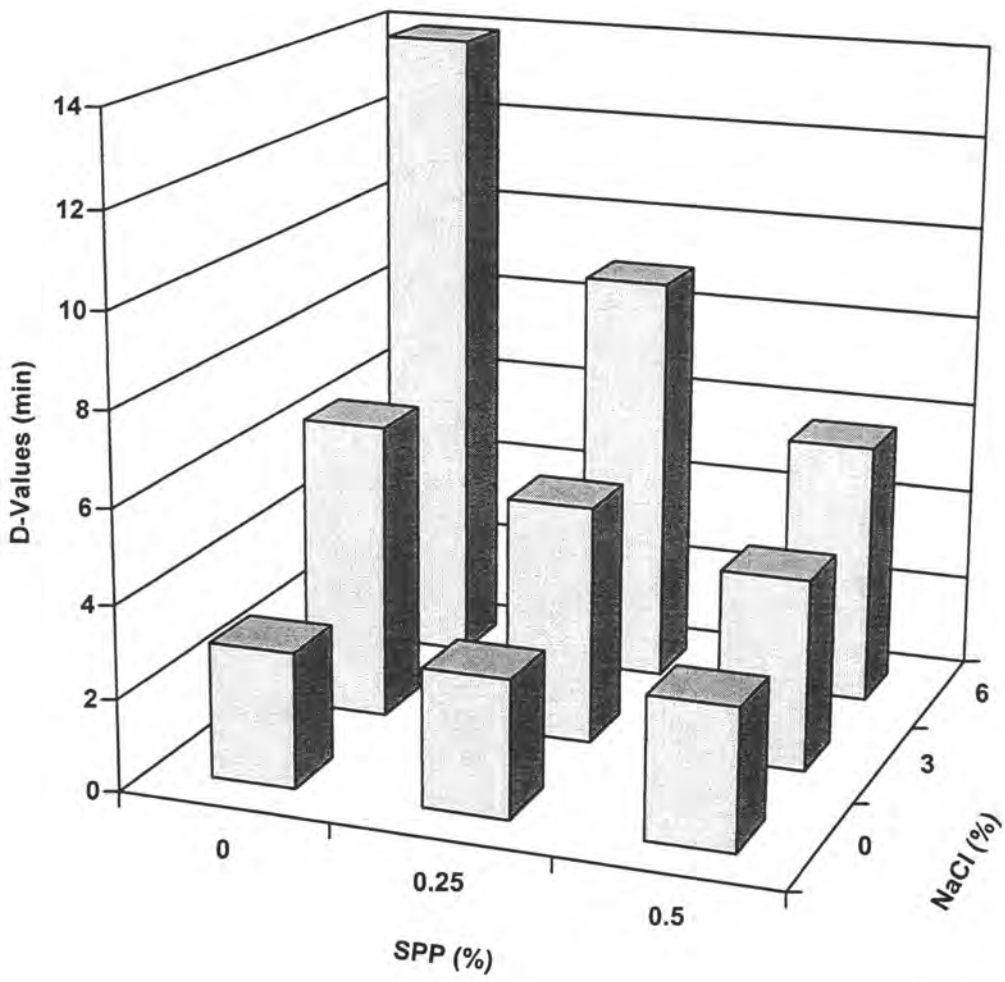

Figure 5a. Effects and interactions of SPP and NaCl on the predicted D-values of starved *L. monocytogenes* ATCC 19116 at 57.5° C.

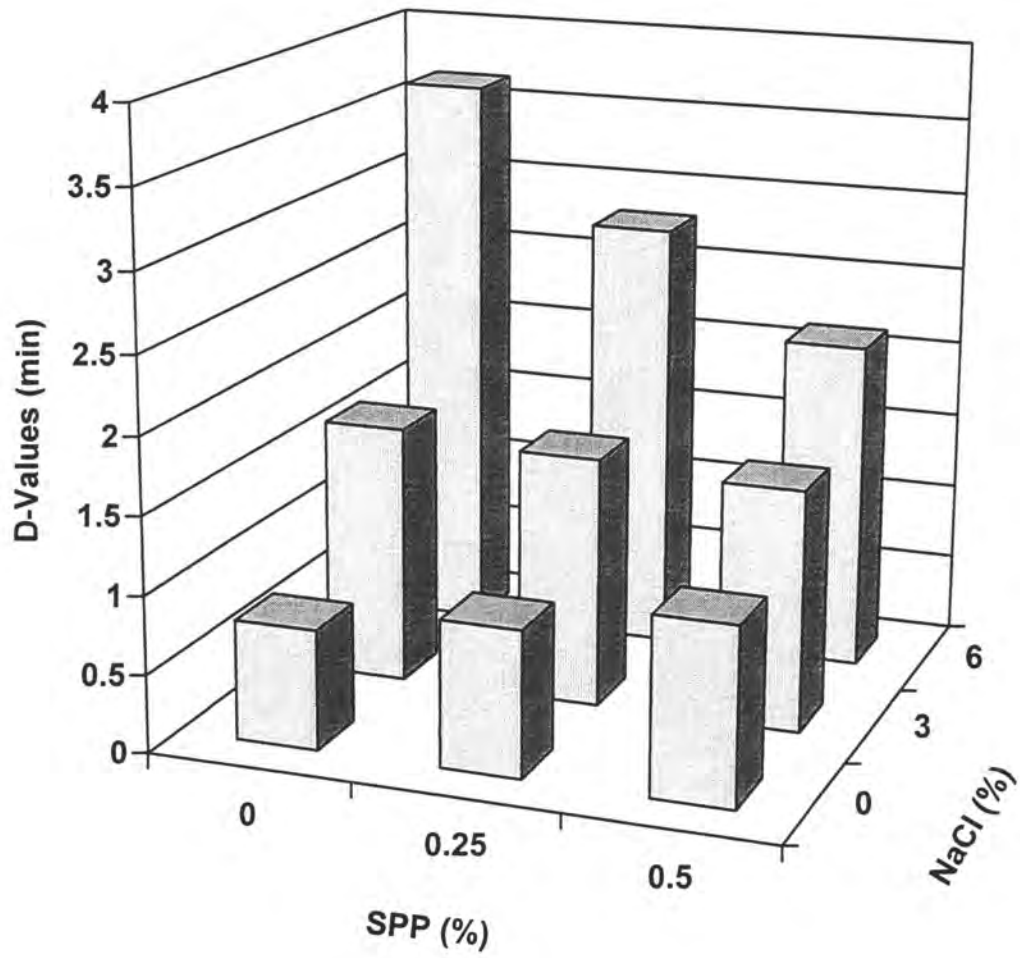

Figure 5b. Effects and interactions of SPP and NaCl on the predicted D-values of starved *L. monocytogenes* ATCC 19116 at 60° C.

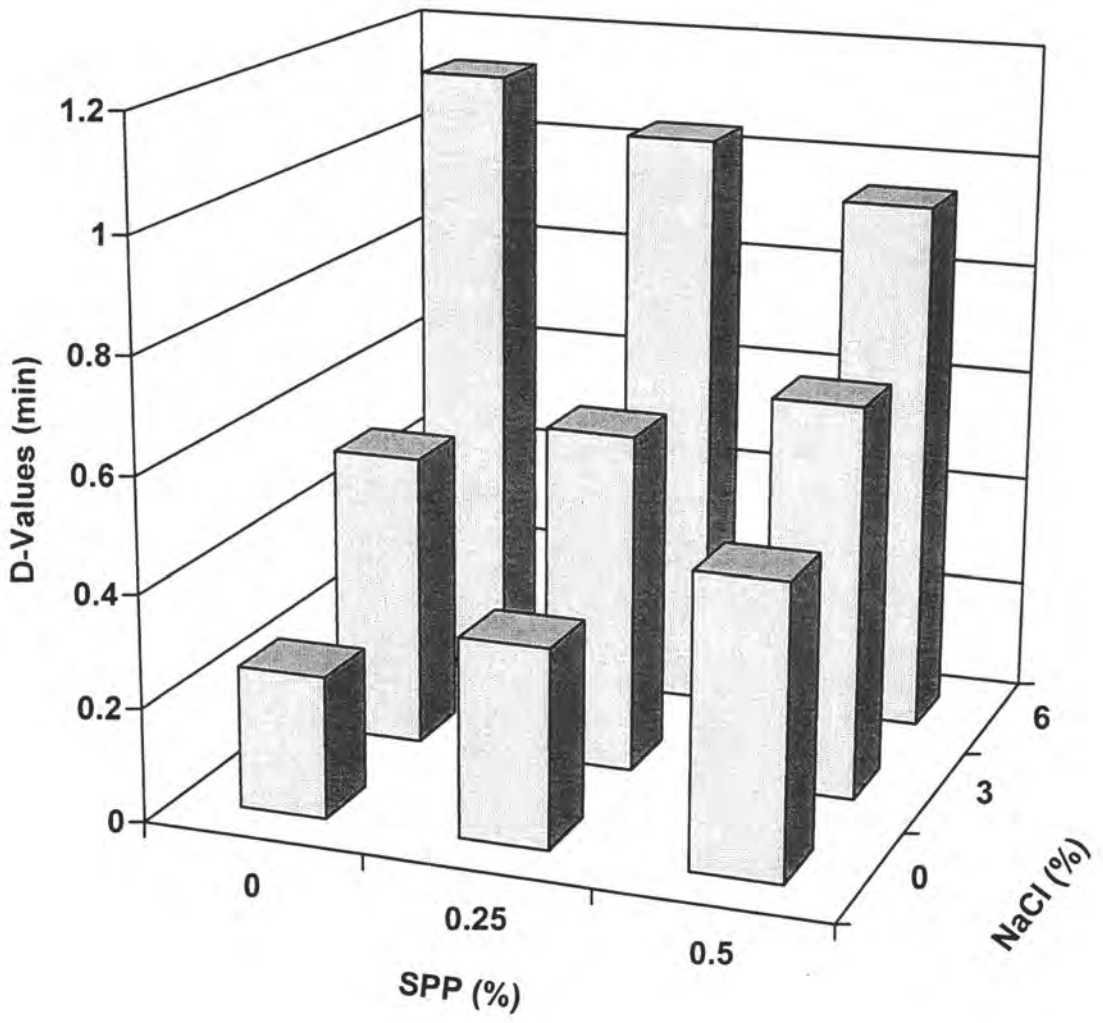

Figure 5c. Effects and interactions of SPP and NaCl on the predicted D-values of starved *L. monocytogenes* ATCC 19116 at 62.5° C.

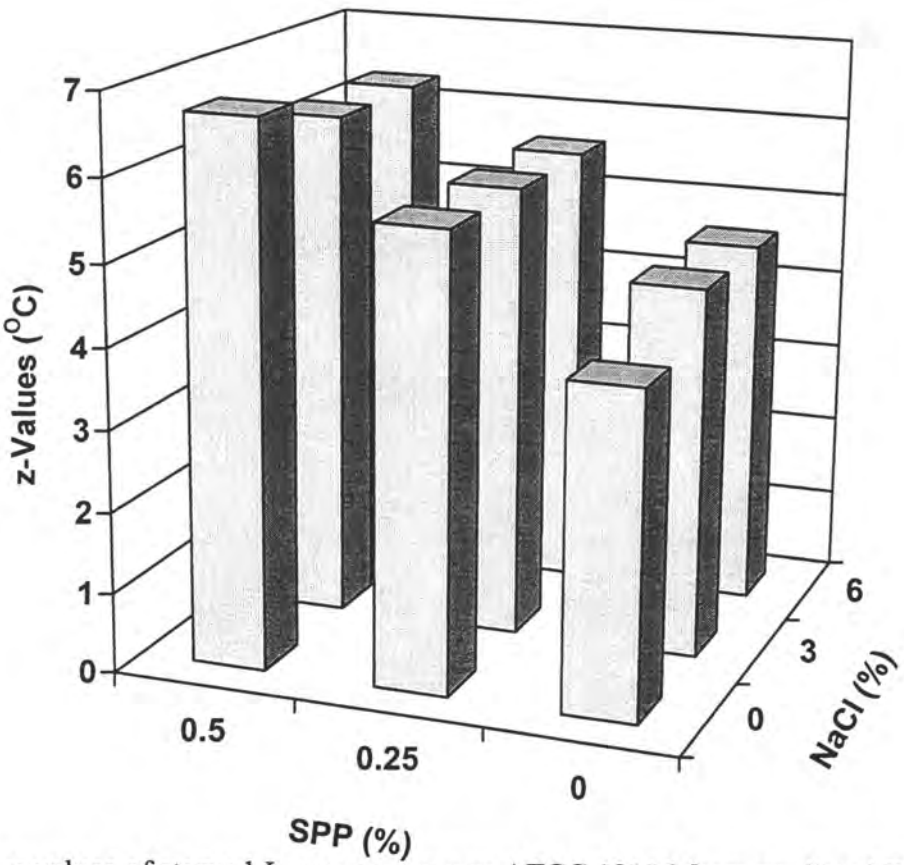

Figure 6. The z-values of starved *L. monocytogenes* ATCC 19116 from predicted D-values obtained in pork slurry with added SPP (0.0 to 0.5%, w/v) and NaCl (0.0 to 6.0%, w/v).

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