ANTIMICROBIAL INTERVENTIONS TO REDUCE *LISTERIA* SPP. CONTAMINATION ON SHRIMP

A Dissertation

by

TSUI-YIN WONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Food Science and Technology

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ABSTRACT

Antimicrobial Interventions to Reduce *Listeria* spp. Contamination on Shrimp.

(August 2009)

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The effects of selected antimicrobials, applied singularly or in combination, and frozen or refrigerated storage conditions on the survival of *Listeria* spp. on inoculated shrimp was evaluated in this study. A combination of 0.5% CPC (Cetylpyridinium Chloride) with a water wash at room temperature and freezing of the shrimp at -22.3°C was the only treatment that had a significant antimicrobial effect on the *Listeria* spp. Antimicrobial effects and the mode of action of PEF (Pulsed Electric Field) and CPC on *Listeria* cells were evaluated in detailed studies. PEF in 0.1% sodium chloride had a bacterostatic effect toward *Listeria* spp. during refrigerated storage, but no immediate or bacteriostatic effect was caused by freezing the samples. A concentration of 1% sodium chloride reduced the *Listeria* spp. population after freezing by 1.1 log; however, the pungent chlorine odor that was generated during treatment might cause discomfort for employees in shrimp processing facilities. Also, chlorine might cause corrosion of metal surfaces of processing equipment. There was no difference in the antimicrobial effects on the survival of *Listeria* spp. by PEF

between the exposure times of 1 or 2 min, as well as in the sodium chloride concentrations of 0.1 and 0.5%. PEF treatment in the presence of 0.1% sodium chloride is recommended. A solution of 0.5% CPC effectively inhibited all of the strains of *Listeria* spp. in the cell suspensions. A treatment of 0.5% CPC combined with PEF treatment in a sodium chloride concentration of 0.1% caused a delayed effect on the *Listeria* spp. after 2 d of refrigerated storage. After 2 d of frozen storage, the formation of ice crystals was decreased in the number of *Listeria* spp. when contaminated samples were treated with water. The results indicated chemicals (e.g. CPC and NaCl) might protect *Listeria* spp. from the formation of ice crystals. TEM (Transmission Electron Microscopy) micrographs revealed that cell membranes were damaged by PEF treatment and that cells were ruptured by CPC treatment. A maximum reduction of 2.76 $\log_{10}$ CFU/g of *Listeria* spp. on shrimp was achieved by a combination of PEF-CPC.

DEDICATION

To my father, Jame, and my mother, Shelly, for their sacrifice, morality, and prayers that helped me to achieve this degree

To the memory of uncle who motivated me to succeed in an academic career

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INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that can survive and even grow at refrigerated temperatures in foods and in food processing environments. Seafood products, including shrimp, are processed in environments that might create potential for cross-contamination with this organism. Therefore, efforts to minimize cross-contamination and to inactivate pathogens in contaminated product are needed.

Selected antimicrobial agents that have been approved by FDA for use in foods were evaluated in this study for their listericidal and bacteriostatic effects with and without the combined application of physical treatments (freezing, ultrasonication and pulse electric fields). Antimicrobial intervention combinations were evaluated for the reduction in levels of *Listeria* spp. inoculated raw shrimp.

Farm-raised shrimp from a Texas processor was used for these experiments. Shrimp were in blocks or IQF (Individual Quick Freezing) and kept frozen until used in experiments. The shrimp were transported to the Microbial Challenge Pilot Plant at the Poultry Science Center at Texas A&M University. Beheaded shrimp samples were inoculated with a five-strain cocktail composed of *L. monocytogenes* ATCC 15313, *L. monocytogenes* Scott A46, *L. monocytogenes* Strain A, *L. innocua* NRRC b33076, and *L. innocua* ATCC 33090.

This dissertation follows the style of *Journal of Food Protection.*

All shrimp samples were treated with one of five defined antimicrobial treatments. Some of these treatments combined chemical interventions with physical treatments such as freezing, ultrasonication, and pulse electric fields in order to evaluate the interactions between antimicrobials. Populations of *Listeria* spp. were enumerated before and after each treatment combination. Separate sets of samples were evaluated to determine their survival under frozen and refrigerated storage.

Transmission Electron Microscopy was used to illustrate morphological changes of *Listeria* cells caused by antimicrobial interventions tested that might explain the mode of actions of any observed effects. The long term goal of this project was to define the optimal combinations of interventions to reduce contamination with *Listeria monocytogenes* in raw samples by the combination of physical and chemical interventions. The mechanisms of inactivation caused by the antimicrobial treatments were described to understand the limitations of the treatments and the commercial applicability in processing settings.

LITERATURE REVIEW

Introduction

It is estimated that approximately 1,800 cases of human listeriosis occur in United States each year, with a fatality rate of 15 to 25% in affected individuals *(89)*. The symptoms of listeriosis, which are often predisposing in pregnant women, newborns, the elderly and the immunocompromised, or immunodeficient can be varied from mild flu-like illness to meningitis and meningoencephalitis *(1)*. Data from a summary of the minimal temperature (ºC) for the growth of *Listeria* spp. in selected foods are in Table 1. *Listeria monocytogenes* can propagate over a temperature range from 0 to 42ºC with optimum growth reported at 30-35ºC. This pathogen can tolerate and grow in food products containing up to 10% sodium chloride *(1)*. This microorganism is almost ubiquitous in the environment *(1)***.** In addition, the organism can be found in wet and low temperature food processing environments *(1)*. Vehicles of transmission often associated with outbreaks or sporadic cases of listeriosis include soft and semi-soft cheeses, hot dogs, deli-meats, and vegetables *(44)*. A few cases of listeriosis have been associated with seafood and seafood products *(24, 25, 36)*. This is important, especially in some countries where seafood is the primary source of protein. Cases of listeriosis associated with seafood products, are most often associated with the ingestion of ready-to-eat (RTE) fishery products *(44)*. Such products include spiced and pickled fish, cold-smoked rainbow trout or salmon, hot-smoked salmon, cooked crabmeat, cooked

shrimp, cooked lobster, surimi-based products, and “ceviche”, which is an acidified fish and shrimp product *(30, 49, 69).*

TABLE 1. *The minimal temperature (ºC) for the growth of* Listeria spp. *in selected foods (46).*

Microorganisms	Minimal growth temperature (ºC)	Food
L. monocytogenes	0.8-1.4	Deli-meat, hot dogs, semi-soft cheese, shredded cabbage and seafood
L. innocua	1.7-3.0	Meats, milk, frozen seafoods, semi-soft cheese, whole egg, and vegetables
L. seeligeri	1.7-3.0	Raw milk, vegetables, cabbage, radishes, and pork
L. welshimeri	1.7-3.0	Raw milk, meat roasts, vegetables, and turkey meat
L. grayi	1.7-3.0	Raw milk

A comprehensive review by the International Commission on Microbiological Specifications for Foods *(41, 45)* stated that if a food product contains ≤100 CFU/g of *L. monocytogenes*, it does not pose a health risk for normal persons. However, U.S. regulatory agencies have adopted a zero-tolerance policy for *L. monocytogenes* in RTE food products due to the lower resistance in susceptible populations and because of its potential to grow in different food matrices at refrigerated temperatures *(74)*. Contamination with *L. monocytogenes* usually occurs after the product has been subjected to a lethal process, generally thermal treatments. The problem arises because the organism is capable of growing in a contaminated cooked and meat or poultry product due to the lack of competitive microflora. Because of the frequent presence of *L. monocytogenes* in contaminated cooked products and the pathogen's lethal effect on susceptible people, the Food Safety and Inspection Service (FSIS) have three alternatives for ready-to-eat meat and poultry processors to control *L. monocytogenes* contamination *(29)*. These alternatives include: 1) both a post-lethality treatment (e.g. post-packaging treatment) that will destroy the organism if the product is contaminated and an antimicrobial agent or a process that inhibits its potential growth if present; 2) either a post-lethality treatment or an antimicrobial agent or a process that inhibits growth that should be combined with a sanitation program specifically to control the organism in areas where contamination could occur; and, 3) a sanitation program specific for *L. monocytogenes* with measures and procedures aimed at

limiting or suppressing the growth of this pathogen or its presence in processing environments *(29)*. No specific regulations have been imposed for seafood or fishery products; however, with the adoption of these alternatives in the meat and poultry industry, there is a possibility that some of these alternations could be applied to seafood processing. Therefore, more research is needed to focus on the effects of single or combinations of antimicrobial treatment (s) or process (es), as well as their effects when used in combination with subsequent freezing or refrigerated storage of commercially processed seafood products.

Listeria monocytogenes **in seafood and seafood products**

Seafood products, especially shrimp, feature a high protein profile and are rich in essential amino acids that are required for proper human nutrition *(20)*. Thus, the seafood products account for a significant share of the protein intake in many populations. Seafood products, including shrimp are harvested from natural sources and are also produced in seafood farms around the world with significant international commercialization. For instance, the international trading of shrimp products accounts for about 20% of the total sales of seafood products worldwide *(20)*. Despite the existence of more than a hundred shrimp species, only a few of these species are used for commercial production purposes. Specific studies on the presence of *Listeria* spp. in different seafood products are summarized below:

Shrimp

Gudmundsdóttir *et al*. *(33)* stated that shrimp has been implicated as a vehicle in epidemiological studies of listeriosis. The prevalence of *Listeria* spp. varies greatly in shrimp products. Reports of prevalence varying from 1.5 to 28.8% in raw shrimp and from 8.1 to 11.4% in cooked shrimp products have been published *(33)*.

Motes *(69)* reported that *Listeria* spp. were recovered more frequently from shrimp samples rather than estuarine water sources and oyster samples. They also found that recovery of *Listeria* spp. from shrimp was not affected by the salinity or fecal coliform count of the harvesting water. Shrimp positive for *Listeria* spp. (5%, 4/78) were all *L. monocytogenes* (100%, 4/4). The authors concluded that *L. monocytogenes* contaminated shrimp can serve as a potential source for the contamination of processing environments and final products *(69)*. McCarthy *(66)* also reported that crustacean exoskeletons are most likely entrapped within the equipment during cleaning and peeling in seafood processing plants. Potential points in shrimp processing where contamination with *L. monocytogenes* can occur are shown in Figure 1.

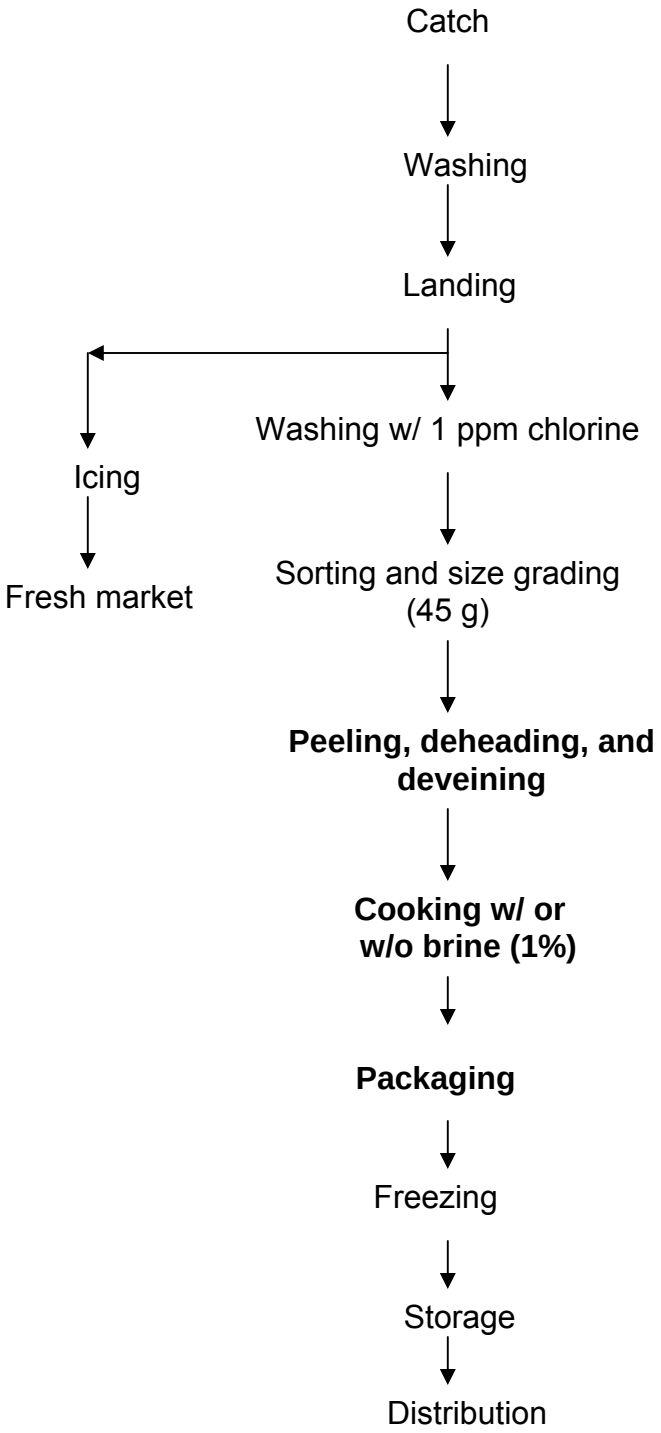


FIGURE 1. *Possible contamination site (bold front) and shrimp processing line (partial flow diagrams) were adapted from Venugopal (100).*

A study by McCathy *(66)* reported that crustacean exoskeletons are most likely entrapped within the equipment during cleaning and peeling in seafood processing plant. Chitin, the structural unit of the exoskeleton of crustaceans, is a major compound of shrimp and is very resistant to chemical solvents and bacterial enzymes. Because of these properties, it has been used in many industrial applications, such as soil treatments, insecticides, livestock, fish feed, fertilizers, and wastewater treatment.

Raw fish

Listeriosis has been rarely linked to consumption of raw tropical or deep water fish (*28*). Nevertheless, *Listeria* spp. can grow or survive at refrigeration temperatures (as low as 1ºC) and tolerate a concentration of 20% sodium chloride *(36)*; hence, these microorganisms can be isolated from contaminated raw fish at very low microbial counts *(36)*. Autio *et al*. *(5)* reported that *L. monocytogenes* was not the most important microorganism in the contamination of cold-smoked rainbow trout. Masuda *et al*. *(64)* reported that *L. monocytogenes* was isolated from only 1.8% (12/683) of fresh seafood sampled at wholesale settings and retail shops in Tokyo during the summers of 1989 and 1990. Although the presence of *L. monocytogenes* on raw seafood is rare, it has been more frequently documented in RTE products. Therefore, additional information is needed on the incidence of this microorganism and the effects of minimal processing (freezing, packaging, etc) on its survival in raw seafood.

Processed seafood products

Processed seafoods, including cooked, smoked, pickled seafood, and surimi, do not normally receive any additional post-process treatment. The growth of *L. monocytogenes* on contaminated processed seafood was not prevented by refrigeration *(66)*. Specific studies on different varieties of cooked seafood are summarized below:

Smoked fish

The Food Agriculture Organization *(28)* reported that cold- smoked fish, e.g. salmon fillets, have been found to be contaminated with *L. monocytogenes* at 100 CFU/g. It was also reported that an increase in the concentration of the contamination is likely to occur during long periods of refrigeration due to the psychrotrophic growth of *L. monocytogenes.* The temperature applied during the smoking process is not sufficient to inactivate all *L. monocytogenes* cells in fish products *(36)* and more validation studies are needed to assess the effects of the commercial cold-smoking process on survival of this organism during refrigerated storage. Cold-smoked (30-40ºC) salmon or hot-smoked (80-100ºC) mackerel, herring, trout, or cod has been shown to be contaminated with *L. monocytogenes* from the processing environment *(30)*. Rørvik *et al*. *(85)* also found that *L. monocytogenes* could be isolated from environmental samples (26%, 42/142) in the fish smokehouse at various processing facilities. Because of this, the evaluation of antimicrobial interventions with the potential activity to

lower the prevalence of *L. monocytogenes* in cold- and hot-smoked fish after processing is warranted.

Seafood salads

Widespread nutritional trends toward low caloric diets have resulted in a significant increase in the consumption of salads, and other dishes featuring fruits and vegetables. Some of these salad products are complemented with a source of high protein, namely poultry products. The addition of seafood components; however, is showing increased consumption as seafood salads. Seafood salads are made from many different raw or cooked seafood ingredients and typically contain some type of salad dressing. A high prevalence of *L. monocytogenes* has been found in seafood salads containing smoked or spiced and picked fish *(36)*. These findings indicate that mayonnaise and other acidic dressings may not necessarily lower the pH of salads sufficiently to inactivate the growth of *L. monocytogenes (44).* Hwang and Tamplin *(44)* found that *L. monocytogenes* (six strains in a cocktail culture) was able to grow slowly (by 2 logs under vacuum; by 4.5 logs under aerobic conditions) in an artificially inoculated shrimp-crabmeat (1:1) seafood salad containing mayonnaise. The mayonnaise product used in this study had a pH of 3.7 and the product was kept for up to 22 d of storage at 4ºC. This finding demonstrated the growth of *L. monocytogenes* in a pre-cooked shrimp salad product mixed with mayonnaise during a long-term refrigerated storage period.

Tracking the sources of contamination

Smoked or raw fish products

Johansson *et al*. *(48)* evaluated the prevalence of *L. monocytogenes* in hot-smoked whitefish or mackerel (n=48), cold-smoked rainbow trout (n=30), and cold-salted rainbow trout (n=32) from Finland. *L. monocytogenes* (20%, 22/110) was found in all three types of RTE retail fish products. Ten of these positive samples (9.0%) were contaminated with *L. monocytogenes* at a concentration > 100 CFU/g. The highest prevalence of *L. monocytogenes* (50%, 16/32) was in cold-salted rainbow trout. Six of these positive samples (19%) were contaminated with *L. monocytogenes* in the range of 100-9900 CFU/g. The second highest prevalence of *L. monocytogenes* (17%, 5/30) was found in cold-smoked rainbow trout. Four of these samples (13.6%) were contaminated with *L. monocytogenes* in the range of 1 X 10^2 to 1.4 X 10^4 CFU/g. The lowest prevalence of *L. monocytogenes* (2%, 1/48) was found in the hot-smoked fish products. On the other hand, Chou *et al*. *(21)* found using repetitive element Polymerase Chain Reaction (rep-PCR) that *L. monocytogenes* was the most predominant *Listeria* spp. in raw channel catfish fillets (n=240) from three different processing plants in the U.S. during four time periods (summer, fall, winter, and spring), with a prevalence range from 25 to 47%. They also found *L. monocytogenes* was most predominant during the winner with a prevalence of 51%. A possible explanation of this finding might be related to its psychotropic characteristic. The prevalence of *L. monocytogenes* in raw and whole crawfish

products and its processing environments were also reported by Lappi *et al*. *(55)*. In their study, the prevalence of *L. monocytogenes* increased from 3.85 (first year) to 10.79% (second year) in raw and whole crawfish products (n=78 for the first year; n=101 for the second year) and 0.65% (n=155, first year) to 0.98% (n=204, second year) for environmental samples. However, the increase of prevalence was not statistically significant. This finding may have been related to the differences in production volumes which was four to five times more in year two than in year one, as well as a mixture (50:50) of wild and farm-raised crawfish during the second year.

Processing environments or steps for smoked fish products

Rørvik *et al*. *(85)* reported the prevalence of *L. monocytogenes* from the environment of a salmon slaughterhouse (16.6%), from a smokehouse (88%), and from equipment used during the smoking process (90%) in Norway using MEE (Multilocus Enzyme Electrophoresis). This indicates that the smokehouse can serve as a reservoir for this microorganism.

Autio *et al*. *(5)* reported that all *Listeria* isolates were characterized by PFGE (Pulsed Field Gel Electrophoresis) at different production stages and in the environment of a cold-smoked rainbow trout facility in Finland. They reported the following prevalence: slaughterhouse (1.6%), machines (24%), brine solution (67%), fish after brining (70%), fish after smoking (80%), fish after slicing (70%), and the final product (100%). Vogel (*101*) noted similar findings using RAPD (Randomly Amplified Polymorphic DNA), isolating *L. monocytogenes* from

products and processing environments in two Danish cold-smoked salmon processing plants. *Listeria monocytogenes* contamination of the cold-smoked salmon in the two plants was 31 to 85% and 0 to 25%, respectively. Results indicated that product contamination in both plants was a result of contamination in the processing environment. They also found that *L. monocytogenes* was capable of surviving in the processing environment for up to four years.

Processing environments or steps for cooked or raw shrimp

Gudmundsdóttir *et al. (33)* found *Listeria* spp. (12.5%, 87/695) in both the raw material (defrosted shrimp) and the environment in two shrimp processing plants. *Listeria monocytogenes* was positively identified in 89.7% (78/87) of these samples. The result of PFGE showed that the percentage of *L. monocytogenes* in the raw material (20.9%, 9/43) increased during the cooking and peeling process to 40.6%, mainly by cross-contamination.

This finding indicated that equipment can serve as a reservoir for *L. monocytogenes*. They also recovered 18.9% from 53 samples collected following the plants equipment in both processing plants indicating that these procedures were not adequate to reduce *L. monocytogenes* in the processing environment.

Another study by Destro *et al*. *(22)* reported that two molecular typing methods, RAPD (two primers: 155, 11 composite profiles and 127, 16 composite profiles) and PFGE (two enzymes: SmaI, 13 composite profiles and ApaI, 15 composite profiles), demonstrated a strong discriminatory power for discerning different *L. monocytogenes* strains (a total of 115 samples) from the environment, fresh samples, and from handlers in a shrimp processing plant. An executive summary on the prevalence (%) of *Listeria* spp. in selected seafood samples, processing steps, and environments is shown in Table 2.

TABLE 2. *The prevalence (%) of* Listeria spp. *in selected samples, processing steps, and environments.*

Sources	Prevalence (%) of *Listeria* spp.	References
Products		
Hot-smoked white or mackerel	2	*(48)*
Raw channel catfish	25-47	*(21)*
Cold-smoked rainbow trout	13.6	*(48)*
Cold-salted rainbow trout	19	*(48)*
Raw fresh shrimp	15-28.8	*(33)*
Cooked shrimp	8.1-11.4	*(33)*
Processing environments for cold-smoked fish products		
Slaughter house	16.6	*(85)*
Smoke house	88	*(85)*
Production steps, equipment, and solution for cold-smoked fish products		
Brining	70	*(5)*
Smoking	80-90	*(5, 85)*
Slicing	70	*(5)*
Machine	24	*(5)*
Brine solution	67	*(5)*
Final product	100	*(5)*

TABLE 2. *Continued.*

Sources	Prevalence (%) of *Listeria* spp.	References
Production steps and environment for RTE shrimp		
Raw material	20.9	*(33)*
Cooking and peeling	40.6	*(33)*
Equipment after cleaning	18.9	*(33)*

Tracking microbial biofilms in seafood processing plant

A comprehensive review of microbial biofilms by Kumar and Anand (*54*) stated that the persistence of accumulated pathogenic bacteria in biofilms may contribute to the contamination of products and processing environments. Blackman and Frank *(12)* reported that the presence of complex growth nutrients (food residues) on various processing surfaces can support the development of biofilms that may harbor *L. monocytogenes*, if sufficient time is given. Their research indicated a need for food processors to decrease the level of complex nutrients on wet surfaces in plant environments.

Electronic and light microscopy techniques have been widely used for identifying the formation of biofilms. Carpentier and Chassaing *(18)* demonstrated interactions between *L. monocytogenes* and other resident microorganisms within biofilms in food industrial settings using epifluroscence microscopy. The formation of *L. monocytogenes* biofilms on chitin incubated for 2 and 7 d at 25ºC after disinfection with or without iodine solution was observed by Scanning Electron Microscopy *(66)*.

Post-harvest interventions to control *L. monocytogenes* in seafood and seafood products

There have been several attempts to use antimicrobial interventions to reduce the prevalence of pathogenic organisms in or on seafood products. Antimicrobial agents currently available for use with raw and cooked seafood products are discussed below:

Raw seafood products

Chemical treatments

Aqueous chlorine dioxide (ClO_2) solutions at concentrations of 40, 100, and 200 ppm, as well as commercial ClO_2 at concentrations of 100, 200, and 400 ppm were more effective than aqueous chlorine in inhibiting a streptomycin-resistant (Str^R) *L. monocytogenes* on cubes of Mangrove snapper *(59)*.

Acidified sodium chlorite (ASC), an oxidative agent, has been approved by the FDA for use as an antimicrobial rinse in the meat and poultry industry as an intervention to control the presence of pathogenic bacteria in raw and cooked products. Su and Morrissey *(95)* reported that ASC at concentrations of 40 to 50 ppm has been approved by the FDA for use in rinsing, thawing, transportation and storage of seafood products. The antimicrobial mechanism of this compound has been explained by Lim *et al. (58)* as a process that inhibits microorganism survival by interfering with cellular protein synthesis functions. This compound specifically attacks amino acid components, such as sulphide and disulphide linkages. Su and Morrissey *(95)* reported a 0.43 $\log_{10}$ CFU/cm^2 reduction of *L. monocytogenes* on whole salmon washed with a 50 ppm acidified sodium chlorite solution for 1 min and then stored at -18ºC for one month, and on salmon fillets stored on ice or at 5°C for 7 d. Moreover, they found a 0.32 to 0.47 $\log_{10}$ reduction in total plate count for salmon fillets similarity washed and stored on ice for 7 d. Researchers also found a 0.62 $\log_{10}$ CFU/g reduction after 7 d of storage in ASC-treated salmon fillets, inoculated with *L. monocytogenes*

(initial inoculum of 10^3 CFU/cm^2 or 10^4 CFU/g). However, there was no significant reduction of *L. monocytogenes* counts found on ASC-treated salmon skin. These results indicated that an ASC solution had a delayed effect on artificially-contaminated *L. monocytogenes* on salmon fillets after 7 d of storage but may not be as effective on the skin.

Cetylpyridinium Chloride (CPC) is a cationic surfactant belonging to the group of quaternary ammonium compounds (QACs). QACs are membrane active antimicrobial agents that have five sequential modes of action on bacteria (*23*). These five mechanisms are: 1) absorption by or penetration of the cell wall; 2) protein-lipid interaction followed by membrane disorganization; 3) release of low molecular weight constituents from the cell; 4) degradation of the protein and nucleic acids; and 5) cell lysis (*87*). At concentrations of 0.05 to 1.0%, this compound has been used for decontamination of *L. monocytogenes* on the surfaces of raw, peeled, and cooked shrimp, as reported by Dupard *et al*. *(23)*. In this study, they found a 1% CPC solution applied with or without subsequent water rinse reduced the level of *L. monocytogenes* V7 on the surface of raw shell-on shrimp by approximately 3.88 $\log_{10}$ CFU/g *(23)*. However, research data in regard to the application of this chemical on seafood products and the combined effect of the interventions subsequent to other further processing operations such as chlorine washes and the freezing of seafood is limited. Acetic acid (0.75-3.0%) has been reported to have listericidal activity in unassociated form *(16)*. However, Bremer and Osborne *(15)* found that the D-value (decimal

reduction time) increased up to 2.3 times in green shell mussels when they were marinated with acetic acid (3.0%) containing *L. monocytogenes*.

Physical treatments

Harrison *et al*. *(35)* reported that vacuum packaging with low density Ethylene Vinyl Acetate (EVA) and Polyvinylidene Chloride (PVDC, Saran™ wrap) films produced 1.12 and 1.05 $\log_{10}$ reductions, respectively, in *L. monocytogenes* Scott A-contaminated brown and white de-headed shrimp during 21 d of storage on ice. Silva *et al. (92)* reported that there was a zero prevalence of *L. monocytogenes* in refrigerated channel catfish (*Ictalurus punctatus*) fillet strips packaged using Modified Atmosphere Packaging (MAP) with a high concentration (63-87%) of carbon dioxide (CO_2) and followed by storage at 2ºC for one month.

Cold-processed seafood products

Chemical treatments

Pelroy *et al*. *(74)* reported that sodium lactate (2-3%) in combination with sodium chloride (3%) and sodium nitrite (125 ppm) was an effective in inhibition of *L. monocytogenes* (10 cells/g) when added to comminuted raw salmon stored at both 5 and 10ºC for up to 50 d. They also found in a separate study that the greatest inhibitory effect on *L. monocytogenes* (10 cells/g) was achieved by combining 190-200 ppm of sodium nitrite with 5% water-phase sodium chloride in a vacuum package and stored at 5ºC for 34 d *(75)*. Pelroy *et al*. *(74, 75)* found that adding a mixture of these chemical agents (sodium lactate, sodium chloride,

and sodium nitrate) to smoked salmon can offer the potential for enhanced shelf-life. However, a total salt content above 4% in cold-smoked fish product is not practical because of acceptability and health concerns for consumers *(74)*.

Physical treatment

Bell *et al*. *(9)* reported that a combination of carbon dioxide (100%) and storage at - 1.5ºC can have a bactericidal effect on the growth of three psychotropic pathogens: *A. hydrophila*, *L. monocytogenes*, and *Y. enterocolitica* on smoked blue cod.

Treatment combinations

Nilsson *et al*. *(71)* reported that a combination of nisin (≥30 IU/ml) and sodium chloride (5%) in vacuum or carbon dioxide (100%) packaging can increase the sensitivity of *L. monocytogenes* to nisin in cold-smoked salmon; therefore, providing a potential anti-listerial agent to this product.

Hot-processed seafood products

Physical treatment

Bremer and Osborne *(15)* determined the thermal death time (TDT) for hot smoking processes on *L. monocytogenes* (10^6 CFU/g) cells in green shell mussels. They found the thermal-time for D values at 56, 58, 59, 60, and 62ºC was estimated to be 48.1, 16.3, 9.5, 5.5, and 1.9 min, respectively. This indicates that small changes in the center temperature of a product during hot smoking can have a pronounced impact on the inactivation of *L. monocytogenes* cells in a hot-smoked product.

Ready-to-eat seafood products

Physical and chemical treatments

Pothuri *et al*. *(79)* reported that counts from a mixture of two *L. monocytogenes* strains (Scott A and F5027) artificially inoculated on crayfish tail meats (10^4 CFU/g) substantially decreased (~a 3 $\log_{10}$ reduction, $P < 0.05$) during 20 d of storage at 4ºC after being treated with lactic acid (2%) and packaged under air, vacuum, or modified atmosphere (78.4% CO_2, 14.8% N_2, and 10.4% O_2). These authors also found that *L. monocytogenes* in crayfish tail meats, treated with 1% lactic acid and packaged under modified atmosphere, started to propagate after 8 d of storage at 4ºC. This indicates that lactic acid solutions have a bacteriostatic effect that delays growth of *L. monocytogenes* in food products maintained in refrigeration.

After treatment with Cetylpyridinium Chloride (CPC, 1.0%), *L. monocytogenes* V7 counts (37°C, 24 h) on the surface of commercially precooked and shell-on shrimp were reduced by approximately 7 $\log_{10}$ CFU units (*23*). However, the effect of further washes on treated and untreated products were not reported in this publication.

A potential biochemical treatment

ε-polylysine, an antimicrobial peptide, has been shown to have significant antimicrobial activity against Gram positive and Gram negative bacteria when used at concentrations ranging from 1 to 8 *μ*g/ml *(91)*. This compound consists of at least ten L-lysine residues of cationic monomers and has antimicrobial

activity against several microorganisms. Shima *et al*, *(91)*, using Transmission Electron Microscopy (TEM), reported that the morphology of *E. coli* K12 cells was changed in the presence of ε-polylysine at a concentration of 50 *μ*g/ml when compared to non-treated *E. coli* K12 cells. Higher concentrations of ε-polylysine (1,000-5,000 ppm) have been used for spraying and dipping sliced fish and fish sushi in Japan *(37)*. The Food and Drug Administration (FDA) indicated that they have no objection to the use of ε-polylysine in seafood, except in cooked or sushi rice where it should be limited to less than 50 ppm *(31)*. Geornaras and Sofos *(31)* reported that ε-polylysine at concentrations of 0.01% at 4°C and 0.02% at 24°C were effective for reducing *L. monocytogenes* strains (10-strain culture mix) to non-detectable levels *in vivo* during 30 d of storage. The antimicrobial effect of this compound was also reported in food extracts. The same authors found this compound at a concentration of 0.02% was effective for reducing 10-strain of *L. monocytogenes* culture mix in rice and vegetable extracts during 6 d of storage at 12°C *(32)*. These experiments indicated that ε-polylysine, a potential antimicrobial agent, can be used for decontamination of *L. monocytogenes* in certain food extracts *(32)*, ready-to-eat fish products, boiled rice, noodles soup stocks, noodles, and cooked vegetable *(37)*.

Control environmental *Listeria* spp. and the biofilms in seafood processing plants

Chemical treatment

Tuncan *(97)* reported the germicidal effectiveness of three common sanitizers (quaternary ammonium compound, iodophor, and chlorine) at different temperatures (2ºC and 25 ºC) for 30 s and various concentrations (25, 50, 100, and 200 ppm) on a cocktail mix (four strains) or on individual strains of *Listeria*. In this study, it was reported that the germicidal effectiveness of chlorine was not influenced by cold temperatures and that the *Listeria* cocktail (>7 $\log_{10}$ CFU/ml) was inactivated after 30 s with chlorine concentrations ranging from 25 to 200 ppm. Exopolysaccharide (EPS) produced by microorganisms during biofilm formation may act to various degrees to interrupt normal diffusion modifier, or may act as a molecular sieve, or an absorbent. The three dimensional structures of biofilms may impart some degree of resistance against antimicrobials but this resistance is lost as soon as this structure is ruptured (*54*). The authors reported that detergents containing chelating agents, e.g. EDTA and ethylene glycol-*bis* (β-aminoethyl ether) N, N, N', N'-tetracetic acid (EGTA) can disrupt EPS structure. However, McCarthy *(66)* suggested that sanitizers (iodine, chlorine, and quaternary ammonium compounds) at the recommended levels and the exposure time of 20 min might not effectively eliminate the attachment of *L. monocytogenes* from contact surfaces. The authors also concluded disinfectants

should be chosen accordingly to control specific contamination problems of individual processors, time (s) of exposure, and concentrations.

Physical treatment

Liu *et al*. *(60)* found that the application of electrolyzed oxidizing water (EO, 0.05-0.2% sodium chloride) following conventional cleaning procedures significantly reduced a *L. monocytogenes* cocktail applied on the surfaces (5 X 5 cm^2) of stainless steel sheets, ceramic tiles, or floor tiles by 3.73, 4.24, and 5.12 log_{10}, respectively. Based on these findings, they concluded that electrolyzed oxidizing water (EO) can potentially be used as a sodium hypochlorite alternative for inactivating *L. monocytogenes* on seafood, and other processing surfaces. Kumar and Anand *(54)* reported a combination of antimicrobials with the use of a low level electric current was successful in controlling biofilms formation.

Biocontrol

Bower *et al. (13)* reported that *L. monocytogenes* cells on silica surfaces can be completely destroyed by a high concentration of nisin (1.0 mg/ml). Contaminated surfaces treated with a lower concentration of nisin (0.1 mg/ml) only exhibited a small degree of inhibition. The authors concluded that the inhibitory activity of nisin on *L. monocytogenes* contaminated surfaces is concentration-dependent.

Antimicrobial interventions to control of *Listeria* spp. in artificially-inoculated headless shrimp

Antimicrobial interventions to control *Listeria* spp. that naturally or artificially contaminate raw or cooked seafood products have been reviewed previously. These interventions were divided into three categories: chemical, physical, and biological intervention that can be used individually or in combination. The interventions that were selected to control *Listeria* spp. in this study were*:*

Biodegradable treatments

Citrate Extracts (Citrosan®)

No data has been published in peer reviewed journals to elucidate the mode of action of this antimicrobial on microorganisms. Based on manufacturer (Diken group, Mexico) information, this natural antimicrobial agent consists of amino acids, acidic sugars, acidic pectins, organic acids, Vitamin C or E, glycerin, and bioflavanoids. A concentration of 0.25% citrate extract for 60 s was also recommended by this manufacturer for use as an antimicrobial agent.

Chemical treatments

Chlorine and chlorinated compounds

Baker *(6)* reported that hypochlorous acid (HOCl) produced from hypochlorites (Cl_2) added to water provided the germicidal function by the formation of the anion hypochlorite OCl^- which is partially dissociated from HOCl (equations 1 and 2).

$Cl_2+H_20 \rightarrow HOCl+ HCl$ (1)

$HOCl \leftrightarrow H^+ + OCl^-$ (2)

Morris *(67)* developed a theoretic curve for the relative germicidal efficiency of HOCl and anion hypochlorite OCl^- to kill 99% of *E. coli* in water within 30 min at 2°C and 5°C within the pH range of 4 to10. His results indicated that HOCl had 80 times more germicidal potency than OCl^-. An advanced theory for the destruction of bacteria by chlorine was reported by Baker *(7)*. The author found that chlorine combined with proteins of cell membranes to form *N*-chloro compounds, and caused the eventual death of microorganisms. Brackett *(14)* reported that sodium hypochlorite of 50-200 mg/L (ppm) applied for up to 20 s was effective for the reduction of both *L. monocytogenes* LCDC 81-861 and *L. monocytogenes* Scott A populations. In the same study, artificially inoculated Brussels sprouts dipped into a 200 mg/L solution of hypochlorite for 30 s reduced *Listeria* populations by two orders of magnitude.

Cetylpyridinum Chloride (CPC, Cecure®)

Cetylpyridinium Chloride (CPC) is a quaternary ammonium salt with hydrophilic and hydrophobic properties (Fig. 2) that has a molecular weight of 358.07 *(4, 42)*. It is also a wetting agent or a low-tension surfactant *(43)*.

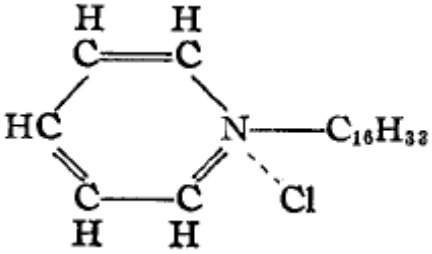
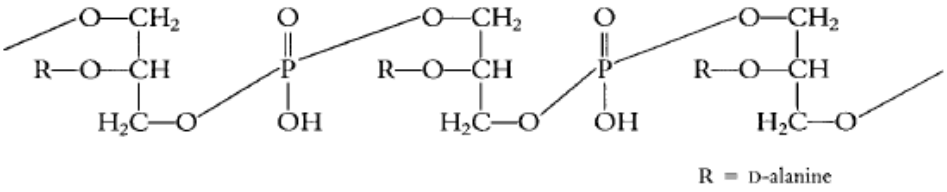


FIGURE 2. *The chemical structure of Cetylpyridinium Chloride (42).*

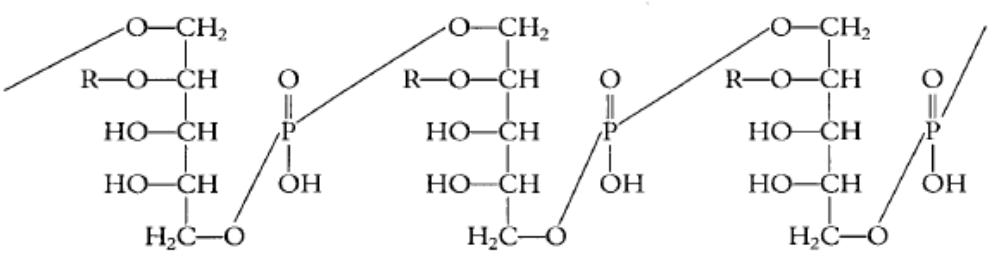
The FDA has approved the use of this quaternary salt as an anti-plaque agent for oral quaternary salt as an anti-plaque agent for oral hygiene and an over-the-counter drug. Common products include mouthwashes and throat lozenges *(4)*. The FDA Plaque Subcommittee allows a concentration level of 0.045% to 0.10% CPC in a highly bioavailable compound and alcohol-free formulation to treat plaque-induced gingivitis *(62)*. Throat lozenges contain 1 to 2 mg CPC and are formulated to dissolve slowly in the back of the mouth *(4)*. The FDA amended the food additive regulations on April 2, 2004 to permit the use of CPC as an antimicrobial agent when applied as spray during the final wash of poultry processing at less than 0.3 g of CPC per pound of poultry *(4)*.

The toxicological effects of the hydrophilic portion and the basic portion of CPC is caused by interactions with negative charges on bacterial cell membrane because the compound causes leakage of the bacterial cell's structure, the disruption of the cell membrane, and the inhibition of cell growth, which subsequently causes cell death (*2,58,102*). Strain sensitivity to CPC may be affected by differences in the cell structure between gram (+) and gram (-)

bacteria. Negatively charged teichoic acids (Fig. 3) are found only in gram (+) bacteria and easily interact with the cell and CPC. The cell surface of G (+) bacteria is more hydrophobic than the cell surface of G (-) bacteria and it permits a greater interaction with the hydrophobic molecules of CPC compared to that of G (-) bacteria. This interaction intervenes in the respiration of G (+) bacteria *(42, 58)*.



(a) Glycerol teichoic acid



(b) Ribitol teichoic acid

FIGURE 3. *The structures of teichoic acids found in G (+) bacteria are the polymers of (a) polyglycerol phosphate and (b) polyribitol phosphate (51).*

Fresh-cut vegetables treated with 0.1% and 0.5% CPC solutions were more effective for reducing G (+) than G (-) bacteria *(102)*. Treatments of 1% CPC showed more germicidal effectiveness than chlorine treatment on fresh-cut vegetables; however, the residual level of 1% CPC FDA limits for human

consumption *(102)*. *Listeria monocytogenes*, *Salmonella* Typhimurium, and *E. coli* O157:H7 treated with 0.5% CPC were reduced ($P \leq 0.05\%$) compared to that treated with 0.1% CPC (*102*). Lim and Mustapha *(58)* also reported that 0.5% CPC reduced the concentration of *L. monocytogenes* on sliced roast beef to an undetectable level (< 1 $\log_{10}$ CFU/cm^2) during storage at 4°C for 10 d, as compared to the inhibitory activity of 0.12% acidified sodium chlorite (ASC) or 0.25% CPC-0.06% ASC *(58)*. The use of a low concentration of CPC was studied in broiler skin. A study by Kim *et al. (52)* reported that the differences of 1.0 to 1.6 $\log_{10}$ in *Salmonella* Typhimurium cell numbers observed between the control and the 0.1% of CPC-treated chicken skins were significant. The authors also found no differences in cell counts between sprayed and immersed chicken skins.

Physical treatments

Ultrasound

The use of ultrasound for the removal or decontamination of spoilage microorganisms and certain food-borne pathogens from fresh produce and raw poultry products has been reported *(57, 88, 90)*. The bactericidal action of this treatment is caused by ultrasonic waves that are generated by mechanical vibrations at frequencies higher than 15 kHz. When such waves propagate in liquid media, alternating compression and expansion cycles are formed. High frequency ultrasound waves causes small bubbles to grow in a liquid media and makes the fluid appear to boil. When the bubbles attain a volume that no longer

absorbs energy, they implode violently. This phenomenon is called cavitation. The temperature of the gas bubbles can exceed 200°C with pressures greater than 300 atm when the bubble is at minimum volume. Under such conditions, the formation of free radicals is induced from water vapor in the bubble. Microorganisms in the liquid suspension may be inactivated by the shock-wave formed when the bubbles collapse or the free radicals *(81, 88)*. Coordinating the FDA-approved interventions with the chilling step in broiler processing, Sams *(88)* found that ultrasonication did not enhance the reduction in Aerobic Plate Count (APC) counts after post-chill drumstick skins were treated with 1% lactic acid (LA). The authors also found that pre-chill ultrasonication did not reduce APC on drumstick skins at any of exposure duration (15 min and 30 min) and temperature combination (25°C and 40°C) during 14 d of storage. However, Lillard *(57)* found that sonication of *Salmonella* cell suspensions produced an extra of 1 o 1.5 $\log_{10}$ reduction per 15 min of treatment in presence of free chlorine at 0.5 ppm. Sams *(88)* concluded that the greatest reduction in counts occurred when sonication and chlorination were used concurrently. The decontamination efficiency of food-borne pathogens using both sonication and washing treatments was also evaluated in fresh produce. Seymour *et al. (90)* reported the effect of ultrasound frequency (32-40 kHz) during small-scale washing trials (2 L) of vegetables. The study found that a further 1 $\log_{10}$ reduction of *S. Typhimurium* populations on cut iceberg lettuce was observed in ultrasound and chlorine combination during washing for 10 min, as compared

with ultrasound or chlorine treatment alone. In contrast to large-scale trials (40 L), four ultrasound frequencies (0, 25, 40, and 62-70 kHz) were tested to access the decontamination efficiency against an ampicillin resistant strain of *E. coli* on cut iceberg lettuce and strawberries compared to chlorine (25 ppm) and water washing treatments. The study found that the frequency of ultrasound had no significant effect on the decontamination of *E. coli* from the fresh produce tested.

Pulsed Electric Field (PEF)

High voltage Pulsed Electric Field (PEF) has been studied intensively in recent years with increased popularity in food applications. This process has been used to inactivate a variety of microorganisms, including *Bacillus subtilis*, *Lactobacillus delbrueckii*, and *Saccharomyces cerevisiae*. PEF treatment involves applying pulses with a magnitude at least 20 kV/cm and a short duration time from 500 ns to 4 μs *(86)* to liquid and semi foods. A severe electro-breakage of cell membrane is most commonly reported at this level. The instabilities in the membranes of treated microorganisms were induced by electrochemical compression and electroporation *(38, 61, 86, 105)*. Two diagrams of electrochemical compression and electroporation on cell membrane are illustrated in Figs. 4 and 5, respectively.

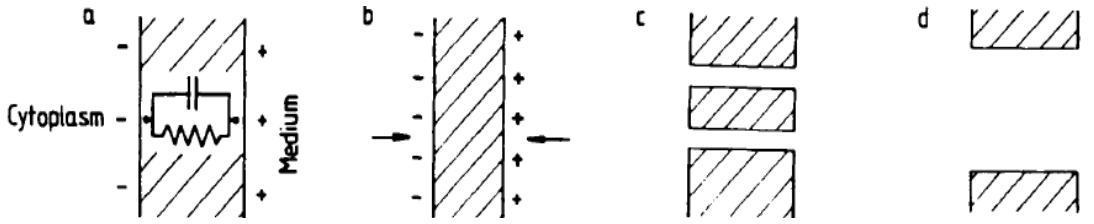


FIGURE 4. *Process of cell membrane compression, reversible and irreversible electric breakdown when exposed to high electric field strength (19, 53, 80, 99). E_C represents the critical electric field intensity. (a) cell membrane in the absence of electric field, E = 0 (b) membrane compression in the presence of electric field, $E < E_C$ (c) reversible pore formation in the presence of electric field, $E > E_C$ (d) irreversible pore formation in the presence of electric field, $E >> E_C$.*

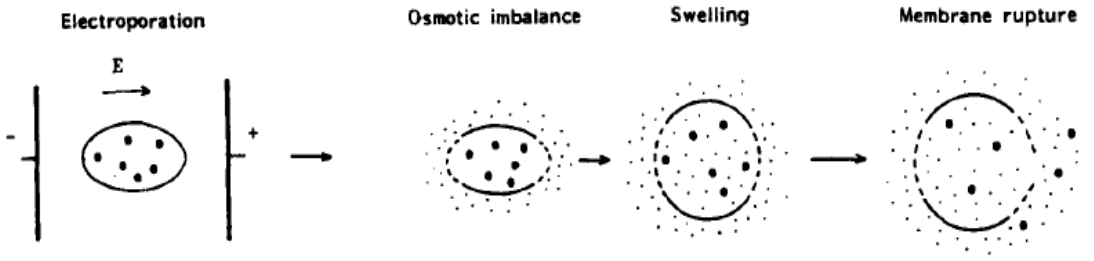


FIGURE 5. *A sequence of electroporation on cell membrane by high intensity PEF (19, 38, 99).*

Most studies indicated that PEF compromises the structural integrity of the cell membrane and affects other metabolic functions as a mode of action to inactivate bacteria. The mode of inactivation on microorganisms by PEF was described in Calderón-Miranda *et al (17).* The trans-membrane potential of the cell membrane is induced when an external field is applied to bacterial cells. Free charges due to the trans-membrane potential are generated on both sides of the cell membrane and then are attracted to each other. Subsequently, cells

are compressed. When the trans-membrane potential of cells is greater than that of natural cells (1 V), the cell membrane subsequently loses its stability and breaks down. As a result, pore formation is generated in cells and causes permanent damage to cell permeability or death occurs. Damaged cell membrane no longer functions properly to regulate the electron transport that controls entrance and exit of small molecules in time.

In a review paper, Wouters *et al. (104)* reported a selective medium plating technique might not be adequate for the detection of sub-lethally injured cells induced by PEF treatment. The authors also concluded that consistent findings can be drawn from other studies published for enumerating *L. innocua* on selective as well as non-selective media. Transmission Electron Microscopy (TEM) revealed that PEF-treatment with 6,000 pulses at 30 kV/cm cause rupture of the cell wall, the loss of intracellular contents (e.g. cytoplasmic and nucleic materials), and the production of cellular debris because irreversible electroporation (*86*). Calderón-Miranda *et al. (17)* reported that significant morphological changes to bacteria and yeast were caused by treatment with PEF. These changes included a significant increase in surface roughness, appearance of craters in the cell wall, elongation and disruption of organelles, cell wall breakage, and pore formation.

Several studies have reported that PEF inactivation is dependent on the electrical field strength, treatment time, pulse number, or temperature alone, or in a combination of factors *(10, 27, 34, 38, 39, 61, 83, 99, 104)*. Jayaram *et al.*

(47) found that 2 or 3 $\log_{10}$ reductions of *Lactobacillus brevis* cells could be achieved with a field strength as low as 5 kV/cm.

Increments of 5 ms (millisecond) in treatment time caused a great reduction of the population of *Lactobacillus brevis* in spite of temperature and media type. All treatments showed that an increase in pulse number (0-5) produced a significant reduction of *E. coli* O157:H7; however, the reductions resulted mostly from the first pulse *(61)*. On the contrary, Ho *et al (38)* reported that an increase in the electrical field strength and pulse number might not achieve a desired microbial decay. The study found that a minimum field strength of 10 kV/cm and pulse number of 10 decreased *Pseudomonas fluorescens* population by 6 $\log_{10}$ cycles.

Two studies *(47,106)* reported that the effectiveness of PEF inactivation could be enhanced by increasing the temperature of the media suspension. The population of *E. coli* O157: H7 decreased more at room temperature than at 0°C when cells were treated with PEF at the field strength of 12.5 kV/cm (*61*). Ravishankar *et al. (83)* reported a greater reduction of *E. coli* O157:H7 (maximum~3.0 $\log_{10}$) when the PEF treatment was applied at a temperature of 55ºC compared to temperatures of 5,15, 35, and 50°C, with a field strength of 30 KV/cm and 10 pulses at a rate of one per minute. Ho *et al. (38)* found that an electric field strength at 10 kV/cm for 10 pulses (2 sec pulse period and 2 μs pulse width) with a spike of reverse polarity produced a significant reduction of *P. fluorescens* population in various aqueous solutions. Fleischman *et al. (27)*

also reported PEF processing applied at the electrical field strength of 20 kV/cm, pulse number of 10, and temperature of 55ºC can achieve reduction of *Listeria monocytogenes* up to 4.5 $\log_{10}$/ml CFU in milk. The electric field strength and pulse numbers from 15 to 30 kV/cm and 5 to 50, respectively, were also evaluated in this study. The results showed that a maximum 1 $\log_{10}$ reduction was observed at 30 kV/cm and no improved reductions (< 1 $\log_{10}$) were found even when using 50 pulses at these conditions.

Inactivation of microorganisms by PEF treatment can also be influenced by the type of microorganism, the physiological conditions of the cells, initial inoculum size, the ionic strength and the conductivity of the suspension medium *(61,104)*. HÜlsherger *et al. (40)* reported the lethal effect of an electric field in the range of 0.2 V/µm to 2.0 V/µm and number of pulses in the range of 2 to 30 on Gram positive bacteria, Gram negative bacteria, and yeast cells. When low pulse numbers were applied to G (+) bacteria, Gram (-) bacteria, and yeasts, the G (+) bacteria and the yeast were less sensitive to electric pulse treatment than Gram negative bacteria. Treatment with high pulse numbers could cause less than 1% survival rates for all microorganisms. The effectiveness of PEF treatment with a mixed population of microorganisms was decreased (*98)*. The authors found that PEF inactivation of *E. coli* reached 6.5 $\log_{10}$ cycles and *B. subtilis* was reduced 3.2 $\log_{10}$ units, while a mixture of *E. coli* and *B. subtilis* substained 4.0 $\log_{10}$ reductions in pea soup after 30 pulses at a field strength of 30 kV/cm. Cells in the stationary phase of growth were generally more resistant

to PEF treatment. Indeed, PEF treatment inactivates young *L. innocua* cultures effectively (*105*). Zhang *et al (106,107)* indicated that inactivation by PEF was not a function of the initial inoculum. However, Matsutomo *et al (65)* demonstrated that the effect of initial inoculation and agitation were directly associated with PEF inactivation. Agitation in the treatment chamber resulted in increased inactivation. Ionic suspension media (e.g. 0.5% sodium chloride and 5 Mm phosphate buffer) caused slightly greater reduction of *E. coli* O157:H7 by PEF treatment than PEF in non-ionic glycerol and sucrose-based media at the field strength of 12.5 kV/cm with the temperature at 25°C (*61*). Ho *et al.* found that the field strength of 25 kV/cm could cause a desirable reduction in *P. fluorescens* only when sodium chloride was added into the solution as an electrical conductor.

PEF technology can inactivate both spoilage and pathogenic microorganisms in liquid or semi-liquid foods without altering the color and flavor in food or reducing the concentration of significant nutrients. As a result, this technology might provide consumers with safe, fresh-like, nutritious food. PEF treatment has been typically applied to fluid foods with intense electric fields in the range of 20 kV/cm to 80 kV/cm and short pulses in the range of 1 μs to 10 μs either in a batch or in a continuous flow system at low temperatures (*68*). A study by Reina *et al. (84)* found a great reduction of viable *L. monocytogenes* cells in three types of inoculated milk (whole, 2%, and skim milk) processed by PEF. The extent of reduction increased as treatment time increased. All milk

samples were inoculated with approximately 10^7 CFU/ml of *L. monocytogenes* and were treated with PEF at the field strength of 30 kV/cm at room temperature for 100, 300, and 600 μs, respectively. An approximate 3 $\log_{10}$ reduction of viable *L. monocytogenes* cells was obtained after 600 μs PEF treatment. The effect of field strength by PEF treatment was also determined in this study. Field strengths of 25 kV/cm and 30 kV/cm were used to evaluate PEF inactivation of viable *L. monocytogenes* cells in whole milk. The authors found that the field strength of 35 kV/cm and treatment times at 300 μs and 600 μs resulted in a greater reduction of viable *L. monocytogenes* cells. These results also indicated that the effect of electric field strength was related to the treatment time. Fernández-Molina *et al. (26)* reported that the survival of *P. fluorescens* cells in whole milk was significantly decreased when the field intensity was increased from 31 to 38 kV/cm and the pulse number was increased from 10 to 30. Microbial inactivation by PEF application was also studied in pea soup, whole egg products, yogurt, and tomato sauce. Martín-Belloso *et al. (63)* found that the inactivation of *E. coli* in liquid egg was a function of total treatment time. The total treatment time was expressed by both pulse duration (2 μs and 4 μs) and pulse numbers (up to 100 pulses). Results showed that the survival fraction of *E. coli* in liquid egg was reduced almost 6 $\log_{10}$ with 100 pulses of 4 μs, whereas the same number of 2 μs pulses reduced the bacterial population less than 5 $\log_{10}$. Mosqueda-Melgar *et al. (68)*; however, reported that an increase from 200 μs to 1000 μs in total treatment time did not enhance the inactivation of

Salmonella enterica Ser. Enteritidis population when 35 kV/cm of electric field intensity was applied to tomato juice.

Multiple antimicrobial treatments

Antimicrobial agents and pulse electric field treatment

A synergistic effect of a combination of high intensity pulsed electric field (HIPEF) and natural antimicrobials or organic acids have been reported in two studies *(61, 68)*. Liu *et al. (61)* found that colony counts of both control and acid (sorbic and benezoic)-treated samples of *E. coli* O157: H7, respectively, decreased progressively, with the field strength of PEF in the range of 6.25 kV/cm to 20 kV/cm. A different study by Mosqueda-Melgar *et al. (68)* found that during 1 h exposure, combinations up to 0.1% of cinnamon bark oil and 2.0% citric acid with HIPEF treatment (35 kV/cm and 1000 μs) were sufficient to reduce a *Salmonella enterica* Ser. Enteritidis population by 5.5-6.0 $\log_{10}$ in tomato juice. These studies suggested that PEF processing could rupture the membrane of the bacterial cell and thus, facilitate the entry of unassociated molecules into the cell and subsequently cause cell death. No research data has been identified that demonstrate a synergetic effect of CPC and PEF against *Listeria* spp. inoculated onto shrimp.

Antimicrobial agents and ultrasound treatment

An extra 10 fold reduction of microbial populations has been reported using a combination of ultrasound with chlorinated water in fresh produce and broiler processing *(57, 90).* These studies indicated that entrapped bacteria can

be easily detached from food products by a high frequency ultrasound wave. The combination of treatments has not been reported for the decontamination of shrimp inoculated with *Listeria* spp.

Antimicrobial agents and storage conditions

The bactericidal or bacterostatic effect of antimicrobial agents on microbial populations in a variety of food products has been studied during refrigerated or frozen storage. These studies *(70, 71, 75, 93, 95)* showed that the effect was dependent on the type of food or the type of microorganisms. A study by Mu et *al. (70)* reported results of treatments to fresh headless shrimp and rainbow trout fillets inoculated with approximately 7 $\log_{10}$ of *L. monocytogenes* and dipped in tap water, 10% Trisodium Sodium Phosphate (TSP), or 20% TSP solution. They were then packaged with Polyvinylidene Chloride (PVDC film) and stored for 0, 3, 6, and 9 d at 4°C. Psychrotrophic plate counts were also examined in this study. The study found that no significant effect of the 20% TSP dipping solution on the psychrotrophic and *L. monocytogenes* populations of headless shrimp after 6 d of storage at 4°C. The 20% TSP dipping treatment significantly lowered the psychrotrophic counts of rainbow trout fillets at day 0 and remained effective during storage. The bacteriostatic effect of CPC against *L. monocytogenes* in Ready-To-Eat (RTE) products during extended storage was published by Singh *et al. (93*). The authors inoculated frankfurters with either a high concentration (7-8 $\log_{10}$) or a low concentration (2-3 $\log_{10}$) of *L. monocytogenes* followed by treatment with 1%

CPC or 1% CPC followed by a water rinse at temperatures of 25, 40, and 55°C and for 30, 40, and 60 s. An immediate antimicrobial reduction of 1.4-1.7 $\log_{10}$ units by 1% CPC treatment was observed when it was sprayed onto frankfurters prior to packaging, but rinsing did not improve the effect of the CPC. The same author found the bacteriostatic effect (increase < 2 $\log_{10}$) was also observed at this level of CPC after 42 d of refrigerated storage at both 0°C and 4°C.

Antimicrobial agents and water wash

Studies reported *(23, 93, 102)* that water rinse after CPC treatment can reduce CPC residues to an acceptable level. Moreover, Dupard *et al. (23)* found that water rinse also removed the immediate antimicrobial effect of CPC, regardless of concentration, on *L. monocytogenes* inoculated cooked or raw shrimp products. The same result was reported by *Singh et al. (93)* in RTE food which showed that no significant reduction of *L. monocytogenes* populations was obtained from either 1% CPC treatment or 1% CPC treatment followed by a wash with water.

Chlorine production and *pH* effect after PEF processing

Free chlorine is generated at the anode and hydrogen at the cathode when electricity is applied to sodium chloride solution through electrodes *(3)*. Hypochlorous acid (HOCl) has a stronger germicidal activity against microorganisms than hypochloric acid (OCl). More HOCl is produced at a low pH compared to a high pH. As a result, chlorine is more active against microorganisms in the lower pH range.

Transmission Electron Microscopy (TEM)

Wouters *et al. (103)* reported that a selective medium plating technique might not be adequate for the detection of sub-lethally injured cells injured by PEF treatment. The authors also concluded that consistent findings can be drawn from other studies for the enumeration of *L. innocua* on selective as well as non-selective media. Hence, Transmission Electron Microscopy (TEM), might provide a better understanding of treatment effects by examining morphological changes that were caused by natural extracts *(72,82)*, bacteriocins *(17,94)*, or pulsed electric field *(17,27,83)*. A combination of PEF and CPC might achieve a better reduction of *Listeria* populations on raw shrimp or in medium as compared to PEF or CPC treatment alone. TEM micrographs might illustrate morphological changes on *Listeria* cells in suspension medium that were treated either by PEF, CPC, or a combination of both treatments. Studies *(17, 26, 77, 83, 86)* have been published that showed changes to cell structures of bacteria that were caused by PEF treatment. Other studies *(2, 58, 102)* fully elucidated antimicrobial actions of CPC on bacterial cells; however, only limited TEM micrographs were published in these papers. Mechanisms of synergism by combining PEF with antimicrobials on bacterial cells were reported in these studies *(76, 96)*. However, no published studies on TEM micrographs are available to illustrate if morphological changes on bacterial cells were induced by a combination of both PEF and CPC treatments.

OBJECTIVES

The goal of this project is to develop antimicrobial intervention strategies using multi-hurdle approach to reduce contamination of raw seafood products with *Listeria monocytogenes and Listeria innocua*. The specific objectives to achieve these goals are:

1. To determine the immediate and residual bactericidal effect of selected chemical antimicrobial intervention strategies applied alone or in combination on raw shell-on, and beheaded shrimp artificially contaminated with *Listeria monocytogenes and Listeria innocua* strains.
2. To determine the immediate and residual bactericidal effect of physical antimicrobial treatments applied alone or in combination on raw shell-on, and beheaded shrimp artificially contaminated with *Listeria monocytogenes and Listeria innocua* strains.
3. To determine the antimicrobial effect of chemical and physical treatments on raw shell-on, and beheaded shrimp artificially contaminated with *Listeria monocytogenes and Listeria innocua* strains.
4. To examine treated cells by electron microscopy to determine if the treatments caused any structural damage to cells that might elucidate the bactericidal effects of the treatments.

MATERIALS AND METHODS

Bacterial cultures

A cocktail consisting of *L. monocytogenes* ATCC 15313, *L. monocytogenes* Scott A46, *L. monocytogenes* Strain A, *L. innocua* NRRC b33076, and *L. innocua* ATCC 33090 was used. These strains were secured from the culture collection at the Department of Poultry Science at Texas A&M University and from Dr. Robin Anderson's laboratory at the Southeast Plains Agricultural Research Center of the United States Department of Agriculture in College Station, Texas.

Inoculum preparation

Each strain from the frozen stock culture was individually inoculated into 18 x 150 mm glass tubes containing 9 ml of Tryptic Soy Broth (Difco, Detroit, MI) and cultured for two consecutive transfers at 37°C for 24 and 18 h, respectively. A 1 ml aliquot of fresh culture from each strain (18 h incubation) was combined and diluted with 5 ml of Buffered Peptone Water (BPW, Difco). The concentration of the inoculum was approximately 9.0-9.5 $\log_{10}$ CFU/ml. This inoculum was used to inoculate the shrimp samples. The same concentration of inoculum was diluted into 9 ml of BPW to reach an approximately 5-6 $\log_{10}$ CFU/ml of *Listeria* spp. for the shelf-life studies.

Raw shrimp samples

Shell and head-on, farm-raised white shrimp (21~25 count per pound) hatched and raised in an artificial pond (~1230 surface acres) with a production capability of 3.5 million pounds per year were obtained from Harlingen Farms in Bayview, TX. The shrimp (~20 lb) were in blocks or IQF as sold by the processor. Frozen samples (-22.4ºC) were transported in refrigerated containers to the Microbial Challenge Pilot Plant at the Poultry Science Center of Texas A&M University and stored in the frozen state until testing. The same lot of head and shell-on shrimp were thawed at 3.3°C for 24 h, manually beheaded and then shrimp of uniform size were weighed for testing and inoculation.

Antimicrobial treatments

Beheaded shrimp samples were inoculated by submerging 25 g or 81 g of shrimp into a Whirl-Pak bag containing 70 ml or 100 ml of inoculum (60 or 90 ml buffered peptone water and 10 ml of bacterial cultures). Inoculated samples were soaked in the bag (Nasco) for 10 min and then air dried for 5 min on a sterile strainer to allow bacterial attachment and distribution. A final concentration of 7.0 to 7.5 $\log_{10}$ CFU/g *Listeria* spp. was recovered from air-dried shrimp. Contaminated samples (three shrimp for each treatment) were treated by immersion into selected treatments. These treatments were: cethylpyridinium chloride (CPC; 40% concentrate, Cecure®, Safe Foods Corp., USA), a chlorine treatment administered as sodium hypochlorite (Bleach; 6% concentrate, Household brand, HEB, USA), citrate extracts (CE; 40%

concentrate, Citrosan®, Diken group, Mexico) and a retail brand of food grade NaCl (0.1% and 0.5%, w/v). Sterile distilled water was used for the control treatment and wash in treatments.

Cell suspension samples were 10 ml of 18 h *L. innocua* NRRC b33076 or *L. innocua* ATCC 33090 culture. Cell suspensions were added into 90 ml of BPW to reach a final concentration of 8.0 $\log_{10}$ CFU/ml, 9.0-10.0 $\log_{10}$ CFU/ml, and 9.0-9.5 $\log_{10}$ CFU/ml, respectively. Cell suspensions were treated either with PEF in 0.5% NaCl for 4 to 5 min, 0.5% CPC, or the combination of both.

Listeria enumeration

Shrimp samples were transferred into a filter Whirl-Pak bag (Nasco, Modesto, CA) containing 25 ml of BPW and homogenized using a Lab-Blender 400 stomacher (Takmar, Cincinnati, OH) for 60s. Ten-fold serial dilutions were prepared in 9 ml of BPW and an aliquot (0.1 ml) of the homogenized sample was surface-plated on Modified Oxford agar (MOX, Oxoid, Basingtoke, UK). The plates were incubated at 30ºC for 24 to 48 h. Characteristic *Listeria* spp. colonies are small, and are surrounded with a black halo. Results were reported as *Listeria* spp. $\log_{10}$ CFU/g of sample. Treated and untreated samples were analyzed within 24 h, 48 h after frozen, and 1, 7 or 8 d after refrigerated storage.

Combination treatments

The effect of multi-hurdle interventions using chemical and physical interventions employed sample preparation methodologies as described. However, the treatments were applied in sequence. Physical treatments

included: refrigerated and frozen storage, water washes, pulse electric fields, and ultrasonication.

Chemical and biochemical interventions

CPC treatment

The final concentration of 0.5% (w/v) CPC was made by mixing 12.5 g of 40% of CPC concentration into 1 L of sterile distilled water for all experiments. Contaminated shrimp were treated with CPC and were followed with or without a water wash. Shrimp without a water wash were immersed in 60 ml or 100 ml of 0.5% CPC for an approximately 20 s at room temperature. Shrimp with a water wash were immersed in 60 ml or 100 ml of 0.5% CPC for an approximately 20 s at room temperature and were followed by a distilled water wash (60 ml or 100 ml) for an approximately 20 s at room temperature. A 1.25 g aliquot of 40% CPC was added to a 100 ml of *Listeria* cell suspensions that did not contain shrimp for 0, 40, 80, 120, 160, 200, 240, and 300 s.

Chlorine treatment

Commercial bleach (6% sodium hypochlorite) was diluted with sterile distilled water to prepare chlorine concentrations of 50, 100, and 200 ppm. The concentration of total chlorine was measured before immersion of the *Listeria* spp. inoculated shrimp into the solution. Free chlorine concentration was measured after immersion of the *Listeria* spp. inoculated shrimp. Total and free chlorine concentrations were determined using a HACH chlorine pocket

colorimeter™ (Loveland, CO). Inoculated shrimp were immersed in a 100 ml of chlorine solution or a 100 ml of water treatment for 20 s.

Citrate Extracts (CE) treatment

For single treatment, inoculated shrimp were immersed in 0.25% (v/v) citrate extracts (2.5 ml/1L) or distilled water for 1 min. For CE treatment in combination with PEF, shrimp were immersed in 0.25% CE for 1 min and followed by 1% NaCl PEF for 1 min or 1% NaCl PEF for 1 min and followed by 0.25% CE for 1 min. Water treatment combinations were applied in the same manner as described previously.

Physical interventions

PEF treatment

An electrical stimulation device from Simmons technology (Model SF7000, Dallas, GA) was used to generate a maximum output of 30-40 volts and 0.26 A for PEF treatments. The remainder of the system consisted of two copper electrodes (the average distance between electrodes was 2.5 cm) and a glass beaker which served as a conductor and a base, respectively. The electrodes were submerged in a Whirl-Pak bag containing samples in the breaker and electrical charges were applied to the top of the electrodes. The treatment, parameters were a 100 ml of NaCl solution at concentrations of 0.1% to 1%, pulse lengths (10 s on and 5 s off), for a duration of 1 to 4 min.

Ultrasound treatment

One liter of 0.5% (w/v) CPC solution or water was added to the ultrasonic cleaning tank (model 5200R-4; Bransonic Ultrasonic Co., Dansbury, CT). CPC- or water-treated shrimp were immersed in the above solutions for 0, 5, 10, 15, and 20 min at an ultrasonic energy of 47,000 Hz with a 200 W output. Shrimp were immersed in a 100 ml of sterile water for an approximately 20 s after exposure to the sonic treatment. The temperature was monitored and recorded during ultrasonic treatment using an Omega digital thermometer (model HH501BT, Stamford, CT) during 20 min of exposure.

Refrigerated and frozen storages

Frozen treated and untreated samples were stored in a Lowe's Holiday® Styrofoam box (model LCH0701PW, North Wikesboro, NC) for 48 h, and then thawed at 3.3°C overnight before enumeration. Samples to evaluate the effect of refrigerated storage on the number of *Listeria* spp. were stored for 1, 7, or 8 d in a J&R Hobert® refrigerator (Model W, Troy, OH) before enumeration. Samples for the shelf-life study were stored in Whirl-Pak bags (one shrimp per bag) in a VWR international incubator (model 2005, Cornelius, OR) at 7°C for 8 d.

Color determinations

A CR-200 Minolta chroma meter (Minolta CR 200, Tokyo, Japan) was used to measure the color parameters of untreated and treated shrimp before and after freezing. Each sample were measured at the head, upper-body, and lower-body regions using the CIE Lab system with tristimulus L* (Lightness), a*

(redness), and b* (yellowness) values. Averages and standard deviations of L* and a* values were calculated *(56).*

Free chlorine production and *pH* effect for PEF treatment

Sodium chloride solutions were prepared by the addition of 0, 0.1, 0.5, and 1.0 g of NaCl to distilled water (100 ml, w/v) and electrodes were submerged in the same manner as described previously. Treatment times for PEF processing were set to 0, 2, and 4 min. After PEF treatment, free chlorine concentration (ppm) and *pH* values were immediately measured using a HACH Pocket colorimeter^{TM} (Loveland, CO) and a Thermo Scientific Orion 2 star pH meter (Beverly, MA), respectively.

Statistical analysis

Bacterial counts were transformed to $\log_{10}$ values before statistical analysis. Three experimental replications were performed for each test. Data were analyzed with the appropriate General Liner Model and SAS (Statistical Analysis Systems Institute, Cary, NC) software. Significant differences between means at a 5% probability level (P = 0.05) were determined using the Tukey test and LS means.

Bacterial preparation for TEM

Listeria innocua NRRC b33076 and *Listeria monocytogenes* ATCC 15313 used in this study. were obtained from Dr. G. Acuff's lab, Kleberg Center, Texas A&M University (College Station, TX). *Listeria innocua* NRRC b33076 or *L. monocytogenes* ATCC 15313 were resuscitated from frozen cultures by two

sequential transfers into 9 ml of Tryptic Soy Broth (TSB, DIFCO) and were incubated for 12 or 18 h at 30°C after each transfer. The active cultures (grown for 12 or 18 h) were added to 90 ml of TSB. Untreated cell suspensions were used as control and the treatment cell suspensions were subjected to 0.1% sodium chloride PEF for 2 min, 0.5% CPC, or the combination of both treatments. All of the suspensions were transported to the USDA Southern Plains Agricultural Research Center (College Station, TX) for preparation to be examined by TEM.

TEM specimen preparation

Untreated and treated suspensions of *Listeria innocua* NRRC b33076 (10^7~10^8 CFU/ml) and *Listeria monocytogenes* ATCC 15313 (10^8~10^9 CFU/ml) were washed 3 to 4 times with fresh TSB and centrifuged at 9200 *X g* for 10 min (rotor type SS-34, Sorvall® RC 5C plus) after each washing. Pellets recovered by centrifugation were mixed with 4% (vol/vol) glutaraldehyde and 4% (wt/vol) paraformaldehyde solution in 0.1 M sodium cacodylate (pH 7.4) and allowed to react for 1 h before another centrifugation which was performed as described above. The bacteria remaining in the supernatant were captured by filtration through nitrite-made filter paper (0.45 microns). Specimens were covered with 2.0% molten agar and reduced to rectangles before the buffer wash was applied. The pellets were washed with 0.1M sodium cacodylate and 0.1M sucrose followed by centrifugation at 7100 *X g* for 5 min (rotor type A-11-8, swing bucket, Eppendorf). Cells were suspended with 2.0% molten agar

(DIFCO) and centrifuged as described. Pellets were recovered and covered with molten agar and reduced to strip sizes.

Agar-coated pellets were post-fixed with 1% (wt/vol) osmium tetroxide in 0.1M sucrose and 0.1M sodium cacodylate for 2 h at 4ºC. These pellets were dehydrated in an ethanol series (vol/vol) (50%, 75%, and 95%). Ethanol was replaced by acetone and the cells were infiltrated and then embedded in epoxy resin (ERL 4206, DER 736, NSA, DMAE with lecithin, and DMAE). Thin sections were cut with glass knives using a Reichert Ultracut S ultramicrotome (Deerland, IN) and picked up on 300 mesh nickel grids. Sections were post stained with 1% (wt/vol) aqueous uranyl acetate followed by Reynold's lead citrate (Reynolds, 1963). Grids were examined and photographed at an accelerating voltage of 100 kV in the Hitachi H7100 TEM (Tokyo, Japan).

RESULTS AND DISCUSSION

Effect of CPC treatment on the survival of *Listeria* spp. on fresh shrimp and shrimp subjected to refrigeration (RF) and frozen (FZ) storage

A total of 1.13 kg of shrimp was used in this study. Contaminated shrimp were washed with water, CPC, a double wash with water, and CPC followed by a water wash. The effects of the treatments were determined immediately and 1 d after refrigeration and after 2 d of frozen storage. CPC alone and the CPC followed by a wash with water immediately reduced *Listeria* spp. populations on shrimp by approximately 2.0 $\log_{10}$ ($P < 0.05$, Fig. 6). However, the effect was not enhanced by double washes with water. There was no difference between these treatments after refrigeration at 3.3°C for 1 d (Fig. 6). The *Listeria* spp. populations of the sample treated with CPC followed by a water wash was reduced approximately 2.2 $\log_{10}$ after 2 d of frozen storage ($P < 0.05$) compared to other treatments (Fig. 6). These results indicated the application of CPC in combination with a wash with water might have improved the antimicrobial effect on *Listeria* spp. contaminated shrimp during freezing. Contrary to our findings, Dupard *et al.* (*23*) reported the application of a 1 min water rinse following a 1 min CPC treatment reduced the bactericidal effectiveness of CPC on *Listeria* spp. inoculated shrimp stored 24 h at 4^{o}C and this effect was more dramatic at concentrations $\geq$ 0.8% CPC than at $\leq$ 0.6% CPC. This suggests that the enhanced bactericidal effect observed with our CPC treatment when followed with water wash may have been due to the presence of residual CPC on the

shrimp during storage following our shorter duration water wash, which was applied for only 20 s.

Significant effects of freezing or refrigeration ($P < 0.05$) were observed for shrimp washed with water only, shrimp subjected to double washes with water, and shrimp treated with CPC followed by a wash with water. Freezing caused a reduction of approximately 0.4 $\log_{10}$ CFU of *Listeria* spp. per gram of shrimp washed with water and on shrimp treated with CPC followed by a water wash. However, the *Listeria* spp. populations on shrimp receiving these treatments increased by 0.5 $\log_{10}$ after 1 d of refrigeration. The use of double washes with water, on the other hand, caused the *Listeria* spp. populations to decrease on shrimp by 0.4 $\log_{10}$ CFU/g under refrigeration. However, this reduction reflects only 1 d of refrigeration. The *Listeria* spp. populations of the shrimp that received only the wash with water increased after 7 d of storage at 3.3°C (Figure on page 75). This indicates that the reduction observed after only 1 d of storage was a temporary effect.

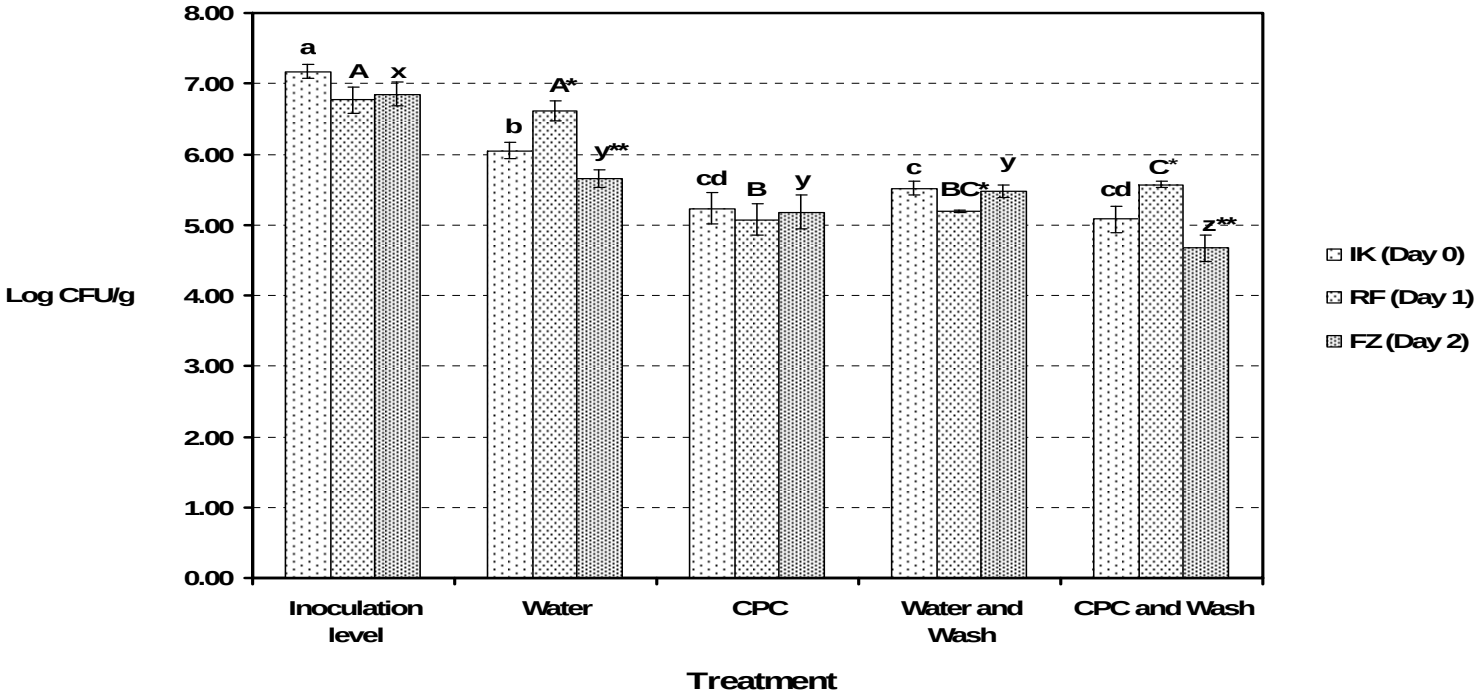


FIGURE 6. *Mean* Listeria spp. *populations on raw shrimp treated with a single or double water wash, 0.5% CPC, a 0.5% CPC and a water wash followed by storage at 3.3°C and -22.3°C for approximately 24 and 48 h. IK= immediate killing (Day 0), RF= Refrigeration (Day 1), and FZ=Frozen storage (Day 2). Bars at the storage day 0 (a, b, c, d), day 1 at 3.3°C (A, B, C), and day 2 at -22.3°C (X, Y, Z) with different letters are significantly different (*P* < *0.05*, n = *3). Asterisks (*, **) between means in the same treatment are significantly different (*P < *0.05*, n = *3) after refrigeration and frozen storage.*

Effect of CPC or water during 20 min of ultrasound washing on the number of *Listeria* spp. on raw shrimp

Contaminated shrimp (n=30) were immersed in 1 L of 0.5% CPC or in water in the tank of the ultrasonicator and were sonicated for 0, 5, 10, 15, and 20 minutes. After sonication, the shrimp were washed with distilled water. Difference in an approximately 0.5 to 1.0 $\log_{10}$ CFU of *Listeria* spp./g of shrimp was found after ultrasonic treatment in water and CPC solution for 15 and 20 minutes *(P* < 0.05). However, the effect of the time of ultrasonic treatment was not significant (*P* > 0.05, Fig. 7). Our findings related to ultrasonic treatment are in agreement with results reported by Sams and Feria *(88)*. They found no effect of sonication on the APC counts of chicken drum sticks treated with water and 1% lactic acid during 14 d and 10 d of storage at 4°C. However, Seymour *et al. (90)*, reported an additional 1 $\log_{10}$ reduction of *S.* Typhimurium (2.7 $\log_{10}$) on iceberg lettuce when ultrasound was combined with a chlorine treatment compared to chlorine treatment without ultrasound treatment. Lillard *(57)* found that sonication of poultry skin in water and in chlorinated water reduced *S.* Typhimurium on poultry skin by 1.1 to 1.5 $\log_{10}$ and 2.0 to 3.2 $\log_{10}$, respectively. The increase in the temperature of the treatment solutions during 20 min of ultrasound treatment is shown in Fig. 8. Our data showed that the temperature increase observed during longer sonication times did not enhance the effect of ultrasound treatment on the number of *Listeria* spp. on shrimp.

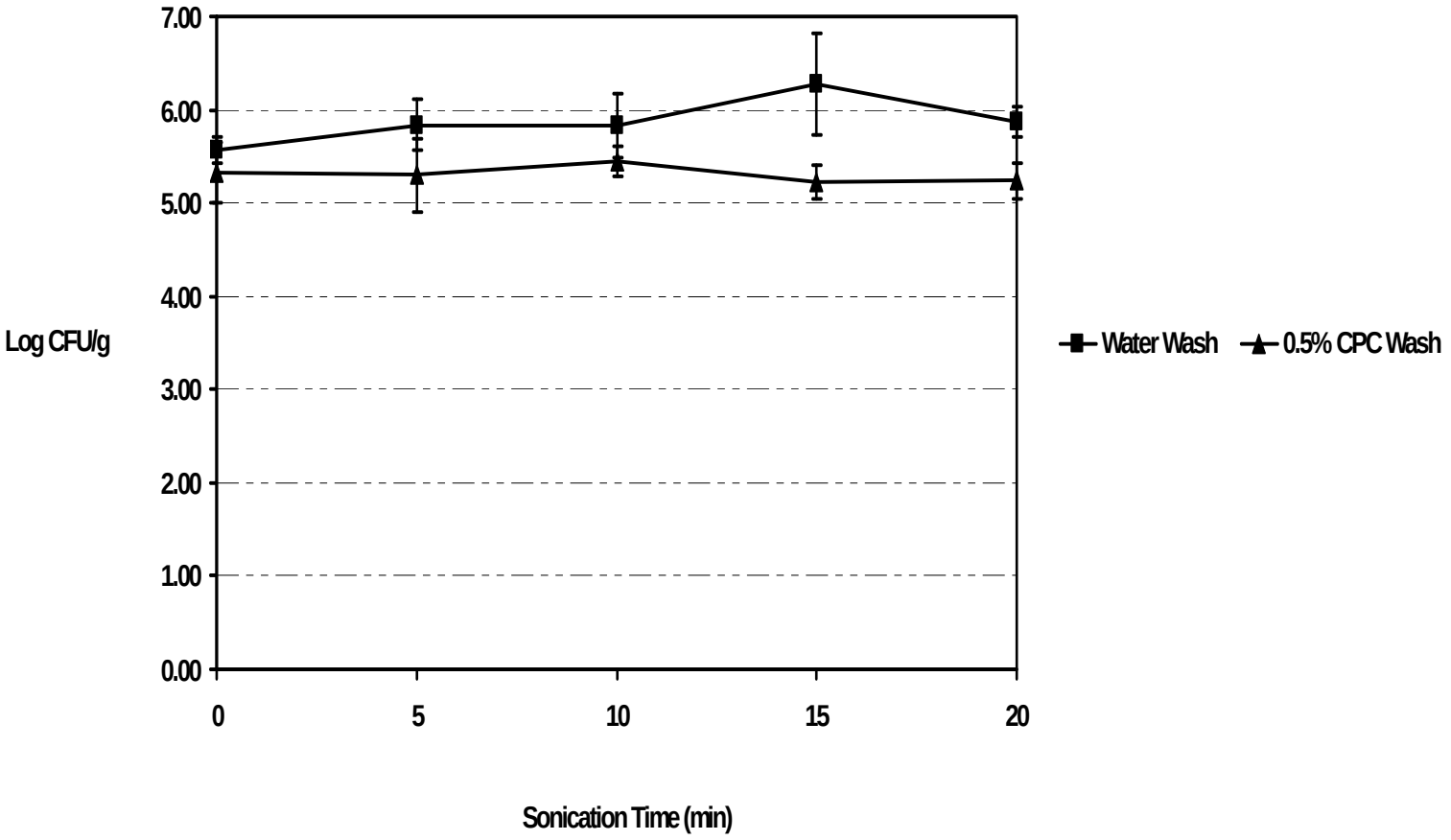


FIGURE 7. *Effect of sonication time on* Listeria spp. *populations of raw shrimp after sonication with 0.5% CPC or after sonication in water*. n = *3*.

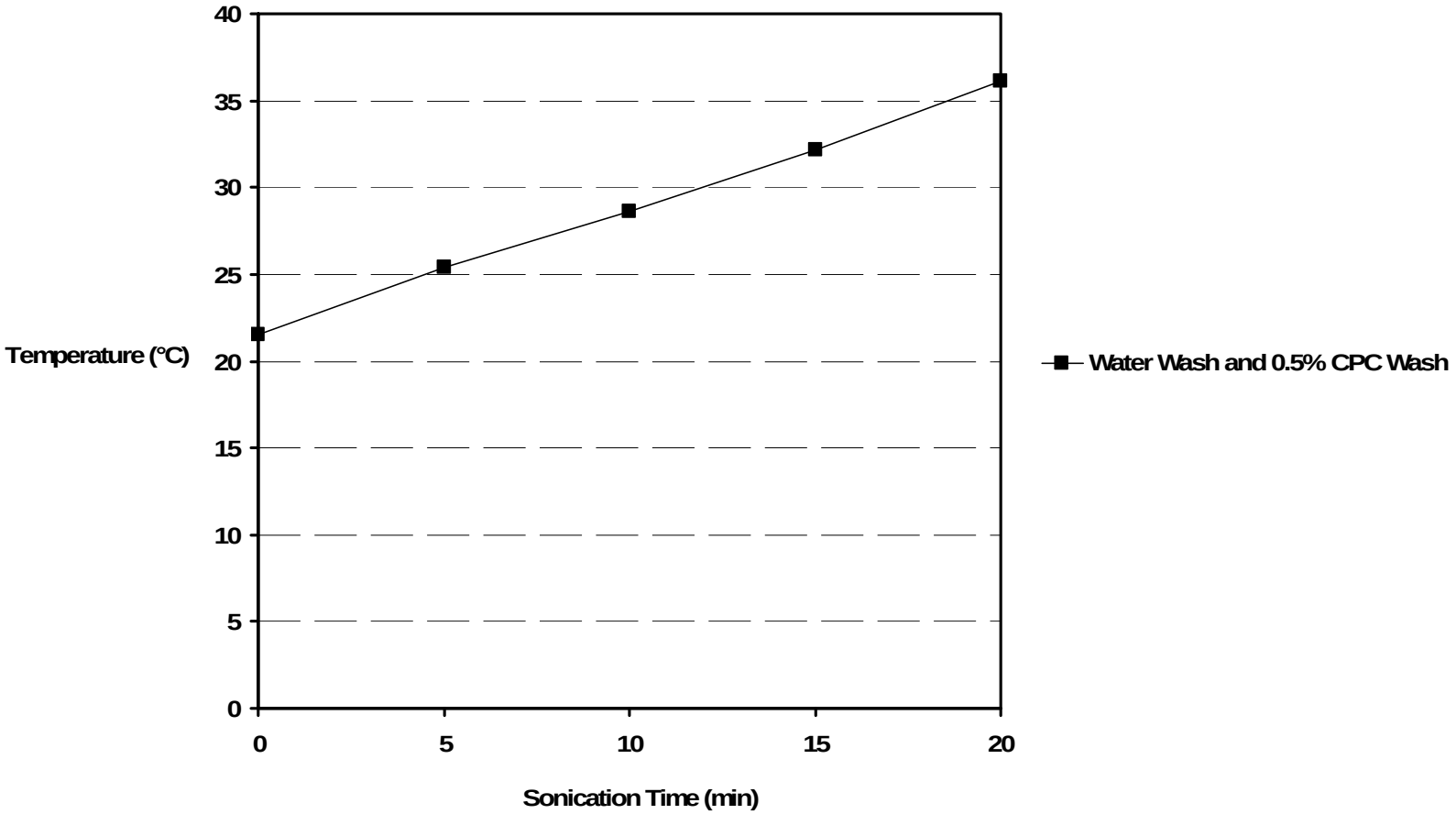


FIGURE 8. *Change in temperature with time during sonciation.*

Effect of chlorine concentration on the survival of *Listeria* spp. on shrimp

A total of 30 shrimp was used in this study. All of the concentrations of chlorine and the washing of shrimp with water caused a significant *(P* < 0.05) decrease in the concentration of *Listeria* spp. on the shrimp before freezing, as compared to the inoculation level (untreated control). Prior to freezing, there was no difference between the numbers of *Listeria* spp. on chlorine and water treated shrimp (*P* > 0.05, Fig. 9). After 2 d of frozen storage, *Listeria* spp. counts on shrimp treated with 50 mg/L (ppm) were not different from the inoculation level (untreated control) (Fig. 9). There was also no difference between the numbers of *Listeria* spp. on shrimp treated with the various levels of chlorine and treated with water after 2 d of frozen storage (Fig. 9). These findings are contrary to the results obtained by Beuchat and Brackett *(11)* who reported that chlorine concentrations of 0.2 and 0.25 ppm caused a significant reduction (*P* < 0.05) of *L. monocytogenes* on contaminated lettuce at both 5 and 10°C.

Brackett *(14)* also reported that chlorine concentrations less than 50 ppm had no antimicrobial effect on cell suspensions of *L. monocytogenes* Scott A and LCDC 81-861. However, when the cells were exposed to chlorine concentrations greater than 50 ppm, no viable cells were observed after 20 s of exposure *(14)*. Approximately 2 to 3 $\log_{10}$ reductions were observed when contaminated Brussels sprouts were dipped into a 200 ppm chlorine solution for 30 s. By comparison; however, a 1.4 $\log_{10}$ reduction was observed when contaminated Brussels sprouts were dipped into water for about the same time period. Injured cells were unable to recover from some batches of Brussels sprouts dipped in chlorine. Freezing caused damage to *Listeria* cells ($P < 0.05$) in the inoculation level (untreated control) and water-treated shrimp in the present experiment, which suggests that untreated and water-treated shrimp might more susceptible to formation of ice crystals than shrimp treated with a chlorine dip.

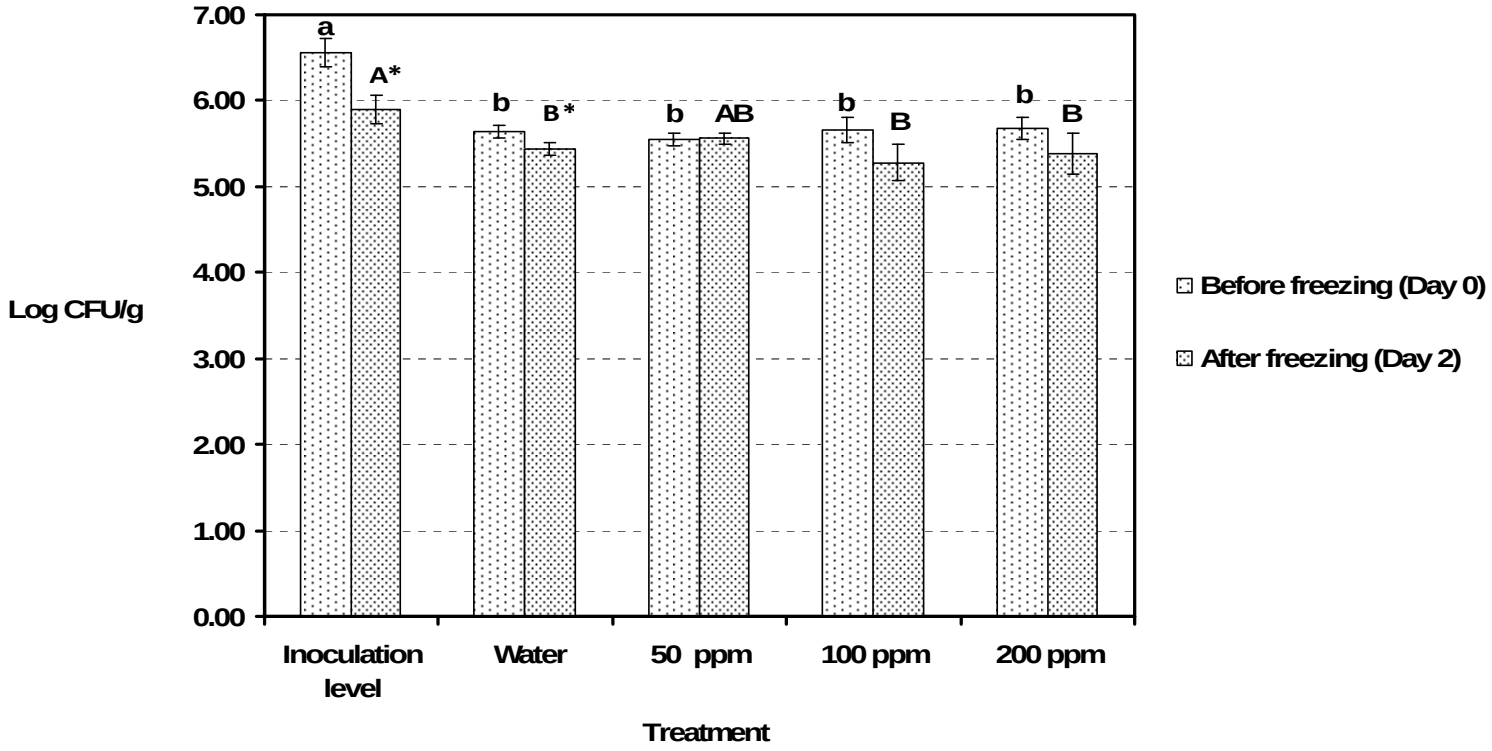


FIGURE 9. *Survival of* Listeria spp. *on raw shrimp immersed in water, 50, 100, and 200 ppm chlorine solution for 20s. Bars in the treatment before freezing (a, b) or after freezing (A, B) with different letters are significantly different (*P < *0.05*, n = 3*). An asterisk (*) between means in the same treatment are significantly different (*P < *0.05*, n = *3) after freezing*.

Effect of application sequence (PEF, CE, and water) and storage on the survival of *Listeria* spp. on inoculated shrimp

A total of 42 shrimp was used in this study. The treatment orders were PEF in 1.0% NaCl followed by CE or water (PEF/CE, PEF/W) and CE or water followed by PEF in 1.0% NaCl (CE/PEF, W/PEF). Shrimp were examined for *Listeria* spp. concentrations immediately after treatment and after storage for 2 d at -22.3^{o}C (Fig. 10). For shrimp examined before freezing, an effect of the order of treatments (physical and chemical, or chemical and physical) on the *Listeria* spp. populations on shrimp was observed, with PEF in 1% NaCl followed by a wash with water-treated (PEF/W), CE followed by PEF-treated (CE/PEF), and PEF followed by CE-treated shrimp (PEF/CE) yielding lower ($P < 0.05$) numbers of *Listeria* spp. than shrimp washed in water followed by PEF in 1% NaCl (W/PEF) (Fig. 10). The reduction of *Listeria* spp. on shrimp treated with 0.25% CE was similar ($P > 0.05$) to the reduction of the *Listeria* spp. populations caused by washing with water (W). A freezing effect was observed for the shrimp treated with water, the combination with PEF, and for the inoculation level (untreated control) ($P < 0.05$, Fig. 10). This indicates ice crystals may have formed faster during freezing in shrimp treated with water than in shrimp treated with CE. Freezing has been observed to damage *Listeria* spp. on shrimp that have been exposed to water in our experiments. CE contains glycerin and glycerides which are known to protect microorganisms from damage during freezing.

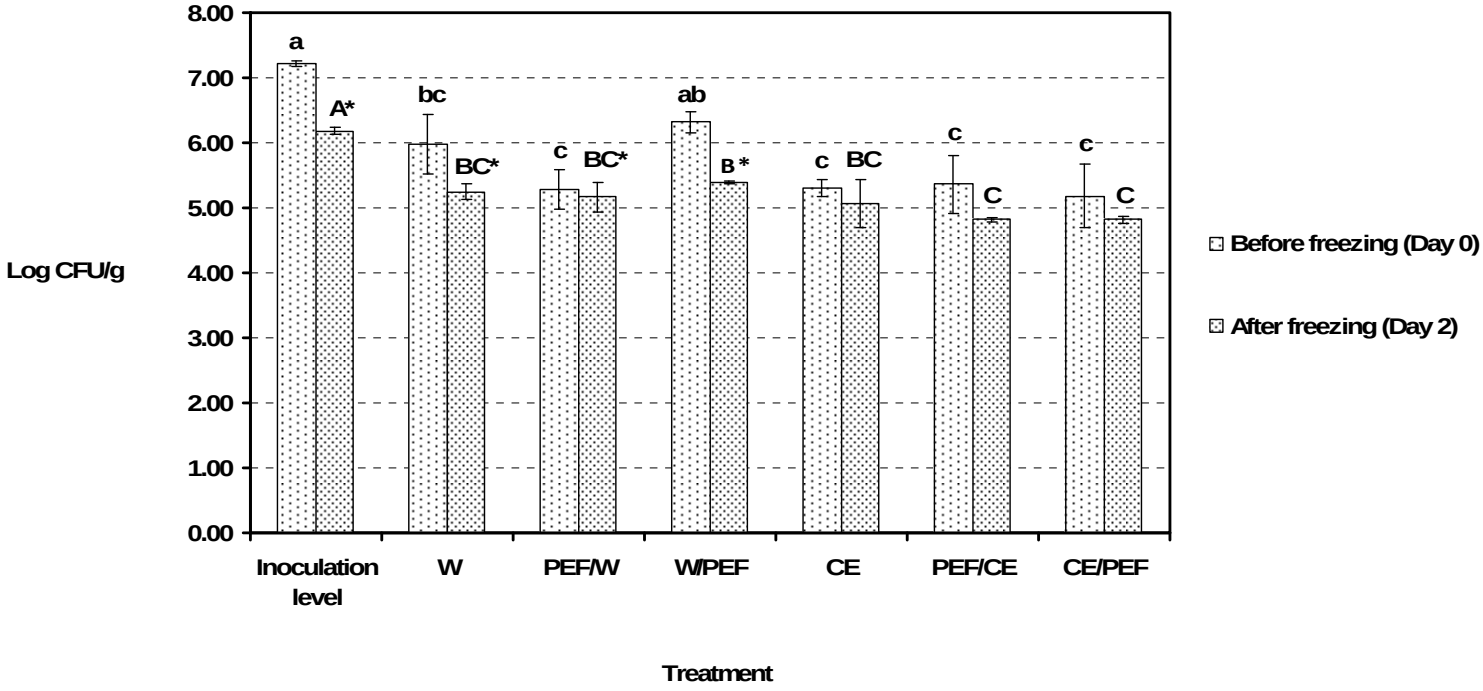


FIGURE 10. *Mean* Listeria spp. *populations on raw shrimp treated with water (W), PEF in 1% NaCl followed by a water wash (PEF/W), a water wash followed by PEF in 1% NaCl (W/PEF), 0.25% Citrate Extracts (CE), PEF in 1% NaCl followed by 0.25% Citrate Extracts (PEF/CE), and 0.25% Citrate extracts followed by PEF in 1% NaCl (CE/PEF). Bars in the treatment before freezing (a, b, c) or after freezing (A, B, C) with different letters are significantly different (*P < 0.05*, n = *3). An asterisk (*) between means in the same treatment are significantly different (*P < *0.05*, n = *3) after freezing.*

Effect of CE and PEF treatments on the color of shrimp before and after frozen storage

A total of 42 shrimp was used in this study. Inoculated shrimp were not used to measure the effect of freezing and CE treatment on the color of shrimp. Prior to freezing, the shrimp that received the water and PEF (W/PEF) treatment were lighter ($P < 0.05$) than the untreated (NT) and PEF-water (PEF/W) treated shrimp (Fig. 11). However, difference in lightness could not be visualized. There was no difference ($P > 0.05$) between L^* values after 2 d of frozen storage for untreated (UT) and treated shrimp.

Similar result were found for a^* (redness) values. The a^* values of shrimp after the water and PEF combination had less redness ($P < 0.05$) than PEF and water, CE, CE and PEF (CE/PEF), and PEF and CE (PEF/CE) combinations (Fig. 12) prior to freezing. However, there was no difference between the a^* values of all treated or untreated shrimp after 2 d of frozen storage ($P > 0.05$). Within the same treatment, the a^* value of PEF and water-treated shrimp was slightly increased after 2 d of frozen storage (Fig. 12). Differences in L^* *and* a^* values found prior to freezing were not found after 2 d of frozen storage.

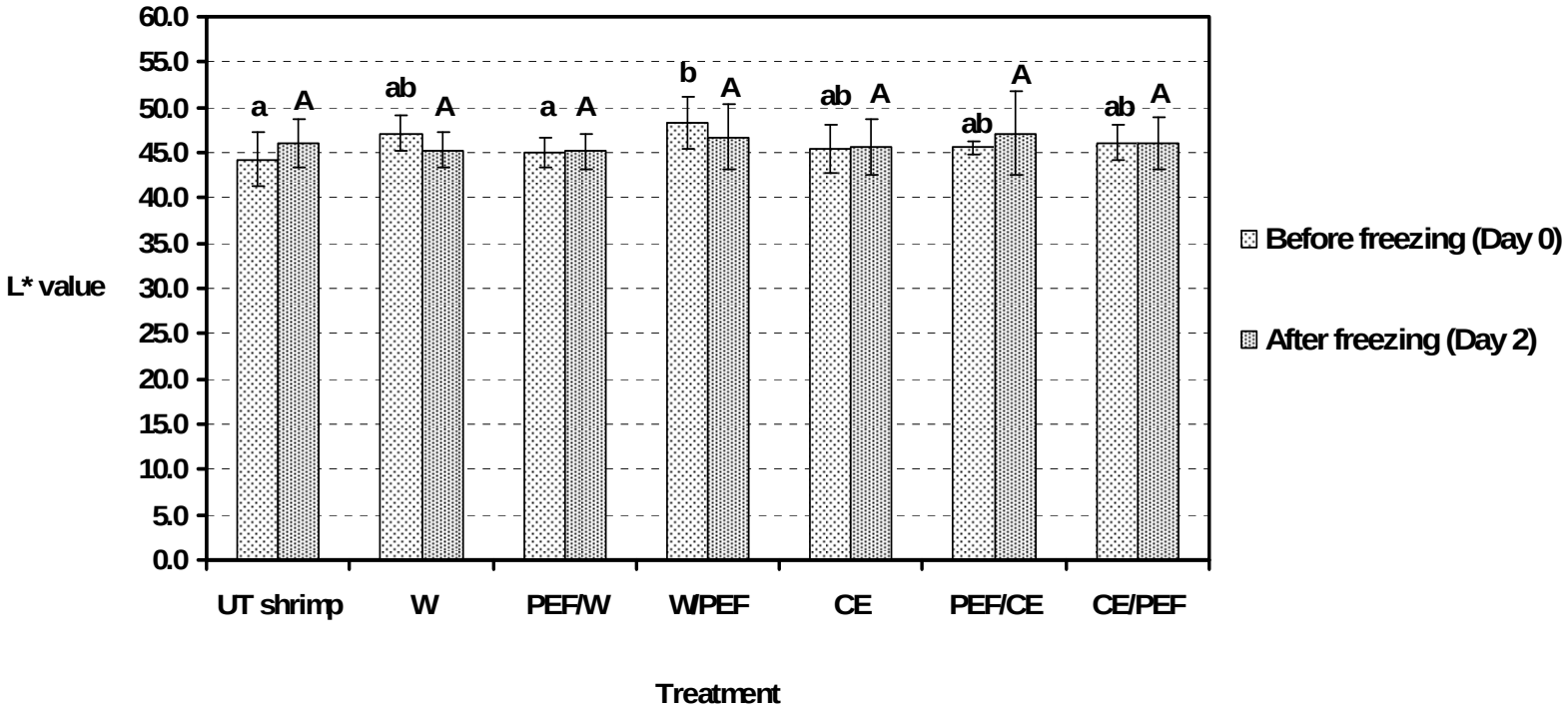


FIGURE 11. *Lightness (L* values) of untreated (UT) shrimp, and shrimp treated with water (W), PEF and water (PEF/W), water and PEF (W/PEF), Citrate Extracts (CE), PEF and Citrate Extracts (PEF/CE), or Citrate Extracts and PEF (CE/PEF), and storage at -22.3°C for 48 h. Bars in the treatment before freezing (a, b) or after freezing (A) with different letters are significantly different (*$P < 0.05$, $n = 9$).

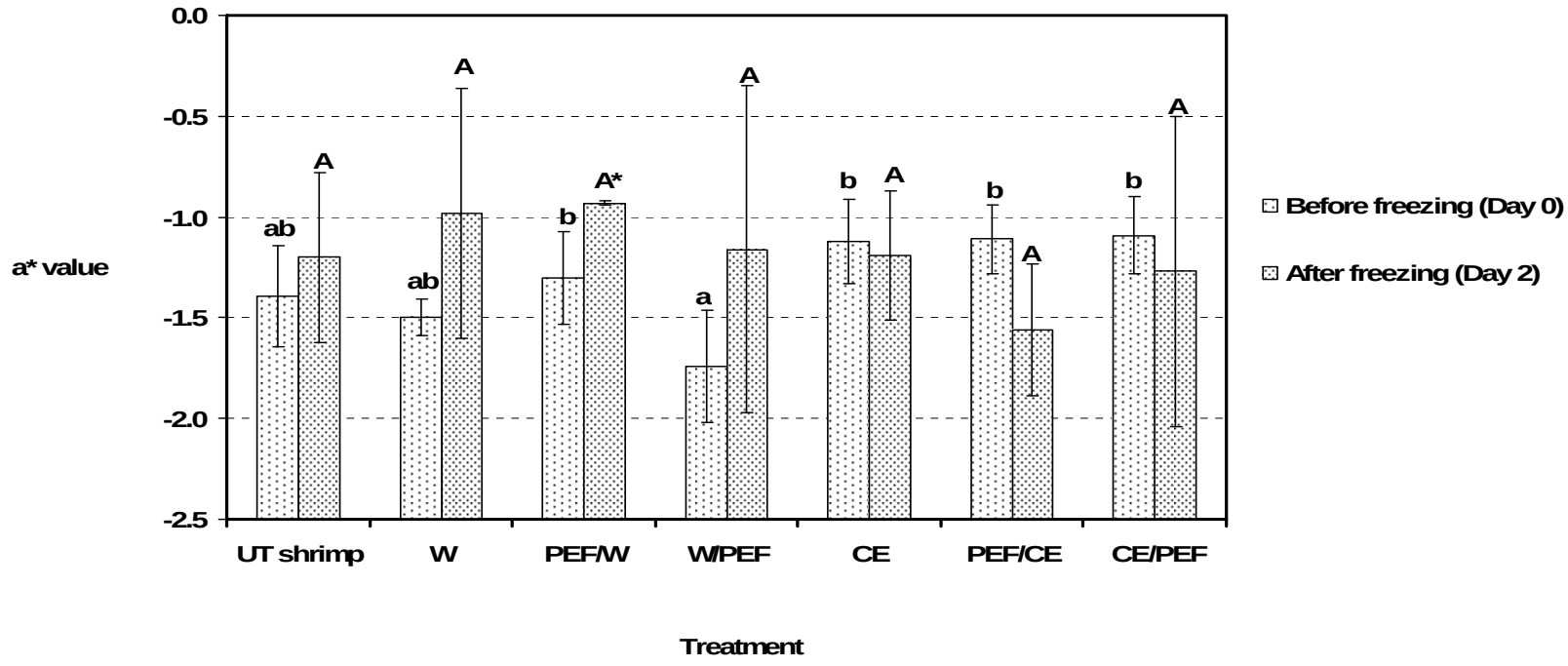


FIGURE 12. *Redness (a* values) of untreated (UT) shrimp, and shrimp treated with water (W), PEF and water (PEF/W), water and PEF (W/PEF), Citrate Extracts (CE), PEF and Citrate Extracts (PEF/CE) or Citrate Extracts and PEF (CE/PEF), and storage at -22.3°C for 48 h. Bars in the treatment before freezing (a, b) or after freezing (A) with different letters are significantly different (P < 0.05, n = 9). An asterisk (*) between means in the same treatment is significantly different (P < 0.05, n = 9) after freezing.*

Antimicrobial effect of PEF in 1% sodium chloride and freezing on the survival of *Listeria* spp. on shrimp

Shrimp (n=18) were used for this study. PEF in a solution of 1% sodium chloride was used to determine if this treatment had an effect on the survival of *Listeria* spp. on shrimp. An effect of 1% added NaCl on the bactericidal effect of PEF treatment against *Listeria* spp. was observed on shrimp only after 2 d of frozen storage ($P < 0.05$, Fig. 13). Before freezing, the concentration of *Listeria* spp. on 1% NaCl PEF-treated shrimp was not different from the concentration of *Listeria* spp. on shrimp that were subjected to PEF in the 0% NaCl (water) only treatment ($P > 0.05$, Fig. 13). Shrimp treated with PEF in the 1% NaCl solution had a lower concentration of *Listeria* spp. than the shrimp that were treated by PEF in water after 2 d of frozen storage. Freezing the shrimp also reduced the number of *Listeria* spp. ($P < 0.05$) on samples treated by PEF in 0% NaCl and inoculation level (untreated control) (Fig. 13). The results indicated that PEF had an antimicrobial effect at these conditions and can be useful for the decontamination of *Listeria* spp. from shrimp.

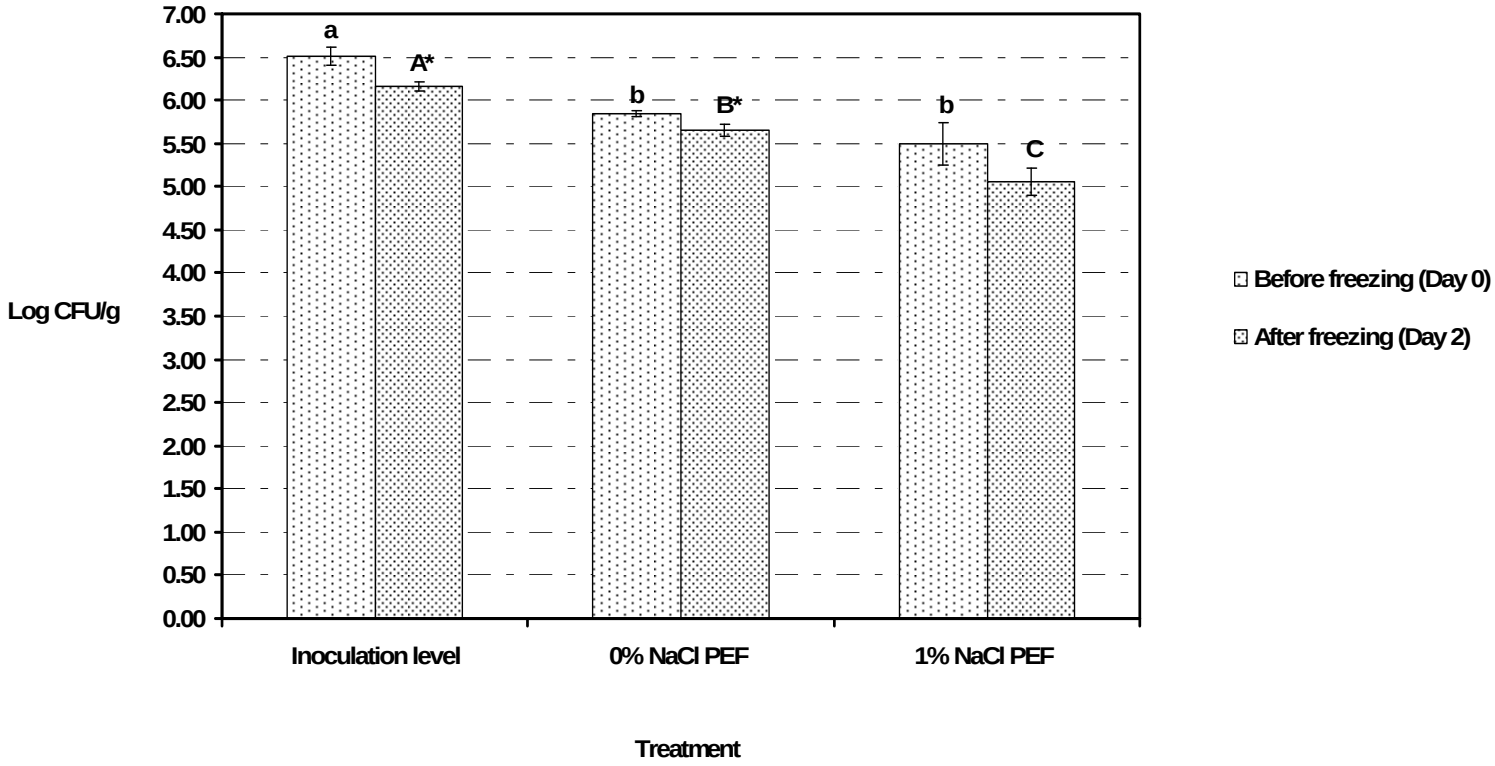


FIGURE 13. *Effect of PEF in 1% NaCl solution on the survival of* Listeria spp. *on raw shrimp for pulse duration time of 1 min. Bars in the treatment before freezing (a, b) or after freezing (A, B, C) with different letters are significantly different (*$P < 0.05$, n = *3). An asterisk (*) in the same treatment are significantly different (*$P < 0.05$, n = *3) after freezing.*

Effect of exposure time of PEF in 0.1% NaCl and frozen storage on the survival of *Listeria* spp. on shrimp

This experiment was conducted using 30 shrimp. These samples were exposed to PEF in a 0.1% solution of NaCl for 1 and 2 minutes. There was a significant ($P < 0.05$) reduction in the number of *Listeria* spp. on shrimp prior to freezing for all PEF-treated shrimp as compared to the inoculation level (untreated control) (Fig. 14). No difference was found between the counts of *Listeria* spp. when the PEF treatment was applied to shrimp in water (0.0% NaCl) and in 0.1% NaCl ($P > 0.05$, Fig. 14). The time of exposure of shrimp to PEF treatment also did not influence the number of *Listeria* spp. remaining on the shrimp. Contrary to our findings, other scientists found a significant reduction (3-6 $\log_{10}$) of *L. monocytogenes* and *E. coli* which reduction increased as treatment time increased in milk and liquid egg, respectively *(63, 84)*. Freezing caused ($P < 0.05$) reductions of *Listeria* spp. on the inoculation level (untreated control) and the shrimp that were exposed to PEF for 2 min in 0% NaCl. The results indicated that shrimp not exposed to PEF treatment and shrimp PEF-treated in water were more susceptible to the formation of ice crystals. Residual sodium chloride on PEF-treated shrimp might slow down the formation of ice crystals or reduce the size of the ice crystals.

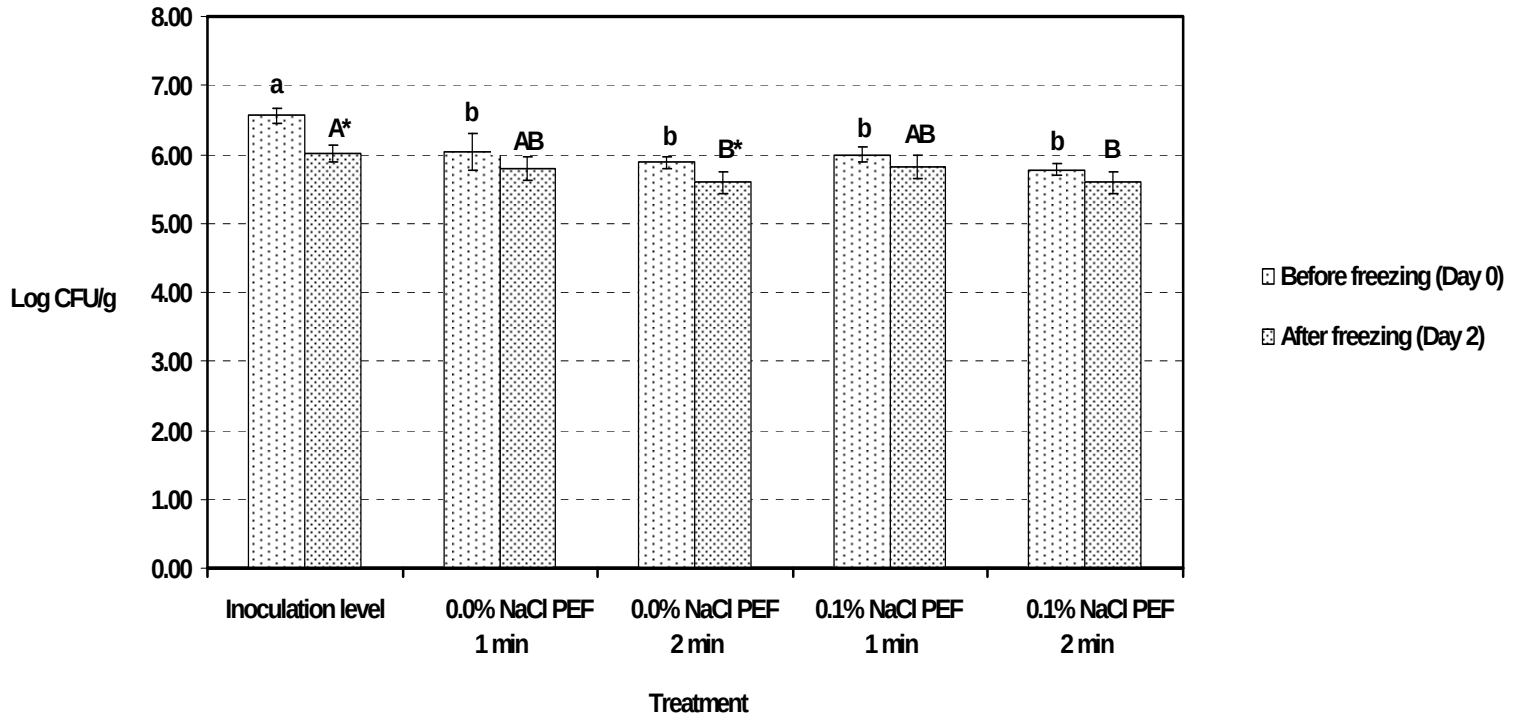


FIGURE 14. *Effect of PEF in 0.1% NaCl on the survival of* Listeria spp. *on raw shrimp for pulse duration times of 1 and 2 mins. Bars in the treatment before freezing (a, b) or after freezing (A, B) are significantly different (*$P < 0.05$, $n = 3$*). An asterisk (*) between means in the same treatment are significantly different (*$P < 0.05$, $n = 3$*) after freezing*.

Antimicrobial and freezing effects on the *Listeria* spp. counts of treated and untreated raw shrimp

The effects of the antimicrobial compound CPC on the counts of *Listeria* spp. on shrimp (n=30) after freezing for 2 d at -22.3°C were evaluated in this study. The combination of PEF in 0.1% NaCl followed by immersion in 0.5% CPC solution and a wash in water (PEF/CPC/W) produced the lowest CFU/g (Fig. 15, $P < 0.05$) of *Listeria* spp. on shrimp. Our results are in agreement with those reported earlier *(61, 68, 76, 96)* that PEF could rupture the membrane of the bacterial cell and allow the entry of antimicrobial compounds into the cell and eventually result in cell death. Freezing reduced ($P < 0.05$) the concentration of *Listeria* spp. on untreated shrimp (Fig. 15), but not the *Listeria* spp. populations of shrimp that were treated with the combination of PEF-CPC-water. The results indicated that ice crystals might damage some *Listeria* cells on untreated shrimp but the number of *Listeria* spp. was not decreased by the combination of other treatments after 2 d of frozen storage. The increase in *Listeria* spp. counts of the PEF-CPC-water combination treatment was statistical significant ($P < 0.05$) after 2 d of frozen storage but this may not be biological significant (< 1 $\log_{10}$ unit).

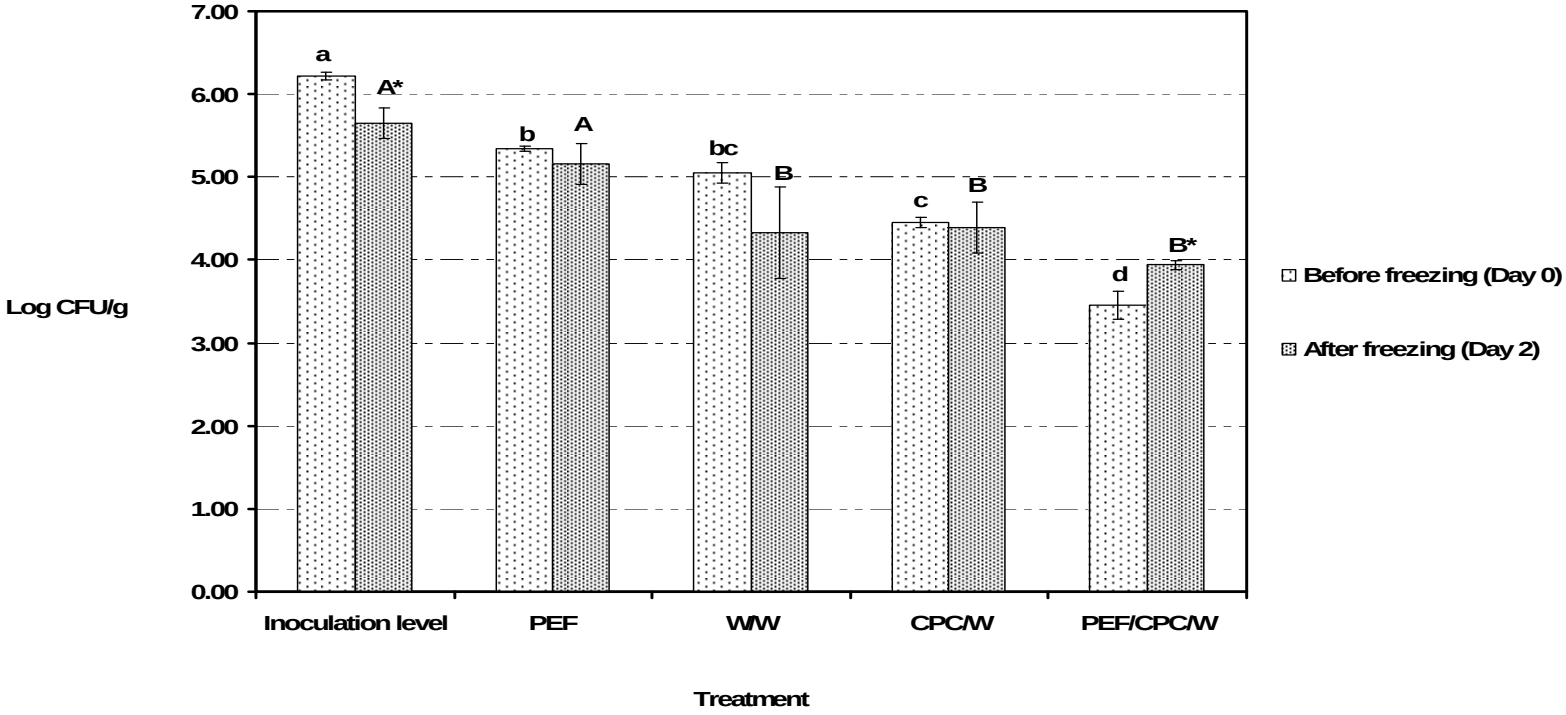


FIGURE 15. *Mean* Listeria spp. *populations on raw shrimp treated with PEF in 0.1% NaCl for 2 min (PEF), a double wash with water (W/W), 0.5% CPC and a water wash (CPC/W), or the combination of the latter two treatments plus a water wash (PEF/CPC/W) and stored at -22.3°C for an approximately 48 h. Bars in the treatment before freezing (a, b, c, d) or after freezing (A, B) with different letters are significantly different (*$P < 0.05$, $n = 3$*). An Asterisk (*) between means in the same treatment are significantly different (*$P < 0.05$, $n = 3$*) after freezing*.

Effect of storage for 7 days at 3.3°C on *Listeria* spp. survival

The effect of antimicrobial treatments on the number of *Listeria* spp. on shrimp (n=30) after refrigerated storage for 7 d at 3.3°C was evaluated in this study. The analysis of the data revealed that treatments and storage days had significant ($P < 0.05$) effects on the survival of *Listeria* spp. At day 0, application of CPC treatments with a following water wash (CPC/W) and the combination of PEF in 0.1% NaCl with a following water wash (PEF/CPC/W) produced greater antimicrobial effects ($P < 0.05$) on *Listeria* spp. on shrimp compared to the water wash and PEF treatments lacking a following water wash (Fig. 16). A wash with double distilled water reduced the *Listeria* spp. populations (~0.4 $\log_{10}$ unit) of the inoculated shrimp more than the PEF treatment. This indicated that the PEF treatment alone was not sufficient to cause a significant change in the *Listeria* spp. populations on the inoculated shrimp. However, after storage at 3.3°C for 7 d; the concentration of *Listeria* spp. on the shrimp increased for all treatments except for the shrimp that received the PEF treatment (Fig. 16). The decrease in *Listeria* spp. populations after 7 d of refrigerated storage of inoculated shrimp that received the PEF treatment may not be biological significant.

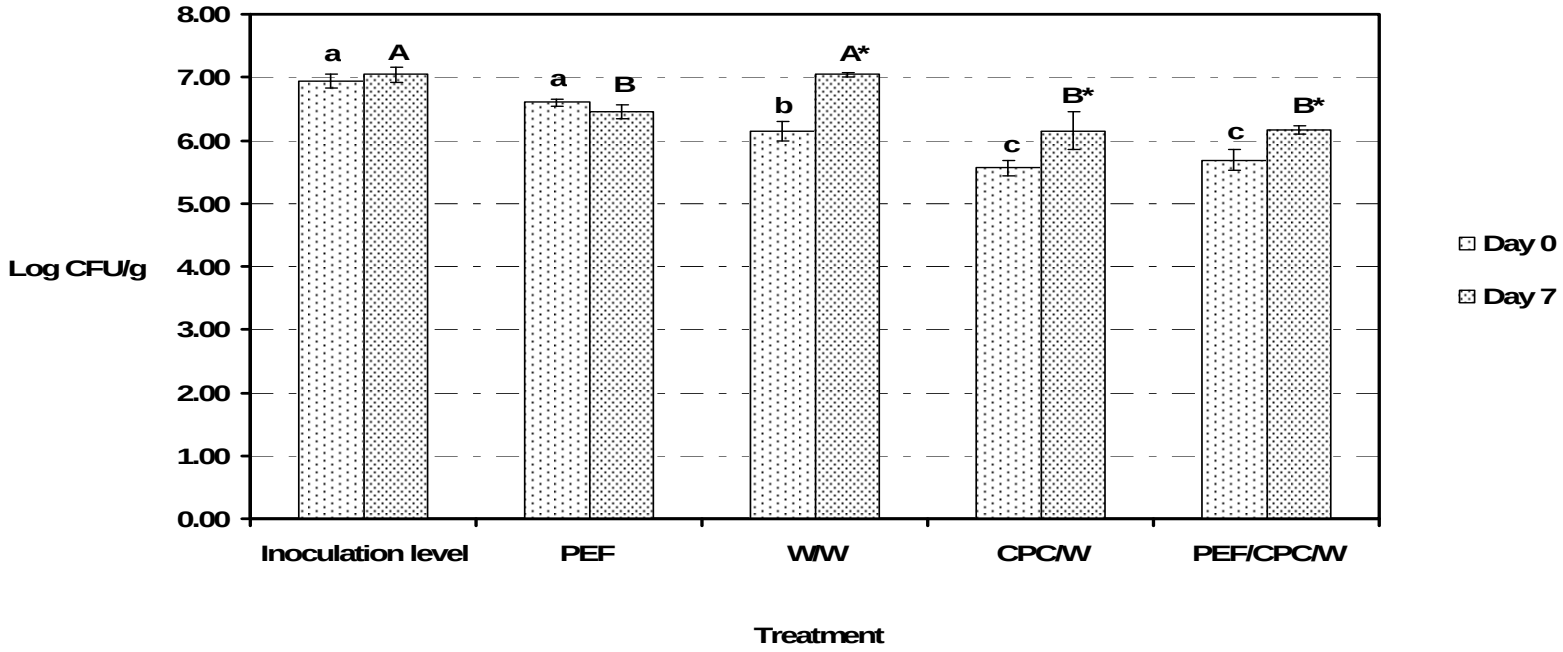


FIGURE 16. *Mean* Listeria spp. *populations on raw shrimp treated with PEF in 0.1% NaCl for 2 min (PEF), double wash with water (W/W), 0.5% CPC followed by a water wash (CPC/W), or the combination of the latter two treatments plus water wash (PEF/CPC/W) and refrigerated at 3.3°C. Bars in the treatment before refrigeration (a, b, c) or after refrigeration (A, B) with different letters are significantly different (*$P < 0.05$, $n = 3$). *An asterisk (*) between means in the same treatment are significantly different (*$P < 0.05$, $n = 3$) *after refrigerated storage.*

Effect of PEF, CPC, and water washes and combinations of these treatments on the concentration of *Listeria* spp. on raw shrimp

The effect of multiple rinses on the removal of the *Listeria* spp. from 30 contaminated raw shrimp was determined before and after 2 d of frozen storage at -22.3°C was evaluated in this study. The data showed that additional washings did not improve the effect ($P > 0.05$) of PEF treatment for the reduction of *Listeria* spp. on contaminated shrimp as compared to other treatments (Fig. 17). Freezing had no effect ($P > 0.05$) on the treated shrimp after storage (Fig. 17). The findings are similar to studies of Dupard *et al. (23)*, Singh *et al. (93)*, and Wang *et al. (102)*. Results of these studies suggested that washing with water did not improve the antimicrobial effects of CPC treatments on contaminated shrimp. This suggests that a water wash for 20 s might have been the boundary for improvement in the effectiveness of CPC treatments on contaminated shrimp.

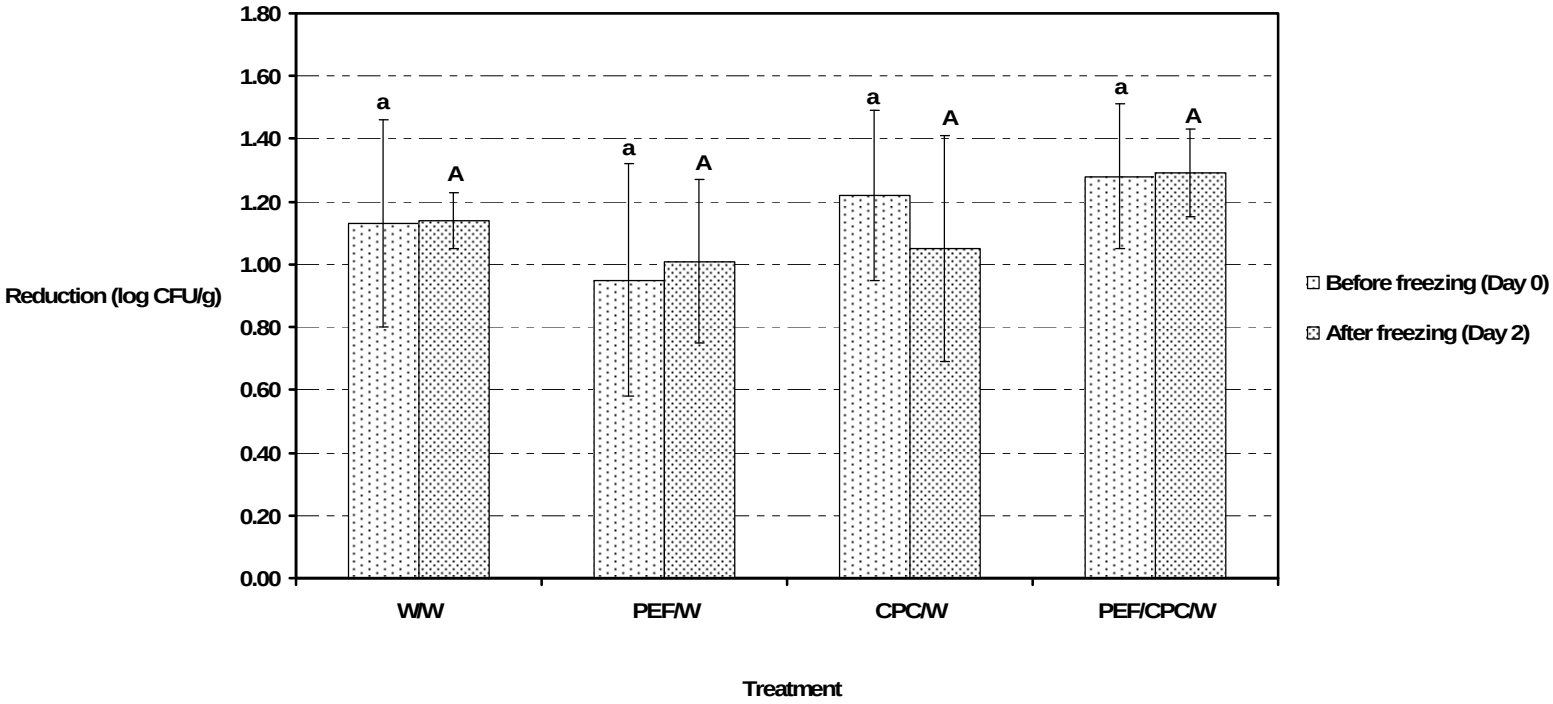


FIGURE 17. *Reduction of* Listeria spp. *populations on raw shrimp treated with a double wash with water (W/W), PEF in 0.1% NaCl for 2 min followed by a water wash (PEF/W), 0.5% CPC followed by a water wash (CPC/W), or a combination of the latter two treatments followed by a water wash (PEF/CPC/W). Bars in the treatment before freezing (a) or after freezing (A) with different letters are significantly different (*P < *0.05*, n = *3)*.

Effect of PEF treatment in 0.1% and 0.5% NaCl on the survival of *Listeria* spp. on shrimp

The effect of PEF treatments in the presence of 0.1% and 0.5% sodium chloride was determined using a total of 18 shrimp. Data for the treatment combinations of PEF in 0.1% and 0.5% NaCl followed by 0.5% CPC and a water wash (PEF/0.1%/CPC/W, PEF/0.5%/CPC/W) or PEF in 0.1% and 0.5% NaCl followed by a water wash (PEF/0.1%/W, PEF/0.5%/W) are presented in Figure 18. The sodium chloride solution of 0.5% did not increase the antimicrobial effect ($P > 0.05$, Fig. 18) of PEF against *Listeria* spp. populations on shrimp compared to PEF treatment in the presence of 0.1% NaCl. This result showed that a concentration of 0.1% NaCl rather than 0.5% NaCl can be used during PEF treatment without reducing the effectiveness of the PEF treatment. The use of lower NaCl concentrations is desired to minimize health and environmental concerns associated with the potential of chlorine gas generation by PEF (*3*). For a single treatment with a water wash, *Listeria* spp. counts in the presence of CPC were significantly lower ($P < 0.05$) than the number of *Listeria* spp. remaining after the PEF treatment. The CPC caused an immediate decrease in the number of *Listeria* spp. on shrimp. Other authors *(58, 73)* have found an immediate reduction in the populations of *L. monocytogenes* on contaminated beef products after the beef was treated with a 0.5% CPC solution.

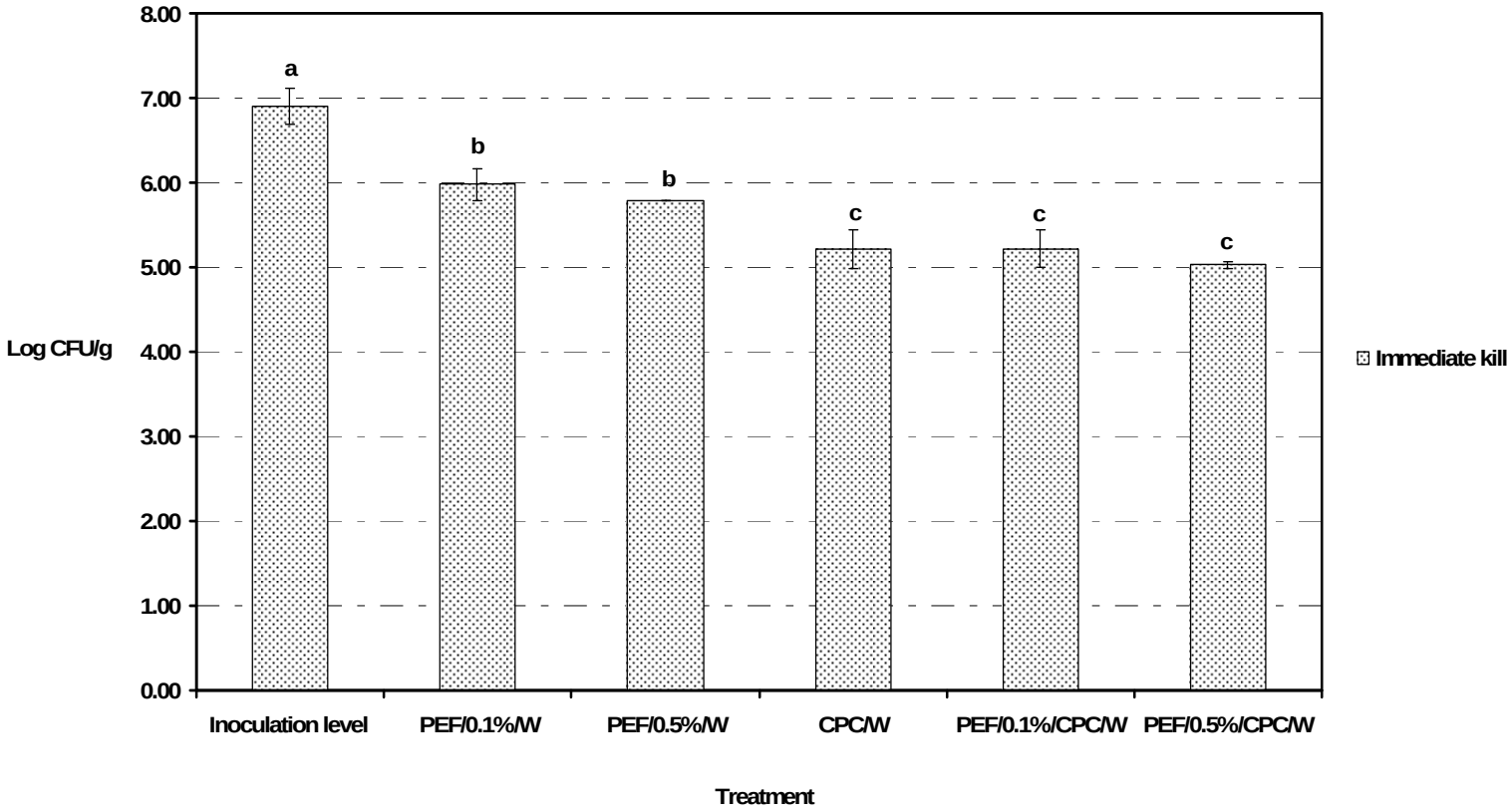


FIGURE 18. *Populations of* Listeria spp. *on raw shrimp after treatment by PEF in 0.1% and 0.5% NaCl for 2 min followed by a water wash (PEF/0.1%/W, PEF/0.5%/W), 0.5% CPC followed by a water wash (CPC/W), or a combination of CPC and PEF at NaCl concentrations of 0.1% and 0.5% followed by a water wash (PEF/0.1%/CPC/W, PEF/0.5%/CPC/W). Bars with different letters are significantly different (*P < *0.05*, n = *3)*.

Effect of PEF treatment in 0.5% NaCl and a 0.5% concentration of CPC on the survival of *Listeria* spp. in buffered peptone water

The PEF and CPC treatments were applied directly to suspensions of *Listeria* spp. in BPW. The CPC treatment and the combination of PEF and CPC (PEF/CPC) caused an immediate reduction of 7 $\log_{10}$ CFU of *Listeria* spp./ml in BPW ($P < 0.05$), as compared to the inoculation level (untreated control) (Fig. 19). Similar reductions in microbial counts were also found using two strains of *Listeria innocua* after treatment with the same concentration of 0.5% CPC (Fig. 20). CPC treatment had an immediate bactericidal effect against *Listeria* cells in suspension and on contaminated shrimp (Figs. 18, 19, and 20). A similar result has been reported by Özdemir *et al.* (*73*) who found an immediate reduction of 2 $\log_{10}$ CFU of *L. monocytogenes*-on contaminated raw beef dipped into 0.5% CPC solution.

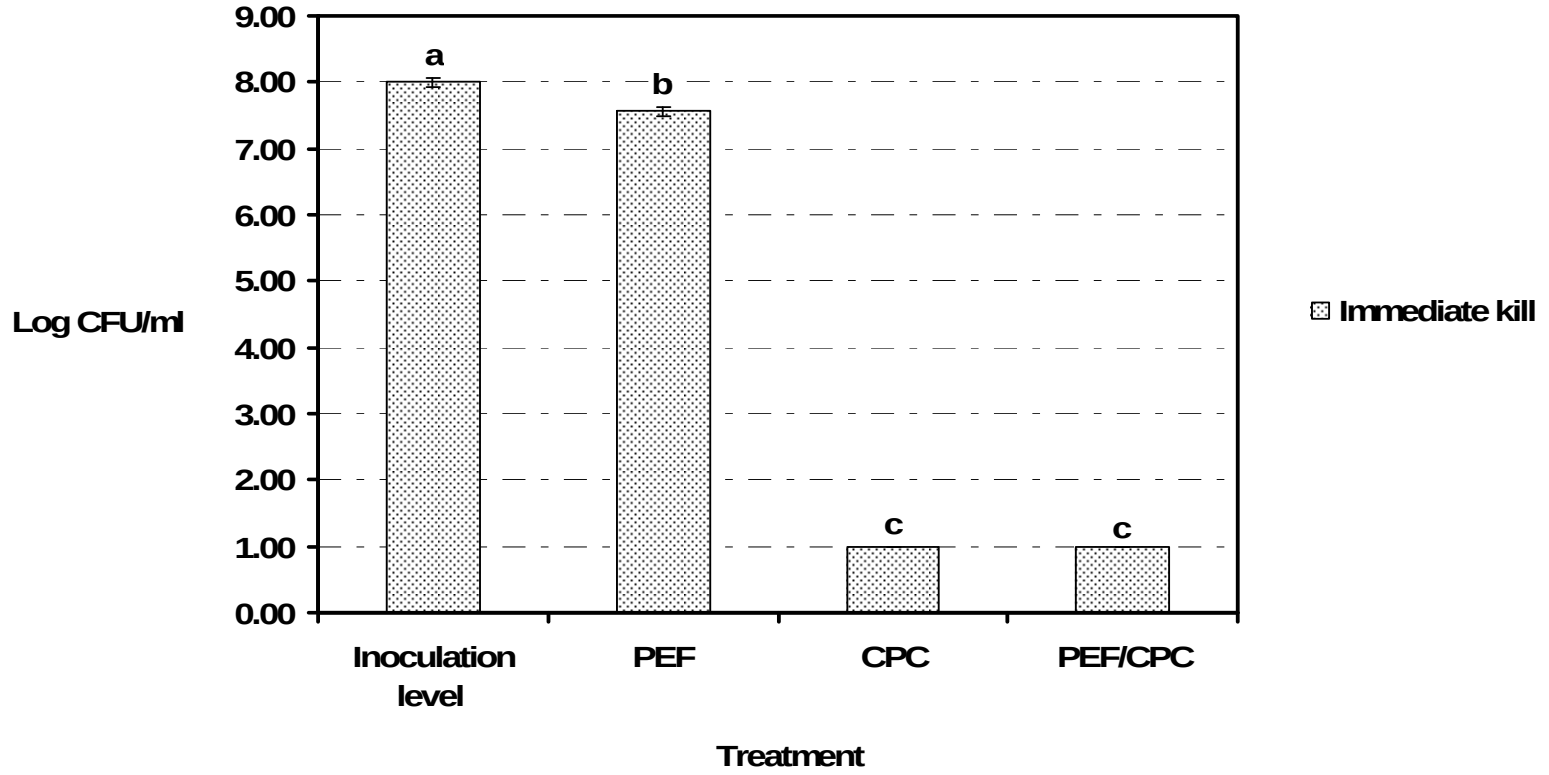


FIGURE 19. *Effect of PEF in 0.5% NaCl for 4 min (PEF), 0.5% CPC (CPC), or a combination of the PEF and CPC treatments (PEF/CPC) on the inactivation of* Listeria spp. *populations in buffer peptone water. Bars with different letters are significantly different (*P < *0.05*, n = *3)*.

Effect of 0.5% CPC or PEF applied in 0.5% NaCl on *Listeria innocua* NRRC b33076 and *Listeria innocua* ATCC 33090

After 18 h of incubation, *Listeria innocua* NRRC b33076 or *Listeria innocua* ATCC 33090 cultures grown in BPW were exposed to 0.5% CPC or PEF applied in 0.5% NaCl for 0, 40, 80, 120, 160, 200, 240, and 300 s, respectively. Differences ($P < 0.05$) in strain susceptibility were observed, with cells of *L. innocua* NRRC b33076 being more susceptible to treatment with PEF in 0.5% NaCl than cells of *L. innocua* ATCC 33090 and this difference was evident immediately upon exposure to the treatment as treated *L. innocua* NRRC b33076 cell concentrations were always lower ($P < 0.05$) than inoculation level (untreated control) (Fig. 20). Conversely, concentrations of *L. innocua* ATCC 33090 were only lower ($P < 0.05$) than inoculation level (untreated control) after 300 s exposure to PEF in 0.5% NaCl (Fig. 20). For both strains, the application of PEF in 0.5% NaCl caused at most 0.3-0.5 $\log_{10}$ decrease in cell concentrations compared to inoculation level (untreated control). CPC-treatment on the other hand, caused an immediate and marked reduction ($P < 0.05$) of approximately 7 to 8 $\log_{10}$ ($P > 0.05$) in cell concentrations of both *L. innocua* NRRC b33076 and *L. innocua* ATCC 33090 (Fig. 20). Thus, whereas 0.5% CPC treatment appeared to be equally effective against both *L. innocua* NRRC b33076 than *L. innocua* ATCC 33090 in this experimental system, its effect was markedly more dramatic than PEF treatment applied in 0.5% NaCl which was more effective against *L. innocua* NRRC b33076 than *L. innocua* ATCC 33090.

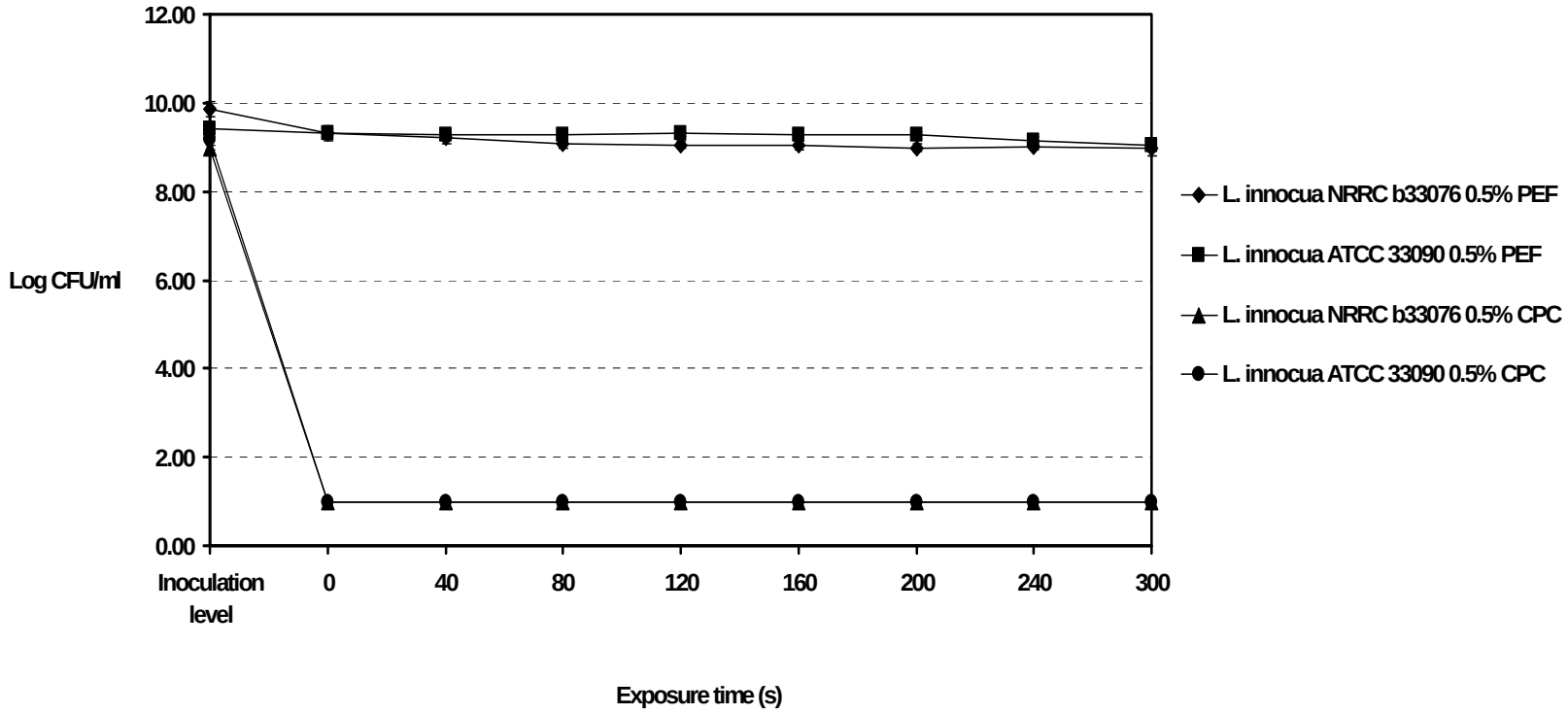


FIGURE 20. *Effect of exposure time on the numbers of* L. innouca NRRC b33076 and L. innocua ATCC 33090 *after treated with PEF in 0.5% NaCl or 0.5% CPC*. n = *3*.

Effect of storage for 8 days at 7°C after antimicrobial treatments on the survival of *Listeria* spp. on shrimp

The effects of antimicrobial treatments on the concentrations of *Listeria* spp. on 48 contaminated shrimp during 8 d of storage at 7°C were evaluated in this study. Rates of *Listeria* spp. growth after 2 d of storage at 7°C were lower on shrimp treated with 0.5% CPC and a water wash (CPC/W), and those shrimp treated with PEF in 0.1% NaCl, 0.5% CPC, and a water wash (PEF/CPC/W) compared to the inoculation level (untreated control) thus indicating a bacteriostatic effect ($P < 0.05$) of these treatments (Fig. 21). However, the bacteriostatic effect appeared to be diminished by 4 d of storage. Singh *et al.* (*93*) also reported an antimicrobial effect of a 1% CPC treatment when sprayed and allowed 30s exposure on polish sausage inoculated with low (3 $\log_{10}$ CFU/g) and high (7 $\log_{10}$ CFU/g) concentrations of *L. monocytogenes* and stored 42 d at 0 and 4°C. Özdemir *et al.* (*73*) reported that the number of *L. monocytogenes* on raw beef muscles treated with 0.5% CPC decreased slightly after 5 d of storage, but the decrease was not significant ($P > 0.05$). However, Wang *et al.* (*102*) found that *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* Typhimurium were able to recover after treatment of fresh-cut vegetables with 0.1 and 0.5% CPC-solutions. As a result, Wang *et al.* (*102*) found no difference ($P > 0.05$) between the number of *L. monocytogenes* and *E. coli* 0157:H7 in samples that were immersed in the CPC solution or in water.

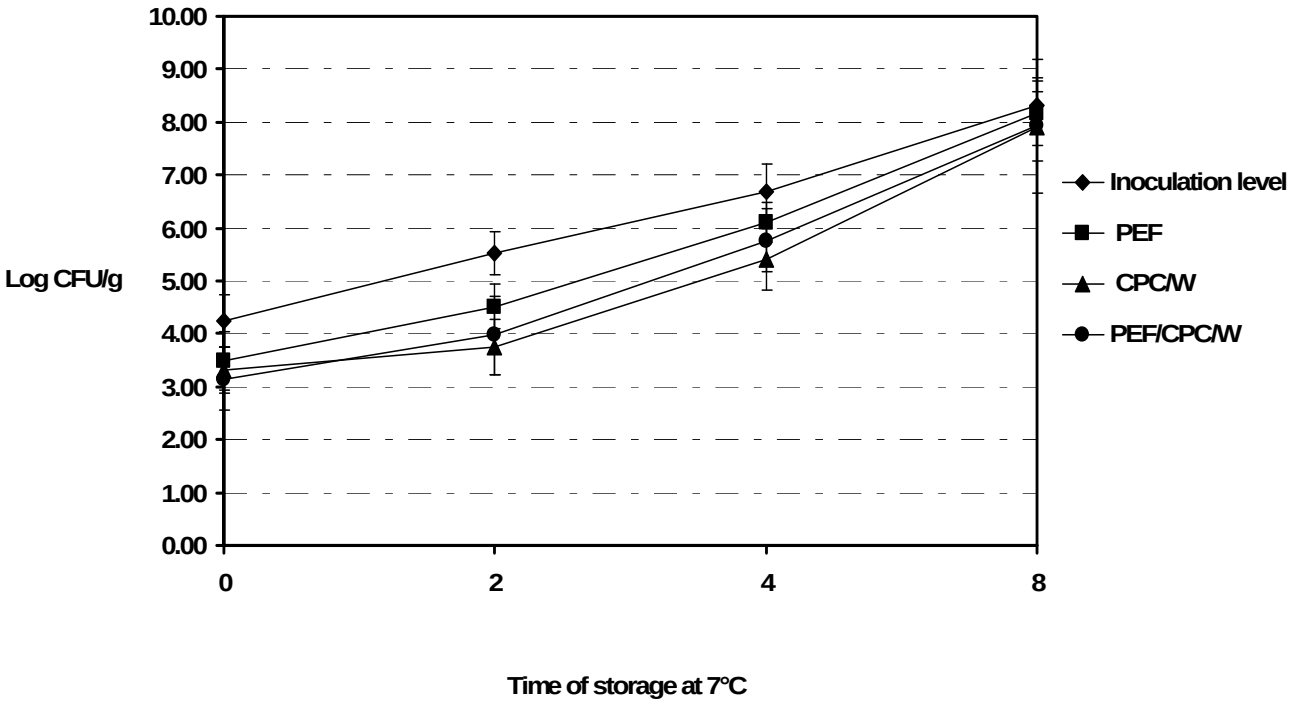


FIGURE 21. Listeria spp. *populations on raw shrimp treated by PEF in 0.1% NaCl for 2 min (PEF), 0.5% CPC with a water wash (CPC), or a combination of the PEF and CPC treatments at 0, 2, 4, and 8 days of refrigeration at 7°C*. n = *3*.

Chlorine production and pH changes during PEF treatment at different concentrations of NaCl and times of exposure

The effects of sodium chloride concentration and the time of treatment on chlorine production were monitored in this study. Free chlorine was not detected in the distilled (control) water. Chlorine production increased ($P < 0.05$) after PEF treatment for 4 min in 0.5% NaCl and after PEF treatment in 1.0% NaCl for 2 and 4 minutes (Fig. 22A). During PEF processing, a pungent chlorine odor was detected when PEF was conducted in 1.0 % NaCl. The smell could cause discomfort for the workers and the chlorine in the air might corrode some metal surfaces (*3*). Sodium chloride concentration and the treatment time (s) affected ($P < 0.05$) the pH of the water; however, the effect was not linear (Fig. 22B). The *pH* of the solutions of 0%, 0.5% or 1.0% NaCl increased after exposure to PEF for 2 min. The changes might have been the result of the sensitivity and the accuracy of *pH* probe. Consequently, further investigation of the effect of PEF treatment on *pH* values in each treatment is recommended.

Electrolyzed water did not increase in acidity at any concentration of sodium chloride used in our study. The *pH* values of this water were in the range of 6 to 8 even after exposure to PEF for 4 min. The maximum NaCl used in our study was 1% and the duration of the PEF treatment was 4 min. Bari *et al.* (*8*) reported that sodium chloride solution at 0.1% (dissolved with tap water) after electrolysis normally has a *pH* ≤2.7 and free chlorine concentrations of 10-80 ppm. With the same concentration of sodium chlorine solution (dissolved with distilled water), an average concentration of 0.50 ppm free chlorine was produced after electrolysis (Fig. 22A). Chlorine is more effective for inactivating microorganisms either at a high concentration or at a low pH (pH ≤ 6). The low concentration of chlorine and an average of pH > 6 in our study (Fig. 22B) indicated that residual chlorine was not the reason *Listeria* cells were damaged by PEF.

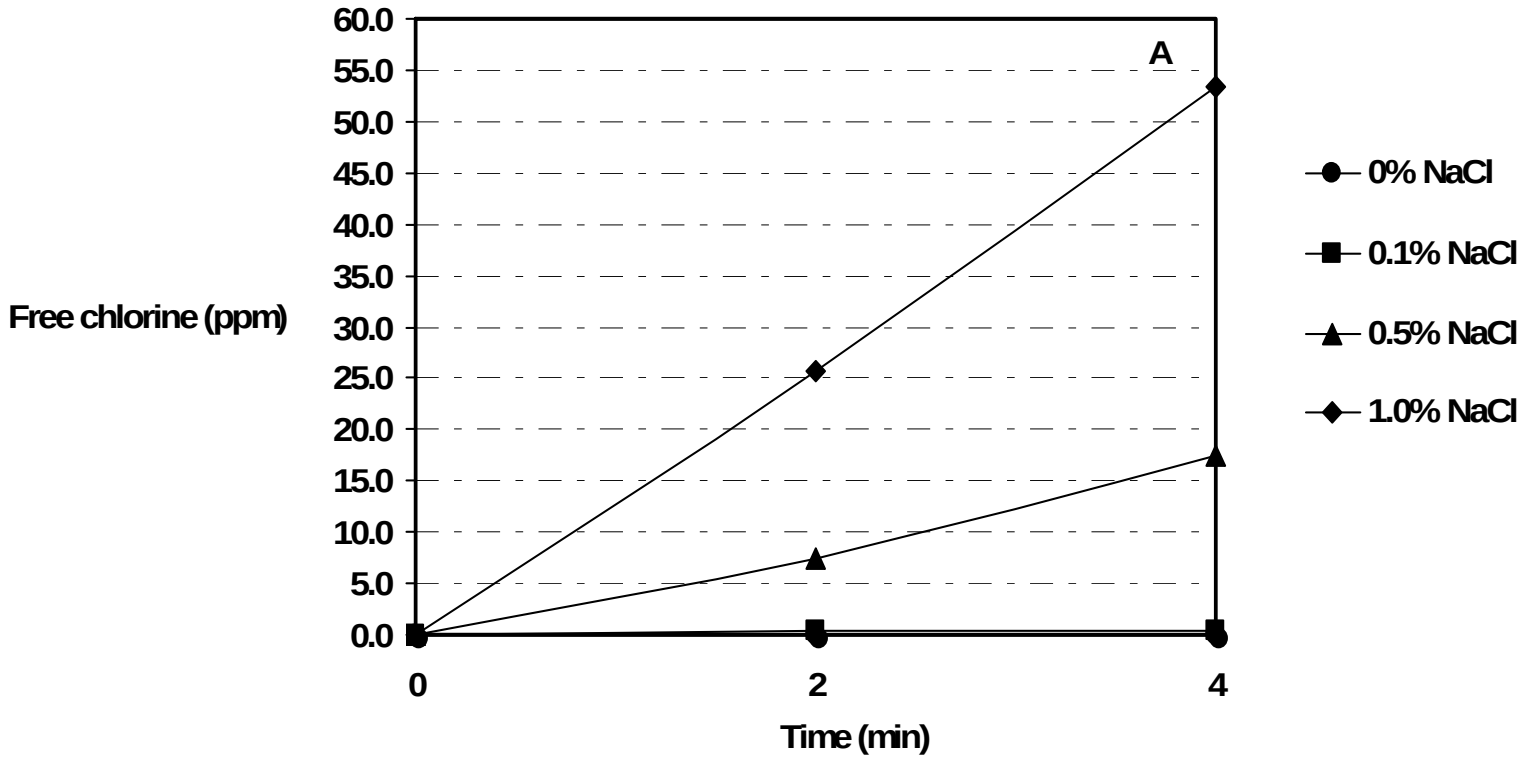


FIGURE 22. *Effect of NaCl concentration on free chlorine (ppm) production and pH change during pulsed electric field treatment (A) free chlorine (ppm) and (B) pH values.* n = *3*.

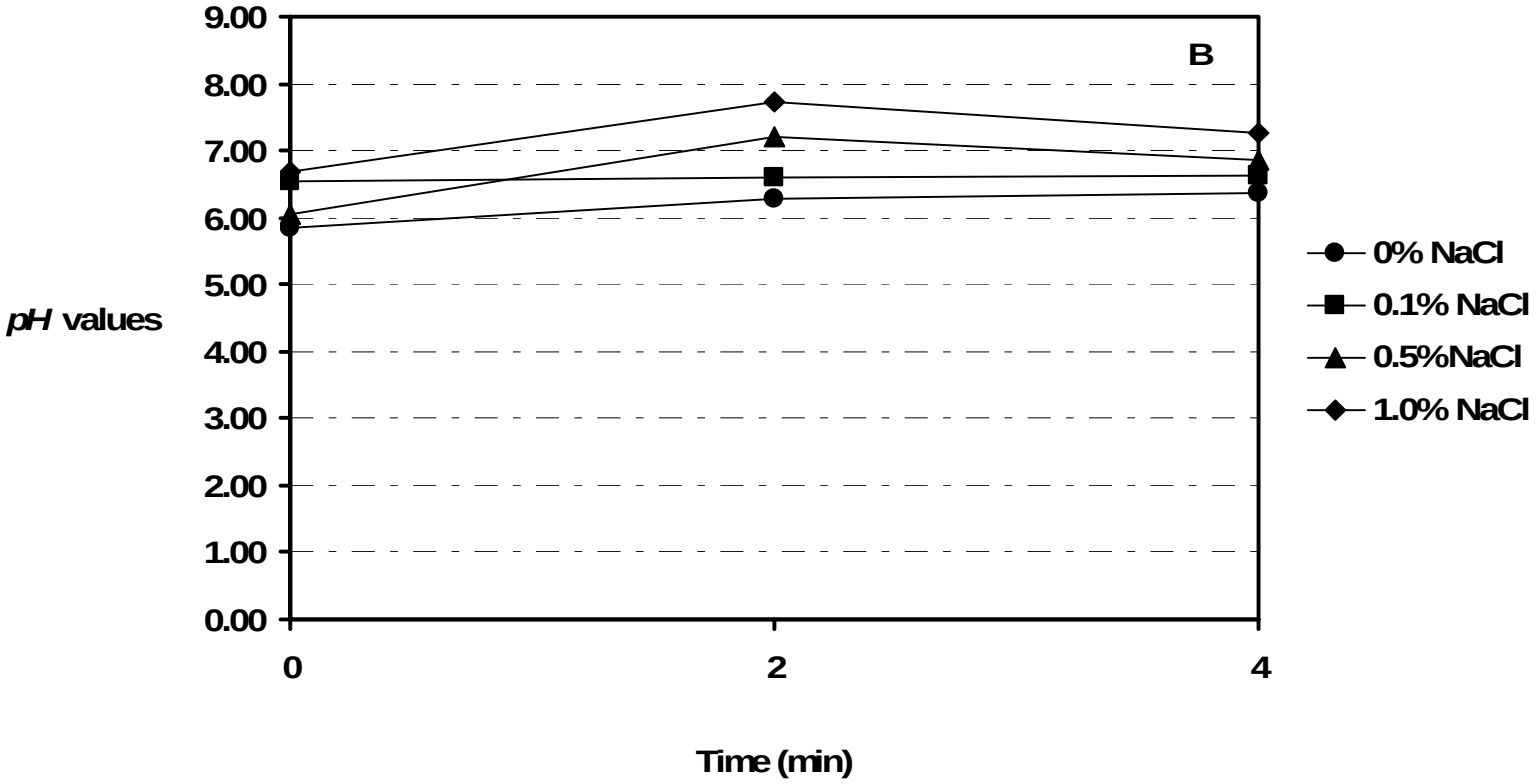


FIGURE 22. *Continued.*

Bacterial cell changes observed by TEM after treatment with PEF and CPC

Untreated cells of *Listeria* innocua NRRC b33076 and *Listeria monocytogenes* ATCC 15313 harvested and examined after incubation for 12 and 18 h, respectively, had clearly defined outer and inner cell membranes and cell wall structure (Figs. 23a and 24a). Outer cell membrane disruption (Figs. 23b and 24b) was observed after cells were added to 0.1% sodium chloride and exposed to PEF treatment at 30.6 volts for 2 min of pulse duration. This type of cell disruption is the beginning of cell morphological changes. The extent of morphological changes in cells was reported in other studies (*17, 34, 77, 86)*. TEM micrographs revealed that cells that were PEF-treated with 6,000 pulses at 30 kV/cm caused the rupture of the cell wall, the loss of intracellular contents (e.g. cytoplasmic and nucleic materials), and the release of cellular debris due to irreversible electroporation (*86*). Calderón-Miranda *et al. (17)* reported that significant morphological changes to bacteria and yeast were caused by treatment with PEF. These changes include a significant increase in surface roughness, appearance of craters in the cell wall, elongation and disruption of organelles, cell wall breakage, and pore formation. TEM micrographs by Pothakamury *et al.* (*77*) showed that cytoplasmic contents leaked from *S. aureus* cells after treatment at with 64 pulses at 60 kV/cm. The findings suggested that damaged cells were the function of electric field strength and the injury to the cells was probably induced by electromechanical breakdown. Hamilton and Sale (*34*) used TEM and observed unimpaired membranes in erythrocytes and *E. coli*

after they were treated with 10 pulses and 20 μs of high voltage electric fields up to 25 kV/cm.

The antimicrobial action of CPC has been reported to involve interaction of CPC with negative charges on bacterial cell membranes. These ionic interactions on the cell surface are thought to eventually result in cell rupture. The bacterial cells in Figs 23c and 24c of our TEM micrographs appear to have experienced cellular breakdown or cell disruption. The micrographs of *Listeria monocytogenes* ATCC 15313 and *Listeria innocua* NRRC b33076 exhibited either cell membrane disruption, cell lysis, or cytoplasmic leakage after the cells were treated with 0.5% CPC.

A synergistic effect of organic acids and PEF has been reported (*76*, *96*) to have enhanced antimicrobial properties that exceed the effect of either treatment applied singularly. These studies indicated that a high intensity PEF might cause disruption of the cell membrane and facilitate entry of antimicrobials into bacterial cells. Those observations were based on plate counts and on a microbiological predictive model. This possible mechanism of synergism was also observed in our finding. Damage to the outer cell membranes was observed after the cells were processed by PEF, as seen in both Figs. 23b and 24b. The extent of cell membranes disruption and cells lysis was visible in both Figs. 23c and 24c after 0.5% CPC treatment. As a result, broken or lysed cell membranes (Figs. 23d and 24d) might be due to a synergistic or additive effect by a combination of both PEF and CPC treatments.

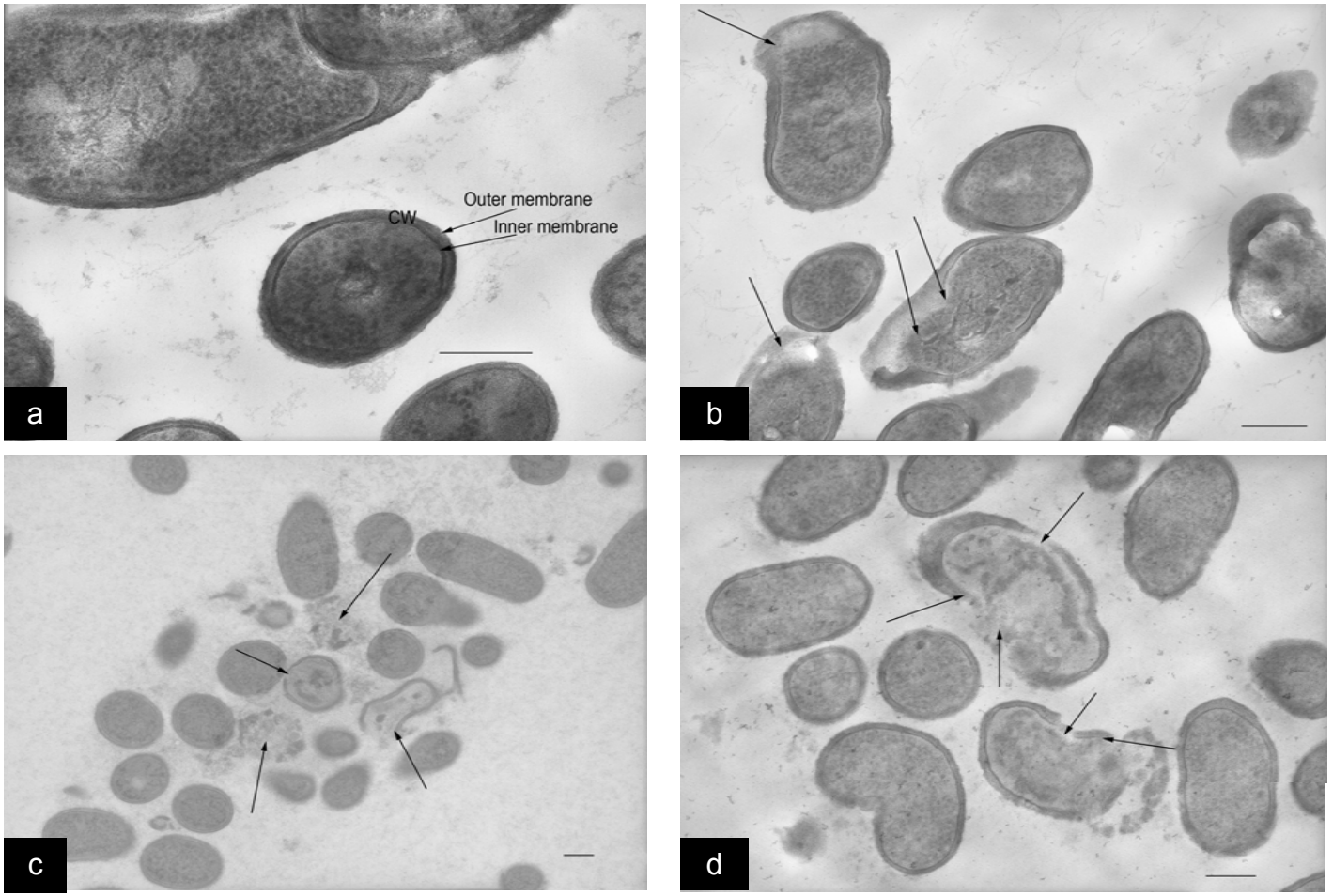


FIGURE 23. *Transmission Electron Microscopy (TEM) of 18 h* Listeria monocytogenes ATCC 15313 *cells: untreated (a), arrows indicate either outer or inner cell membrane, CW=Cell Wall; treated with 0.1% salt PEF for 2 min (b), arrows indicate outer cell membrane disruption; treated with 0.5% CPC (c), arrows indicate either cell membranes disruption and cells lysis; and treated with a combination of both treatments (d), arrows indicate cell membranes disruption*. *Bar = 200 µm*.

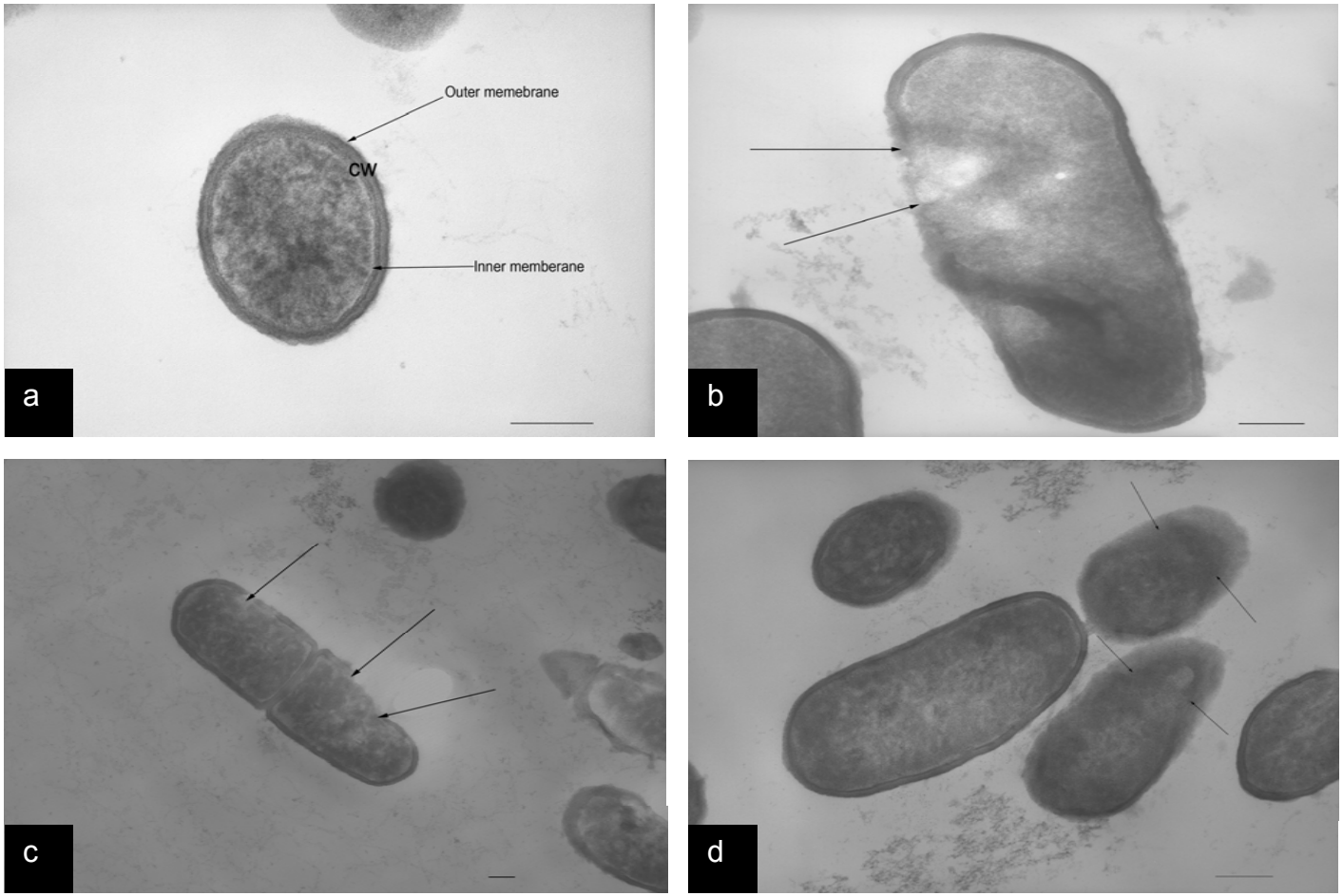


FIGURE 24. *Transmission Electron Microscopy (TEM) of 12 h* Listeria innocua NRRC b33076 *cells: untreated (a), arrows indicate either outer or inner cell membrane, CW=Cell Wall; treated with 0.1% salt PEF for 2 min (b), arrows indicate outer cell membrane disruption; treated with 0.5% CPC (c), arrows indicate either cell membranes disruption or broken membrane; and treated with a combination of both treatments (d), arrows indicate lysis of cell membranes. Bar = 100 µm*.

CONCLUSIONS

Results from all experiments were concluded as shown below:

- *Listeria* spp. counts were reduced on shrimp by a combination of CPC and a water wash after frozen storage.
- *Listeria* spp. counts could not be reduced on shrimp by ultrasonication.
- Chlorine treatment did not reduce *Listeria* spp. on shrimp.
- Citrate extracts did not reduce *Listeria* spp. on shrimp.
- *Listeria* spp. counts were reduced on shrimp treated with PEF in 1% NaCl and freezing.
- PEF in 0.1% NaCl was as effective as PEF in 1.0% NaCl for reducing the number of *Listeria* spp. on shrimp.
- CPC was the most effective treatment for reducing the number of *Listeria* spp. on shrimp.
- CPC was the most effective treatment for reducing the number of *Listeria* spp. in buffer peptone water.
- PEF caused damage to cell membranes of *Listeria*.
- CPC caused structural damage to *Listeria* cells.
- CPC can be used to reduce *Listeria* spp. on contaminated shrimp.

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