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Research Note

Effect of Prestorage Treatments and Storage Conditions on the Survival of *Salmonella* Enteritidis PT4 and *Listeria monocytogenes* on Fresh Marine and Freshwater Aquaculture Fish

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ABSTRACT

The effect of prestorage treatments, such as immersion in a sorbate solution (5%, wt/vol), heating (60°C, 1 min), and a combination of the two treatments, and the subsequent storage in air or under modified atmosphere packaging (MAP; 40% CO_2, 30% O_2, and 30% N_2) at chill temperatures (0 ± 1°C), on *Listeria monocytogenes* and *Salmonella* Enteritidis PT4 was studied. The prestorage treatments affected the pathogenic bacteria, and in all cases, there was a decrease in their population, with the sorbate and combination (hot water and sorbate) treatment being most effective. The beneficial effect of the prestorage treatments, which was more pronounced in storage under MAP conditions, suggests an interaction of the treatments with the CO_2 of MAP against injured bacterial cells.

Seafood products are highly perishable foods, which, unless correctly stored, processed, packaged, and distributed, spoil quickly and can potentially become unsafe because of microbial growth. In the last decade, the incidence of foodborne diseases has increased in Europe, despite the introduction of hazard analysis critical control point (HACCP) and new food safety regulations *(39).* The increasing number and severity of food-poisoning outbreaks worldwide has increased public awareness about the safety of seafood, which is a vehicle for most of the known bacterial pathogens *(21, 39).* The annual health care costs, traced to a few selected foodborne pathogens such as *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* spp. was estimated at €5 to 6 billion per year, of which €4 billion were attributed to meat and seafood products. For example, among the 22,386 investigated outbreaks in which food items were identified, fish and shellfish products were involved in 5% *(39).* With regard to the causative microorganisms, *Salmonella* Enteritidis was involved in more than 75% of all cases.

Red mullet (*Mullus barbatus*) and carp (*Cyprinus carpio*) are fish of economic importance in Greece, representing the marine and aquaculture species, respectively. They are stored and distributed in a traditional way in wooden or plastic boxes in ice, which reduces their quality significantly *(16).* It is well established that a longer shelf life of fish can be achieved with storage in low temperature (0 to 5°C) combined with vacuum packaging or modified atmosphere packaging (VP or MAP) *(23).* However, both of these hurdles could not guarantee the control of pathogenic bacteria like *Salmonella enterica* subsp. *enterica* serotype Enteritidis PT4 and *L. monocytogenes,* which have been found in fish and fish products *(8, 35).* Indeed, these pathogenic bacteria can survive in food products held at low temperatures *(2, 28).* On the other hand, concerns have arisen about the controversial findings related to the potential inhibitory effect of VP or MAP systems on the growth and survival of these pathogens in model systems or foods *(5, 6, 14, 25, 28, 31).*

Thus, there is a need to apply additional hurdles. This is the case in the meat industry, in which different decontamination procedures (i.e., hot water, steam, and acid or nonacid approaches) have been applied extensively, in comparison with the limited available information related to fish *(22, 33).* Treatments with chlorinated water, ozone, or organic acids can reduce the total aerobic flora or *L. monocytogenes* but could not ensure a product free of *Listeria* or *Salmonella (3, 10, 29).*

Sorbic acid and its salts are widely used as food preservatives *(33).* However, there is little information, if any, related to their application on fish *(11, 37).* Among its salts, potassium sorbate was reported to be the most effective from a microbiological and sensory point of view *(11, 37).* Although the microbial association of fish (e.g., pseudomonads, *Shewanella putrefaciens,* lactic acid bacteria, *Brochothrix thermosphacta,* and *Enterobacteriaceae*) have

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been found to be inhibited by sorbate in fresh fish stored under aerobic conditions or MAP systems *(11),* no data are available on the potential reduction of the above-mentioned pathogenic bacteria.

Thus, the present study sought the most appropriate pretreatement (i.e., immersion in hot water with or without the addition of sorbate) in order to evaluate the effect on the reduction of pathogenic bacteria in inoculated marine and freshwater aquaculture fish.

MATERIALS AND METHODS

Organisms and preparation of inocula. *Salmonella* Enteritidis PT4 was maintained on slants of nutrient agar at 4°C. The inoculum was an overnight culture in nutrient broth (1.05443, Merck, Darmstadt, Germany) at 37°C. After harvesting the cells by centrifugation (3,000 × *g,* 15 min, 4°C), washing twice, and resuspending with saline (NaCl, 0.85%, wt/vol), 1 ml of the dense suspension (CFU $> 10^8$ ml^{-1}) was used for inoculation of fish. *L. monocytogenes* 4B was maintained on slopes of tryptone soya agar (CM131, Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) at 4°C, and an inoculum was prepared as above, but in Lactobacillus MRS broth with acetate and citrate omitted *(20).*

Fish treatment, packaging, and storage. Two replicated storage experiments were carried out with fresh red mullet (*M. barbatus*) and carp (*C. carpio*). In every experiment, 300 whole fish from each species were used. Red mullet fish (weight ≈ 150 g) were caught off-shore at around 0100 to 0200 h. After catch, fish were kept in ice and transported to a local fishery shop until they were bought (i.e., within 4 to 9 h after catch). Then they were transported in ice within 30 min from their purchase to the laboratory. Carp (weight ≈ 200 g) were provided by a Greek company (HELPA, Preveza, Greece) and transferred to the laboratory in ice within 16 h after catch. Both fish on arrival at the laboratory were gutted. The fish were dipped for 60 s in sterilized water containing *S. enterica* or *L. monocytogenes* at populations of 8×10^6 and 3×10^5 CFU/ml, respectively. The fish were left to dry for 10 min. In both cases (red mullet and carp), the whole fish were dipped (four to five fish) either in a 10-liter tank containing a solution of potassium sorbate (5% wt/vol) with or without hot water (60°C) for 1 min or just in hot water (60°C). The pH value of the solutions was adjusted to 6.0 with HCl (research grade) at 20°C. This pH value did not affect the appearance of the skin of the fish *(11).* After draining, the fish were placed in a wooden box in the traditional way or in aluminum trays (two fish per tray) and then packed under a modified atmosphere (40% CO_2, 30% O_2, and 30% N_2) in Suprovac polyamide laminate bags (thickness, 90 mm; gas permeability at 20°C; and 50% relative humidity, ~25, 90, and 6 $cm^3/m^2/d$ for CO_2, O_2, and N_2, respectively) with a vacuum packaging machine (Howden Food Equipment B.V., The Netherlands). The fish samples of both species were stored at 0 ± 1°C. Untreated fillets (60 fish samples, dipped in sterile water) were placed in a wooden box covered with ice (traditional way) and stored aerobically or under MAP conditions at 0 ± 1°C. Two sampling units were analyzed per treatment on each sampling day (individual packs).

Microbiological analysis and enumeration. Samples (10 g) from inoculated fish were weighed aseptically, added to sterile quarter-strength Ringer's solution (90 ml), and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. Decimal dilutions in quarter-strength Ringer's solution were prepared, and duplicate 1- or 0.1-ml samples of appropriate dilutions were poured or spread plated on the following media: (i) Palcam Listeria-selective agar (Merck, 1.11755) supplemented with Palcam Listeria-selective supplement (Merck, 1.12122) for the enumeration of *L. monocytogenes,* incubated at 30°C for 24 h and (ii) xylose lysine desoxycholate (XLD) agar (Merck, 1.05287) for enumeration of *Salmonella* Enteritidis PT4, incubated at 37°C for 24 h. When colonies of *Salmonella* Enteritidis PT4 and *L. monocytogenes* were not evident on plates from the 10-fold dilution (below the plating threshold), the enrichment technique was used for the resuscitation of possibly injured living cells as follows. For *Salmonella,* a 10-g sample of fish was suspended in 90 ml of selenite cystine enrichment broth (Merck, 1.07709) and incubated at 35°C for 12 to 18 h; then, 1 ml of the above medium was serially diluted and spread plated on XLD plates. For *L. monocytogenes,* a 10-g sample of fish was suspended in 90 ml Listeria enrichment broth (Oxoid, CM897), incubated at 30°C for 24 h, then spread on Palcam agar. Three replicates of at least three appropriate dilutions were enumerated. All plates were examined visually for typical colony types and morphologic characteristics associated with each growth medium.

RESULTS AND DISCUSSION

Although *Salmonella* Enteritidis is not indigenous to the aquatic environment, it has occasionally been isolated from fish and is usually found in fish contaminated through poor handling practices *(8).* The ability of this pathogen to survive in food products held at low temperatures has been well established *(7, 21, 28).*

L. monocytogenes is a widespread microorganism that has been isolated from a variety of foods, including fish *(18, 21, 27, 30).* From seafood, it has been isolated on a regular basis since 1987. A relatively high incidence of the organism (6 to 36%) in ready-to-eat cold smoked salmon and cooked fish products has raised concern about the survival and growth potential of this organism in seafood *(4).* Several studies refer to the isolation of this pathogen from a variety of fish species: rainbow trout, frozen fish and seafood, and frozen lobsters *(8, 17, 21, 27, 28, 30, 38).*

The present study sought to investigate the inhibitory effect of 5% (wt/vol) potassium sorbate in combination with other treatments (e.g., hot water) on fresh-caught marine fish red mullet and fresh water aquaculture carp deliberately inoculated with pathogenic bacteria and stored under either aerobic or MAP conditions. The results are shown in Tables 1 through 4.

In particular, it was found that there was a significant 1-log reduction of the initial load of *Salmonella* in carp treated with 5% (wt/vol) potassium sorbate with or without hot water (60°C). During storage of carp either in air or under MAP, the *Salmonella* population survived in all cases. In MAP conditions, a gradual decrease of the *Salmonella* counts was observed, being more pronounced in samples treated with sorbate or with sorbate in combination with hot water (Table 2). MAP conditions also extended the storage period of carp to 30 days compared with storage in air (results not shown). Similar observations were made with red mullet. Treatment of fish with 5% (wt/vol) potassium sorbate and sorbate in combination with hot water was quite effective in reducing the initial population of *Salmonella* almost 1 log (Table 2). During a 15-day storage pe-

TABLE 1. *Changes in* Salmonella *Enteritidis and* Listeria monocytogenes *inoculated in carp and stored aerobically at 0 ± 1°C without treatment or treated with potassium sorbate, hot water at 60°C, or their combination*[a]

	Pathogen count (log CFU/g)							
	Control		Potassium sorbate		Hot water		Combination	
Day	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.
0	6.2 ± 0.1	4.8 ± 0.1	5.3 ± 0.2	4.0 ± 0.1	5.9 ± 0.1	4.0 ± 0.2	4.9 ± 0.2	3.9 ± 0.1
1	4.8 ± 0.3	4.6 ± 0.2	5.2 ± 0.2	4.3 ± 0.1	5.2 ± 0.1	4.6 ± 0.2	4.8 ± 0.1	3.9 ± 0.1
3	4.9 ± 0.2	4.6 ± 0.3	5.0 ± 0.1	4.0 ± 0.1	4.7 ± 0.1	4.7 ± 0.2	4.9 ± 0.1	3.3 ± 0.3
5	4.8 ± 0.2	4.7 ± 0.3	4.9 ± 0.1	4.2 ± 0.2	5.0 ± 0.2	4.8 ± 0.2	4.8 ± 0.1	3.6 ± 0.3
7	5.2 ± 0.1	6.1 ± 0.2	4.8 ± 0.2	4.2 ± 0.2	5.2 ± 0.2	4.9 ± 0.2	4.9 ± 0.1	4.0 ± 0.2
11	5.9 ± 0.2	5.9 ± 0.3	4.6 ± 0.1	4.2 ± 0.1	5.1 ± 0.2	4.7 ± 0.1	4.8 ± 0.2	3.8 ± 0.2
15	6.2 ± 0.2	6.5 ± 0.2	4.7 ± 0.1	4.0 ± 0.2	5.2 ± 0.2	4.7 ± 0.1	4.9 ± 0.1	3.8 ± 0.1

[a] S.E., *Salmonella* Enteritidis; L.m., *L. monocytogenes.*

riod, the counts of *Salmonella* remained at the same level for fish stored in air. The MAP conditions suppressed the *Salmonella* counts in the samples subjected to combined treatment in the first 3 days of storage, whereas MAP proved to be more effective on the sorbate-treated samples at the end of storage, when the difference in the pathogen counts among these samples and the other treatments was apparent. The immersion of red mullet in hot water had no effect on *Salmonella* during storage under air or MAP (Table 2).

The effect of pretreatment on the *Listeria* population in both red-mullet and carp are shown also in Tables 1 through 4 and were found to be similar to those reported for *Salmonella.* The initial reduction of population in samples treated with sorbate (with or without hot water) was about 1 log unit. For example, in the samples stored in air, the combination treatment and that of potassium sorbate suppressed the bacterial population more compared with heating, in which the counts increased slightly, and with the control, in which *Listeria* seems to be favored to grow. It is apparent (Tables 1 and 2) that MAP conditions for the same period of storage of carp compared with that in air (15 days) were effective in keeping the *Listeria* population at constant levels in all cases. Growth of the pathogen was observed in the control samples and, to a lesser extend, in the treated samples after 15 days of storage. At the end of storage, the sorbate-treated samples had the lowest counts compared with other treatments. Similar results have been reported by Slade and Davies *(32),* who observed that numbers of *L. monocytogenes* remained static during the early stage of a 20-day storage period of cod (*Gadus morhua*) when stored at 0°C but increased slightly (~0.5 to 1 log) toward the end, whereas trout numbers declined slightly over the 14-day storage period. Marginal growth was obtained with a pool of *L. monocytogenes* strains on channel catfish fillets stored at 4°C for 16 days *(24)* and on raw salmon stored at 5°C for 6 days *(4).* The work of Bajard et al. *(2)* has also indicated the ability of *L. monocytogenes* to grow at very low temperatures.

The beneficial effect of sorbate has been shown with other pathogenic bacteria, such as *Aeromonas caviae* and *Aeromonas sobria* on fish *(1).* It has also been reported that treatment of fish with potassium sorbate has a beneficial effect on microbiological quality by reducing the initial counts and extending the shelf life of refrigerated fish stored in air or under MAP *(11, 12, 15, 36).* Heat treatment is also reported to be effective in reducing the initial microbial flora of fish, and its combination with sorbates increases the effect *(22).* However in this study, heat treatment alone had minor inhibitory activity.

The effect of these prestorage treatments (sorbate, sorbate in combination with heating) was more beneficial under MAP than storage in air. Statham *(34)* has also reported that CO_2 enhanced the action of sorbates. This enhanced

TABLE 2. *Changes in* Salmonella *Enteritidis and* Listeria monocytogenes *inoculated in carp and stored under a modified atmosphere at 0 ± 1°C without treatment or treated with potassium sorbate, hot water at 60°C, or their combination*[a]

	Pathogen count (log CFU/g)							
	Control		Potassium sorbate		Hot water		Combination	
Day	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.
0	6.2 ± 0.1	4.8 ± 0.1	5.3 ± 0.2	4.0 ± 0.1	5.9 ± 0.1	4.0 ± 0.2	4.9 ± 0.2	3.9 ± 0.1
5	5.6 ± 0.2	4.7 ± 0.1	5.2 ± 0.2	4.5 ± 0.2	5.5 ± 0.1	4.5 ± 0.2	4.8 ± 0.2	4.4 ± 0.2
10	5.7 ± 0.1	4.3 ± 0.2	5.3 ± 0.2	4.6 ± 0.2	5.4 ± 0.2	4.8 ± 0.1	4.9 ± 0.2	4.2 ± 0.2
15	4.7 ± 0.1	5.2 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	4.9 ± 0.1	4.7 ± 0.1	4.7 ± 0.2	3.8 ± 0.2
20	4.3 ± 0.2	5.4 ± 0.1	4.4 ± 0.0	4.4 ± 0.3	4.8 ± 0.2	5.2 ± 0.1	4.4 ± 0.1	3.5 ± 0.2
25	4.3 ± 0.1	5.3 ± 0.2	4.3 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	5.6 ± 0.1	4.3 ± 0.1	3.5 ± 0.2
30	4.3 ± 0.1	5.1 ± 0.2	4.2 ± 0.1	4.6 ± 0.2	4.5 ± 0.1	5.8 ± 0.1	4.1 ± 0.1	3.5 ± 0.2

[a] S.E., *Salmonella* Enteritidis; L.m., *L. monocytogenes.*

TABLE 3. *Changes in* Salmonella *Enteritidis and* Listeria monocytogenes *inoculated in red mullet and stored aerobically at 0 ± 1°C without treatment or treated with potassium sorbate, hot water at 60°C, or their combination*[a]

	Pathogen count (log CFU/g)							
	Control		Potassium sorbate		Hot water		Combination	
Day	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.
0	5.4 ± 0.1	5.8 ± 0.1	4.4 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	5.4 ± 0.2	4.7 ± 0.1	4.9 ± 0.1
2	5.4 ± 0.1	5.6 ± 0.2	4.4 ± 0.2	4.9 ± 0.1	4.9 ± 0.2	5.3 ± 0.1	4.3 ± 0.3	4.8 ± 0.2
4	5.3 ± 0.2	5.6 ± 0.3	4.5 ± 0.1	4.8 ± 0.1	5.3 ± 0.2	5.4 ± 0.1	5.0 ± 0.3	4.9 ± 0.1
6	6.0 ± 0.1	5.7 ± 0.3	4.9 ± 0.2	4.9 ± 0.2	5.8 ± 0.3	5.2 ± 0.1	4.8 ± 0.2	4.8 ± 0.1
8	5.8 ± 0.2	6.1 ± 0.2	4.4 ± 0.2	4.8 ± 0.3	5.2 ± 0.1	5.3 ± 0.2	5.0 ± 0.2	4.9 ± 0.1
10	5.8 ± 0.2	5.9 ± 0.3	4.5 ± 0.1	4.9 ± 0.1	6.1 ± 0.3	5.3 ± 0.2	5.2 ± 0.1	4.9 ± 0.1
12	6.2 ± 0.1	6.5 ± 0.2	4.6 ± 0.2	4.9 ± 0.1	5.5 ± 0.2	5.3 ± 0.2	4.9 ± 0.2	4.7 ± 0.2
14	6.0 ± 0.1	6.3 ± 0.2	4.6 ± 0.1	5.1 ± 0.1	5.6 ± 0.1	5.0 ± 0.1	5.0 ± 0.2	4.6 ± 0.2

[a] S.E., *Salmonella* Enteritidis; L.m., *L. monocytogenes.*

effect might be a result of the additive or synergistic actions of CO_2 and low temperature on injured bacteria, according to the hurdle concept. The nature of action of carbon dioxide on bacteria is not clear, but it is speculated to be related to cell membranes, acidification of cytoplasm, and inhibition of enzymes *(9).* On the other hand, the action of sorbic acid against microorganisms is attributed to reactions with enzymes containing sulfydrylic groups *(33),* the elimination and altering of the electrochemical gradient and structure, respectively, of cell membranes *(19),* or both. Heat is also known to cause dissipation and destruction of cell membranes. The common site of action of these three factors is the cell membrane. Injury of the cell membrane by prestorage treatments presumably brings about increased sensitivity to subsequently applied CO_2. Damaged cells might be inactivated, and the recovery is further delayed, resulting in lower counts after prolonged storage in contrast to unharmed cells.

The CO_2-dependent bacteriostasis of *Salmonella* at chill temperatures is well known *(13, 28, 35).* The growth and survival of *Salmonella* Typhimurium was also examined by Slade and Davies *(32)* on cod and farmed rainbow trout (*Oncorhynchus mykiss*) stored at 0, 5, and 12°C under air, 60% CO_2–40% N_2, 80% CO_2–20% N_2, and 40% CO_2–30% N_2–30% O_2. They reported that there was no growth greater in any MAP treatment than in aerobic control, and frequently growth was reduced. Moreover, although the effectiveness of the modified atmosphere was greatly diminished at higher temperatures, even at 12°C, which would be a severe abuse temperature, pathogen growth was still inhibited slightly compared with that in the aerobically stored product, as was also observed in this study. The population of *Salmonella* declined or remained at the initial levels on both inoculated fish during storage under MAP. On the other hand, reports about the effect of atmospheric composition on the growth of *L. monocytogenes* are controversial. Some studies have reported that the pathogen is not greatly inhibited by vacuum or CO_2-enriched atmospheres and can predominate in refrigerated meat or meat products packaged in this way, whereas others have shown that packaging in these atmospheres inhibits listerial growth *(14, 20, 26).* In our experiments, it is apparent that MAP acted bacteriostatically on *Listeria* in comparison with the control samples, in which *Listeria* was able to grow. The initial effect of these treatments was evident in all cases; however, the use of MAP does not guarantee the control of this pathogen. In other words, the above-mentioned observations demonstrated that although both *Salmonella* Enteritidis and *L. monocytogenes* cannot grow in modified atmospheres at refrigeration temperatures (e.g., 1°C) it might constitute a risk

TABLE 4. *Changes in* Salmonella *Enteritidis and* Listeria monocytogenes *inoculated in red mullet and stored under a modified atmosphere at 0 ± 1°C without treatment or treated with potassium sorbate, hot water at 60°C, or their combination*[a]

	Pathogen count (log CFU/g)							
	Control		Potassium sorbate		Hot water		Combination	
Day	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.	S.E.	L.m.
0	5.4 ± 0.1	5.8 ± 0.1	4.4 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	5.4 ± 0.2	4.3 ± 0.1	4.9 ± 0.1
3	5.0 ± 0.2	5.6 ± 0.2	4.3 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	5.3 ± 0.1	4.3 ± 0.1	4.9 ± 0.2
6	5.1 ± 0.1	5.6 ± 0.2	3.9 ± 0.1	4.8 ± 0.1	5.1 ± 0.2	5.4 ± 0.1	4.3 ± 0.1	4.5 ± 0.1
9	5.1 ± 0.1	5.7 ± 0.1	3.8 ± 0.2	4.6 ± 0.2	5.5 ± 0.2	5.2 ± 0.1	4.8 ± 0.1	4.5 ± 0.2
12	5.1 ± 0.2	5.6 ± 0.2	3.8 ± 0.1	4.4 ± 0.3	5.5 ± 0.2	5.3 ± 0.2	4.8 ± 0.1	4.4 ± 0.1
15	5.1 ± 0.1	5.9 ± 0.2	3.5 ± 0.2	4.5 ± 0.1	5.0 ± 0.1	5.3 ± 0.2	4.5 ± 0.2	4.3 ± 0.1
18	5.1 ± 0.1	6.1 ± 0.1	3.6 ± 0.1	4.5 ± 0.1	5.0 ± 0.1	5.3 ± 0.2	4.0 ± 0.1	4.4 ± 0.1
21	5.1 ± 0.2	5.8 ± 0.1	3.4 ± 0.1	4.5 ± 0.1	5.0 ± 0.1	5.0 ± 0.1	3.9 ± 0.1	4.5 ± 0.1

[a] S.E., *Salmonella* Enteritidis; L.m., *L. monocytogenes.*

when temperature abuse is produced in the commercial chain. This situation can also occur with other nonpsychrotrophic organisms.

Previous studies *(11)* have shown that the concentration of potassium sorbate (initial concentration 5%, wt/vol) in fish throughout the storage period ranged from 200 to 300 mg/100 g fish. This concentration is about 10-fold lower than the acceptable daily intake (25 mg/kg of body weight) stipulated by the World Health Organization. The lethal dose of sorbates is about 10 g/kg of body weight, whereas common salt (NaCl) is about 5 g/kg of body weight.

Although information on MAP applied to fish and fish products, as well as adjunct treatments (e.g., sorbates, heat treatment, etc.), is rather limited and has confirmed the above-mentioned findings *(11),* this report extends our knowledge to new fish species and of the behavior of pathogenic bacteria on muscle foods (e.g., fish) treated with compounds that could be useful to the fish industry.

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