Fate of *Staphylococcus aureus, Salmonella enterica* Serovar Typhimurium, and *Vibrio vulnificus* in Raw Oysters Treated with Chitosan

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ABSTRACT

The fate of *Staphylococcus aureus, Salmonella enterica* serovar Typhimurium, and *Vibrio vulnificus* in oysters treated with chitosan was investigated. Three concentrations (0.5, 1.0, and 2.0%) of chitosan in 0.5% hydrochloric acid were prepared and coated onto raw oysters, which were then stored at 4°C for 12 days. Untreated oysters and oysters coated with 0.5% hydrochloric acid without chitosan were used as controls. *S. aureus* cells were most sensitive to 2.0% chitosan followed by 0.5 and 1.0%. In general, chitosan treatment of oysters produced a decline in the population of *S. aureus* by 1 to 4 log CFU/ml compared with the untreated control. Chitosan treatment had no influence on the reduction of *Salmonella* Typhimurium over the 12-day storage period; inhibition of *Salmonella* Typhimurium growth was similar in both the control samples and the chitosan-treated samples. However, time of storage had a major effect on the survival of *Salmonella* Typhimurium on oysters. Neither time nor chitosan concentration had a significant effect on the growth of *V. vulnificus* during storage. All treatments were similar in inhibiting *V. vulnificus* growth.

Chitosan (poly-β-(1→4)N-acetyl-d-glucosamine) is the N-deacetylated form of chitin, a high-molecular-weight polysaccharide found in abundance in shellfish processing wastes (2). Chitosan is usually manufactured from the shells of crustaceans (crabs, shrimps, and crayfish) either by chemical or microbiological treatments (27). It has various applications in biomedical, pharmaceutical, agricultural, food, and biotechnological fields (15). The utilization of chitosan is of commercial interest because of its high nitrogen content (6.89%) and its utility as a chelating agent to recover protein and polysaccharide from food processing waste water (17, 24).

Seafood has been implicated in 10 to 19% of the estimated 76 million annual U.S. cases of foodborne illness (5, 6, 31). The factor most commonly associated with infection is consumption of raw or undercooked seafood, including oysters, mussels, shrimp, fish, and clams. Millions of Americans enjoy eating raw oysters; however, people with certain medical conditions, particularly liver disease, are at high risk for becoming seriously ill or dying from eating raw oysters (5, 31). Although several oyster treatments such as pasteurization, high hydrostatic pressure, and irradiation and reliance on approved growing waters have reduced the risk of human infections, there is an emerging need for developing new, simple, and inexpensive methods to enhance the safety of raw oysters (14, 19, 21, 27).

Simpson et al. (26) claimed that chitosan exhibited strong antimicrobial properties against several microorganisms found on raw shrimp at concentrations ranging between 0.0075 and 0.01%. Results of their study indicated that *Bacillus cereus* required concentrations of ≥0.02% chitosan for a bactericidal effect, whereas *Escherichia coli* and *Pseudomonas vulgaris* were completely inhibited by concentrations of ≥0.0075% chitosan. No evidence of growth was found for *Staphylococcus aureus* at chitosan concentrations of ≥0.01%, whereas the pseudomonads required concentrations in excess of 0.01% to inhibit growth. In the same study, *Salmonella* Typhimurium and *P. vulgaris* required chitosan concentrations of about 0.01% to inhibit growth. Jeon et al. (16) found that chitosan coatings on herring and cod inhibited microbial growth by 2 to 3 log units during 12 days of refrigerated storage. Coma et al. (10) reported that chitosan inhibited the growth of *Listeria monocytogenes* in tryptose agar medium after 8 days of incubation at 37°C.

Several techniques and disinfectants have been used for preservation and for extension of the shelf life of raw oysters. However, in today’s health-conscious society, there is great concern about the potential harmful effects of some food preservatives. Chitosan, a natural preservative, has several advantages over other types of antimicrobial compounds; it has high antibacterial activity, a broad spectrum of activity, and a rapid killing rate, it is biodegradable, and toxicity for mammalian cells is low (13, 30). However, there is little information available on the use of chitosan as an antimicrobial agent on raw oysters. The purpose of this study was to examine the effect of chitosan on three pathogens, *S. aureus, Salmonella* Typhimurium, and *Vibrio vulnificus*, in raw oysters.
MATERIALS AND METHODS

Preparation of chitosan solutions. Commercial food-grade chitosan (T.C. Union Company, Smvtriprakran, Thailand) derived from shrimp shells (deacetylation degree [DD] of 80%) was dissolved in 0.5% (vol/vol) HCl (0.6 M) with medium heat and continuous mechanical stirring (stirring hotplate, Fisher Scientific, Pittsburgh, Pa.) for 2 to 4 h, depending on the solubility. Four concentrations of chitosan dissolved in 0.6 M HCl were prepared: 0.0, 0.5, 1.0, and 2.0% (2 g of chitosan in 100 ml of 0.6 M HCl). Untreated raw oysters were used as controls. The pH of all solutions was adjusted to 5.6 (32) with 1 N NaOH solution to increase the antimicrobial activity by allowing chitosan to be in the protonated form (10) and increasing the solubility of chitosan (26). Solutions were autoclaved at 121°C for 15 min and stored at 4°C until used (28).

Cultures for antimicrobial tests. S. aureus (ATCC 12600) was obtained from the Department of Food Science and Technology at the University of Georgia (Athens). Salmonella enterica Typhimurium (nalidixic acid–resistant strain) was obtained from the U.S. Department of Agriculture Agricultural Research Service (Athens, Ga.). V. vulnificus (ATCC 27562) was purchased from the American Type Culture Collection (Manassas, Va.). These cultures were stored on cryobeads (Micro Bank, Pro-Lab Diagnostics, Austin, Tex.) at −80°C. Culture beads were transferred to 5 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.), vortexed, and incubated at 35°C for 24 h to activate the bacterial cells. After activation, the culture was transferred into a fresh TSB tube and incubated at 35°C for another 24 h. The bacterial strains were subcultured twice to achieve a final inoculum concentration of 10⁶ CFU/ml.

Coating of oysters with chitosan. Oysters (Crassostrea gigas) were coated with chitosan solutions applied as described by Tsai et al. (30) with some modifications. Fresh shucked oysters packaged in a plastic container (Willapoint Oysters, South Bend, Wash.) were purchased at a retail market in Athens, Ga. The oysters had a 1-week “sell by” date. They were washed with cold deionized water for 30 s and then dipped in chitosan solutions of 0.0, 0.5, 1.0, and 2.0% for 1 h at 4°C. The ratio of oysters to solutions was set at 1:2 (wt/vol). The treated samples were then removed and dried for 20 min under air in a safety hood and then placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, Wis.). Oysters were individually inoculated with 0.5 ml of 24-h bacterial cultures of S. aureus, Salmonella Typhimurium, and V. vulnificus and stored at 4°C for 12 days. All experiments were performed in triplicate, with two observations for each treatment after 0, 4, 8, and 12 days of refrigerated storage. An additional control group of untreated oysters also was maintained. These negative control samples, which were neither coated nor inoculated, were maintained for detection and enumeration of naturally present S. aureus, Salmonella Typhimurium, and V. vulnificus.

Microbial analysis: homogenization of samples. Two samples from each treatment were removed at 4-day intervals for microbial examination. Samples were homogenized with 25 ml of buffered peptone water (Difco, Becton Dickinson) by blending for 30 s on high speed in a stomacher (Stomacher 400, Seward, Tekmar Company, Cincinnati, Ohio).

Microbial analysis: S. aureus. S. aureus concentrations were determined using the method described by the U.S. Food and Drug Administration (3) with modifications. Serial dilutions of homogenized samples up to 10⁵ were made in 9-ml tubes of buffered peptone water. Selected dilutions were spiral plated on Baird Parker agar (Difco, Becton Dickinson) plates and then incubated at 35°C for 24 h. Positive colonies, which appeared black, shiny, and convex with a yellowish brown halo, were counted. Ten percent of the positive colonies were confirmed with the latex agglutination test (Staphytest Plus, Oxoid Ltd., Basingstoke, Hampshire, UK). This test differentiates S. aureus from other staphylococci by detecting clumping factor, protein A, and certain polysaccharides found in methicillin-resistant S. aureus. Gram stains of cultures were also used for confirmation of identity.

Microbial analysis: Salmonella Typhimurium. Serial dilutions of homogenized samples up to 10⁶ were made in 9-ml tubes of buffered peptone water. Selected dilutions were spiral plated on tryptic soy agar (Difco, Becton Dickinson) containing 2 mg/liter nalidixic acid (Sigma Chemical Company, St. Louis, Mo.) and incubated at 35°C for 24 h.

Microbial analysis: V. vulnificus. V. vulnificus was isolated and enumerated according to the method described by Kayser and DePaola (18). A 1-ml aliquot of homogenate was inoculated into three 10-ml tubes of alkaline peptone salt (APW; Sigma). A three-tube most-probable number (MPN) serial dilution was employed with incubation at 35°C for 18 h. Without shaking the tubes after incubation, 3-mm loopfuls from the top 1 cm of the APW tubes with growth were streaked onto modified cellobiose-polymyxin B–colistin (mCPC) agar, which was prepared in the laboratory according to the method described in the Bacteriological Analytical Manual (1). Plates were incubated at 40°C for 24 h. Positive colonies on mCPC agar appeared round, flat, opaque, and yellow and were 1 to 2 mm in diameter. The MPN was calculated using tubes presumed positive for V. vulnificus (4). The identity of V. vulnificus colonies was confirmed with API 20E diagnostic strips (bioMérieux, Inc., Hazelwood, Mo.) according to the manufacturer’s directions. Cultures from 10% of the positive tubes were confirmed.

Microbial analysis: aerobic plate counts. The aerobic plate count of all oyster samples was determined by using plate count agar (Difco, Becton Dickinson) that was incubated at 35°C for 24 h.

Statistical analysis. Data were analyzed using an SAS Software analysis of variance (25), with treatment and day of sampling as the independent variables. All experiments were performed in triplicate, with two observations for each treatment (n = 6). Means were separated by the Duncan multiple range test. Differences were considered significant at P ≤ 0.05 (25).

RESULTS AND DISCUSSION

Various preliminary experiments were included as part of the present study. Initially, the chitosan was diluted into 0.5% acetic acid (83 mM). However, the acetic acid solvent alone caused approximately 6-log reductions in cell counts during the 15-day storage period, so the antimicrobial effect of chitosan could not be determined. Therefore, 0.6 M HCl was used as the solvent for chitosan. Preliminary studies also included preenrichment of the oyster homogenate at 35°C for 24 h. Results indicated that preenrichment lowered the pH of the oyster homogenate from 6.6 to 4.2, probably from growth of lactic acid bacteria. Therefore the preenrichment step was omitted.

Aerobic plate counts of chitosan-treated samples. The aerobic plate counts of the untreated oyster samples ranged from 6.3 to 8.9 log CFU/ml of homogenate throughout the 12 days of storage.
TABLE 1. Changes in the population of Staphylococcus aureus on raw oysters treated with various concentrations of chitosan and stored at 4°C for 12 days

<table>
<thead>
<tr>
<th>Coating</th>
<th>S. aureus concn (log CFU/ml)a</th>
<th>0 days</th>
<th>4 days</th>
<th>8 days</th>
<th>12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6.63 ± 0.85 A a</td>
<td>5.70 ± 2.10 Ab ab</td>
<td>4.61 ± 0.37 A b</td>
<td>4.77 ± 0.17 A b</td>
<td></td>
</tr>
<tr>
<td>0.0%</td>
<td>6.24 ± 0.85 A a</td>
<td>6.17 ± 1.52 A a</td>
<td>5.00 ± 0.94 A ab</td>
<td>4.76 ± 0.16 A b</td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>5.08 ± 0.39 B a</td>
<td>4.06 ± 1.27 B b</td>
<td>3.36 ± 0.48 B b</td>
<td>1.22 ± 0.45 C c</td>
<td></td>
</tr>
<tr>
<td>1.0%</td>
<td>4.62 ± 0.66 B a</td>
<td>3.49 ± 1.33 C ab</td>
<td>3.27 ± 1.16 B b</td>
<td>2.49 ± 0.31 B b</td>
<td></td>
</tr>
<tr>
<td>2.0%</td>
<td>4.54 ± 1.10 B a</td>
<td>3.59 ± 0.60 C ab</td>
<td>3.41 ± 0.59 B b</td>
<td>0.69 ± 0.77 D c</td>
<td></td>
</tr>
</tbody>
</table>

a Values are mean ± standard deviation (n = 6) population of S. aureus in oyster homogenate (oysters were homogenized in 25 ml of buffered peptone water for 30 s). Within a column, mean values not followed by the same capital letter are significantly different (P < 0.05). Within a row, mean values not followed by the same lowercase letter are significantly different (P < 0.05).

Effect of chitosan on S. aureus populations. Data on the effect of chitosan on S. aureus in raw oysters is presented in Table 1. The S. aureus population gradually decreased by >1 log CFU/ml (90%) in both control groups (0.0% chitosan and untreated) during the 12 days of storage. The addition of chitosan to the oysters caused significant inactivation of S. aureus; a 1- to 2-log reduction occurred immediately upon application (0 days) and was followed by a 1- to 2-log reduction after 4 days of storage. Inactivation continued thereafter. Treatment with 2.0% chitosan produced more than 4-log reduction in S. aureus by day 12 compared with the untreated control. Less inactivation occurred with lower chitosan concentrations, e.g., 0.5% chitosan produced a 3.9-log reduction. Regardless of the chitosan concentration, treatment of oysters produced a decline in the population of S. aureus by 1 to 4 log CFU/ml compared with the untreated control. The time between chitosan application and testing was approximately 1 h.

Chitosan is believed to inhibit S. aureus by the interaction of the protonated positive charge of chitosan and the negative charge on the microbial cell surface, increasing the permeability of the cell membrane and leading to the death of the cell (30, 32). Various researchers have demonstrated the antimicrobial activity of chitosan against S. aureus in suspension studies. Wang (32) examined the effect of chitosan concentrations between 0.5 and 2.5% dissolved in 2.0% acetic acid on the inhibition and inactivation of S. aureus in nutrient broth at 30°C for 8 days. Chitosan prevented the growth of S. aureus after 1 day of incubation, and there was a 3-log reduction after 8 days. Darmadji and Izumimoto (11) determined the antimicrobial effects of chitosan in yeast extract peptone glucose broth and found that S. aureus was inhibited by 0.01% chitosan after incubation at 30°C for 24 h. Papineau et al. (22) found that chitosan hydroglutamate caused a 4-log decrease in the population of S. aureus within 2 min of exposure and an approximately 6-log decrease within 60 min of exposure.

When chitosan is used in a food matrix or as a coating on various foods, the antimicrobial activity is influenced by the type of solvent used to dissolve chitosan, the DD of chitosan, the pH and composition of food matrix, the length and temperature of storage, and the species of microbial contaminant (9, 10, 12, 20, 30). Devlieghere et al. (12) tested the antimicrobial activity of chitosan (94% DD dissolved in 2.5% [wt/vol] acetic acid at concentrations of 40 to 750 mg/liter) in the presence of oil, starch, whey protein, and NaCl. Gram-negative bacteria were sensitive to chitosan treatment (0.006%, wt/vol), while activity was variable against gram-positive bacteria. For example, L. monocytogenes was less susceptible to chitosan than were lactic acid bacteria, for which the MIC was 0.05%. That study revealed that the antimicrobial activity of chitosan is dependent on pH, matrix composition, and ionicity. As pH decreased, the chitosan became more protonated and the antimicrobial activity increased. Food matrix composition also influenced the antimicrobial activity; starch reduced the activity, but the effect on protein depended on the pH or charge of the protein. High ionicity (NaCl concentration)

TABLE 2. Changes in the population of Salmonella Typhimurium on raw oysters treated with various concentrations of chitosan and stored at 4°C for 12 days

<table>
<thead>
<tr>
<th>Coating</th>
<th>Salmonella Typhimurium concn (log CFU/ml)a</th>
<th>0 days</th>
<th>4 days</th>
<th>8 days</th>
<th>12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.2 ± 0.8 A a</td>
<td>6.1 ± 1.2 A ab</td>
<td>5.2 ± 0.7 A b</td>
<td>2.8 ± 2.3 A c</td>
<td></td>
</tr>
<tr>
<td>0.0%</td>
<td>7.5 ± 0.2 A a</td>
<td>6.4 ± 0.6 A b</td>
<td>5.6 ± 0.3 A b</td>
<td>2.3 ± 1.4 A c</td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>6.5 ± 0.8 B a</td>
<td>6.4 ± 0.7 A a</td>
<td>5.4 ± 0.3 A a</td>
<td>3.8 ± 1.6 A b</td>
<td></td>
</tr>
<tr>
<td>1.0%</td>
<td>7.4 ± 0.3 A a</td>
<td>6.0 ± 0.9 A b</td>
<td>5.3 ± 0.2 A b</td>
<td>3.3 ± 1.8 A c</td>
<td></td>
</tr>
<tr>
<td>2.0%</td>
<td>7.1 ± 0.3 A b</td>
<td>6.0 ± 0.4 A ab</td>
<td>5.1 ± 0.3 A b</td>
<td>2.9 ± 2.4 A c</td>
<td></td>
</tr>
</tbody>
</table>

a Values are mean ± standard deviation (n = 6) population of Salmonella Typhimurium in oyster homogenate (oysters were homogenized in 25 ml of buffered peptone water for 30 s). Within a column, mean values not followed by the same capital letter are significantly different (P < 0.05). Within a row, mean values not followed by the same lowercase letter are significantly different (P < 0.05).
TABLE 3. Changes in the number of *Vibrio vulnificus* on raw oysters treated with various concentrations of chitosan and stored at 4°C for 12 days

<table>
<thead>
<tr>
<th>Coating</th>
<th>0 days</th>
<th>4 days</th>
<th>8 days</th>
<th>12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2.891 ± 4.412 A a</td>
<td>1.488 ± 3.479 AB a</td>
<td>13.7 ± 9.5 A a</td>
<td>21.3 ± 24.7 A a</td>
</tr>
<tr>
<td>0.0%</td>
<td>2.870 ± 4.429 A a</td>
<td>49 ± 94 B a</td>
<td>9.1 ± 14.8 A a</td>
<td>373 ± 562 A a</td>
</tr>
<tr>
<td>0.5%</td>
<td>4.299 ± 4.697 A a</td>
<td>4.295 ± 4.701 A a</td>
<td>9.7 ± 16.5 A a</td>
<td>11.4 ± 5.2 A a</td>
</tr>
<tr>
<td>1.0%</td>
<td>2.867 ± 4.430 A a</td>
<td>1.513 ± 3.470 AB a</td>
<td>7.9 ± 10.9 A a</td>
<td>239 ± 425 A a</td>
</tr>
<tr>
<td>2.0%</td>
<td>2.879 ± 4.421 A a</td>
<td>189 ± 446 B a</td>
<td>2.6 ± 5.1 A a</td>
<td>23.8 ± 35.3 A a</td>
</tr>
</tbody>
</table>

*Note: Values are mean ± standard deviation (n = 6) number of *V. vulnificus* in oyster homogenate (oysters were homogenized in 25 ml of buffered peptone water for 30 s). Within a column, mean values not followed by the same capital letter are significantly different (P < 0.05). Within a row, mean values not followed by the same lowercase letter are significantly different (P < 0.05).*

decreased microbial activity, contrary to the observations of Chung et al. (8). Coma et al. (9) found that chitosan (DD 98%) was more effective against gram-positive bacteria (*S. aureus* and *L. monocytogenes*) than against gram-negative bacteria (*Pseudomonas*) in an agar model; total population reduction was observed for gram-positive bacteria, but the *Pseudomonas* population was reduced by only 77%. Chitosan treatment was also effective in inhibiting *Pseudomonas* growth over a 6-day period when applied as a coating to cheese. Lopez-Caballeria et al. (20) found that chitosan (DD 97% in 0.5 M acetic acid) applied as a coating for fish patties had some inhibitory effect on gram-negative bacteria (*Pseudomonas*, *Enterobacter*, and aerobic bacteria) during a 12-day storage period, but there was resistance among lactic acid bacteria (20).

**Effect of chitosan on Salmonella Typhimurium populations.** The population of *Salmonella* Typhimurium was significantly reduced (2 to 5 log CFU/ml) in all samples (control and treated) during the 12 days of storage (Table 2). The population of the pathogen was reduced in the control samples at the same rate as in the treated samples. Statistical analysis indicated that time of storage was the main factor influencing the population reduction.

Although the effect of chitosan on the inhibition of *Salmonella* Typhimurium was not evident in this study, Tsai (29) found that a chitosan-oligosaccharide mixture inhibited the growth of *Salmonella* in raw milk. Wang (32) found a greater than 3-log reduction in the population of *Salmonella* Typhimurium in a nutrient broth system treated with chitosan at concentrations of 1.0, 1.5, 2.0, and 2.5% at pH 5.5 during a storage period of 8 days at 30°C.

**Effect of chitosan on *V. vulnificus* populations.** No growth of *V. vulnificus* was observed on oysters in either the control or treatment groups during the first 8 days of storage; during this time there was a 1- to 2-log reduction in the population (Table 3). However, between day 8 and day 12, some growth was evident. Even though the effect of chitosan on *V. vulnificus* was not clear in this study, Park et al. (23) found that chitosan acetate inhibited growth of *V. vulnificus* in Luria-Bertani broth after overnight incubation at 37°C, with an MIC of 0.04% (wt/vol). This discrepancy may be due to the survival of *Vibrio* in oysters but not in a laboratory medium. Our results also are not in agreement with those of Chen et al. (7), who found the MICs of 200 and 100 ppm for *Vibrio cholerae* and *Vibrio parahaemolyticus*, respectively, for 69% deacetylated chitosan on oysters stored at 5°C for 14 days; i.e., the chitosan used (DD 69%) effectively inhibited growth of *Vibrio* species. Similarly, Tsai (29) observed about a 1-log inhibition of *Vibrio* by chitosan with a DD of 98% that was dissolved in HCl.

The results of the present study confirmed the antimicrobial properties of chitosan and indicated that chitosan coating may be useful for controlling proliferation of pathogenic bacteria on raw oysters. The bactericidal activity of chitosan was most effective against *S. aureus* and was increasingly effective against *S. aureus* at the three concentrations studied (0.5, 1.0, and 2.0%). Storage temperature is key for reducing microbial risks associated with raw oysters by preventing the growth of *Salmonella* Typhimurium and *V. vulnificus*. Other factors such as the structural composition of the chitosan used (degree of deacetylation), source of chitosan, pH of the medium, water activity, storage temperature of the oysters, and degree of contamination can influence the effectiveness of chitosan as a microbial inhibitor (32). This study and others have demonstrated the complex process involved in determining the antimicrobial activity of chitosan because the effects of chitosan differ depending on the species of microbial contaminant.

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