Growth and toxigenesis behavior of *Clostridium botulinum* type E in Persian sturgeon (*Acipenser persicus*) Caviar prepared with various preservatives

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Abstract: Growth behavior of *Clostridium botulinum* type E beluga was studied in Acipenser persicus granular caviar treated with 5% NaCl, 5%NaCl plus 0.3% boric acid and 0.4%borax and 5%NaCl plus 0.15% methyl paraben incubated at temperatures -2, 5 and 15 °C for 224 days. The initial number of inoculated bacterial spore was 5.1×10^4 cfu/g caviar in each treated sample. The number of bacterial spores in samples treated with 5% NaCl changed to 3.59×10^4, 1.02×10^5 and 9.9×10^5 cfu/g at -2 °C, 5 °C and 15 °C, respectively, while those samples treated with 5%NaCl plus 0.3% boric acid and 0.4%borax changed to 1.56×10^4, 3.65×10^4 and 9.22×10^4 cfu/g, respectively. Also, number of bacterial spores in samples treated with 5% NaCl plus 0.15% methyl paraben changed to 1×10^4, 2.86×10^4 and 3.56×10^4 cfu/g at the above storage temperatures, respectively. Fourteen days post-inoculation, toxin production was positive in samples treated with 5% NaCl incubated at 5 °C and 15 °C, while those samples treated with 5%NaCl plus 0.3% boric acid and 0.4% borax were positive for toxin production only at 15 °C. Toxin production was negative in samples treated with 5%NaCl plus 0.15% methyl paraben incubated at all three above mentioned temperatures. These data showed that 5%NaCl plus methyl paraben had more inhibitory effect than boric, borax and NaCl. Also, such inhibitory effects can be increased at lower storage temperature of -2 °C. Therefore, due to restricted usage of boric acid and borax in granular caviar, use of methyl paraben can be recommended as a safe preservative with a strong antimicrobial effect at caviar pH.

Key words: Caviar,*Clostridium botulinum*, preservative.

Introduction

Iran is one of the major exporters of sturgeon caviar in the world producing 1.89 and 2.6 7mt meat and caviar in 2004, respectively (Deputy of production and exploration 2005, Iran Fisheries Organization). Chemical spoilage and bacterial contamination of sturgeon caviar is high because of its composition in high protein (22-28%) and lipid contents (15-78%), presence of tissue materials, and lack of pasteurization during its processing (Bruner et al., 1995; Suny 1996). Therefore, caviar quality improvement is one of the fundamental matters during its processing. Physical (freezing) and chemical (preservatives) treatments plus packaging in anaerobic condition are current methods used for the control of contamination and spoilage of caviar

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(Sternin and Dore, 1993). However, under such conditions the risk of contamination may still remain high.

Some of the most important preservatives used in food are benzoate derivatives (parabens), sorbet, nitrite, borax and boric acid. The Persian caviar is traditionally processed using two additives of salt without any particular preservative and a combination of salt plus a preservative such as boric acid and borax. However, use of the later preservative is restricted or prohibited in many countries (Pelroy *et al.,* 1982; European Parliament and the Commission of the European Communities, 1995).

Clostridium botulinum, the causative agent of botulism is widely distributed in soil, sediments and aquatic environment (Dodds, 1993a). The foodborne botulism outbreak is estimated to be 20% per year (Hatheway, 1995). Among the seven recognized serotypes of the bacterium (Hatheway, 1995) type E is the most common detectable serotype in aquatic environments (Hatheway, 1995), particularly in freshwater sediments of various parts of the world such as the United States, Greek (Bott *et al.,* 1968), Japan (Yamakawa and Nakamura, 1992), Sweden (Johannsen, 1963) and Finland (Hielm *et al.,* 1998c). Most of the fish-borne botulism outbreaks have been also recorded in Canada, USA, former USSR, Europe, Japan and Iran. This food poisoning was linked to the consumption of canned or fermented fish usually eaten without further cooking (Hauschild, 1993).

Because the botulinal spores are widely distributed in the environment, they may be introduced into processed foods through raw materials or by post-processing contamination (Dodds, 1993b). *Clostridium botulinum* type E grows well in carrion of fin fish, marine mammals and invertebrates and can be a part of aquatic animal alimentary tracts (Lalitha, 2000). The bacterium is also capable to grow well at low temperature of 3.3 °C, different salinity concentrations of 4.5-5% NaCl and anaerobic condition (Elliott and Schaffner 2001). Since the present caviar collection and processing is very often under the risk of contamination with this highly toxic bacterium, the application of some safe and friendly preservatives in the caviar are essential to prevent the growth and toxin production by *C. botulinum* type E. The aim of this study was to evaluate the effect of various preservative formulations on the growth behavior and toxigenesis of this bacterium in Persian sturgeon caviar.

Materials and Methods

Sample preparation of Caviar and preservatives: Tins (totally 63 samples in duplicate) containing 50 g each of Persian sturgeon (*Acipenser persicus*) caviar were obtained and processed in a caviar plant in Mazandaran province, Iran. Samples were divided in three groups randomly, following preservative formula added to each group under aseptic condition: 5% NaCl, 5% NaCl plus 0.3% boric acid and 0.4% borax; and 5% NaCl plus 0.15% methyl paraben.

Bacterial preparation and inoculation: *C.botulinum* type E was grown in Cooked Meat medium (Difco) at 30 °C for 4-12 days.

Primary suspension was then filtrated by multilayer scour and the residual suspension was centrifuged at 10000 × g for 25 min 3 times. To inactivate the vegetative cells ethanol (70%) (Merck) was added to the centrifuged sediment 1h before further centrifuging. Counting of the free spores was carried out using egg yolk agar (EYA) [nutrient agar (Merck) plus yolk sac (Merck)] and brain heart infusion agar (BHI) (Merck) (Solomon *et al.,* 1995; Nordic Committee on Food Analysis, 1991b). The bacterial spores were then added to each sample at a concentration of 5.1×10^4 cfu/g prior to cap the tins. Each sample group was then kept at temperatures -2, 5 and 15 °C for a period of 224 days.

Detection of bacterial growth and toxin analysis in samples: The bacterial count was examined immediately after bacterial inoculation (time zero) and on days 14, 28, 56, 112, 168 and 224 post-inoculation by culturing of samples on EYA and BHI at 30°C for 48-72 h under anaerobic condition (Solomon *et al.,* 1995; Nordic Committee on Food Analysis 1991b). The procedure by the Nordic Committee on Food Analysis protocol (1991a) was used for the assay of botulinum toxin in the caviar samples. Briefly, 5 g sample was homogenized with

Table 1. Mean and standard deviation of *Clostridium botulinum* type E strain beluga spores (cfu/ml) in Persian caviar processed with different preservatives and incubated at -2 °C, 5 °C and 15 °C for 224 days. (A= 5%NaCl , B= 5% NaCl + 0.3% boric acid + 0.4%borax , C= 5% NaCl + 0.15% methyl paraben).

Temperature	Formula	Time (days)						
		0	14	28	56	112	168	224
-2 °C	A	5.1×10^4	$4.7\times10^4 \pm 1.6\times10^4$	$4.5\times10^4 \pm 5.7\times10^3$	$4.3\times10^4 \pm 2.9\times10^4$	$4\times10^4 \pm 2.8\times10^4$	$3.8\times10^4 \pm 1.3\times10^4$	$3.5\times10^4 \pm 2.6\times10^4$
	B	5.1×10^4	$4.4\times10^4 \pm 3.2\times10^4$	$4.0\times10^4 \pm 2.8\times10^4$	$3.4\times10^4 \pm 1.1\times10^4$	$2.5\times10^4 \pm 1.5\times10^4$	$2.1\times10^4 \pm 1.5\times10^4$	$1.5\times10^4 \pm 2.8\times10^3$
	C	5.1×10^4	$4.1\times10^4 \pm 1.4\times10^4$	$3.7\times10^4 \pm 2.8\times10^4$	$3.1\times10^4 \pm 1.4\times10^4$	$2.2\times10^4 \pm 1.2\times10^4$	$1.6\times10^4 \pm 5\times10^3$	$1.0\times10^4 \pm 7.0\times10^3$
5 °C	A	5.1×10^4	$5.5\times10^4 \pm 5.5\times10^4$	$5.9\times10^4 \pm 1.5\times10^4$	$6.6\times10^4 \pm 4.5\times10^4$	$7.7\times10^4 \pm 1.3\times10^4$	$8.2\times10^4 \pm 1.2\times10^4$	$1.0\times10^5 \pm 4.2\times10^3$
	B	5.1×10^4	$4.9\times10^4 \pm 1.5\times10^4$	$4.7\times10^4 \pm 1.2\times10^4$	$4.5\times10^4 \pm 2.5\times10^4$	$4.2\times10^4 \pm 1.6\times10^4$	$3.8\times10^4 \pm 2.8\times10^4$	$3.6\times10^4 \pm 9.8\times10^3$
	C	5.1×10^4	$4.6\times10^4 \pm 3.2\times10^4$	$4.4\times10^4 \pm 1.1\times10^4$	$4.1\times10^4 \pm 1.5\times10^4$	$3.7\times10^4 \pm 1.6\times10^4$	$3.2\times10^4 \pm 2.6\times10^4$	$2.8\times10^4 \pm 1.1\times10^4$
15 °C	A	5.1×10^4	$9.5\times10^4 \pm 9.8\times10^3$	$1.2\times10^5 \pm 1.4\times10^4$	$2.1\times10^5 \pm 1.5\times10^4$	$4.6\times10^5 \pm 1.6\times10^5$	$6.6\times10^5 \pm 8.4\times10^4$	$9.9\times10^5 \pm 2.6\times10^5$
	B	5.1×10^4	$5.3\times10^4 \pm 3.1\times10^3$	$5.5\times10^4 \pm 2.4\times10^4$	$5.9\times10^4 \pm 2.6\times10^4$	$6.5\times10^4 \pm 9.8\times10^3$	$7.5\times10^4 \pm 1.5\times10^4$	$9.2\times10^4 \pm 1\times10^4$
	C	5.1×10^4	$4.9\times10^4 \pm 1.4\times10^4$	$4.6\times10^4 \pm 9.8\times10^3$	$4.3\times10^4 \pm 2.5\times10^4$	$4.1\times10^4 \pm 2.9\times10^4$	$3.9\times10^4 \pm 1.5\times10^4$	$3.5\times10^4 \pm 1.5\times10^4$

gelatin phosphate buffer in a ratio 1:2 (w/v) for two minutes in a Stomacher 400 Lab Blender. The homogenated materials were centrifuged at 10000 x g for 15 minutes and for 20 min at 4 °C. The methodology outlined by FDA (Solomon *et al.,* 1995) was employed for the trypsinized, untrypsinized and toxin neutralization tests. Toxicity of the supernatants was then tested using mouse bioassay. All mice were observed periodically for 96 h for symptoms of botulism and death. Toxin neutralization tests were then made using type specific monovalent antitoxin E (Pastor Institute, France).

The obtained data were statistically analyzed using SPSS (SPSS version 6.1). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test was used to identify the significant difference between the used formula ($p<0.05$).

Results

The obtained results are shown in Table 1 and Figures 1-3. At storage temperature of -2 °C, the number of bacterial spores in the samples treated with 5% NaCl, the combination of 5% NaCl, 0.3% boric

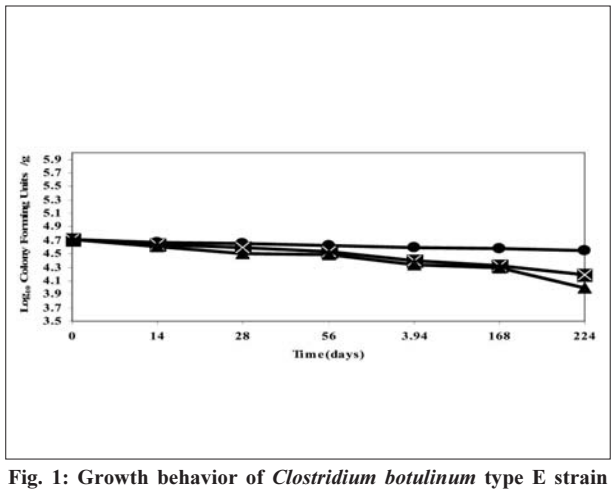

Fig. 1: Growth behavior of *Clostridium botulinum* type E strain beluga spores (cfu/ml) in Persian caviar processed with different preservatives and incubated at -2 °C for 224 days. —●— 5% Na C1, —⊠— 5% Na C1 + 0.3% Boric acid + 0.4% Borax, —▲— 5% Na C1 + 0.15% Methyl paraben.

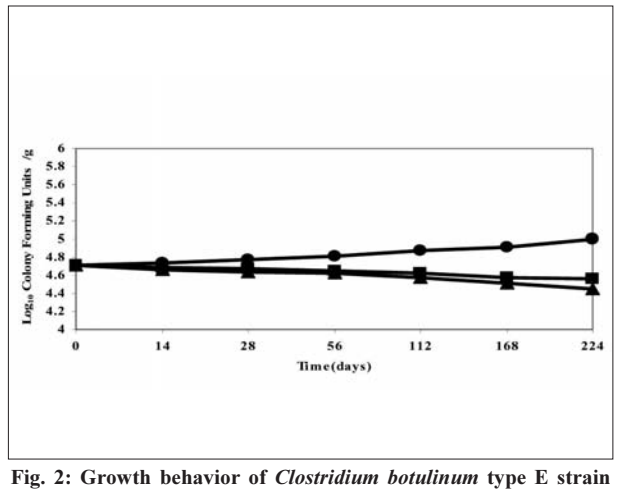

Fig. 2: Growth behavior of *Clostridium botulinum* type E strain beluga spores (cfu/ml) in Persian caviar processed with different preservatives and incubated at 5 °C for 224 days. —●— 5% Na C1, —⊠— 5% Na C1 + 0.3% Boric acid + 0.4% Borax, —▲— 5% Na C1 + 0.15% Methyl paraben.

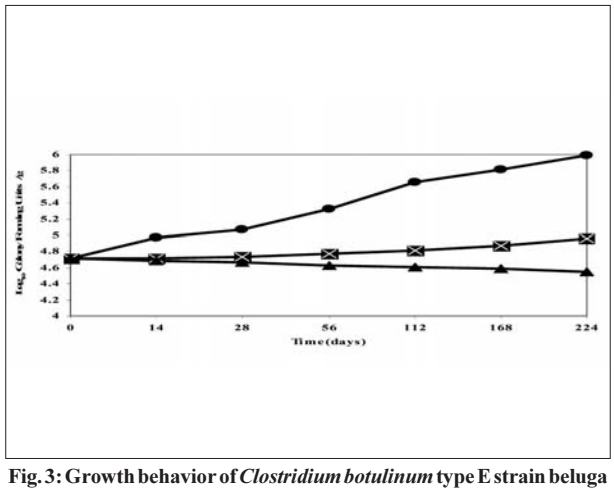

Fig. 3: Growth behavior of *Clostridium botulinum* type E strain beluga spores (cfu/ml) in Persian caviar processed with different preservatives and incubated at 15 °C for 224 days. —●— 5% Na C1, —⊠— 5% Na C1 + 0.3% Boric acid + 0.4% Borax, —▲— 5% Na C1 + 0.15% Methyl paraben.

acid and 0.4% borax and 5% NaCl plus 0.15% methyl paraben, reduced from 5.1×10^4 cfu/g to 3.59×10^4 cfu/g ($p<0.05$), 1.56×10^4 cfu/g ($p<0.05$) and 1×10^4 cfu/g ($p<0.05$) respectively, 224 days post-inoculation (Table 1, Figure 1). Also at storage temperature of 5 °C, number of bacterial spores in samples treated with 5% NaCl increased to 1.02×10^5 cfu/g ($p<0.05$), while the number of bacterial spores in samples treated with the combination of 5% NaCl, 0.3% boric acid and 0.4% borax and 5% NaCl plus 0.15% methyl paraben decreased to 3.65×10^4 cfu/g ($p<0.05$) and 2.86×10^4 cfu/g ($p<0.05$), respectively 224 days post-inoculation(Table 1, Figure 2). In addition, the counting of bacterial spores at 15 °C in the samples treated with 5% NaCl and the combination of 5% NaCl, 0.3% boric acid and 0.4% borax increased to 9.9×10^5 cfu/g ($p<0.05$) and 9.22×10^4 cfu/g ($p<0.05$), respectively while those samples treated with 5% NaCl plus 0.15% methyl paraben decreased to 3.56×10^4 cfu/g ($p<0.05$). (Table 1, Figure 3). Toxin production was positive in 5% NaCl treatment at 5 °C and 15 °C 14 days post-inoculation. Samples treated with 5% NaCl plus 0.3% boric acid and 0.4% borax were positive for toxin production at 15 °C 14 days post-treatment. Toxigenesis was negative at -2 °C in all treated groups. Furthermore, no toxin production was detected in samples treated with 5% NaCl plus methyl paraben.

Discussion

Vacuum packaging is one of the treatment regimes currently used in granular caviar. This anaerobic condition provides growth of anaerobic bacteria such as *C. botulinum* specially type E. Several studies have indicated that vacuum-packaging is more conducive to botulinum toxin production than CO_2-enriched modified atmospheres or storage in air (Reddy *et al.,* 1996 and 1997; Garcia *et al.,* 1987; Baker *et al.,* 1990b). However, Baker *et al*., (1990a) found that growth and

toxin production by type E were only slightly faster in fish homogenate packaged in vacuum than that packaged in 100 % CO_2-enriched modified atmospheres (Baker *et al.,* 1990a). The ability of type E to grow and produce toxin has been studied in vacuum-packaged raw fish of several different species (Baker and Genigeorgis, 1990; Garren *et al.,* 1995), in vacuum-packaged cold-smoked herring (Cann *et al.,* 1980), vacuum-packaged hot-smoked salmon (Pelroy *et al.,* 1982), mackerel and Tialpia (Reddy *et al.,* 1996). However, in several of these studies the type E inoculums were grossly above the natural contamination level encountered in fish and often the inoculation was performed after the processing.

Although the refrigerated storage of food products at < 3 °C is an adequate control for growth and toxin production by *C. botulinum* type E (Graham *et al.,* 1997), the produced toxin is stable at refrigerated temperatures. This is because the bacterial spores can germinate relatively rapidly at temperatures as low as 2 °C, with an optimum temperature at 9 °C (Grecz and Arvay, 1982); Graham *et al.,* 1997). In this study, toxin production was positive in caviar samples treated with 5% NaCl at 5 °C and 15 °C, 14 days-post inoculation. Also, caviar samples treated with combination of 5% NaCl, 0.3% boric acid and 0.4% borax were positive for toxin production at 15 °C 14 days post-inoculation, while no toxin production was detected in samples treated with salt plus methyl paraben. Therefore, it seems that use of a combination of salt plus methyl paraben is more inhibitory than boric acid and borax.

As the shelf life of refrigerated foods is increased, more time is available for the growth and toxin production by *C. botulinum*. As the storage temperature increase, the time required for toxin formation is significantly shortened (Graham *et al.,* 1997). The results of this study showed that methyl paraben had more inhibitory effect than boric acid, borax and NaCl alone, until 224 days post-inoculation. In addition, with a decrease in the storage temperature, the inhibitory effect of this preservative was increased.

Sperber (1982) reported that the bacterial growth and toxin production do not occur above a water phase of 5% NaCl. The preserving action of salt in most foods is closely related to other factors in the curing process. For example, use of nitrite and acidic pH and an increase in the processing temperature can decrease the level of salt required to inhibit the growth of type E strains (Lynt *et al.,* 1982). Graham *et al*., (1997) studied the combined effect of pH (5-7.3), NaCl concentration (0.1-5.0%) and temperature (4-30ºC) on growth of non-proteolytic *C. botulinum* in laboratory media. The results showed that *C. botulinum* type E was able to grow in all used treatments. In this study, the pH of all treatment groups was acidic and in a range of 5.5-6.3, which is suitable for growth of these bacterial spores. However, the growth of bacterial spore in all treatment groups was significantly decreased at lower temperature of -2ºC. In conclusion, it seems that a combination use of low temperature less than -2ºC plus use of methyl paraben can be chosen as a suitable method to inhibit the growth and toxin production by *C. botulinum* type E in the granular caviar.

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