

**EFFECT OF POLYPHOSPHATES AND NaCl ON
AEROMONAS HYDROPHILA K144**

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ABSTRACT

Polyphosphates are multifunctional ingredients added to many foods, particularly meat products. In addition to their moisture-binding properties, polyphosphates have been reported to inhibit various bacteria. In the current study, four food-grade polyphosphates were evaluated for their effects on the growth of Aeromonas hydrophila K144 in both a model system and a food system. Since polyphosphates can interact with other food factors, effect of these compounds were studied at different NaCl levels and temperatures. The model system was BHI Broth modified by the addition of polyphosphates (sodium pyrophosphate, sodium tripolyphosphate, Hexaphos, or Sodaphos) and NaCl; incubation was aerobic at 5 or 28C. The food system was ground pork with NaCl and polyphosphate added and inoculated with A. hydrophila. Individually, the polyphosphates were relatively noninhibitory in both BHI broth and ground pork, but NaCl and temperature interacted with the polyphosphates in the model system to increase generation and lag times of A. hydrophila. In BHI broth, a combination of 2% of any of the polyphosphates tested and 3.5% NaCl inactivated the bacterium; this inactivation was temperature-dependent. By both a plating system and electron microscopy, the polyphosphate-NaCl combination was shown to injure the bacterium. In the ground pork, the polyphosphate-NaCl combination limited growth of the bacterium during refrigerated storage. These results suggest that polyphosphates could be useful to control the presence of A. hydrophila in certain foods.

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²Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

INTRODUCTION

Aeromonas hydrophila is a facultatively anaerobic, Gram-negative, oxidase-positive bacterium widely distributed in the environment, particularly in water supplies (Hazen *et al.* 1978) and foods (Palumbo *et al.* 1985). Its ability to grow at $\leq 5\text{C}$ (Palumbo and Buchanan 1988), a temperature traditionally used to prevent the growth of foodborne pathogens, indicates that factors other than low temperature holding must be used to control the growth of this organism in foods. Previous work from our laboratory has indicated that low pH values and increased NaCl levels combined with low holding temperature can be used to limit the growth of *A. hydrophila* (Palumbo *et al.* 1991, 1992).

Polyphosphates function in meat products and other foods to enhance moisture binding (Ellinger 1972). Polyphosphates have been shown to inhibit the growth of various bacteria (Elliott *et al.* 1964; Firstenberg-Eden *et al.* 1981; Knabel *et al.* 1991; Lee *et al.* 1994; Post *et al.* 1963; Zaika and Kim 1993; Zessin and Shelef 1988). Other factors such as storage temperature and NaCl level affect the antimicrobial activity of polyphosphates (Zaika and Kim 1993). The effect of four food-grade polyphosphates on the growth of *A. hydrophila* was studied in both a model system and a food system. The model system was BHI broth modified by adding different levels of polyphosphates and/or NaCl with incubation at temperatures from 5 to 42C. The food system was ground pork with 2% polyphosphate and salt equivalent to 3½% brine. Using both the model system and the food system, we investigated the effect of various combinations of polyphosphate, temperature and NaCl on the growth, survival, injury, and inactivation of *A. hydrophila*.

MATERIALS AND METHODS

Microorganism

Aeromonas hydrophila K144 was used throughout these studies. The experimental flasks were inoculated from a dilution (made in 0.1% peptone water) of an overnight culture (BHI Broth, Detroit, MI; 18 h at 28C, 150 rpm).

Medium and Variables

BHI Broth was modified by the addition of NaCl and/or polyphosphate. The 0.5% NaCl present in BHI was taken into account when determining the NaCl level. The polyphosphates added were: sodium pyrophosphate (analytical reagent, Mallinckrodt, Paris, KY), Sodaphos (sodium hexametaphosphate [sodium polyphosphate-glassy], food grade, FMC Corporation, Philadelphia, PA), sodium

tripolyphosphate (food grade, FMC Corporation), and Hexaphos (food grade, FMC Corporation). The polyphosphates and NaCl were added to the BHI Broth and heated with stirring to dissolve the polyphosphate; the broth was then sterilized in an autoclave.

Protocol

A $3 \times 3 \times 2$ factorial design was used for the growth studies: NaCl levels of 0.5%, 2.0%, and 3.5% (W/V); phosphate levels of 0%, 1%, and 2% (w/v); incubation temperatures of 5 and 28C. All experiments were repeated twice and similar responses observed.

Procedure

Duplicate flasks (50 ml of a specific variable combination in 250-ml flasks) were inoculated as indicated above to achieve a starting population density of ca 2×10^3 cfu/ml. Viable counts were determined at appropriate intervals by surface plating onto duplicate plates of Tryptic Soy Agar (TSA, Difco) using a Spiral Plater (Model D, Spiral Biotech, Bethesda, MD); dilutions were made as needed in phosphate-buffered saline. Colonies were counted either manually or with a Laser counting system (Spiral Biotech, Bethesda, MD) after incubation for 24–36 h at 28C. For variable combinations that inactivated the microorganism, sampling was discontinued when two successive viable counts of < 21 cfu/ml (the lower limit of detection) were observed.

Injury

The TSA-TSAS plating system described by Iandolo and Ordal (1966) was used to demonstrate the presence of injured cells during treatment with NaCl-polyphosphate combinations. For our studies with *A. hydrophila*, the TSAS had a final (total) NaCl concentration of 2%.

Inactivation

The kinetics of inactivation of *A. hydrophila* by the combination of 3.5% NaCl and 2% polyphosphate was studied at 42, 37, 28, 19, 12, and 5C. At intervals during treatment, aliquots were removed for viable cell counting on TSA as described above. The data (as \log_{10}) were analyzed by the declining model of Whiting (1993) and the results expressed as D-values (time for a 10-fold decline in viable count).

Data Processing and Analysis

Viable count data were converted to Log_{10} . Growth curves for the individual experiments were then generated using Abacus (an iterative, nonlinear regression program (Damert 1995) to fit the data to the Gompertz equation (Gibson *et al.* 1987). Lag and generation times were also calculated from the Gompertz equation.

pH

The initial pH values of the different culture combinations were determined on separate aliquots of each broth after sterilization. The pH was measured with a combination electrode attached to an Orion pH meter (model 601A/digital Ionalyzer).

Electron Microscopy

A. hydrophila was grown overnight (ca 18 h) in BHI broth at 28C. An appropriate dilution of this culture (made in 0.1% peptone water) was added to fresh BHI containing 2% Sodaphos and 3.5% NaCl; final concentration of the bacterium was ca 10^5 CFU/ml. At intervals during incubation, 4-ml aliquots were removed for viable cell count and electron microscopy. Viable cell count was determined as described above. For electron microscopy, the cells in the remaining aliquot were fixed in the culture medium by the addition of glutaraldehyde to a final concentration of 1% (v/v). After standing for 2 h at room temperature, the samples were stored at 4C before embedding. In preparation of thin sections, aliquots of fixed cells were sedimented in an Eppendorf centrifuge (model 5413) for 10 min at 14,000 rpm. The supernatant fluid was decanted and the pellet was washed with a solution of 2% osmium tetroxide in cacodylate buffer for 1 hr, then washed in water, dehydrated in a graded series of ethanol solutions, and finally embedded in epoxy resin. Thin sections of cell pellets were cut with diamond knives and stained with solutions of uranyl acetate and lead citrate. Observations were made with a Philips CM12 scanning-transmission electron microscope operating at 60 kv, and photographic images were made at 28,000 \times magnification.

Food Studies

Two food systems were chosen to investigate whether the effect of high polyphosphate/high NaCl on *A. hydrophila* seen in BHI broth could be demonstrated in a food product: scallops containing naturally occurring *A. hydrophila* and the ground pork system described by Palumbo (1988). For the

scallops study, scallops were purchased at a local market and held at 5C for three days to increase the number of naturally occurring *A. hydrophila*. These scallops were then treated by dipping in a solution of 2% polyphosphate and 3.5% NaCl for two min. Total aerobic microflora was determined by surface plating dilutions (made in 0.1% peptone water) onto TSA and incubating the plates at 28C for 24 h. *A. hydrophila* was counted by surface plating onto starch ampicillin agar (Palumbo *et al.* 1985). Amylase-+ colonies (*A. hydrophila*) were counted after 24 h at 28C by flooding the plates with Lugol's iodine (4 ml). The treated scallops were sampled at zero time and during several days storage at 5C. For the ground pork studies, center portions of fresh pork shoulders (purchased from a local market) were removed as to minimize bacterial contamination. This center portion was then coarse ground (¼ in. plate) in a sterile grinder, 2% polyphosphate and NaCl equivalent to 3.5% brine and a dilution of an overnight culture of *A. hydrophila* K144 (grown in BHI Broth) were added and this mixture was fine ground (1/8 in. plate) through a sterile grinder. The ground pork was then held aerobically in sterile plastic bags at 5C. Samples were taken at zero time and at intervals during storage for total aerobic plate and *A. hydrophila* counts as described above.

RESULTS AND DISCUSSION

The effect of polyphosphates on the growth kinetics of *A. hydrophila* K144 was studied at refrigeration temperature (5C) and the bacterium's optimum temperature (28C) in the presence of 0.5, 2.0 and 3.5% NaCl in BHI broth. The presence of NaCl or polyphosphate individually caused relatively small increases in lag and generation times (data not shown). The major effect seen, however, was the complete inhibition of growth of *A. hydrophila* exposed to the combination of 2% polyphosphate and 3.5% NaCl at both temperatures; plate counts of these cultures revealed that no viable cells were present. The intermediate level (2% NaCl and 1% polyphosphate) also inhibited growth, but since highest NaCl-polyphosphate level had the most pronounced effect, this combination was further investigated. The lack of a major effect of polyphosphate alone on the growth of *A. hydrophila* was not unanticipated because there are several reports that indicate that Gram-negative bacteria, in contrast to Gram-positive bacteria, are relatively unaffected by polyphosphates (Chen *et al.* 1973; Knabel *et al.* 1991; Post *et al.* 1963).

It has been proposed that polyphosphates exert their antibacterial effect by elevating the pH or chelating essential metal ions or both. We determined the initial pH of various combinations of NaCl and polyphosphate in BHI broth (control pH, 7.2). The pH values of BHI broth containing polyphosphate-NaCl combina-

tions are as follows: Hexaphos, 5.9–6.5; Sodaphos, 6.3–6.6; pyrophosphate, 7.5–8.0; and tripolyphosphate, 6.9–7.0. Previous work (Palumbo *et al.* 1991, 1992) has indicated that *A. hydrophila* can readily grow at pH values from 7.2 to 5.9 at various NaCl levels. In addition, we tested BHI Broth with 2.0 and 3.5% NaCl adjusted to pH 7.8 and 8.2 (with NaOH) and observed that the bacterium could grow at these higher pH values even at the higher NaCl levels (data not shown). This suggests that the effect of polyphosphates on *A. hydrophila* was not due to the alteration in pH. Firstenberg-Eden *et al.* (1981) reported increased inhibitory activity of sodium phosphates against *Moraxella-Acinetobacter* when combined with sodium chloride. Elliott *et al.* (1964) reported that a polyphosphate mixture was effective against pseudomonads isolated from poultry meat. They indicated that the inhibition was not due to high pH, but rather to chelation of metal ions needed for bacterial growth. The chelation effect is further supported by the observation of Zessin and Shelef (1988) who determined that the antibacterial activities of polyphosphates were highest in low mineral media.

As indicated, there is a consistent effect at the higher polyphosphate level combined with the highest NaCl level. The viable count decreased to below the lower limit of detection occurred when *A. hydrophila* was exposed to this combination. This decline was studied at 42 to 5°C, temperatures at which the bacterium is normally able to grow. The decline or inactivation data, presented as D-values at the various temperatures, are shown in Fig. 1. At the higher temperatures, the polyphosphates had similar rates of inactivation; at the lower temperatures, the short chain compounds (tripolyphosphate and pyrophosphate) were more active than the longer chain Hexaphos and Sodaphos. The basis for the differential effect based on chain length is not known. When the inactivation data in Fig. 1 were plotted as an Arrhenius plot (not shown) (log inactivation rate versus the reciprocal of the absolute temperature), all four polyphosphates gave similar energies of inactivation of 22,140 cal. In a plot of log inactivation rate versus temperature (not shown), similar Z-values of 21.0°C were calculated for the four polyphosphates.

The presence of injured cells during exposure to high polyphosphate/high NaCl was investigated using the TSA-TSAS plating system. A typical response is shown in Fig. 2; as evidenced by the lowered count on TSAS, injured cells were detected at the first sampling time and by 5½ h there were no colonies on TSAS, whereas the count on TSA had declined only about 2 log cycles. As will be shown by electron microscopy, there are also changes in cellular morphology after 2 h exposure to the combination of 2% polyphosphate and 3½% NaCl.

When studied in foods, the high polyphosphate/high NaCl combination had only a limited effect on *A. hydrophila* in scallops. At the time of treatment, the number of *A. hydrophila* was reduced about one log cycle compared to a water rinse; during refrigerated storage, the number of *A. hydrophila* on the polyphosphate (all four were evaluated individually)/NaCl-treated scallops increased, though not

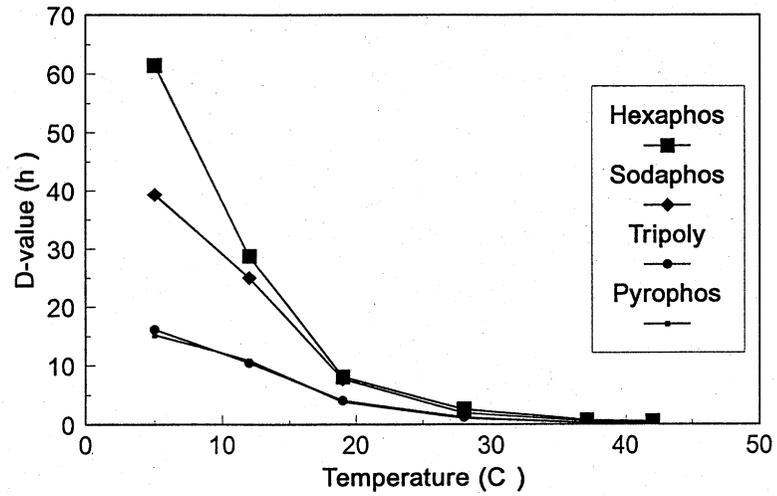


FIG. 1. THE EFFECT OF TEMPERATURE AND SPECIFIC POLYPHOSPHATE ON THE INACTIVATION OF *A. HYDROPHILA*

Ca 10⁵ CFU/ml initial count of *A. hydrophila* in BHI Broth containing 2% of specific polyphosphate and 3.5% NaCl; data shown are the average of duplicate flasks and counted on duplicate plates.

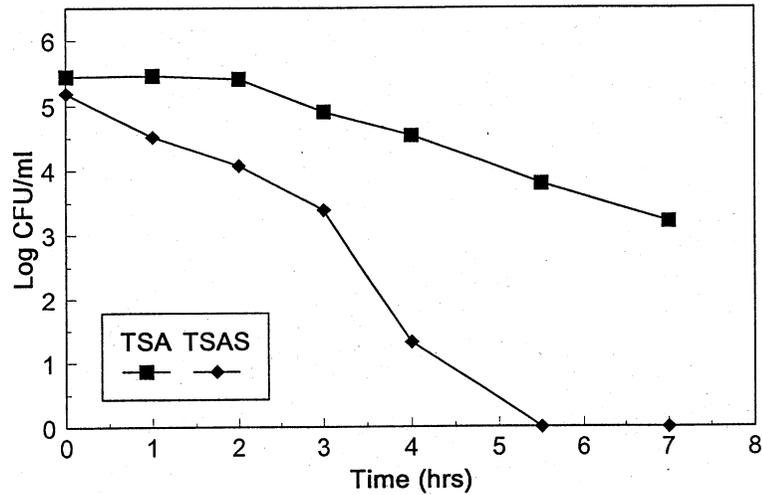


FIG. 2. RESPONSE OF *A. HYDROPHILA* K144 TO 2% SODAPHOS AND 3.5% NaCl IN BHI BROTH AT 28C

Survivors were plated on TSA and TSAS (TSA containing 2% NaCl).

quite as rapidly as on the control (data not shown). This limited effect is undoubtedly due to the limited contact time (as seen in Fig. 1): the D-value for the bacterium is fairly high compared to the contact time. However, in ground pork the combination of tripolyphosphate/NaCl, while it does not seem to have any lethal effect, does limit the outgrowth of *A. hydrophila* during refrigerated storage (Fig. 3) possibly because of continuous exposure to the combination. When the bacterium does increase in the ground pork treated with the combination of high polyphosphate/high NaCl, numbers were considerably less than in the ground pork treated with either high polyphosphate or high NaCl individually (Fig. 3). pH would not appear to be an explanation of the effect in the two food systems because the pH of the treated scallops was 6.94 and that of the treated pork was 6.75, both well within the growth range of the bacterium. None of the treatments had any effect on the total aerobic microflora (data not shown).

Exposure of *A. hydrophila* to a combination of 2% Sodaphos and 3.5% NaCl at 28C damaged the cell (as evidenced by the difference in count between TSA and TSAS) within the first 2 h (Fig. 2). Alterations in cellular structure during exposure to 2% Sodaphos and 3.5% NaCl were examined by transmission electron microscopy (Fig. 4). Changes in the surfaces of cells exposed to the combination were seen after 2 h (Fig. 4a (control) versus Fig 4b. (combination)). In the control (Fig. 4a), surface appendages (arrows) can be observed; the com-

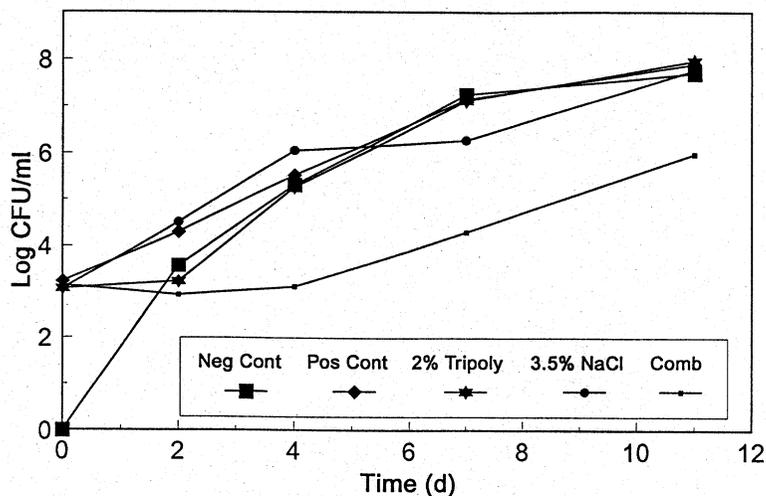


FIG. 3. EFFECT OF POLYPHOSPHATE AND NaCl ON *A. HYDROPHILA* K144 INOCULATED INTO GROUND PORK HELD AT 5C
A. hydrophila count was determined on starch ampicillin agar. Control, bacterium alone; tri, bacterium + 2% tripolyphosphate; NaCl, bacterium + 3.5% NaCl; comb, bacterium + 2% tripolyphosphate and 3.5% NaCl.

combination of 2% Sodaphos and 3.5% NaCl has removed surface appendages from the cells (Fig. 4b). By 7 h of exposure to the combination, there is essentially

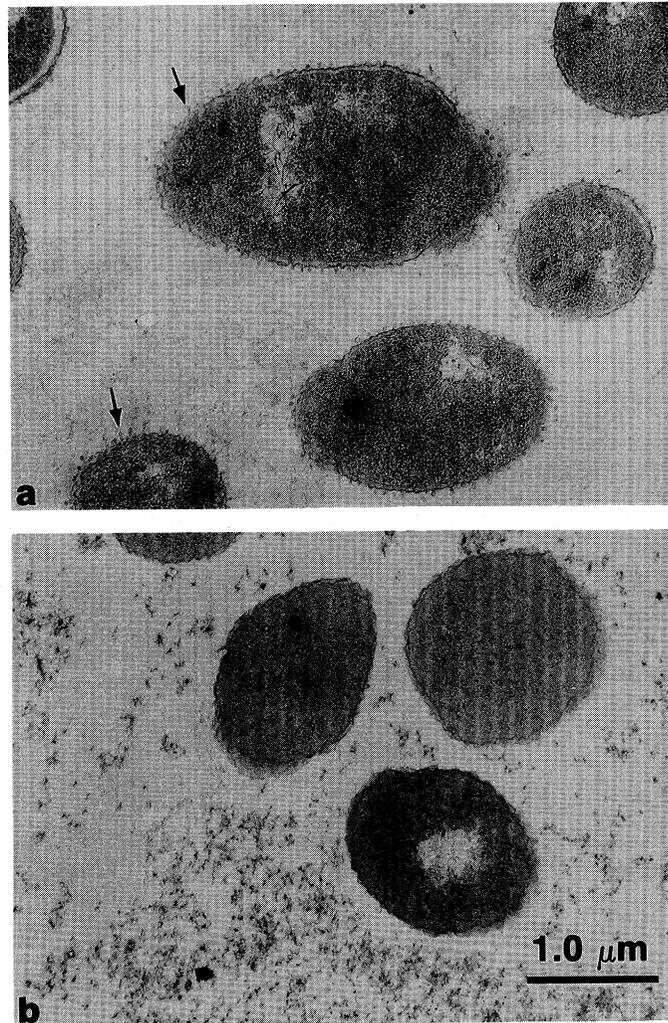


FIG. 4. CHANGES IN CELLULAR STRUCTURE OF *A. HYDROPHILA* K144 EXPOSED TO 2% SODAPHOS AND 3.5% NaCl IN BHI BROTH AT 28C ($\times 28,000$)

Zero time (control) TSA count was log 7.03 CFU/ml and TSAS count was 6.41 CFU/ml: (a) 2 h (control); TSA count was log 6.74 CFU/ml, TSAS count was 5.32 CFU/ml. (b) 2 h (2% sodaphos and 3½% NaCl); TSA count was log 5.26 CFU/ml, TSAS count was 3.51 CFU/ml.

a complete breakdown of cellular structure and organization (not shown; both TSA and TSAS counts decreased dramatically) and there were too few cells for electron microscopy. Cells exposed to 2% Sodaphos or 3.5% NaCl individually were similar in appearance to the control cells at all sampling times (not shown).

The basis of the lethality of the high polyphosphate-high NaCl combination on *A. hydrophila* is not known. The combination first injured the cells and then caused complete destruction of cellular structure and death of the cell. Individually, high polyphosphate and high NaCl levels supported growth, though at slower rates. Further studies are needed to ascertain the mechanism for the destruction of *A. hydrophila* by the polyphosphate-NaCl combination. The data in this report suggest that the growth and survival of *A. hydrophila* can be controlled in refrigerated foods by the addition of appropriate polyphosphates when combined with sodium chloride.

REFERENCES

- CHEN, T.C., CULOTTA, J.T. and WANG, W.S. 1973. Effects of water and microwave energy precooking on microbiological quality of chicken parts. *J. Food Sci.* 38, 155-157.
- DAMERT, W. 1995. EERC. Personal communication.
- ELLINGER, R.H. 1972. Phosphates as food ingredients. CRC Press, Cleveland, OH, 190 pp.
- ELLIOTT, R.P., STRAKA, R.P. and GARIBALDI, J.A. 1964. Polyphosphate inhibition of growth of pseudomonads from poultry meat. *Appl. Microbiol.* 12, 517-522.
- FIRSTENBERG-EDEN, R., ROWLEY, D.B. and SHATTUCK, G.E. 1981. Inhibition of *Moraxella-Acinetobacter* cells by sodium phosphates and sodium chloride. *J. Food Sci.* 46, 579-582.
- GIBSON, A.M., BRATCHELL, N. and ROBERTS, T.A. 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized port slurry. *J. Appl. Bacteriol.* 62, 479-490.
- HAZEN, T.C., FLIERMANS, C.B. HIRSCH, R.P. and ESCH, G.W. 1978. Prevalence and distribution of *Aeromonas hydrophila* in the United States. *Appl. Environ. Microbiol.* 36, 731-738.
- IANDOLO, J. J. and ORDAL, Z. J. 1966. Repair of thermal injury of *Staphylococcus aureus*. *J. Bacteriol.* 91, 134-142.
- KNABEL, S.J., WALKER, H.W. and HARTMAN, P.A. 1991. Inhibition of *Aspergillus flavus* and selected gram-positive bacteria by chelation of essential metal cations by polyphosphates. *J. Food Protect.* 54, 360-365.

- LEE, R.M., HARTMAN, P.A., OLSON, D.G. and WILLIAMS, F.D. 1994. Bactericidal and bacteriolytic effects of selected food-grade phosphates, using *Staphylococcus aureus* as a model system. *J. Food Protect.* 57, 276-283.
- PALUMBO, S.A. 1988. The growth of *Aeromonas hydrophila* K144 in ground pork at 5C. *Int. J. Food Microbiol.* 7, 41-48.
- PALUMBO, S.A. and BUCHANAN, R.L. 1988. Factors affecting growth or survival of *Aeromonas hydrophila* in foods. *J. Food Safety* 9, 37-51.
- PALUMBO, S.A., MAXINO, F., WILLIAMS, A.C. BUCHANAN, R.L. and THAYER, D.W. 1985. Starch ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. *Appl. Environ. Microbiol.* 50, 1027-1030.
- PALUMBO, S.A., WILLIAMS, A.C., BUCHANAN, R.L. and PHILLIPS, J.G. 1991. Model for the aerobic growth of *Aeromonas hydrophila* K144. *J. Food Protect.* 54, 429-435.
- PALUMBO, S.A., WILLIAMS, A.C., BUCHANAN R.L., and PHILLIPS, J.G. 1992. Model for the anaerobic growth of *Aeromonas hydrophila* K144. *J. Food Protect.* 55, 260-265.
- POST, F.J., KRISHNAMURTY, G.B. and FLANAGAN, M.D. 1963. Influence of sodium hexametaphosphate on selected bacteria. *Appl. Microbiol.* 11, 430-435.
- WHITING, R. C. 1993. Modeling bacterial survival in unfavorable environments. *J. Ind. Microbiol.* 12, 240-246.
- ZAIKA, L.L. and KIM, A.H. 1993. Effect of sodium polyphosphates on growth of *Listeria monocytogenes*. *J. Food Protect.* 56, 577-580.
- ZESSIN, K.G. and SHELEF, L.A. 1988. Sensitivity of *Pseudomonas* strains to polyphosphates in media systems. *J. Food Sci.* 53, 669-670.
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