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Application of antimicrobial ice for reduction of foodborne pathogens (*Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*) on the surface of fish

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ABSTRACT

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Aims: The efficacy of antimicrobial ice was evaluated for the reduction of foodborne pathogens on the surface of fish.

Methods and Results: Antimicrobial ice containing chlorine dioxide (ClO_2) was utilized to control foodborne pathogens in laboratory media and on fish skin. *Escherichia coli* O157:H7, *Salmonella* serotype Typhimurium and *Listeria monocytogenes* strains were treated with antimicrobial ice for 30 min on plates of selective agar and for 120 min on fish skin at room temperature, and then incubated for enumeration. After treatment with 100 ppm ClO_2 for 30 min, 5·4, 4·4 and 3·2 $\log_{10}$ reduction was obtained with *E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes* on laboratory media, respectively. When antimicrobial ice (100 ppm ClO_2) was applied to fish skin for 120 min, total reduction of *E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes* was 4·8, 2·6 and 3·3 $\log_{10}$, respectively.

Conclusion: The initial load of foodborne pathogens was reduced by antimicrobial ice and the lowered microbial level was maintained during treatment.

Significance and Impact of the Study: The application of antimicrobial ice is a simple and effective method for the safe preservation of fish.

Keywords: antimicrobial ice, chlorine dioxide, fish, foodborne pathogen, preservation.

INTRODUCTION

World fishery production reached 126·2 million tons in 1999, an increase of 7·2% above the 1998 level. The value of world total fishery production grew by 7% to an estimated US$ 125 billion (FAO 2001). In 2001, total fishery production was reported to be 130·2 million tons (FAO 2003). Fishery products are distributed with minimal treatment before sale to the consumer. In the retail establishments, seafoods are regularly displayed on ice to prevent spoilage of products and growth of pathogens. There are many possibilities for contamination during harvest, processing and distribution owing to improper handling and storage (Kim *et al.* 1999). According to data of foodborne disease outbreaks (1983–1993) in the United States, fishery products were the third highest source of disease vectors (Lipp and Rose 1997).

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It is well recognized that microbial concerns are widely associated with seafoods (FDA 1998). Various seafoods and the marine environment have been revealed as sources for the isolation of foodborne pathogens, such as *Escherichia coli* O157:H7 (Ayulo *et al.* 1994; Fernandes *et al.* 1997; Teophilo *et al.* 2002), *Salmonella* spp. (Wilson and Moore 1996; Heinitz *et al.* 2000; Martinez-Urtaza *et al.* 2003) and *Listeria monocytogenes* (Weagant *et al.* 1988; Colburn *et al.* 1990; Jemmi 1993; Ben Embarek 1994; Huss *et al.* 1995; van Schothorst 1996; Ericsson *et al.* 1997; Jørgensen and Huss 1998; Ward 2001).

The rising threat of foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* demands effective detecting and control methods to make sure that these pathogens are not present in raw foods.

The water used for preparing ice must be free from solids, bacteria, flavours, odours and dissolved minerals must be at the lowest possible level (WHO 1976), and should not contain pathogenic bacteria that could remain viable during storage (Dickens *et al.* 1985). Commercial ice should be safe and of the same quality as drinking water. Ice is in direct contact with juices or soft drinks, and indirectly by refrigerating foods such as fish and seafood (Falcão *et al.* 2002). Many reports have demonstrated an association between contaminated ice and enteric diseases and the association between infections and contaminated ice used in hospitals (Moore *et al.* 1953; Dickens *et al.* 1985; Murphy and Mepham 1988; Ries *et al.* 1992; Burnett *et al.* 1994; Wilson *et al.* 1997). Dickens *et al.* (1985) examined the survival of *Salmonella* Typhi, *Shigella flexneri*, *Shigella sonnei* and enterotoxigenic *Escherichia coli* (ETEC) in thawed ice and in ice allowed to melt in various popular drinks.

Chlorine dioxide (ClO_2) is becoming more widely used in the food industry and is obtaining regulatory approval for direct food contact. The Food and Drug Administration (FDA) allows the use of ClO_2 for controlling microbial populations in poultry processing water (FDA 1995) and for washing fruits and vegetables (FDA, USDA, CDC 1998). Concentration of ClO_2 is regulated under 21 Code of Federal Regulations (CFR) 173·325. Process water for poultry, red meat, fruits and vegetables should contain ClO_2 ranging from 500 to 1200 ppm, and water or ice in contact with seafood should contain ClO_2 ranging from 40 to 50 ppm. Chlorine dioxide has a strong oxidizing and sanitizing activity against a broad range of pathogenic micro-organisms. Chlorine dioxide showed a constant bactericidal effect over a pH range of 3·0–9·0, but liquid chlorine did so only at neutral pH (Huang *et al.* 1997). The oxidizing capacity of ClO_2 is 2·5 times as high as that of liquid chlorine (Benarde *et al.* 1965). Chlorine dioxide was less reactive than aqueous chlorine in interacting with organic compounds (Wei *et al.* 1987; Sen *et al.* 1989). Many research studies reveal that ClO_2 has more antimicrobial activity than aqueous chlorine in seafood (Andrews *et al.* 2002) and poultry products (Thiessen *et al.* 1984).

The object of this study was to develop an antimicrobial ice for the preservation of seafoods. An antimicrobial ice formulation capable of releasing gaseous ClO_2 was evaluated against foodborne pathogens on selective media and on fish skin.

MATERIALS AND METHODS

Culture cocktail

Three isolates each of *Escherichia coli* O157:H7 (ATCC 35150, 43889, 42890), *Salm.* Typhimurium (ATCC 19585, 363755, 43174) and *L. monocytogenes* (ATCC 19114, 7644, 19113) were used to prepare a culture cocktail. Each strain of *E. coli* O157:H7 and *Salm.* Typhimurium were inoculated into sterile tryptic soy broth (TSB; Difco, Detroit, MI, USA) tubes using a 10 μl inoculation loop. *Listeria monocytogenes* strains were inoculated into sterile TSB containing 1% yeast extract. Cultures were incubated for 20 h at 37°C. To prepare the culture cocktail, 1 ml of each strain was combined into a sterile 50 ml centrifuge tube and centrifuged at 25 000 *g* for 30 min. The supernatant was removed after centrifugation, and the pellet resuspended in 0·2% buffered peptone water (pH 7·2 ± 0·2; Difco, Sparks, MD, USA), and then centrifuged at 25 000 *g* for 30 min again. This washing procedure was repeated three times to remove residual TSB. After the final centrifugation, the pellet was resuspended in 9 ml of 0·2% buffered peptone water and stored at 4°C until use.

Ice preparation

To make 100 ppm ClO_2 solution, Oxine® (38·1 ml; Biocide International, Norman, OK, USA) was added to citric acid (3·94 g) and allowed to dissolve for a few (<5) minutes, then the beaker was swirled gently to ensure complete dissolving of citric acid crystals. A small amount (*ca* 200 ml) of deionized water was then added, and 3–5 min was allowed for the production of ClO_2 before the remaining water was added to make a final volume 800 ml. For other concentrations, chemical amounts were adjusted in proportion. After stirring, the ClO_2 solution was divided between two domestic ice cube trays, and stored in a freezer to form ice cubes. Ice was then weighted into 700 ± 10 g batches, crushed using an iron hammer, sealed in polyethylene bags, and stored at −20°C until use. The concentration of ClO_2 was determined by the iodometric method using a commercial ClO_2 test kit (Bio-cide International).

Experiment on laboratory media

The culture cocktail had *ca* 10^6 CFU ml^{-1} of each strain. One hundred microlitre was plated onto duplicate plates of selective media, yielding *ca* 10^5 CFU per plate. The cocktail was enumerated by spread plating 100 μl of 10^{-4}–10^{-7} 10-fold serial dilutions onto duplicate plates of each selective medium. The following media were used for selective growth of each foodborne pathogens: MacConkey sorbitol agar (SMAC) was incubated at 37°C for 24 h to culture *E. coli* O157:H7, and enumerated; xylose lysine deoxycholate agar (XLD) and Oxford medium base supplemented with antibiotics (OAB) were incubated at 37°C for 48 h to cultivate *Salm.* Typhimurium and *L. monocytogenes*, respectively. Colonies were counted immediately after incubation.

Antimicrobial ice containing 25, 50 and 100 ppm ClO_2 was tested for time-intervals of 10, 20 and 30 min. For each

time-interval, five plastic storage boxes (Rubbermaid® SnapToppers; 34·5 × 21·6 × 10·6 cm; Rubbermaid, Wooster, OH, USA), each containing 700 ± 10 g crushed ice of each ClO_2 concentration, were prepared. Crushed ice from an ice machine was used for the control. Lids were removed from duplicate inoculated Petri dishes (described previously) before placing them bottom side down on the ice. The plastic boxes were tightly sealed. After each treatment interval Petri dishes were removed, lids put in place, and incubated at 37°C for 24 h and enumerated for *E. coli* O157:H7 (SMAC), and for 48 h and enumerated for *Salm.* Typhimurium (XLD) and *L. monocytogenes* (OAB). This experiment was replicated three times.

Fish skin preparation

Frozen mackerels were obtained from a local market and defrosted under refrigeration. Mackerel skin was peeled using a sterile scalpel, cut into 4 × 6 cm strips, and air dried for 10 min in a biosafety hood with the fan running to remove surface moisture. The nine culture pathogen cocktail was inoculated (100 μl) onto the scale side of the skin with a pipette and air dried for 30 min.

Fish skin experiment

Crushed ice containing 0, 25, 50 and 100 ppm ClO_2 was tested for time intervals of 10, 20, 30, 60 and 120 min. For each time-interval, four plastic storage boxes, each containing 700 ± 10 g crushed ice of each ClO_2 concentration, were prepared. Inoculated mackerel skin was placed scale side up onto the ice in the boxes, and the boxes tightly sealed. After each treatment interval, the mackerel skins were removed, placed into stomacher bags (Model 80 type®; Seward, Norfolk, UK), and stomached with 50 ml 0·2% buffered peptone water (Difco, Detroit, MI, USA) at medium speed for 2 min (Seward Stomacher® 80 Biomaster, Seward, Norfolk, UK). The stomached fish skin samples were 10-fold serially diluted using buffered peptone water to yield *ca* 10^2 CFU per plate. Diluted sample (100 μl) was plated onto duplicate plates of SMAC, XLD and OAB. Selective medium plates were incubated at 37°C for 20–24 h to enumerate *E. coli* O157:H7 and *Salm.* Typhimurium, and 48 h to enumerate *L. monocytogenes*. This experiment was repeated three times.

Statistical analysis

The averages of the duplicate plate counts from three replications were converted to units of $\log_{10}$ CFU ml^{-1}. Data were evaluated by analysis of variance using the GLM procedure of SAS (Version 8·1; SAS Institute Inc., Cary, NC, USA) for a completely randomized design. Inoculum counts were used as a covariant to normalize data between treatment replications. When the main effect was significant ($P < 0·05$), mean separation was accomplished with probability option (PDIFF, a pairwise *t*-test).

RESULTS

To evaluate the effectiveness of antimicrobial ice, viable foodborne pathogens on agar surfaces were enumerated following treatment. As shown in Figures 1–3, foodborne pathogens on agar plates were reduced by more than 3 $\log_{10}$ after 10 min treatment. *Escherichia coli* O157:H7

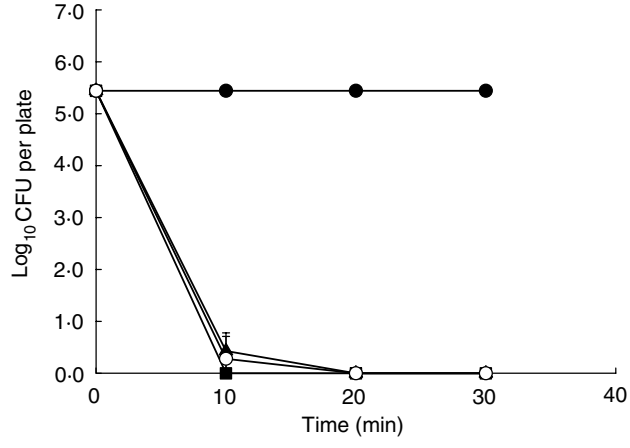

Fig. 1 Effect of antimicrobial ice against *Escherichia coli* O157:H7 inoculated on MacConkey sorbitol agar selective medium. Plates were placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 30 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

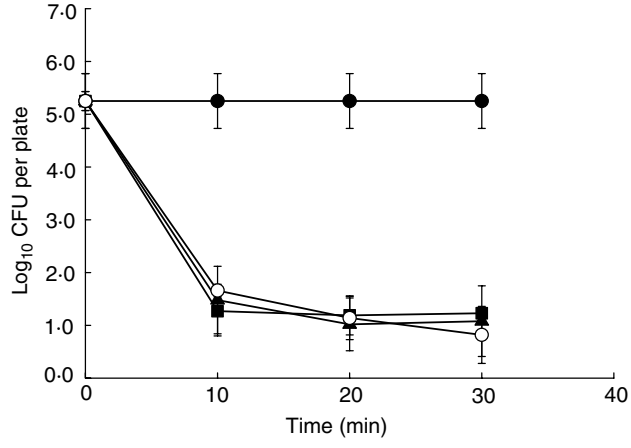

Fig. 2 Effect of antimicrobial ice against *Salmonella* Typhimurium inoculated on xylose lysine deoxycholate selective medium. Plates were placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 30 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

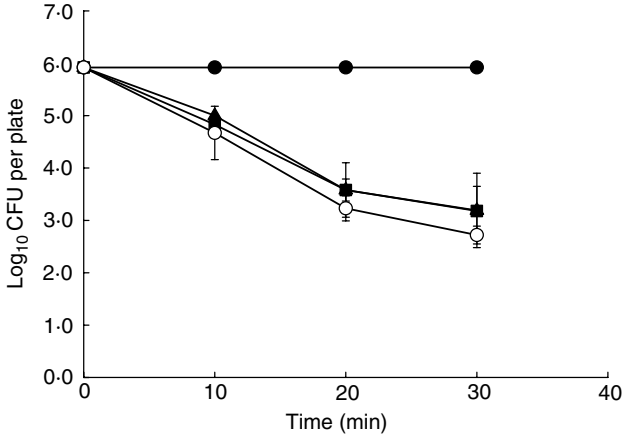

Fig. 3 Effect of antimicrobial ice against *Listeria monocytogenes* inoculated on Oxford medium base supplemented with antibiotics selective medium. Plates were placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 30 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

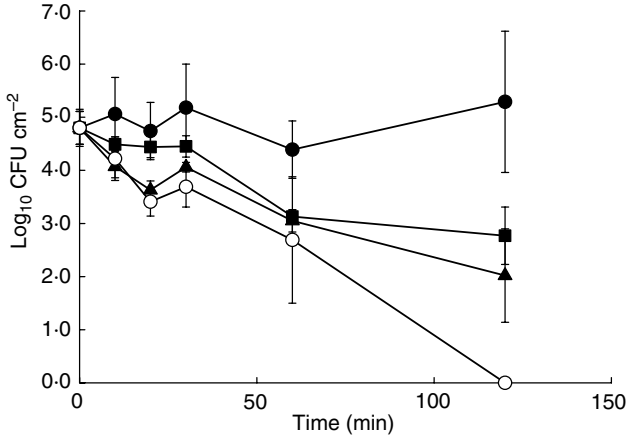

Fig. 4 Effect of antimicrobial ice against *Escherichia coli* O157:H7 inoculated on mackerel skin. Fish skin was placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 120 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

showed a 5 $\log_{10}$ reduction after 10 min on antimicrobial ice (Fig. 1), and no detectable *E. coli* cells were found after 20 min treatment (detection limit was 1 CFU per plate). Antimicrobial ice applied at all concentrations was significantly different ($P < 0{\cdot}01$) from the control. In Fig. 2, *Salm.* Typhimurium was reduced by 4 $\log_{10}$ after 10 min treatment with antimicrobial ice. Microbial levels remained at about 1×10^1 CFU per plate thereafter. As in the case of *E. coli*, *Salmonella* cells were significantly inhibited by antimicrobial ice ($P < 0{\cdot}01$). *Listeria monocytogenes* was more resistant to antimicrobial ice treatment than were *E. coli* or *Salmonella*. As shown in Fig. 3, only a one log

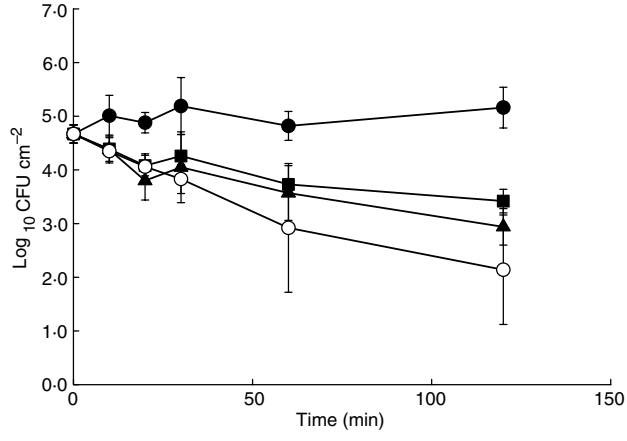

Fig. 5 Effect of antimicrobial ice against *Salmonella* Typhimurium inoculated on mackerel skin. Fish skin was placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 120 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

reduction of *Listeria* was observed after 10 min. After 30 min, the estimated reduction ranged from 2·7 to 3·2 $\log_{10}$. Although the reduction levels of *Listeria* were lower than those of *E. coli* and *Salmonella*, antimicrobial ice showed significant ($P < 0{\cdot}01$) bactericidal activities at all concentrations tested.

In addition to laboratory media, mackerel skin was used to simulate the antimicrobial effect of ClO_2 ice on seafood. The populational changes of *E. coli* O157:H7 are shown in Fig. 4. *E. coli* remained at 5·3 $\log_{10}$ CFU cm^{-2} after storage on the control ice for 120 min. At 25 ppm ClO_2, the *E. coli* levels started to decrease after 10 min, and were reduced to 3·1 $\log_{10}$ CFU cm^{-2} after 60 min, whereas the control remained at 4·4 $\log_{10}$ CFU cm^{-2}. After 120 min, 25 ppm ClO_2 significantly ($P < 0{\cdot}01$) inhibited *E. coli* compared with the control. Antimicrobial activity was observed at the outset with 50 ppm ClO_2. The final population of *E. coli* was significantly ($P < 0{\cdot}001$) reduced to 2·0 $\log_{10}$ CFU cm^{-2} after 120 min. When 100 ppm was used, populational change was similar to that of 50 ppm up through 30 min, but levels were reduced to 2·7 $\log_{10}$ CFU cm^{-2} after 60 min. Final count of *E. coli* O157:H7 with 100 ppm antimicrobial ice was below detection limit (0·3 $\log_{10}$ CFU cm^{-2}) after 120 min treatment. The total reduction on 100 ppm ClO_2 ice was 4·8 $\log_{10}$.

Results from the application of antimicrobial ice for the control of *Salm.* Typhimurium on mackerel skin are shown in Fig. 5. *Salmonella* levels remained constant during treatment with the control ice. When antimicrobial ice was applied, levels of *Salmonella* were reduced. After 120 min treatment with 25 ppm antimicrobial ice, the *Salmonella* level was significantly ($P < 0{\cdot}001$) reduced compared with the control. The final *Salmonella* count after treatment with

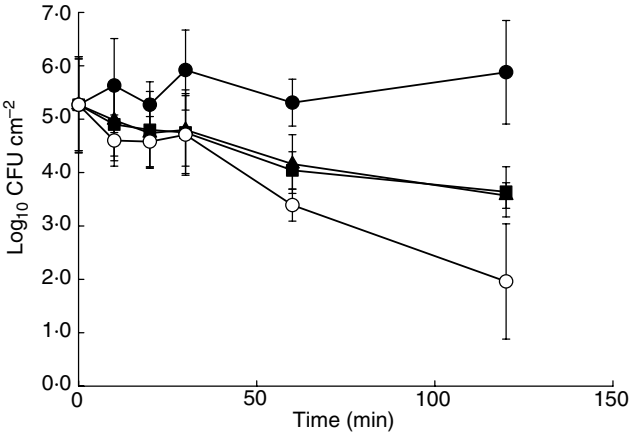

Fig. 6 Effect of antimicrobial ice against *Listeria monocytogenes* inoculated on mackerel skin. Fish skin was placed on antimicrobial ice containing chlorine dioxide (0, 25, 50 and 100 ppm) for 120 min (●, 0 ppm; ■, 25 ppm; ▲, 50 ppm; ○, 100 ppm)

50 ppm antimicrobial ice was 2·9 $\log_{10}$ CFU cm^{-2} while that of the control was remained at 5·2 $\log_{10}$ CFU cm^{-2}. With 100 ppm antimicrobial ice, the level was decreased to 2·1 $\log_{10}$ CFU cm^{-2} after 120 min indicating 2·6 $\log_{10}$ total reduction.

Population levels of *L. monocytogenes* on mackerel skin affected by antimicrobial ice are shown in Fig. 6. With the ice control, the level of *Listeria* increased slightly and remained constant above 5·9 $\log_{10}$ CFU cm^{-2} throughout the treatment interval. *Listeria* populations gradually decreased during treatment with 25 ppm ClO_2, but exposure for at least 30 min was required before a significant difference ($P < 0{\cdot}05$) from the control could be detected. Storage for 120 min reduced the final population of *Listeria* to 3·6 $\log_{10}$ CFU cm^{-2}. When 50 ppm ClO_2 was used, the reduction pattern was similar to that of 25 ppm. The effect of 100 ppm ClO_2 was similar to that of other concentrations until 30 min, but the level was decreased to 2·0 $\log_{10}$ CFU cm^{-2} after 120 min. Antimicrobial ice (100 ppm) reduced *L. monocytogenes* by 3·3 $\log_{10}$ after 120 min exposure.

DISCUSSION

After obtaining regulatory approval for direct food contact (FDA 1995; FDA, USDA, CDC 1998), many researches have been conducted to examine the antimicrobial activity of ClO_2 against major foodborne pathogens e.g. *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes*, on various foods. Han *et al.* (2000) reported a 6·5 $\log_{10}$ reduction of *E. coli* O157:H7 on injured green pepper surfaces after treatment with 1·24 ppm ClO_2 gas. Singh *et al.* (2002a) showed that *E. coli* O157:H7 on lettuce was reduced by 1·7 and 2·2 $\log_{10}$ after treatment with 10 ppm aqueous and 1 ppm gaseous ClO_2, respectively. Singh *et al.* (2003) reported aqueous ClO_2 (25 ppm for 5 min) produced a 0·96 $\log_{10}$ reduction of *E. coli* O157:H7 on alfalfa seed, and Taormina and Beuchat (1999) also reported that acidified ClO_2 (500 ppm for 3 min) reduced populations of *E. coli* O157:H7 on alfalfa seeds by more than 2 $\log_{10}$. When *E. coli* O157:H7 was sprinkled onto lettuce, a higher log reduction (1·6 $\log_{10}$) was observed after treatment with 100 ppm for 10 min (Singh *et al.* 2002b). *Salmonella* Typhimurium and aerobic plate counts (APC) in ground beef were reduced 1·4 and 1·2 $\log_{10}$ by 200 ppm ClO_2 followed by cetylpyridinium chloride (0·5%) treatment, respectively. *Salmonella* Typhimurium populations declined 1·2 $\log_{10}$ after 7 days of refrigerated display while simultaneously retarding aerobic bacterial growth (Pohlman *et al.* 2002). Spray rinsing or chilling with ClO_2 (20 ppm) significantly reduced the incidence of Salmonellae from turkey carcasses (Villarreal *et al.* 1990). Several research studies using ClO_2 against *Listeria monocytogenes* have been reported. With 3 ppm gaseous ClO_2, *L. monocytogenes* on green peppers was reduced by 7·4 $\log_{10}$, whereas 3 ppm aqueous ClO_2 and water washing resulted in reductions of 3·7 and 1·4 $\log_{10}$, respectively (Han *et al.* 2001). Zhang and Farber (1996) reported a washing effect of ClO_2 on fresh cut lettuce. A 10-min treatment with 5 ppm ClO_2 resulted in a maximum reduction of *L. monocytogenes* by 1·1 and 0·8 $\log_{10}$ at 4 and 22°C, respectively.

Chlorine dioxide has been recognized as having the highest bactericidal activity against several bacteria in comparison with sodium hypochlorite, iodine and quaternary ammonium compounds (Tanner 1989) and aqueous chlorine (Thiessen *et al.* 1984; Andrews *et al.* 2002). Either gaseous or aqueous ClO_2 can be used for food sanitation. Gaseous treatment was more effective than aqueous ClO_2 in reducing numbers of micro-organisms on surfaces. According to the results of Han *et al.* (2001), the antimicrobial activity of gaseous ClO_2 was twice better than that of aqueous ClO_2. This may be due to the greater surface penetration ability of gas than that of liquid. Although gaseous treatment with ClO_2 resulted in higher log reductions in comparison with aqueous solutions of that compound, some micro-organisms penetrated into inaccessible parts of various food samples, such as protective hydrophobic pockets, folds, or cracks in the samples, where the sanitizer could not get through (Adams *et al.* 1989; Babic *et al.* 1996; Singh *et al.* 2002b). This hypothesis can explain the difference of activity of ClO_2 against pathogens on laboratory media (Fig. 1) vs fish skin (Fig. 4). *Escherichia coli* O157:H7 cells could receive greater exposure to ClO_2 gas on laboratory media than on fish skin, because of the places on the skin (e.g. interstices between scales) where pathogens could be protected from the sanitizer.

According to our results, *Listeria* (Figs 3 and 6) was more resistant to ClO_2 than were *E. coli* O157:H7 (Figs 1 and 4) and *Salmonella* (Figs 2 and 5), in both the laboratory media and mackerel skin experiments. *Listeria* is a Gram-positive bacterium while *E. coli* and *Salmonella* are Gram-negative. As a result of the differences in cell wall peptidoglycan structure, susceptibility to ClO_2 between Gram-negative and Gram-positive bacteria is different (Huang *et al.* 1997). Chlorine dioxide, and other disinfectants, is more effective on Gram-negative bacteria. According to the study conducted by Huang *et al.* (1997) the amount of ClO_2 required to destroy 95% of Gram-positive and Gram-negative bacteria was 1·3 and 1·18 ppm, respectively.

Although certain antimicrobial agents have good activity, concentration of the active ingredients should remain constant during treatment. Kim *et al.* (1999) reported that treatment with ClO_2 up to 200 ppm slightly reduced the numbers of mesophilic microfloa. Bacterial populations of untreated and treated salmon skin were 3·3 and 2·9 $\log_{10}$ CFU g^{-1} at day 0, respectively. These populations increased to 6·4 and 6·0 $\log_{10}$ CFU g^{-1} at day 7. Bacterial numbers in treated samples increased, but the amount of increase was lower than for that of untreated samples. Andrews *et al.* (2002) observed a 3 $\log_{10}$ reduction of total aerobic count in shrimp using 40 ppm ClO_2. However, they also had the same phenomenon of total mesophilic counts increasing after 14 days. Although a one time treatment with ClO_2 could lower the growth rate, it could not reduce numbers of mesophilic microfloa during storage. According to the other workers (Kim *et al.* 1999; Andrews *et al.* 2002), simultaneous washing with ClO_2 should be needed to maintain lowered levels of micro-organisms. If antimicrobial ice is used for the preservation of seafood, microbial populations will be reduced effectively and kept at a lower level for the duration of storage.

Traditional application of gaseous ClO_2 requires a gas generator and encounters difficulties in maintaining a constant level of ClO_2. Chlorine dioxide gas easily evaporates and loses effectiveness during the storage interval. Using antimicrobial ice containing ClO_2 provides sustained release of ClO_2 as the ice melts. The advantage of using antimicrobial ice is as follows: (i) to destroy water-borne pathogens that can be transmitted to ice. Seafood products are generally stored and displayed on ice. The direct contact between seafood and ice can cause cross-contamination of seafood products; (ii) to maintain a constant level of ClO_2 gas. Chlorine dioxide is easily evaporated and eventually loses its antimicrobial activity. Although frequent washing with ClO_2 solution could maintain antimicrobial activity, the antimicrobial efficiency of aqueous ClO_2 is still low compared with the gaseous form. Using antimicrobial ice containing ClO_2 could solve this problem by continuously releasing the chemical during melting.

The results of this study indicate that antimicrobial ice can successfully reduce the initial load of foodborne pathogens and maintain the lowered microbial level during treatment. This information can help seafood product industries ensure and maintain seafood safety.

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