Research Note

Botulism Challenge Studies of a Modified Atmosphere Package for Fresh Mussels: Inoculated Pack Studies

C. R. NEWELL,1* LI MA,2 AND MICHAEL DOYLE3

1Maine Shellfish R + D, 7 Creek Lane, Damariscotta, Maine 04543; 2National Institute for Microbial Forensics and Food and Agricultural Biosecurity, Department of Entomology and Plant Pathology, Oklahoma State University, 127 Noble Research Center, Stillwater, Oklahoma 74078; and 3Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223-1797, USA

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ABSTRACT

A series of botulism challenge studies were performed to determine the possibility of production of botulinum toxin in mussels (Mytilus edulis) held under a commercial high-oxygen (60 to 65% O2), modified atmosphere packaging (MAP) condition. Spore mixtures of six strains of nonproteolytic Clostridium botulinum were introduced into mussel MAP packages receiving different packaging buffers with or without the addition of lactic acid bacteria. Dye studies and package flipping trials were conducted to ensure internalization of spores by packed mussels. Inoculated mussel packages were stored at normal (4°C) and abusive (12°C) temperatures for 21 and 13 days, respectively, which were beyond the packaged mussels’ intended shelf life. Microbiological and chemical analyses were conducted at predetermined intervals (a total of five sampling times at each temperature), including total aerobic plate counts, C. botulinum counts, lactic acid bacterial counts, package headspace gas composition, pH of packaging buffer and mussel meat, and botulinum toxin assays of packaging buffer and mussel meat. Results revealed that C. botulinum inoculated in fresh mussels packed under MAP packaging did not produce toxin, even at an abusive storage temperature and when held beyond their shelf life. No evidence was found that packaging buffers or gas composition influenced the lack of botulinum toxin production in packed mussels.

World aquaculture production of edible blue mussels in 2007 was greater than 1.6 million metric tons (9). While market trends include increased sales and consumption of modified atmosphere packaged (MAP) mussels, most studies on MAP mussel quality and safety have concentrated on the effects of oxygen concentration and mussel size (4, 18), and the effects of gas mixtures (primarily O2, CO2, and N2) on microbiological, biochemical, and sensory attributes of blue mussels during refrigerated storage (10, 11). Because of the potential for nonproteolytic spores of Clostridium botulinum to grow at temperatures warmer than 3°C in seafood MAP packages, questions have been raised regarding the safety of live mollusks from the hazard of botulism under time and abusive temperature conditions (12, 19–21). However, no previous inoculated pack studies have been conducted to determine if C. botulinum growth and toxin production can occur as a function of temperature abuse. Such studies are needed to determine if raw (live) mussels packaged under MAP conditions are a potential hazard (22). The primary objective of this study was to determine if nonproteolytic C. botulinum could produce toxin in MAP-packaged live mussels under normal and abusive temperature storage conditions. In addition, we investigated whether the addition of lactic acid bacteria (LAB), a low pH buffer, or headspace gas composition affected toxin production in our inoculated pack studies. We investigated the addition of LAB because LAB are known to produce bacteriocins inhibitory to C. botulinum (17).

MATERIALS AND METHODS

Preliminary studies on optimization of inoculation procedure. At the beginning of this study, dye and inoculation studies were conducted to identify the best inoculation procedure that would ensure the even uptake of the C. botulinum spores (introduced into the mussel package in the buffer solution) by mussels throughout the package, not just those on the bottom layer. Fluorescent (yellow, with a green cast) water-soluble dye DFWB-K73-52 (Risk Reactor, Santa Ana, CA) was evaluated to determine the best concentration (0.001, 0.01, 0.1, and 1%) for tracing based on the cold (4°C overnight) and heat (steamed in capillary tube for 5 min) stability of the dye.

Mussels were packed according to the protocol described below and including those containing 0.1% fluorescent dye in the packaging buffer. The packages were held at 4°C overnight before mussels (top and bottom layers) were dissected to determine dye uptake. Mussels (10 from the top and 10 from the bottom layers) were boiled in water for 1 to 2 min (until the shell opened) to firm the mussel meat for easy separation of the intestinal tract (i.e., digestive gland) from the tissue. The intestinal tract was removed by dissection and transferred to a sterile, transparent petri dish cover for examination under a UV light for yellow-green fluorescence. The intestinal tract was chopped, a drop of distilled water was added, and the preparation was again examined under UV light to determine if the dye had been taken into the gastrointestinal tract.
Mussels were packed according to the protocol described above, with packaging buffer containing 0.1% fluorescent dye. The packages were held upside down at 4°C for predetermined intervals then turned right side up and held at 4°C for predetermined intervals before mussels (top and bottom layers) were dissected for dye uptake evaluation as described above. This protocol was performed to determine if flipping the packages would provide the best distribution of the inoculated dye in all mussels in each package.

**Fresh mussels.** For all experiments, freshly harvested live mussels (*Mytilus edulis*) were shipped overnight from The Great Eastern Mussel Farms (Tenants Harbor, ME) to the University of Georgia Center for Food Safety at Griffin. On arrival, mussels were transferred into baskets and submerged in Instant Ocean sea water (www.instantocean.com), with an air stone bubbler operating in a cooler (4°C) for 2 h, and then the water was drained 45 min prior to inoculation of mussels. This protocol was used to simulate packing of mussels in a commercial facility.

*C. botulinum* spores preparation. A six-strain spore mixture of *C. botulinum* Beluga (type E), Minnesota (type E), 70 E (type E), 2 B (type B), 17 B (type B), and F202 (type F) was prepared from individual spore stocks at a concentration of ca. 10⁶ spores per ml in sterile distilled water as stock. On the day of each inoculation study, the six-strain spore mixture was heated to 60°C for 10 min, then cooled in cold water before being diluted to a final concentration ca. 2,000 spores per ml.

*Pedioococcus pentosaceus* culture preparation. *P. pentosaceus* ATCC 43201 was cultured in de Man Rogosa Sharpe (MRS) broth at 37°C for 24 h for 2 days consecutively. Cells were washed once with phosphate-buffered saline solution (PBS; pH 7.2), resuspended to the original volume with PBS, and enumerated by plating dilutions onto MRS plates (37°C, 24 h).

Packaging buffer preparation. Packaging buffers that were tested included normal packaging buffer (NPB) containing 7% NaCl and 0.2% Hamulbac FMC (which contains sodium citrate and citric acid); PBS (pH 7.2); and low-pH PBS solution (pH adjusted to 2.5 with 6 N HCl). In the first two trials, only NPB that was prepared with tap water was used, whereas for the last two trials all buffers were prepared with distilled water that had been filter (0.22-μm pore size) sterilized.

Inoculation, packaging, storage, and sampling plan of mussels. Mussels were packed in a standard blue 0.8-mm-thick tray weighing ca. 2 lb (900 ± 5 g), and then 35 ml of packaging buffer was added to the package, and the packages were filled with O₂-N₂ (65:35) and sealed with a barrier film under a vacuum (532 mm Hg) with a Koch KATS 400 packaging unit (Koch Equipment Co., Kansas City, MO). Tray dimensions were 4.5 by 23.5 by 13.3 cm. The packaging buffers and packaging treatments varied among the trials: In trial 1, only NPB was used, and the treatments included control (mussels with NPB only), treatment 1 (mussels with NPB plus 5 ml of *C. botulinum* spore mixture), and treatment 2 (mussels with NPB plus 5 ml of *C. botulinum* spore mixture and 1 ml of washed overnight culture of *P. pentosaceus*); in trial 2, the packaging buffer (35 ml) included NPB, PBS, and LPBS, and 5 ml of *C. botulinum* spore mixture was added to each of the packages.

All packages of mussels were held upside down at 3 or 12°C for 5 h before being turned right side up (180°) for the remaining storage time. This flipping procedure was selected based on a preliminary study that revealed that packed mussels in both layers could internalize *C. botulinum* spores introduced through the packaging buffer (see study described below in “Materials and Methods”). There were five sampling times for each storage temperature. MAP mussels held at 12°C were sampled at 1, 6, 8, 10, and 13 days postinoculation, and those held at 3°C were sampled at 1, 7, 14, 17, and 21 days postinoculation. In the first study, nine samples were tested at each sampling interval for each storage temperature, including five samples inoculated with both *P. pentosaceus* and *C. botulinum*, two inoculated with *C. botulinum* only, and two controls. In the second study, two PBS, two LPBS, and three NPB samples were used for each storage temperature at each sampling time.

Headspace gas composition of each package was determined with an O₂-CO₂ headspace gas analyzer (model 6600, Illinois Instruments, Johnsburg, NY) for each sample. NaCl content was determined in trial 2 for all samples by using a Salinity Conductivity Temperature meter (YSI, Yellow Springs, OH).

Quantitative microbial analyses. Microbial analyses of mussel meat and juices for each package were conducted according to the following protocols. Packaging juice in each package was transferred to a sterile cup. A total of 12 to 15 mussels (a variety of closed, slightly opened, and opened) from each package were selected and placed onto a sterile sheet of aluminum foil. Mussel meat was removed with a sterile scalpel, and a total of 55 g was collected and transferred to a sterile blender jar and blended for 1 min at high speed. Five grams of the blended meat was used for pH determination, and 50 ml of gel phosphate buffer (pH 6.2) was added to the remaining mussel meat and blended for another 1 min. The entire content of the blender was then transferred into a stomacher bag with a filter and macerated by stomaching for 2 min at high speed before storing on ice. Mussel meat and juice samples were serially diluted (1:10) with 0.1% peptone water and plated onto plate count agar, McClung-Toabe egg yolk agar (McClung), and Rogosa SL agar plates (only in trial 1) to obtain aerobic plate counts, presumptive *C. botulinum* counts, and presumptive *P. pentosaceus* counts, respectively. Plate count agar was incubated at 23°C for 48 h, whereas McClung and Rogosa SL agar plates were incubated in Coy anaerobic chambers (Coy Laboratory Products, Grass Lake, MI) at 31°C for 48 h.

Botulinum toxin bioassay. A portion (10 ml) of mussel tissue sample, after blending and stomaching according to the protocol described above, was sedimented by centrifugation at 14,000 rpm (23,000 × g) for 10 min. The supernatant was collected and filtered through a 0.8-μm-pore-size filter and then a 0.45-μm-pore-size filter before trypsinization at 37°C for 1 h and injection into mice (BALB/c strain). A portion of the filtered samples was saved and held at −20°C for confirmation testing. Injected mice were observed for symptoms of botulism for 48 h after injection, according to the U.S. Food and Drug Administration’s (FDA) *Bacteriological Analytical Manual* protocol.
Uptake study with \textit{C. botulinum} spores and \textit{P. pentosaceus}. Mussels were packed according to the protocol described in the ‘‘Inoculation, packaging, storage, and sampling plan of mussels’’ section. For trial 1, the treatments included the control (mussels with NPB only), treatment 1 (mussels with NPB plus 5 ml of \textit{C. botulinum} spore cocktail), and treatment 2 (mussels with NPB plus 5 ml of \textit{C. botulinum} spores and 1 ml of \textit{P. pentosaceus} washed overnight culture). The inoculated and uninoculated MAP mussels were stored upside down at either 3 or 12°C for 5 h before being turned right side up for the remaining storage time of 1 day at 3°C and 7 days at 12°C.

On day 1 (3 and 12°C) and day 7 (12°C only), mussels from each group (control and treatment) were sampled (meat and packaging juice) and assayed for presumptive \textit{P. pentosaceus} (Rogosa SL agar) and \textit{C. botulinum} spore (McClung agar and \textit{C. botulinum} isolation agar) counts. The top and bottom layers of mussels in each package were sampled separately; specifically, 10 mussels from the top layer were collected as the top layer sample, and 10 mussels from the bottom layer were collected as the bottom layer sample. In addition, presumptive \textit{P. pentosaceus} and \textit{C. botulinum} spore counts in mussel intestinal tracts were determined for day 1 mussel packages stored at 12°C. Mussel meat from 10 mussels (top and bottom) from each package (control and treatments) was collected, soaked in 10% bleach for 5 min with agitation, washed with PBS three times (10 min each), drained, and weighed before the addition of gel phosphate buffer for blending. These counts were determined to confirm initial spore concentrations in the treated samples as well as the uptake of spores by the mussels in their gastrointestinal tracts. In addition, botulinum toxin bioassays were conducted on the mussel meat according to the protocol described above.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Storage temp (°C) & Days of storage & Mussel group & Sample site & \textit{C. botulinum} & \textit{Pediococcus} & Toxicity test \textsuperscript{b} & \\
& & & & McClung & CBI & Rogosa & Botulinum toxin \\
\hline
3 & 1 & Control & Juice & ND & ND & ND & N \\
& & Meat & Top & ND & ND & ND & N \\
& & & & Bottom & ND & ND & N \\
& & Treatment 1 & Juice & 2.45 & 2.55 & ND & N \\
& & Meat & Top & 2.27 & 2.27 & ND & N \\
& & & & Bottom & 2.05 & 2.15 & ND & N \\
& & Treatment 2 & Juice & 2.58 & 2.39 & 6.85 & N \\
& & Meat & Top & 2.2 & 2.03 & 6.12 & N \\
& & & & Bottom & 1.98 & 1.83 & 6.45 & N \\
& 12 & Control & Juice & ND & ND & ND & N \\
& & Meat & Top & ND & ND & ND & N \\
& & & & Bottom & ND & ND & ND \\
& & Intestine & & ND & ND & ND & N \\
& & Treatment 1 & Juice & 3.08 & 3.05 & ND & N \\
& & Meat & Top & 2.15 & 2.24 & ND & N \\
& & & & Bottom & 1.88 & 2.36 & ND & N \\
& & Intestine & & 1.5 & 1.34 & ND & N \\
& & Treatment 2 & Juice & 2.36 & 2.26 & 7.24 & N \\
& & Meat & Top & 2.26 & 2.26 & 7.01 & N \\
& & & & Bottom & 2.28 & 2.18 & 6.95 & N \\
& & Intestine & & 1.68 & 1.62 & 5.31 & N \\
& 12 & 7 & Control & Juice & ND & ND & 5.1 & N \\
& & Meat & Top & ND & ND & 1.73 & N \\
& & & & Bottom & ND & ND & 2.68 & N \\
& & Intestine & & ND & ND & N & N \\
& & Treatment 1 & Juice & 1.53 & 2.21 & 4.59 & N \\
& & Meat & Top & 1.83 & 1.73 & 2.32 & N \\
& & & & Bottom & 1.51 & 2.04 & 2.97 & N \\
& & Intestine & & 1.34 & 2.21 & 6.42 & N \\
& & Treatment 2 & Juice & 1.86 & 2.05 & 6.31 & N \\
& & Meat & Top & 1.87 & 1.96 & 5.82 & N \\
& & & & Bottom & 1.87 & 1.96 & 5.82 & N \\
\hline
\textsuperscript{a} ND, not detected. The detection limits were <2 CFU/g for meat and <1 CFU/ml for juice. \\
\textsuperscript{b} N, no botulinum toxin was detected by the mouse bioassay.
\end{tabular}
\caption{Microbial counts of \textit{Clostridium botulinum} and \textit{Pediococcus pentosaceus} and toxicity tests of live MAP-packaged mussels at two times and temperatures for internalization study, treated with normal packaging buffer (control), LAB (treatment 1), and \textit{C. botulinum} spore mixture (treatment 2).}
\end{table}

Determination of potential antimicrobial effects of packaging atmosphere. The purpose of this study was to examine whether high oxygen in the modified atmosphere package had an inhibitory effect on the germination of spores. Mussels were packed as described in the ‘‘Inoculation, packaging, storage, and sampling plan of mussels’’ section for trial 1 above. The packages were then filled with 100% \textit{N}_2 (negative control; Treat-N) whereas treatment Treat-O packages were filled with \textit{O}_2-\textit{N}_2 (65:35) and
sealed by a Koch KATS 400 packaging unit. Packages (negative control and treatments) were held inverted at 12°C for 5 h before being turned right side up for the remaining storage time. The two negative controls and five of each treatment samples were assayed at each sampling time. Designed as a preliminary study, there were just two sampling times, day 7 and day 14, and the negative controls were sampled on days 6 and 12.

Headspace gas composition and the pH of each package, quantitative microbial analyses of mussel meat and juices in each package, and botulinum toxin in the mussels were determined according to the protocol described above.

### RESULTS

**Preliminary studies on optimization of the inoculation procedure.** The initial dye study revealed that even uptake of the dye by mussels was only achieved when the mussels were in contact with the packaging buffer for more than 4 h; therefore, a flipping time at 5 h was selected. For the uptake study conducted with *C. botulinum* spores and *P. pentosaceus*, in general, there were no substantial differences in presumptive *P. pentosaceus* and *C. botulinum* spore counts in juice and meat (top and bottom layers)

<table>
<thead>
<tr>
<th>3°C trial</th>
<th>Environment</th>
<th>Treatment</th>
<th>Values on day:</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>Headspace gas composition&lt;sup&gt;b&lt;/sup&gt;</td>
<td>O&lt;sub&gt;2&lt;/sub&gt; (%)</td>
<td>Control</td>
<td>39</td>
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<td></td>
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<td>Treatment 1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Treatment 2</td>
<td>54.2</td>
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<tr>
<td></td>
<td>CO&lt;sub&gt;2&lt;/sub&gt; (%)</td>
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<td></td>
<td></td>
<td>Treatment 1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment 2</td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td>Mussel meat</td>
<td>Control</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment 1</td>
<td>6.55</td>
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<tr>
<td></td>
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<td>Treatment 2</td>
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<td>Packaging juice</td>
<td>Control</td>
<td>7.18</td>
</tr>
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<td>Treatment 2</td>
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<td>Treatment 2</td>
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<tr>
<td></td>
<td></td>
<td>Treatment 2</td>
<td>4.26</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> count&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mussel meat</td>
<td>Control</td>
<td>&lt;0.30&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>Treatment 1</td>
<td>&lt;0.30&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>&lt;0.30&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Packaging juice</td>
<td>Control</td>
<td>&lt;0.00&lt;sup&gt;f&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>Treatment 1</td>
<td>&lt;0.00&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment 2</td>
<td>&lt;0.00&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
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<td>Mussel meat</td>
<td>Control</td>
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<td></td>
<td></td>
<td>Treatment 1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Treatment 2</td>
<td>ND</td>
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</table>

<sup>a</sup> Values are means of two or three independent replications.

<sup>b</sup> O<sub>2</sub> contents on day 14 and thereafter could be lower than the recorded value because of error in the gas analyzer setting.

<sup>c</sup> APC, aerobic plate counts, expressed in log CFU per milliliter (juice) or gram (meat).

<sup>d</sup> Rogosa LAB plate counts; presumptive *P. pentosaceus* ATCC 43210, expressed in log CFU per milliliter (juice) or gram (meat).

<sup>e</sup> Detection limit for meat samples was 2 CFU/g.

<sup>f</sup> Detection limit for packaging juice samples was 1 CFU/ml.

<sup>g</sup> McClung plate counts; only *Clostridium*-like colonies were counted, expressed in log CFU per milliliter (juice) or gram (meat).

<sup>h</sup> ND, not detected.
samples obtained on day 1 held at 3 or 12 °C, indicating that these inocula were well distributed among the mussels by the turning procedure (Table 1).

When the mussel meats and intestinal tracts were treated by washing with 10% bleach, which should eliminate spores as well as vegetative cells outside of the intestinal tracts, the *P. pentosaceus* and *C. botulinum* spore counts inside the intestinal tracts were generally 0.5 to 1 log CFU/g less than those on the meat.

Some bacteria other than *P. pentosaceus* also grew to large numbers on Rogosa SL agar plates containing samples of the controls and other treatment groups that were not inoculated with *P. pentosaceus* when held for 7 days at 12 °C. Most (if not all) of the bacteria growing on these plates were not *P. pentosaceus*, but apparently were part of the indigenous microflora of the mussels.

Botulinum toxin was not detected in any of the samples, including the controls and those of treatments...
inoculated with *C. botulinum* alone or in coinoculation with *P. pentosaceus*.

**Challenge studies with commercial high-oxygen (60 to 65% O₂) MAP and inclusion of LAB in the packaging buffer.** No botulinum toxin was detected in any packages (Tables 2 and 3). However, presumptive *C. botulinum* counts were obtained for treatment groups that were inoculated with *C. botulinum* spores (ca. 10,000 spores per package), but not in negative control samples. In addition, botulinum toxin was detected in mussel meat that had been inoculated with *C. botulinum* spores and incubated within a Coy anaerobic chamber at 31°C for 48 h.

**Table 4. Effects of packaging buffer on microbial counts, toxicity, and acceptance in a high-oxygen MAP package with LAB and Clostridium botulinum spore mixture at 3°C, over five sampling dates**

<table>
<thead>
<tr>
<th>3°C trial</th>
<th>Environment</th>
<th>Treatment&lt;sup&gt;h&lt;/sup&gt;</th>
<th>1</th>
<th>7</th>
<th>14</th>
<th>17</th>
<th>21</th>
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<td>O₂ (%)</td>
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<td>51.45</td>
<td>30.1</td>
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<td></td>
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<td>LPBS</td>
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<td>CO₂ (%)</td>
<td>PBS</td>
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<td>Mussel meat</td>
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<td>LPBS</td>
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<sup>a</sup> Values are means of two or three independent replications.
<sup>b</sup> PBS, phosphate-buffered saline; LPBS, low-pH PBS (pH 2.5); NPB, normal packaging buffer.
<sup>c</sup> NA, not done.
<sup>d</sup> APC, aerobic plate counts; expressed in log CFU per milliliter (juice) or gram (meat).
<sup>e</sup> McClung plate counts; only *Clostridium*-like colonies were counted, expressed in log CFU per milliliter (juice) or gram (meat).
<sup>f</sup> Before, before package being opened; After, after package being opened.
<sup>g</sup> ND, not detected.
TABLE 5. Effects of packaging buffer on microbial counts, toxicity, and acceptance in a high-oxygen MAP package with LAB and Clostridium botulinum spore mixture at 12°C, over five sampling dates

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a Values are means of two or three independent replications.
b PBS, phosphate-buffered saline; LPBS, low-pH PBS (pH 2.5); NPB, normal packaging buffer.
c APC, aerobic plate counts; expressed in log CFU per milliliter (juice) or gram (meat).
d McClung plate counts; only Clostridium-like colonies were counted, expressed in log CFU per milliliter (juice) or gram (meat).
e Before, before package being opened; After, after package being opened.
f NA, not done.
g ND, not detected.

during mixing with the buffer before transfer into the treatment group packages. The treatment group was the last group of mussels to be packaged. It is likely that packages of mussels having counts on Rogosa agar were those packed earlier in the study, as the mussels in these packages neutralized the packaging buffer to increase the pH soon after being packed. We conducted a follow-up study in which an overnight culture of *P. pentosaceus* was inoculated into packaging buffer, and a time-course survival determination was carried out by enumerating *P. pentosaceus* in the packaging buffer at 0, 1, 2, 3, and 24 h after inoculation. Results revealed that a roughly 3-log reduction of *P. pentosaceus* occurred during the first hour of exposure to packaging buffer, more than a 6-log reduction occurred after 2 h of exposure, and *P. pentosaceus* was no longer detectable (<1 CFU/ml) after 3 h of exposure.

Interestingly, some counts were obtained on Rogosa SL agar from some mussels in the negative control group that
were not inoculated with *P. pentosaceus*. These counts were obtained only with the mussel meat samples. Microscopic examination of these colonies revealed they were rod-shaped bacteria (not cocci like *P. pentosaceus*), similar to those observed in a preliminary uptake study and were part of the acid-tolerant indigenous microflora of mussels.

Based on our results (bacterial counts on McClung agar of negative control samples, both mussel meat and packaging juice), there were naturally occurring *Clostridium*-like bacteria present in the mussels but the number decreased to an undetectable level at 7 days and thereafter. Similarly, the total numbers of inoculated *C. botulinum*, both in positive control samples and treatment groups, either in mussel meat or packaging juice, held at either 3 or 12°C, also decreased with storage time. It appeared that the packaging conditions were inhibitory to the growth of *C. botulinum* in general. This could explain why we did not detect botulinum toxin even in the positive control samples. The positive controls were designed based on the assumption that mussels inoculated with *C. botulinum* and packed under the commercial conditions with packaging buffer, as in this study, would produce botulinum toxin.

**Effects of packaging buffers.** No botulinum toxin was detected in mussels packaged in (i) commercial packaging buffer containing 7% salt, pH 2.5; (ii) PBS containing 0.8% salt, pH 7.2; or (iii) LPBS containing 0.8% salt, pH 2.5 (Tables 4 and 5). It is likely that the initial high-oxygen concentration in the package, initially ca. 60 to 65%, and the subsequent high–carbon dioxide concentrations provided an inhibitory effect on *C. botulinum* spore germination and toxin production.

**Determination of potential antimicrobial effects of packaging atmosphere.** No botulinum toxin was detected in any packages packed under either pure N₂ (Treat-N) or commercial high-O₂ (Treat-O) atmosphere and the *C. botulinum* spore counts were very low, although the initial inoculation level was 4,150 spores per package (Table 6).

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<td><strong>APC</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mussel meat Negative</td>
<td>&gt;5.70</td>
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<td>4.22</td>
<td>5.6</td>
<td>&gt;6.70</td>
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<td>Packaging juice Negative</td>
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<td><strong>McClung counts</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
<td>Treatment N</td>
<td>&lt;0.00</td>
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<td><strong>Toxicity</strong></td>
<td>Mussel meat Negative</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
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<sup>a</sup> Values are means of two or three independent replications.

<sup>b</sup> Headspace gas composition was determined using an O₂-CO₂ headspace gas analyzer.

<sup>c</sup> APC, aerobic plate counts, expressed in log CFU per milliliter (juice) or gram (meat).

<sup>d</sup> McClung plate counts; only *Clostridium*-like colonies were counted, expressed in log CFU per milliliter (juice) or gram (meat).

<sup>e</sup> Detection limit for meat samples was 2 CFU/g.

<sup>f</sup> Detection limit for packaging juice samples was 1 CFU/ml.

<sup>g</sup> ND, not detected.
DISCUSSION

These experiments, which were replicated several times, revealed that live mussels, when packed in an initially oxygen-enhanced MAP package, were safe from the production of botulinum toxin, even under abusive-temperature storage (12°C) conditions for at least 12 days, which is beyond their shelf life. Interestingly, as the relatively high-oxygen concentration in the package declined, the carbon dioxide concentration concomitantly increased to high concentrations, i.e., greater than ca. 20% at 8 days. Perhaps these atmospheric gas combinations provided antibotulinic activity in the packages. Mussels stored at a normal storage temperature of 3°C did not develop botulinum toxin, even at 14 to 21 days in the absence of oxygen and with ca. 10 to 14% CO₂. The cold storage temperature was likely the primary contributing factor to preventing botulinum toxin production in the 3°C trials.

Preliminary studies were conducted to assure that the live mussels received uniform inocula of sufficient concentrations of nonproteolytic C. botulinum spores throughout the packages. These studies were followed by several experiments examining the safety of a commercial MAP formula by using enhanced oxygen with a low pH buffer to reduce odor. Studies were also performed to examine the growth of P. pentosaceus ATCC 43201 in inoculated packs using mussels, buffer, LAB, and a C. botulinum spore mixture held under normal (3°C) and abusive (12°C) temperatures. The purpose of these experiments was to determine if LAB could be added to mussel packages as an additional barrier to potential botulinum toxin production.

Millions of packages of MAP-packaged mussels with a variety of MAP gas mixtures and buffer formulas (enhanced oxygen, oxygen and carbon dioxide, with and without a buffer) have been sold in Europe during the past decade, and there have been no reported cases of botulism. For example, in The Netherlands, over 50 million kg of live mussels has been sold annually in 2009 and 2010 in MAP packages (5). C. botulinum toxin was not produced in our studies of fresh, live mussel MAP packages (even when packaged in nitrogen), even under extreme spoilage conditions. In our experiments, we examined gas composition and the composition of the buffer solution (salt and pH) as possible contributing factors, and other researchers (19) mention “unquantified controlling factors,” resulting in the lack of botulism in short shelf life commercial foods that are stored chilled.

Perhaps antimicrobial activity of unheated mussel juice contributes to preventing botulinum toxin production in mussel MAP packages. It is widely known that live bivalve mollusks, such as mussels, clams and oysters, possess strong antimicrobial agents in their hemolymph (6) and extrapallial fluid. These agents include hemocytes and humoral defense factors (lysozymes, bacteriocidins, defensins, mytilins, and myticins), which are active against both gram-negative and gram-positive bacteria (1, 2, 3, 7, 8, 13–15), although no studies to date have evaluated their activity in relation to nonproteolytic C. botulinum spores. This could explain why C. botulinum produced botulinum toxin only in cooked mussel meat (16) (in which the antimicrobial peptides are denatured) but not in fresh mussel meat. Further studies are needed to isolate and validate such a C. botulinum antimicrobial agent from unheated mussel juice or tissue homogenates.

ACKNOWLEDGMENTS

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REFERENCES


