Research Note

Effect of Lime Juice on *Vibrio parahaemolyticus* and *Salmonella enterica* Inactivation during the Preparation of the Raw Fish Dish Ceviche

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**ABSTRACT**

Ceviche is a raw fish dish common in Peru and other Latin American counties. The most characteristic feature of ceviche is the use of lime juice for marinating or “cooking” the raw fish. Confirmed cases of cholera in Peru, New Jersey, and Florida have been associated with ceviche. Although the effect of organic acids on pathogenic bacteria has been well characterized, few data exist on the effect of these acids in seafood systems. The objective of the study was to evaluate the effects of lime juice marination on pathogens likely to be present in ceviche. Tilapia (*Oreochromis niloticus*) fillet pieces were inoculated with *Vibrio parahaemolyticus* and *Salmonella enterica* (>7 log CFU/g) and incubated at 25 and 4 °C for 30 or 120 min in the presence of fresh lime juice at concentrations typical for the preparation of ceviche. Similar levels of cells were also inoculated into fresh lime juice without tilapia. Surviving cells were enumerated on selective (xylose lysine Tergitol 4 and thiosulfate-bile-citrate-sucrose) and nonselective (tryptic soy agar) media. *V. parahaemolyticus* levels were reduced to below detection limits (~5-log reduction) under all conditions studied. *Salmonella* strains on tilapia were much more resistant to inactivation and were only slightly reduced (~1- to 2-log reduction). *Salmonella* and *V. parahaemolyticus* inoculated directly into lime juice without tilapia were all reduced to below detection limits (~5-log reduction). A typical ceviche recipe reduces *V. parahaemolyticus* risk significantly but is less effective for control of *S. enterica*.

Ceviche is a raw marinated fish dish popular in South American countries and similar to products consumed on many Pacific Islands. Ceviche recipes vary across countries and cultures. Most recipes published in the United States use tilapia (*Oreochromis niloticus*) as the seafood of choice. Lime juice or a mixture of lime and other citrus juices are always used to marinate the raw fish. Other major recipe variables include the temperature (either room or refrigerator) and time of marination.

Ceviche poses the same inherent health threats associated with any raw seafood. The Academy of Nutrition and Dietetics (1) has issued an advisory against consumption of raw seafood, including ceviche, for pregnant women, elderly people, children, and immunocompromised individuals. A wide number of pathogens are associated with the consumption of seafood, including *Salmonella*, which is the most frequent cause of seafoodborne illness in the United States, and *Vibrio parahaemolyticus*, which was responsible for several highly publicized seafoodborne outbreaks in the late 1990s (11).

The spread of endemic diseases such as cholera due to consumption of uncooked fish remains a persistent threat in cultures where ceviche is consumed and sanitation is poor (17). Cholera epidemics occurred simultaneously in many South American countries in 1991 (19). Cholera cases were reported in New Jersey and Florida in 1991 (4) in individuals who had eaten crab meat brought from South America and in individuals who had traveled to South America and had consumed raw seafood, including ceviche, while there.

Because ceviche is not cooked, the microbiological safety of the dish relies solely on the effect of the lime juice. Citric acid is a major constituent of lime juice and is naturally present at about 0.056 g/g (1.4 g/oz) (13). Organic acids such as citric acid have historically been used in food preparation and formulation because of their flavors and antimicrobial effects. The antibacterial properties of organic acids are attributed to their undissociated (i.e., uncharged or protonated) forms, which can penetrate bacterial membranes (6). The precise effectiveness of organic acids against foodborne pathogens can be difficult to study because of the multiple factors involved (14). *V. parahaemolyticus* is susceptible to low pH but can develop acid tolerance (2, 9, 20), and *Salmonella* is more susceptible to propionic and acetic acids than to citric or lactic acids (5).

Although the effect of organic acids on pathogenic bacteria has been studied extensively, few data exist on the effect of these acids in seafood systems, especially under conditions that simulate those in ceviche production (11).
Given the popularity of this dish, the objective of the study was to study the effects of lime juice marination on two pathogens likely to be present in ceviche during preparation.

**MATERIALS AND METHODS**

**Bacterial strains and culture methods.** Four strains of *S. enterica* (Dr. Jianghong Meng, University of Maryland, Baltimore) and five clinical isolates of *V. parahaemolyticus* (Dr. Haiqiang Chen, University of Delaware, Newark) were used in all experiments (Table 1). Strain stock cultures were stored in 24% glycerol at −80°C until used. Tryptic soy broth (TSB; Difco, BD, Sparks, MD) was used to prepare the overnight cultures of the bacterial strains. Antibiotic-resistant strains of *Salmonella* were cultured in TSB containing 0.01 g/liter tetracycline. *V. parahaemolyticus* strains were cultured in TSB containing 3% (wt/vol) laboratory grade extrapure sodium chloride (Acros Organics, Fair Lawn, NJ). Peptone water (0.1%) was used for all dilutions. Peptone water used for *V. parahaemolyticus* also contained 3% (wt/vol) laboratory grade extrapure sodium chloride.

All *Salmonella* and *V. parahaemolyticus* strains were individually grown in TSB (with or without salt or antibiotic) in 15-ml falcon tubes (BD, Franklin Lakes, NJ) overnight at 37°C. Aliquots (2 ml) from each overnight culture were added to fresh tubes of TSB and incubated for 24 h at 37°C, and then 300 μl of each of these cultures was added to fresh TSB in 15-ml falcon tubes and incubated for an additional 24 h at 37°C. Duplicate tubes of this overnight cocktail were centrifuged for 5 min at 3,500 rpm at 4°C. The pellets obtained were resuspended in 9 ml of 0.1% peptone buffer (with 3% [wt/vol] sodium chloride for *V. parahaemolyticus* strains) and centrifuged again. The buffer was then discarded, and the pellets were resuspended in fresh 0.1% peptone water and combined to form the tilapia inoculum.

Xylose lysine Tergitol 4 (XLT4) agar (Difco, BD) was used as a selective medium for *Salmonella*. Thiosulfate-bile-citrate-sucrose agar (Difco, BD) was used as the selective plating medium for *V. parahaemolyticus*. Additional agar powder was added to enhance the solidification in plates. Tryptic soy agar (Difco, BD) was used as the nonselective medium for both species, supplemented with 3% (wt/vol) sodium chloride for *Vibrio*.

**Preparation of tilapia and lime juice.** Frozen tilapia (*Oreochromis niloticus*) fillets were purchased from local supermarkets, transported with ice packs, and stored at −10°C until used. Fillets were thawed for ~20 min at room temperature. Thawed fillets were cut into cubes ~2.5 cm on a side and each weighing ~4.5 g. Surface pH was measured with a bench-top pH meter (Accumet AB 15/15+, bench-top meter, Fisher Scientific, Fair Lawn, NJ). Limes were purchased from local supermarkets and refrigerated until used. Freshly squeezed lime juice was prepared on the day of each experiment.

**Experiments with both lime juice and fish.** A surface inoculation technique was used in this study. Fish cubes of ~2.5 cm on each side were inoculated with 400 μl of the appropriate tilapia inoculum, and the inoculum was spread evenly on the fish surface with a sterile plastic loop. Samples were held for less than 1 min before lime juice treatment. Starting bacterial levels were generally ~7 log CFU/g of fish.

A standardized ratio of 1 ml of lime juice to 1.5 g of fish was used for all experiments, based on a survey of on-line and cookbook ceviche recipes. Ceviche recipes commonly involve tossing the fish in ceramic containers after applying the lime juice. In our study, we used two plastic weigh boats (Fisher Scientific) sealed with masking tape to toss the individual fish pieces. Marination treatments of 30 and 120 min under both room temperature (25°C) and refrigeration (4°C) conditions were evaluated.

After lime juice treatment, the fish samples were placed in a sterile filter bag (Fisher Scientific) and diluted with 0.1% peptone water (with 3% [wt/vol] sodium chloride as required). Samples were then stomached (Stomacher 400 Lab-Blender, Tekmar Co., Cincinnati, OH) for 15 min. Samples were spread plated in duplicate on the respective selective and nonselective media and incubated at 37°C overnight, and typical colonies were enumerated.

**Experiments with lime juice alone.** The ability of lime juice to inactivate *V. parahaemolyticus* and *Salmonella* (in the absence of fish) also was investigated. Bacterial inocula were prepared as specified above and mixed with lime juice, taking care that the bacteria to lime juice ratio was kept the same as that with the experiments involving fish. The solutions were then incubated for the same time and temperature combinations, diluted, and plated in duplicate.

**RESULTS AND DISCUSSION**

**pH values of fish and lime juice.** Lime juice pH was 2.2 to 2.5 during the study. The surface pH of the fish was 6.8 to 7.2 before lime juice treatment but dropped to ~3.6 within 10 min of exposure to lime juice. The final surface pH of the fish was not affected by the temperature of incubation or by incubation times longer than 10 min. The lime juice treatment also changed the appearance of the fish pieces, which became opaque and whitish after several minutes. The appearance and observed texture change was not significantly affected by the time or temperature of exposure.
TABLE 2. Effects of temperature, time, and recovery medium on inactivation of Salmonella on tilapia by lime juice used in preparation of ceviche

<table>
<thead>
<tr>
<th>Recovery medium and treatment time</th>
<th>Mean ± SD Salmonella reduction (log CFU/g)</th>
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<tbody>
<tr>
<td></td>
<td>Room temp</td>
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<tr>
<td>Xylose lysine Tergitol 4 agar</td>
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<tr>
<td>30 min</td>
<td>1.53 ± 0.10</td>
</tr>
<tr>
<td>120 min</td>
<td>1.45 ± 0.21</td>
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<tr>
<td>Tryptic soy agar</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>1.37 ± 0.17</td>
</tr>
<tr>
<td>120 min</td>
<td>1.12 ± 0.02</td>
</tr>
</tbody>
</table>

Results for *V. parahaemolyticus*. All treatments with lime juice (with and without fish, at room and refrigeration temperatures, and for any time studied) resulted in *V. parahaemolyticus* inactivation to below the detection limit on both selective and nonselective agars. Based on the difference between the starting level and the detection limit, a minimum ~5-log reduction in the level of *V. parahaemolyticus* was generally observed, although this reduction differed among the experimental trials.

The susceptibility of *V. parahaemolyticus* to inactivation under acidic conditions agrees with findings reported by Vanderzant and Nickelson (18) in shrimp homogenate. In shrimp homogenates adjusted to pH values of 4 or less, no surviving bacteria were detected after 5 min, whereas in shrimp homogenate at pH 5, no survivors were detected after 15 min. These authors also reported that *V. parahaemolyticus* levels in shrimp homogenates at pH 6 and above were stable for at least 2 h. The results in the present study also are consistent with those for *Vibrio cholerae* O1. Mata et al. (10) reported a 3-log reduction of the organism in ceviche made from mahi-mahi fish 5 min after the addition of lime juice, and Rodrigues et al. (15) found that *V. cholerae* levels were reduced within 3 h to below the limit of detection in peanut sauces made with two or more limes.

Results for *Salmonella*. Table 2 shows the effects of temperature, time, and recovery medium on inactivation of *Salmonella* during the preparation of ceviche. Treatment time (30 or 120 min) appears to have little effect on the inactivation of *Salmonella*, with only slight differences (always less than 0.5 log CFU/g) and no clear trends. Refrigeration slightly enhanced inactivation, with ~0.5-log greater reductions when cells are recovered on XLT4 and similar results for recovery on TSA, but only for the longer (120 min) treatment time. Measured reductions were slightly greater on XLT4 than on TSA, as expected, because XLT4 is a selective medium and selective media recover cells (especially stressed cells) with a lower efficiency.

*Salmonella* can survive in environments with pH below 4 through activation of an acid tolerance response (8). *Salmonella* is also protected from organic acids when attached to poultry skin (16), and a similar mechanism may be working here with respect to the tilapia flesh. The survival of *Salmonella* in our experiments is consistent with the findings of Escartín and Vitela (7), who surveyed fish ceviche in Guadalajara, Mexico, for *Salmonella* and found that 16% of more than 200 samples were positive for *Salmonella*, including two of eight samples with pH below 4.

Experiments with lime juice alone. The reductions obtained by treatment of each pathogen with lime juice without fish were ≥5 log CFU/g for both *Salmonella* and *V. parahaemolyticus*. These results are consistent with the observations of Nogueira et al. (12), who found a 5-log reduction of *Salmonella* in lime juice concentrates within 15 min under frozen conditions. Similar results were reported by Bradshaw et al. (3), who studied the survival of *V. parahaemolyticus* in cooked seafood at refrigeration temperatures. These researchers found that when an acidic tomato-based cocktail sauce was added to surface-inoculated shrimp at a ratio of 2:1, a 2- to 3-log reduction in *V. parahaemolyticus* occurred over 48 h but in the cocktail sauce alone that reduction occurred in 6 h or less.

*Salmonella* risk was reduced but not eliminated and *V. parahaemolyticus* risk was significantly reduced when ceviche was prepared according the conditions used in this study. Lime juice alone (without the fish) was effective for inactivating both pathogens. Refrigeration during marination had a slight impact on *Salmonella* reduction, but time of marination did not influence the reductions of either pathogen.

REFERENCES


