**Staphylococcus aureus Growth and Toxin Production in Nitrogen-Packed Sandwiches**

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**ABSTRACT**

Plastic-enclosed sausage, hamburger and turkey sandwiches were inoculated with enterotoxigenic *Staphylococcus aureus* to evaluate the potential hazard of staphylococcal food poisoning in sealed foods maintained in an N$_2$ environment. The effect of such food storage on staphylococcal growth and enterotoxin production was determined under varying conditions of time (1-31 days) and temperature (8, 12, and 26 C). At 8 and 12 C, none of the sandwiches became toxic after 31 days of storage; however, at 26 C, sausage and hamburger sandwiches became toxic at days 2 and 4, respectively, while remaining organoleptically acceptable. Turkey sandwiches did not support sufficient growth of staphylococci to allow the production of detectable amounts of enterotoxin at any of the temperatures tested.

Advances in packaging technology, including the use of oxygen-modified environments to preserve the aesthetic quality of foods and retard the growth of spoilage organisms, have been extended to sandwiches and other prepared convenience foods. Of the various trends in food packaging (17), vacuum packaging has been used with increasing frequency and has generally received favorable consumer response.

In the 1960s the effect of vacuum packaging on growth and toxin formation by *Clostridium botulinum* and *Staphylococcus aureus* was studied in fish (19) and selected cured meats (8). Studies on staphylococcal growth and production of enterotoxin type B in vacuum-packed bacon (7) after incubation at 25 C for 14 days had negative results, while those on vacuum-packed hams (18) showed staphylococcal growth to be generally inhibited by the presence of large numbers of competing bacteria. Studies on the preservation of fresh apples and potatoes (12) by gas exchange indicated that CO, N$_2$ or vacuum packaging had little effect on the destruction of *S. aureus*. Although studies of the incidence of *S. aureus* on vacuum-packed bologna (15) provided little evidence of staphylococcal contamination, later studies (16) indicated that bologna sandwiches in plastic bags supported the growth of staphylococci after incubation at 21 C for 25 h. Interest in extending the shelf-life of foods by gas exchange has recently increased, hence, studies are being done to determine what effect various gas atmospheres (9,10) have on organisms that cause spoilage as well as those that cause foodborne illness (13).

Some foods currently marketed in impermeable plastic wrappers are stored in anaerobic or gas-modified environments. Generally, sandwiches as well as other food products hermetically sealed in plastic and preserved in N$_2$ will remain in satisfactory condition for a month or more if refrigerated. In some cases, however, retailers and vendors may, for economic reasons, sell foods that were temperature-abused. In other cases, food products stored in vending machines or other refrigeration systems may be subjected to less than ideal conditions, e.g., improperly functioning or inoperative refrigeration systems. In 1977, staphylococcal intoxications accounted for 15.9% of all outbreaks of foodborne illness in the United States (7). Of the 25 outbreaks caused by staphylococci, 18 were traced to improper holding temperatures.

The purpose of this study was to determine whether staphylococci could grow and produce toxins in N$_2$-packed sandwiches while the sandwiches remained organoleptically acceptable.

**MATERIALS AND METHODS**

**Cultures**

The strains of *S. aureus* used in this study represented five toxin serotypes that have been established as serological entities (4). Our laboratory designations and the sources of the organisms used in the 5-strain inoculum composite were as follows: strain 743 from scrambled eggs; strain 778 from chantilly cake; strain 834 from a leg abscess; strain 315 from turkey; and strain 790 from chicken tetrazzini. With the exception of strain 834, all of the strains of *S. aureus* were isolated from foods iniminated in food poisoning outbreaks. Each of the five strains produced one of the staphylococcal enterotoxin types: A (SEA), B (SEB), C (SEC), D (SED), and E (SEE). The single strain inoculum designated 485, which was used in this study, was obtained from wiener meat and produced enterotoxins A, B, and D.

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Sandwiches

The selection of the three types of sandwiches used in this study was based on their popularity among consumers as well as on the pH that might permit the growth of staphylococci. The selected sandwiches were turkey on wheat bread, sausage on biscuit, and hamburger on a roll. Sandwiches frozen in hermetically sealed wrappers were obtained from the manufacturer and maintained in the frozen state until the day of study. They were thawed before inoculation with staphylococci.

Preparation of inocula

 Cultures of *S. aureus* were grown in BHI broth, pH 6.8, using a nursing bottle rotation method (5). Cells from each of the six strains of staphylococci were collected by centrifugation and washed 3x with normal physiological saline. Cell suspensions were prepared and turbidities were matched according to the No. 1 McFarland standard (4), as described in the *Bacteriological Analytical Manual* (11), using a spectrophotometer (Spectronic 20, Bausch and Lomb) at a wavelength of 480 nm.

The S-strain inoculum was prepared by adding 0.2 ml of each adjusted culture suspension to 99.0 ml of Butterfield buffer, giving an equivalent of 3 x 10^6 organisms/ml. Appropriate dilutions were further prepared in Butterfield buffer to inoculate the sandwiches.

Inoculation and incubation of sandwiches

The average weights of the sandwiches were determined by weighing at least 10 sandwiches of each type. *Staphylococcus aureus* was then added to the thawed sandwiches at a rate of 30 organisms/g. Inoculum concentrations were confirmed by duplicate plating onto Baird-Parker agar (11).

After inoculation, the sandwiches were placed in separate plastic bags ("Baggies," Colgate-Palmolive Co., New York, NY). The bags were closed, but not sealed, marked for identification, and placed in Case-Anaero jars. The jars were closed, evacuated, and flushed 5x with N₂ before being filled with N₂. Inoculated sandwiches were incubated in triplicate or duplicate at temperatures simulating conditions possible at the retail level: good (8 C) and poor (12 C) refrigeration from 7 to 31 days; and conditions of abuse (26 C) from 1/2 to 7 days for sausage and hamburger sandwiches. Turkey sandwiches were incubated from 7 to 31 days. As controls, similar sandwiches were inoculated and held under aerobic conditions at 26 C. Inoculated sandwiches were incubated in temperature-controlled environments at 8 C and 12 C (Freas 815, Low Temperature Incubator, GCA/Precision Scientific) and 26 C (Reach-In Incubator, Forma Scientific). All temperatures were monitored with a temperature recorder (Tempscribe, Bacharach Instrument Co.) and the temperature recorder charts were changed at weekly intervals.

Enumeration of organisms

Growth of *S. aureus* in each type of sandwich held under the various time-temperature conditions was enumerated on Baird-Parker agar using the surface streak method as described in the *Bacteriological Analytical Manual* (11). Sausage and turkey sandwiches stored at 8 C and 12 C were removed from the N₂ environment and growth was enumerated at 7, 14, 21 and 31 days. Hamburger sandwiches were sampled at 7, 14 and 21 days. To determine the earliest time for toxin detection at 26 C, hamburger and sausage sandwiches were sampled at 1/2, 1-2, 4, 6-7 or 7, 14, 21 and 31 days.

Organoleptic examination

All sandwiches were observed and judged by the authors for their consumer acceptability based on color, odor, texture, and the presence or absence of mold before staphylococcal growth was enumerated.

Toxin extraction and serological assay

Since a consumer usually eats all of an organoleptically acceptable sandwich, the whole sandwich was extracted and examined for *S. aureus* and enterotoxin. Enough 0.2 M NaCl was added to each sandwich to give a 1:5 dilution; the mixture was blended in a Waring Blender at high speed for 3 min and the resultant slurries were enumerated for *S. aureus* then stored frozen until the number of *S. aureus* was determined. Samples with high staphylococcal growth levels were examined further for the presence of enterotoxin by the modified Casman and Bennett method as described by Bennett and McClure (3) and by microslide gel diffusion (2).

RESULTS AND DISCUSSION

The frozen sandwiches used in this study had been prepared for retail distribution through convenience food outlets and vending machines. Although the labels advocate refrigeration before sale, failure to maintain proper refrigeration of these sandwiches during storage is common; thus there is potential for the growth of pathogens. In this study, the N₂-packed sandwiches inoculated with enterotoxigenic staphylococci were stored under conditions which simulated good refrigeration (8 C), poor refrigeration (12 C), and temperature abuse (26 C) at the retail level.

*Staphylococcus aureus* growth profiles are shown in Fig. 1 for N₂-packed sausage and turkey sandwiches stored at 8 C for 31 days and for hamburger sandwiches stored at 8 C for 21 days. Under good refrigeration conditions, sandwiches showed low numbers of staphylococci; thus enterotoxin formation would be unlikely. In turkey sandwiches, particularly, the staphylococcal count decreased from 1.5 x 10^4 organisms/g after 14 days to 1 x 10^2 organisms/g after 31 days. Similar growth profiles were obtained with turkey sandwiches stored at 12 C. In turkey sandwiches stored at 26 C, the staphylococcal count ranged from 6 x 10^3 organisms/g at 7 days to 1 x 10^4 organisms/g at 31 days. Possible factors which may have contributed to the inhibition of *S. aureus* growth in turkey sandwiches were not investigated.

Figure 2 compares *S. aureus* growth and toxin production in sausage sandwiches incubated at 26 C in both N₂ and aerobic atmospheres. Growth of *S. aureus* organisms in sausage sandwiches stored in N₂ for 12-24 h (3 x 10^3 organisms/g) was insufficient for toxin detection. However, SEA and SED were detected in 2 days at which time the staphylococcal population was 2.3 x 10^6 organisms/g. When sausage sandwiches were held at 26 C in an aerobic environment, toxin formation occurred at 30 h with an associated count of 2.9 x 10^4 organisms/g.

SEB was detected in sausage sandwiches held in N₂ or in aerobic environments after longer incubation periods and with larger numbers of *S. aureus* than those necessary for production of SEA or SED. In N₂-stored sandwiches, SEB was detected after 4 days of incubation and counts of 2 x 10^5 organisms/g. In sandwiches held in aerobic environments, detectable amounts of this toxin were evidenced when counts averaged 6 x 10^7 organisms/g. Under laboratory conditions, *S. aureus* usually produces more SEB than SEA or SED (5); however, in foods a number of factors influence the production time and amount of these enterotoxins.

Staphylococcal growth and toxin production in hamburger sandwiches held at 26 C in both N₂ and aerobic environments are shown in Fig. 3. In hamburger sandwiches stored aerobically, SEA and SED were detected after 30 h, when the staphylococcal count reached 5.1 x 10^6 organisms/g. SEB was produced after

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7 days, with the number of *S. aureus* at 6.7 x 10^7 organisms/g. In contrast, when hamburger sandwiches were held at 26 C in an N_{2} environment, the staphylococcal counts reached 7 x 10^5 and 5 x 10^6 organisms/g at 2 and 4 days, respectively, with no detectable toxin. However, SEA and SED were detected after 7 days with lower staphylococcal counts (2.5 x 10^4 organisms/g) than were observed after 4 days of storage. This particular pattern of growth suggests that the level of staphylococci (5 x 10^6 organisms/g) at 4 days may be responsible for the presence of detectable toxins after 7 days. It also suggests that toxin was produced but not released during the first 4 days when hamburger sandwiches were stored in N_{2} at 26 C. This pattern of growth and production of enterotoxin contrasts with that observed in sandwiches stored aerobically. Evidence of intracellular toxin formation with subsequent release into the environment has been observed in other laboratories (Tatini, personal communication).

Figure 4 shows the results of growth and toxin production in inoculated sausage sandwiches held at 26 C in an N_{2} environment. These sandwiches were inoculated either with a single enterotoxigenic strain (*S. aureus* 485) or with a 5-strain composite. The *S. aureus* growth ranged from 1.2 x 10^5 to 2 x 10^5 organisms/g in 1 day; however, no enterotoxins were detected. After 2 days of storage, SEA and SED were produced in the sandwiches by the single strain of *S. aureus* at a level of 2.3 x 10^6 organisms/g; SEA, SED, and SEE were detected in sandwiches with the 5-strain composite at a count of 9 x 10^5 organisms/g. SEB was produced in 4 days at staphylococcal levels ranging from 2 x 10^7/g for the single strain to 3.2 x 10^7/g for the 5-strain composite. SEC was detectable only after 7 days of storage at a count of 4 x 10^7 organisms/g.

Figure 5 presents a comparison of staphylococcal growth and toxin production by the single strain, *S. aureus* 485, and the 5-strain composite inoculated into hamburger sandwiches and stored at 26 C in an N_{2} environment. SEA was detectable in 4 days in hamburger sandwiches inoculated with the 5-strain composite at a staphylococcal count of 7 x 10^6 organisms/g. SED and SEE were detected in hamburger sandwiches at 7 days.
with a count of $2.7 \times 10^6$ organisms/g, although the population of staphylococci was at its maximum after 4 days of incubation regardless of inoculum type. Both growth profiles were essentially the same except that trace amounts of SEA were detected in sandwiches inoculated with the 5-strain composite at 4 days of storage. SEB and SEC were not detected in hamburger sandwiches after 7 days of storage in an N$_2$ environment.

Sausage and hamburger sandwiches stored in N$_2$ and held at 26°C were still acceptable when enterotoxin(s) reached detectable levels. Toxins detected in sausage sandwiches on day 2 were serotypes A, D, and E ($9 \times 10^5$ organisms/g). Type A enterotoxin was detected in hamburger sandwiches ($7 \times 10^6$ organisms/g) on day 4.

Results from surveys have indicated a higher incidence of enterotoxin A- and D-producing staphylococci than B- and C-producing staphylococci in foods. However, our studies showed that the A and D serotypes are generally produced earlier or in the presence of fewer staphylococci than are serotypes B and C. Our studies also suggested that the greater frequency of enterotoxin types A and D in food poisoning outbreaks (6,20) may be due to their earlier production in some foods.

Although an N$_2$ environment may effectively retard the growth of spoilage organisms, it does not inhibit the growth of S. aureus. Staphylococci may proliferate under these conditions and, if enterotoxigenic, may produce toxins during storage. A potential hazard to health could exist, therefore, if refrigeration is not properly maintained.

REFERENCES

Figure 5. Comparative growth and toxin production in hamburger sandwiches stored at 26 C in a nitrogen environment after inoculation with a single strain (---SS---) and a 5-strain composite (-----SSC-----) of enterotoxigenic Staphylococcus aureus. CFU = colony-forming units. Solid and broken lines indicate the growth profile; symbols represent earliest detection of enterotoxin serotypes.


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