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Potential C. botulinum Risks in Muscle Food Stored under Modified Atmospheres

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Recently there has been a renewed interest in the use of modified atmospheres (vacuum packaging, hypobaric atmospheres or atmospheres based on blends of CO_2, O_2, N_2) to extend the storage shelf-life of fresh muscle foods at reasonable cost. Successful application of these technologies reduces losses due to spoilage, makes products available for long distance transportation and allows utilization of remote but rich resources (5,6,8,10,11,19).

One of the major food safety concerns which has limited the application of modified atmospheres, especially to fish which is more sensitive to spoilage than red meat and poultry, is the potential risk from C. botulinum growth. This risk is amplified by the fact that the non-proteolytic types B, E and F can grow at 3.3 - 4.0 (6). Also the retardation of the normal aerobic spoilage flora of fresh muscle food by modified atmospheres (MA) may increase the probability (P) for C. botulinum growth and toxigenesis during prolonged storage at abused temperatures (6,7,10,12,23,26). Because of the high prevalence of C. botulinum in seafood, especially in fish coming from fish farms, as compared to red meat and poultry, the concern by the fish industry is even greater (7,12,13,18,26). Also, the question of whether of not spoilage may precede toxigenesis and alarm the processor or the consumer, has not been fully clarified (2,6-8,10,16,17,22, 23).

In recent years a number of investigators have explored the potential growth of C. botulinum in muscle foods stored under MA (2,5-8,10,12,15,17, 18,22-24). The low prevalence of C. botulinum in red meat and poultry has contributed to the expansion of the MA use for such products (11,21,23). The picture with respect to fresh fish and shellfish remains unclear.

Some of the most recent findings with respect to the risk of C. botulinum growth in fish stored under MA have as follows: 1) Vacuum packaging is not a requirement for C. botulinum to grow in muscle foods

(7,6,7,12,22). Fish contaminated with type E spores may become toxic under aerobic conditions, but reduced O_2 pressure will increase the risk of C. botulinum toxigenesis (2,16); 2) As the storage temperature is increased from 0 to 10°C, the time interval between an acceptable spoilage and detectable toxin production in fish stored under MA shortens and thus the safety margin decreases. Below 10°C it seems that spoilage by the normal microbes precedes toxigenesis (7,22) whether or not the fish is stored under MA. Toxigenesis before obvious spoilage at $\leq$ 10°C under 60-90% CO_2 has been reported (6,16); 3) Fish from the tropics last longer under refrigeration, probably because of lower incidence of psychrophilic bacteria. In such fish, toxigenesis may precede spoilage. Rates of spoilage and toxigenesis seem to be similar at temperatures $\geq$ 10°C; 4) Carbon dioxide selectively inhibits muscle food spoilage bacteria (8,10,11). The degree of inhibition increases with increased CO_2 concentration, decreased temperature and bacterial levels and earlier application (3,8,10,11,20). While CO_2 contentrations of up to 25% are sufficient for shelf-life extension of red meat, concentrations of 40-100%, depending on temperature, are optimal for fish (10,25); 5) CO_2 concentrations of <1At can stimulate germination of C. botulinum spores. At >1At CO_2 may inhibit germination, delay toxigenesis and promote death of C. botulinum cells (5,10). Increased rate of toxigenesis with decreasing CO_2 level from 90 to 60% to storage in air has been observed at 10°C (6). Toxigenesis was accelerated with increased temperature and spore load (6,12). Dipping fish fillets in sorbate and tripolypoly-phosphate solutions and subsequent storage under CO_2 has been shown to delay toxigenesis for a longer time as compared to the use of CO_2 only (22). Although the above mentioned studies have extended our knowledge, we are still lacking a quantitative evaluation of the risk of C. botulinum toxigenesis in fish as it relates to type of fish, type and load of C. botulinum spores, temperatures of storage, MA of storage and selected preservatives shown to delay toxigenesis. During the last three years we have been dealing with such quantitative aspects first in model broth systems and then in fish. Significant findings will be discussed briefly in this presentation. For details the reader should consult references (9,10,14,15) and future papers from this group.

Material and Methods

Probability of C. botulinum growth in broth. The experiments were arranged in a factorial fashion. They included 17 individual (cells and spores) C. botulinum strains (3 type A, 6 type B, 5 type E and 3 type F), 9 temperatures of incubation (4,8,12,16,20,30,37,42 and 47°C) for up to 28

days with one replication. Brain heart infusion broth (Difco) with 0.25% ascorbate of 0.1% cysteine was found suitable as the growth medium. Using a 9 ten-fold dilution-27 ampule most probable number (MPN) technique, the decimal reduction (DR) of a microbial cell or spore population inoculated into broth and incubated at various temperatures was determined. The reciporcal of the antilog of the DR gave an estimate of the P of growth initiation of one C. botulinum cell or spore in the broth at the test incubation temperatures. A vaspar overlay on broths maintained anaerobicity during incubation. In every experiment we included a number of temperatures and 30°C. The P (%) of growth at 30°C was set arbitrarily to 100 and a P relative to 30°C was calculated for the other temperatures.

Probability of C. botulinum growth in fish. Fresh dressed red snapper (Sebastes paucispinis) and King salmon (Oncorhynchus tshawytscha) were purchased from a wholesaler. Whole fish muscle homogenates were prepared aseptically and then frozen until use. Defrosted (overnight at 4°C) ground fish was packed in tissue culture plates (ca 1.5g/well, 24 wells/plate). Each sample was inoculated with 20µl of spore suspension. Additional (1.5g/well) homogenate was added to each well, the plates were covered with the lid and then placed in 20x50 cm barrier bags (Cryovac type B540 with O_2 transmission of 30-50 $cc/m^3/24$ hrs at 1 atm and 22.8°C). Appropriate MA of vacuum, 100% CO_2 and 70% CO_2 + 30% air were created with the use of Multivac A300 model 22 packaging machine. The experiment was arranged in a factorial design including 7 ten-fold dilutions (10^4 to 10^{-2} spores/sample) of a pool of non-proteolytic spores (equal levels of 4B, 5E and 4F) and control, triplicate inoculations, three MA, five temperatures of incubation (4,8,12,17 and 30°C) and multiple samplings (0,1,2,3,6,9,12,15,18 and 21 days for red snapper and up to 60 days for salmon). At a particular day and for each temperature and MA condition, a set of 21 samples representing the 7. inoculation levels plus control in triplicate were removed for total plate counts, pH, gas analysis, toxicity testing and estimation of the probability of toxigenesis. For toxicity the samples were kept forzen until analysis, which was done according to the standard mouse test. The lowest inoculum level was typed to verify that toxicity was due to the inoculum. Probability of growth and toxigenesis was calculated by the 7x3 MPM method as done in the broth studies.

Fillets of 25g ± 5 (without skin) were prepared aseptically from whole fish. Two fillets, one on top of the other, were placed in each barrier bag. A 20µl spore inoculum was delivered in the center between the two fillets, which were next packed at the desired MA. This experiment

included 5 inoculum levels (10^4 to 10^0 spores/sample) of the non-proteolytic pool, 3 controls, 5 temperatures of incubation (1,4,8,12 and 30°C), 3 MA (vacuum, 100% CO_2, 70% CO_2 + 30% air) and multiple samplings. The analytical methods were similar to these used for the fish homogenate studies.

Statistical Methods

Statistical analysis and modeling was based on computer programs (4) BMDP2V for ANOVA, BMDP2R for stepwise regression, BMDP9R for all possible subsets regression, BMDP1R for linear regression of the lag phase and BMDPLR for the logistic regression model to describe the probability curves after the lag periods.

Results and Discussion

Probability of C. botulinum growth in broth. A total of 612 experiments were performed. The studies have demonstrated that the P of growth is affected by the variables, cell type, proteolytic ability, temperature and time. The relationship of temperature to P is a bell-shaped curve indicating the need of a heavy inoculum to initiate growth at the extreme upper and low temperatures. For proteolytic A, B and F types, incubation temperatures of 16 to 37°C will require in all but one strain 1 to 50 cells/ml to initiate growth. At 12 and 47°C, inocula > 10^6 to 10^8 cells/ml and 50 to 10^8 cells/ml, will be required respectively. No growth occurred at 8°C with inocula of 10^8 cells/ml. In general, spores behaved similarly to vegetative cells with the exception of the upper temperature limits and for types A and B only. Non-proteolytic types E, B and F can grow at lower temperatures and can initiate growth with fewer cells than the proteolytic types. Thus, at the same level of inoculum, the non-proteolytic types can initiate growth at 8°C while the proteolytics require 16°C. Also, much lower levels of inoculum would suffice to initiate growth of non-proteolytic strains at 4°C than for proteolytic strains at 12°C. Depending on strain, growth of non-proteolytics at 4°C required between 17 to 10^6 cells/ml and from 16 days to 4 weeks of incubation. Using multiple linear regression analysis, formulas were derived which related the P of one cell or spore to grow after a certain time, at a particular temperature. The $\log_{10}$ P (%) for a spore of the most capable non-proteolytic B. E and F strains to grow at 4 to 30°C after 28 days incubation is $P = -6.09+1.56(T)-0.09(T)^2+0.0015(T)^3$ where T Temperature. The $\log_{10}$ P (%) with respect to T and storage time (ST) in days is $P = 93.95-0.3(T)-82.22(e)^{1/T}-8.64(1/ST)$. The respective formulas for the most capable proteolytic A, B and F strains are: $P = 25.02+2.41(T)-0.05(T)^2$ and $P = 296.09-0.43(T)-271.39(e)^{1/T}-3.53(1/ST)$. Temperatures with

P > 100% reflect environments more conducive to growth than 30°C.

Probability of growth in fish. As in the broth experiments these studies demonstrated the increased chance of C. botulinum growth in fish with increasing level of contamination. Higher inoculums were needed to initiate toxin production at lower than higher temperatures. Analysis of variance for the red snapper tissue homogenate experiment demonstrated that the effect of temperature (T) and storage time (ST) on the P of 1 spore to initiate toxigenesis was highly significant ($P<0.005$). The effect of the interactions T x ST, MA x T and MA x ST was significant ($p<0.05$) while the effect of MA was nonsignificant. For the maximum inoculum of 1×10^4 spores/sample, a linear relationship existed between the $\log_{10}$ of the lag time (time before toxigenesis starts) and the temperature of storage. The best fit equation for the lag period in days was: $\log_{10}(\text{days}) = 1.63 - 0.1T \pm 0.008$, $r^2 = 0.93$.

A combination of the lag period formula and logistic regression analysis permitted the derivation of predictive equations relating the P of 1 non-proteolytic spore to grow and produce toxin (based on a maximum inoculum of 1×10^4 spores/sample) and storage time (up to 21 days), temperature of storage (4 - 30°C) and MA in red snapper tissue homogenate. The derived equation has as follows: $\log_{10} P\ (\%) = \frac{5\ (e^Y)}{(1+e^Y)} - 3$

$$\text{vacuum}, Y = -1.93-0.048T-0.37(ST-10^{1.63-0.1T})+0.1T(ST-10^{1.63-0.1T})$$

$$100\%\ CO_2, Y = -3.2+0.014T+0.323(ST-10^{1.63-0.1T})+0.635T(ST-10^{1.63-0.1T})$$

$$70\%\ CO_2 + 30\%\ \text{air}, Y = -2.6-0.022T-0.054(ST-10^{1.63-0.17})+0.0877(ST-10^{1.63-0.1T})$$

$$\text{Combined MA}, Y = -2.47-0.032T-0.068(ST-10^{1.63-0.1T})+0.0877(ST-10^{1.63-0.1T})$$

An excellent agreement was found between predicted and observed P for toxigenesis. According to the combined formula for all MA the probability of 1 spore to grow and produce toxin in fish after 18 and 21 days at 4°C is 0.0029% and 0.0092% respectively. This means that only 2.9 and 9.2 spores per 10^5 will grow and produce toxin after 18 and 21 days at 4°C or you need at least 34,482 and 10,869 spores respectively to initiate toxigenesis in red snapper tissue homogenate. The highest reported incidence of C. botulinum spores in fish is 5.3/g (13). In most cases it is <1/g. We found that all red snapper lots contained residential type A spores at 0.09-2.4 spores/g and one lot had A and E (at 0.09 spores/g). Based on our studies red snapper kept at 4°C will not become toxic under natural spore load and storage of up to 21 days. Yet slight temperature abuse to 8°C will result in 97.7% and 87% toxigenesis by 1 spore/g in 21 and 18 days

according to the predictive model.

A general agreement, especially at the low temperature was observed between the P for red snapper tissue homogenates and fillets. The earliest time to observe toxicity at 4°C at the maximum inoculum (1 x 10^4/sample) was 19 days. This was observed in one of two tissue homogenate experiments but not in fillets incubated for up to 21 days.

Factorial design experiments based on the use of salmon tissue homogenate, the same inoculum, as for the red snapper experiments, T, and MA but extending the ST to 60 days have been completed with the exception of statistical analysis and modeling. Raw data from these experiments demonstrated the earliest production of toxin at 4,8,12,17 and 30°C and maximum inoculum of 1 x 10^4/sample after 15,9,6,2 and 1 day of storage respecticely. The estimated $\log_{10}$ P(%) for 1 spore to grow and produce toxin at the same temperatures and times was -2.76, -2, 0, -1 and 2. Vacuum packaging seemed to support earlier production of toxin than CO_2 MA. Few vacuum packed samples became toxic after 15 days at 4°C but not in CO_2 MA even after 60 days at 4°C.

Figure 1 presents: 1) The calculated best fit curve relating the length of the lag phase and the temperature of storage ($\log_{10}$ days = 1.45-0.085 x T). The curve is based on the shortest lag phase times obvserved in all of our experiments at 10^4 spores/sample inoculum levels; 2) The combinations of storage time and temperature which resulted in toxigenesis in our studies or reported by others. Only in 4 reported cases toxigenesis was observed at times equal or shorter than the predicted by the curve lag times. Toxigenesis occurred in herring inoculated with 10^4 E spores/g and incubated at 5°C for 9 days (1), whiting inoculated with 50 E spores/g (5000/package) and incubated at 8°C for 5 days (Solberg pers. comm.), mackerel inoculated with 10^4 non-proteolytic B and E spores/package and incubated at 10°C for 4 days (2) and herring inoculated with 10^2 E spores/g and incubated at 15°C for 1 day(12). The above inoculums are from 20 to 2000 times greater than the maximum natural load of C. botulinum found in fresh fish.

In summary, MA have been proven beneficial in extending the shelf-life of muscle foods at reasonable cost.. The potential risk of C. botulinum growth in fish stored under MA has remained a limiting factor to expansion. Our studies have laid the foundations for a more realistic approach in quantitating the risk and then setting workable standards.

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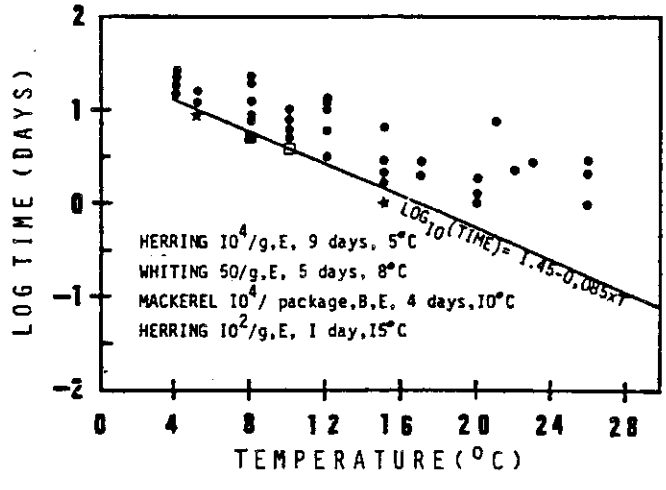

Fig. 1. Comparison of temperature and time combinations which resulted in toxigenesis, based on this study and literature, with the calculated best fit curve relating the shortest lag phases (recorded in this study) to corresponding temperatures.

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