Fate of Selected Food-borne Pathogens during the Fermentation of Squid Shiokara

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Shiokara, a traditional fermented seafood in Japan, has never been reported to cause bacterial food poisoning. In this study, shiokara products were inoculated with Staphylococcus aureus, Vibrio parahaemolyticus, and Clostridium botulinum type E and the fates of these pathogens were determined during the fermentation of squid shiokara. V. parahaemolyticus declined rapidly and was not detectable after 12 days of fermentation. When shiokara was inoculated with mixtures of vegetative cells and spores of C. botulinum type E, the number of vegetative cells declined rapidly within 2 days, though the viable spores remained. S. aureus survived but did not grow or produce enterotoxin during the fermentation. These results confirmed the safety of traditional shiokara with respect to bacterial food hygiene. However, strict control of contamination by S. aureus throughout the manufacturing process is necessary, because the organism remained viable during fermentation.

Key words: food-borne pathogen; squid; shiokara; Staphylococcus aureus; Vibrio parahaemolyticus; Clostridium botulinum

Introduction

Shiokara is a representative fermented seafood consumed in Japan. The materials of shiokara are non-sterilized raw squid mantle muscle, liver of squid, and NaCl (more than 10% weight of muscle and liver). It has been reported that the dominant microorganisms present during fermentation of shiokara are Staphylococcus and Micrococcus. With 10% NaCl, generally, Staphylococcus grows steadily. However, there has never been any report of food poisoning after consumption of shiokara. In general, S. aureus is a poor competitor with other spoilage bacteria. Nishimura and Shinano found that S. aureus decreased rapidly within a few days in Wood & Baird medium supplemented with 8–10% NaCl and 500–600 mg/mL of trimethylamine oxide (TMAO) to imitate the contents of shiokara; and similar results were found in inoculated shiokara. Another similar study was performed by Yamazaki et al. who determined the inhibitory effects of squid liver in shiokara on the survival of S. aureus, and concluded that 3% squid liver would reduce S. aureus rapidly. However, no study on the fates of Vibrio parahaemolyticus and Clostridium botulinum type E has been reported.

The objective of this study was to determine the fates of S. aureus, V. parahaemolyticus, and C. botulinum inoculated into squid shiokara.

Materials and Methods

Microorganisms

Staphylococcus aureus IAM 12544, ATCC 12600 was obtained from the Institute of Applied Microbiology, University of Tokyo. Vibrio parahaemolyticus T-77-4 was obtained from Tokyo Metropolitan Research Laboratory of Public Health, Japan. Clostridium botulinum type E (5545) was obtained from Dr. Igimi (the National Institute of Infectious Diseases, Japan).

Preparation of shiokara

Three shiokara products inoculated with S. aureus, V. parahaemolyticus, or C. botulinum, and
one control sample without inoculation were prepared simultaneously. The mixture of raw squid mantle muscle, squid liver (5% weight of mantle muscle), and NaCl (10% weight of mantle muscle plus liver contents) was stored in a container at 20°C for 28 days and stirred sufficiently once a day.

Recovery test in shiokara
Each bacterial strain was grown overnight (S. aureus in TSB (BBL, USA), V. parahaemolyticus in TSB containing 3% NaCl, and C. botulinum in GAM broth (Nissui, Tokyo, Japan) grown anaerobically) at 30°C. Then 5 mL liquots of cultures were inoculated into shiokara (500 g), which was incubated at 20°C for 14 days. Fifteen mL of TSB containing 10% NaCl (for S. aureus and V. parahaemolyticus) was inoculated with 0.15 mL of culture and incubated at 20°C for 14 days while GAM broth containing 10% NaCl (for C. botulinum) was incubated anaerobically in an Anaerobic Jar (BBL Microbiology Systems, Cockeysville, MD, USA), as positive controls.

Enumeration of pathogens and background bacteria
Samples of 5 g were taken from the shiokara products inoculated with S. aureus and V. parahaemolyticus, and blended in 45 mL sterilized diluent (0.85% w/v sodium chloride water) by using a Stomacher Lab-blender 80 (Seward UAC House, London, UK) for 2 min, while for TSB alone, a 1 mL sample was taken and mixed in 9 mL sterilized diluent by using a mixer (Automatic Labo-Mixer NS-8, Pasolina) for 30 sec. Serial dilutions were made in the same diluent and then 0.1 mL sample dilutions were plated on Mannitol Salt Egg-Yolk Agar (MSEY agar, Eiken, Tokyo, Japan) and TCBS Agar (Eiken, Tokyo, Japan) respectively. After incubation of the plates at 37°C for 48 h, S. aureus and V. parahaemolyticus were enumerated by counting all typical colonies on the media. Shiokara product and GAM broth inoculated with C. botulinum were sampled, blended and diluted by the same method mentioned above except that anaerobic diluent (KH2PO4, 4.5 g; Na2HPO4, 6 g; L-cysteine, 0.7 g; Tween 80, 0.7 g; agar, 1 g; 1,000 mL distilled water, pH 7.6. sterilized by autoclaving at 115°C for 20 min) was used. To differentiate the viable vegetative cell and spore counts, sample dilutions heated at 60°C for 1 h were used for spore counting. Ten mL of sample dilutions and 15 mL of Clostridia Count Agar (Nissui, Tokyo, Japan) (heated at 50°C) were mixed in a P. T. Pouch (Sakami Medical Instrument, Tokyo, Japan) and sealed to prevent contamination with air bubbles. After incubation at 30°C for 24 h, black colonies formed by sulfate-reducing clostridia were counted. Total bacterial counts of shiokara products inoculated with V. parahaemolyticus and C. botulinum were enumerated by a surface plating method using TSA (BBL, USA) containing 10% NaCl.

Antimicrobial activity test
A total of 345 culture fluids of isolates from shiokara product (control sample) and the extract from the same shiokara product were investigated for antimicrobial activity against S. aureus by a paper disk diffusion method. Aliquots of 1 mL of the culture of isolates, grown in TSB at 20°C for 14 days, and 1 mL of the extract of shiokara, stored at 20°C for 14 days and blended with an equal quantity of 10% NaCl solution by using a Stomacher Lab-blender 80 (Seward UAC House, London, UK) for 2 min, were each centrifuged by using a high-speed refrigerated centrifuge SRX-201 (Tomy, Tokyo, Japan) at 15,000 rpm for 20 min twice. The supernatant from the culture fluids and extract mentioned above were added to paper disks (size 8 mm, Advantec, Tokyo, Japan), and the growth inhibition zone around the paper disks was observed after incubation at 37°C for 48 h.

Determination of organic acid
The organic acid contents in TSB containing 10% NaCl and shiokara product, which had been inoculated with S. aureus, were extracted with 6% perchloric acid at 0 and 14 days of incubation. After neutralization and quantification, the organic acid contents in extracts were determined with an HPLC Organic Acid Analysis System (Shimadzu, Kyoto, Japan).

Determination of Staphylococcal enterotoxins
The staphylococcal enterotoxin type A-D products in TSB containing 10% NaCl and shiokara product inoculated with S. aureus were deter-
mined by SET-RPLA (Denkaseiken, Tokyo, Japan) according to the manufacturer’s protocols at 0 and 14 days of incubation. TSB that remained intact and the sample dilution of shiokara product mentioned above, which was blended with an equal quantity of 10% NaCl water by using a Stomacher Lab-blender 80 (Seward UAC House, London, UK) for 2 min, were each centrifuged by using a high-speed refrigerated centrifuge SRX-201 (Tomy, Tokyo, Japan) at 3,000 rpm for 20 min. The supernatant from the TSB and sample dilution of shiokara product were added to V type microplates and latex sensitized with anti-enterotoxins type A–D, and the reversed passive latex agglutination was observed after incubation at room temperature for 24 h.

Recovery test in shiokara

Figs. 1–3 illustrate the population changes of Staphylococcus aureus, Vibrio parahaemolyticus, and Clostridium botulinum inoculated into squid shiokara stored at 20°C. Only a negligible decrease in the number of S. aureus during shiokara fermentation was observed. At the end of
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Table 1. Changes in Organic Acids, Staphylococcal Enterotoxins (SEs), S. aureus Counts, and pH in TSB (containing 10% NaCl) and Shiokara

<table>
<thead>
<tr>
<th>Source</th>
<th>TSB</th>
<th>Shiokara</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Lactic acid (mg/100 mL)</td>
<td>11.5</td>
<td>70.5</td>
</tr>
<tr>
<td>Acetic acid (mg/100 mL)</td>
<td>11.5</td>
<td>17.4</td>
</tr>
<tr>
<td>SEs (ng/mL)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S. aureus counts (log CFU/mL)</td>
<td>5.2</td>
<td>8.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Discussion

The results in this study indicated that the rapid reduction of Clostridium botulinum in shiokara was due to the high NaCl concentration, but the fates of the other two pathogens (Vibrio parahaemolyticus and Staphylococcus aureus) were not simply attributable to the NaCl concentration. During the fermentation of shiokara product, the population of S. aureus remained constant. The inhibitory effect might be due to the presence of other inhibitory substances produced in shiokara, such as trimethylamine oxide (TMAO) and squid liver. It is beyond the scope of our study to identify the most inhibitory factor. Perhaps the synergistic effect of these factors as well as Aw, pH, NaCl, NaNO2, and organic acids would be the most likely explanation of the inhibition.

An important finding in this study was that a rapid decline in S. aureus population, as reported by Nishimura and Shinano and Yamazaki et al., was not observed. The reason for this difference is not clear. In the previous studies, no selective medium was used for enumerating S. aureus, making the interpretation of the results difficult. The difference between our results and others may also be attributed to differences in the type and initial quality of squid muscle and liver, TMAO concentrations (not determined in this study), and strains of S. aureus used. In this study, it was confirmed that there was no inhibitory effect of culture fluid of isolates from shiokara product or the extract of shiokara product against S. aureus (data not shown). The results suggested that S. aureus can survive during fermentation of shiokara.

Antimicrobial activity test

No inhibitory effect against S. aureus was detected either in the extract of shiokara or in the 345 culture fluids of isolates from shiokara product (data not shown).

Determination of organic acid

After incubation at 20°C for 14 days, considerable amounts of both lactic acid and acetic acid accumulated in shiokara product. In TSB containing 10% NaCl, lactic acid accumulated, but acetic acid accumulation was less evident (Table 1).

Determination of staphylococcal enterotoxins

After incubation at 20°C for 14 days, no staphylococcal enterotoxin type A–D was detected in shiokara product or TSB inoculated with S. aureus (Table 1).
when present, and continues to be a potential hazard.

In this study, no staphylococcal enterotoxin (type A–D) was detected in TSB containing 10% NaCl and shiokara product inoculated with S. aureus. These results were in accordance with the previous studies\(^\text{11}^\text{–13\text{\textperiodcentered}}}\). McLean \textit{et al.}\(^\text{11\text{\textperiodcentered}}}\) have reported that staphylococcal enterotoxin is usually produced in the initial part of the stationary phase. Morse \textit{et al.}\(^\text{12\text{\textperiodcentered}}}\) have concluded that the maximum production of staphylococcal enterotoxin occurs during the last period of the log phase. Genigeorgis & Sadler reported that when \textit{S. aureus} was kept below pH 6.5 and at over 10% NaCl concentration, production of staphylococcal enterotoxin would be inhibited\(^\text{13\text{\textperiodcentered}}}\).

In conclusion, the results of our study suggest that the potential for growth of \textit{S. aureus}, \textit{V. parahaemolyticus}, and \textit{C. botulinum} in traditional squid shiokara at 20°C is very low. However, the survival of \textit{S. aureus}, when introduced as a contaminant during the production process, represents a potential hazard if storage is improper. Control of \textit{S. aureus} at low levels during production will be necessary.

\textbf{References}