FACTORS INFLUENCING GROWTH AND TOXIN PRODUCTION BY S. AUREUS*

CONSTANTIN A. GENIGEORGIS
Department of Epidemiology and Preventive Medicine
School of Veterinary Medicine
University of California, Davis, California 85616

Definition

Staphylococcal food poisoning is a food-borne intoxication due to the consumption of foods containing preformed exotoxins (enterotoxins) which are produced by certain strains of Staphylococcus aureus. Under certain conditions, however, the symptoms of this common type of food poisoning may occur as a result of growth of staphylococci in the intestinal tract (3).

National Statistics

Together with Salmonella and Clostridium perfringens food poisonings, staphylococcal food poisoning is among the most common food-borne illnesses. Actually, considering the number of food poisoning outbreaks, staphylococcal food poisoning occupied the number one position in the last decade.

Types of Foods Involved

Certain types of foods are more suitable than others for staphylococcal growth and production of enterotoxins. During the period 1961-1968, 301 staphylococcal food poisoning outbreaks were recorded by the National Communicable Disease Center (5). Of these 40.6% were due to meats (21.4% to hams, 3.7% to pork, 11.1% to beef, 4.4% to meat mixtures and processed meats), 21.8% to poultry (10.7% to turkey, 11.1% to chicken), 13.7% to cream-filled products, 5.9% to fish and shellfish, 5.5% to potato and macaroni salad, 3.7% to milk and milk products, 2.6% to eggs and egg products, and finally 6.3% to other foods.

Of the total number of outbreaks due to meats, 74 were due to cured meats (58 to ham, 8 to corned beef, 1 to jerky, 5 to luncheon meat and 2 to combinations of ham and corned beef sandwiches).

Conditions Necessary for Outbreaks

The epidemiology of staphylococcal food poisoning has been recently reviewed (5,33). Bryan (5) indicates that staphylococcal food poisoning occurs only when all of the following five conditions are met:

---

1. A source of an enterotoxigenic strain of Staphylococcus aureus must be in the food preparation environment. The source can also be the raw meat or milk (i.e., meat abscesses and mastitis).

2. The organism must be transferred from the source to the food either directly or indirectly.

3. The contaminated food must be capable of supporting the growth of S. aureus.

4. The contaminated food must remain in the temperature range suitable for the growth of S. aureus for enough time to allow proliferation of the organism and production of enterotoxins.

5. The quantity of toxin produced in the food and the volume of food consumed should be large enough to produce symptoms of food poisoning.

In this review I will limit myself to conditions No. 3, 4 and 5. I will emphasize the effect of various food and environmental parameters on the growth of staphylococci and enterotoxin production in meats. Four recent reviews by Bergdoll (4), Baird-Parker (2), Minor and Marth (32-35), and Riemann et al. (40) are valuable sources of additional information on these conditions and on other aspects of this food poisoning.

Factors Influencing Growth and Enterotoxin Production by S. aureus

1. Level of Contamination. The size of the initial inoculum (contamination) is the factor which most affects the initiation of growth of food poisoning bacteria, including staphylococci, in foods (13,15,16,26,39).

For instance, an initial staphylococcal inoculum of $10^3$ cells/g of meat will have less chance to initiate growth and produce enterotoxins than $10^5$ cells/g inoculum. We have accumulated extensive experimental data with regard to the initiation of staphylococcal growth in both culture media and meats. Our studies have indicated that the initial contamination level of the food is a very important parameter determining the range of other environmental parameters which will permit growth and enterotoxin production by staphylococci (12-18,49) (figures 1, 2, 3 and 4).

2. Effect of pH. The response of S. aureus to acidity varies with strain and is influenced by the size of the inoculum, the type of medium, the salt concentration, the temperature, the type of acid, and the atmosphere (12-18,32-35,45).

In recent experiments we found that the decreased pH of commercially prepared cured meats and of certain broths required that a greater number of staphylococcal cells must be present for growth to be initiated. This is demonstrable under both aerobic or anaerobic conditions (14-16).
Fig. 1. Effect of initial inoculum (log cells/ml) and temperature on the ranges of pH and NaCl concentrations of PHP-NAK (3% + 3%) broth inoculated with $1 \times 10^3$ to $1 \times 10^8$ cells/ml of S. aureus strain 137 and incubated aerobically at the indicated temperatures for up to 10 days.
Fig. 2. Effect of pH, temperature, and initial inoculum (log cells/ml) on the yield of enterotoxin C production in PHP-NAK (3% + 3%) broth inoculated with 1 x 10³ to 1 x 10⁸ cells/ml of S. aureus strain 137 and incubated aerobically for 48 hours at the indicated temperatures.
Fig. 3. Combination of pH values and brine concentrations which supported (closed symbols) or did not support (open symbols) production of enterotoxin A (squares), B (triangles) and C (circles) aerobically in laboratory media and foods at inoculum levels up to $1 \times 10^7$ cells/ml or g (solid line) or $1 \times 10^8$ cells/ml or g (broken line). Reproduced from Riemann et al. (40).
Fig. 4. Combinations of pH values and brine concentrations which supported (closed circles) or did not support (open circles) aerobic staphylococcal growth in laboratory media and foods at inoculum levels up to $1 \times 10^4$ cells/ml or g. Reproduced from Riemann et al. (40).
Enterotoxin B can be produced in cured meats with initial pH 5.0 to 9.0 (3,12,14,29). Broth inoculated with $10^6$ cells/ml and incubated at 37°C has supported aerobic enterotoxin C production at initial pH values from 4.0 to 9.8 (17). This initial pH range becomes narrower when the inoculum and the temperature is decreased and the NaCl concentration is increased as shown in figure 1. Aerobic enterotoxin C production has been demonstrated in cured meats with an initial pH above 4.7 (16). However, we have been unable to detect anaerobic enterotoxin C production in cured meats with pH 4.9 to 7.1 (18) or enterotoxin B production below pH 5.3 (13). The rate of enterotoxin B production and the amount of enterotoxin finally produced is greater in media in which the pH is not controlled (2). Production of minute amounts of enterotoxin C was demonstrated in culture media inoculated with two S. aureus strains and incubated anaerobically at 37°C for 5 days.

In contrast to its effect on production of enterotoxin B and C, pH from 5.0 to 8.0 did not appreciably affect production of enterotoxin A (23,38). Production of enterotoxin A in reconstituted nonfat milk solids with an initial pH of 4.5 has been reported (46).

3. Effect of Sodium Chloride. The effect of sodium chloride on staphylococcal growth and survival has been studied extensively. Staphylococci survived in brine with 23% NaCl. Aerobically staphylococci multiplied in media with 16-18% brine (12,15,26,42); anaerobically staphylococci multiplied in media with 14-16% salt (13,42, unpublished data).

Our most recent studies on the effect of NaCl and pH on the probability of initiation of staphylococcal growth in culture media and meat environments indicated the following effects and interactions under both aerobic and anaerobic conditions: (1) The probability of initiation of growth is significantly affected by strain, pH and NaCl concentration, (2) The effect of NaCl varies with pH levels and strains, and (3) The probability of initiation of growth is linearly related to NaCl concentration when many strains are studied. As the NaCl concentration increases the probability of initiation of growth decreases (13,15,16).

Apparently, 10% NaCl in brine is the highest concentration which permits production of enterotoxins A, B and C in laboratory media and semi-preserved meat products. Growth to levels of more than $10^7$ cells/g or ml of medium occurs above this NaCl concentration (12,14,17,26). As the concentration of the NaCl increases from 0 to 10% the yields of enterotoxins B and C decrease to undetectable amounts. On the other hand, Markus and Silverman (28) reported NaCl up to 10% did not essentially alter the amount of enterotoxin A produced. The inhibitory effect of NaCl on enterotoxin production becomes more obvious at lower temperatures of incubation and extreme pH values. Sodium chloride and pH interactions and their effects on staphylococcal growth and enterotoxin production based on literature reviews are presented in figures 3 and 4.
4. **Effect of Water Activity.** The minimal water necessary for microbial growth is best expressed as water activity rather than as relative water content or solute concentration (42).

Scott (42) was the first to study extensively the effect of water activity on staphylococcal growth. He determined the lowest aw at which growth would occur. In several nutrient media he controlled water activity by adding salts or sugar or mixtures of both. Similar experiments were performed in three dried foods reconstituted with water to give the required range of aw. In every case growth occurred aerobically at levels of aw down to 0.86 or 0.88. In contrast, the lowest water contents at which growth was observed in the various substrates ranged from 16% to 37% of the dry weight. Growth at these aw levels was not affected appreciably by the nature of the major solutes present. Under anaerobic conditions staphylococci grow at a minimum aw of 0.90 (42).

Recent experiments by Labuza et al. (24) on the microbiology of intermediate moisture foods indicate that staphylococci can grow in a "baby food pork" with an aw depressed down to 0.75-0.84 by glycerol. The same food in a freeze-dried preparation was rehydrated. The rehydrated product supported staphylococcal growth at a minimum of aw 0.90. The conclusion was that aw alone was not controlling growth, but that one must think in terms of total water content and aw.

Most recently Troller (50) has reported the effect of aw on enterotoxin B production. The aw of two broths was adjusted to desired values by either additions of glycerol or of dehydrated protein hydrolysate. The minimum aw supporting aerobic enterotoxin B production at 30 C was 0.97. Lowering the aw of the medium from 0.99 to 0.97 caused a marked reduction in toxin synthesis despite attainment of high numbers of S. aureus.

With the addition of a hydrometer indicator in our laboratory (model 15-3000, Hydrodynamics, Inc.) our research in aw effects expanded. I decided to determine the aw of various broths I have used in the past to study the effect of NaCl and pH on enterotoxin B and C production. The minimum aw which supported enterotoxin B production aerobically at 37 C in brain heart infusion broth (BHI) (12) and enterotoxin C in PHP-NAK (3% + 3%) (17) broth was 0.96-0.97. The concentration of NaCl in brine in both broths was about 10% (12,17). The minimum aw which supported enterotoxin C production in the same medium at 30 C was 0.97 or 8% NaCl (figure 1). The aw of the broths measured at 30 C and 27 C was found to be lower than at 37 C. So PHP-NAK with 12% salt had water activities of 0.91, 0.92, and 0.95 at 27 C, 30 C, and 37 C, respectively. The aw of the broths with pH of 4.5 to 8.5 varied by no more than 0.01.

These results indicate that it is not only the lowering of the temperature of incubation which decreases the rate of growth of staphylococci and the yield of the enterotoxin produced, but also the decrease of aw. Additional experiments are now in progress.
5. **Effect of Temperature and Time.** The minimum reported temperature permitting staphylococcal growth is 6.7°C (1). Aerobic production of enterotoxin B in BHI broth at 16°C has been reported by Lilly et al. (25). Marland (29) reported enterotoxin B production in broths at temperatures from 15.2°C to 43.2°C. Our studies have indicated production of enterotoxin B in cured meats incubated anaerobically up to 16 weeks at 10°C. Production was greater at 30°C than at 22°C or 10°C (14). In the presence of increasing amounts of NaCl, higher temperatures were needed to support enterotoxin production in broths (21). Recent data have indicated that the upper temperature limit for production of enterotoxins is 45-46°C (41,47). The optimum temperature is 40°C for enterotoxins B and C production (51).

The minimum time needed for staphylococci to grow and produce enough toxin to cause poisoning is not well defined. It has been reported that it takes about a million cells to produce this amount of toxin. On the other hand, the generation time of *S. aureus* under optimum conditions (growth in BHI broth, pH 7.4 at 37°C) is about 30 minutes. Since both the yield of toxin and the generation time are affected by a number of parameters (initial level of contamination, pH, temperature, *aw*, competition with other bacteria, air, etc.), prediction of the minimum time required for staphylococci to produce enough toxin to cause poisoning is difficult.

Dack (9) has reported experimental induction of poisoning in human volunteers. According to his data, cream became toxic after 5 hours of exposure to 24°C, mashed potatoes with milk after 6 hours, shellfish after 72 hours at 37°C, and bacon sandwiches after 22 hours at 37°C. A number of studies (10,14,45,46,48) based on serological detection of enterotoxins indicated production of the toxins in a variety of food items after 4 or more hours incubation.

The interactions and effects of pH, NaCl concentrations, inoculum size and temperature on enterotoxin C production in PHP-NAK (3% + 3%) broth combinations with pH 4.0 to 9.3, NaCl 0 to 10% inoculated with 1.5 x 10^3, 1.5 x 10^5 and 1.5 x 10^7 per ml and incubated aerobically at 20°C for 10 days in a shaker indicated no enterotoxin C production after a 4X concentration of the samples. Toxin was detected in a broth (0.09 μg/ml) with pH 6.4 and NaCl 0% after 80X concentration but not at pH 7.2. Toxin was detected in a broth with 0% NaCl and pH 7.0, but not at pH 6.0, inoculated with 7 x 10^5 cells/ml and incubated aerobically at 15°C for up to 16 days and concentrated 80X. The effect of pH, inoculum and temperature on the yield of enterotoxin C in PHP-NAK broth incubated aerobically for 48 hours is shown in figure 2.

6. **Effect of Atmosphere and Packaging.** Staphylococci are facultative anaerobes that grow best in the presence of oxygen but no growth occurs in complete absence of CO_2 (19). An atmosphere containing 10-40% CO_2 was used in the past for rich staphylococcal growth and high yield of enterotoxin in culture media. Today high yields of enterotoxins are obtained by growing cultures in semisynthetic media and with intensive aeration.
Packaging can have a definite influence on the safety of foods such as semi-preserved meats. If the meat is vacuum packed, the gaseous phase in the package will change because of the consumption of oxygen and the resulting accumulation of carbon dioxide (22). This change is much more favorable for facultative anaerobic and carbon-dioxide resistant lactobacilli than for staphylococci and micrococci. One result of this is a change in the spoilage pattern. The meats often turn sour and develop upon prolonged storage a flavor somewhat similar to that of sour milk or buttermilk.

Another consequence is that the growth of S. aureus is limited because of competition with other bacteria in the meat (8,11,14, 22,40). It must be emphasized that staphylococci in the absence of competition can grow and produce enterotoxins A and B in meats under anaerobic conditions (13,14,49). However, neither the extent of growth nor the amount of enterotoxin produced is as prolific as it is under aerobic conditions (14). Another important aspect is that staphylococci can grow anaerobically and produce enterotoxins in meats without appreciable change in the color or smell even after 2 months storage at 10 C and the presence of over 1 x 10^9 cells/g. We have been unable to demonstrate anaerobic enterotoxin C production in 60 different types of cured meats inoculated with 1 x 10^8 staphylococci per gram and incubated at 26 C for 20 days. On the contrary, growth to up to 10^7-10^9 cells/g was supported in these meats with pH to 4.85 to 7.05, 1.14 to 1.38% NaCl, 9 to 876 ppm nitrite and 0.02 to 7.45 ppm HNO2. Small yields of enterotoxin C were produced anaerobically on PHP-NAK agar (cellophane membrane culture) and cooked sirloin inoculated with 1 x 10^8 cells per membrane on granis and incubated at 37 C for 5 and 10 days, respectively.

7. Effect of Nitrite. Recent studies on nitrite inhibition of S. aureus in BHI indicate that it involves extension of the adjustment phase, decrease in growth rate, and damage or destruction of some cells. The magnitude of inhibition is dependent on the interaction of NaN02 concentration, initial pH and oxygen pressure (6). The inhibition is more effective at a lower pH, more than 200 ppm nitrite and anaerobic conditions.

Neither NaN03 in concentrations up to 1000 ppm nor NaNO2 at levels up to 200 ppm appears to affect growth of staphylococci or production of enterotoxin A (28) and enterotoxin B (31) under aerobic conditions. We have found that under anaerobic conditions, levels of undissociated HNO2 above 0.54 ppm inhibited enterotoxin B production in cured meats. Unfortunately levels of up to 7.45 ppm HNO2 (pH 4.9) and 876 ppm NaN02 (pH 7.0) did not inhibit aerobic production of enterotoxin C in cured meats (18).

8. Effect of Food Flora and Glucose. The poor ability of staphylococci to compete with other food bacteria has been well documented and reviewed (33-35). The microbial inhibition of staphylococci is due mainly to a low pH, production of H2O2 or other inhibitory substances (antibiotics) or competition for essential nutrients. Decrease of
pII and production of H$_2$O$_2$ are the best documented factors. It has been suggested that the majority of staphylococcal food poisonings are due to food in which the microbial flora is substantially reduced (36,37) and consequently the inhibitory factors are reduced.

Thus, the contaminating staphylococci remain without serious competition, grow very well, and produce high yields of enterotoxins. A good example of staphylococcal activity when competition is reduced is that which occurs frequently in cured meats (especially ham) (5). The high salt content of these cured meats inhibits a lot of fresh meat bacterial flora and selects for staphylococci, which in the absence of effective inhibition grow well and produce enterotoxins. Of two pieces of fresh meat, one raw and one cooked, both inoculated with the same number of staphylococci, the cooked will support a far better growth and toxin production because of the absence of competition with other food bacteria.

It must be emphasized, however, that staphylococci are not always inhibited by other bacteria (44). Some bacteria even boost enterotoxin production (30; Genigeorgis, unpublished). Fortunately, inhibition has been found to be more common than stimulation (30,43).

The inhibitory effect of competing organisms on S. aureus depends on storage temperature and NaCl concentration (20,30,36,37). Higher temperatures and brine concentrations tend to favor the mesophilic salt-tolerant staphylococci.

Riemann et al. (40) have recently studied the possibility of adding glucose to semi-preserved meats to form lactic acid by the food flora in case of temperature mishandling and so inhibit C. botulinum and S. aureus growth. Experiments in which 1% glucose was added to commercially produced semi-preserved meat products indicated the feasibility of this method of inhibition. In most cases, but not all, glucose addition resulted in a pH drop to below 5.3 during 4 days of incubation at 20-30 C and no toxin was produced by S. aureus or C. botulinum.

Additional studies based on the addition of glucose and irradiated Pediococcus cerevisiae cells in semi-preserved meats indicated a rapid production of lactic acid, a drop in pH in a few hours to well below 5.0, and the inhibition of C. botulinum, C. perfringens, salmonella and S. aureus growth (Al-Mashat, Ph.D. Thesis, 1972). The effect of 0.2 to 10% glucose on the growth and enterotoxin B production by S. aureus in BHI base agar medium (cellophane membrane cultures) aerobically and anaerobically at 37 C has been evaluated (Genigeorgis, unpublished). It was found that both the amount of glucose used in the medium and the conditions of incubation (aerobic versus anaerobic) had a significant effect on the growth, the yield of enterotoxin B produced, and the final pH of the medium. Under aerobic conditions, the yield of enterotoxin at the 0.2 and 1.0% glucose levels was 70 µg and 250 µg/membrane within 24 hr, while
the pH of the medium increased from 7.4 to about 9.0. When the amount of glucose was increased to 2-10% the yield of enterotoxin after 6 days incubation decreased to less than 4 μg and in most samples the toxin was undetectable. The pH also decreased to values below 5.5. Under anaerobic conditions levels of 0.2 and 1.0% glucose in the medium resulted in 2 and 9 μg enterotoxin per membrane in 6 days. The pH decreased slightly to 7.0. When the glucose level was increased to 2-10%, no significant difference with regard to the yield of enterotoxin was found between aerobic and anaerobic incubation.

9. **Mechanism of Enterotoxin Synthesis.** Recently available information on the mechanism of enterotoxin synthesis has been reviewed adequately and will not be discussed further here (2,33). I will refer only to some of our own data on the biosynthesis of enterotoxin C (Genigeorgis and Cahalan, unpublished).

A number of experiments were completed to obtain information on the bio-synthesis of enterotoxin C. Enterotoxin C is produced throughout the log phase of growth, but the highest amount is released by the cells into the surrounding medium during the late log and early stationary phase. Studies on the aerobic enterotoxin production by replicating and non-replicating cells in media with different nitrogen source, glucose, chloramphenicol and phosphate support the theory of the presence of a toxin precursor pool in exponentially growing cells. On the basis of the experimental data accumulated it is believed that the biosynthesis of enterotoxin C is similar to enterotoxin B (27) rather than to enterotoxin A (26). A comparison of the amounts of enterotoxin C produced by log phase cells in (1) PHP-NAK broth, (2) metabolically exhausted PHP-NAK broth (stationary culture supernatant) plus fresh nutrients, and (3) exhausted PHP-NAK broth showed decreasing yields of enterotoxin from broth No. 1 to No. 3. The experimental data indicated that the presence of certain nutrients and the absence of certain waste products have a stimulatory effect on toxin production.

**Staphylococcal Gastroenteritis Attributed to Genoa Italian Style Dry Salami.**

Last year a large number of staphylococcal food poisoning outbreaks were reported from a number of states. Eventually the USDA and USPHS pinpointed the causal food as being Genoa-type dry salami, which is a cold-smoked uncooked pork product sliced and packaged under vacuum.

The salami was produced by two companies in two separate plants. Samples of salami analyzed indicated coagulase-positive staphylococcal counts from $1 \times 10^3$ to $1 \times 10^6$ cells/g. Many of the samples were positive for enterotoxin A even though the contamination level was $1 \times 10^3$ to $3 \times 10^4$/g. Some samples, in addition to staphylococci, were positive for salmonellae.
To my knowledge, no staphylococcal food poisoning due to dry salami has been previously reported. We can assume that in this case of poisoning either something unusual happened in the plants or that a better reporting system of food poisoning outbreaks picked up cases which were previously unreported. Another possibility is that growth of staphylococci and enterotoxin production occurs quite frequently in these products, but in rather low yields, and since very few people eat large amounts of these salamis, they are not receiving enough toxin to get sick. The unusual event is that the plants permitted larger than usual amounts of enterotoxin to be produced---enough to precipitate the outbreaks.

I have learned that the processing of similar types of dry sausages is based on a natural inoculation of the emulsions with lactic acid bacteria. Immediately after mixing, the emulsions are stuffed in casings and placed at 27-30 C for 3-4 days. This incubation is followed by a certain degree of dehydration at 20 C for 20 or more days. The product is then sent to the market in the casings or sliced and vacuum packaged.

I have not analyzed Genoa-type salami, but I have analyzed other dry salami sold at room temperature. The analysis indicated:

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Salt in meat</th>
<th>Salt in brine</th>
<th>% H₂O</th>
<th>A₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.15</td>
<td>5.30</td>
<td>12.7</td>
<td>36.4</td>
<td>0.94</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>5.15</td>
<td>15</td>
<td>29</td>
<td>0.905</td>
</tr>
</tbody>
</table>

The problem which needs answering is: Can staphylococci grow in the final products and produce enterotoxins or is the toxin formed during the processing? On the basis of the above analysis and the data I gave you before, we can make a safe conclusion that growth might take place in the final product but no toxin can be produced because of the high brine concentrations. In the case of the outbreaks, staphylococci possibly grew during the processing and most probably during the first incubation at 27-30 C for 3-4 days. If the raw meat had unusually high initial staphylococcal counts, then the natural lactic acid flora was unable to compete efficiently through a decrease of pH. In this case then inhibition of staphylococcal growth and production of enterotoxin could not take place. The contaminating staphylococci were able to multiply to at least a level of 10⁶/g. They produced enterotoxin. Subsequent dehydration did not affect the amount of preformed toxin but had some killing effect on staphylococci to decrease their numbers to levels as low as 1 x 10³/g. A number of vacuum packaged Italian style salamis have been analyzed in the past. They have been found to have pH and brine concentration values around 5 (14,40). Staphylococci surviving the no-heat treatment process of such products will be able to grow and produce enterotoxin when exposed to optimum temperatures and aerobic conditions.
REFERENCES


