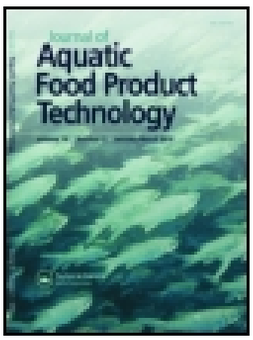

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Influence of Temperature and pH on Growth and Toxin Production from Spores of *Clostridium botulinum*

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ABSTRACT. This study was undertaken to determine the influence of temperature and pH on growth and time to toxicity of spores of *Clostridium botulinum* types isolated from tropical fish. Anaerobic meat medium with varying temperatures (4-30°C) and pH values (4.5 to 7.0) were used and toxicity was tested by mouse bioassay. An increase in lag phase was noticed by lowering the growth temperature from 30°C to 4°C and the pH from 7.0 to 4.6. The probability of toxin production increased with storage time, but decreased as either the pH or storage temperature and pH (combined stress) was decreased. The results indicated the effect of pH in controlling toxin production by *C. botulinum* spores in refrigerated foods with pH 5.0 or < 5.0. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com>*

KEYWORDS. *Clostridium botulinum*, growth, toxin production, pH, temperature, combined stress

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INTRODUCTION

The ability of *Clostridium botulinum* to survive under adverse conditions in the environment, adapt and eventually multiply in foods makes it a major food safety hazard (Gaze, 1992; Notermans et al., 1995). Non-proteolytic *C. botulinum* is recognized as a potential hazard in minimally processed food that is designed for extended storage (Conner et al., 1989; Gaze, 1992). This concern is due to the fact that all type E and non-proteolytic type B and F strains (metabolic group II) can grow at 3.3°C (Schmidt et al., 1961; Eklund et al., 1967) and spores of these psychrotrophic strains are less heat-resistant. All type A and proteolytic type B and F strains (group I) and all types C and D strains (group III) do not multiply at temperatures below 10°C (Smith, 1977). Groups I and II have been associated with human botulism while group III is responsible for animal botulism (Hauschild, 1993).

In recent years, there has been an increasing demand for minimally or partially processed seafoods that rely on good refrigeration to ensure product quality and safety. It is not possible to guarantee refrigerated storage temperatures less than 3.0°C of products throughout the distribution, marketing and also by consumers. The concept of hurdles or multiple barriers to inhibit microbial growth is gaining importance in the food industry (Scott, 1989; Leistner and Gorris, 1995). *C. botulinum* is a world wide contaminant of fish (Sakaguchi, 1979; Huss, 1981; Dodds, 1993; Lalitha and Gopakumar, 2000). *C. botulinum* types C and D are the predominant serotypes in tropical fish (Dodds, 1993; Lalitha and Gopakumar, 2000; Lalitha and Surendran, 2002). These types are associated with animal botulism. However, type C toxin has been linked to recent infant botulism in Japan (Oguma et al., 1990). Several investigators have incorporated secondary safety barriers in conjunction with refrigeration temperature to inhibit *C. botulinum* types A, B and E growth and toxin formation (Baird-Parker and Freame, 1967; Eklund et al., 1967; Emodi and Lechowich, 1969a, b; Graham and Lund, 1987; Lund et al., 1985, 1990; Eklund, 1993; Graham et al., 1996). Similar studies on *C. botulinum* types C and D are scarse. Because growth and toxin production by *C. botulinum* types C and D have been reported in fish and shellfish tissue homogenates (Lalitha and Gopakumar, 2001), effect of stress factors on the predominant *C. botulinum* types in nutrient rich medium was investigated and such information is needed to extend shelf-life of minimally processed fish products.

The objective of this study was to determine the risk of survival of, and toxin production by *C. botulinum* types isolated from fish at mild

temperature abuse storage conditions and to compare the behavior of Group III types C and D with Group I types A and B and Group II type E. The effect of additional barriers (pH) to control the growth of *C. botulinum* in processed fish products was also studied.

MATERIALS AND METHODS

Clostridium botulinum Inoculum

The following strains of *Clostridium botulinum* were used in the study: first, strains isolated from wild and farmed fish–*C. botulinum* Group I type A strain (53A), Group I type B strain (90B), Group III type C strain (18C, 275C), Group III type D strain (2693D, 2131D); second, reference strains of Group III type D strain (ATCC 27517) and Group II type E strain (NCIB 10660). They were maintained in Cooked Meat Media (CMM). The CMM tubes were heated, cooled, inoculated and overlayered with 10-15 ml of pre-sterilized paraffin oil to enhance anaerobiosis. For studying the influence of pH on *C. botulinum*, types A, B, C and D isolates from fish and types D and E reference strains were used. To study the combined effect of temperature and pH, type A strain 53A, type C strain 18C, type D strain 2131D and type E reference strain were used.

The spore crop was prepared by inoculating *C. botulinum* strains into deoxygenated CMM and overlayered with pre-sterilized paraffin oil. After incubation at 30°C for 1-2 weeks, spores were harvested, suspended and washed (10000 × g for 20 min at 4°C) with sterile distilled water (25 ml) and resuspended in 50% ethanol for 1 h to kill the vegetative cells (Tsang et al., 1985; Jensen et al., 1987). Spores were then stored in sterile distilled water at 1°C until needed. The numbers of viable spores in the suspensions were calibrated by plate counts on anaerobic Tryptone Soytone Glucose Yeast extract (TSGY) Agar (Lalitha and Iyer, 1991). Final density of spore inoculum was 1.0×10^4 to 3.0×10^6 viable spores ml^{-1} for *C. botulinum* types A, B, C and D and 10^2 viable spores ml^{-1} for type E.

Microcosm for Individual and Combined Stress

The effect of temperature was studied in anaerobic CMM tubes (pH 7.0). Tubes were steamed for 20 min to drive off residual oxygen, cooled and overlayered with 10-15 ml of sterile paraffin oil to enhance

anaerobiosis. Ten tubes of each spore type and temperature were made. The tubes were held at 30°C, 15°C, 10°C and 4°C for 24 h before inoculation. The single tubes of CMM were inoculated with 1 ml of a single type spore inoculum as described above. The tubes were incubated at the required temperature. The tubes were observed for growth (turbidity and gas production) daily for the first ten days of incubation and afterwards at intervals up to 6 weeks. Toxicity was tested using mouse bioassay.

The influence of pH was studied in freshly prepared CMM tubes. The pH was measured using a pH meter (Systronics, Bombay). The pH values of CMM tubes were adjusted from pH 6.0 to 4.6 at 0.2 pH unit intervals by the addition of an appropriate volume of 2 N HCl and boiled. The pH was measured after cooling and, if necessary, pH was adjusted by adding HCl before autoclaving. The pH of three tubes from each batch of sterile medium was measured using pH meter before boiling to remove oxygen. Ten tubes of each spore type and pH were made for each strain. The single tubes of CMM were inoculated with 1 ml of a single type spore inoculum as described above. Each tube was sealed with sterile paraffin oil and incubated at 30°C. Examinations for growth were made daily for the first two weeks and afterwards at intervals up to 5 weeks and toxicity was tested.

To study the combined effect of temperature and pH, CMM was adjusted to pH 7.0, 6.0, 5.5, 5.0 and 4.5 as described above. Ten tubes of each spore type and pH were made. Inoculation of the tubes were made as described previously. Each tube was overlayered with sterile paraffin oil and tubes of spore types A, C, and D were incubated at 30°C, 15°C and 10°C and tubes of type E were incubated at 30°C, 15°C, 10°C and 4°C. Examinations for growth were made daily for the first two weeks, afterwards at intervals up to 5 weeks. Toxicity was tested.

Mouse Bioassay

The mouse bioassay described by FDA (Solomon and Lilly, 1998) was used to detect the presence of toxins in growing culture of *C. botulinum.* Two ml of the liquid portion of CMM was mixed with equal volume of Gelatin Phosphate buffer (pH 6.2) and centrifuged 20 min at 10000 × g at 4°C. The pH of the supernatant was adjusted to 6.2. Supernatant was kept frozen until tested.

The methodology outlined by Solomon and Lilly (1998) was employed for trypsinization, toxicity test, estimation of toxin and toxin neutralization tests. Toxin neutralization tests were made using type-spe-

cific monovalent antitoxins A-E obtained from US Centers for Disease Control (Atlanta, GA).

The lag time, i.e., the incubation time up to the sampling period before the first observation of *C. botulinum* toxin (Baker and Genigeorgis, 1990), was estimated.

RESULTS AND DISCUSSION

The effect of temperature, pH and combined stress on growth and toxin production by *C. botulinum* was examined on the basis of increase in lag phase and time to toxicity. The effect of sub-optimal temperature on growth and toxin production by *C. botulinum* in anaerobic meat media was studied and the data are summarized in Table 1. The outgrowth time was substantially short at ambient temperature (30°C) for *C. botulinum* Group I, II and III strains. The lag phase increased from < 24 h to almost 5-7 d with the lowering of storage temperature from 30°C to 15°C for Group I type A (53A) and B (90B) and Group III type C (18C, 275C) and D (2693D, 2131D and ATCC 27517). Similar observations were made earlier for type A (Ohye and Scott, 1957; Sperber, 1982; Jensen et al., 1987) and Group III (Segner et al., 1971). The behavior of Group III strains were similar to Group I strains. Only psychrotrophic *C. botulinum* Group II type E strain grew and produced toxin at refrigerated storage. For type E, the lag time increased from < 1 d to 2 d, 7 d and to 28 d when the storage temperature was lowered from 30°C to 15°C, 10°C and 4°C, respectively. At the lower end of the growth range, growth of type E strain occurred after several weeks of incubation as reported earlier (Ohye and Scott, 1957; Solomon et al., 1977; Jensen et al., 1987).

Table 2 shows the effect of sub-optimal pH on growth and time to toxicity of *C. botulinum*. The lag time increased from < 24 h to almost 4 d with the lowering of pH from 7.0 to 5.0 at 30°C for *C. botulinum* type A to D strains. At pH values approaching the limit that allowed growth, the incubation time required increased (Lund et al., 1987). At the lowest pH at which growth was detected, pH 4.8, a lag time of 20 d was noticed for Group I type A strain 53 A. In medium adjusted to pH 4.6 and incubated at 30°C for 35 days, neither growth nor toxin production was noticed. *C. botulinum* type E did not grow below pH 5.4. While a pH < 4.6 is considered to prevent growth and toxin formation by *C. botulinum* in most conditions (Ohye and Christian, 1967; Ito and Chen, 1978; Odlaug and Pflug, 1978), there are reports of growth of type A and B (Raatjes

TABLE 1. Effect of storage temperature on growth and toxin production by *Clostridium botulinum* types incubated 42 days in anaerobic meat medium.

C. botulinum **spore type**	**Inoculum level per 10 ml**	**Storage temperature (°C)**	**Earliest time to toxicity in days**
Group I			
Type A-53 A	1.1×10^5	30	1
		15	6
Type B-90 B	3.2×10^6	30	1
		15	7
Group III			
Type C-18 C	3.0×10^6	30	2
		15	7
-275 C	6.0×10^5	30	2
		15	8
Type D-2693 D	5.0×10^5	30	2
		15	7
-2131 D	3.0×10^7	30	18
		15	8
-ATCC 27517	1.0×10^6	30	2
		15	8
Group II			
Type E-NCIB 10660	1.0×10^3	30	1
		15	3
		10	8
		4	34

and Smelt, 1979; Smelt et al., 1982; Tanaka, 1982; Young-Perkins and Merson, 1987) and type E strains (Tsang et al., 1985) at pH values lower than this. It has been attributed to the rise in pH brought about by the growth of other organisms such as bacteria, yeasts or molds, high concentration of proteins in the medium or sufficiently anaerobic growth medium. It has also been shown that the pH tolerance of *C. botulinum*

TABLE 2. Effect of pH on growth and toxin production by *Clostridium botulinum* incubated 35 days at 30°C in anaerobic meat medium.

***C. botulinum* spore type**	**Inoculum level per 10 ml**	**Earliest time to toxicity in days**								
		pH of CMM								
		7.0	**6.0**	**5.8**	**5.6**	**5.4**	**5.2**	**5.0**	**4.8**	**4.6**
Group I										
Type A-53 A	1.1×10^5	1	2	4	4	4	4	4	21	–
Type B-90 B	3.2×10^6	1	2	2	4	4	4	4	–	–
Group III										
Type C-18 C	3.0×10^6	2	2	2	4	4	11	–	–	–
-275 C	6.0×10^5	2	3	5	5	5	26	–	–	–
Type D-2693 D	5.0×10^5	1	4	5	6	6	6	–	–	–
-2131 D	1.0×10^6	1	2	2	4	4	4	16	–	–
-ATCC 27517	3.0×10^7	1	2	4	4	4	4	15	–	–
Group II										
Type E-NCIB 10660	1.0×10^3	1	3	3	4	6	–	–	–	–

may be greatly modified by the acidulants used, and the concentration, type and strain of *C. botulinum* spores (Ito and Chen, 1978). Segner et al. (1966) observed that the minimum pH, adjusted with HCl, that supported growth from an inoculum of 2×10^6 type E spores in a variety of culture media to be in the range of 5.24-5.36. The differences between our results and those of Segner et al. (1966) may be due to the fact that they used a higher inocula.

The lower pH limit that allowed growth of *C. botulinum* type D strains was 5.0 and a lag time of 15-16 d was noticed. The limiting pH of *C. botulinum* type C was 5.2 in the present study. Data on the minimum pH that allows growth of *C. botulinum* type D is very limited. Segner et al. (1971) found growth of marine strains of *C. botulinum* type C in FEM medium at pH 5.1 and not below that. Lund et al. (1990) reported different rates of germination in sub-optimal conditions for different spore crops of the same strain of *C. botulinum*. Variations in acid tolerance among *C. botulinum* type E strains have been reported (Emodi and Lechowich, 1969a; Ito and Chen, 1978; Smelt et al., 1982).

The effect of combined stress (temperature and pH) on growth and toxin production by *Clostridium botulinum* was examined and the results are presented in Table 3. Although the minimum pH for growth of type A was between 4.8 to 5.0 at 30°C, when the temperature was sub-optimal, as in the case of foods stored at chill temperatures, the minimum pH for growth was raised to 5.5 as reported earlier (Graham and Lund, 1987). Similar observations were made for types C, D and E. At pH 5.5, a lag time of 14 d was noticed for type A, whereas, for types C and D, it was 10 d. Graham and Lund (1987) observed growth of type A after 14 d at pH 5.5 and 16°C in PYGS medium. The difference in the time to toxicity at pH 5.5 between Group I and III strains may be due to the higher inocula of types C and D spores used. There is a lack of previously published data for the combined effect of low temperature and low pH on growth of types C and D strains. Following the transfer of tubes from 10°C and 4°C to 30°C, growth and toxin production oc-

TABLE 3. Combined effect of pH and temperature on growth and toxin production by *Clostridium botulinum* in anaerobic meat medium stored for 35 days.

***C. botulinum* spore type**	**Inoculum level per 10 ml**	**Storage temperature (°C)**	**Earliest time to toxicity in days pH of CMM**				
			7.0	**6.0**	**5.5**	**5.0**	**4.5**
Group I							
Type A-53 A	1.1×10^5	30	1	2	4	11	–
		15	4	5	14	–	–
Group III							
Type C-18 C	3.0×10^6	30	1	2	5	–	–
		15	4	5	10	–	–
Type D- 2131 D	3.0×10^7	30	1	2	5	16	–
		15	4	6	10	–	–
Group II							
Type E-NCIB 10660	1.0×10^3	30	1	2	3	–	–
		15	3	3	5	–	–
		10	12	15	-	–	–
		4	28	-	-	–	–

curred in all tubes inoculated with types A, C and D within 12-18 d at pH 5.0 but not at pH 4.5. Lund et al. (1985) did not find multiplication and formation of toxin by type A at 16°C over the pH range 5.1-4.5.

C. botulinum type E strain failed to grow and produce toxin at 10°C and 4°C at pH 5.5 as reported earlier (Segner et al., 1966; Graham and Lund, 1987). Emodi and Lechowich (1969a) found that the minimum pH permitting growth of six strains of type E from an inoculum of 1.5×10^6 spores was 5.4-5.6 at 15.6°C, 5.6-6.0 at 10°C and 6.2-6.4 at 5°C.

Toxin was detected only in those cultures that showed evidence of growth (turbidity or gas production). Similar observations were made earlier (Graham et al., 1996; Whiting and Oriente, 1997; Elliot and Schaffner, 2001).

In conclusion, the study indicates that the combination of sub-optimal temperature and sub-optimal pH inhibit *C. botulinum*. Fish products with a pH > 5.0 could obviously pose a public health hazard at refrigerated temperature and additional barriers (e.g., water activity reduction and antimicrobial agents) are necessary in medium acid products (pH 5-6) to prevent the growth and toxin production by *C. botulinum* at mild temperature abuse conditions. The behavior of *C. botulinum* Group III type C and D cultures at sub-optimal temperature and sub-optimal pH was similar to Group I type A. Our results revealed that despite the apparent inhibition of growth and toxin production at 4°C and 10°C and at pH 5.0 and below, spores remained viable and capable of growth and toxin production under favorable conditions. Temperature abuse of refrigerated products during distribution or retailing or in the hands of the consumer would increase the potential for a health hazard situation. Stability against *C. botulinum* can best be achieved through proper refrigerated storage of seafood products along with product reformulation to suitable pH or thorough additional barriers to ensure the public health safety.

REFERENCES

Baird-Parker, A.C. and Freame, B. 1967. Combined effect of water activity, pH and temperature on the growth of *Clostridium botulinum* from spore and vegetative cell inocula. J. Appl. Bacteriol. 30: 420-429.

Baker, D.A. and Genigeorgis, C. 1990. Predicting the safe storage of fresh fish under modified atmospheres with respect to *Clostridium botulinum* toxigenesis by modeling length of the lag phase of growth. J. Food Prot. 53: 131-140.

Conner, D.E., Scott, V.N., and Bernard, D.T. 1989. Potential *Clostridium botulinum* hazards associated with extended shelf-life refrigerated foods: A review. J. Food Safety 10: 131-153.

Dodds, K.L. 1993. *Clostridium botulinum* in the environment. In: *Clostridium botulinum: Ecology and Control in Foods*. Hauschild, A.H.W. and Dodds, K.L. (Eds.) pp. 21-52. Marcel Dekker Inc., New York.

Eklund, M.W., Wieler, D.I., and Poysky, F.T. 1967. Outgrowth and toxin production of non-proteolytic type B *Clostridium botulinum* at 3.3 to 5.6°C. J. Bacteriol. 93: 1461-1462.

Eklund, M.W. 1993. Control in fishery products. Chap. 7. In: *Clostridium botulinum*: *Ecology and Control in Foods*. Hauschild, A.H.W. and Dodds, K.L. (Eds.) Marcel Dekker Inc., New York.

Elliot, P.H. and Schaffner, D.W. 2001. Germination, growth, and toxin formation of non-proteolytic *Clostridium botulinum* as affected by multiple barriers. J. Food Sci. 66: 575-579.

Emodi, A.S. and Lechowich, R.V. 1969a. Low temperature growth of type E *Clostridium botulinum* spores. I. Effects of sodium chloride, sodium nitrite and pH. J. Food Sci. 34: 78-81.

Emodi, A.S. and Lechowich, R.V. 1969b. Low temperature growth of type E *Clostridium botulinum* spores. 2. Effects of solutes and incubation temperature. J. Food. Sci. 34: 82-87.

Gaze, J.E. 1992. The importance and control of *Clostridium botulinum* in processed foods. British Food J. 94: 8-15.

Graham, A.F. and Lund, B.M. 1987. The combined effect of sub-optimal temperature and sub-optimal pH on growth and toxin formation from spores of *Clostridium botulinum*. J. Appl. Bacteriol. 63: 387-393.

Graham, A.F., Mason, D.R., and Peck, M.W. 1996. Inhibitory effect of combinations of heat treatment, pH and sodium chloride on growth from spores of non-proteolytic *Clostridium botulinum* at refrigeration temperature. Appl. Environ. Microbiol. 62: 2664-2668.

Hauschild, A.H.W. 1993. Epidemiology of human foodborne botulism. In: *Clostridium botulinum*: *Ecology and Control in Foods*. Hauschild, A.H.W. and Dodds, K.L. (Eds.) pp. 69-104. Marcel Dekker Inc., New York.

Huss, H.H. 1981. *Clostridium botulinum* type E and botulism. 58 pp. Technical Univ. of Denmark, Lyngby, Denmark.

Ito, K.A. and Chen, J.K. 1978. Effect of pH on growth of *Clostridium botulinum* in foods. Food Technol (Champaign). 32: 71-72, 76.

Jensen, M.J., Genigeorgis, C., and Lindroth, S. 1987. Probability of growth of *Clostridium botulinum* as affected by strain, cell and serological type, inoculum size and temperature and time of incubation in a model broth system. J. Food Safety 8: 109-126.

Lalitha, K.V. and Gopakumar, K. 2000. Distribution and ecology of *Clostridium botulinum* in fish and aquatic environments of a tropical region. Food Microbiol. 17: 535-541.

Lalitha, K.V. and Gopakumar, K. 2001. Growth and toxin production by *Clostridium botulinum* in fish (*Mugil cephalus*) and shrimp (*Penaeus indicus*) tissue homogenates stored under vacuum. Food Microbiol. 18: 651-657.

Lalitha, K.V. and Iyer, K.M. 1991. Isolation and characterization of a putrefactive anaerobe *Clostridium sporogenes* from fishes and shellfishes. Fish. Technol. 28: 38-41.

Lalitha, K.V. and Surendran, P.K. 2002. Occurrence of *Clostridium botulinum* in fresh and cured fish in retail trade in Cochin (India). Int. J. Food Microbiol. 72: 169-174.

Leistner, L. and Gorris, L.G.M. 1995. Food preservation by hurdle technology. Trends Food Sci. Technol. 6: 41-46.

Lund, B.M., Graham, A.F., and Franklin, J.G. 1987. The effect of acid pH on the probability of growth of proteolytic strains of *Clostridium botulinum*. Int. J. Food Microbiol. 4: 215-226.

Lund, B.M., Graham, A.F., George, S.M., and Brown, D. 1990. The combined effect of incubation temperature, pH and sorbic acid on the probability of growth of non-proteolytic type B *C. botulinum*. J. Appl. Bacteriol. 69: 481-492.

Lund, B.M., Wyatt, G.M., and Graham, A.F. 1985. The combined effect of low temperature and low pH on survival and growth and toxin formation from spores of *Clostridium botulinum*. Food Microbiol. 2: 135-145.

Notermans, S., Gallhoff, G., Zwietering, M.H., and Mead, G.C. 1995. The HACCP concept: Specification of criteria using quantitative risk assessment. Food Microbiol. 12: 81-90.

Odlaug, T.E. and Pflug, I.J. 1978. *Clostridium botulinum* and Acid foods. J. Food Protect. 41: 566-573.

Oguma, K., Yokotta, K., Hayashi, S., Takeshi, K., Kumagai, M., Itoh, N., Tachi, N., and Chiba, S. 1990. Infant botulism due to *Clostridium botulinum* type C toxin. Lancet 336: 1449-1450.

Ohye, D.F. and Christian, J.H.B. 1967. Combined effects of temperature, pH and water activity on growth and toxin production by *Clostridium botulinum* type A, B and E. In: *Botulism* 66. Ingram, M. and Roberts, T.A. (Eds.) pp. 217-223. Chapman and Hall Ltd., London.

Ohye, D.F. and Scott, W.J. 1957. Studies on the physiology of *Clostridium botulinum* type E. Australian J. Biol. Sci. 10: 85-94.

Raatjes, G.J.M. and Smelt, J.P.P.M. 1979. *Clostridium botulinum* can grow and form toxin at pH lower than 4.6. Nature 281: 398-399.

Sakaguchi, G. 1979. Botulism. In: *Food-Borne Infections and Intoxications*. Riemann, H. and Bryan, F.L. (Eds.) pp. 389-442. Academic Press, London.

Schmidt, C.F., Lechowich, R.V., and Folinazzo, J.F. 1961. Growth and Toxin production by type E *Clostridium botulinum* below 40°F. J. Food Sci. 26: 626-630.

Scott, V.N. 1989. Interaction of factors to control microbial spoilage of refrigerated foods. J. Food Protect. 52: 431-435.

Segner, W.P., Schmidt, C.F., and Boltz, J.K. 1966. Effect of sodium chloride and pH on the outgrowth of spores of type E *Clostridium botulinum* at optimal and suboptimal temperatures. Appl. Microbiol. 14: 49-54.

Segner, W.P., Schmidt, C.F., and Boltz, J.K. 1971. Minimal growth temperature, sodium chloride tolerance, pH sensitivity and toxin production of marine and terrestrial strains of *Clostridium botulinum* type C. Appl. Microbiol. 22: 1025-1029.

Smelt, J.P.P.M., Raatjes, G.J.M., Crowther, J.S., and Verrips, C.T. 1982. Growth and toxin formation by *Clostridium botulinum* at low pH values. J. Appl. Bacteriol. 52: 75-82.

Smith, L.D. 1977. *Botulism. The Organism. Its Toxins. The Disease.* Charles C. Thomas, Publisher, Springfield, IL, USA.

Solomon, H.M., Lynt, R.K., Lilly, T. Jr., and Kautter, D.A. 1977. Effect of low temperature on growth of *Clostridium botulinum* spores in meat of the blue crab. J. Food Protect. 40: 5-7.

Solomon, H.M. and Lilly, T. 1998. *Clostridium botulinum*. In: *FDA Bacteriological Analytical Manual*. AOAC International, Gaithersburg, MD, USA. pp. 1-10.

Sperber, W.H. 1982. Requirements of *Clostridium botulinum* for growth and toxin production. Food Technol. 36: 89-94.

Tanaka, N. 1982. Toxin production by *Clostridium botulinum* in media at pH lower than 4.6. J. Food Protect. 45: 234-237.

Tsang, N., Post, L.S., and Solberg, M. 1985. Growth and toxin production by *Clostridium botulinum* in model acidified systems. J. Food Sci. 50: 961-965.

Whiting, R.C. and Oriente, J.C. 1997. Time-to-turbidity model for non-proteolytic type B *Clostridium botulinum*. Int. J. Food Microbiol. 36: 49-60.

Young-Perkins, K.F. and Merson, R.L. 1987. *Clostridium botulinum* spore germination, outgrowth and toxin production below 4.6: Interactions between pH, total acidity and buffering capacity. J. Food Sci. 52: 1084-1088, 1096.