original article

Risk assessment of the growth of *Clostridium botulinum* and spores germination induced by high hydrostatic pressure in seafood

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ABSTRACT

Aims: In the present study, a risk assessment for growth of *Clostridium botulinum* in model seafood deli salads was performed along with the effect of growth of resident microbes on the intrinsic and extrinsic properties of the product. Furthermore, high hydrostatic pressure (HHP) was applied to induce the germination of the surrogate spores.

Materials and Methods: Five batches of seafood deli salads were stored at 4°C for up to 42 days, with samples being withdrawn periodically for microbial and chemical analysis. The extrinsic (oxygen content of the headspace) and intrinsic (redox potential, pH, and water activity) properties were determined along with microbial counts (total viable counts [TVCs], yeast and mold, lactic acid bacteria) over the product shelf-life. The data generated were then uploaded into a predictive model, and the potential growth of *C. botulinum* was assessed under different storage conditions. Furthermore, product inoculated with *Bacillus atrophaeus* was pressure treated at 400, 500, and 600 MPa for 1, 2, and 3 min at 20°C.

Results: From analysis of the deli salads during storage at 4°C, the TVC remained below 2 log colony forming unit (CFU)/g with no lactic acid bacteria being detected. The yeast and mold count progressively increased during the storage period attaining 6 log CFU/g at the end of the 45-day storage period. There was no change in the pH and oxidation-reduction potential of the deli salads during storage. Predictive model indicated that in storage at 4°C and pH 5.1 and a water activity of 0.974, the generation time was 561.37 h. Only one log spore germination induction was observed when HHP treatment was performed at 600 MPa for 3 min.

Conclusions: From predictive modeling, it was determined that based on the intrinsic and extrinsic factors of stored deli salad, the growth of *C. botulinum* was unlikely. In addition, the application of HHP did not significantly induce germination of the surrogate spores.

Key words: *Clostridium botulinum*, food, high hydrostatic pressure, modeling, risk assessment

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INTRODUCTION

Clostridium botulinum is a Gram-positive, obligate anaerobic, endospore-forming rod.[1] The spores are highly resistant to stresses such as drying, freezing, heat, and high acidic condition and to antimicrobials.[1] The germination and outgrowth of spores can occur in low acid (pH > 4.6) and high water activity (>0.92) foods stored under anaerobic conditions.[2,3]

There are seven strains or serotypes (A–G) of *C. botulinum* based on differences in antigenicity activity among the toxins. Types A, B, E, and F are primarily associated with foodborne botulism.[1] Foodborne botulism develops as a result of the ingestion of preformed *botulinum* neurotoxin in food. The toxin is one of the most poisonous biological substances, with 30 ng/kg bodyweight of neurotoxin being able to cause potentially fatal intoxication.[4] Foodborne botulism can be caused by two types of physiologically and genetically distinct *Clostridia*. Specifically, proteolytic *C. botulinum* is a mesophile and contains strains for the toxins type A, B, and F. The minimum growth temperature of this type is 10°C–12°C. The nonproteolytic *C. botulinum* is a psychrotroph and can produce the B, E, and F toxins under refrigerated conditions,[4] hence a concern in low acid ready-to-eat refrigerated food.

Given the *C. botulinum* is the most virulent pathogen encountered in foods, there are regulations in place to minimize the risk from the pathogen. Specifically, high-risk foods (vacuum packed, ready-to-eat products) need to have two hurdles in place (e.g., water activity <0.92, pH <4.5) to prevent growth of *C. botulinum*.[3] Although intrinsic and extrinsic factors can be set at the processing stage, the activity of endogenous microflora can result in changes over prolonged storage. For example, the growth of microbes can reduce the acidity of the product through catabolism of organic acids leading to a raise in the pH into the zone that would support *C. botulinum*.[3-5]

The high virulence of *C. botulinum* means that challenge trials involving the pathogen can only be performed in specialized laboratories. Consequently, to aid risk assessment studies, different growth prediction models have been constructed and made available to processors. One such model is ComBase that was developed by the UK Institute of Food Research, Australian Food Safety Centre, and USDA Agriculture Research Service (http://www.combase.cc). The model inputs are the initial spore level, water activity, and pH. The models were constructed using actual growth data of *C. botulinum* under different conditions and on occasion within actual food systems. Given the generic nature of predictive models, there is the risk of underestimating and overestimating pathogen growth. Yet, the model data are accepted by regulators as evidence of conducting a risk analysis study.

The purpose of the current study was to investigate the changes in the intrinsic parameters of selected seafood product over the stated shelf-life. Then, the measured parameters were inputted into growth prediction models and generated time-to-toxin production data. Moreover, high hydrostatic pressure (HHP) was applied to induce the germination of the surrogate pores.

MATERIALS AND METHODS

Product description

Two seafood salads with minor difference in ingredients formula were selected for assessing a risk of *C. botulinum*. Formulas 1 and 2 were both packed in 3 kg pail and plastic bag, respectively. Formula 1 ingredients were Alaskan pollock, water, liquid egg whites, corn starch, wheat starch, mirin (water, glucose, alcohol, sweet rice extract, rice koji, salt), potato starch, snow crabmeat, natural and artificial flavors, modified corn starch, soybean oil, salt, sugar, sorbitol, soy protein isolate, carrageenan, sodium phosphates, paprika, color, dressing (soybean and/or canola oil, water, sugar, vinegar, frozen egg yolk, modified corn starch, salt, sodium benzoate, mustard flour, xanthan gum, sodium citrate, dehydrated onion, calcium disodium ethylenediaminetetraacetic acid [EDTA], spices), celery, chili sauce (tomatoes, vinegar, sugar, salt, dehydrated onion, spices, garlic powder), green peppers, and citric acid.

Formula 2 ingredients were Alaskan pollock, water, wheat flour, sugar, wheat starch, soybean oil, salt, fresh egg white, snow crab meat, natural and artificial flavor, sorbitol, mirin (rice extract, alcohol, salt, dextrose, corn syrup solids), maltose, calcium carbonate, soy protein isolate, potato starch, calcium carbonate, disodium inosinate, disodium guanylate, monoglyceride, hydrolyzed corn/soy/wheat/whey (milk) protein, autolyzed yeast extract, sodium phosphate, color, dressing (soybean and/or canola oil, water, sugar, vinegar, frozen egg yolk, modified corn starch, salt, sodium benzoate, mustard flour, xanthan gum, sodium citrate, dehydrated onion, calcium disodium EDTA, spices), celery, citric acid, and ascorbic acid.

Product sampling

Five batches (three of Formula 1 and two of Formula 2) of the seafood products were stored in 3 kg containers at 4°C. Samples ($n = 3$) were withdrawn periodically over the stated shelf-life (35 days) for microbial sampling as in accordance with the Health Canada guidelines.[6] Further sampling was taken beyond the shelf-life to determine what would happen if users would hold and consume the product beyond its intended shelf-life.

Chemical analysis

Redox potential/oxidation-reduction potential (ORP) (Eh) was measured at each sampling point using microprocessor

ORPTestr 10 (Oaktan, Vernon Hills, USA) probe according to the manufacturer's instruction. The electrode was placed approximately 4 inches below the food's surface to take a measurement of the central part of the packaging. In addition, water activity was measured at each sampling point using AquaLab 4TEV (USA) in accordance with manufacturer's instruction. The salt and preservative content were recorded from the label on the pack. Oxygen content of the headspace of sample was measured at Guelph Food Science and Technology, Guelph, Canada (GFST). An Orion Model 290A pH meter was used to measure the pH of the food. The headspace oxygen and carbon dioxide concentration were determined in the first sampling point at the Guelph Food Technology Center, Guelph, Canada, using a headspace gas analyzer. These were allowed evaluation of changes in intrinsic characteristics and its effect on the growth and toxin production of *C. botulinum*.

Microbial analysis

The number of total aerobic bacteria, yeast/mold, and lactic acid bacteria were determined using triplicate pour plates technique.[7] Ten-fold serial dilutions were prepared in 0.1% sterile buffered peptone water (Oxoid, USA). From each dilution, a 1 mL aliquot was inoculated to Petrifilm plate (3M Company, USA), Sabouraud dextrose agar (Oxoid, USA), and MRS agar (Oxoid, USA) for enumeration of total viable count (TVC), yeast/mold, and lactic acid bacteria, respectively. Petrifilm plate and MRS agar were incubated at 37°C for 24–48 h. Sabouraud dextrose agar was incubated at 25°C for 3–5 days. Following incubation, the colony forming units (CFUs) per gram of food were determined and results were expressed as CFU/g of food.[7]

High hydrostatic pressure treatment

Bacillus atrophaeus was used as a surrogate of *C. botulinum*. Endospores were prepared on nutrient agar (Oxoid Ltd.). The plates were incubated at 30°C for 14 days and spores recovered by scraping off the agar surface using sterile distilled water along with a spreader. The spores were harvested by centrifugation (4000 ×*g*, 15 min at 4°C) and washed twice with distilled water. The concentration of spores was determined by heat activating (70°C for 10 min) a 1 ml aliquot of the preparation that was subsequently diluted with sterile distilled water, followed by plating onto nutrient agar. The plates were incubated at 30°C for 48 h and colonies were enumerated. The spore suspension was diluted to give 8 log CFU/ml and stored at 4°C until required.

The pouches containing 25 g of seafood salad were inoculated with 1 ml of the spore to give a final inoculum level of approximately 10^6 spores/g. Pouches were pressure treated at 400, 500, and 600 MPa for 1, 2, and 3 min at 20°C. After pressure treatment, the pouches were opened aseptically and suitable dilutions prepared in 0.1% sterile buffered peptone water (Oxoid, USA). The appropriate dilutions were enumerated in duplicate as described above. The degree of spore germination was estimated by heating the samples at 70°C for 10 min before plating onto *Clostridia* agar. The difference between controls (non-HHP treated) and treated samples represents the degree of spore germination. Reported spore counts are averages of duplicate or triplicate samples.

Predictive modeling

The various parameters of each sample were placed into the ComBase predictor model (http://www.combase.cc/index.php/en/) to predict growth model and used to predict the time for toxin production under different storage temperature conditions. Models were generated under different storage conditions to provide worse case scenarios.

Experimental plan and statistics

Each experiment used at least duplicate samples, and in the majority of cases, triplicate sampling was applied. The data were statistically tested for significant differences (95% confidence levels) using ANOVA and test.

RESULTS

Microbial analysis

The total aerobic count of all five batches was below the level of detection (<1 log CFU/g) at the start of the storage periods [Table 1]. No lactic acid bacteria were detected during the 45 days sampling period although yeasts and molds were encountered [Table 1].

Chemical analysis

Headspace oxygen and carbon dioxide concentration for Formula 1 were 19.1% and 0.7% respectively. These statistics for Formula 2 were 18.9% and 1.7%.

Predictive modeling

An example of model generated is shown in Figure 1. As shown, at storage at 4°C and pH 5.1 and a water activity of 0.974, the generation time was 561.37 h. The maximum growth rate was 0.001 log/concentration/h.

High hydrostatic pressure

There was no significant induce in spore germination [Figure 2]. Only one log spore germination induction was observed when HHP treatment was performed at 600 MPa for 3 min.

DISCUSSION

Five batches of seafood deli salads were stored at 4°C for up to 42 days, with samples being withdrawn periodically for microbial and chemical analysis. TVCs did not change during storage up to the final sampling when low levels (<2 log CFU/g) were detected. Although levels of yeast and molds were below, the level of detection (<1 log CFU/g) on the first storage day levels progressively increased over time. The different batches were considered acceptable

Table 1: Microbial counts of seafood deli salad stored at 4°C

Seafood Salad batch	Storage day/(Log CFU/g ± SE)						
	0	7	14	21	28	35	42
Total aerobic count							
F1 1[a]	<1	<1	<1	<1	<1	<1	1.24±0.34
F1 2	<1	<1	<1	<1	<1	<1	1.30±0
F1 3	<1	<1	<1	<1	<1	<1	1.63±0.89
F2 1[b]	<1	<1	<1	<1	<1	<1	1.81±0.47
F2 2	<1	<1	<1	<1	<1	<1	1.75±0.39
Lactic acid bacteria							
F1 1	<1	<1	<1	<1	<1	<1	<1
F1 2	<1	<1	<1	<1	<1	<1	<1
F1 3	<1	<1	<1	<1	<1	<1	<1
F2 1	<1	<1	<1	<1	<1	<1	<1
F2 2	<1	<1	<1	<1	<1	<1	<1
Yeast and molds							
F1 1	<1	<1	0.59±0.83	1.01±1.43	1.86±0.11	2.70±0.88	4.60±0.03
F1 2	<1	<1	0.76±1.07	2.22±0.40	2.30±0.25	2.80±0.77	5.06±2.04
F1 3	<1	<1	<1	2.32±0.31	2.37±0.55	3.79±1.00	5.04±0.93
F2 1	<1	<1	1.91±0.12	1.85±0.13	2.63±0.21	5.60±0.09	6.58±0.28
F2 2	<1	<1	1.16±1.64	2.31±0.05	2.41±0.03	3.34±0.59	4.87±0.81

[a]Formula 1, [b]Formula 2. SE: Standard error

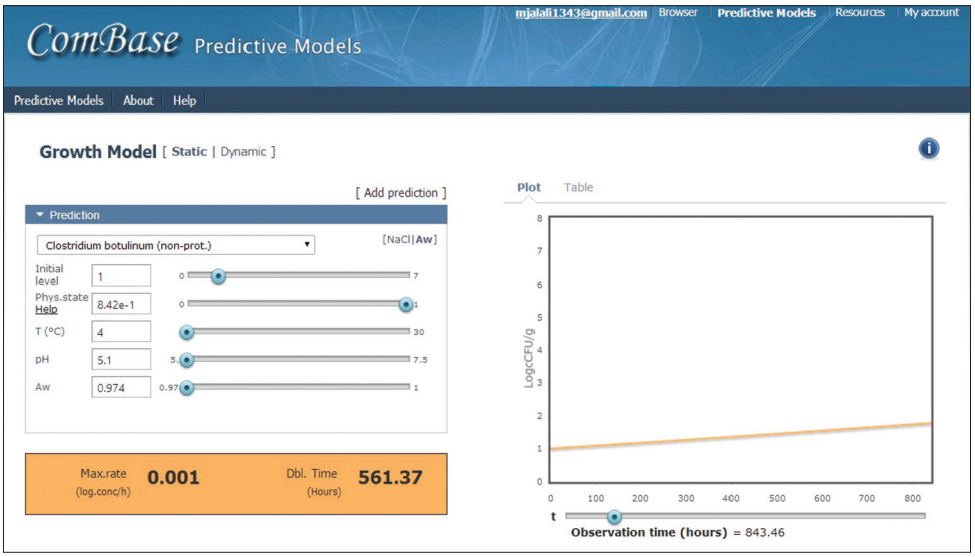

Figure 1: ComBase predictive model output for the growth of *Clostridium botulinum* under conditions found in the seafood deli salad used in the current study

from a visual and aroma standpoint up to the final storage day. Yet, at the end of storage, the yeast and mold counts had approached 6 log CFU/g that is typically sufficient to generate off-odors. Therefore, it is unlikely that the storage period would go beyond 42 days at 4°C.

Despite the growth of yeast and molds, the pH of the deli salad did not significantly change over the 45-day shelf-life. In addition, there was no change in the water activity or ORP readings in the five deli salad batches. It is generally accepted that nonproteolytic *C. botulinum* does not multiply and produce a toxin at pH ≤5.0, at a NaCl concentration ≤5% and that the minimum water activity allowing growth is 0.94–0.97 [Table 1]. In the present study, the highest value for pH and water activity was 4.85 and 0.970, respectively. These statistics indicate that the growth and toxin production in the food under study are unlikely. The effect of ORP and oxygen concentration on growth *C. botulinum* has been quantified (Lund, 1993). High ORP usually indicates the presence of O_2. The optimum ORP for growth of *C. botulinum* is about −350 mV.[8] Peck (2000) reported that an initial aerobic atmosphere (20% oxygen) in meat slurry did not restrict growth of *C. botulinum* and stated that the food was highly reduced and consequently supported the growth of nonproteolytic *C. botulinum*. Therefore, the presence of oxygen in headspace should be considered with caution. However, in the present study, the ORP readings remained positive potential throughout suggesting negligible growth potential of *C. botulinum* [Table 2].

Table 2: Changes in deli salad pH, water activity, and oxidation-reduction potential of seafood salad stored at 4°C

Seafood salad batch	Storage day						
	0	7	14	21	28	35	42
pH							
F1 1[a]	4.28	4.21	4.16	4.23	4.15	4.02	4.11
F1 2	4.09	4.07	4.17	4.16	4.08	3.93	3.99
F1 3	4.11	4.22	4.19	4.34	4.24	4.09	4.11
F2 1[b]	4.59	4.65	4.58	4.74	4.64	4.55	4.55
F2 2	4.65	4.67	4.85	4.83	4.64	4.51	4.54
Water activity							
F1 1	0.957	0.952	0.965	0.967	0.967	0.965	0.969
F1 2	0.960	0.957	0.964	0.961	0.962	0.963	0.966
F1 3	0.955	0.945	0.9663	0.961	0.966	0.967	0.967
F2 1	0.957	0.955	0.960	0.962	0.963	0.964	0.963
F2 2	0.952	0.970	0.961	0.962	0.961	0.969	0.965
Oxidation-reduction potential							
F1 1	-	308	308	309	272	358	337
F1 2	-	313	325	324	285	340	308
F1 3	-	302	311	336	288	302	305
F2 1	-	104	108	111	85	151	141
F2 2	-	108	96	103	105	138	119

F1: Formula 1, F2: Formula 2

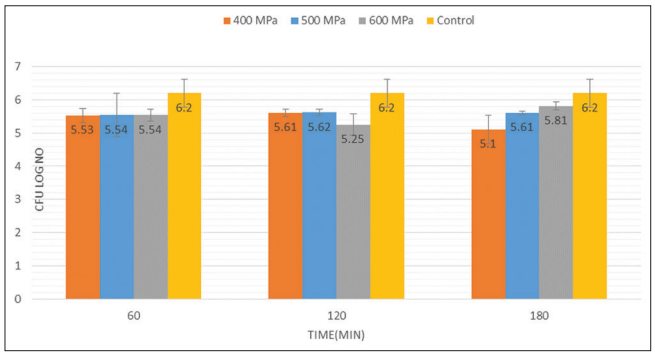

Figure 2: Effect of high hydrostatic pressure on *Bacillus atrophaeus* spore germination in seafood salads

Foods have very complex matrices and safety related to nonproteolytic *C. botulinum* dependent upon multifactor. Although suboptimal intrinsic factors (pH, ORP, water activity) might not prevent growth individually, they might in combination in a hurdle.[4] Given the high virulence of *C. botulinum*, the predictive model was used for describing the effect of combinations of preservative factors in seafood under study. From the intrinsic parameters of the seafood deli salad, the high acidity coupled with positive ORP and moderate water activity, it can be concluded that the growth of *C. botulinum* (proteolytic and nonproteolytic strains) would be unlikely. This was confirmed using the ComBase predictive growth model which under even the worst case scenario identified from Table 1 would lead to minimal growth with a doubling time over 500 h. The model assumed that the *C. botulinum* was in an active -growing state when introduced into the deli salad. A number of similar studies in which foods have been inoculated with spores of nonproteolytic *C. botulinum* and stored at 4°C the time-to-toxin production reported to be relatively high. For examples, times to toxin formation in cod, red snapper, salmon, and catfish reported to be 432, 504, 1008, and 504 h, respectively.[9] Of note, both pH and water activity of fish are significantly higher than seafood in the present study. It should be noted that most of the models provide data on combination between two or more preservative factors and can be implemented to minimize the amount of challenge testing required to ensure food safety. However, predictive models may predict well for some food groups; they may be of limited use in other types of food. In addition, some models have been developed in culture media and may be rather more generic in application.[9,10]

Deli salads were pressure treated at 400, 500, and 600 MPa for 1, 2, and 3 min at 20°C. The application of HHP did not induce germination of *B. atrophaeus* spores. Spores of *C. botulinum* are highly resistance to HPP. Hence, the safety of food subjected to HPP alone has not yet been guaranteed.[11] Therefore, a number of hurdles have been used to extend spores inactivation. For instance, about 3 log reduction of spores of *C. botulinum* type A at 827 MPa and 75°C for 15 min has been reported.[12] Six log reduction of nonproteolytic *C. botulinum* spores with a large variation among strains at similar pressure and temperature has also been reported.[13] However, HPP is able to induce the spore germination by either activating nutrient receptors (moderately high pressures 50–300 MPa) or Ca-diaminopimelic acid release (very high pressures 400–800 MPa).[14] It seems that not all germinated spores are killed by pressure and not all spores are germinated under HHP; hence, safety of this method is questionable.[11,14]

CONCLUSION

At the course of seafood deli salad storage, there was no increase in TVC or lactic acid bacteria counts although yeasts and molds progressively increased in number. However,

growth of the latter did not result in significant changes in the pH, water activity, and ORP of the product. One of the limitations of the current study is the size of the food package used; it is difficult to generalize the data to smaller size. The application of HHP did not induce germination of *B. atrophaeus* spores. Based on the intrinsic and extrinsic factors measured, it can be concluded that growth of *C. botulinum* in the seafood deli salad is highly unlikely.

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Conflicts of interest

There are no conflicts of interest.

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