Initiation of Staphylococcal Growth in Laboratory Media

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Received for publication 28 December 1970

The effects of pH and NaCl concentration on the probability of aerobic growth initiation in Brain Heart Infusion broth at 30 C by five staphylococcal strains producing enterotoxins A, B, C, and D were studied in a factorial design experiment. Statistical analysis of the data indicated: (i) significant effects of pH, NaCl, and strain on the probability of growth; (ii) diverse effects of NaCl with various pH levels and strains; (iii) essentially a linear relationship between NaCl concentration and probability of growth initiation when data for all strains were pooled; (iv) the relationship between NaCl concentration and probability of growth initiation varies from linear to sigmoid, depending on the pH of the broth, when the statistically untreated (raw) data are plotted for each strain. Equations were derived which relate the decimal reductions of the number of cells of a staphylococcal population to the concentration of NaCl and pH of broth to which the population was exposed. From the equations, the probability that one cell is capable of initiating growth can be calculated. The impact of these types of studies on the development of realistic staphylococcal standards in foods is discussed.

The effects of heat and radiation on bacterial destruction have been studied extensively, and probabilities for survival and growth, especially of Clostridium botulinum spores, have been estimated. As a result of such studies, a standard heat treatment for low-acid canned foods has been established (18) which will reduce C. botulinum spores by a factor of 10¹⁴ (12 decimal reductions or 12D values). This 1:10¹⁴ probability of C. botulinum spore survival is considered a minimum safety requirement. The effect of other preservation methods, such as acidification, drying, curing, and smoking, have not been expressed in terms of decimal reduction or inactivation values. If such values were known, it would be possible to estimate minimum standards for cured and smoked vacuum-packed foods that would achieve a safety level comparable to that of canned foods.

Several researchers (2, 6, 10-16) have described the effect of NaCl and pH on Staphylococcus aureus; but quantitative data are still lacking. Some preliminary work which included C. botulinum has been published (6, 17).

The present study is concerned with estimating the probability of initiation of aerobic staphylococcal growth in broths. Similar studies of growth and enterotoxigenesis in various meats are published separately in the following note (8).

MATERIALS AND METHODS

Preparation of experimental broths. To prepare the broths, 37 g of dehydrated Brain Heart Infusion (BHI) broth (Difco) was dissolved in 700 ml of boiling distilled water. After the broth was cooled to room temperature, an additional 100 ml of water was added to make the basal medium. To provide the broths with various concentrations of salt, 80-ml portions were placed in beakers, and NaCl was then added in amounts of 0, 4, 8, 12, and 16 g per beaker. After the NaCl was dissolved, the pH was adjusted to the desired values by adding 0.5 N NaOH or 0.5 N HCl. The values were checked with a Beckman Expandomatic pH meter equipped with a Beckman H2 glass electrode. The broths were autoclaved in 100-ml volumetric flasks (15 min at 121 C under 15 psi of pressure) and then cooled. The volume was brought to 100 ml with sterile distilled water.

Inoculation and incubation. S. aureus strains 243, S-6, 264, 137 and 472, producing enterotoxins B, AB, A, C, and D, respectively, were used in this study. Overnight BHI broth cultures of these strains were inoculated into 25 ml of BHI broth containing 0.25% Tween 80. The fresh cultures were incubated at 37 C on a reciprocal shaker for 4 hr. The cultures were then centrifuged, the cells were washed once with saline, and concentration of cells was adjusted to an optical
density (OD) of 0.3 to 1.0 at 660 nm, by using a Spectronic-20 colorimeter (Bausch & Lomb). Nine tubes each containing 9 ml of broth were prepared from each type of experimental broth. A 1-ml amount of the cell suspension was added in the first tube, and 10-fold serial dilutions of the suspension were added to the other tubes.

Three portions of 2 ml each were transferred with a sterile syringe from each of the nine tubes to 2-ml screw-cap vials. The caps were put on loosely and the vials were placed in 2-lb coffee cans. In each can, there was a vessel containing a brine of the same NaCl concentration as the broth. The cans were closed with plastic lids and placed in the aerobic incubator at 30°C where they were left for 20 days. Every other day, vials with growth (turbidity) were removed and recorded. From the presence or absence of growth in the 27 vials prepared for each salt-pH combination, the most-probable number of cells that had initiated growth was calculated by the technique of Fisher and Yates (5). The number of staphylococcal cells present in the cell suspension used as inoculum was determined by plating on cow blood-agar (BHI base) in duplicate. This number was always between 10⁵ and 9 × 10⁸.

**Statistical methods.** The experiments, arranged in a factorial design (19), involved five staphylococcal strains, five NaCl concentrations (1, 4, 8, 12, and 16%), and six pH values (4.7, 5.1, 6.1, 7.0, 7.8, and 8.9). For the statistical evaluation of the effects of strain, pH, NaCl, and their interaction upon staphylococci, the logarithm (log) of the ratio R₁/R₀ was used for each broth and strain condition, where R₁ is the number of cells in the inoculum and R₀ is the number initiating growth. This log represents the number of decimal reductions of a staphylococcal population resulting from its exposure to a particular broth environment. The probability that one cell will initiate growth in such an environment can be calculated from the formula

\[ P = \frac{1}{\text{antilog} \left[ \log \left( \frac{R_1}{R_0} \right) \right]} = \frac{1}{R_1/R_0} = R_0/R_1. \]

The logs determined in the factorial experiments were evaluated by using the biomedical computer programs (3) for factorial design and multiple regression analysis. Equations representing the trend surface (response surface) were constructed for each strain under these aerobic conditions. Each equation related the effects of NaCl and pH levels on log decrease for that strain. The model for the trend surface was

\[ Y = a + b_1 x_1 + b_2 x_2 + b_3 x_1^2 + b_4 x_2^2 + b_5 x_1 x_2, \]

where \( Y \) is estimated log decrease (decimal reductions) of the bacterial population, \( a = \) intercept, \( x_1 = \) per cent NaCl \((w/v)\), \( x_2 = \) pH level, \( b_1, b_2, \ldots, b_5 = \) regression coefficients. Although the effect \( b_5 x_1^2 \) [(per cent NaCl)²] was not generally significant, it was retained in the model for purposes of symmetry.

**RESULTS**

Statistical analysis of the data indicated the following effects and interactions. (i) Strain, pH, and concentration of NaCl all significantly affected the size of the log decrease of the staphylococcal population. (ii) The effect of NaCl varied with pH levels and by strains. (iii) When data for all strains were pooled, NaCl concentration and size of log decrease were related linearly. (iv) When the raw data for each strain were plotted, the relationship between NaCl concentration and log decrease of the population varied from linear to sigmoid, depending on the pH of the broth (Fig. 1). The above four observed effects and interactions apply also to the probability of growth.

From the factorial and multiple regression analysis, the following five equations were derived for the five staphylococcal strains used, based on the first four levels of NaCl: strain 243, \( Y = \log \text{reduction} = 44.03 + 0.37 \text{(salt)} - 14.03 \text{(pH)} + 0.003 \text{(salt)}^2 + 1.09 \text{(pH)}^2 - 0.02 \text{(salt)} \times \text{(pH)}; \) strain S-6, \( Y = 31.81 + 0.53 \text{(salt)} - 9.33 \text{(pH)} + 0.003 \text{(salt)}^2 + 0.66 \text{(pH)}^2 - 0.02 \text{(salt)} \times \text{(pH)}; \) strain 264, \( Y = 34.03 + 0.36 \text{(salt)} - 10.63 \text{(pH)} - 0.0015 \text{(salt)}^2 + 0.80 \text{(pH)}^2 - 0.004 \text{(salt)} \times \text{(pH)}; \) strain 137, \( Y = 53.85 - 0.80 \text{(salt)} - 15.02 \text{(pH)} - 0.00008 \text{(salt)}^2 + 1.03 \text{(pH)}^2 + 0.064 \text{(salt)}^2 \times \text{(pH)}^2 \).

**FIG. 1.** Statistically untreated data indicating the effect of pH and NaCl on the log decrease of populations of five staphylococcal strains.
(pH); strain 472, $Y_e = 32.73 - 0.04 \text{(salt)} - 10.28 \text{(pH)} + 0.01 \text{(salt)}^2 + 0.03 \text{(salt)} \text{(pH)}$.

For strains 243, S-6, 264, 137, and 472, the standard errors (SE) were 2.78, 1.99, 1.83, 1.98, and 2.47, respectively. Approximate 95% confidence contours for a specified log reduction can be obtained by using $Y_e \pm 2 \text{SE}$. Using the equations, response curves have been constructed for each strain relating pH, NaCl, and log reductions. In Fig. 2 the curves indicate the combinations of pH and NaCl which will reduce populations of strains S-6, 137, 243, and 472 by 1, 2, 3, 4, 5, and 6 logs. The range of NaCl and pH combinations which will decrease populations of all five strains by 3, 4, 5, and 6 logs is indicated in Fig. 3. It is obvious from the figure that the response of the various strains to NaCl concentrations is becoming more uniform (narrow) as the pH approaches values between 7 and 8. This more uniform response of strains exposed to optimum pH is also apparent in Fig. 1. Response curves and the approximate 95% confidence limits for 6- and 3-log reductions of strain 243 are presented in Fig. 4 for comparison. As indicated in this figure, there is a zone of pH and NaCl combinations which will result in 3- to 6-log reductions, whereas the other two zones combinations are likely to result only in large (6-log) and in small (3-log) reductions of populations of strain 243.

**DISCUSSION**

The effect of various environmental conditions on staphylococcal growth in culture media and in foods has been studied extensively (2, 7, 9-11, 13-15), and certain limited standards for staphylococci in foods have been established (1, 4).

The present study is one of a series aiming toward the accumulation of data which eventually will help in establishing realistic staphylococcal standards. To obtain basic information, we have studied the effects of two important factors, NaCl and pH, on the log reduction of staphylococcal populations inoculated in Brain Heart Infusion broth. The probability that one cell can initiate growth can be calculated from the derived equations which relate NaCl concentration and pH of the medium to log reductions of a staphylococcal population exposed to this environment. Thus, for a broth at pH 6.0 with 5% NaCl, the log decrease for the strain S-6 incubated aerobically is 1.69, the antilog is 49, and the probability of initiating growth is $\frac{1}{249}$ or 0.204%. On the basis of data reported previously (7), the same strain incubated anaerobically in the same broth will
STAPHYLOCOCCAL GROWTH IN LABORATORY MEDIA

Fig. 3. Range of NaCl and pH combinations which will decrease populations of all five staphylococcal strains by 3, 4, 5, and 6 logs.

Fig. 4. Response curves, with approximate 95% confidence limits, of pH and NaCl combinations resulting in 3- and 6-log decreases in populations of staphylococcal strain 243.

have a log decrease of 2.92, and the probability of initiating growth is only 0.12%. The effects of aerobiosis (present findings) versus anaerobiosis (7) on the combinations of pH and salt which will decrease populations of strains 137, 243, S-6, and 472 by 3 and 6 logs are further compared in Fig. 5.

In general, there is close agreement between the present findings and the data published previously on the effects of NaCl and pH on staphylococcal growth, indicating that the experimental techniques are comparable. The findings of the present and earlier studies are summarized as follows. (i) There is a decreased rate of growth of food-poisoning staphylococci when exposed to media with concentrations of NaCl increased from 0 to 20% (7, 10, 13, 14). (ii) Increasing the NaCl concentration of a broth requires more concentrated inocula for initiation of growth (14). (iii) Higher concentrations of NaCl and extreme pH values prevent growth, or delay it, or diminish the total amount, depending on concentration of inoculum and time of incubation (6). (iv) Smaller concentrations of NaCl are required to inhibit initiation of staphylococcal growth at pH values remote from optimum (12). (v) There is better staphylococcal growth aerobically than anaerobically. The magnitude of the effects of the two conditions varies with strain and, at some limited pH values, appears to be reversed. (vi) The effects of pH and NaCl on
FIG. 5. Comparison of the effect of aerobic (○) and anaerobic (●) incubation of broths upon the combinations of pH and NaCl needed for 2 (broken lines) and 6 (continuous lines)-log decreases of populations of staphylococcal strains 243, 137, S-6, and 472.

Staphylococcal growth vary with the strain and the type of medium used (2, 14-16). (vii) Different investigators have used different staphylococcal strains, inocula, and media (2, 7, 10, 11, 13-16). Consequently, minor disagreements in present and past findings in the reported values of upper NaCl concentrations and of pH limits permitting staphylococcal growth are understandable.

Certain limited standards for staphylococci in foods have been established (1, 4). These standards tolerate 0 to 1,000 staphylococcal cells per g of food. However, they vary with agency and with state and country and are based on research data not analyzed statistically. A suggested tolerance of less than 1,000 cells/g probably represents a contamination level that can easily be met in most commercial food preparations. Any NaCl-pH combination (level) that will reduce the probability of growth initiation by a factor of 10^6, therefore, seems reasonable. The problem then is to find what combinations of NaCl and pH will have such an effect with a probability of safety of over 95%.

Some results concerning only strain 243 are presented in Fig. 4. In this figure, any combination of NaCl and pH outside the outer curve has a 95% probability of giving at least a 6-log decrease in the number of staphylococcal cells. Such calculations may be biased, of course, because we have extrapolated results from our data to an area where we do not have experimental data. In the experiments, our most concentrated inoculum was 9 × 10^7 cells/ml, and here we are talking about log decreases in the range of 6 + 2 SE (7.83 to 8.78 logs) for all five strains used. This was the reason that decimal reductions due to the effect of 16% NaCl were not considered in the derivation of the equations. The maximum inoculum used was not enough to measure the full effect of 16% NaCl concentration on the initiation of staphylococcal growth in broths.

The present data can only indicate, with 95% confidence limits, the combinations of NaCl and pH which will decrease populations of all five strains by 6 logs, less 2 SE (6 logs - 2 SE = 3.22 to 4.17, depending upon strain).

To obtain more realistic results, the present
studies should be supplemented not only with other studies in which concentrated inocula have been used but also with studies in which appropriate food items serve as culture media. The use of such media will eventually allow the development of equations based on quantitative characteristics of foods and permit accurate predictions of the probability of initiating staphylococcal growth. The results of the first series of these types of experiments have been reported (8). Studies on a third food factor, nitrite, have been interrupted until further data are accumulated on the reactions and stability of nitrite in laboratory media and foods.

ACKNOWLEDGMENTS

This investigation was sponsored by the Food Protection and Toxicology Center of the University of California and was supported by Public Health Service grant FD 00103 from the Food and Drug Administration.

LITERATURE CITED

1. Baltzer, J. 1969. The impact of standards on the meat industry in Western Europe, p. 91–106. Proc. Meat Ind. Res. Conf. Amer. Meat Inst. Foundation.
2. Buttiaux, R., and J. Moriance. 1958. Le comportement des germes tests de contamination fœcale dans les saumures de viandes, p. 247–262. Proc. Int. Symp. Food Microbiol., 2nd, Cambridge, England, 1937.
3. Dixon, W. T. 1967. Biomedical computer programs. Univ. of California Press, Berkeley.
4. Elliott, R. P., and H. D. Michener. 1961. Microbiological process report. Microbiological standards and handling codes for chilled and frozen foods. A review. Appl. Microbiol. 9:452–468.
5. Fisher, R. A., and F. Yates. 1947. Statistical procedures for biological and agricultural research, 2nd ed. Oliver and Boyd, Ltd, Edinburgh.
6. Genigeorgis, C. 1969. Interaction between curing factors and staphylococci in culture media and food.

Proc. 5th Symp. World Ass. Vet. Food Hygienists, Opatija, Yugoslavia.
7. Genigeorgis, C., and W. W. Sadler. 1966. Effect of sodium and pH on enterotoxin B production. J. Bacteriol. 92:1383–1387. (Erratum, vol. 93, p. 772, 1967.)
8. Genigeorgis, C., M. Savoukidis, and S. Martin. 1971. Initiation of staphylococcal growth in processed meat environments. Appl. Microbiol. 21:940–942.
9. Hovat, S. A., and H. Jackson. 1969. Effects of sodium chloride and temperature on the growth and production of enterotoxin B by Staphylococcus aureus. Can. Inst. Food Technol. J. 2:56–59.
10. Hucker, G. J., and W. C. Haynes. 1937. Certain factors affecting the growth of food poisoning micrococci. Amer. J. Pub. Health 27:590–594.
11. Iandolo, J. J., Z. J. Ordal, and L. D. Witter. 1964. The effect of incubation temperature and controlled pH on the growth of Staphylococcus aureus MF 31 at various concentrations of NaCl. Can. J. Microbiol. 10:808–811.
12. Ingram, M., and A. G. Kitchell. 1967. Salt as a preservative for foods. J. Food Technol. 2:1–15.
13. Lechowich, R. V., J. B. Evans, and C. F. Niven, Jr. 1956. Effect of curing ingredients and procedures on the survival and growth of staphylococci in and on cured meats. Appl. Microbiol. 4:360–363.
14. Maitland, H. B., and G. Martyn. 1948. A selective medium for isolating staphylococci based on the differential inhibiting effect of increased concentrations of sodium chloride. J. Pathol. Bacteriol. 68:553–561.
15. Nuneheimer, T. D., and F. W. Fabian. 1940. Influence of organic acid, sugar, and sodium chloride upon strains of food poisoning staphylococci. Amer. J. Pub. Health 30:1040–1049.
16. Parfentiev, I. A., and A. R. Catelli. 1964. Tolerance of Staphylococcus aureus to sodium chloride. J. Bacteriol. 88:1–3.
17. Riemann, H. 1966. The effect of the number of spores on growth and toxin formation by C. botulinum type E in inhibitory environments, p. 150–157. In M. Ingram and T. A. Roberts (ed.), Botulism. Chapman and Hall, London.
18. Riemann, H. 1969. Food processing and preservation effects, p. 489–541. In H. Riemann (ed.), Food-borne infections and intoxications. Academic Press Inc., New York.
19. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods. Iowa Univ. Press.