j.Egypt.net.med.Assoc 79, no 4. 1029 – 1045 (2019)

FACTORS AFFECTING THE VIABILITY OF *CLOSTRIDIUM BOTULINUM* IN SMOKED FISH DISPLAYED IN MARKETS

By

Ammar M.A.M. [1]and Azza S. El-Demerdash[2*]

[1]Agriculture Research Center (ARC), Animal Health Research Institute (AHRI), Assuit, Egypt.

[2] Agriculture Research Center (ARC), Animal Health Research Institute (AHRI), Zagazig, Egypt.

ABSTRACT

Cold smoked fish products can be naturally contaminated with *C. botulinum*.That could represent a serious hazard for consumers due to the probability of germination and toxin production. Therefore, the present study estimated the intrinsic hurdles (pH, sodium chloride, sodium nitrite, moisture, and competing microflora) and extrinsic hurdles as temperature concerning the presence of viable *C. botulinum* spores in 100 vacuum-packaged cold-smoked fish (renga) samples, mechanical defects were detected in 27% of the samples while 100% of the samples showed gross temperature abuse and only 2% were contaminated by viable non-proteolytic *C. botulinum* spores.The isolates were characterized by a polymerase chain reaction (PCR) to type B (1%) and type E (1%). Samples contained detectable levels of anaerobic spores; consequently, *C. botulinum* spores showed low values for most examined intrinsic hurdles and a high level of mechanical defects compared to those with non-detectable levels of anaerobic spores.Such data serve to reinforce the conclusion that manufacturing practices were inadequate to eliminate *C. botulinum* spores from smoked fish (renga) marketed in Sharkia retail markets. The salt content cannot stand alone as a control measure, and the potential for botulinal toxin production exists for such samples, especially over prolonged periods of storage with temperature abuse,. This is a warning sign that introduces further surveillance programs to ensure the safety of the product before it reaches consumers.

Keywords:

Smoked fish, C. *botulinum*, PCR, Toxins, Food safety.

INTRODUCTION

Smoked fish are the popular type of fish products that have been consumed on a large scale in Egypt **(Elgazzar *et al.* 2005)** and mainly prepared from the imported raw material of frozen

herring fish **(Salem 2004)**. This frozen herring product is also known as "renga" which is commercially produced by using three main steps, the first step is dry salting with NaCl, the second is partially air drying and the final step is smoking whether by using cold or hot smoking **(Osheba 2013).**

Although smoking increases the shelf life of the fish products, the hygienic standards of the fish products before, during and after it is suspected. Fish smoking helps in slowing down fish deterioration**,** thereby giving the commodity a longer shelf-life. However, investigations have shown the presence of microbial contaminants even on smoked fish **(Nyarko *et al.* 2011).** The contamination of live fish by *C. botulinum* has been recognized for many years. Worldwide prevalence studies in temperate geographical areas have shown C. *botulinum* type E as the most prevalent toxin type in aquatic environments, fish, and fishery products **(Kim *et al.* 2001)**. Post-processing microbial contaminants originate mainly from poor handling practices while some could be from the air, the source of the fish, or other degrading substances **(Jeyasanta *et al*. 2015)**.

Most vegetative cells of bacteria can be destroyed during the smoking process. However, their spores may be hard to eliminate **(ICMSF 1996)**. In most foods, botulinum spores are of no consequence unless they can grow and produce a toxin **(Aberoumand 2010).**

Growth and toxin production by *C. botulinum* in foods are affected by numerous aspects that some of which can be considered as "hurdles" comprising preliminary levels of spores, temperature,salt (water phase), acidity (pH),atmosphere (oxygen) and preservatives **(Merialdi *et al*. 2016)**. The inhibition of botulinum toxigenesis in fishery products relies almost solely on NaCl and refrigeration below 3°C **(Aberoumand 2010)**.

A vast range of studies has shown that hot-smoked and cold-smoked fish are good substrates for *C. botulinum* and that the organisms may grow and produce a toxin, depending on salt and temperature levels **(FDA 2001 [a])**. Thus, *C. botulinum* may become an important hazard to the food safety of aquaculture products **(Hastein *et al*. 2006)**. Two of the *C. botulinum* groups are responsible for foodborne botulism. Group I *C. botulinum* is proteolytic, mesophilic, and strains produce toxins of types A, B or F. Group II *C. botulinum* is nonproteolytic, psychrotrophic, strains produce toxins of types B, E or F, and in particular strains that produce neurotoxin of type E,is a major concern for cold-smoked fish **(FDA2001[a]).**

The organism can produce the most potent biological neurotoxins known, which if ingested,

results in 'botulism'. Botulism is a severe disease of humans and animals, with a high fatality rate. Typical symptoms are flaccid muscle paralysis; often initially blurred vision, followed by an acute symmetrical descending bilateral paralysis, and if not treated, ultimately paralysis of the respiratory or cardiac muscles. If severe cases are not fatal, then full recovery may take months or even years **(Carter and Peck 2015)**. The amount of type A toxin required to cause death in humans varies between 0.1 and 1.0 mg **(Hocking*et al*., 2003).** A lower lethal dose is estimated at 1ng/kg body weight by **FAO Joint *et al.*, (2002).**

Temperature abuse, inadequate processing, unspecified controlling factors were the main probable causes of foodborne outbreaks of botulism reported worldwide **(Carter and Peck 2015).** The potential for large outbreaks is illustrated by an outbreak of 91 botulism cases with 18 deaths related to type E which were associated with a traditionally salted fish dish (fesaikh) in Egypt in 1991 **(Weber *et al.* 1993).** In the United States, 161 cases of botulism were laboratory-confirmed and reported to CDC (Centre for Disease Control) in 2014. Foodborne botulism accounted for 9 %, infant botulism for 80 %, wound botulism for 10 % of the cases, and botulism of unknown or other etiology for 1%. In Europe, 133 botulism cases including three deaths were reported to the European Centre for Disease Prevention and Control (ECDC) in 2015. The average case fatality rate in Europe from 2010 to 2015 was 5.4 % **(Clauwers *et al*., 2017).** Besides, the costs for treatment, investigation, food recalls, and any legal actions are likely to be in millions of dollars/pounds even for a relatively small outbreak**.**

The successful prevention of foodborne botulism depends on (i) identifying appropriate control measures when new processing technologies are introduced or modified, and (ii) ensuring that these effective control measures are applied correctly. This requires the control of two physiologically distinct bacteria *C. botulinum* Groups I and II **(Carter and Peck 2015).** Concerning Egypt, the area of smoked fishery products and *C. botulinum* has not been studied extensively and very few data are available on the prevalence of *C. botulinum* in smoked fish reaching the consumer and the applied control measures. The search aims to assess the factors controlling the viability of *C. botulinum* in smoked fish reaching the consumer and documenting their relation to the detected types.

MATERIAL AND METHODS

Sample collection:

A total of 100 smoked renga samples were purchased randomly from different supermarkets in Zagazig, Sharkia Province, Egypt.

External examination of samples:

Gross evaluation of the soundness of package, the general condition of contents as well as evidence of spoilage was carried out according to **EOS (2005).**

Physico-Chemical Analysis:

A. Determination of pH.

The pH value was estimated according to **Goulas and Kontominas(2005)**as follows.

Ten gram**s** of sample were homogenized in 100 ml of distilled water and the mixture was filtered. The pH of the filtrate was measured using a pH meter (Gallenhamp No.101284) at ambient temperature.

B. Moisture Content:

Moisture content was determined by drying of 5 g of minced smoked fish in a convection oven at 105°C until constant weight **(AOAC 2000).**

C. Salt Content.

The sodium chloride content in smoked fish samples was determined by the volumetric method of Volhard **(AOAC 2000).**

D. Water phase salt.

Water phase salt was calculated by the formula

% NaCI = [(g NaCl) X 100]/[(g NaCl) + (g H_2o)] **(Rowswell 2017).**

E. Nitrites

Samples were assayed for nitrite as sodium nitrite using the **(AOAC 1990)** colorimetric method.

Bacteriological examination:

1. Samples were prepared according to **(FDA 2001[b]).** Briefly, it was applied as 10g quota of each sample was aseptically weighed into 90 ml of 0.1% peptone water in a sterile plastic bag, and then mingled in a Stomacher for thirty seconds. Ten-fold serial dilutions were used for microbiological examination.

2. Enumeration of total viable counts of aerobes, anaerobes, anaerobic spores was done according to **Vanderzalt and Splittstoesser (1992) and Harmon and Kautter (1978**), respectively.

3. Isolation and identification of *Clostridium botulinum* through the detection of typical Gram-positive Bacilli with sub-terminal oval spores grow on cooked meat medium and producing turbidity, gas, and digestion of the meat particles. Proteolytic activity of the obtained isolates was detected by inoculation into cooked meat medium anaerobically at 42 ºC. Proteolytic strains of *C. botulinum* (Type A, B, and F) in cooked meat media caused the blackening of the meat, decomposing it, reducing its volume with the formation of foul-smelling **(Solomon and Lilly, 2001)**.

Molecular Detection:

A. DNA extraction and PCR amplification.

The genomic DNA of *C. botulinum* isolates was extracted using the QIAamp DNA Mini Kit (Cat. No. 51304- Qiagen) according to the instructions of the manufacturer. Primers used for PCR reaction are mentioned in (Table 1).Then, PCR was performed with 50µl of reaction mixture containing 25 µL of DreamTaq TM Green MasterMix (2X) (Fermentas, USA), 0.3 µL of 100 pmole of each primer (Sigma, USA), 6 µL of template DNA and water nuclease-free up to 50 µL. Each PCR cycle consisted of initial denaturation at 95°C for 5 min followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 25 s, and extension at 72°C for 1 min 25 s. Final extension at 72°C for 3 minutes followed **(Lindström *et al.* 2001).**

B. PCR products visualization and analysis:

The amplified PCR products were separated by electrophoresis on 2% agarose gel (Applichem, Germany, GmbH) by running 20 µl of the PCR products. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed by software.

Statistical analysis:

Data were subjected to analysis of variances (one way-ANOVA) according to **(Knapp and Miller 1992)** using (SPSS Statistics 17.0) software program.

RESULTS AND DISCUSSION

Botulism is a devastating disease caused by botulinum neurotoxins (BoNTs) secreted primarily by *Clostridium botulinum* **(Bever *et al.* 2019).** Fish products are exquisitely prone to be the source for food botulism because of primary contamination with *Clostridium botulinum* spores, lack of heat inactivation of *C. botulinum* spores, frequently used air-tight packaging, lack of preservatives, and lack of heat inactivation of already produced botulinum toxin **(Dressler 2005)**. The natural contamination level of food products with *C. botulinum* spores is generally low and ranges from 10 to 1,000 spores/g **(Lindström and Korkeala 2006)**. Spores represent a metabolically dormant form of the organism derived from vegetative cells. They are more resistant to environmental challenges and control measures than vegetative cells. Such challenges include freezing, drying, pressure, radiation, ultraviolet light, chemicals, and heat **(Setlow and Johnson 2013).**

Growth of *C. botulinum* in suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of sodium chloride, an inhibitory concentration of sodium nitrite or other preservatives, or two or more of these conditions in combination **(FDA 2017)**.

The obtained results in (Table 2) indicate that 27% of cold-smoked fish samples show mechanical defects due to faulty processing by manufacturers. Also, the collected samples showed gross temperature abuse at their marketing sites that manifested by loose packages. The **EOS (2005)** recommended that mechanical defects must not be higher than 5% in the case of the whole fish product.

Although the collected samples were vacuum packaged, they showed gross temperature abuse at their marketing sites that manifested by loose packages. The process of vacuum packaging removes the air and prevents its return using an airtight seal that surrounds the food within the packaging material. But vacuum packaging does not eliminate the need for refrigeration. Failure to refrigerate is potentially dangerous as smoked fish can allow the toxin to develop whether the product is vacuum packaged or not. If storage temperatures rise above 3 °C, there is a risk that *C. botulinum* may grow and produce toxins in some types of smoked fish.

For this reason, the use of such packaging for smoked fish should be restricted to frozen products **(Hudson and Lake 2011**, **Novoslavskij *et al.* 2016).**

The values of microbiological examinations in (Table3) cleared that, the count of viable

aerobic bacteria ranged from 2 to 7.8 log CFU/g with a mean of 4.69 ± 0.37. Within samples that did not reveal anaerobes, six samples (6%) contained aerobic bacteria of more than 5 log CFU/g (Table 6). This revealed the role of poor processing and improper handling in contaminating the product as proved previously by **Jeyasanta *et al*. (2015).**

Moreover, the samples that contained an undetectable level of anaerobic spores showed a low aerobic count ranging from 2 to more than 5 log compared to samples that showed detectable anaerobic spores (3.5-7.9 log), (Tables 5,6). Forty-seven samples (47%) were positive for anaerobic spores and contained viable anaerobic spores that ranged from 1 to 2.5 log CFU/g. Besides, 22 (46.8%) of them were mechanically defected (Table 5). This data are supporting the suggestion of **Szabo and Gibson, (2003)** who reported that, the metabolic activity of high concentrations of spores germinating may favor the growth of *C. botulinum***.**

Of all spore-forming tested samples, only two *C. botulinum* isolates were detected (2%) and both of them were non-proteolytic. There is a concern that this may become an emerging issue as recently botulism outbreaks frequently associated with these clostridia.

The assessment done with multiplex PCR permits a simultaneous, sensible and rapid detection of the neurotoxin types of two *C. botulinum* isolates. The PCR products were visualized in agarose gels; 205bp for B type and 389bp for E enabled an easy distinction between the fragments as shown in Fig. (1).

These recovery results were lower than those of **Sarvestani *et al.* (2016)** who detected *C. botulinum* in 5 fish samples out of 120 ones in which, types A,B were identified in two and three cases,respectively. Also**,Lindström *et al*.(2001)**detected the presence of non-proteolytic *C.botulinum* type E which resulted in elevated viable counts in vacuum packaged hot-smoked fish.

Survival of type B, E spores through the smoking process is contrary to the generally accepted low heat resistance of these spores and represents a great hazard on public health as documented previously by **Peck (2006) and Aberoumand (2010).**

Data in table 4 revealed that sodium chloride content varied from 3.2 to 15.2% with a mean of 10.88±1.17. The **EOS (2005)** recommended the sodium chloride content for smoked fish must be within the range of 2.5-8%. The salt content slightly higher in the present study concluded that prolonged salting time and approximate salting are not at the correct proportion in the processing center. **Hudson and Lake (2011)** revealed that traditional fish

products preserved by salting alone have a high salt content 20% or more. A lower salt content requires chilling or freezing to preserve the final product. **Osheba,(2013)** revealed that, the main problem in commercial smoked herring is the high salt or sodium content which leads to many health problems. The positive correlation between sodium intake and the incidence of hypertension in old people and diabetes was cleared by **Hermansen,(2000).** Moreover, **Tuomilehto *et al.*,(2001)** found that high sodium intake correlated with mortality and the risk of coronary heart disease.

Regarding moisture content, it was beyond 48.4 to 66.4% with a mean of 57.03±1.50 (Table 4). **Burt, (1988)** stated that the moisture level must not exceed 15 % in smoked fish and any raise in this level facilitates microbial infestations. Also, industrial specifications for smoked finished products generally are recommended with water content in the fish flesh of less than 65% **(Cardinal *et al* 2001).** In related studies, **Kolodziejska *et al.*,(2002) and Goulas and Kontominas, (2005)** reported that, the moisture content of smoked mackerel samples varied from 56.7% to 59%, respectively. Variation in moisture content maybe because of the various treatments before smoking such as salting and drying **(Jeyasanta *et al*. 2015).** The percentage of sodium chloride in the aqueous phase of samples (WPS %) varied from 5.3 to 22.5% with a mean of 15.72±1.53 (Table 4). If the salt content is the controlling factor for safety, a concentration of 3.5% or above should be achieved throughout the aqueous phase of food. This should be monitored for every production batch **(Rowswell 2017).** Water phase salt guidelines vary depending on the smoking process, packaging, and nitrites. For vacuum-packaged hot- or cold-smoked fish, water phase salt must be at least 3.5% when no nitrites are used and at least 3.0% with at least 100 ppm nitrite **(FDA 1998)**. Consequently, all examined samples were within the safety limit of WPS%. Meanwhile, According to **FDA (2001[a]) guide** and **Huss *et al.*, (2004)**, brined products containing >10% WPS are usually safe for most pathogens except *S. aureus* at both ambient and cold storage because of the level of water activity (^{a}w). Although low ^{a}w is another prospect of preventing infectious agent growth, it is less seemingly to drop ^{a}w to the low levels for brined merchandise. *L. monocytogenes* and *C.botulinum* (type E and non-proteolytic type B and F) are other pathogens that present health hazards even at cold storage conditions if the salt content is lower than 10 % WPS, especially under reduced oxygen packing (e.g.vacuum packing), **(Köse 2010).**

Other investigators revealed that there is no minimum water phase salt needed to control

C. botulinum in vacuum packaged (VP) fish products stored at ≤ -18 ^{0}C **(Gilbert *et al.*, 2006).** Sodium nitrite, it could be detected in 88% of samples. Their amounts ranged from 0.1 to 10.51 ppm with a mean of 1.78±0.72. The samples showed detectable anaerobic viable spores contained nitrite level varied from undetectable level to 0.26 ppm (Table 5).

The corresponding values for those contained the undetectable level of spores were 0.26 to 10.51 ppm (Table 6).

Nitrite levels decrease during storage, depending on temperature and product formulation. **Hyytiä *et al.* (1997)** have studied the depletion rates for sodium nitrite and potassium nitrate in vacuum-packed cold-smoked rainbow trout over eight weeks stored at 4 and 8°C. Nitrite depletion was more rapid at 8°C while nitrate depletion was not significantly affected by temperature. Overall the concentrations of nitrite and nitrate used did not completely inhibit toxigenesis of non-proteolytic *C. botulinum* over the six-week storage although numbers of toxic samples were considerably reduced using nitrite and nitrate in the curing process.

Also, a recent study of **Rowswell (2017)** investigated the importance of nitrite usage as a controlling factor for *C. botulinum* and concluded that nitrite depletes readily from the product during storage thereby reducing the antimicrobial effect.

Concerning pH values, they vary from 7 to 7.6 with a mean of 7.33±0.03 (Table 4). In related studies, pH in vacuum packaged cold-smoked salmon varies between 6 and 6.3 **(Gram and Huss 1996).** Meanwhile**, Goulas and Kontominas, (2005)** recorded a mean pH value of 6.12±0.02 for smoked chub mackerel. The increase in pH values may be attributed to the production of volatile basic components such as ammonia, trimethylamine and total volatile nitrogen **(Ruiz-Capillas and Moral 2005).**

It is clear that all strains of *C. botulinum* grow and produce toxin down to pH 5.2 (when other conditions are optimal). Group I grow**s** slowly down to pH 4.6. Group II strains grow slowly down to pH 5.0. At this point, cells generally undergo sporulation. In some circumstances, germination and growth can take place below an initial pH of 4.6 **(Szabo and Gibson 2003).** Spore germination of Group II *C. botulinum* is optimum at pH 5.5-8.0 **(Plowman and Peck 2002).**

Results of the current and previous work elsewhere revealed the association between consumption of seafood, especially the traditionally processed ones (salted and smoked) and botulinal food outbreaks poisoning.

CONCLUSION

From previous results, the current fish processing practices were inadequate to eliminate *C. botulinum* spores from vacuum-packaged smoked fish (renga) that constitutes a consumer health risk because it is ready-to-eat food. Therefore, these products should be routinely tested for the presence of *C. botulinum*. The management of current risk requires industry inherence to good manufacturing practice**s**, good hygiene, appropriate product formulation (e.g. pH, levels of salt, sodium nitrite) and development of reliable time-temperature indicators for frozen long-storage fishery products.

REFERENCES

Aberoumand A. (2010): Occurrence of *Clostridium botulinum* in fish and fishery products in retail trade, a review article. World J Fis Mar Sci. 2:246-250.

AOAC. (1990): Association of Official Analytical Chemists. 15th Ed, Sect 97331, 95208, 93709 Arlington, VA.

AOAC. (2000): Official Methods of Analysis of Association of Analytical Chemist. 17th Ed, AOAC Int Gaithersburg MD USA.

BeverCS,ScotcherM,Cheng LW,HnaskoRM,StankerLH.(2019): Development and characterization of monoclonal antibodies to botulinum neurotoxin type E. Toxins (Basel). 11:407.

Burt JR. (1988): Fish smoking and drying: the effect of smoking and drying on the nutritional properties of fish. Elsevier Applied Science.

Cardinal M, Knockaert C, Torrissen O, Sigurgisladottir S, Mørkøre T, Thomassen M, Vallet JL. (2001): Relation of smoking parameters to the yield, colour and sensory quality of smoked Atlantic salmon (Salmo salar). Food Res Int. 34:537-550.

Carter AT, Peck MW. (2015): Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. Res Microbiol. 166:303 - 317.

Clauwers C,Lood C, van Noort V, MichielsCW.(2017): Canonical germinant receptor is dispensable for spore germination in *Clostridium botulinum* group II strain NCTC 11219. Sci Rep.;7 (1):15426. Doi: 10.1038/s41598-017-15839-y.

DresslerD.(2005): Botulism caused by consumption of smoked salmon.Nervenarzt. 76:763 -766.

Elgazzar MM., Hassan M., Farghaly RM, Sallam KI. (2005): Mycological and related studies on smoked fish. 4th IntSciConfMansoura:762-793.

EOS. (2005): Smoked Fish, Egyptian Organization for Standardization and Quality, Arab Republic of Egypt.

FAO Joint, others (2002): Evaluation of certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organ Tech Rep Ser. 1-62. Available from: https://www.ncbi.nlm.nih.gov/pubmed/11969182

FDA. (2017): Bacteriological analytical manual for examination of foods. Food and Drug Administration of the U.S. Department of health and human services.

FDA F. (1998): Fish and Fisheries Products Hazards and Controls Guide. FDA, Off Seaf 2nd ed Washington, DC. 276.

FDA US. (2001[a]): Guidance for industry: bioanalytical method validation. US Department of Health and Human Services. Food Drug Adm Cent Drug Eval Res (CDER), Cent Vet Med.

FDA US. (2001[b]): Bacteriological analytical manual. Chapter 17: *Clostridium botulinum.*

Gilbert S, Lake R, and Hudson A and Cressey P. (2006): Risk Profile: *Clostridium botulinum* in ready-to-eat smoked seafood in sealed packaging. Client Report FW0625. A reported for the New Zealand Food Safety Authority. Institute of Environmental Science and Research Ltd.

Goulas AE, Kontominas MG. (2005): Effect of salting and smoking-method on the keeping quality of chub mackerel (Scomber japonicus): biochemical and sensory attributes. Food Chem. 93:511-520. Available from: http://www.sciencedirect.com/science/article/pii/S0308814604007769

Gram L, Huss HH. (1996): Microbiological spoilage of fish and fish products. Int J Food Microbiol. 33:121–137.

Harmon SM, Kautter DA. (1978): Media for confirming *Clostridium perfringens* from food and feces. J Food Prot. 41:626 - 630.

Hastein T, Hjeltnes B, Lillehaug A, Utne Skare J, Berntssen M, Lundebye AK. (2006): Food safety hazards that occur during the production stage: challenges for fish farming and the fishing industry. Rev Sci Tech. 25:607- 625.

Hermansen K. (2000): Diet, blood pressure and hypertension. Br J Nutr. 83:S113-S119.

Hocking AD, others (2003): Foodborne microorganisms of public health significance. Australian Institute of Food Science and Technology Incorporated (AIFST Inc.).

Hudson A, Lake R. (2011): Risk profile, *Clostridium botulinum* in ready to eat smoked fish and shellfish in sealed packaging . NewZealand Government, Ministry for Primary Industries. Technical Paper No: 2012/27. Available from: https://www.mpi.govt.nz/dmsdocument/35337/direct

Huss HH, Ababouch L, Gram L. (2004): Assesment and management of seafood safety and quality FAO Fischeries Technical Paper. Available from: http://www.fao.org/3/a-y4743e.pdf

Hyytiä E, Eerola S, Hielm S, Korkeala H. (1997): Sodium nitrite and potassium nitrate in control of nonproteolytic *Clostridium botulinum* outgrowth and toxigenesis in vacuum-packed cold-smoked rainbow trout. Int J Food Microbiol. 37:63-72.

ICMSF. (1996): Micro Organisms in Foods 5: Microbiological Specifications of Food Pathogens: Blackie Academic and Professional.

Jeyasanta IK, Giftson H, Saritha K, Patterson J. (2015): Studies on the nutritional and microbiological quality of smoked tuna fish (Auxis thazard) in Tuticorin , South East Coast of India. Int J Food Qual Saf. 1:28-38.

Kim S-H, Field KG, Chang D-S, Wei C-I, An H. (2001): Identification of bacteria crucial to histamine accumulation in Pacific mackerel during storage. J Food Prot. 64:1556 -1564.

Knapp RG, Miller MC. (1992): Clinical epidemiology and biostatistics.: Harwal Publishing Company.

Kolodziejska I, Niecikowska C, Januszewska E, Sikorski ZE. (2002): The microbial and sensory quality of mackerel hot smoked in mild conditions. LWT-Food Sci Technol. 35:87-92.

Köse S. (2010): Evaluation of seafood safety health hazards for traditional fish products: preventive measures and monitoring. Turkish J Fish Aquat Sci J Fish Aquat Sci. 10:139 - 160. Available from: http://www.trjfas.org.

Lindström M. (2003): Diagnostics of *Clostridium Botulinum* and thermal control of Nonproteolytic *C.Botulinum* in refrigerated processed foods. J Food Prot.:11. Available from: http://ethesis.helsinki.fi/julkaisut/ela/elint/vk/lindstrom/diagnost.pdf.

Lindström M, Keto R, Markkula A, Nevas M, Hielm S, Korkeala H. (2001): Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. Appl Environ Microbiol. 67:5694 -5699.

Lindström M,Korkeala H.(2006): Laboratory diagnostics of botulism.Clin Microbiol Rev19:298-314.

Merialdi G, Ramini M, Parolari G, Barbuti S, Frustoli MA, Taddei R, Pongolini S, Ardigò P, Cozzolino P. (2016): Study on potential *Clostridium botulinum* growth and toxin production in Parma ham. Ital J Food Saf. 5.

Novoslavskij A,Terentjeva M, Eizenberga I, Valcicna O, Bartkevičs V, Berzicnš A. (2016): Major foodborne pathogens in fish and fish products: a review. Ann Microbiol.66:1-15.

Nyarko HD, Obodai EA, Coomson LK, Coomson SS, Aniwe Y. (2011): Microbial profile of smoked sardine (Sardilella aurita) at smoking sites and market centres of Tema, Ghana-1. Arch Appl Sci Res. 3:443 - 453.

Osheba AS. (2013): Technological attempts for production of low sodium smoked herring fish (renga). Adv J Food Sci Technol. 5:695 -706.

Peck MM. (2006): *Clostridium botulinum* and the safety of minimally heated, chilled foods: an emerging issue, Journal of Applied Microbiology, 101: 556 -570

Plowman J, Peck MW. (2002): Use of a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. J Appl Microbiol. 92:681- 694.

Rowswell C. (2017): The safety and shelf-life of vacuum and modified atmosphere packed chilled foods with respect to non-proteolytic *Clostridium botulinum*. Food Stand Agency United Kingidm.

Ruiz-Capillas C, Moral A. (2005): Sensory and biochemical aspects of quality of whole bigeye tuna (Thunnus obesus) during bulk storage in controlled atmospheres. Food Chem. 89:347–354.

Salem DAI. (2004): Microbiological profile of local smoked fish. Fac. Vet. Med. Moshtohor, Zagazig Univ. Benha Branch.

Sarvestani S, Poormontaseri M, Fazeli M, Hosseinzadeh S. (2016): Occurrence of *Clostridium botulinum* in Four Species of Fish in the Persian Gulf. Int Arch Med. 9.

Setlow P, Johnson EA. (2013): Spores and their significance. In Food Microbiol. American Society of Microbiology:45-79.

Szabo EA, Gibson AM. (2003): *Clostridium botulinum*. Foodborne Microorg Public Heal Significance, :505-542.

Solomon HM and Lilly T. (2001): Bacteriological Analytical Manual Chapter 17 *Clostridium botulinum.* Jackson, GJ, Merker, RI, Bandler, R., Eds, Available from: https://www.fda.gov/food/laboratory-methods-food/bam-clostridium-botulinum.

Tuomilehto J,Jousilahti P, Rastenyte D,Moltchanov V,Tanskanen A, Pietinen P,Nissinen A. (2001): Urinary sodium excretion and cardiovascular mortality in Finland: a prospective study. Lancet. 357:848 - 851.

Vanderzalt C, Splittstoesser DF. (1992): Compendium of methods for microbiological examination of food. 3rd. Washingt Publ by Amer Public Heal Assn.

Weber JT, Hibbs Jr RG, Darwish A, Mishu B. Corwin AL, Rakha M, Hatheway CL, Sharkawy S El, Rahim SA El, Al Hamd MFS, others (1993): A massive outbreak of type E botulism associated with traditional salted fish in Cairo. J Infect Dis. 167:451–454.

Table (1): Primers used for Multiplex PCR amplification of the *C. botulinum* types.

Toxin type	Sequence (5=‹3)	Size of PCR amplicon (bp)
A	**GGG CCT AGA GGT AGC GTA RTG TCT TYA TTT CCA GAA GCA TAT TTT**	**101**
B	**CAG GAG AAG TGG AGC GAA AA CTT GCG CCT TTG TTT TCT TG**	**205**
E	**CCA AGA TTT TCA TCC GCC TA GCT ATT GAT CCA AAA CGG TGA**	**389**
F	**CGG CTT CAT TAG AGA ACG GA TAA CTC CCC TAG CCC CGT AT**	**543**

Table (2): External examination characteristics of examined samples.

External characters	Affected samples	
	+ve out of 100	%
Grossly damaged package	**0**	**0**
Gross temperature abuse (loose package)	**100**	**100**
Presence of scales	**0**	**0**
Mechanical defects (incised muscle, crutched or skinless area)	**27**	**27**
Abnormal consistency	**0**	**0**
Evidence of spoilage (abnormal odor, color or taste)	**0**	**0**
Presence of foreign matter	**0**	**0**

Table (3): Statistical values of indicator microorganisms in examined samples.

Indicators	+v out of 100	%	Min.	Max.	Log Mean
Log total viable aerobic bacteria	**100**	**100**	**2**	**7.8**	**4.69±0.37**
Log total viable anaerobic bacteria	**47**	**47**	**1.6**	**2.5**	**2.14±0.10**
Log total viable anaerobic spores	**47**	**47**	**1**	**2.5**	**1.83±0.16**

Table (4): Statistical values of intrinsic controlling factors in examined samples.

Indicators	+v out of 100	%	Min.	Max.	Mean
pH	**100**	**100**	**7**	**7.6**	**7.33±0.03**
Moisture %	**100**	**100**	**48.4**	**6.4**	**57.03±1.50**
Sodium chloride %	**100**	**100**	**3.2**	**15.2**	**10.88±1.17**
Water phase salt %	**100**	**100**	**5.3**	**22.5**	**15.72±1.53**
Sodium nitrite (ppm)	**88**	**88**	**0.1**	**10.51**	**1.78±0.72**

Table (5): Distribution of indicators, control parameters and *Clostridium botulinum* in samples containing detectable level of viable anaerobic spores.

Log total anaerobic viable spores	Log total viable anaerobes		Log total viable aerobic		pH		Moisture %		Sodium chloride %		Water phase salt %		Sodium nitrite (ppm)		+ve / 100	%	Mechanical defects		C. botulinum	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max			Affected	%	No.	%
1- <2	1.8	2.2	3.5	6.4	7	7.67	60.23	66.83	3.61	3.92	5.91	7.24	0	0.1	19	19	8	8	0	0
2- 2.5	2.3	2.5	5.8	7.9	7.01	7.67	66.22	69.01	3.23	13.76	5.32	9.22	0	0.26	28	28	14	14	2	2
Total	-	-	-	-	-	-	-	-	-	-	-	-	-	-	47	47	22	22	2	2

Table (6): Distribution of indicators and control parameters in samples containing the undetectable level of viable anaerobic spores.

Log total viable aerobic count	Log total Viable anaerobes	Log total anaerobic viable spores	pH		Moisture%		Sodium chloride%		Water phase salt %		Sodium nitrite (ppm)		+ve / 100	%	Mechanical defects		C. botulinum
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max			Affected	%	
2-<3	<1	<1	7.05	7.51	49.8	61.8	13.8	14.5	19.2	21.7	2.5	10.61	14	14	0	0	-
3-<4	<1	<1	7.29	7.31	44.7	63.1	13	15	19.4	22.5	3.2	6.43	24	24	0	0	-
4-5	<1	<1	7.33	7.41	60.9	66.4	13.9	14.9	17.4	19.6	1.6	2.5	9	9	5	5	-
>5	<1	<1	7.47	7.6	57.7	62.8	13.3	13.8	17.5	18.9	0.25	0.56	6	6	-	-	-
Total	-	-	-	-	-	-	-	-	-	-	-		53	53	5	5	-

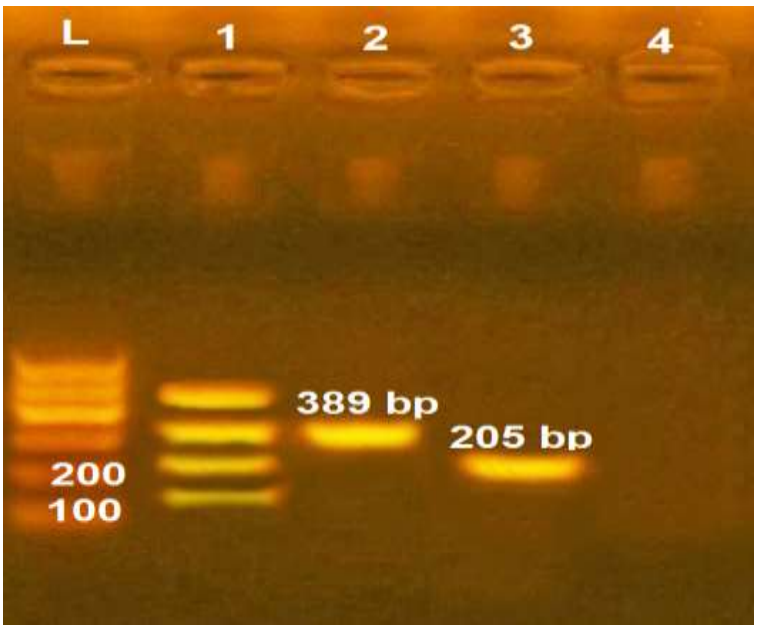

Fig. (1) Agarose gel electrophoresis of multiplex PCR detection of *C. botulinum*. Lanes L: 100 bp ladder (marker); lane 1: Control positive strains (*C. botulinum* type A, *C. botulinum* type B, *C. botulinum* type E and *C.botulinum* type F), lane 2: *C. botulinum* isolate type E, lane 3: *C. botulinum* isolate type B. lane 4: control negative

العوامل المؤثرة في حيوية الكلوستريديم بتيولينم في الاسماك المدخنة المعروضة بالأسواق

د.محمود عمارمحمد عمار[1], د.عزه صلاح الدمرداش[2]

[1] مركز البحوث الزراعيه، معهد بحوث الصحه الحيوانيه، أسيوط، مصر

[2] مركز البحوث الزراعيه، معهد بحوث الصحه الحيوانيه، الزقازيق، مصر

الملخص العربى

يمكن أن تكون منتجات السمك المدخن علي البارد ملوثة بشكل طبيعي بـالكلوستريديم بتيولينم. وهذا يمثل خطرا كبيرا على المستهلكين بسبب احتمال نموها وقدرتها علي انتاج السموم. لذلك ، قدرت هذه الدراسة العقبات الداخليه (الرقم الهيدروجيني ، كلوريد الصوديوم ، نتريت الصوديوم ، الرطوبة ، والميكروفلورا المتنافسة) والعقبة الخارجية (درجة الحرارة) فيما يتعلق بوجود جراثيم البتيولينم القابلة للحياة في ١٠٠ عينه من الأسماك المدخنة على البارد والمعبئه في عبوات مفرغه في الهواء. تم الكشف عن عيوب ميكانيكية في ٢٧ ٪ من العينات ، في حين أظهرت ١٠٠ ٪ من العينات سوء التحكم في درجة الحرارة اثناء التسويق ٢ ٪ فقط احتوت جراثيم الكلوستريديم بتيولينم قابلة للحياة. تميزت العزلات بتفاعل البلمرة المتسلسل إلى ١ ٪ من النوع B و١ ٪ من النوع E . كما وجد ان العينات تحتوي على مستويات محدده من الجراثيم اللاهوائية ؛ وبالتالي ، أظهرت جراثيم الكلوستريديم بتيولينم قيمًا منخفضة لمعظم العقبات الداخلية المدروسة ومستوى عالٍ من العيوب الميكانيكية مقارنة بتلك ذات المستويات غير القابلة للاكتشاف للجراثيم اللاهوائية. تعمل هذه البيانات على تعزيز الاستنتاج القائل بأن ممارسات التصنيع كانت غير كافية للتخلص من جراثيم الكلوستريديم بتيولينم من الأسماك المدخنة (الرنجه) التي يتم تسويقها في أسواق البيع بالتجزئة بمحافظه الشرقيه.ولا يمكن أن يعتبر محتوى الملح بمفرده كمقياس تحكم ، وتوجد إمكانية لإنتاج التوكسين البوتوليني لمثل هذه العينات (خاصة خلال فترات التخزين الطويلة مع إساءة استخدام درجة الحرارة). فهذه علامة تحذير لتقديم برامج مراقبة إضافية لضمان سلامة المنتج قبل وصوله إلى المستهلكين.