Effect of pH, Sodium Chloride, and Sodium Nitrite on Enterotoxin A Production

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The combined effects of pH, sodium chloride, and sodium nitrite were studied by using a dialysis sac technique in brain heart infusion broth. Growth and enterotoxin A production by Staphylococcus aureus strain 100 were found to decrease with the addition of sodium nitrite, with a decrease in pH from 7.0, and with an increase in sodium chloride concentration. The significance of these results is discussed in relation to cured meats.

McLean et al. (12) reported that neither NaNO₂ in concentrations up to 1,000 μg/ml nor NaNO₃ up to 200 μg/ml affected growth of Staphylococcus aureus or enterotoxin B production in brain heart infusion (BHI) broth at pH 7.0. Their results indicated that a combination of 2% NaCl, 120 μg of NaNO₂ per ml, and 200 μg of NaNO₃ per ml in BHI broth reduced the amount of enterotoxin B produced.

Markus and Silverman (13) investigated growth and enterotoxin A production by S. aureus strains 100 and S-6 in shake flask cultures held at 37°C for 48 h. They found that up to 10% NaCl did not essentially alter the ratio of enterotoxin A formation to growth, although the quantity of enterotoxin decreased with increasing salt level. Sodium chloride concentrations higher than 10% inhibited bacterial growth. The addition of 200 μg of NaNO₂ per ml and 1,000 μg of NaNO₃ per ml affected neither growth nor toxin formation by strain 100. They observed no synergistic effect on growth or enterotoxin A production in a medium containing 10% NaCl, 200 μg of NaNO₂ per ml, and 1,000 μg of NaNO₃ per ml. The initial pH of their medium was 6.6 to 6.8.

Of the known enterotoxins, type A is most commonly the cause for staphylococcal food poisoning. However, research on type A enterotoxin has been retarded due to the small quantity of enterotoxin A normally produced in broth culture. Casman and Bennett (3) described a cellophane sac technique which concentrated enterotoxin and permitted detection of weakly enterotoxigenic strains. Their method was selected for the research described herein to study certain conditions influencing enterotoxin A produced by S. aureus strain 100.

MATERIALS AND METHODS

Staphylococcus strain. Strain 100, obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin, Madison) was used throughout. This strain produces only enterotoxin A (11). Preparación of inoculum. The culture was maintained on stock culture agar (Difco) slants. Fresh-18 to 24-h slants were washed with 10 ml of phosphate buffer (pH 7.2). The suspension was diluted to obtain the desired inoculum level in 0.5 ml.

Media and incubation conditions. The cellophane sac technique of Casman and Bennett (3) was used. Sodium chloride was added to BHI broth (Difco) and autoclaved in Roux bottles with sacs in place. The concentrations of NaCl described are in addition to the NaCl present in the dehydrated medium. Adjustments in pH were made with HCl. Sodium nitrite was filter sterilized and added to the autoclaved broth. Sac cultures were incubated for 96 h at 37°C.

Harvesting growth and toxin. The quantity of fluid in the sacs after incubation varied from 0.0 to 2.5 ml. A known quantity of sterile saline (≤2.5 ml) was added to wash out each sac. This material was then used for total count determinations. The culture suspension was then centrifuged to remove the bacterial cells. Tenfold serial dilutions were made of the supernatant for toxin assay.

Assay for total counts and toxin. Standard plate count agar (Difco) was used to determine levels of S. aureus. Enterotoxin A was assayed by the microslide gel diffusion technique as described by Casman et al. (4). By working with known standardized material, it was possible to consistently detect 1 μg/ml by the microslide technique. On this basis, a positive test of the undiluted supernatant and a negative test at the 1:10 dilution was recorded as having 1 μg of enterotoxin per ml. Enterotoxin A and homologous antisera were supplied by M. S. Bergdoll.

RESULTS

Table 1 summarizes a series of tests on the effect of NaCl, pH, and nitrite on enterotoxin A
production in dialysis sacs with an *S. aureus* inoculum of 10,000 per sac. Enterotoxin levels decreased as the NaCl concentration was increased in BHI broth at pH 7.0. A decrease in toxin production occurred as pH decreased from pH 7.0 to 4.5. Nitrite had only a slight effect at pH 7.0. The effect of nitrite at 0 and 3% NaCl became more evident as the pH decreased.

Growth occurred at pH 7.0 with 12% NaCl regardless of nitrite level. Growth occurred at pH 4.9 and 4.5 at 0 and 3% NaCl but only in the absence of nitrite.

The effect of inoculum level in dialysis sacs was studied relative to NaCl level in the broth. Toxin was produced at 7, 10, and 9% NaCl for the low (7.5 x 10^4 cells/sac), medium (7.6 x 10^4 cells/sac), and high (3.1 x 10^5 cells/sac) inoculum levels, respectively (Table 2). Growth occurred at 9, 11, and 10% NaCl for the low, medium, and high inoculum levels.

The relationship between the level of *S. aureus* attained in the sacs after 96 h at 37 C and the presence of enterotoxin is shown in Table 3. The lowest level of *S. aureus* in which toxin was detected was 10^7/ml.

The effect of NaCl (0 and 8 to 16%) on the growth of *S. aureus* strain 100 when inoculated in the conventional manner into BHI broth without dialysis sacs was tested. After 96 h at 37 C, a 10-fold increase had occurred at 15% NaCl. A decrease from the inoculum level of 7.5 x 10^8/ml occurred at 16% NaCl to 50/ml. The counts were progressively higher as the NaCl level decreased with a maximum viable concentration of 4.9 x 10^6/ml in BHI broth without added NaCl.

**DISCUSSION**

The results in Table 1 show that addition of NaNO_2_ influences growth of *S. aureus* and decreases the quantity of enterotoxin A produced as the pH of the medium is decreased below pH 7.0. The general ineffectiveness of NaNO_2_ observed by McLean et al. (12) and Markus and Silverman (13) may be due to the use of media having an initial pH near neutrality. The relative merit of NaNO_2_ in cured meats should be considered in terms of pH 5.8 to 6.2 as being typical for most nonfermented, nonpickled cured meats. The pH of fermented and pickled cured meats would normally be well below pH 5.4.

Castellani and Niven (5), Lechowich et al. (11), and Buchanan and Solberg (2) demonstrated that nitrite inhibition of *S. aureus* growth in broth media is dependent upon pH. Available data suggest that it is HNO_2_ which is the active antimicrobial form of nitrite (5, 16). Nitrite becomes very bactericidal within the pH range of 4.5 to 5.5, because nitrite exists principally as HNO_2_ (16). It is possible that inhibition of *S. aureus* by nitrite may be due to interference with a metabolic system (5).

Genigeorgis et al. (7), studying the effect of pH, salt, and nitrite on enterotoxin B production under anaerobic conditions in laboratory cured ham, found that undissociated HNO_2_ affected enterotoxin production at 10 C. The probability of ham with an initial pH of 5.58 or higher becoming toxic decreased as the HNO_2_ concentration increased. Enterotoxin B was not produced below pH 5.58 anaerobically at 10 C.

It has been demonstrated many times that pH or NaCl, alone, influence both growth and enterotoxin production. Our findings corroborate earlier reports (1, 6, 8-10, 13, 17) that enterotoxin production ceases before growth is prevented by pH or NaCl.

The conventional method of inoculating directly into broth results in relatively low levels of enterotoxin A on a per milliliter basis. Marcus and Silverman (13) and Reiser and Weiss (14) found strain 100 to produce 15 µg or less enterotoxin A per ml in broth media.

Kato et al. (10) used strain 100 for large-scale production of enterotoxin A. Under optimum conditions, they obtained 4 to 6 µg of enterotoxin A per ml. The amount of enterotoxin produced in the range of pH 5.0 to 8.0 did not vary markedly.

Reiser and Weiss (14) reported strain 100 to be the only one of six strains tested wherein the amount of enterotoxin produced was not influenced by the medium. They also concluded that enterotoxin production appeared to be little influenced by initial pH (5.3, 6.0, 6.8) or length of incubation time beyond 24 h at 37 C.

Barber and Deibel (1) found that *S. aureus* strain 100 grew at pH 4.8 and enterotoxin A was produced at pH 5.0 in buffered BHI broth under aerobic conditions. Under anaerobic conditions, growth occurred at pH 5.7 but not at 5.4. Enterotoxin A was produced at pH 6.0 but not at 5.7 anaerobically.

By using the sac technique, we observed enterotoxin levels as high as 120 µg/ml. The higher levels obtained with the sac technique allowed for the effects of pH, salt, and nitrite on enterotoxin production to be more obvious.

It appears that, despite the level of inoculum, *S. aureus* strain 100 is more sensitive to NaCl when grown in dialysis sacs than when inoculated directly into broth. The highest NaCl level permitting growth in dialysis sacs was 12% (Table 1), whereas growth occurred up to 15% when the broth was inoculated in a conventional manner.
Table 1. Effect of NaCl, pH, and nitrite on enterotoxin production by S. aureus strain 100 in dialysis sacs suspended in BHI broth and inoculated with 10,000 per sac

| NaCl (%) | pH 7.0 | pH 6.1 | pH 5.7 | pH 5.3 | pH 4.9 | pH 4.5 |
|----------|--------|--------|--------|--------|--------|--------|
| 0        | 100/120 | 50/80  | 50/100 | 60     | 10     | 30     | 10/40  | 1/50   | 7/10/40 | 10/30 | 10         | 1/10 | 1/10       | 1/10 | 1/10       | 1/10 | 1/10       |
| 3        | 110     | 20     | 20     | 70     | 1      | 20     | 30     | 1      | 1       | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 4        | 80      | NT     | 40     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 5        | 60      | 80     | 20/40  | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 6        | 60      | 40     | 40/40  | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 7        | 40      | 60     | 10/10  | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 8        | 10      | 10     | 1      | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 9        | 1       | 1      | 1      | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 10       | 0       | 0      | 0      | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 11       | 0       | 0      | 0      | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |
| 12       | 0       | 0      | 0      | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     | NT     |

* Nitrite (µg/ml).
+ Results from two separate experiments.
+ No toxin detected.
+ NT, No test at this variable.
+ Highest NaCl level showing growth in levels tested.
+ NG, no growth at any salt level.
The sac technique was a satisfactory procedure for the purpose intended. The procedure does have the inherent problem of variable amounts of fluid in the sacs after the incubation period. This and the fact that we were working with a small amount of fluid for quantitating enterotoxin and viable cell counts does lead to some loss of precision. For these reasons the concentrations of enterotoxin, in particular, are approximations and not absolute values. The data are sufficient, however, to show trends of influence for the factors tested. It is of interest that 10⁷ cells/ml were necessary before enterotoxin was detected in the sac cultures. This compares with other reports for foods and conventional broth cultures. We found too few samples at 10⁸ cells/ml to state that enterotoxin might not be detected at this cell level in sac cultures.

It is generally conceded that the levels of salt, pH, and nitrite normally found in most nonfermented, nonpickled, cured meats which are not dried extensively will not prevent growth or enterotoxin formation by S. aureus under aerobic conditions. However, these levels may become inhibitory under anaerobic conditions such as with vacuum packaging (1; C. Genigeorgis and J. Prucha, Bacteriol. Proc., A102, 1971).

Fermented cured meats have been implicated as a cause of staphylococcal food poisoning. Our results suggest that in the case of fermented sausage the possible benefit of nitrite should not be ignored as a contributing factor for inhibiting enterotoxin formation. This may be of most importance in borderline instances and could be one factor in preventing enterotoxin formation, although high levels of staphylococci may be attained in the product. For this reason the maximum allowable level of NaNO₃ (156 μg/g) should be considered during the manufacture of fermented cured meats. Additional research in this area would be of interest.

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