**Staphylococcus aureus Growth and Thermostable Nuclease and Enterotoxin Production in Canned Salmon and Sardines**


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**ABSTRACT**

*Staphylococcus aureus* growth, thermostable nuclease (TNase) and enterotoxin production in inoculated canned salmon incubated at 22 ± 1°C for 4 d were dependent on the size of inoculum, and on the amount of oxygen present in the headspace; under nitrogen with an inoculum of 7 cfu/can, 10^2-10^3 cfu/g, no TNase and traces of enterotoxins (A, B, C_2) were observed; under oxygen with the same inoculum >10^9 cfu/g, >6.0 μg TNase and up to 5.2 μg total enterotoxins (A, B, and C_2)/100 g of salmon were observed. Values were intermediate under atmospheric air. After 1 week, 2 months and 4-24 months of incubation of salmon under nitrogen, mitomycin C was observed; after 1 week, counts declined to 10^5 cfu/g.

Enterotoxins/100 g; after 1 week, counts declined to 10^5 cfu/g.

Under nitrogen with an inoculum of 7 cfu/can, 10^2-10^3 cfu/g, no TNase and traces of enterotoxins (A, B, C_2) were observed; under oxygen with the same inoculum >10^9 cfu/g, >6.0 μg TNase and up to 5.2 μg total enterotoxins (A, B, and C_2)/100 g of salmon were observed. Values were intermediate under atmospheric air. After 1 week, 2 months and 4-24 months of incubation of salmon under nitrogen, mitomycin C was observed; after 1 week, counts declined to 10^5 cfu/g.

Enterotoxins/100 g; after 1 week, counts declined to 10^5 cfu/g.

**Enterotoxin in canned food causing illness have rarely been reported and only a few experiments have been conducted to determine toxic levels in artificially inoculated canned food. In none of these enterotoxin quantitatively assayed. Segalove et al. (23) added several levels of *S. aureus* into various types of canned foods; after incubation of the incubated cans for 2 to 60 d at 22 and 37°C the authors failed to detect enterotoxin by human volunteers, monkey or kitten tests, although *S. aureus* counts reached 10^7/ml in the fluid of canned salmon. Davidson and Dack (7), by employing human volunteers, were able to demonstrate the presence of enterotoxins in *S. aureus*-inoculated canned corn and oysters after 3 d of incubation but not in salmon. Bailozov (1), by using intravenous injection of cats, detected enterotoxin in extracts of artificially inoculated canned meat and fish after 1 to 120 d of incubation at 18-25°C. Mansfield et al. in 1983 (12) examined the growth, thermostable nuclease (TNase) and enterotoxin production of a *S. aureus* strain added to 6-lb. cans of corned beef; after 1 to 3 weeks of incubation, *S. aureus* counts reached 10^9/g of meat. Further incubation for 1 year reduced the counts to 10^7/g. Enterotoxin and TNase were present when counts exceeded 5 x 10^9/g.

Davidson and Dack (7) demonstrated that enterotoxin is not produced as readily under anaerobic conditions as it is aerobically. Baird-Parker (2) found that the amount of enterotoxin B in static cultures incubated in air was about 10 times higher than in static cultures incubated under a 95:5 mixture of N_2 + CO_2. Thatcher et al. (29), however, detected enterotoxin in extracts of *S. aureus*-inoculated vacuum-packed bacon incubated at 37°C under nitrogen.

Even though Surgalla and Dack (26) showed that *S. aureus* spread from the point of inoculation to distant parts in canned meat in <2 to 60 d, cans of corn, oysters, salmon and corned beef inoculated with *S. aureus* were normal in odor, appearance and taste even after several months (7,12). These authors (26) also noticed that excessive *S. aureus* growth resulted in yellowish discoloration and “ropy” consistency of canned meat products.
but no change in odor. In staphylococcal enterotoxin food poisonings involving contaminated canned kippered herring in oil and canned mackerel, the product smelled and tasted normal (13,15).

The purpose of our investigation was to determine S. aureus growth, enterotoxin and TNase production under anaerobic conditions resembling PPL in canned salmon and sardines, and follow olfactory and visual changes of the products for up to 24 months of storage at room temperature.

MATERIALS AND METHODS

Inoculation
 Adequate numbers of cans of salmon and sardines from identical code lots for each experiment were inoculated with single or pooled enterotoxigenic Staphylococcus strains anaerobically (under nitrogen) in duplicate according to a previously published method (24). For the study of the effect of air and oxygen on growth of S. aureus and enterotoxin and TNase production, the vacuum in the headspace of canned salmon was replaced with nitrogen, air or oxygen before inoculation.

Gas analysis
 After incubation periods, headspace gas was analyzed for the presence of carbon dioxide by inserting into the can through the silicon sealant (Drägerwerk, A. G., Lubeck, Germany) and pressing both ends of the can to create a pressure and thereby passing the gas through the gas detection tube. When this method failed to show gas, the can was inflated with a carbon dioxide and oxygen-free nitrogen gas and then the pressure was released through the inserted needle connected to the gas detection tube by the rubber tubing. For the demonstration of absence of carbon dioxide gas in non-contaminated can controls, the same method was employed.

Media
 All bacteriological media were obtained from Difco Laboratories, Detroit, Michigan.

Determination of S. aureus counts
 At zero time and after the incubation periods, cans were opened aseptically and their contents were poured into sterile plastic bags to be homogenized by the Colworth Stomacher 400 (A. J. Seward and Co., Ltd., Blackfriars Road, London, England) for 5 min. Appropriate dilutions were made in 0.1% peptone water, and S. aureus colony forming units per 1 g (cfu/g) of product were determined on Mannitol Salt Agar (MSA), and Baird-Parker (BP) agar. Inocula are expressed as cfu/can.

Sources of S. aureus strains used for inoculation
(a) MF 31 (phage type 42E/47/53/83A/84/85) producing enterotoxin A, - Z. J. Ordal, University of Illinois, IL.
(b) S6 (phage type 29/80/47/75/77/83A) producing enterotoxin B, - M. S. Bergdoll, University of Wisconsin, WI.
(c) ST 361 (non typable) producing enterotoxin C2 - M. S. Bergdoll, University of Wisconsin, WI.
(d) AB (phage type 54 (42E +/-/47 +/-/83A +/-/81 +/-/82 +)) producing enterotoxins A, B and C2, - J. Y. D’Aoust and R. Szabo, Health and Welfare Canada, Ottawa, Ontario.

Growth and preparation of the S. aureus cultures
 S. aureus strains were inoculated into tryptic soy broth (TSB) from 0.1 ml of an overnight stationary culture, and these were shaken at 35±0.5°C for 18±1 h, the cells were centrifuged and washed five times, resuspended and diluted in 0.1% sodium thioglycolate in 0.1% peptone water. S. aureus strains, inoculum sizes, products, incubation periods, at 22±1°C and gas atmospheres for Trials I-VIII are shown in Table 1.

Storage of cans
 Duplicate (unless stated otherwise) samples for each time period of examination were stored at 22±1°C.

Olfactory evaluation
 At each time period of storage at 22±1°C, the contents of two inoculated cans and an uninoculated control were presented to a five- to seven-member untrained panel of judges who recorded the olfactory acceptability on a five-point hedonic scale, with the value of one for extremely disliked samples and a value of five for extremely liked samples.

Trained judges were blind-folded to separate two contaminated cans from the single control. No taste panels were conducted.

Determination of TNase
 TNase was quantitated by the microslide method as described by Lachica et al. (10) and Cords and Tatini (6), and expressed as µg/100 g of product.

Extraction of enterotoxin from foods
 Enterotoxins were extracted from canned salmon and sardines by the method of Roberts et al. (22).

Radioimmunoassays for staphylococcal enterotoxins A, B and C
 Two methods were used to determine enterotoxins: (a) Double-antibody radioimmunoassay (20,21,22) was used to assay enterotoxins in canned salmon and sardines stored up to 4 months (Trials IV to VIII). (b) Solid phase radioimmunoassay (8) was used in Trials I, II, and III, and in Trials IV to VIII to assay enterotoxins in canned salmon and sardines from 5 up to 24 months of storage.

The individual toxins (A, B and C2) or their sums as total enterotoxins are presented as µg/100 g of product.

RESULTS

Enterotoxin production of experimental strains in brain heart infusion broth (BHIB)
 At 35°C 48 h BHIB shake cultures of the S. aureus strains used in the experiments produced toxins as shown in Table 2.

Effects of headspace atmosphere
 Trials I, II, and III show the effects of three levels of inoculum in salmon under nitrogen, air and oxygen atmosphere on growth, TNase and enterotoxin production of S. aureus AB strain (Table 3). During the 4 d of incubation, increasing inoculum size from 7 cfu to 81 cfu/can

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### TABLE 1. Storage trials of S. aureus-inoculated cans.

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Inoculum: strains ratio, cfu/can</th>
<th>Canned product</th>
<th>Incubation period and atmospheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>AB, 7</td>
<td>220-g can pink salmon</td>
<td>4d 4 replicates for each of three gases: nitrogen, air, oxygen</td>
</tr>
<tr>
<td>II</td>
<td>AB, 64</td>
<td>220-g can pink salmon</td>
<td>0,1,2,4 weeks, and 2, 4, 5, 6, 8, 10, 12, 17 months under nitrogen</td>
</tr>
<tr>
<td>III</td>
<td>AB, 81</td>
<td>220-g can pink salmon</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
<tr>
<td>IV</td>
<td>MF31, S6, ST361, 1:1:1, 66</td>
<td>220-g can pink salmon</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
<tr>
<td>V</td>
<td>MF31, S6, ST361, 1:1:1, 1600</td>
<td>220-g can pink salmon</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
<tr>
<td>VI</td>
<td>AB, 148</td>
<td>220-g can pink salmon</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
<tr>
<td>VII</td>
<td>MF31, S6, ST361, 1:1:1, 45</td>
<td>Sardines in soya oil</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
<tr>
<td>VIII</td>
<td>AB, 130</td>
<td>Sardines in soya oil</td>
<td>0,1,2,4 weeks, and 2, 3, 4, 5, 6, 8, 9, 10, 12, 17, 24 months under nitrogen</td>
</tr>
</tbody>
</table>

*35°C incubation in BHIB for 48 h.


<table>
<thead>
<tr>
<th>Strain</th>
<th>A (μg/ml)</th>
<th>B (μg/ml)</th>
<th>C (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6</td>
<td>0.44</td>
<td>2.0</td>
<td>0.076</td>
</tr>
<tr>
<td>ST361</td>
<td>0.088</td>
<td>0.0</td>
<td>4.2</td>
</tr>
<tr>
<td>AB</td>
<td>0.704</td>
<td>0.073</td>
<td>0.0</td>
</tr>
<tr>
<td>MF31</td>
<td>9.72</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Four replicate cans were used for each inoculum level and atmosphere.*

and increased amounts of oxygen resulted in better growth of S. aureus and higher TNase and enterotoxin levels.

**Growth, TNase and enterotoxin production in salmon**

Trials IV, V and VI measured S. aureus growth and production of TNase and enterotoxin in canned salmon stored at 22±1°C from 1 week up to 24 months (Fig. 1); S. aureus growth reached ≥10^7-10^8/g or greater by the first week and began to decrease after 2 months to

### TABLE 3. The effect of size of inoculum, nitrogen, air and oxygen on S. aureus growth, enterotoxin and TNase production in canned salmon after four days of incubation at 22±1°C.

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Size of inoculum cfu/can</th>
<th>Gas in headspace</th>
<th>Range of cfu/g</th>
<th>Range of TNase (μg/100g)</th>
<th>Range of total enterotoxins (μg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7</td>
<td>Nitrogen</td>
<td>10^2-10^3</td>
<td>0.0</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Air</td>
<td>10^2-10^5</td>
<td>trace</td>
<td>0.0-0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>10^2-10^8</td>
<td>0.1-2.4</td>
<td>0.1-0.35</td>
</tr>
<tr>
<td>II</td>
<td>64</td>
<td>Nitrogen</td>
<td>10^5-10^6</td>
<td>0.0-0.4</td>
<td>trace-0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Air</td>
<td>10^5-10^8</td>
<td>0.0-6.4</td>
<td>trace-1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>≥10^8</td>
<td>2.5-6.4</td>
<td>1.0-2.1</td>
</tr>
<tr>
<td>III</td>
<td>81</td>
<td>Nitrogen</td>
<td>10^5-10^8</td>
<td>0.0</td>
<td>0.3-1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Air</td>
<td>10^5-10^8</td>
<td>trace</td>
<td>2.0-2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>≥10^9</td>
<td>≥16.0</td>
<td>3.6-5.2</td>
</tr>
</tbody>
</table>

*Four replicate cans were used for each inoculum level and atmosphere.*
the $10^5$-$10^6$/g range with further decreases to $10^3$/g after 4-8 months. Levels of TNase, in spite of the high counts ($10^6$ to $10^7$/g), were relatively low after 1 week of incubation in Trial VI, 0.06 and 1.0 µg/100 g in the duplicate cans; whereas in Trial IV at the same period salmon containing *S. aureus* in the $10^8$/g range contained 3.0, and 16.0 µg of TNase/100 g. Throughout the entire storage study, TNase showed a great deal of variability from can to can, and from one storage period to another. This variability may be due to inherent shortcomings in the method of TNase determination or may be due to product variation. Enterotoxins were present after 1 week in Trials IV (1.2 µg/100 g) and VI (2.1 µg/100 g). These concentrations are considered sufficient to cause food poisoning. After 2-4 weeks, most cans contained over 1 µg of total enterotoxins/100 g and some had levels reaching as high as 3.8 µg/100 g (Trial IV), and 6.2 µg/100 g (Trial V) by the 12th month of storage. In Trials IV and V, enterotoxins A, B and C₂ were present at various concentrations, A and C being the most prominent. This could be expected on the basis of results obtained in shake cultures of the pure strains (Table 2). In Trial VI, where cans were inoculated with the single AB strain, enterotoxin A was predominant from the first week to 24 months, enterotoxins B and C appeared sporadically, and enterotoxin C₂ mainly after 5 months. The AB strain in the overnight BHIB culture produced mostly A and some B, but no C toxin (Table 2). The longer incubation

**Figure 1.** Effect of storage on *S. aureus* growth, TNase and enterotoxin production in canned salmon inoculated under nitrogen with MF 31, S6, and 361 strains in 1:1:1 ratio at 66 cfu/can in Trial IV, and 1600 cfu/can in Trial V; and with AB strain at 148 cfu/can in Trial VI and incubated at 22 ± 1°C for 1 to 4 weeks and from 2 to 17 months. For all periods of incubation duplicate cans were analyzed, and both results are presented.
period and/or different substrate or cultural conditions may account for these differences.

Growth, TNase and enterotoxin production in sardines

Trials VII and VIII measured *S. aureus* growth and production of TNase and enterotoxin in canned sardines stored from 1 d to 12 months (Fig. 2). In Trial VII, analysis after 1 d showed an increase in cfu to $10^3$ to $10^4$/g in duplicate cans and the presence of 0.55 μg of enterotoxin B/100 g in one can only but no TNase; after 2 d counts were over $10^8$/g. TNase was 6.2-16 μg/100 g and the total value for enterotoxins A, B and C2 was about 2.6 μg/100 g. Counts reached their peak after 1 week at $10^9$/g and the same cans had 3.7 to 3.9 μg of enterotoxins/100 g. In this trial, enterotoxins A, B and C2 were present in most cans in various proportions up to the 12th month of storage. Proportions and presence of these toxins are consistent with the results obtained in BHIB cultures (Table 2). TNase showed a great degree of variability with 16 μg/100 present after 2 d but <0.1 μg after 1 week when the *S. aureus* counts were at their maximum ($10^9$/g), and rising again to 16 μg/100 g after 8 months when the cfu declined to <$10^7$/g.

In Trial VIII (Fig. 2), *S. aureus* counts were at $10^2$ to $10^3$/g in sardines after 1 d of incubation, but neither TNase nor enterotoxin was detectable. After 4 weeks, counts were in the $10^7$-$10^8$/g range. TNase was detected in only one can (1.3 μg/100 g), but enterotoxins were present in both (3.75 and 2.95 μg/100 g, respectively). *S. aureus* counts began to decline gradually after 2 weeks to $10^3$/g and the decline continued to the 12th month but never below $10^5$/g. At the same time TNase remained around the 2 μg/100 g concentration, occasionally reaching 6.4 and 16 μg/100 g (at 1 week and 6 months, respectively). Enterotoxin A was predominant after 4 d; the concentration of enterotoxin A in most samples was over 2 μg/100 g after 1 week, and by the 12th month of storage was 4.8-5.2 μg/100 g. Enterotoxin B was detected in smaller concentrations from (0.40 to 0.55 μg/100 g) and enterotoxin C2 was detected only after 8 months of storage in low concentrations (0.19 to 0.22 μg/100 g). The proportion and development of the presence of the toxins is consistent with results obtained in Trial VI.

Olfactory and visual evaluation of *S. aureus* contaminated canned salmon and sardines

Three trained judges were able to detect in all instances an acidic smell resulting from *S. aureus* growth in canned salmon and sardines after 2 d and up to 24 months of incubation at 22 ± 1°C. In contrast, an untrained panel consisting of 6 people were divided in their ability to detect olfactory changes in these products (2-3 persons would usually consider a spoiled product acceptable). For a product incubated for 2 weeks, 4-5 persons would accept the spoiled product; after 2 and up to 24 months of incubation, 2-3 persons would still consider the *S. aureus* and enterotoxin contaminated product acceptable as judged by smell.

The inability of some people to detect *S. aureus*-related spoilage of canned salmon and sardines is probably enhanced by the fishes' characteristic odor which tended to mask slight changes, such as a poignant acidic smell detected by the trained judges. For each can measured we found small amounts of carbon dioxide by using gas detection tubes after 2 d of incubation and up to 17 months; the amount of gas produced, however, was never sufficient to cause the cans to swell. Figures 3 and 4 show the averaged organoleptic response represented on a five-point hedonic scale, of six people to *S. aureus*-contaminated canned salmon (of Trials IV, V and VI) and sardines (of Trials VII and VIII). As shown by Fig. 3 and 4, at 0 time, people were not able to distinguish between inoculated cans and uninoculated ones because the product appeared the same. This is borne out by the close values (sometimes crossing over) for control and inoculated cans for salmon (Fig. 3) and sardines (Fig. 4). Figure 3 shows that the average response of 6 people on duplicate *S. aureus* contaminated cans of salmon was usually well below the value for the noncontaminated control; that is, most people could tell most of the time up to 17 months of storage in all three Trials IV, V, VI, and up to 24 months in Trial V, that something was not quite right with the contaminated cans. Similar conclusions can be drawn from the olfactory evaluation results (Fig. 4) of *S. aureus* contaminated sardines in Trials VII and VIII.

DISCUSSION AND CONCLUSIONS

Low inoculum *S. aureus* contamination (resembling PPL) of canned salmons and sardines stored at room temperature 22 ± 1°C for less than a week is expected to result in $10^2$-$10^3$ cfu/g and over 1.0 μg of total enterotoxin per 100 g of product although there was considerable variation in amounts of enterotoxins A, B and C produced. An aerobic headspace atmosphere, when compared to an anaerobic one, will also encourage toxin formation. Lower inoculum and absence of oxygen in cans of salmon tended to retard growth of *S. aureus* and production of TNase and enterotoxin during 4 d of incubation at 22 ± 1°C. Under a nitrogen atmosphere, *S. aureus* counts may be expected to decline to $10^3$/g or less but the toxins will remain stable and may increase to over 6.0 μg/100 g after 12 months in both canned salmon and sardines. These levels of toxins are considered in excess to evoke the symptoms of enterotoxin food poisoning. Although various degrees of fair to good correlation between the presence of *Staphylococcus* enterotoxins and TNase have been reported in a great variety of food products (3,6,10,11,12,14,17,18,19,27,28,33) our findings showed a great variability of TNase concentration from can to can or from period to period, and no apparent correlation with concentrations of enterotoxins or cfu/g in the same samples.

The average response to the organoleptic evaluation showed that some undesirable odor change in *S. aureus*-contaminated salmon and sardines occurred after 2 d and
Figure 2. Effect of storage on S. aureus growth, TNase and enterotoxin production in canned sardines inoculated under nitrogen with MF 31, S6 and 361 strains in 1:1:1 ratio at 45 cfu/can in Trial VII, and with AB strain at 130 cfu/can in Trial VIII and incubated at 22±1°C for 1 to 4 d, for 1 to 4 weeks and for 2 to 12 months; for all periods of incubation duplicate cans were analyzed.
up to 12 and 24 months of storage, and this change was detectable by some people when they compared the spoiled product with a non-contaminated control. However, this difference may be difficult to determine by a consumer opening one can and choosing between eating it or not, especially if there has been no prior indication of a problem. Certainly for canned mackerel (13) and canned corned beef (12) organoleptic change could not be detected, and in many staphylococcal food-poisoning outbreaks involving canned fish products, appearance (5,9,13,15,16) and taste (5,15) were found to be normal, only occasionally tasting salty (5), dubious (16), putrid (13), bitter (31), or “funny” (32). Even if organoleptic warning signs are present, however, these do not necessarily prevent consumption of the food (30). Therefore, every precaution should be taken by the industry, distributor and consumer to prevent PPL: the industry by providing reliable seams, uncontaminated cooling water and safe handling practices; the distributor by providing proper handling (for instance, elimination of case-cutter damage, denting or rusting of cans); and the consumer by avoiding any abuses of the cans in the home.

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REFERENCES


