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Evaluation of Factors Involved in Antibotulinal Properties of Pasteurized Process Cheese Spreads

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ABSTRACT

Pasteurized process cheese spreads with various levels of sodium chloride, disodium phosphate, moisture and pH were challenged with spores of *Clostridium botulinum* types A and B. Response surface methodology was used to design experiments that would yield maximum results with the minimum number of trials. Supplemental experiments were added to further clarify the response and to examine combinations of special interest. A total of 304 treatment combinations (batches) was incubated at 30°C, and five samples from each batch were taken at predetermined intervals up to 42 wk of incubation and tested for botulinal toxin. Sodium chloride and disodium phosphate inhibited botulinal toxin production with similar effectiveness. The inhibitory effect of low pH (<5.7) and low moisture (<54%) levels on botulinal toxin production was as expected, i.e., as either pH or moisture went up, it was necessary to increase sodium chloride and/or phosphate concentrations to compensate. Differences in water activity between cheese spreads with different compositions were observed but they were too small to use for controlling the properties of the products, e.g., a range of 9% in moisture level (51 to 60%) produced only 0.022 variation in water activity. Combinations of the above factors were developed for safe pasteurized process cheese spreads containing up to 60% moisture.

Pasteurized process cheese spreads (cheese spreads) have been on the market for many years, mostly as shelf-stable products in hermetically sealed containers. They have a relatively high pH (generally pH 5.4 to 6.0) and a moisture content of about 50%. They are not sterile, yet they have an excellent safety record with regard to the hazard of botulism. Studies addressing the botulism hazard of such products have been published by Wagenaar et al. (*16-19*), Kautter et al. (*8,9*), Tanaka et al. (*12,13*) and Briozzo et al. (*2*). These studies revealed that growth and toxin production by *Clostridium botulinum* are dependent on the variety of cheese, moisture, salt, pH and added sodium phosphate or sodium citrate, but the relationship of these factors in preventing botulinal toxin production is not clear in these references.

Cheese spreads do not conform to the federal regulation for low acid canned foods (*1*) which prescribes that foods in hermetically sealed containers with pH above 4.6 and water activity (a_w) higher than 0.85 are required to undergo a heat treatment sufficient to destroy spores of *C. botulinum*. Such a heat treatment would destroy cheese spreads as they are currently produced.

This experiment was designed to clarify the effects of sodium chloride, disodium phosphate, pH and moisture, singly and in combination, on toxin production by *C. botulinum*. The experiments were designed and results were analyzed statistically, and mathematical models were derived using logistic regression (*4*) to organize the results and to enable prediction of the effect of formulation changes on safety. Mathematical models predicting the safety of cheese spreads will be presented elsewhere. In addition, a similar study with bacteriological media, the relationship of cheese spreads components and water activity, and an improved procedure for water activity determination will be reported elsewhere.

MATERIALS AND METHODS

Cheese blend

A blend of 72.5% American cheese, 1.24% butterfat, 3.03% nonfat dry milk, 5.42 whey powder, 1.01% whey protein concentrate, 16.8% water and 0.04% mixed color, with a target moisture of 44%, was prepared and thoroughly blended for uniformity, and ca. 1.36-kg (3-lb) quantities were sealed in moisture-tight plastic containers. A total of 688 1.36-kg units was kept frozen until used.

Pasteurized process cheese spreads (cheese spreads)

The cheese blend was thawed at 4°C just before preparing the cheese spreads. The quantity of cheese spread prepared for each experimental batch was 1.75 to 2.5 kg, depending on the experiment. Cheese blend, sodium chloride, disodium or-

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thophosphate, and water were added to a steam-jacketed stainless steel kettle equipped with an agitator with plastic scrapers, the cover was closed, and the agitator and steam were turned on. As the contents melted, an amount of hydrochloric acid or sodium hydroxide required to adjust the pH was dissolved in the additional water needed to achieve the target level of moisture, and was added slowly to the melted mixture. Where lactic acid was used, the acid was added at this point by diluting it in water used to adjust moisture. The mixture was brought to 88°C with agitation, and held for 2 min before spores of *C. botulinum* were added. The temperature of the mixture was kept at 88°C for an additional 2 min, then the product was poured hot into 20-ml glass vials, capped tightly, and cooled at 4°C.

Concentrations of ingredients

Sodium chloride (total, i.e., NaCl from cheese blend plus NaCl added), disodium phosphate (added) and hydrochloric acid or sodium hydroxide concentrations were calculated (*3*) as "percent solids in water phase":

$$\text{Percent in water phase} = \frac{\text{Percent in whole} \times 100}{\text{Percent in whole} + \text{Percent in water}}$$

The crystalline water in disodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), water from hydrochloric acid (37% HCl, specific gravity 1.18), and the water from the cheese blend were all calculated into the target moisture level. Sodium chloride was adjusted by adding the required amount of NaCl to NaCl from the cheese blend to achieve the "total" target level. Hydrochloric acid and sodium hydroxide were used to adjust the pH to minimize inhibitory effects other than those due to pH on *C. botulinum*. Many organic acids are known to have antibacterial properties beyond the effect of pH.

Preparation of C. botulinum *spores*

Five strains each of *C. botulinum* types A (56A, 62A, 69A, 77A and 90A) and B (53B, 113B, 213B, 13983B and Lamanna-okra B) were used (*14*). Spores suspended in sterile water (ca. 5 ml; exact quantity depended on the spore concentration and the amount of cheese spread prepared) were added dropwise to the heated (88°C) cheese spread under agitation. The target spore concentration was 1,000 spores/g of product.

Extraction and assay of botulinal toxin

Each test sample taken from a vial (3 g) was mixed with 6 ml of gelatin-phosphate buffer, pH 6.2. After thorough mixing, the preparation was centrifuged at 5,000 × *g* for 10 min and the aqueous supernatant fluid was tested for toxin by mouse bioassay (*5,13*). Each of two mice was injected intraperitoneally with 0.5 ml of the extract from the test sample. Mice were held up to 4 d. Deaths were confirmed with two additional mice that were challenged with a sample-antitoxin mixture (types A and B) which was preincubated at 37°C for 30 min. Unprotected controls were used together with the protected mice.

Trypsinization

Montville (*10*) and Wagner and Busta (*20*) have reported that the mouse bioassay can be made more sensitive by treating samples with trypsin. Trypsinization was done according to the *Bacteriological Analytical Manual* procedure (*5*) using duplicate samples for all trials.

Enumeration of C. botulinum

The five-tube most probable number (MPN) method using Trypticase peptone glucose yeast extract broth as the growth medium (*5*) was used to determine the initial numbers of *C. botulinum*. Tubes showing bacterial growth were tested for the presence of toxin by the mouse bioassay. Only toxin-positive tubes were counted as growth-positive in determining the MPN of *C. botulinum*. In some cases, an anaerobic plate count using Wynne-EY agar (McKnur Laboratories, Ottawa, Canada) with dithiothreitol overlay described by Hauschild and Hilsheimer (*6*) was used with satisfactory results (Tanaka, unpublished data).

Incubation and sampling schedule

Samples were incubated at 30°C. Five samples from each batch were taken for botulinal toxin assay after each of 4, 5.5, 8, 11, 15, 21, 30 and 42 wk of incubation and pH was determined on at least three of the five samples. A composite of five samples of each batch was assayed for botulinal toxin before the start of incubation (0-time). At 1 d after preparation, the pH was determined on at least three individual samples from each batch. This 1 d delay for 0-time reading of pH was necessary for pH levels to become stable. Enumeration of *C. botulinum* was done on one sample from each batch at 0-time.

Other analyses

pH was measured with an Altex (Model 3560, Beckman Instruments, Irvine, CA) digital pH meter with a Futura combination electrode that was immersed directly into the sample. The pH meter was standardized using pH 7.00 and 4.00 buffers (Fisher Scientific Co., Chicago, IL).

Chemical constituents were measured by Association of Official Analytical Chemists procedures (*7*): moisture, item 16.233; chloride with KSCN, item 16.272; phosphorus; gravimetric quinolinium molybdophosphate procedure, item 2.026-2.028; fat, item 16.255.

Water activity was determined using a Beckman Hygroline apparatus (Beckman Industrial, Cedar Grove, NJ) with an improved procedure which accurately determined water activity to the third digit. The procedure was developed in our laboratory (manuscript in preparation).

Statistical treatment

Response surface methodology (RSM) (*11*) was used to design many of the experiments, often supplemented by a grid design. Logistic regression (*4*) was used to analyze the results and to produce mathematical models for predicting the botulinal safety of cheese spreads with different compositions.

RESULTS AND DISCUSSION

Composition of the cheese blend

The cheese blend was assayed for sodium chloride, phosphorus and moisture content. Three samples were randomly taken and duplicate determinations were done on each. Averages and standard deviations (in parentheses) of the six measurements for each of the three analyses were as follows: NaCl, 1.415% (0.008%); moisture, 43.67% (0.34%); and phosphorus, 0.421% (0.007%). All calculations for the added ingredients of the cheese spreads were based on these moisture and sodium chloride contents.

Water activity of cheese spreads

Water activity (a_w) of cheese spreads did not change much with changing compositions. This was evident

when large changes in moisture, up to 9%, resulted in small changes in a_w, maximum of 0.022 units. Water activity ranged from 0.940 to 0.962, moisture from 51 to 60%, NaCl from 1.23 to 3.95% (in whole) and Na_2HPO_4 from 1.31 to 3.35% (in whole).

Effect of water activity on toxin development

As mentioned earlier, a_w of cheese spreads prepared in this study ranged from 0.940 to 0.962. When a_w was at or below 0.944, no toxin formation was observed, and with a_w at higher than 0.957, all the products developed toxicity. Either a positive or negative response was observed when the a_w was between these two ranges (Fig. 1). While these observations indicate that a_w might play an important role in determining the safety of cheese spreads, two factors must be kept in mind: (a) the uncertainty of a_w measurements in cheese systems and (b) these observations were made on limited ranges of other variables. For instance, the highest pH we tested when a_w was at or below 0.943 was pH 5.98. One cannot predict if a safe product would still be made at pH higher than 5.98. Water activity levels, like other parameters such as pH, NaCl and phosphate concentrations, must be considered in relationship to other factors. Also, as mentioned above, a_w is a function of a combination of ingredients, hence it is unreasonable to consider a_w as an isolated entity.

Spore distribution

Distribution of spores within the samples was relatively uniform. With the inoculation target level of 1,000 spores/g, the 0-time count was generally between 1.7×10^2 and 1.1×10^3 spores/g. Spores also were enumerated in 0.2-g samples taken from four vials of each of two batches of cheese spread to see if the spores were uniformly distributed. Counts of the eight samples were consistent, averaging 3.2×10^2 spores/g, with a range of 1.7×10^2 to 7.0×10^2 spores/g, and a standard deviation of 1.87.

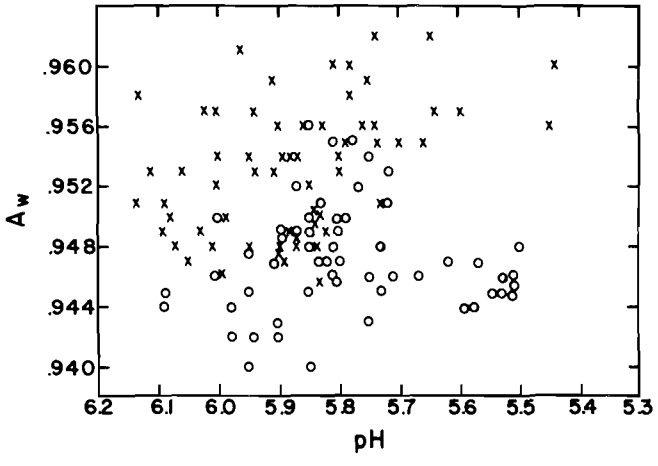

Figure 1. *Development of toxicity in each batch was plotted relative to the a_w and pH of process cheese spreads. Open circles indicate those batches that did not produce toxic samples throughout 42 wk at 30°C. X's indicate batches that produced one or more toxic samples during incubation.*

Trypsinization

Montville (*10*) and Wagner and Busta (*20*), working with low protein systems, showed that the sensitivity of the mouse assay can be increased by treating samples with trypsin. Although pasteurized process spreads have a substantial amount of protein, trypsinization was tried.

Ten samples of the cheese blend used in our experiments, and ten samples of a commercial cheese spread were trypsinized to learn if there might be a problem with nonspecific deaths. All samples were done in duplicate for a total of 40 trials. None of the untrypsinized controls gave mouse deaths, but 18 of 20 trypsinized cheese blend samples, and 17 of 20 trypsinized commercial cheese spread samples killed mice. It was apparent this procedure would not be suitable for the botulinal trials.

The problem of non-specific deaths with trypsinization has occurred twice before in this laboratory. Tanaka et al. (*15*) reported trypsin-treated bacon samples leading to non-specific deaths. M. P. Doyle (personal communication) reports the same problem in studies on pasta.

Botulinal toxin development in cheese spreads and estimate of the safety of products

All four factors examined (sodium chloride, disodium phosphate, pH and moisture content) had significant effects on toxin production by *C. botulinum* in cheese spreads. As expected, the tendency for toxicity development increased as NaCl or phosphate level was lowered, pH was raised, or moisture content was increased.

The results of the study are summarized in Figures 2 through 7. Each figure represents products with a fixed level of moisture. The pH is plotted vs. "NaCl + phosphate" concentration. Combined "NaCl + phosphate" was used because statistical analyses revealed that the effect of sodium chloride on toxin production was quite similar to that of disodium phosphate, and the effects of NaCl and phosphate were additive within the range of the study. Graphical representation thus becomes much simpler when these two entities are treated together. In these graphs, percent "NaCl + phosphate" in whole cheese spread was plotted rather than their percentage in the water phase. This was done for convenience of usage. When one or more samples of a batch of product were confirmed to be toxic during the 42-wk incubation, the batch was considered toxic and marked with an "X" in the figures. When none of the forty samples of a batch was toxic, the batch was considered to be safe and marked with an "O" in the figures. The curved line within each of the figures results from a mathematical model estimating the boundary that separates the combinations that are likely to produce safe products from those for unsafe products.

This mathematical model was developed using logistic regression. Logistic regression is appropriate when the response is binary (e.g., toxic or non-toxic) and can be used to predict the probability of a given response (e.g.,

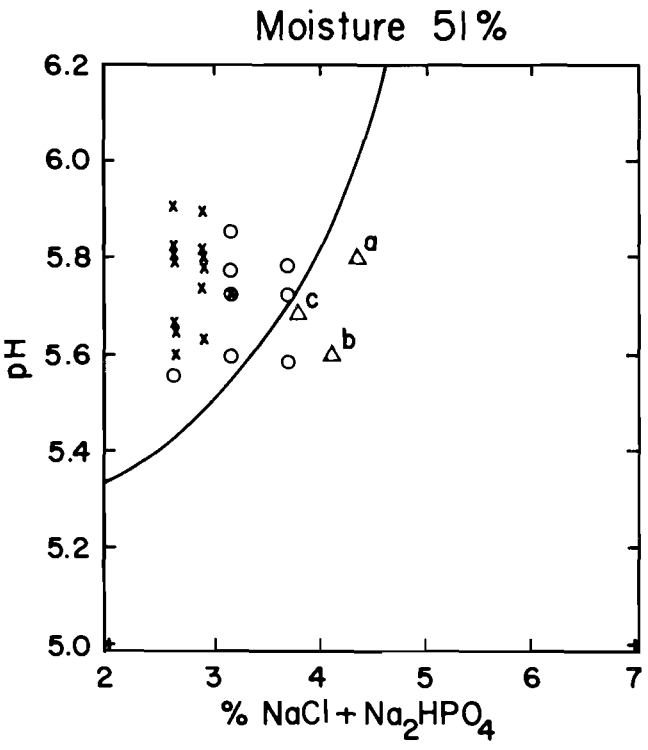

Figure 2. *Development of botulinal toxicity in each batch of cheese spread was plotted against percent "NaCl + phosphate" in whole vs. pH. The moisture content of the products was 51%. The solid line represents the boundary between the treatment combinations that would be free of a botulinal hazard from those combinations that would be potentially hazardous. A quadratic mathematical model was used to locate the line. Open circles indicate batches that did not produce toxic samples during 42 wk of incubation at 30°C. X's indicate batches that produced one or more toxic samples during incubation. Three triangles (marked a, b and c) represent results of three commercial cheese spreads.*

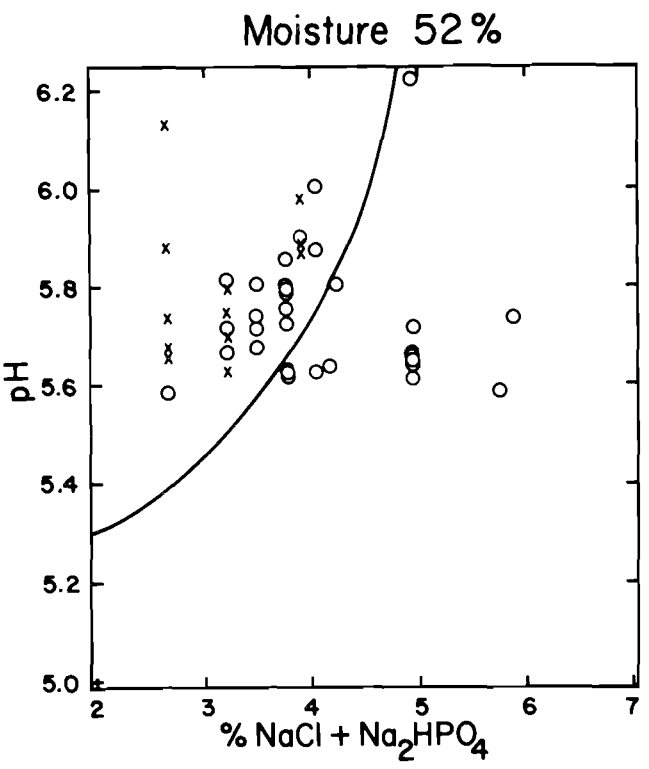

Figure 3. *Same as Figure 2, except moisture is 52%.*

toxicity). This quadratic logistic model was developed by determining which terms (ingredients, squares of the ingredients and interactions between the ingredients) contributed significantly to the correct prediction of toxicity. The model, once developed, can be used to predict at what time a given proportion of a combination of ingredients will become toxic. Because this experiment lasted 42 wk, the line in the plots displays which combinations of ingredients can be expected to become toxic within 42 wk of incubation at 30°C.

All the products that produced toxic samples are located to the upper left side of the boundary line. Because a conservative estimate was used to determine the boundary, any combination of the ingredients that lies to the lower right of this line can be expected to produce products that are free of a botulism hazard. The model presented here is the most conservative estimate in which experimental deviation within our study is considered. For this reason many combinations that did not produce toxic samples fell above and to the left of the boundary curves. As the moisture level increases, this boundary moves toward the lower right, meaning that it becomes more difficult to produce safe combinations when mois-

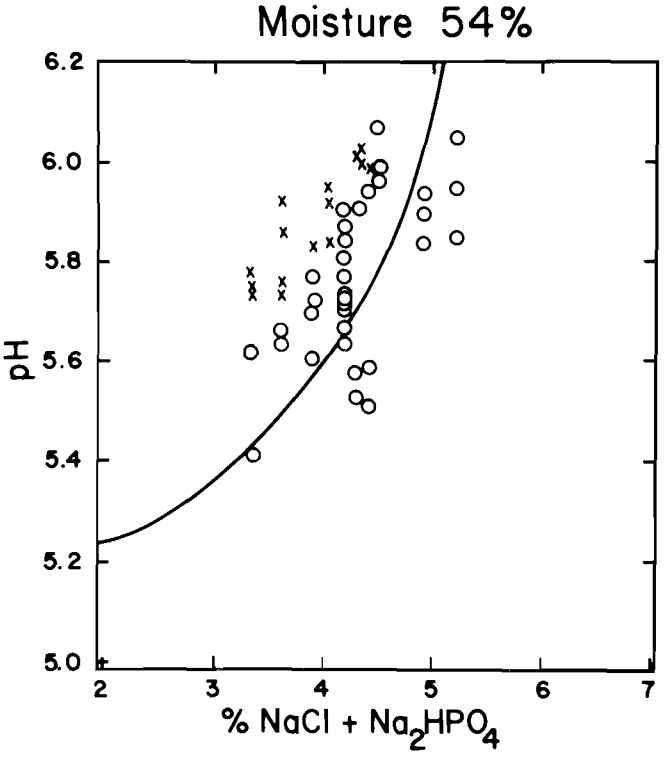

Figure 4. *Same as Figure 2, except moisture is 54%.*

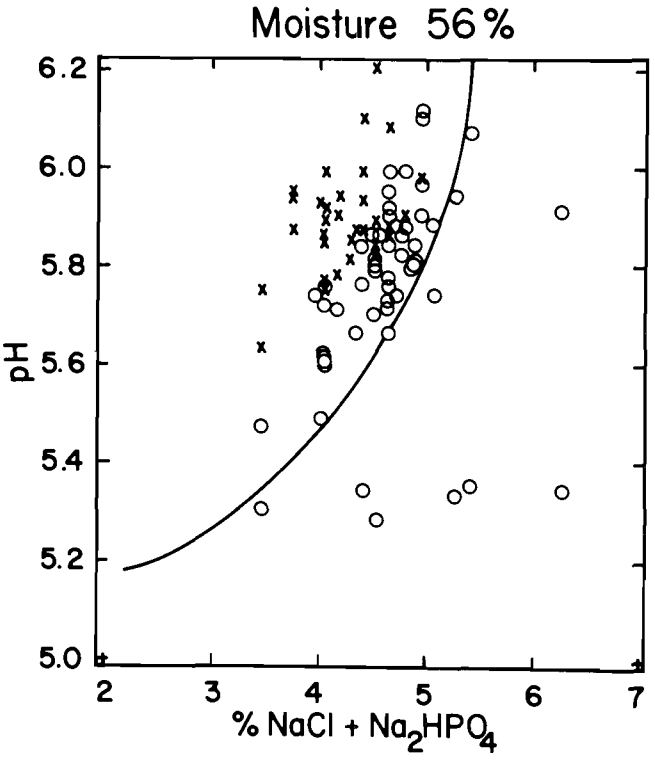

Figure 5. *Same as Figure 2, except moisture is 56%.*

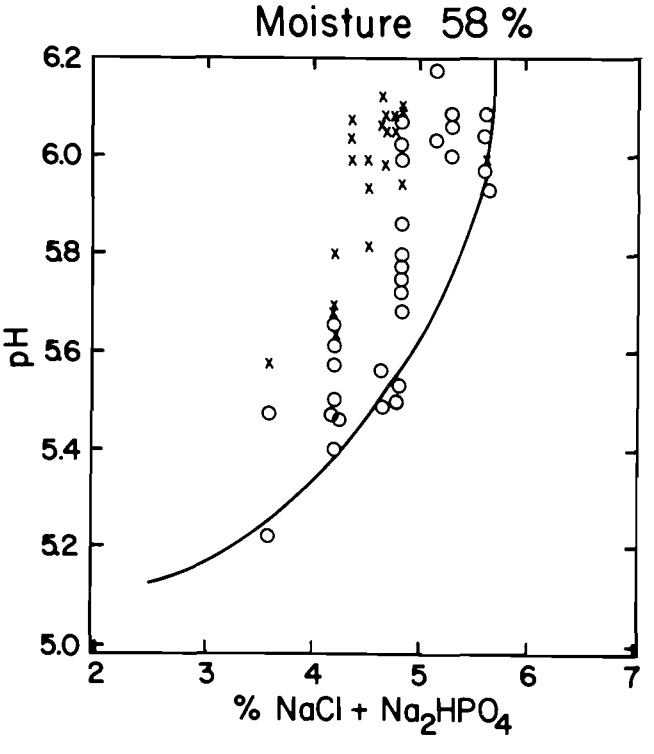

Figure 6. *Same as Figure 2, except moisture is 58%.*

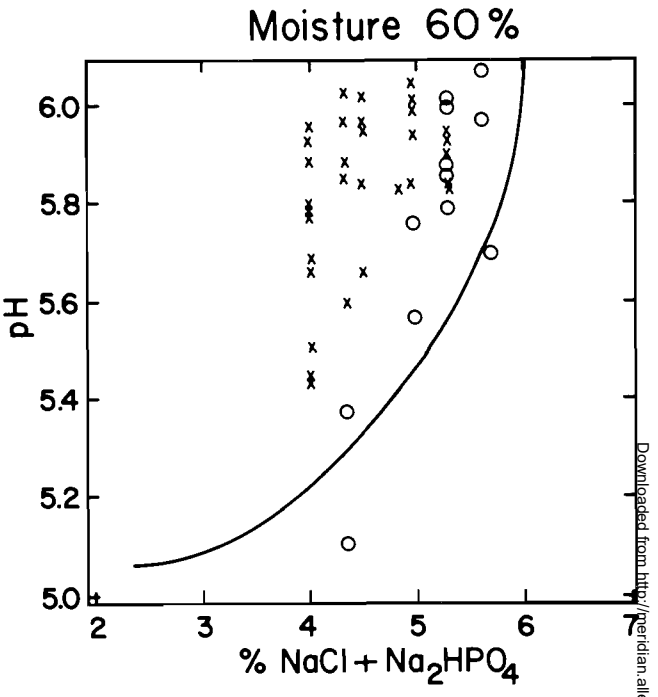

Figure 7. *Same as Figure 2, except moisture is 60%.*

ture levels increase. Results of three current commercial products containing ca. 51% moisture are plotted in Figure 2. Their ingredient combinations are within the safe area, and the history of these products indicates consistent safety.

Effect of lactic acid on toxin development

The addition of lactic acid (0.25%) helped inhibit toxin formation. However, this amount of lactic acid reduced pH by an average of ca. 0.17 pH units. Statistical analysis revealed that lactic acid likely had a slight additional inhibitory effect on botulinal toxin production beyond its effect on pH, but the observed inhibition was largely attributed to the reduction of pH. Therefore, the addition of lactic acid should be considered mainly as a convenient way to achieve reduced pH levels.

Comparison with other studies

Results obtained in this study agree well with data reported by Briozzo et al. (*2*), Kautter et al. (*8,9*), Tanaka et al. (*12,13*) and Wagenaar and Dack (*16*). Briozzo et al. (*2*) reported detecting botulinal toxin in a medium composed of 1.52% NaCl, 5.08% Joha S9 emulsifying salt or sodium tripolyphosphate, and 9.66% cheese whey powder, with pH 5.7 and a_w 0.965, inoculated with 10^2 to 10^4 *C. botulinum* spores/g and incubated at 30°C. When NaCl and Joha salt concentrations were increased to 2.37% and 8.97%, respectively, and 1.97% glycerol was added to lower the a_w to 0.949, no toxin formation was observed. In our study, when a_w was as high as 0.960, all the products became toxic (Fig. 1), but at a_w 0.950, toxin formation depended upon other factors in-

cluding NaCl, phosphate, moisture level and pH. Tanaka et al. (*13*) reported that toxin was formed in cheese spread with pH 5.97, 58.28% moisture and 4.5% NaCl plus phosphate, but no toxin formation was observed in cheese spread with pH 6.00, 53.57% moisture and 4.64% NaCl plus phosphate. The former composition was well above and to the left of the safety boundary curve shown in Figure 6 where we observed toxin formation in this study also, and the latter can be placed in the area slightly above and to the left of the safety boundary curve in Figure 4, where we observed many nontoxic combinations. Most of the data presented by Wagenaar and Dack (*16*) were of cheese spreads in higher pH areas than the pH range used in our study, but when pH values were relatively low (<pH 6.0) where we have most of our data, brine concentrations had to be below 6.5% for cheeses to produce toxic samples. The brine concentration of 6.5% corresponds to 3.75% salts in whole if the moisture content is 54%; this is an area where we observed toxin formation in some cases (Fig. 4). The data from the "post-process" inoculation of botulinal spores study reported by Tanaka et al. (*13*) also agree well with these new data if added moisture with "post-process" inoculation is considered.

Conclusion

It is apparent that a single factor is not responsible for the safety of unsterilized pasteurized process cheese spreads. The lower the moisture, the safer the product becomes. Higher NaCl plus phosphate concentrations produce safer products. Lower pH levels increase safety. High moisture products can be produced safely if the pH is low enough and the NaCl plus phosphate concentrations are high enough. Our data indicate conditions to the right of the curves (Figs. 2 to 7) that are safe. When moisture levels are as high as 58 to 60%, a small change in formulation can lead to unsafe conditions, hence it is prudent to allow a reasonable margin for error by not crowding the curves too closely. If new ingredients or changes in processing conditions are introduced, a new safety study should be conducted.

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REFERENCES

1. Anonymous. Code of federal regulations, No. 21 (revised Apr. 1977). Section 108. Emergency permit control, and Section 113, thermally processed low-acid foods in hermetically sealed containers. U. S. Government Printing Office, Washington, DC.
2. Briozzo, J., E. A. de Lagarde, J. Chirife, and L. Parada. 1983. *Clostridium botulinum* type A growth and toxin production in media and process cheese spread. Appl. Environ. Microbiol. 45:1150-1152.
3. Cerveny, J. G. 1980. Effects of changes in the production and marketing of cured meats on the risk of botulism. Food Technol. 34(5):240-243.
4. Cox, D. R. 1970. The analysis of binary data. Methuen, London.
5. Food and Drug Administration. 1976. Bacteriological analytical manual for foods, 5th ed. Association of Official Analytical Chemists, Arlington, VA.
6. Hauschild, A. H. W., and R. Hilsheimer. 1977. Enumeration of *Clostridium botulinum* spores in meats by a pour-plate procedure. Can. J. Microbiol. 23:829-832.
7. Horwitz, W. (ed). 1980. Official methods of analysis, 13th ed. Association of Official Analytical Chemists, Washington, DC.
8. Kautter, D. A., T. Lilly, Jr., R. K. Lynt, and H. M. Solomon. 1979. Toxin production by *Clostridium botulinum* in shelf-stable pasteurized process cheese spreads. J. Food Prot. 42:784-786.
9. Kautter, D. A., R. K. Lynt, T. Lilly, Jr., and H. M. Solomon. 1981. Evaluation of the botulism hazard from imitation cheeses. J. Food Sci. 46:749-750, 764.
10. Montville, T. J. 1983. Dependence of *Clostridium botulinum* gas and protease production on culture conditions. Appl. Environ. Microbiol. 45:571-575.
11. Myers, R. H. 1971. Response surface methodology. Allyn and Bacon, Boston.
12. Tanaka, N. 1982. Challenge of pasteurized process cheese spreads with *Clostridium botulinum* using in-process and post-process inoculation. J. Food Prot. 45:1044-1050.
13. Tanaka, N., J. M. Goepfert, E. Traisman, and W. M. Hoffbeck. 1979. A challenge of pasteurized process cheese spread with *Clostridium botulinum* spores. J. Food Prot. 42:787-789.
14. Tanaka, N., E. Traisman, M. H. Lee, R. G. Cassens, and E. M. Foster. 1980. Inhibition of botulinum toxin formation in bacon by acid development. J. Food Prot. 43:450-457.
15. Tanaka, N., L. Meske, M. P. Doyle, E. Traisman, D. W. Thayer, and R. W. Johnston. 1985. Plant trials of bacon made with lactic acid bacteria, sucrose and lowered sodium nitrite. J. Food Prot. 48:679-686.
16. Wagenaar, R. O., and G. M. Dack. 1955. Studies on canned cheese spread experimentally inoculated with spores of *Clostridium botulinum*. Food Res. 20:144-148.
17. Wagenaar, R. O., and G. M. Dack. 1958. Factors influencing growth and toxin production in cheese inoculated with spores of *Clostridium botulinum* types A and B. I. Studies with surface-ripened cheese type I. J. Dairy Sci. 41:1182-1190.
18. Wagenaar, R. O., and G. M. Dack. 1958. Factors influencing growth and toxin production in cheese inoculated with spores of *Clostridium botulinum* types A and B. II. Studies with surface-ripened cheese type II. J. Dairy Sci. 41:1191-1195.
19. Wagenaar, R. O., and G. M. Dack. 1958. Factors influencing growth and toxin production in cheese inoculated with spores of *Clostridium botulinum* types A and B. III. Studies with surface-ripened cheese type III. J. Dairy Sci. 41:1196-1200.
20. Wagner, M. K., and F. F. Busta. 1985. Inhibition of *Clostridium botulinum* 52A toxicity and protease activity by sodium acid pyrophosphate in media systems. Appl. Environ. Microbiol. 50:16-20.