Contribution of Composition, Physicochemical Characteristics and Polyphosphates to the Microbial Safety of Pasteurized Cheese Spreads

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ABSTRACT

Pasteurized process cheese spread was manufactured with moisture contents of 52, 54, 56 and 60%. Three different types of phosphate emulsifier were used, disodium ortho-phosphate and two commercially-available polyphosphates, S9 and S9H. Pasteurized, processed cheese spreads were inoculated with approximately $1 \times 10^6$ Clostridium botulinum spores/gram cheese in the cook kettle, held 3 min at 80°C, hot-filled into glass containers, and incubated at 30°C. Samples were analyzed over 30 weeks for growth of C. botulinum and toxigenesis. Toxin was first detected in 60% moisture cheese with disodium ortho-phosphate as the emulsifier at 8 weeks and in 60% moisture cheese with the test polyphosphates as the emulsifier when tested at 20 weeks. None of the other cheese formulations were toxic at 20 weeks. Toxin production correlated statistically to time, moisture, pH and phosphate type.

Key Words: Clostridium botulinum, polyphosphates, phosphates, processed cheese

MATERIALS AND METHODS

Clostridium botulinum cultures and spores

Five strains of C. botulinum Type A (A1, A36, A52, A62, A69) and two proteolytic Type B (SLR 1364 and SLR 1365) from the Silliker Laboratories Research Culture Collection (SLR) were used. These organisms were propagated on Liver Veal Agar (LVA) incubated anaerobically at 35°C. Spores were harvested from the LVA plates by swabbing with cotton swabs into phosphate buffered saline (PBS). Spores were resuspended in PBS and centrifuged twice. Spores were stored frozen at -20°C, heat-shocked for 10 min at 80°C, cooled rapidly in an ice water bath at 1°C, and stored at 4°C for a maximum of 6 h. Spore counts were determined by Direct Microscopic Count (5) and Most-Probable-Number (MPN) analysis (2).

Cheese blend

The cheese base blend was prepared according to Tanaka et al. (15). The cheese blend consisted of 72.5% Cheddar cheese (Rochester Cheese, Rochester, MN), 1.24% butterfat (Land O'Lakes, Minneapolis, MN), 3.03% non-fat dry milk (Continental Colloids, Inc., West Chicago, IL), 5.42% whey powder (Continental, 1.01% whey protein concentrate (Continental), 16.8% water and 0.04% mixed color (Continental). The target moisture was 43.67%. The mixture was blended at 60°C (Stephan UCM-12, Hameln, FRG) until homogeneous and then sealed in 3 lb quantities in moisture-tight plastic containers. The base blends were frozen at -20°C until used.

Pasteurized processed cheese spreads

The cheese spread consisted of the cheese blend, NaCl, one of the phosphate types and water. The cheese base blend was thawed at 4°C for 16 h before use. The phosphates used were disodium ortho-phosphate (S9763, Sigma Chemical Co., St. Louis, MO) and two polyphosphates (S9 and S9H; BK Ladenburg GmbH, FRG). The NaCl (S9625, Sigma) target level was 1.415%. Phosphorus, from the disodium phosphate or poly-phosphate, was added at a target level of 0.421%. The ingredients were heated to 80°C and blended (Stephan UCM-12 with vacuum pump 006-316, digital thermometer PDAW 48/100/B3, probe 12.08-12, control D8, Hameln, FRG). The pH was adjusted to pH 5.6 with hydrochloric acid or sodium hydroxide mixed with the additional deionized water necessary to achieve the target moisture as well as the target pH values of the cheese spreads. The cheese mixture was held at 80°C under continuous slow agitation (500 RPM) for 3 min before packaging into sterile glass containers with wax-
coated, cardboard lids and plastic caps (Qorpak® 2 oz. bottles 147-637, Curtis Matheson Scientific, Inc., Houston, TX).

Cheese spread composition

The compositions of the finished cheese spreads were analyzed (1) for protein (AOAC 920.123), fat (AOAC 933.05), moisture (AOAC 926.08), ash (AOAC 935.42), total NaCl (as chloride ion, AOAC 975.20), sodium (AOAC 975.03), phosphate (AOAC 958.01) and magnesium (AOAC 975.03). All values are reported on a whole cheese spread wet basis. Percentages reported are also on a wet basis for the cheese spread and not as percent in the water phase.

Inoculation of cheese spread with C. botulinum spores

The molten cheese spread was held under slow agitation (500 RPM) for 3 min at 80°C to ensure even temperature throughout the cheese mass. Spores of C. botulinum Types A and B were added at a level of 10,000 spores/g cheese spread. The C. botulinum spores were incorporated into the cheese spread by agitation at 80°C for an additional 3 min. The cheese spread was poured at 80°C into sterile glass containers with wax-coated, cardboard lids and plastic caps. Samples were inverted, slowly cooled to room temperature, and then placed under incubation at 30°C.

Sample analysis

The samples of cheese spread were analyzed as per Tanaka et al. (15). Samples were taken initially and at 2, 4, 6, 8, 10, 12, 15, 20 and 30 weeks. All samples were analyzed for pH, water activity, moisture, salt, total aerobic and anaerobic population levels. Total aerobic population levels were determined using All-Purpose Tween agar (APT) with incubation at 35°C for 48 h. Anaerobic plate counts for C. botulinum were performed with liver veal agar (LVA) with incubation at 35°C for 48 h and representative colonies confirmed with An-IDENT® strips (API® Analytab Products, Sherwood Medical, Plainview, NY). Inoculated samples analyzed for C. botulinum were tested in duplicate.

Samples were analyzed for C. botulinum toxin by mouse pathogenicity bioassay initially and at 8 and 20 weeks. Toxin production was assessed using the mouse bioassay (3) with slight modifications in the i) number of samples taken, ii) no trypsinization treatment and iii) number of mice injected. A composite of five independent replicate samples were used to obtain a larger sample size from a larger sample population. Although research has indicated that trypsinization of samples can improve the sensitivity of the mouse assay (11,21), samples were not trypsinized based on previous research indicating non-specific death induced by the treatment in the case of cheese spreads (15).

All mice were observed for symptoms and death for 72 h. Samples were declared positive if mice presented typical symptoms and there were no instances of death in protected mice (3,5).

Phosphatase analyses

A Fluorophos® Test System (Advanced Instruments, Needham Heights, MA) was used for phosphatase analysis. The system consisted of a fixed filter fluorometer (excitation 439 nm, emission 560 nm) with a constant temperature controlled sample chamber, round-bottom, borosilicate cuvettes (12 mm x 75 mm), fixed volume mechanical pipettes (2.0 and 0.075 ml), and a 20-well, dry, cuvette incubation block equipped with a thermostat and regulator. Emitted light was detected by a photomultiplier tube and digitized by an analog-digital converter over a 3-min period, and then compared to a standardized regression curve programmed into the software.

Cuvettes containing 2.0 ml of Fluorophos reagent were warmed for 10 min to 38 ± 0.1°C in the heating block. A 0.5 g cheese sample was added to a 16 mm x 100 mm screw-cap, borosilicate glass test tube. A 5.0 ml volume of cheese extraction buffer (FLA005; Advanced Instruments) was added to the test tube and the cheese sample was macerated with a sterile glass rod. The cheese-buffer mixture was cooled for 10 min in an ice bath and then centrifuged at 1,000 g for 10 min. A 0.075 ml volume was removed from the supernatant layer, dispensed into the Fluorophos reagent, mixed by vortexing, placed into the fluorometer, the appropriate product test program chosen and the assay run. After 3 min the average increase in fluorescence and phosphatase activity in mU/L was displayed and recorded by an internal printer on thermal paper.

Statistical analysis

An analysis of variance (α = 0.05) was performed using a multiple regression model analyzing toxigenesis as a function of time (week of analysis), pH, water activity, moisture, percent salt and phosphate, and phosphate type. A second analysis of variance (α = 0.05) eliminating the variable of time was performed using a multiple regression model analyzing toxigenesis as a function of pH, water activity, moisture, percent salt and phosphate, and phosphate type.

RESULTS AND DISCUSSION

This study agrees well with and confirmed findings of earlier research in several studies using disodium phosphate as an emulsifying salt in cheese spreads.

Composition

The cheese spread compositions at each moisture level are summarized in Table 1. All processed cheeses had moisture levels close to target values given in previous research (14). The increase in percent moisture levels caused a small proportional decrease in the percent fat, protein and salt.

Statistical analysis

The data were first analyzed by evaluating toxin as a function of pH, water activity, moisture, percent salt and phosphate, and phosphate type using a multiple regression model and an analysis of variance (α = 0.05 level). When all data and variables were grouped together for analysis, time (week of analysis), moisture level and pH had a significant effect (p < 0.05) on toxin production (Table 2). The type of phosphate used in the cheese spread was statistically significant at 8 weeks. At 8 weeks only the

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control disodium phosphate cheese spreads at 60% moisture were toxic (Fig. 1), whereas all 60% moisture cheese spreads were positive at 20 weeks. The statistical significance of time was expected since toxin could be elaborated during product incubation and would likely be detected the longer samples are incubated. The period of time until toxin was detected in the control disodium phosphate cheese spread was toxic at Week 8. At Week 20 all 60% moisture cheese spreads were toxin-positive, which weighted the data towards moisture level because the toxin results were equivalent regardless of phosphate type. Tanaka et al. (14,15) concluding that 54 to 56% moisture is the borderline formulation with regard to risk of C. botulinum toxigenesis in pasteurized, processed cheese spreads. At these moisture levels some samples developed toxicity. There were instances where samples developed toxicity at 51, 52 and 54% moisture, but these tended to be at higher pH values, lower salt and lower phosphate levels.

In the present study, toxin production was observed in the cheeses made to contain 60% moisture (Fig. 1). However, toxigenesis was delayed for an undetermined period between the 8 and 20-week analyses in 60% moisture cheese spreads using polyphosphates S9 and S9H as the emulsifier. The exact length of the delay in toxigenesis was not determined except to note that control samples became toxic within 8 weeks and polyphosphate samples became toxic between Week 8 and Week 20. No toxin was detected in cheese spreads with 52, 54 or 56% moisture. This could be due to the shorter duration of this study vis-à-vis Tanaka et al. (14,15); 20 weeks versus 42 or 48 weeks for the last toxin analysis.

The study by Tanaka et al. (15) also indicated that changes in moisture level had little effect on water activity (aw). In that study, a difference of 9% moisture corresponded to an aw difference of only 0.022. A similar result was noted in this study. Although there was an 8% moisture difference between the cheeses in this study, the water activity ranged only from 0.91 to 0.96 aw units over the duration of the study. Tanaka et al. (15) observed no toxin production at aw below 0.944, and all cheese supported toxin development when the aw was above 0.957. Findings
in our study indicate toxin can be produced in the control disodium phosphate 60% moisture cheese spreads with an $a_w$ of 0.93 to 0.94; and in the experimental polyphosphate S9 and S9H 60% moisture cheese spreads having an $a_w$ of 0.94 to 0.95.

Briozzo et al. (4) examined growth and toxigenesis of C. botulinum using polyphosphate S9 in media with three different salt concentrations and water activities at pH 5.7 to 5.8 and in cheese. Toxin production was observed after 14 d in the medium having a water activity of 0.965 with 1.52% NaCl and 5.08% polyphosphate S9. Relating the findings of this study to Briozzo et al. (4), no toxin was detected at 20 weeks in the 56% moisture cheeses having similar water activity, pH values and NaCl amounts as the medium of Briozzo et al. (4). However, the media did not have the same components and composition as the cheese even though it possessed similar water activity, pH values and NaCl levels.

In a study by Kautter et al. (9), cheese spreads with bacon and Limburger cheese became toxic. Although the composition of the cheese spreads was not stated, the results were similar to the present study. The cheese spread with bacon, with a pH of 5.7 and an $a_w$ of 0.94, became toxic at 50 days (7 weeks) when inoculated with $2.4 \times 10^4$ spores per jar. The pH, $a_w$ and time to toxicity were very similar to results in this study evaluating polyphosphates. Unfortunately, the type of phosphate emulsifier and the moisture levels were not stated. A subsequent study (15) indicated toxin production could have been an artifact of surface-ripened cheeses. It is impossible to directly relate our findings to Kautter et al. (9) due to the differences in inoculation procedure and lack of information regarding phosphate type and moisture levels.

**Water activity ($a_w$)**

In this study, $a_w$ did not correlate to toxin production at the significance level chosen (0.05) at either 8 or 20 week sampling periods. The $a_w$ remained stable for the duration of the study. However, there is no direct correlation between percent moisture and water activity (6). There are uncertainties associated with measuring $a_w$ in cheeses and the results are only valid within the range of variables tested (15). Use of water activity as the only factor for determining food stability or safety is discouraged for nondilute solutions and complex or intermediate moisture systems (7).

Furthermore, other experimental variables like phosphate type used for emulsification, pH, and salt also influence growth of microorganisms (9) and can have an additive or synergistic effect (12). It would be imprudent to rely on $a_w$ alone in determining the safety of a cheese spread formulation.

**pH**

The effect of the cheese pH was also analyzed statistically. Although the pH was initially set to 5.6, pH values generally increased during the study. This phenomenon has been observed before and the cause, protein breakdown, is part of the normal aging of cheese and cheese spreads (8). At Week 8, the pH of the cheese was observed to statistically influence the production of toxin, but not at Week 20. This observation is likely coupled to the detection of toxin in the control disodium phosphate cheese spread, but not in the two experimental cheese spreads at the 8 week point. Interestingly, the experimental cheeses possessed a higher pH, which would be expected to be less inhibitory to C. botulinum (Fig. 1). However, earlier research indicated that pH did not affect toxigenesis in heated, bacterially surface-ripened cheeses (16,18,20).

**Phosphate type**

Phosphate type was significant at Week 8 but not at Week 20. This result showed that the experimental phosphates S9 and S9H delayed toxigenesis for an undetermined period between Week 8 and Week 20 when compared to the control disodium phosphate. Toxin developed...
in the control samples between Week 0 and Week 8. Toxin formation occurred in the polyphosphate samples between Week 8 and Week 20. Based on the delay of toxigenesis, use of polyphosphates S9 and S9H may increase the margin of safety in formulation and during typical storage and distribution. However, it will not prevent toxigenesis in high moisture formulations.

Cheeses with higher moisture, a lower concentration of salt and phosphate, and a higher pH would be expected to be at a greater risk of toxigenesis. As seen in Fig. 1, cheese samples in the upper left area with high moisture, high $a_w$, lower concentrations of salt + phosphate and higher pH should be at greater risk. However, although the S9 and S9H cheese spreads had equal or higher moisture and pH values, coupled with equal or lower concentrations of salt + phosphate than the control disodium phosphate, indicating a greater potential risk, toxigenesis was delayed.

Phosphatase activity

Related to phosphate and inhibition, it was noted that failure of the phosphates to inhibit C. botulinum in the 60% moisture cheese spreads was not due to phosphatase degrading the emulsifiers. Phosphatase levels were almost always below the sensitivity of the phosphatase assay used even though a highly sensitive fluorometric assay was used with a detection limit of 10 mU of activity/Kg cheese. This indicated that there was no residual phosphatase activity in the product or phosphatases of microbial origin, which could have degraded the protective effect of the phosphatases used in the cheese formulations.

Salt + phosphate levels

The last variable, percent salt+phosphate, was not statistically significant over the range tested. Earlier research indicated the percent sodium chloride and phosphate influenced toxin production (15). Although it likely plays a role via water activity, phosphate type, and moisture level, it was not found to be statistically significant in this study.

Clostridium botulinum population levels

Regardless of the type of phosphate used, higher numbers of anaerobic microbes were observed than aerobic microorganisms. The come up time for the product and the hold time of 6 min at 80°C should be lethal for most vegetative cells. The aerobic plate count likely represented both native aerobic and facultatively anaerobic, spore-forming microflora of the cheese and other dry ingredients used to make the processed cheese, whereas the anaerobic count represented the added C. botulinum organisms. The C. botulinum spores were expected to survive the cooking process, as would aerobic spore-formers. In general, C. botulinum counts exceeded aerobic plate counts by approximately 2 log cycles. C. botulinum counts generally remained level over the course of the study, but there was some fluctuation associated with individual sample variation.

Physical observations

The use of polyphosphates S9 and S9H resulted in cheese of altered appearance and altered rheological properties. Upon manufacture, a greater quantity of acid was required to adjust the pH to 5.6 with the control disodium phosphate. A 20-30 ml volume of 1 N HCl was required to lower the pH to 5.6 when the polyphosphates were used whereas, the disodium phosphate cheese spreads required 90 ml. Based on this repeated observation, it appeared the buffering capacity of the polyphosphates S9 and S9H was markedly less than the control disodium phosphate.

Secondly, the viscosity of the control disodium phosphate cheese was markedly less than the experimental cheeses. It appeared the polyphosphate salts can bind greater quantities of moisture than disodium phosphate, although this was not reflected in water activity measurements. The effect could also be due to the additional 60 ml of fluid added through the extra acid needed to lower the pH of the control cheese spread.

Finally, it appeared that the color of the cheeses was intensified or protected by the polyphosphate salts. It is not known whether the greater quantities of acid in the control cheeses affected the color or whether it is related to light dispersion of the products instead of the polyphosphate salts protecting or intensifying the color. However, the cheeses made with polyphosphates were markedly firmer and possessed a more intense orange color initially and throughout the duration of the 30-week study based on visual assessment. The color of the cheese spreads darkened during the study as observed in previous studies (14), likely due to the Maillard-reaction of lactose with the milk proteins. The control phosphate cheeses exhibited a darker brown color based on visual assessment. The cheeses were not sampled for organoleptic testing, so the effect of the polyphosphates on flavor is not known.

CONCLUSIONS

Polyphosphate salts were able to delay toxicity in processed, pasteurized cheese spreads at high moisture levels better than disodium phosphate, although none of the phosphates were able to prevent toxigenesis completely. However, the test polyphosphates appeared to have a greater margin of safety in formulation and during storage and distribution. This was demonstrated by the delay of toxin production in the 60% moisture cheese samples compared to the cheese spread made with disodium phosphate. Cheeses with higher moisture, a lower concentration of salt and phosphate and a higher pH would be expected to be at a greater risk of toxigenesis. Moisture and water activity did not correlate significantly for formulation purposes. From a quality standpoint, the polyphosphates gave a product with more intense color and greater viscosity.

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