CHALLENGE STUDIES WITH *CLOSTRIDIUM BOTULINUM* TYPE E IN A VALUE-ADDED SURIMI PRODUCT STORED UNDER A MODIFIED ATMOSPHERE

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ABSTRACT

Challenge studies were carried out on raw, cooked, and sterilized surimi nuggets, inoculated with 10^4 spores/g of C. botulinum *type E spores. All products were packaged in air and air with an Ageless SS oxygen absorbent and stored at 4, 12 and 25C. Toxin was not detected in any raw product throughout storage (28 days). The absence of toxigenesis was attributed to the low pH (4.1-4.3) due mainly to the growth of lactic acid bacteria (10^7CFU/g). Toxin was also not detected in any cooked product after 28 days. Product pH did not decrease as previously (due to the absence of LAB), but counts of* C. botulinum *still decreased throughout storage.*

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In sterile nuggets, C. botulinum *counts increased to 10^6 cfu/g at both 12 and 25C, respectively, by 28 days. Lactic acid bacteria and* Bacillus *spp. were not detected throughout the 28 days storage period. Toxin was detected by days 28 and 14 at 12 and 25C, respectively, and toxigenesis preceded spoilage. The absence of toxin in cooked nuggets was attributed to the anti-botulinal role by* Bacillus *species, the predominant spoilage bacteria in cooked nuggets.*

INTRODUCTION

Value-added battered and breaded seafood products are very popular in industrialized countries and are becoming increasing popular in many southeast Asian countries. For long term storage (>6 months), freezing is the only viable method to extend the shelf life and keeping quality of these products. Recently, modified atmosphere packaging (MAP) has been gaining in popularity as an alternative preservation technique to freezing. However, the success of MAP technology depends on strict temperature control at all stages of processing, storage and distribution. Temperature abuse of MAP products can enhance spoilage, reduce shelf-life and compromise the microbiological safety of these products.

Clostridium botulinum is one of the major public health concerns with regard to MAP foods. Seafood products are particularly important, since nonproteolytic strains of *C. botulinum* are widely distributed in marine and aquatic environments. To date, commercial use of MAP to extend the shelf-life of seafood products has been limited by the potential growth of, and toxin production by, nonproteolytic strains of *C. botulinum*. This is due to the fact that (1) nonproteolytic *C. botulinum* can grow under anaerobic conditions at refrigerated storage (4C); (2) the normal aerobic spoilage microorganisms are inhibited by the modified atmosphere; and (3) there is a possibility of temperature abuse. Furthermore, products could be toxic, yet may still be judged acceptable by the consumer.

Several studies have indicated that *C. botulinum* can produce toxin in MAP seafood products (Stier *et al.* 1981; Post *et al.* 1985; Rhodehamel *et al.* 1991; Reddy *et al.* 1996). However, very little data is available on the growth of *C. botulinum* in value-added, battered and breaded seafood products packaged under MAP conditions.

The purpose of this study was to monitor the physical, chemical, microbiological, and sensorial changes in control/inoculated studies with *C. botulinum* type E in value-added surimi-shrimp nuggets packaged in air and under MAP conditions and to determine the levels of additional barriers required (if any) to ensure the public health safety of nuggets, especially at mild/severe temperature abuse conditions (12 and 25C).

MATERIALS AND METHODS

Formulation of Value-added Nuggets

Value-added battered and breaded nuggets were formulated from appropriate mixtures of surimi, kamaboko, shrimp broth prepared from shrimp processing waste, water, shrimp flavor, salt, wheat flour, onion powder, garlic powder, sugar, monosodium glutamate, pepper, oil, bread crumbs, and Calpro (gelling agent). All dry ingredients, with the exception of shrimp flavor, were obtained from Bluewater Seafoods, Lachine, Quebec. Shrimp flavor was obtained from Quest International, Dorval, Quebec. High grade Alaska Pollock surimi was purchased from H. Aida, Valleyfield, Quebec while frozen shrimp waste was obtained from Les Fruits de Mers Matane, Matane, Quebec.

Kamaboko, a surimi gel, was prepared from appropriate mixtures of surimi, salt, water, wheat flour, Calpro, monosodium glutamate and potato starch. The mixture was then set for 15 min at 40C and then cooked for 15 min at 90C. Kamaboko was used primarily to enhance the texture of the product.

Shrimp broth was prepared by placing 10 kg of frozen waste into a large kettle with approximately 30 L of water. The slurry was allowed to boil for approximately 1 h, until 8-10 L of broth remained after evaporation. The broth was then strained through cheese cloth, boiled again for 10 min, cooled and then packed into low density polyethylene (LDPE) bags and frozen until use.

The value-added nuggets were formed in ice-trays, frozen overnight and removed from the trays the following morning for battering and breading. The batter mix was prepared by adding 320 mL of water to 200 g of dry batter mix. Nuggets were dipped in the batter mix, rolled in bread crumbs, and then deep fried in vegetable oil for ~2 min in order to set the bread crumbs. The nuggets were then drained to remove excess oil, cooled, packed in LDPE bags, and stored frozen until use.

Prior to use, one batch of nuggets was cooked in canola oil (180C) for 5 min to an internal temperature of ~95C to completely destroy all LAB present in the products. Another batch was sterilized in an autoclave at 121C for 15 min to destroy all microorganisms present. Each batch was checked for the presence/absence of LAB/*Bacillus* species immediately after cooking/sterilization using the methodology described below.

Inoculum Preparation

The spore inoculum was prepared from a four strain mixture of *C. botulinum* type E (strains 8550, Gordon, Bennett, and Russ). Cultures were grown in TPGY broth for 10 days at 25C in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). The spores were then harvested in distilled water by centrifuging at 17500 × g for 20 min at 4C and

then stored at 4C in gelatin phosphate buffer at pH 6.6 until use. Equal numbers of spores of each strain were mixed to form a single suspension of ~2×10^5 spores per mL. The spore mixture was heat shocked at 60C for 20 min prior to inoculation of nuggets.

Product Inoculation and Packaging

Nuggets (~30g) were inoculated with 20 μL of inoculum in four different locations in the interior of the nugget, to give a final inoculum level of 10^4 CFU/g. Control nuggets were inoculated with a similar volume of sterile 0.1% peptone water. Water activity (a_w) was checked prior to packaging with a Decagon water activity meter (model CX-1, Decagon Devices, Inc., Pullman, WA).

All nuggets were packaged (2 per bag) in 210 mm × 210 mm high gas barrier Cryovac bags (OTR: 3-6 cc/m^2/day at 4.4C and 0% RH; Cryovac, Mississauga, Ontario, Canada). Air packaged nuggets were sealed using an Impulse heat sealer. Anaerobic conditions were created by taping Ageless SS oxygen absorbents (Mitsubishi Gas & Chemical Co., Japan) inside appropriate bags which were then sealed as described above. These absorbents were used since they were designed to scavenge headspace oxygen to low levels (<0.01%) at refrigerated storage conditions in less than 24 h. All packaged raw nuggets were stored at 4, 12 or 25C, while all packaged cooked and sterilized were only stored at 12 and 25C. All nuggets were monitored for physical, chemical, microbiological, and sensorial changes throughout 28 days or until shelf-life was terminated.

Headspace Gas Analysis

Packages were analyzed for changes in headspace gas composition by withdrawing gas samples using a 0.5 mL gas-tight pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, FL) through silicone seals (Mocon, Minneapolis, MN) attached to the outside of each package. Headspace gas was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector using a Porapack Q column with molecular sieve 5A (80-100 mesh) columns in series (Supelco Inc, Bellefonte, PA, USA). Helium was used as the carrier gas at a flow rate of 20 mL min^{-1}. The column oven was set at 80C. The injector and detector were set at 100C. Peaks were recorded and analyzed with a Hewlett-Packard integrator (model 3390A, Hewlett-Packard Co., Avondale, PA).

Sensory Analysis

All packaged nuggets were evaluated subjectively, and in a random sequence, for odor and overall appearance at each sampling time (day 0, 3, 7, 14, 21, and 28) by a six member untrained panel using the method of Larmond (1979). Nuggets

were scored using a five point scale (1=unacceptable, 5=very acceptable). For each attribute, a score of 3 was considered to be the lower limit of acceptability, implying that shelf-life was terminated when a score of less than 3 was obtained.

Changes in pH

The pH of surimi nuggets was measured with a previously calibrated Corning pH meter (Model 220, Corning Glass Works, Corning, NY) with a gel filled polymer body combination electrode with Ag/AgCl reference (model 13-620-104, Fisher Scientific, Montreal, Quebec) by placing the electrode into a 1:2 dilution of nugget:distilled water after being stomached for 1 min. Analysis (in duplicate) was carried out at each sampling time (day 0, 3, 7, 14, 21, and 28).

Microbiological Analysis

Nuggets were only evaluated for lactic acid bacteria and *Bacillus* species based on previous storage studies and for *C. botulinum* type E. At each sampling day, packaged nuggets were aseptically opened and emptied into a stomacher bag on a tared balance. Sterile peptone water (0.1%) was added to give an initial 1:3 dilution and the bag was stomached in a Colworth Stomacher (Model 400, A.J. Seward, London) for 2 min. All subsequent dilutions were made from this dilution.

Lactic acid bacteria were enumerated on MRS agar (Difco, Michigan) using a spread plate technique. All plates were incubated at 35C for 48 h (DeMan *et al.* 1960).

Bacillus counts were done by heat shocking a portion of the initial dilution at 75C for 15 min, cooling, further diluting and spread plating on PEMBA (Holbrook and Anderson 1980). All plates were incubated at 35C for 24-48 h.

C. botulinum was enumerated by spread plating 0.1 mL of the appropriate dilutions in duplicate on *Clostridium botulinum* Isolation agar (CBI) (Dezfulian *et al.* 1981). Plates were incubated anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 25C for 72 h. Plating efficiency of CBI agar was compared to plating efficiency on trypticase-peptone-glucose-yeast extract (TPGY) agar and TPGY egg yolk agar by plating dilutions of stock cultures on each medium and counting the number of colonies.

Toxin Assay

At each sampling time, the remaining initial homogenate from each packaged sample was weighed and an equal weight of gelatin phosphate buffer (pH 6.6.) added. The mixture was again stomached for 2 min and then centrifuged at 17,500 × g for 20 min at 4C. The supernatant was then filter sterilized, trypsinized and 0.55 mL were injected intraperitoneally into each of two mice (20-25 g), and

observed for up to 72 h for symptoms of botulism. Mice showing severe distress (pinched waist, labored breathing, limb paralysis) were sacrificed immediately. Two additional mice were injected if only one mouse died. Samples were considered positive for toxin if 2/2 or ≥2/4 mice died (Hauschild *et al.* 1975). Control samples were prepared and injected in a similar manner.

RESULTS AND DISCUSSION

Headspace Gas Analysis

Changes in headspace gas composition of raw control and inoculated surimi nuggets (a_w 0.98) packaged in air or air with an oxygen absorbent and stored at 4, 12, and 25C are shown in Table 1.

For air packaged raw control nuggets stored at 4C, headspace O_2 decreased gradually over 28 days to less than 1%, while CO_2 increased to ~30% (Table 1). For raw control nuggets packaged with an oxygen absorbent, headspace O_2 decreased rapidly to less than 1%, while headspace CO_2 increased to 4% (Table 1). Similar trends were observed for products stored at 12C. In both raw control nuggets packaged in air and in air with an oxygen absorbent, headspace O_2 decreased to less than 1% within 14 days and 2 days while CO_2 increased to 44 and 10%, respectively, by day 28 (Table 1). At 25C, similar but more dramatic trends were observed for all raw control packaged nuggets. In all packaging treatments, headspace O_2 decreased to less than 1% after ~5 days, while CO_2 increased to approximately 60% for air packaged nuggets and 80% for nuggets packaged with an oxygen absorbent by day 14. All packages stored at 25C had a blown appearance at the end of storage due to in-package CO_2 production.

Similar changes in headspace gas composition were observed for all raw nuggets inoculated with *C. botulinum* and packaged in air and in air with an oxygen absorbent and stored at 4, 12, and 25C, respectively (Table 1). Again, all inoculated packages stored at 25C had a blown appearance after 28 days. These changes in headspace gas composition are consistent with those observed in MAP pork and can be attributed to the growth and metabolism of spoilage bacteria in the product (Lambert *et al.* 1991a, b).

Changes in headspace gas composition of cooked, control nuggets packaged in air or in air with an oxygen absorbent and stored at 12, and 25C are shown in Table 2. In air packaged nuggets stored at 12C, headspace O_2 decreased to < 1% by day 28, while headspace CO_2 increased to ~25% (Table 2). In cooked nuggets packaged with an oxygen absorbent at 12C, headspace O_2 and CO_2 were < 1% by 7 days and remained at this level throughout storage (Table 2). At 25C, headspace

TABLE 1.
SUMMARY OF HEADSPACE GAS AND SENSORY ANALYSIS IN RAW SURIMI NUGGETS

Packaging Treatment	Inoculation[1]	Storage Temp (°C)	Headspace Gas (%v/v) Initial O_2	Initial CO_2	Final O_2	Final CO_2	Sensory Scores[2] Odor	Appearance
Air	-	4	20	<1	<1	30	3	4.5
Air	+	4	20	<1	<1	30	3	4.5
Air	-	12	20	<1	<1	44	2	3
Air	+	12	20	<1	<1	45	3	3
Air	-	25	20	<1	<1	60	2	2
Air	+	25	20	<1	<1	80	2	2.5
Air + oxygen absorbent	-	4	<1	<1	<1	4	3.5	4.5
Air + oxygen absorbent	+	4	<1	<1	<1	5	4	4.5
Air + oxygen absorbent	-	12	<1	<1	<1	10	2	4
Air + oxygen absorbent	+	12	<1	<1	<1	32	3	3.5
Air + oxygen absorbent	-	25	<1	<1	<1	80	3	3
Air + oxygen absorbent	+	25	<1	<1	<1	40	3	3

[1] Samples inoculated with a mixture of *C. botulinum* type E spores
[2] Scale of 1 to 5: 5=most acceptable, 1=least acceptable
All results average of triplicate samples

O_2 decreased to < 1% in both packaging treatments after 7 days. Headspace CO_2 increased to ~40% in air packaged nuggets, while in nuggets packaged with an oxygen absorbent, CO_2 levels initially increased to 40%, then decreased to ~ 15%, possibly due to package leakage (Table 2).

Similar trends were observed for headspace gas changes in cooked inoculated nuggets at 12 and 25C (Table 2). The lower CO_2 production in all cooked nuggets at both 12 and 25C, compared to similarly packaged raw nuggets, was probably due to the reduced background flora of LAB, which were destroyed in the cooking process.

Changes in headspace gas composition of sterilized control and inoculated nuggets are shown in Table 3. In control nuggets packaged in air, headspace gas remained constant, i.e., ~20% O_2 and 80% N_2, at 12 and 25C throughout the storage period (Table 3). In nuggets packaged with an oxygen absorbent, headspace O_2 decreased to <1% while N_2 increased to ~99% (Table 3). These results i.e., lack of CO_2 production, can be attributed to the destruction of all microorganisms by the sterilization process.

In all nuggets inoculated with *C. botulinum* and stored under air at 12C, CO_2 levels increased to ~40%, while headspace O_2 decreased to approximately 1% (Table 3). For nuggets packaged in air with an oxygen absorbent, O_2 levels decreased to approximately 1%, while CO_2 remained below 1% (Table 3).

At 25C, higher levels of CO_2 were detected. For air packaged inoculated nuggets, headspace CO_2 increased to 55%, while the CO_2 concentration in nuggets packaged in air with an oxygen absorbent increased to 24% (Table 3). In all cases, headspace O_2 decreased to less than 1% and remained at this level throughout storage (Table 3). Since the nuggets were sterile, CO_2 production in the inoculated nuggets can only be attributed to the growth and metabolism of *C. botulinum*.

Sensory Analysis

Changes in sensory analysis scores (odor & overall appearance) of both control and inoculated raw nuggets packaged in air or air with an oxygen absorbent and stored at 4, 12, and 25C are also summarized in Table 1. For raw control nuggets stored at 4C, both odor and appearance scores remained acceptable throughout 28 days (Table 1). At 12C, nuggets developed acidic odors by day 28, while their appearance remained acceptable (Table 1). Control nuggets packaged in air and stored at 25C developed similar, but stronger, acidic odors by day 21 while their texture and appearance was mushy. These nuggets were judged to be unacceptable at the termination of the storage trial (Table 1). However, control nuggets packaged with an Ageless SS absorbent were still acceptable to panelists after 28 days (Table 1). Although most products developed a sharp acidic odor, this odor was not unpleasant and dissipated within a few minutes upon opening the packages. Similar sensory trends were observed for raw nuggets inoculated with 10^4 spores/g of *C. botulinum* in all packaging conditions (Table 1).

TABLE 2.
SUMMARY OF HEADSPACE GAS AND SENSORY ANALYSIS IN COOKED SURIMI NUGGETS

Packaging Treatment	Inoculation[1]	Storage Temp (°C)	Headspace Gas (%v/v) Initial O_2	Initial CO_2	Final O_2	Final CO_2	Sensory Scores[2] Odor	Sensory Scores[2] Appearance
Air	-	12	20	<1	<1	25	5	5
Air	+	12	20	<1	<1	23	5	5
Air	-	25	20	<1	<1	40	3	4
Air	+	25	20	<1	<1	80	3	4
Air + oxygen absorbent	-	12	<1	<1	<1	<1	5	5
Air + oxygen absorbent	+	12	<1	<1	2	<1	5	5
Air + oxygen absorbent	-	25	<1	<1	<1	40	3	4
Air + oxygen absorbent	+	25	<1	<1	<1	40	4	4

[1] Samples inoculated with a mixture of *C. botulinum* type E spores
[2] Scale of 1 to 5: 5=most acceptable, 1=least acceptable
All results average of triplicate samples

TABLE 3.
SUMMARY OF HEADSPACE GAS AND SENSORY ANALYSIS IN STERILE SURIMI NUGGETS

Packaging Treatment	Inoculation[1]	Storage Temp (°C)	Headspace Gas (%v/v) Initial O_2	Initial CO_2	Final O_2	Final CO_2	Sensory Scores[2] Odor	Appearance
Air	-	12	20	<1	20	<1	5	5
Air	+	12	20	<1	<1	40	4	4
Air	-	25	20	<1	20	<1	3.5	4
Air	+	25	20	<1	<1	55	2.5	4
Air + oxygen absorbent	-	12	<1	<1	<1	<1	5	5
Air + oxygen absorbent	+	12	<1	<1	<1	<1	3.5	4
Air + oxygen absorbent	-	25	<1	<1	<1	<1	4	4
Air + oxygen absorbent	+	25	<1	<1	<1	24	3	4

[1] Samples inoculated with a mixture of *C. botulinum* type E spores
[2] Scale of 1 to 5: 5=most acceptable, 1=least acceptable
All results average of triplicate samples

Changes in sensory characteristics of all packaged cooked, control and inoculated surimi nuggets stored at 12 and 25C are summarized in Table 2. Sensory evaluation scores for odor and appearance of cooked, control nuggets at 12C under both packaging conditions remained at 5 throughout the 28 days storage period (Table 2). At 25C, odor scores for nuggets packaged in air with and without an oxygen absorbent remained acceptable until day 28. The appearance of nuggets stored at 25C under both packaging treatments was also still acceptable and all nuggets had scores of ~4 after 28 days (Table 2).

Inoculated cooked nuggets had almost identical changes in sensory characteristics over the 28 day storage period. At 12C, nuggets maintained odor and appearance scores of 5 throughout the 28 day storage period (Table 2). At 25C, nuggets maintained acceptable color and appearance, however musty, earthy odors developed by day 14. However, all nuggets still had acceptable scores of 3 or above (Table 2).

These results are significant since nuggets maintained an acceptable appearance at the end of the storage period, even at 25C. Although a strong musty odor was present, it was much less potent after opening. Consumer acceptability of the cooked product could result in potential problems if *C. botulinum* was capable of growth in the packaged nuggets.

Sensory analysis changes of sterilized control and inoculated surimi nuggets stored at 12 and 25C are shown in Table 3. In control nuggets stored at 12C under both packaging conditions, nuggets maintained acceptable odor and appearance scores (scores = 5) throughout 28 days (Table 3). At 25C, nugget odor and appearance scores decreased slightly to 3.5-4 for both packaging treatments. Sterilization of the nuggets had an immediate deteriorative effect on the texture (results not shown). Nuggets were slightly softer following sterilization, however there were no effects on appearance or odor of the sterilized nuggets.

In inoculated sterilized nuggets, stale fish-like odors developed by day 28, with odor scores decreasing to 3.5-4 in both packaging treatments stored at 12C (Table 3). At 25C, these off odors developed more rapidly and were evident by day 14. Odor scores decreased to unacceptable levels by day 28. All nuggets also fell apart more easily, especially the crumb layer, probably due to the effect of sterilization. However, there were no noticeable effects on color of the nuggets, which had scores of ~5 throughout the storage period.

Changes in pH

Changes in pH values of both control and inoculated raw nuggets packaged in air or air with an Ageless SS oxygen absorbent and stored at 4, 12, and 25C are shown in Fig. 1.

In all raw control nuggets stored at 4, 12, and 25C, pH values decreased from ~6.5 to approximately 4.9-4.0 by 21, 14, and 7 days, respectively (Fig. 1 a-c).

Similar trends were observed in raw inoculated nuggets stored at 4, 12, and 25C (Fig. 1 a-c). In nuggets stored at 4, 12, and 25C, pH decreased to 4.9-4.0 by days 28, 14, and 7, respectively.

These changes in pH can again be attributed to the growth of lactic acid bacteria, increased CO_2 production, and dissolution of the CO_2 in the aqueous phase of the product (Lambert *et al.* 1991a, b). The rapid decrease in pH of nuggets stored at 12 and 25C may also have created unfavorable conditions for the growth of *C. botulinum*, since nonproteolytic strains of *C. botulinum* are unable grow below pH 5.0.

The changes in pH for cooked control and inoculated nuggets packaged in air or air with an oxygen absorbent and stored at 12 and 25C are shown in Fig. 2 a,b. The pH in all packaged control nuggets did not decrease below 6.0 (Fig. 2 a,b). Similar, almost identical trends, were observed for inoculated nuggets (Fig. 2 a,b). This is of significance since the pH remained optimal for the growth of *C. botulinum* throughout the 28 day storage period. In raw nuggets, the pH decreased to < 5.0 by day 14 at 12C, creating unfavorable conditions for the growth of *C. botulinum*. The differences in pH between raw and cooked nuggets can be attributed to the destruction of the predominant spoilage and acid producing LAB in the raw product.

Changes in pH of all sterilized control and inoculated nuggets under similar and storage conditions are shown in Fig. 2 c,d. In control nuggets stored at both 12 and 25C, there was no change in pH in any of the treatments over the 28 day storage period. This can again be attributed to the destruction of background spoilage microorganisms, i.e., LAB and *Bacillus* spp., by the sterilization process (Fig. 2 c, d). In inoculated nuggets, the pH decrease was greater in nuggets stored at 25C than at 12C. However, the pH never decreased below 5.0 in any of the packaging treatments. At 12C, the pH decreased to approximately 5.8 by day 28 in nuggets packaged in both air and air with an oxygen absorbent (Fig. 2 c). For nuggets stored at 25C, pH decreased to 5.1-5.4 in both packaging treatments by day 28 (Fig. 2 d). The decrease in pH observed in inoculated nuggets can be attributed to CO_2 production by *C. botulinum* and dissolution of this CO_2 into the aqueous phase of the product. The fact that product pH in all samples did not decrease below 5.0 was significant since pH conditions were again favorable for the growth of nonproteolytic *C. botulinum*.

Microbiological Analysis

Since changes in microbiological counts in both control and inoculated raw nuggets packaged in air and in air with an oxygen absorbent and stored at 4, 12, and 25C were similar, only air packaged inoculated results are shown in Fig. 3. Both LAB and *Bacillus* species were identified on the basis of their colony

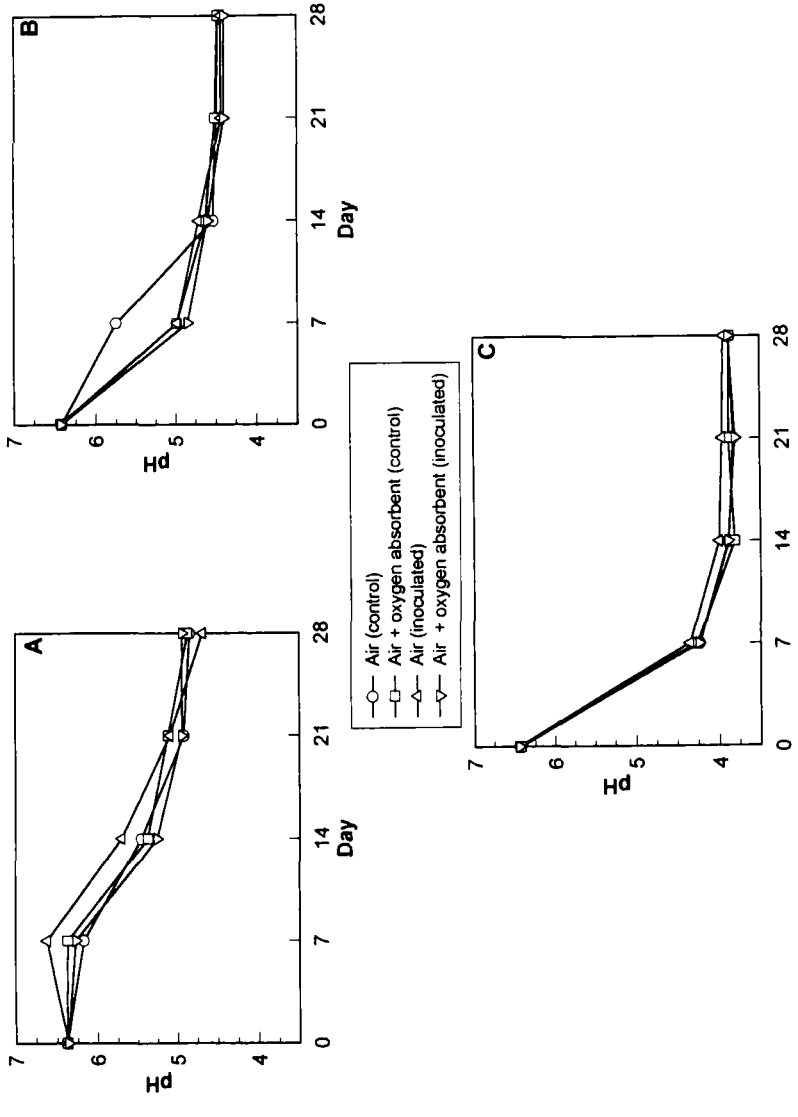

FIG. 1. CHANGES IN pH OF RAW CONTROL AND INOCULATED SURIMI NUGGETS PACKAGED IN AIR AND AIR WITH AN OXYGEN ABSORBENT OVER 28 DAYS AT (A) 4C, (B) 12C, AND (C) 25C

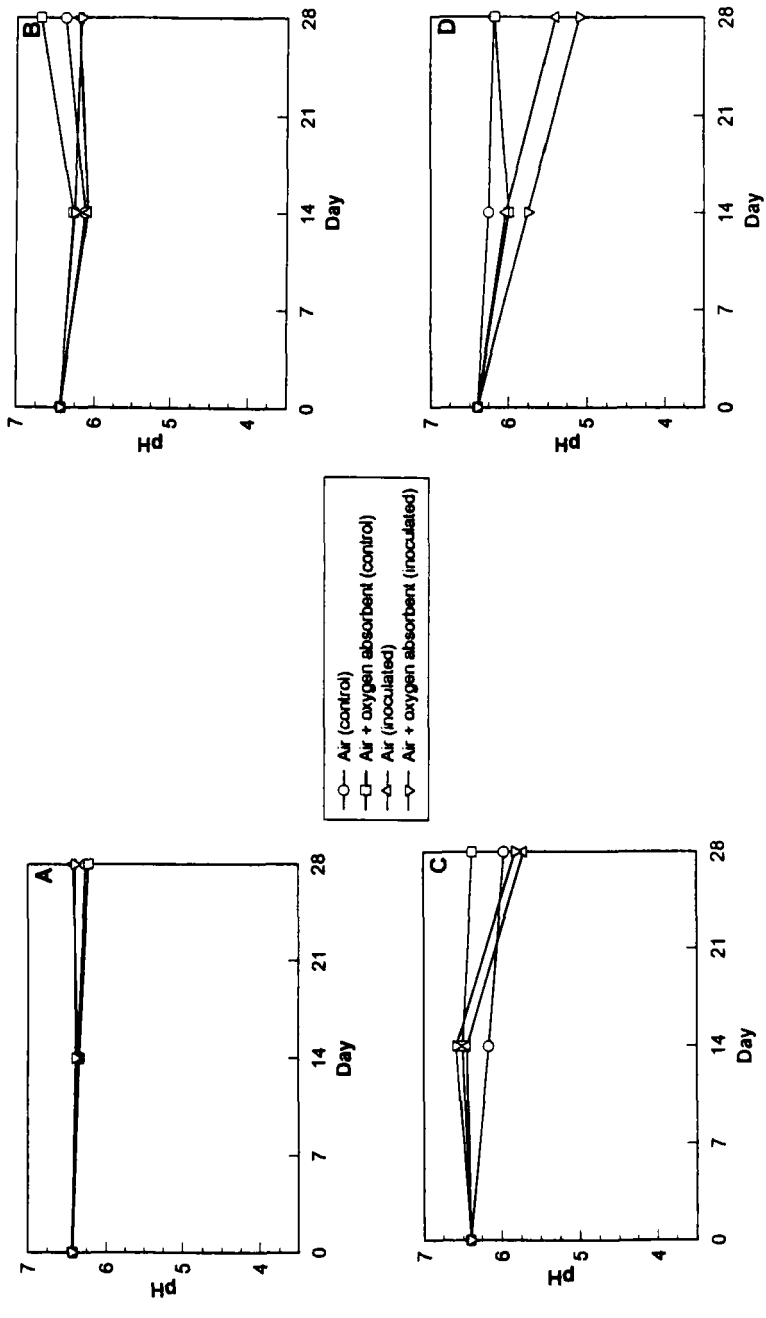

FIG. 2. CHANGES IN pH OF COOKED AND STERILIZED, CONTROL AND INOCULATED SURIMI NUGGETS PACKAGED IN AIR AND AIR WITH AN OXYGEN ABSORBENT OVER 28 DAYS: (A) COOKED (12C), (B) COOKED (25C), (C) STERILIZED (12C), AND (D) STERILIZED (25C)

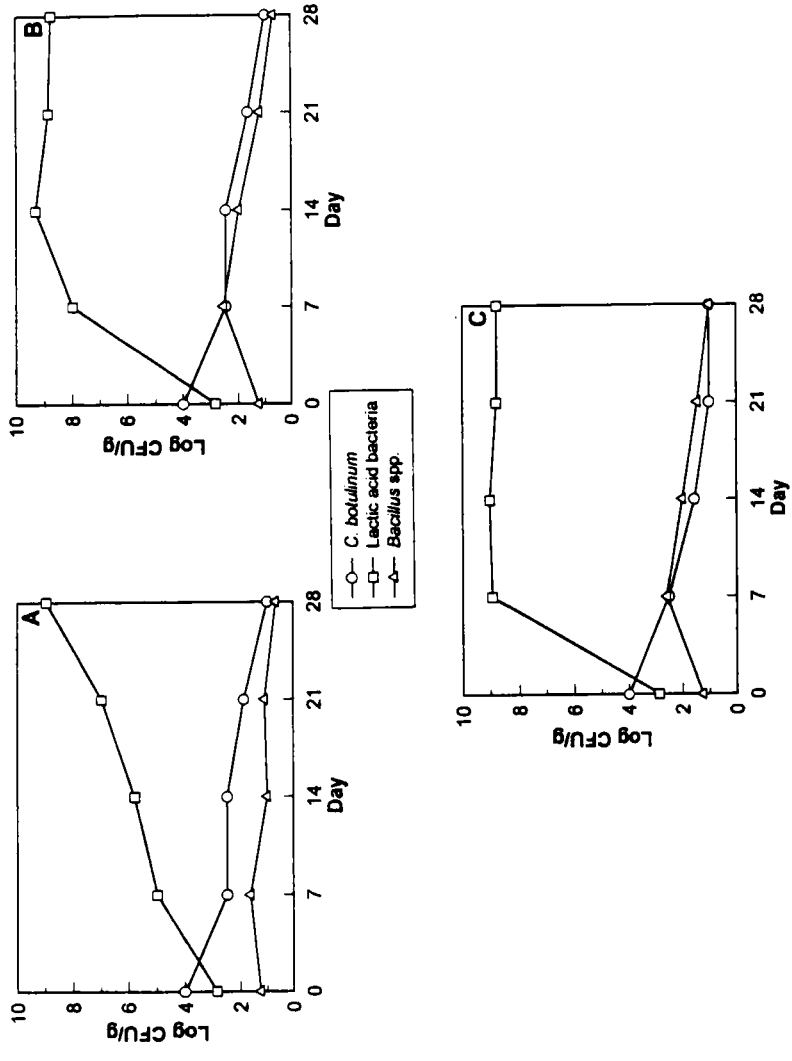

FIG. 3. CHANGES IN MICROBIOLOGICAL COUNTS OF RAW INOCULATED SURIMI NUGGETS PACKAGED IN AIR OVER 28 DAYS AT (A) 4C, (B) 12C, AND (C) 25C

characteristics on each media as well as Gram stain and catalase reaction.

Dezfulian *et al.* (1981) found that only 1 out of 3 strains of *C. botulinum* type E grew on CBI agar. This led them to suggest the simultaneous use of CBI agar and nonselective egg yolk agar, to avoid missing drug-susceptible type E strains when attempting to isolate *C. botulinum* from clinical samples. All strains of *C. botulinum* type E used in the present study were checked for growth on CBI agar and were found to grow as efficiently on CBI agar as they did on TPGY agar with and without egg yolk (data not shown). The combination of the selectivity of CBI agar and the appearance of the lipase reaction suppressed most of the background microflora and allowed a distinction to be made between *C. botulinum* type E and background flora. The use of CBI agar to detect growth of nonproteolytic strains of *C. botulinum* in foods has been reported previously (Austin *et al.* 1998).

Microbiological counts in raw control nuggets comprised mainly of lactic acid bacteria and *Bacillus* spp. at the onset of storage; however, LAB became the predominant spoilage bacteria as storage progressed. At 4C, LAB counts increased from ~10^3 CFU/g to ~10^7 CFU/g by day 14, while *Bacillus* counts decreased slightly during the 28 day storage period (results not shown). Similar trends were observed in all packaging treatments at 12 and 25C. However, growth of LAB was more rapid at higher temperatures (results not shown). *C. botulinum* was not detected in any of the control nuggets.

In inoculated raw nuggets packaged in air or air with an oxygen absorbent and stored at 4, 12, and 25C, counts for *C. botulinum* actually decreased to < 10^2 CFU/g during the 28 day storage period (Fig. 3 a-c). Again, lactic acid bacteria became the predominant spoilage bacteria in all packaging treatments, increasing to ~ 10^7 CFU/g by 7 days (Fig. 3 a-c). LAB have been shown to become the predominant spoilage microorganisms in many muscle foods packaged under MAP conditions (Lambert *et al.* 1991 a, b). The lack of growth of *C. botulinum* in all inoculated nuggets was probably due to the decrease in pH produced mainly by the growth of LAB, which resulted in unfavorable pH conditions for the growth of *C. botulinum*. The fact that *C. botulinum* is a poor competitor and that the limiting pH for the growth of nonproteolytic strains of *C. botulinum* is approximately 5.0 confirms these results. The slight decrease in *Bacillus* counts could also be attributed to the decrease in pH and hence unfavorable growth conditions.

Again, since microbiological changes in both control and inoculated cooked surimi nuggets were almost identical (with the exception of *C. botulinum* counts in inoculated nuggets), only changes in inoculated nuggets are shown (Fig. 4 a,b).

Lactic acid bacteria were never detected in cooked inoculated nuggets confirming the efficacy of the cooking process to destroy all LAB. *Bacillus* counts increased gradually from ~10^1 to ~10^4 and 10^6 CFU/g at 12 and 25C, respectively, throughout the 28 day storage period (Fig. 4 a,b). These results also explain the differences in CO_2 production, pH reduction, and sensory analysis between raw and

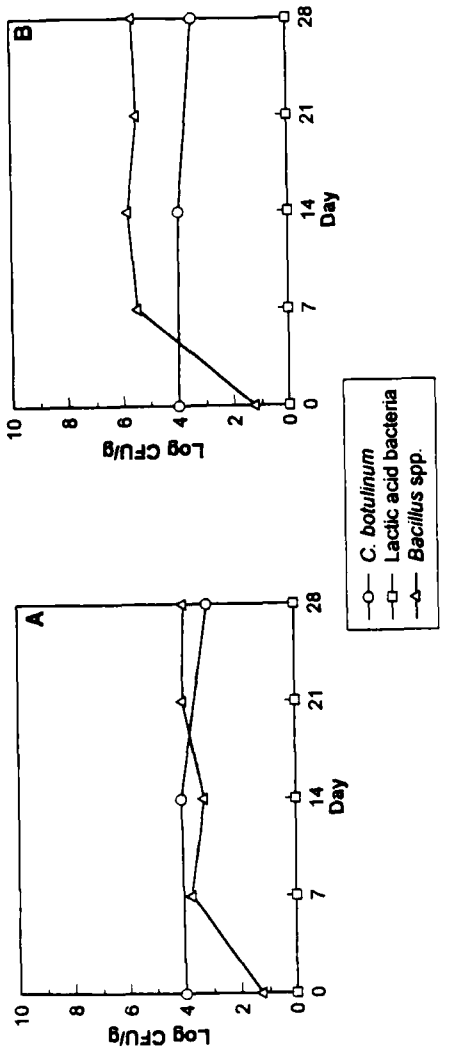

FIG. 4. CHANGES IN MICROBIOLOGICAL COUNTS OF INOCULATED COOKED AND STERILIZED SURIMI NUGGETS PACKAGED IN AIR OVER 28 DAYS: (A) COOKED (12C), (B) COOKED (25C), (C) STERILIZED (12C), AND (D) STERILIZED (25C)

cooked nuggets. At 12C, counts of *C. botulinum* actually decreased by day 28 in nuggets packaged in air and in air with an oxygen absorbent (Fig. 4 a). At 25C, *C. botulinum* counts decreased in all air packaged nuggets, while counts remained at ~10^4 CFU/g in nuggets packaged with an oxygen absorbent (Fig. 4 b). These results were surprising since it was anticipated that, in the absence of pH reducing strains of LAB, counts of *C. botulinum* would increase. This lack of growth may be due to the inhibitory effect of *Bacillus* species which increased throughout storage.

Microbiological changes in sterilized and inoculated nuggets are shown in Fig. 4 c, d. In nuggets inoculated with 10^4 spores/g of *C. botulinum*, counts in all packaging treatments reached ~10^6 CFU/g by day 28 (4 c, d). Again, LAB and *Bacillus* spp. were not detected in any packaged, sterilized nuggets throughout storage. *C. botulinum* was able to grow in nuggets packaged in air since its growth is dependent on the redox potential of the medium, and not on the gas atmosphere within the package. Previous studies by Christiansen and Foster (1965), Ajmal (1968), Sugiyama and Yang (1975), and Lambert *et al.* (1991 a, b) have all shown the ability of *C. botulinum* to produce toxin in both aerobic and anaerobic conditions. The growth of *C. botulinum* in sterilized surimi nuggets can therefore be attributed to the absence of *Bacillus* spp., which may have had an inhibitory effect on the growth of *C. botulinum*.

Toxin Assay

Toxin was not detected in any of the packaged raw nuggets throughout the 28 day storage period. The absence of toxin is directly related to the decrease in the counts of *C. botulinum* at all storage temperatures. Generally, counts of ~10^6 CFU/g are required for toxin development in foods (Smith and Sugiyama 1988). Counts of *C. botulinum* in raw, inoculated surimi nuggets never exceeded the inoculation level, i.e., 10^4 CFU/g, and decreased throughout storage (Fig. 3). Again, the absence of growth of, and toxin production by, *C. botulinum* was probably due to the production of acid by LAB and a decrease in pH to <5.0, resulting in unfavorable growth conditions. The minimum pH at which nonproteolytic *C. botulinum* will grow is ~pH 5.0 (Dodds 1994). Although *C. botulinum* was inoculated at a higher level than the initial LAB count, i.e., 10^4 CFU/g, *C. botulinum* was still unable to grow and produce toxin since it is a poor competitor.

These results are in agreement with Crandall and Montville (1993) who studied the inhibition of *C. botulinum* in a model gravy system by coinoculation with bacteriocin producing strains of lactic acid bacteria. They reported that strains of *Lactococcus lactis*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus*, when inoculated at 10^4 CFU/mL, were able to inhibit the growth of *C. botulinum* type A and B inoculated into the broth at 10^2, and 10^4 spores/mL, and stored at 15C. Although it was initially thought that bacteriocin production was responsible for inhibition, samples inoculated with nonbacteriocinogenic strains of LAB were still

capable of inhibiting the growth of *C. botulinum*. Therefore, production of acid was determined to be the mode of inhibition. At severe temperature abuse conditions (25 and 35C), the antibotulinal effect of LAB tested was reduced and *C. botulinum* was capable of growth and toxin production at these temperatures. This temperature effect was not observed in our study. Although acid production by lactic acid bacteria is the most likely cause of inhibition of *C. botulinum* in our studies, bacteriocin production by LAB could also play a secondary role. Okereke and Montville (1991) found that various strains of *L. plantarum*, *L. lactis*, *P. pentosaceus*, and *Lactobacillus acidophilus* were capable of inhibiting *C. botulinum* at 4, 10, 15, and 35C in agar systems.

However, the results observed in this study are in disagreement with the observations of Reddy *et al.* (1996), who studied toxin development by *C. botulinum* in fresh Tilapia fillets packaged under a modified atmosphere (75% CO_2:25% N_2). Although total aerobic and anaerobic counts in MAP fresh Tilapia fillets increased to 10^8 CFU/g at 4 and 8C, it had no effect on the growth of *C. botulinum*. Furthermore, product pH increased to 6.6 in air packaged samples, and remained in the range of 6.3-6.5 in MAP samples stored at 4, 8, and 16C. Toxin was detected in all samples, however toxin development did not precede spoilage in any samples. These differences could be attributed primarily to differences in the product under study and the background microflora of fresh Tilapia fillets, which consisted mainly of *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Moraxella*, *Acinetobacter*, and *Vibrio* spp., i.e., more proteolytic species which resulted in pH being maintained near neutral. However, raw surimi nuggets have a background microflora consisting mainly of lactic acid bacteria, and *Bacillus* species resulting in a rapid pH decrease and inhibition of *C. botulinum*.

With respect to cooked nuggets, toxin was not detected in any nuggets throughout the 28 day storage period. The absence of growth of, and toxin production by, *C. botulinum* in cooked surimi nuggets was surprising, since the conditions in cooked nuggets appeared favorable for the growth of *C. botulinum*, compared to the unfavorable conditions, i.e., low pH, observed in raw nuggets after 28 days. In cooked nuggets, the pH did not decrease below 6.0, while a_w remained relatively unchanged, and no competitive LAB were present. Based on these results, it was concluded that the inhibition of growth of, and toxin production by *C. botulinum* in all cooked nuggets could be due to the presence of heat resistant *Bacillus* species, since these were the only organisms found in relatively high numbers (~10^4 and 10^6 CFU/g at 12 and 25C, respectively) in cooked nuggets (Fig. 4 a,b). Inhibition by *Bacillus* spp. could be possibly due to competition or by production of other compounds, such as secondary metabolites, toxins, bacteriolytic enzymes, bacteriophages, bacteriocins and bacteriocin-like molecules.

TABLE 4.
SUMMARY OF TOXIN DEVELOPMENT IN STERILE SURIMI NUGGETS

Packaging Treatment	Inoculation[1]	Storage Temperature (°C)	Days to toxin development[2]		
			0	14	28
Air	-	12	0/2[3]	0/2	0/2
Air	+	12	0/2	0/2	2/2
Air	-	25	0/2	0/2	0/2
Air	+	25	0/2	2/2	2/2
Air + oxygen absorbent	-	12	0/2	0/2	0/2
Air + oxygen absorbent	+	12	0/2	0/2	2/2
Air + oxygen absorbent	-	25	0/2	0/2	0/2
Air + oxygen absorbent	+	25	0/2	2/2	2/2

[1] Samples inoculated with a mixture of *C. botulinum* type E spores

[2] Trypsinized extract

All results average of triplicate samples

This hypothesis was confirmed in challenge studies in sterilized nuggets. Toxin was detected in all inoculated sterilized nuggets stored at 12 and 25C by days 28 and 14, respectively (Table 4). These results confirm an inhibitory effect of *Bacillus* spp., on the growth of, and toxin production by, *C. botulinum*. The production of toxin is of concern since nuggets were determined to be of acceptable odor and appearance at the onset of toxigenesis at both temperatures. At 12C, nuggets had odor and appearance scores of 3.5-4 when toxin was formed at 28 days. At 25C, nuggets had odor and appearance scores of 4-5 when toxin was formed at 14 days. These results are also important since they suggest that *Bacillus* spp. may be a natural bio-preservative in the surimi-nuggets. However, this is of concern, since there is no way of guaranteeing the presence of these organisms in the raw ingredients. Therefore, surimi-nuggets manufactured with different ingredients under different conditions could, perhaps, present a public health hazard to consumers, particularly at temperature abuse storage conditions. The occurrence of *Bacillus* organisms in food is also cause for concern, since some species, such as *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis*, have been implicated in various foodborne diseases (Kramer and Gilbert 1989). Further studies are now underway to identify the isolated *Bacillus* species from nuggets and their source. Studies are also underway to confirm the anti-botulinal role of the isolated *Bacillus* species.

In conclusion, this study has shown that raw, value-added battered and breaded surimi nuggets packaged under a modified atmosphere do not appear to be a public health risk with respect to the growth of *C. botulinum,* the major concern of MAP seafood products. This inhibition was attributed to the competitive growth of, and pH reduction by background microorganisms, specifically lactic acid bacteria.

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