Journal of Food Protection, Vol. 50, No. 11, Pages 952-955 (November 1987)
Copyright© International Association of Milk, Food and Environmental Sanitarians

Production of Staphylcoccal Enterotoxin A and Thermonuclease in Cream Pies

ELISA YOKO HIROOKA[1], SONIA PRESA C. DE SALZBERG[2] and MERLIN S. BERGDOLL[3*]

Departmento de Pathologia Geral, Centro de Ciências Biológicas, Fundaçao Universidade Estadual de Londrina, Caixa Postal 6001, 86.100, Londrina, PR, Brazil, Departmento de Ciência de Alimentos, Facultade de Engenharia de Alimentos, Universidade Estadual de Campinas, Caixa Postal 6121, 13.100, Campinas, SP, Brazil, and Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, WI 53706.

(Received for publication March 31, 1987)

ABSTRACT

The change in pH, growth of mesophilic bacteria and growth, thermonuclease (TNase) and enterotoxin A (SEA) production by *Staphylococcus aureus* in artificially inoculated cream pies were investigated. The mesophilic count varied from 10^4 CFU/g at 20°C to 4.0×10^7 CFU/g at 37°C after 12 h of incubation and from 2×10^6 CFU/g at 20°C to 7×10^8 CFU/g at 37°C after 35 h of incubation. The *S. aureus* count varied from $<10^2$ at 20°C to 1.6×10^4 CFU/g at 37°C after 12 h of incubation and from 8×10^2 at 20°C to 5.4×10^6 at 37°C after 35 h of incubation. TNase was detectable after incubation for: 35 h at 20°C (2.9 ng/g), 12 h at 30°C (9.4 ng/g), and 12 h at 37°C (72 ng/g). SEA was detectable after incubation for: 35 h at 20°C (3.9 ng/g), 18 h at 25°C (3.9 ng/g) and 14 h at 30°C (4.8 ng/g).

Cream pie is a perishable product that can support rapid microbiological growth because of its high nutritional value. The possibility for cream to be contaminated with staphylococci is good because bovine mastitis due to staphylococci is quite common in Brazil *(11)*. Approximately 50% of the pasteurized milk used for cheese manufacture is contaminated with staphylococci *(15)*. This contamination could be because of either inadequate pasteurization or recontamination after pasteurization. Considering that Brazil is a tropical country, and that it is not uncommon for dairy products to be held at room temperature in most commercial places, conditions are ideal for growth of staphylococci in these products with the possible production of enterotoxin. This problem is aggravated by the poor sanitary conditions in Brazil, particularly in the rural areas. Staphylococcal enterotoxin A (SEA) is the enterotoxin most frequently involved in staphylococcal food poisoning outbreaks and the one generally associated with staphylococci isolated from foods *(19)*.

The main objective of the research reported here was to study the conditions necessary for staphylococcal growth and enterotoxin production in cream pies.

[1]*Universidade Estadual de Londrina.*
[2]*Universidade Estadual de Campinas.*
[3]*University of Wisconsin.*

MATERIALS AND METHODS

Microorganism

Staphylococcus aureus strain FRI-100, a producer of SEA, was used in this investigation.

Cream filling

The cream filling was prepared according to the procedures used by Brazilian bakeries: 1 L of milk, 400 g of sugar, 100 g of corn flour, 8 ml of 1% yellow dye (Tintanil) and 1 egg yolk. The filling was boiled, allowed to cool, weighed, and inoculated.

Inoculum

The inoculum was prepared by placing 1 ml of an overnight culture (grown in 4% N-Z Amine NAK + 10 μg of niacin/ml and 0.5 μg of thiamin) in an "L" form test tube containing 5 ml of the same medium and incubating at 37°C with shaking until the optical density was 0.3 at a wavelength of 600 nm. This corresponds to approximately 5×10^7 CFU/ml. Serial dilutions of the culture were prepared in 0.1% peptone water to obtain inocula corresponding to approximately 1, 10, and 100 CFU/ml. Counts were determined by plating on plate count agar (PCA).

Incubation conditions

The inoculated and uninoculated cream fillings were incubated at 20, 30 and 37°C and at room temperature (20-30°C). Samples were withdrawn after different incubation periods for pH, total plate count, staphylococcal count, thermonuclease (TNase) and SEA determinations. All samples were done at least in duplicate. An uninocualted sample was run with each inoculated sample.

pH determination

The pH of the cream fillings was determined after the fillings were blended with the same weight of distilled H_2O for 1 min.

Total count of staphylococci and mesophilic organisms

The cream fillings were homogenized by blending with an equal amount of 0.1% peptone H_2O. The homogenates were serially diluted and 1 ml of each dilution was plated on PCA for mesophilic count and on Baird-Parker agar for staphylococcal count. The colonies on PCA agar were identified by gram staining of at least three colonies per plate.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/50/11/952/1657817/0362-028x-50_11_952.pdf by guest on 25 January 2021

Determination of TNase activity

The cream fillings were extracted according to Tatini et al. *(17)*, and the TNase activity was determined by the method of Lachica et al. *(7)*. The approximate concentration of TNase was calculated from a standard curve prepared using different concentrations of purified nuclease (EC 3.1.31.1 with a specific activity of 221 units/mg of protein, Sigma Chemical Co., St. Louis, MO) dissolved in 0.05 *M* Tris buffer containing 0.1% bovine serum albumin (Fluka AG, Buchs SG, 05480).

Determination of SEA

SEA was determined by the enzyme-linked immunosorbent assay (ELISA) of Freed et al. *(4)*. The cream fillings were extracted as follows: 50 g was homogenized with an equal quantity of H_2O, the pH was adjusted to 4.5 with 6 *N* HCl, and the extract centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant fluid was adjusted to pH 7.5 with 5 *N* NaOH, $CHCl_3$ was added (10% of volume), agitated for 5 min and centrifuged. The supernatant fluid was adjusted to pH 7.5, Tween 20 was added (0.5%) and the samples were assayed for SEA. A standard curve was prepared by adding 0.625, 1.25, 2.50, 5.0, 10.0, 15.0 and 20.0 ng of SEA/ml to phosphate-saline buffer at pH 7.2 (PBS) and with 0.1% Tween 20 to the extract of uninoculated cream fillings.

RESULTS

pH

The final pH of the cream samples varied from 6.0 to 6.7; this was only a slight change from the pH of 6.6 before incubation.

Staphylococcal and mesophilic counts

The staphylococcal and mesophilic counts for an *S. aureus* inoculum of 10 CFU/g are given in Table 1. There was very little difference in the staphylococcal counts when an *S. aureus* inoculum of approximately 1 CFU/g was used. Staphylococcal counts were not made for the 100 CFU/g inoculum. There did appear to be an increase of approximately 1 $\log_{10}$ in the mesophilic counts with the smaller inoculum. Gram staining revealed that at incubation temperatures of 30 and 37°C, gram-positive cocci predominated over gram-positive sporulated bacilli, whereas this was reversed at incubation temperatures of 20 and 25°C. The mesophilic counts of the uninoculated product varied from 2.6×10^6/g at room temperature to 2.4×10^7/g at 37°C. No staphylococcal counts were observed.

TNase and SEA

The TNase and SEA concentrations for an *S. aureus* inoculum of 10 CFU/g are given in Table 2. At an *S. aureus* inoculum of approximately 1 CFU/g, TNase was first detectable after 35 h of incubation at 25°C, after 21 h of incubation at 30°C, and after 12 h of incubation at 37°C. SEA was not analyzed for in the samples in which the lower inoculum was used nor was it analyzed for before 14 h because none was detectable at 30°C, the temperature above which the cream pies would be held commercially. The amounts of SEA detected in the samples incubated at room temperature were essentially the same as those given in Table 2 for 30°C. Analysis for SEA in cream samples inoculated with 100 CFU of *S. aureus*/g revealed the following: SEA was first detectable after 35 h incubation at 20°C (3.9 μg/g), after 18 h of incubation at 25° (2.2 μg/g), and after 14 h of incubation at 30°C (4.8 μg/g). Fifty micrograms of SEA/g was present in the cream after 14 h of incubation at 37°C.

TABLE 2. *TNase and SEA detected in cream inoculated with 10 CFU/g of* S. aureus *strain FRI-100.*

Temp. (°C)	TNase (ng/g)			SEA (ng/g)		
	12 h	21 h	35 h	14 h	18 h	35 h
20	ND[a]	ND	2.9	ND	NA[b]	ND
25	ND	ND	15.1	ND	ND	15.1
30	2.9	9.4	320.0	ND	3.6	32.4
37	71.7	146.3	320.0	9.3	10.7	81.1

[a]ND = Not detectable.
[b]NA = Not analyzed.

DISCUSSION

Cream fillings are excellent substrates for growth of *S. aureus* and production of enterotoxin. Iaria and Nascimento (Congresso Brasileiro de Microbiologia, Sao Paulo, 1983, Sociedade Brasileira de Microbiologia) showed that cream pies from commercial establishments in Sao Paulo had high microbial contamination with 55% contaminated with staphylococci, 19.5% of which had staphylococcal counts above 10^5 CFU/g. Commercially, sales of cream pies are begun immediately after they are made and continue through the day; however, if any are not sold they are kept overnight (often unrefrigerated) for

TABLE 1. *Staphylococcal and mesophilic counts in cream inoculated with 10 CFU of* S. aureus *strain FRI-100/g.*

Temp. (°C)	Staphylococcal count			Mesophilic count		
	12 h	18 h	35 h	12 h	18 h	35 h
20	$<10^2$	ND[a]	8.0×10^2	1.0×10^4	ND	2.0×10^6
25	1.0×10^2	1.1×10^3	6.0×10^3	1.7×10^5	1.4×10^6	4.8×10^7
30	3.6×10^3	1.5×10^4	6.0×10^4	4.5×10^6	6.5×10^6	1.7×10^8
37	1.6×10^4	1.7×10^5	5.4×10^6	4.2×10^7	6.3×10^7	6.8×10^8

[a]ND = Not done.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/50/11/952/1657817/0362-028x-50_11_952.pdf by guest on 25 January 2021

sale the next day. We obtained an increase in growth of *S. aureus* from approximately 10 to 10^4 after 18 h at 30°C and 10^5 after 18 h at 37°C. Both TNase and enterotoxin were detectable in these samples although the enterotoxin level was quite low in the 30°C sample (3.6 ng/g); however, the amount of enterotoxin produced would be sufficient to make a sensitive consumer ill, providing the individual ate at least 50 g of the cream filling.

Freshly prepared creams in Brazil have low bacterial counts with the staphylococci being introduced during the handling of the cream. Staphylococci are not highly competitive organisms *(16,18)*; however, elimination of other vegetative organisms during pre-heating of the cream favors growth of the staphylococci. The predominant growth of sporulated organisms in the cream during incubation indicates that cooking of the cream during preparation serves as a thermic shock which activates the germination of the sporulated forms but destroys the vegetative ones as no vegetative cells could be detected in our heated cream filling. According to Eibler and Kessler *(3)* creams heated at 80 to 100°C could be kept at 5°C for 4 weeks, while storage at 10°C resulted in an increase in count to 10^6 CFU/ml after 8 d with predominance of sporulated microorganisms.

The sporulated microorganisms found in the cream filling may be introduced through some of the ingredients such as the sugar and starch. Even when the sporulated forms predominated, TNase and SEA production were detectable. Malburg and Noleto *(8)* showed that incubation of *S. aureus* with *Bacillus cereus* did result in decreased growth of *S. aureus* strain FRI-100; however, SEA production was detectable both in laboratory medium and meat medium even when the *B. cereus* CFU/ml was greater than the *S. aureus* CFU/ml.

The CFU/g level at which SEA production was first noted was approximately 10^4 which is at least 2 $\log_{10}$ values lower than other investigators have reported for detection of SEA production at 1 ng/g or ml *(13)*. The relatively low inocula used here compared to those used by other investigators could account for detection of SEA at lower total counts. The other possibility is that the major area of growth was the surface with the counts reaching much higher levels, levels nearer those others have found necessary for enterotoxin production. The low inocula levels should be investigated although Noleto et al. *(13)* were unable to detect SEA production in laboratory media at initial counts of 10^2 CFU/ml in the presence of counts of 10^2 CFU/ml of competing organisms when *S. aureus* strain FRI-100 growth reached 10^6 CFU/ml.

The high sucrose content of the cream fillings favors the growth of staphylococci because these organisms can grow at relatively high osmotic pressures and low water activities *(5,8,11,18)*, whereas growth of other microorganisms is partially inhibited under such conditions. Staphylococci utilize carbohydrate as an energy source in preference to proteins or amino acids, and as a result of carbohydrate metabolism acid is released which tends to keep the pH from rising. Thus lack of change in pH is not an acceptable indicator of staphylococcal growth in the cream fillings.

The best test for the safety of a product is to analyze for the presence of enterotoxin; however, this is not possible on a routine basis at the present time in Brazil because of the lack of the necessary enterotoxin reagents. This may be possible in the future as enterotoxin detection kits are becoming available for testing foods for the presence of enterotoxin. Until it is feasible to do routine enterotoxin testing, the only other possible guide available is the testing for TNase. TNase is detectable in most foods before detection of enterotoxin, and, thus, can be used as a screening mechanism for enterotoxin detection in those foods *(14,18)*. Even though TNase testing is not an acceptble screening mechanism for some foods *(1,2,6,9)*, the fact that TNse was detectable in all samples of cream fillings in which SEA was detectable, indicates this test could be used as a screening technique for cream pies.

The possibility of staphylococcal food poisoning occuring from the consumption of cream pies, even from small *S. aureus* inocula, cannot be ignored as is shown by the results presented here. The fact that such foods are kept for a number of hours without refrigeration in many commercial establishments, for example, in Sao Paulo City, is of concern. The situation in rural areas is even more of a problem because of inadequate sanitary conditions. The fact that year-round temperatures in most areas in Brazil range from 20 to 30°C or above indicates the problem to be a continuing one. One can conclude from the results presented here that consumption of baked goods containing cream fillings may result in staphylococcal food poisoning, unless the product is kept refrigerated.

ACKNOWLEDGMENTS

This investigation was supported in part by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison.

REFERENCES

1. Bouwer-Hertzberger, S. A., H. Sol-Vos, D. A. A. Mossel, and H. Mol. 1981. False-negative results in examining foods for staphylococcal thermonuclease. Ant. van. Leeuvoenhoek 47:245-246.
2. Daoud, S. M., and J. M. Debevere. 1984. Effect of growing bacteria isolated from food on staphylococcal growth and thermonuclease activity. Int. J. Food Microbiol. 1:197-204.
3. Eibel, H., and H. G. Kessler. 1984. The storage stability of pasteurized cream. Milchwissenschaft 39:648-651.
4. Freed, R. C., M. L. Evenson, R. F. Reiser, and M. S. Bergdoll. 1982. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in foods. Appl. Environ. Microbiol. 44:1349-1355.
5. Genigeorgis, C., H. Reimann, and W. W. Sadler. 1969. Production of enterotoxin B in cured meats. J. Food Sci. 34:62-68.
6. Gudding, R. 1980. Nucleases of some udder pathogenic organisms. Acta Vet. Scand. 21:256-266.
7. Lachica, R. V. F., C. Genigeorgis, and P. D. Hoeprich. 1971. Metachromatic agar - diffusion methods for detecting staphylococcal nuclease activity. Appl. Microbiol. 21:585-587.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/50/11/952/1657817/0362-028x-50_11_952.pdf by guest on 25 January 2021

8. Malburg, L. M., Jr., A. C. Noleto, and M. S. Bergdoll. 1987. Production of staphylococcal enterotoxin in mixed cultures. (In preparation).
9. Markus, Z. H., and G. J. Silverman. 1970. Factors affecting the secretion of staphylococcal enterotoxin A. Appl. Microbiol. 20:492-496.
10. Medwid, R. D., and D. W. Grant. 1980. Inactivation of staphylococcal thermonuclease by an enzyme-like factor produced by *Streptococcus faecalis* subsp. *liquefacians*. J. Food Prot. 43:201-202.
11. Muller, E. E., O. H. Neto, J. M. Souza, F. A. C. Marques, A. L. Macuco, and W. D. Giacometti. 1978. Estudos da prevalência de mastite bovina. Semina 1:47-48.
12. Niskanen, A. 1977. Staphylococcal enterotoxins and food poisoning. Production/properties and detection of enterotoxins. Technical Centre of Finland. 83 p.
13. Noleto, A. L., and M. S. Bergdoll. 1980. Staphylococcal enterotoxin production in the presence of non-enterotoxigenic staphylococci. Appl. Environ. Microbiol. 39:1167-1171.
14. Park, C. E., H. B. Elderea, and M. K. Rayman. 1978. Evaluation of staphylococcal thermonuclease (TNase) assay as a means of screening food for growth of staphylococci and possible enterotoxin production. Can. J. Microbiol. 24:1135-1139.
15. Santos, E. C., C. Genigeorgis, and T. B. Farver. 1981. Prevalence of *Staphylococcus aureus* in raw and pasteurized milk used for commercial manufacturing of Brazilian minas cheese. J. Food Prot. 44:172-176.
16. Tatini, S. R. 1973. Influence of food environments on growth of *S. aureus* and production of various enterotoxins. J. Milk Food Technol. 36:559-563.
17. Tatini, S. R., B. R. Cords, and J. Gramo. 1976. Screening for staphylococcal enterotoxins in foods. Food Technol. 30:64-74.
18. Troller, J. 1976. Staphylococcal growth and enterotoxin production factors for control. J. Milk Food Technol. 39:449-503.
19. Wieneke, A. A. 1974. Enterotoxin production by strains of *Staphylococcus aureus* isolated from foods and human beings. J. Hyg. 73:225-262.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/50/11/952/1657817/0362-028x-50_11_952.pdf by guest on 25 January 2021

Bentley, et al., *con't. from p. 951*

3. Cox, N. A., J. S. Bailey, and J. E. Thomson. 1983. Evaluation of five miniaturized systems for identifying *Enterobacteriaceae* from stock cultures and raw foods. J. Food Prot. 46:914-916.
4. Cox, N. A., and A. J. Mercuri. 1978. Comparison of two minikits (API and R-B) for identification of *Enterobacteriaceae* isolated from poultry and meat products. J. Food Prot. 41:107-110.
5. Cox, N. A., A. J. Mercuri, M. O. Carson, and D. A. Tanner. 1979. Comparative study of Micro-ID, Minitek and conventional methods with *Enterobacteriaceae* freshly isolated from foods. J. Food Prot. 42:735-738.
6. Cross, H. R., I. Tennent, and D. A. Muse. 1979. Storage properties of primal cuts of hot and cold-boned beef. J. Food Qual. 2:289-296.
7. Davidson, W. D., R. L. Cliplef, R. J. Meade, and L. E. Hanson. 1968. Post-mortem processing treatment on selected characteristics of ham and fresh pork sausage. Food Technol. 22:772-774.
8. Duncan, D. B. 1955. New multiple range and multiple F tests. Biometrics 11:1-7.
9. Ferguson, E. J., and R. L. Henrickson. 1979. Final report on energy conservation in the meat processing industry. U.S. Dept of Energy, Contract EY-76-5-05-5097.
10. Fung, D. Y. C., C. L. Kastner, M. C., Hunt, M. E. Dikeman, and D. H. Kropf. 1980. Mesophilic and psychrotrophic bacterial populations on hot-boned and conventionally processed beef. J. Food Prot. 43:547-550.
11. Krieg, N. R., and J. G. Holt. (ed.). 1984. Bergey's manual of systematic bacteriology, 9th ed. The Williams and Wilkins Co., Baltimore, Maryland.
12. Lee, C. Y., D. Y. C. Fung, and C. L. Kastner. 1982. Computer assisted identification of bacteria on hot-boned and conventionally processed beef. J. Food Sci. 47:363-367.
13. Lin, H. S., D. G. Topel, and H. W. Walker. 1976. Influence of pre- and postrigor processing on the quality of pork sausage. J. Anim. Sci. 42:1346.
14. Lin, H. S., D. G. Topel, and H. W. Walker. 1979. Influence of prerigor and postrigor muscle on the bacteriological and quality characteristics of pork sausage. J. Food Sci. 44:1055-1057.
15. Reagan, J. O., F. H. Liou, A. E. Reynolds, and J. A. Carpenter. 1983. Effect of processing variables on the microbial, physical and sensory characteristics of pork sausage. J. Food Sci. 48:146-149.
16. Siegel, D. G., D. M. Theno, and G. R. Schmidt. 1976. Protein extraction during ham massaging. J. Anim. Sci. 42:1347.
17. Snedecor, G. W. 1957. Statistical methods, 5th ed. The Iowa State College Press, Ames, Iowa.
18. Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dougan, Jr. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J. Am. Oil Chem. Soc. 37:44-48.
19. Theno, D. M., D. G. Siegel, and G. R. Schmidt. 1976. Micro structure of sectioned and formed ham. J. Anim. Sci. 42:1347.