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Growth and Toxin Production of Proteolytic *Clostridium botulinum* in Aseptically Steamed Rice Products at pH 4.6 to 6.8, Packed under Modified Atmosphere, Using a Deoxidant Pack

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ABSTRACT

Demand for aseptically steamed rice products has been increasing rapidly in Japan over the past 10 years. In our previous study, we showed that proteolytic *Clostridium botulinum* produce toxins in steamed rice products packaged under a modified atmosphere of ≤0.3% oxygen. In the present study, we examined the effect of pH to control botulism risk in steamed rice products packaged under modified atmosphere (5% CO_2 and 95% N_2 as the balance) with the inclusion of a deoxidant pack to produce an oxygen concentration of ≤0.3%. A mixture of 10 strains of proteolytic *C. botulinum* (5 type A strains and 5 type B strains) was inoculated into steamed rice products at pH values between 4.6 and 6.8 prior to packaging. All samples were stored at 30°C for 24 weeks. Samples at higher pH showed earlier starts of neurotoxin production. Neurotoxin was detected after 2 weeks of incubation in samples at pH 5.4 or above, whereas it took 4 weeks for the toxin to be detected in samples at pH 5.2 to 5.3 and 12 weeks in samples at pH 5.0 to 5.1. In samples at pH 4.9 or below, no toxin was detected during the experimental period. Apparent sample spoilage did not occur before *C. botulinum* produced neurotoxin in most of the samples. Based on these results, we conclude that aseptically steamed rice products must be packaged at pH 4.9 or below under modified atmosphere containing ≤0.3% oxygen, with the inclusion of a deoxidant pack.

Aseptic packaging, which is commonly used for milk, soup, pasta, and other foods, has also been used for steamed rice products in Japan. Packaged rice products can be stored for 6 months, or even longer, at room temperature and only require microwave oven heating preparation. These products are often packed under modified atmosphere: some contain 5 to 10% oxygen, and some are packed under modified atmosphere with the inclusion of a deoxidant pack so that oxygen concentration nearly reaches 0%. Modified atmosphere packaging (MAP) has become a popular means of extending shelf life of precooked foods, such as fresh pork *(12),* fish fillets *(10, 13),* vegetables *(6, 11, 16),* and other food items *(3, 17),* by preventing growth of aerobic bacteria, molds, and yeasts. However, MAP may permit growth of and toxin production by anaerobic spore-forming bacteria such as *Clostridium botulinum.* Although aseptically steamed rice products are packaged after cooking and steam sterilization, using an ultrahigh-temperature flash-heating process (generally ≥8 s at 135°C; $F_0 > 3.1$) to eliminate primary contamination, these products still carry a slight risk of secondary contamination of *C. botulinum* spores. In fact, production of neurotoxin by proteolytic *C. botulinum* was demonstrated in commercially manufactured steamed rice with MAP (pH 6.5) *(8).* In our previous study *(8),* therefore, we examined the effect of oxygen concentration on preventing toxin production by this pathogen in these products, and we determined 10% oxygen to be most effective. However, we still considered lower oxygen concentrations to be desirable in order to inhibit the growth of aerobic microorganisms. In fact, atmospheric conditions of nearly 0% oxygen with the inclusion of a deoxidant pack has already been adapted for the manufacture of some of these products in Japan in order to prevent the growth of aerobes, including molds.

When *C. botulinum* spores alone were inoculated into a medium made from cooked meat medium, toxin production was strictly limited below pH 4.6 *(21).* However, since aseptically steamed rice products with pH adjusted below 4.6 taste acidic, most products currently distributed in Japan have a higher pH, mostly around 6.5.

In this study, we examined the pH threshold for controlling the risk of *C. botulinum* packaging that achieves ≤0.3% oxygen concentration through the used of modified atmosphere and the inclusion of deoxidant pack. To determine the safety level of pH that prevents *C. botulinum*–toxin production, proteolytic *C. botulinum* (types A and B) were inoculated into steamed rice adjusted to pH values between 4.6 and 6.8.

MATERIALS AND METHODS

Bacterial strains. Five type A strains (56A, 62A, 97A, Hall A, and Renkon-1 A) and five type B strains (9B, 213B, 407-1 B,

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Fukuyama B, and Okra B) of proteolytic group I were used in this study. The 56A, Hall A, Renkon-1 A, 213 B, 407-1 B, and Fukuyama B strains were kindly provided from the culture collection of H. Nakano (Laboratory of Food Microbiology and Hygiene, Hiroshima University, Hiroshima, Japan). The other *C. botulinum* strains (62A, 97A, 9B, and Okra B (proteolytic, group I) were kindly provided by the National Institute of Infectious Diseases (Tokyo, Japan).

Rice samples. Steamed, sterilized rice was prepared in a pilot plant. Briefly, after the rice bran had been sufficiently removed, the rice was washed and soaked in water for 30 min. After removing the water, the rice was steam sterilized using an ultrahigh-temperature flash-heating process (generally ≥8 s at 135°C; $F_0 >$ 3.1), followed by cooling. Packaging and the entire processing beyond the sterilization were carried out in a clean room (class 1,000). The sterilized rice was soaked in sterilized cooking water adjusted using gluconic acid to yield steamed rice with a pH of 4.6 to 6.8. The pH of water before cooking was equal to the pH of rice after cooking. And the rice was cooked at a rice:water ratio of 1:1 to produce a final cooked product with a water content of 60% (water activity of 0.98 to 0.99), which is representative of commercial products currently distributed in Japan. The soaked rice was then steam cooked at approximately 100°C, without controlling pressure for 30 min. The color of the acidically adjusted rice did not change after cooking, and it was identical to rice cooked normally. The steam-cooked rice was then aseptically packed in commercial packaging, with sample sizes of approximately 110 g, using a plastic plate and aseptically sealing with plastic film. These rice samples were stored at 30°C for 1 week and were visually inspected prior to inoculation with *C. botulinum.*

Preparation of *C. botulinum* spores. Preparation of *C. botulinum* spores and the subsequent enumeration were conducted under anaerobic conditions. *C. botulinum* strains were precultured in cooked meat medium (Eiken Chemical Co., Tokyo, Japan) at 37°C overnight. Spores of each strain were produced at 37°C over 7 days in Trypticase Peptone yeast broth (pH 7.0) with 5% (wt/vol) Trypticase Peptone (Difco, Becton Dickinson, Sparks, Md.), 0.5% (wt/vol) Bacto Peptone (Difco, Becton Dickinson), and 0.1% (wt/vol) Bacto yeast extract (Difco, Becton Dickinson). Before harvest, spores were checked with a phase contrast microscope (Olympus Co., Tokyo, Japan) for the dominance of refractile spores (>90%). Spore crops of each strain were centrifuged at 21,480 × *g* for 10 min at 4°C (SRX-201, Tomy Seiko Co., Tokyo Japan), and pellets were washed with sterile distilled water. Each crop was resuspended in sterile distilled water and frozen at −20°C until use. Spores of each strain were counted by the three-tube most-probable-number method after heat-shock treatment (80°C for 10 min, followed by rapid cooling).

Inoculation with *C. botulinum.* Equal numbers of *C. botulinum* spores from each of 10 different strains were mixed and adjusted to a concentration of approximately 4 log spores per ml. The spore mixtures were heat shocked (80°C for 10 min, followed by rapid cooling) and then 100 μl of the spore mixture was inoculated into each rice sample at each of 10 points (total of 1 ml of inoculum per rice sample) to avoid contingency differences among the samples. Cell counts of the samples and time-zero inoculum were determined by the serial dilution and pouch method *(2, 5),* using clostridia count agar (Nissui Pharmaceutical Co., Tokyo, Japan). Inoculated samples were packaged in high-gas-barrier film bags (Basela, Kureha Chemical Industry Co., Tokyo, Japan), using a Tospack V 400 gas changer (Tosei, Ohito, Shizuoka, Japan) along with a deoxidant pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) under atmospheric conditions of 0% oxygen, 5% carbon dioxide, and 95% nitrogen. All samples were incubated at 30°C. The samples were tested at weeks 0, 2, 4, 12, and 24. All the experiments were conducted in triplicate.

pH and atmospheric composition measurements. Headspace gas was analyzed for the concentration of carbon dioxide and oxygen, using a G5000A gas chromatograph (Hitachi, Tokyo, Japan). Subsamples (10 g) were placed in plastic bags (80 ml; Organo Co., Tokyo, Japan) with 10 ml of sterilized distilled water and mixed by hand for approximately 1 min to achieve a sufficiently homogeneous mixture without damaging the rice grains. Prior to the experiments, we confirmed that hand mixing was sufficient to mix the samples for microbiological counts while avoiding shredding rice grains as occurs when samples are mixed with a stomacher. pH was measured with a glass electrode pH meter (Horiba, Kyoto, Japan). The remainder of each sample (approximately 100 g) was placed in a stomacher bags (400 ml; Organo) with 100 ml of sterilized phosphate buffer (0.4% [wt/vol] Na_2HPO_4 and 0.2% [wt/vol] gelatin, pH 6.2) and mixed by hand (1 to 2 min). A portion of the mixture was used for microbiological analysis, and approximately 13 ml was transferred to sterilized centrifuge tubes (15 ml; Labcon, Petaluma, Calif) and stored at −20°C until neurotoxin assay.

Sensory evaluation. After incubation, the packages were opened, and the appearance and odor of the samples were assessed for spoilage by a panel of five judges with no special training. A sample was regarded as spoiled when all five people judged the sample as inedible, but an edible judgment by just one person was sufficient to consider the sample unspoiled.

Microbiological analysis. Anaerobic cell counts were carried out by serial dilution and pouch methods *(2, 5)* with Clostridia count agar. Portions of the same samples were used for contamination checks on PCA (Eiken) by the pour-plate method. Uninoculated samples were also checked for aerobic and anaerobic counts to confirm the absence of all bacteria.

Neurotoxin assay. Frozen rice samples were thawed, mixed, and centrifuged at 2,190 × *g* for 10 min. Two mice (approximately 20 g) were each intraperitoneally injected with 0.5 ml of supernatant. Samples were considered toxic if typical respiratory symptoms of botulism occurred and both mice died during the 48-h observation period *(19).* For samples producing toxic reactions, neurotoxin was tested for serum type by inoculating sample into mice protected with A and/or B antiserum (two mice for each treatment; six mice total) (Chiba Serum Institute, Chiba, Japan). Following the manufacturer's instructions, antiserum protection was established with a 0.5-ml intraperitoneal injection of 2 U/ml adjusted type A and/or B antiserum and the same volume of sample incubated for 1 h at room temperature. If mice protected with B antiserum died while those protected with A antiserum lived during the 48-h observation period, then the toxin was considered to be type A. Conversely, if mice protected with A antiserum died while those protected with B antiserum lived, then the toxin was considered to be type B. If mice protected with both A and B antiserum lived while the other mice died, then both toxin types A and B were considered present.

RESULTS

Measurements of pH and atmospheric composition. Atmospheric composition and pH values are shown in Table 1. The oxygen concentration at the time of packaging

TABLE 1. *Analysis of rice samples inoculated with* Clostridium botulinum *(type A, B) spores*[a]

Initial pH	Incubation time (wk)	No. of samples spoiled/no. of samples tested	Sample with toxin	Sample parameters				
				pH	O_2 (%)	CO_2 (%)	Anaerobic count (log CFU/g)	Anaerobic count (log CFU/g)[b]
4.6–4.7	0	0/1	ND[c]	4.7	0.8	1.8	2.1	ND
	2	0/3	ND	4.7 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	2.1 ± 0.1	ND
	4	0/3	ND	4.7 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	ND
	12	0/3	ND	4.8 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	1.8 ± 0.1	ND
	24	0/3	ND	4.8 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 0.0	ND
4.8–4.9	0	0/1	ND	4.9	0.8	1.8	2.1	ND
	2	0/3	ND	4.8 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	2.3 ± 0.1	ND
	4	0/3	ND	5.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	ND
	12	0/3	ND	5.0 ± 0.1	0.3 ± 0.2	0.0 ± 0.0	1.9 ± 0.1	ND
	24	0/3	ND	5.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	2.5 ± 0.2	ND
5.0–5.1	0	0/1	ND	5.2	0.8	1.5	2.1	ND
	2	0/3	ND	5.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	2.3 ± 0.3	ND
	4	0/3	ND	5.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.1	ND
	12	0/2	ND	5.5 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.1	ND
		0/1	A	5.6	0.3	0	3.8	ND
	24	0/3	A (3/3)[d]	5.4 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	4.2 ± 0.2	ND
5.2–5.3	0	0/1	ND	5.4	0.8	1.6	2.1	ND
	2	0/3	ND	5.3 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	4.4 ± 0.1	ND
	4	0/3	A (1/3)[e]	5.4 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	4.5 ± 1.1	ND
	12	0/3	ND	5.5 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	2.7 ± 0.6	ND
	24	0/3	A (3/3)	5.5 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	4.4 ± 0.0	ND
5.4–5.5	0	0/1	ND	5.5	0.8	1.6	2.1	ND
	2	0/3	A (3/3)	5.5 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	4.9 ± 0.0	ND
	4	0/3	A+B (3/3)	5.6 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	4.7 ± 0.1	ND
5.6–5.7	0	0/1	ND	5.6	0.8	1.6	2.1	ND
	2	0/3	A+B (3/3)	5.7 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.4 ± 0.1	ND
	4	ND	ND	ND	ND	ND	ND	ND
5.8–5.9	0	0/1	ND	5.9	0.8	1.7	2.1	ND
	2	0/3	A+B (3/3)	5.9 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.6 ± 0.0	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.0–6.1	0	0/1	ND	6.1	0.8	1.6	2.1	ND
	2	0/3	A+B (3/3)	6.0 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.6 ± 0.2	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.2–6.3	0	0/1	ND	6.2	0.8	1.7	2.1	ND
	2	0/3	A+B (3/3)	6.2 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	6.2 ± 0.0	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.8–6.9	0	0/1	ND	6.8	0.8	1.7	2.1	ND
	2	3/3	A+B (3/3)	6.8 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	6.8 ± 0.1	ND
	4	ND	ND	ND	ND	ND	ND	ND

[a] Rice sample had 60% water content.
[b] The limit of detection of viable count is 2 CFU/g.
[c] ND, not detected.
[d] Toxin was detected (0.1% oxygen, pH 5.4) in all three samples.
[e] Toxin was detected (0.3% oxygen, pH 5.4) in one of three samples.

was 0.8%, and it dropped to ≤0.3% after 2 weeks in all samples. The initial carbon dioxide concentration was 1.8%, but no CO_2 was detected after 2 weeks of incubation in all sample types. There was no significant change in the sample pH value over 24 weeks.

Growth of *C. botulinum* in steamed rice. Growth of *C. botulinum* in steamed rice at 30°C is shown in Table 1. Anaerobic counts were assumed to equal the *C. botulinum* counts because rice samples were supposed to be sterile in the absence of *C. botulinum* inoculation. Initial *C. botulinum* counts were an average 2.1 log CFU/g for all sample types. Counts after 2 weeks of incubation increased significantly in samples initially adjusted to pH 5.2 or above, ranging from 4.4 log CFU/g in samples at pH 5.2 to 6.8 log CFU/g in samples at pH 6.8. The only exception was samples at pH 5.2 to 5.3 collected at week 12, in which the anaerobic counts were lower than the counts of those collected at week 4. Sample spoilage occurred only in samples at pH 6.8. Increases in anaerobic counts were also detected in samples of lower pH later in the experimental period. One of the samples at pH 5.0 showed an increased anaerobic count of 3.8 log CFU/g after 12 weeks of incubation

and an anaerobic count of 4.2 log CFU/g was detected in all three samples.

No bacterial growth was detected by aerobic cell counts in any of the samples, as expected.

Neurotoxin assay. Neurotoxin detection data are shown in Table 1. The higher the sample pH value, the earlier the start of neurotoxin production was observed. Neurotoxin was detected after 2 weeks of incubation in samples at pH 5.4 or above, while it took 4 weeks for the toxin to be detected in samples at pH 5.2 to 5.3, and 12 weeks in samples at pH 5.0 to 5.1. In samples at pH 4.8 or below, no toxin was detected during the experimental period (24 weeks).

Type A toxin was detected in samples of pH $\geq$ 5.0. Type B toxin was detected only in samples of pH $\geq$ 5.4.

Uninoculated samples were not included in the neurotoxin assay as negative control because they were confirmed to be free of bacteria in aerobic and anaerobic counts.

DISCUSSION

When *C. botulinum* spores alone were inoculated into rice samples at a concentration of 2 log CFU/g, counts of this pathogen increased, and neurotoxin was detected in samples at initial pH 5.4 or above after 2 weeks of storage at 30°C. This result indicates that MAP-steamed rice products, which are usually at pH 6.5, are at risk of *C. botulinum*–toxin formation when secondary contamination with this organism occur. On the other hand, Kazama et al. *(9)* reported that at least 1 month was needed before the toxin could be detected in the same type of sample stored at 30°C, with much higher inoculation load (4.2 log CFU/g). The only difference between their experimental design and ours was the control of atmospheric conditions in the packaging. They used a deoxidant pack to decrease the O_2 content but did not pack the samples under a modified atmosphere. Their method might have allowed high initial and subsequent O_2 content in the package, as our previous experiments showed that the use of deoxidant pack alone is not sufficient to produce a completely anaerobic atmosphere (data not shown). In fact, our data show that a long incubation time (2 to 3 months) is required for *C. botulinum*–toxin production and growth in rice products stored at oxygen concentration of 0.4% (data not shown). Since Kazama et al. *(9)* did not indicate the oxygen concentration in their report, it is not known what O_2 content prevents the growth of *C. botulinum.* In contrast to their experiments, our experiments were based on the worst-case scenario, using oxygen concentration of $\leq$0.3%, since we think that risk assessment of any foodborne pathogen must be carried out based on the worst-case scenario.

In samples at pH 4.9 or below, anaerobic counts did not increase from the initial counts, and neurotoxin was not detected during the entire experimental period used in this study. It is well reported that toxin production is not limited above pH 4.6 in pure cultures of *C. botulinum* isolated from food contamination cases *(21).* This inconsistency of pH threshold may be attributed to the unique properties of aseptically steamed rice samples. Rice is composed almost entirely of starch, with little protein *(18),* and proteolytic *C. botulinum* has limited ability to utilize starch as a carbon source *(15),* although under optimized conditions, at least some strains could utilize starch *(20),* and high-starch foods such as potatoes could be the source of an outbreak *(1).* Moreover, when *C. botulinum* and *Bacillus subtilis* were grown together on rice, *B. subtilis* was reported to promote the growth and toxin production of *C. botulinum (8),* as amylase produced by *B. subtilis* digests starch that then stimulates the growth of *C. botulinum.* Other researchers have also reported that the presence of other microbial species allows *C. botulinum* to grow and produce neurotoxins *(6, 14).* For example, the presence of molds results in increased *C. botulinum* growth and allows neurotoxin production at pH 4.2 *(7).* This is possible because molds not only produce amylase, but also locally increase the pH *(7).* In the present study, we inoculated *C. botulinum* alone into rice samples. Thus, the effects of co-inoculation with other microorganisms should be studied further. Also, since botulinum toxin type A was produced at pH 4.75 and water activity of 0.97 in vacuum-packed potatoes acidified with organic acid, other factors contributing to inhibition remain to be identified *(4).*

Unexpectedly low anaerobic count corresponding with no toxin production was observed for samples at pH 5.2 to 5.3 collected at week 12 (Table 1). All three samples had similar anaerobic counts, and the pH values and atmospheric compositions were sufficient for *C. botulinum* to multiply and produce toxins. Therefore, the reason for these results is not known.

In conclusion, we demonstrated the worst-case scenario of botulism in steamed rice products after inoculation of *C. botulinum* spores alone under an atmosphere of $\leq$0.3% oxygen. We conclude that aseptically steamed rice products must be adjusted to pH 4.9 or below to avoid the risk of botulism from consumption of these MAP foods.

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REFERENCES

1. Angulo, F. J., J. Getz, J. P. Taylor, K. A. Hendricks, C. L. Hatheway, S. S. Barth, H. M. Solomon, A. E. Larson, E. A. Johnson, L. N. Nickey, and A. A. Ries. 1998. A large outbreak of botulism: the hazardous baked potato. *J. Infect. Dis.* 178:172–177.
2. Bladel, B. O., and R. A. Greenberg. 1965. Pouch method for the isolation and enumeration of clostridia. *Appl. Microbiol.* 13:281–285.
3. Daifas, D. P., J. P. Smith, B. Blanchfield, and J. W. Austin. 1999. Effect of pH and CO_2 on growth and toxin production by *Clostrid-*

ium botulinum in English-style crumpets packaged under modified atmosphere. *J. Food Prot.* 62:1157–1161.
4. Dodds, K. L. 1989. Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked, vacuum-packed potatoes. *Appl. Environ. Microbiol.* 55:656–660.
5. Fujisawa, T., K. Aikawa, I. Furukawa, and T. Takahashi. 2000. Occurrence of clostridia in glass bottled foods. *Int. J. Food Microbiol.* 54:213–221.
6. Hotchkiss, J. H., M. J. Banco, F. F. Busta, C. A. Genigeorgis, R. Kochiba, L. Rheaume, L. A. Smoot, J. D. Schuman, and H. Sugiyama. 1992. The relationship between botulinal toxin production and spoilage of fresh tomatoes held at 13 and 23°C under passively modified and controlled atmospheres and air. *J. Food Prot.* 55:522–527.
7. Huhtanen, C. N., J. Naghski, C. S. Custer, and R. W. Russell. 1976. Growth and toxin production by *Clostridium botulinum* in moldy tomato Juice. *Appl. Environ. Microbiol.* 32:711–715.
8. Kasai, Y., B. Kimura, S. Kawasaki, T. Fukaya, K. Sakuma, and T. Fuji. 2005. Growth and toxin production by *Clostridium botulinum* in steamed rice aseptically packed under modified atmosphere. *J. Food Prot.* 68:1005–1011.
9. Kazama, A., T. Muta, and N. Matsuda. 1994. Effects of pH on toxin production in hermetically packaged steamed rice by *Clostridium botulinum* types A and B and heat resistance of those spores. *Jpn. J. Food Microbiol.* 11:165–171.
10. Kimura, B., S. Kuroda, M. Murakami, and T. Fujii. 1996. Growth of *Clostridium perfringens* in fish fillets packaged with a controlled carbon dioxide atmosphere at abuse temperatures. *J. Food Prot.* 59: 704–710.
11. Koseki, S., and K. Itoh. 2002. Effect of nitrogen gas packaging on the quality and microbial growth of fresh-cut vegetables under low temperatures. *J. Food Prot.* 65:326–332.
12. Lambert, A. D., J. P. Smith, and K. L. Dodds. 1991. Effect of initial O_2 and CO_2 and low-dose irradiation on toxin production by *Clostridium botulinum* in MAP fresh pork. *J. Food Prot.* 54:939–944.
13. Lyver, A., J. P. Smith, J. Austin, and B. Blanchfield. 1998. Competitive inhibition of *Clostridium botulinum* type E by *Bacillus* species in a value-added seafood product packaged under a modified atmosphere. *Food Res. Int.* 31:311–319.
14. Odlaug, T. E., and I. J. Pflug. 1979. *Clostridium botulinum* growth and toxin production in tomato juice containing *Aspergillus gracilis*. *Appl. Environ. Microbiol.* 37:496–504.
15. Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.). 1986. Bergey's manual of systematic bacteriology, 8th ed. Williams & Wilkins, Baltimore, Md.
16. Solomon, H. M., E. J. Rhodehamel, and D. A. Kautter. 1998. Growth and toxin production by *Clostridium botulinum* on sliced raw potatoes in a modified atmosphere with and without sulfite. *J. Food Prot.* 61:126–128.
17. Torre, M. D., M. L. Stecchini, and M. W. Peck. 1998. Investigation of the ability of proteolytic *Clostridium botulinum* to multiply and produce toxin in fresh Italian pasta. *J. Food Prot.* 61:998–993.
18. U.S. Department of Health, Education, and Welfare. 1972. Food composition table for use in east Asia 1972. U.S. Department of Health, Education, and Welfare, Food and Agriculture Organization, Food Policy and Nutrition Division, Bethesda, Md.
19. U.S. Food and Drug Administration. 2001. Bacteriological analytical manual. AOAC International, Gaithersburg, Md.
20. Whitmer, M. E., and E. A. Johnson, 1987. Development of improved defined media for *Clostridium botulinum* serotypes A, B, and E. *Appl. Environ. Microbiol.* 54:753–759.
21. Wong, D. M., K. E. Young-Perkins, and R. L. Merson. 1988. Factors influencing *Clostridium botulinum* spore germination, outgrowth, and toxin formation in acidified media. *Appl. Environ. Microbiol.* 54:1446–1450.