Evaluation of Lactic Acid Bacterium Fermentation Products and Food-Grade Chemicals To Control Listeria monocytogenes in Blue Crab (Callinectes sapidus) Meat

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Fresh blue crab (Callinectes sapidus) meat was obtained from retail markets in Florida and sampled for viable Listeria monocytogenes. The pathogen was found in crabmeat in three of four different lots tested by enrichment and at levels of 75 CFU/g in one of the same four lots by direct plating. Next, crabmeat was steam sterilized, inoculated with a three-strain mixture of L. monocytogenes (ca. 5.5 log10 CFU/g), washed with various lactic acid bacterium fermentation products (2,000 to 20,000 arbitrary units [AU]/ml of wash) or food-grade chemicals (0.25 to 4 M), and stored at 4°C. Counts of the pathogen remained relatively constant in control samples during storage for 6 days, whereas in crabmeat washed with Perlac 1911 or Microgard (10,000 to 20,000 AU), numbers initially decreased (0.5 to 1.0 log10 unit/g) but recovered to original levels within 6 days. Numbers of L. monocytogenes cells decreased 1.5 to 2.7 log10 units/g of crabmeat within 0.04 day when washed with 10,000 to 20,000 AU of Alta 2341, enterocin 1083, or Nisin per ml. Thereafter, counts increased 0.5 to 1.6 log10 units/g within 6 days. After washing with food-grade chemicals, modest reductions (0.4 to 0.8 log10 unit/g) were observed with sodium acetate (4 M), sodium diacetate (0.5 or 1 M), sodium lactate (1 M), or sodium nitrite (1.5 M). However, Listeria counts in crabmeat washed with 2 M sodium diacetate decreased 2.6 log10 units/g within 6 days. In addition, trisodium phosphate reduced L. monocytogenes counts from 1.7 (0.25 M) to >4.6 (1 M) log10 units/g within 6 days. These results demonstrate that numbers of L. monocytogenes on crabmeat may be reduced appreciably by “washing” with select antimicrobial agents.

Listeria monocytogenes is a facultative intracellular pathogen responsible for several outbreaks and numerous sporadic cases of food-borne listeriosis (15, 27). This organism is frequently associated with fresh, frozen, and ready-to-eat seafood products and is epidemiologically implicated in a limited number of seafood-related listeriosis episodes (12, 15, 25). The association of L. monocytogenes with seafood (incidence from 12 to 26%), notably crabmeat, smoked fish, lobster, shrimp, and surimi, has also resulted in several product recalls (summarized and reported by Ryser and Marth [25]). Further studies to monitor and subsequently control L. monocytogenes in seafoods are justified by the clinical and commercial significance of listeriosis outbreaks, as well as by a zero-tolerance action level set by the U.S. Food and Drug Administration for ready-to-eat foods.

L. monocytogenes is difficult to control in seafood, and in foods in general, because it can grow at refrigeration temperatures, survive in brine solutions, and tolerate extremes in heat and pH (25). Regarding the prevalence of L. monocytogenes in the seafood-processing environment, a 1988 survey conducted in Oregon revealed that 60% (10 of 17) of the seafood-processing facilities examined showed evidence of Listeria contamination (22). Similarly, a survey of crab-processing plants in Virginia estimated the prevalence of L. monocytogenes from 1989 to 1993 at 4 to 9% (8). In another study, L. monocytogenes was found in 7 of 24 (29%) samples of cooked crabmeat (29). Regardless of the source, the presence of L. monocytogenes in seafoods, primarily as a result of post-processing contamination, represents a significant problem, particularly for cooked ready-to-eat seafoods, such as crabmeat (1, 24, 28).

The frequency at which L. monocytogenes is isolated from seafoods and its growth and survival properties warrant the investigation of additional barriers to supplement traditional control practices. The purpose of the present study was to evaluate lactic acid bacterium (LAB) fermentation products and food-grade chemicals for their ability to control L. monocytogenes in crabmeat. Blue crab meat was selected as the test substrate because the prevalence of L. monocytogenes is higher in this product than in other seafoods (13). Also, a recent study by Farber (14) demonstrated that L. monocytogenes grows better on crabmeat than on certain other seafoods. Lastly, there have been no reports on the use of biopreservatives (i.e., LAB fermentation products) to control L. monocytogenes in crabmeat and relatively little information on other intervention strategies (i.e., organic acids and chemical preservatives).

Portions of this work have been presented [9] at the Joint Meeting of the Atlantic Fisheries Technology Society and Tropical and Subtropical Fisheries Technology Society, 29 August to 1 September 1993, Williamsburg, Va., and at the Annual Meeting of the Institute of Food Technologists, 25 to 29 June 1994, Atlanta, Ga.)

MATERIALS AND METHODS

Listeriae. L. monocytogenes 101M (serotype 4b; meat isolate), NCTC 5105-3 (serotype 3a; human isolate; hypohemolytic variant of NCTC 5105), and V7 (serotype 1/2a; raw-milk

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isolate) were grown at 37°C in tryptose phosphate (Difco Laboratories, Detroit, Mich.) broth and maintained as previously described (30). The three-strain listeria mixture was prepared essentially as described previously (21).

**Microbiological sampling of crabmeat.** Fresh, shelled blue crab (Callinectes sapidus) meat obtained from retail markets in Florida was shipped on ice to Wisconsin and sampled within 4 h of arrival for the total bacterial load by direct plating and for L. monocytogenes by both direct plating and enrichment. Samples (25 g) were homogenized for 60 s with deionized H2O (225 ml) in a sterile commercial blender (model 34BL97; Waring Products Division, New Hartford, Conn.). To determine the total bacterial load, the homogenate was diluted in 0.1% peptone and 0.1 ml of each dilution was added to 5 ml of tempered (48°C) plate count agar (Difco) medium, mixed, and then overlaid onto plate count agar plates. The agar plates were incubated at 37°C for 24 to 48 h before colonies were counted. For direct plating of listeriae, appropriate dilutions of crab homogenate were spread onto Listeria Enrichment (LE; Difco) agar (1.5%) plates and incubated at 37°C for 24 to 48 h before colonies were counted. To achieve the minimum detection level of 10 CFU/g of crabmeat, a 0.2-ml portion of the homogenate was spread onto each of five LE agar plates. Samples in which listeriae were not detected by direct plating were examined by using the Food and Drug Administration enrichment procedure (18). L. monocytogenes colonies were confirmed by standard biochemical tests (18).

**Inoculating crabmeat.** Fresh blue crab meat (ca. 2 kg) was sterilized in a 4-liter glass beaker by autoclaving (121°C for 25 min). Following sterilization, the meat was cooled to 4°C in an ice bath and then inoculated with 1 liter of the Listeria cocktail to levels of 5 × 105 CFU/g. The beaker was inverted three times to distribute the inoculum throughout the meat and then held at 4°C for 5 min. The crab-Listeria slurry was then poured (aseptically) into a large funnel containing a sterile stainless steel sieve that drained into a 1-liter graduated cylinder. The crabmeat was pressed with a large sterile pestle until 1 liter of fluid (the original volume of inoculum) was recovered. L. monocytogenes levels in the inoculated crabmeat were determined by homogenizing 20-g samples of meat with 180 ml of 0.1% peptone, spread plating dilutions onto LE agar, and incubating the plates for 24 to 48 h at 37°C. Numbers of L. monocytogenes in the original inoculum and the fluid collected from the crabmeat were also determined by spread plating appropriate dilutions on LE agar plates as above.

**Washing crabmeat with antimicrobial agents.** The LAB fermentation products tested included Alta 2341 (Quest International, Sarasota, Fla.), enterocin (Microgard (Wesman Foods, Inc., Beaverton, Ore.), Nisin (Sigma Chemical Co., St. Louis, Mo.), and PerLae 1911 (Quest). Each was tested at levels of 0, 2,000, 10,000, and 20,000 arbitrary units (AU)/ml (defined below). The food-grade chemicals tested included sodium acetate (Sigma), sodium diacetate (International Sourcing, Inc., Upper Saddle River, N.J.), sodium lactate (calculated on an anhydrous basis [60% solution of sodium 1-lactate; Purac, Inc., Arlington Heights, Ill.]), sodium nitrite (Mallinckrodt, Inc., Paris, Ky.), and trisodium phosphate (TSP; Rhone-Poulenc, Inc., Cranberry, N.J.). Each of the food-grade chemicals was tested at 0, 0.25, 0.5, 1, 2, or 4 M depending on their activity or the levels permitted in foods or both. Control samples were washed with deionized water. As shown in Table 1, stock solutions of each antilisterial agent were adjusted to equivalent arbitrary units (fermentation products) or molarity (chemicals) and prepared at three different application levels.

Crabmeat inoculated with L. monocytogenes was divided into several 200-g lots for “washing” with antilisterial agents. Each wash solution (200 ml) was separately added to individual lots of crabmeat in a 600-ml beaker. The beakers were inverted three times to distribute the antilisterial agents before the meat was drained by using a sterile funnel and sieve as described above. The entire washing procedure required 30 to 60 s for each antilisterial agent. Treated crabmeat was divided into 20-g aliquots and placed into sterile petri dishes. At appropriate intervals during storage at 4°C, duplicate samples were monitored for viable listeriae as described above.

**Crabmeat pH.** The pH of the crabmeat was monitored with a sanitized electrode both before and after washing with antilisterial agents. In a second set of experiments, the pH of inoculated crabmeat (in the absence of antilisterial agents) was adjusted with 1 N HCl or 5 N NaOH to values ranging from 2 to 12. A 100-g sample of inoculated crabmeat (ca. 105 CFU/g) and 200 ml of deionized water were combined in a 500-ml beaker. The crab-Listeria slurry was then adjusted to a target pH with gentle stirring with a magnetic stir bar. Excess fluid (200 ml) was drained from the crabmeat as described above, and the pH of the crabmeat was recorded. For each pH increment, five 20-g samples of crabmeat were placed into petri plates and stored at 4°C. Counts of the pathogen in the crabmeat were determined just before and 60 min after adjusting the pH of the meat.

**Monitoring antilisterial activity.** The antimicrobial activity of fermentation products and chemicals was measured and compared by using the “spot-on-lawn” method as described previously (10). Antilisterial activity was reported in arbitrary units as the inverse of the highest dilution (5 μl spotted) showing a definite inhibition zone on a lawn of sensitive indicator cells (i.e., L. monocytogenes 101M, V7, or NCTC 5105-3), multiplied by 200 to convert to a per milliliter basis.

**Statistical analyses.** The data reported are average values from duplicate samples and two separate trials. Data were analyzed statistically with the SAS program (SAS Institute, Inc., Cary, N.C.), wherein analysis of variance and separation tests of the least significant difference of the means were used to compare antilisterial agents on the basis of \( \log_{10} \) CFU counts of Listeria cells. All analyses were done at the 95% confidence level.

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### Table 1. Concentrations of stock solutions of LAB fermentation products and food-grade chemicals used in this study

<table>
<thead>
<tr>
<th>Application level</th>
<th>Concentration of fermentation products (AU)</th>
<th>Concentration of food-grade chemicals (M)</th>
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<tr>
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<td>Alta</td>
<td>Enterocin</td>
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* Numbers within brackets represent the percent solution of antimicrobial agents.
RESULTS

Occurrence of L. monocytogenes in crabmeat. Pursuant to evaluating the effect of various antimicrobial agents on the behavior of L. monocytogenes in blue crab meat, the prevalence of the pathogen in the fresh product was determined by direct plating and the Food and Drug Administration enrichment method. L. monocytogenes was found in three of four lots tested by enrichment and at levels of 75 CFU/g in one lot by direct plating. In comparison, the total bacterial load in fresh crab meat ranged from 4.8 to 5.3 log_{10} CFU/g (mean, 5.1 [four samples]). These results provided further justification for exploring strategies to better prevent the contamination of crabmeat with L. monocytogenes.

LAB fermentation products as antilisterial agents in crabmeat. The behavior of L. monocytogenes in fresh blue crab meat in the presence of three levels of LAB fermentation products was examined (Fig. 1). Counts of the pathogen remained relatively constant and were not significantly different (P < 0.05) from the control in samples washed with each of the fermentation products used at their lowest level (level I; 2,000 AU/ml). Similarly, counts of L. monocytogenes remained relatively constant compared with the control in samples washed with 10,000 AU of MicroGard or Perlac per ml (level II) (Fig. 1B). At the highest level tested (20,000 AU/ml), MicroGard and Perlac had no appreciable effect (P < 0.05) on pathogen numbers after 6 days compared with the control. In contrast, washing crabmeat with Alta, enterocin, or Nisin at 10,000 AU/ml (level II) and 20,000 AU/ml (level III) resulted in significantly lower L. monocytogenes counts (P < 0.05) compared with control treatments. For example, counts of L. monocytogenes decreased 1.2 (Alta), 2.1 (enterocin), and 2.6 (Nisin) log_{10} units/g within 0.04 day in crabmeat washed with 10,000 AU/ml. Thereafter, counts of the pathogen increased somewhat over 6 days but did not return to initial inoculum levels. An initial decrease of 2 to 3 log_{10} units was also observed with wash solutions containing Alta, enterocin, and Nisin at 20,000 AU/ml. More important, the recovery and resurgence of L. monocytogenes were lessened over the 6-day sampling period when the crabmeat was treated with 20,000 AU/ml (ca. 0.5 to 1.1 log_{10} units) compared with 10,000 AU/ml (ca. 0.8 to 1.6 log_{10} units). These data identified the potential of Alta, enterocin, and Nisin as antilisterial agents for crabmeat.

Food-grade chemicals as antilisterial agents in crabmeat. The efficacy of food-grade chemicals as antilisterial agents in crabmeat was also evaluated (Fig. 2). With the exception of TSP (0.25 and 0.5 M), when these chemicals were used at low (level I; Fig. 2A) or medium (level II; Fig. 2B) concentrations, L. monocytogenes numbers were not significantly different (P < 0.05) in controls compared with those in controls of crabmeat washed with acetate, lactate, diacetate, or nitrite and stored at 4°C over the 6-day sampling period. However, when used at level I concentrations, both diacetate (0.5 M) and TSP (0.25 M) reduced L. monocytogenes numbers by 0.8 log_{10} unit after 2 days at 4°C. After 6 days, counts of L. monocytogenes in diacetate-washed crabmeat increased (0.8 log_{10} unit) whereas counts in TSP-washed crabmeat decreased (0.9 log_{10} unit). Likewise, at level II concentrations, washing with diacetate (1 M) decreased listeria counts 0.8 log_{10} unit by day 2, but populations recovered to near-original levels within 6 days. In contrast, TSP (0.5 M) appreciably reduced counts of L. monocytogenes within 0.04 day (1.9 log_{10} unit), and counts decreased an additional 2.6 log_{10} units over the 6-day sampling period.

When used at the highest concentrations (level III; Fig. 2C), acetate (4 M), nitrite (1.5 M), and lactate (1 M) decreased initial counts of L. monocytogenes ca. 0.8 log_{10} unit within 2 days, but the counts increased ca. 0.5 log_{10} unit within 6 days. In contrast, washing crabmeat with 2 M diacetate significantly (P < 0.05) reduced (2.6 log_{10} units) L. monocytogenes numbers during storage at 4°C for 6 days. Washing crabmeat with 1 M TSP significantly (P < 0.05) reduced L. monocytogenes numbers by 2.4 log_{10} units/g within 0.04 day of treatment. The pathogen was not detectable (reduction of >4.6 log_{10} units/g) by direct plating after 6 days at 4°C. Results from washing crabmeat with food-grade chemicals revealed that diacetate and TSP displayed the greatest antilisterial activity.

Effect of pH on the behavior of L. monocytogenes in crabmeat. These strategies for crabmeat are shown in Fig. 2C, which depicts the behavior of L. monocytogenes in crabmeat washed with four pH-adjusting agents and Nisin at pH 5.1. A control (no treatment) and an inoculum of 20,000 CFU/g were used in each experiment and checked daily for 6 days. The pH of each treatment was measured at 0, 6, and 12 hours and noted to be stable at approximately 6.3 for all trials. In each experiment, wash solutions containing Alta, enterocin, or Nisin were added at levels of 10,000 AU/ml to crabmeat (level II) and 20,000 AU/ml (level III). Crabmeat washed with acetate, lactate, diacetate, or nitrite and stored at 4°C over the 6-day sampling period. However, when used at level I concentrations, both diacetate (0.5 M) and TSP (0.25 M) reduced L. monocytogenes numbers by 0.8 log_{10} unit after 2 days at 4°C. After 6 days, counts of L. monocytogenes in diacetate-washed crabmeat increased (0.8 log_{10} unit) whereas counts in TSP-washed crabmeat decreased (0.9 log_{10} unit). Likewise, at level II concentrations, washing with diacetate (1 M) decreased listeria counts 0.8 log_{10} unit by day 2, but populations recovered to near-original levels within 6 days. In contrast, TSP (0.5 M) appreciably reduced counts of L. monocytogenes within 0.04 day (1.9 log_{10} unit), and counts decreased an additional 2.6 log_{10} units over the 6-day sampling period.

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meat. In another component of this study, we conducted experiments to distinguish between the antilisterial activity of the antimicrobial agents used to wash crab meat and any pH effects contributed by these antimicrobial agents. The pH of the crab meat as received was 8.1 (average of 12 samples). The pH was monitored both before and after washing the crab meat with the six most potent antimicrobial agents: Alta, enterocin, and Nisin (each at 20,000 AU/ml), and acetate (4 M), diacetate (2 M), and TSP (1 M). The pHs of the individual wash solutions (in ascending order) were as follows: Nisin, 3.8; Alta, 5.1; diacetate, 5.1; enterocin, 5.8; acetate, 8.2; and TSP, 12.7.

The pHs of crab meat samples immediately after being washed with these antimicrobial agents (in ascending order) were as follows: Nisin, 5.2; diacetate, 5.3; Alta, 7.4; enterocin, 7.5; acetate, 8.1; and TSP, 11.5. In general, the pH of the crab meat (pH 8.1) decreased somewhat (pH 5.2 to 7.5) after washing with LAB fermentation products and diacetate. Washing with acetate did not alter the pH, whereas washing with TSP initially raised the pH to 11.5. Typically, over the 6-day sampling period, the pH of all treatments remained relatively constant (±0.4 pH unit), that is, similar to the pH immediately following treatment.

After establishing the baseline pH data reported above, the survival of *L. monocytogenes* in crab meat was examined within 60 min of adjusting the pH to values ranging from 2 to 12 to ascertain the effect of pH on pathogen survival in the absence of any antimicrobial agents (Fig. 3). Pathogen numbers remained relatively constant (±0.2 logFU/unit) compared with control values over the range of pH 4.4 to 10. As depicted in Fig. 3, Alta, enterocin, Nisin, acetate, and diacetate did not alter the pH of crab meat to inhibitory levels, but TSP (1.0 M) elevated the pH to ca. 11.5, a level which is detrimental to *L. monocytogenes* survival.

**DISCUSSION**

Cooked shelled crab meat is specifically subject to cross-contamination with raw product by personnel and from raw crab in the processing environment (22); thus, it serves as a possible carrier of *L. monocytogenes*. Although *L. monocytogenes* is inactivated by proper thermal processing of crab meat (16, 17), the results herein and those reported by other investigators (6) revealed that the pathogen is present in retail product. With the exceptions of listeriosis episodes in New Zealand in 1980 and 1992 and one in Connecticut in 1989, as well as a few sporadic cases, seafoods are not a common vehicle of listeriosis compared with other food vectors (3, 15, 25). However, the association of listeriosis with the consumption of seafood and the ability of *L. monocytogenes* to grow and survive in crab meat represent a potential threat to the consumer and, as such, prompted us to evaluate LAB fermentation products and food-grade chemicals as interventions.

Little is known about the incidence and fate of *L. monocytogenes* in fish or seafood compared with other foods, except that the organism is capable of growth and survival on a variety
of fish products during refrigerated storage (25). For example, the level of pathogen increased ca. 5 log units in 2 weeks at 7°C following challenge (10^3 CFU/g) of sterile shrimp, crab, surimi, and white fish (20). Similarly, L. monocytogenes numbers increased ca. 1 to 3 log units in 7 days at 4°C on artificially inoculated shrimp, crab, smoked salmon, and lobster (14). In contrast, 14 days of storage at 5°C was required to demonstrate appreciable growth (increase from 10^4 to 10^6 CFU/g) of the pathogen on crabmeat (5). The same authors reported substantial growth (increase from 10^4 to 10^8 CFU/g) on crabmeat in 8 days during storage at 10°C. Our results are in general agreement with those of other investigators. In artificially inoculated crabmeat, L. monocytogenes numbers increased slightly (ca. 0.2 to 0.4 log_{10} CFU/g) in 6 days at 4°C.

To our knowledge, there have been few reports detailing the use of LAB fermentation products or food-grade chemicals to control L. monocytogenes in crabmeat. Of 10 agents tested in the present study, Nisin, enterocin, Alta, diacetate, and TSP were the most effective in reducing numbers of L. monocytogenes. Despite a significant initial decrease in pathogen numbers after washing with Alta, enterocin, or Nisin at 10,000 or 20,000 AU/ml, counts of L. monocytogenes increased slightly after 6 days at 4°C. Similarly, when used at high concentrations (2 M), diacetate reduced counts of L. monocytogenes by 2.6 log_{10} units/g within 6 days without allowing any resurgence. Overall, at all levels tested TSP was the most effective antilisterial agent: the pathogen was not detected by direct plating within 4 days at 4°C (>5.0 log_{10} unit drop) when 1 M TSP was used for washing. The data also indicated that the antilisterial activity of TSP was due in large measure to the elevated pH (pH < 11) of crabmeat washed with 1 M TSP, whereas the listericidal activity of fermentation products and diacetate was not due solely to pH. Additional studies are warranted to characterize the mechanism(s) by which the aforementioned antimicrobial agents inhibit L. monocytogenes on crabmeat. The results of the present study agree with and expand upon our previous investigations, and those of others, that established the efficacy of bacteriocins of LAB and/or sodium diacetate for controlling L. monocytogenes in food (for examples, see references 7, 11, 19, 21, 23, and 26). Sensory properties notwithstanding, the results indicate that the utility of TSP may be expanded for controlling undesirable bacteria associated with foods other than poultry (4). However, some of the antilisterial wash agents tested herein await regulatory approval.

The presence and growth of L. monocytogenes in ready-to-eat fish products such as crabmeat heightens the potential for listeric illness in certain subpopulations. Our results indicate that some fermentation products and food-grade chemicals may serve as additional factors in decreasing the likelihood of illness caused by crabmeat contaminated with L. monocytogenes. To fully exploit these antilisterial agents, we are presently identifying additional antilisterial agents, developing simple yet effective application strategies for such agents, testing the efficacy of these safety assurance systems in a processing plant, and evaluating the effect, if any, of the agents on the nutritional, textural, and sensory properties of crabmeat. A multifaceted approach to seafood safety as described herein is required to strive for a Listeria-free product from harvest to consumption.

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REFERENCES

CONTROL OF L. MONOCYTOGENES IN CRABMEAT


