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Short communication

Inability of non-proteolytic *Clostridium botulinum* to grow in mussels inoculated via immersion and packaged in high oxygen atmospheres

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ABSTRACT

A series of botulism challenge studies were conducted to determine if botulinum toxin would be produced in mussels (*Mytilus edulis*) inoculated with non-proteolytic *Clostridium botulinum* spores and held under modified atmosphere (MA) packaging conditions at normal (4 °C) and abusive (12 °C) temperatures. Spore mixtures of six strains of non-proteolytic *C. botulinum* were introduced into live mussels through immersion in a seawater solution with cultured algae. Mussels were packed in a commercial high-oxygen (60–65% O_2) MA-package with a buffer, and also packed under a vacuum. Feeding live mussels cultured algae (10^4 cells/ml) with a *C. botulinum* spore suspension (10^3 spores/ml) in seawater at 4 °C for 6 h resulted in the uptake of spores into mussel tissue (500/g) and the mussel GI tract (100/g). Under all of the experimental conditions evaluated, none of the fresh mussels became toxic, even after spoilage and in the absence of oxygen. However, control samples using tuna or cooked mussel meats became toxic in the absence of oxygen. Botulinum toxin was not produced in fresh mussels packaged under the MA-packaging conditions evaluated, even at an abusive storage temperature (12 °C) for at least 12 days or at normal storage temperate (4 °C) for at least 21 days, which is beyond their shelf life.
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1. Introduction

In 2010, aquaculture production of mussels worldwide approached 2 million metric tons (FAO, 2012), and hermetically sealed, enhanced-oxygen modified atmosphere packaging (MAP) is becoming the preferred method of packing mussels for better quality and extended shelf life (Bernardez and Pastoriza, 2011). While millions of live mussel MA-packages are sold in Europe each year (e.g., greater than 50 million kg/year in the Netherlands, J. Bol, pers. commun.), the package is prohibited in the U.S. due to the potential risk of botulism, which is a serious illness that can be fatal. Foodborne botulism has occurred in a few instances in cooked and home-prepared mussels (Lecour et al., 1988; Hauschild, 1993), and avian type E botulism has been associated with the consumption of fresh water mussels from anoxic sediments by birds (Getchell and Bowser, 2006). Botulism spores have been detected in clams and oysters from the Oregon coast (Craig et al., 1968), but no cases of human botulism have been observed from the consumption of packaged live mussels (not previously cooked and preserved). However, with the exception of studies by Newell et al. (2012), no previous inoculated-pack studies have been reported to determine if *Clostridium botulinum* growth and toxin production can occur in MA fresh live mussel packages under temperature abuse conditions. Such studies are needed to determine if raw (live) mussels packaged under MAP conditions are a potential risk for botulism (United States Food and Drug Administration, 2011). The main objective of this study was to determine if MA-packaged live mussels were prone to the development of botulism as a seafood hazard. In a previous study, inoculated pack studies were performed with live mussels that received uniform inocula of sufficient concentrations of non-proteolytic *C. botulinum* spores throughout the packages (Newell et al., 2012). However, in those studies, spores were introduced externally into the mussel package via a buffer solution. In these studies with the objective of simulating natural contamination of *C. botulinum* spores in mussels (*Mytilus edulis*) to ensure consistent uptake of *C. botulinum* spores in the digestive tracts, feeding studies were performed using cultured algae and a spore suspension in seawater.

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Since no toxin was produced by any live mussels (even the controls) in the previous studies using live mussels (Newell et al., 2012), experiments were performed not only using an enhanced oxygen modified atmosphere, but also under a vacuum using cooked mussel meat and tuna as positive controls. Tuna was included in this study to serve as a control as it is prone to production of botulinum toxin by *C. botulinum* when the conditions are permissive (Lynt et al., 1975). Botulism challenge studies were performed at normal storage (4 °C) and abuse (12 °C) temperatures, with the objective of determining if botulism is a seafood risk in live mussels in an enhanced oxygen MAP package.

2. Materials and methods

2.1. Uptake of C. botulinum spores by mussels using an immersion method

A mixture of 6 non-proteolytic *C. botulinum* strains was used: Beluga (type E), Minnesota (type E), 70 E (type E), 2 B (type B), 17 B (type B), and F202 (type F). A spore crop for each individual strain was prepared according to the method described by Peck et al., 1992. Approximately equal numbers of spores of each strain were mixed to prepare a master stock of 10^8 spores/ml and stored at −20 °C in sterile distilled water. To prepare the inocula, a master stock was diluted in sterile distilled water to a final concentration of ca. 10^6 spores/ml and heat-shocked at 60 °C for 10 min before inoculation into the seawater.

Fresh mussels (*M. edulis*) were shipped overnight to the University of Georgia Center for Food Safety from Great Eastern Mussel Farms in Tenants Harbor, Maine. The mussels were bottom cultured in Maine, and shipped under refrigeration from their wet storage tanks. Upon arrival, mussels were transferred into baskets and submerged in seawater with an air stone aerating in coolers (4 °C) with or without *C. botulinum* spores.

Mussels were submerged in Instant Ocean® (www.Instantocean.com) seawater (control), or Instant Ocean water containing *C. botulinum* spores (final concentration at ca. 1000, 100, and 10 spores/ml) without or with algae (final concentration was 10^4 algal cells/ml). The algae were included to stimulate the natural filtering feeding activity of mussels (Newell et al., 2001). All containers were held at 4 °C and mussels (12 in each group) were sampled at 2, 4, and 6 h after submersion to enumerate *C. botulinum* spores in mussel tissue and mussel GI tracts (=digestive gland).

Mussel inner tissue (MT) and the digestive gland or GI tract (GI) were assayed for *C. botulinum* spore counts. For the MT samples, mussel tissue was aseptically removed from each of 5 mussels and surface-disinfected by washing in a 10% sodium hypochlorite solution for 5 min, followed by rinsing three times with tap water. Mussel tissue from each mussel was dried on sterile paper towels before being weighed, blended in phosphate buffered saline (PBS, 1:5 dilution), and enumerated for presumptive *C. botulinum* spores on McClung-Toabe egg yolk agar plates (Atlas, 2004). For the GI samples, each mussel was dipped into boiling water for 15 s to harden the meat for easy separation of the GI tract from the other tissue. The temperature of the GI content was ca. 45–47 °C, indicating such treatment would not destroy *C. botulinum* spores which may be present. The hardened tissues were removed from each mussel, and the surface was disinfected and rinsed as described above for mussel inner tissue. The GI content was aseptically removed from each mussel's GI tract and weighed, mixed with PBS (1:5 dilution), and enumerated for presumptive *C. botulinum* spores on McClung-Toabe egg yolk agar plates.

2.2. Positive-control study

Precooked, chuck, light tuna in pouches (12 oz. StarKist™ flavor fresh pouch, Starkist.com) was purchased from a local store and the entire content was used in the study. Mussels were handled according to the protocol described above.

Drained mussels were placed briefly (ca. 15 sec.) in previously boiling water for easy removal of mussel tissue from the shells. The mussel meat (removed from briefly boiled shells) was then autoclaved at 121 °C for 30 min to produce the cooked mussel treatments.

The 6-strain mixture of *C. botulinum* spores was heated to 60 °C for 10 min, and then cooled in cold water to room temperature before inoculation. The spore concentration of the mixture was 2.4×10^4 spores/ml.

Mussels were packed in a standard blue 0.8 mm thick polypropylene 1 kg MA-package in two layers per tray, weighing ca. 2 lbs (900 ± 5 g) and a count of ca. 20 mussels per lb, then 35 ml of packaging buffer was added to the package, and the packages were filled with O_2/N_2 (65:35) and sealed with a barrier film under a vacuum (532 mm Hg) using a Koch KATS 400 packaging unit (Koch Equipment Co., Kansas City, Mo.). Tray dimensions were $4.5 \times 23.5 \times 13.35$ cm. In each package, 35 ml of packaging buffer containing 7% NaCl and 0.2% Hamulbac FMC (which contains sodium citrate and citric acid) was prepared with distilled water that was filter-sterilized (0.22 um) and added to the package to prevent odors. Additional details regarding package preparation and sampling have been published previously (Newell et al., 2012).

Fresh mussels containing *C. botulinum* spores in the GI tract content were prepared by two approaches: 1) Flip-flop method (Newell et al., 2012): fresh mussels (ca. 850–860 g) were packed into each tray, then 35 ml of packaging buffer and 5 ml of *C. botulinum* spore mixture were added to the package, and the package was filled with O_2/N_2 (65:35) and sealed by a Koch CATS 400 packaging unit. The mussel packages were held upside down at 12 °C for 5 h before turning them right side up for the remaining overnight storage. Previous studies revealed that fresh mussels would take up some of the spores into their GI tract from the packaging buffer (Newell et al., 2012) if the packages were flipped. Mussels were removed from the packages the next morning and repacked (2/3 of the original mussels in each package) under vacuum using packaging film (used for sealing the trays). One-third of the remaining mussels in each package (12-15 mussels) were used for enumerating *C. botulinum* spores in the tissues for the initial inoculation (day 1) levels. 2) Submersion method: fresh mussels were submerged in seawater containing *C. botulinum* spores (4.4×10^3 spores/ml) overnight for uptake of the spores into their GI tract. The next morning, mussels were drained, then vacuum packaged. A portion (12–15) of the mussels was used to enumerate *C. botulinum* spores in the tissues. Mussel tissues were also removed from fresh mussels containing *C. botulinum* spores in the GI tract content prepared by the submersion method described above and vacuum packaged (ca. 100 g in each bag). For tuna and cooked mussel meat packages, 300 g of meat (either tuna or cooked mussel meat) was packed into each tray and 2.5 ml of *C. botulinum* spore mixture was added to each tray of mussels or tuna meat. Packaging buffer (10 ml) was added to 2 trays of each type of meat that were inoculated with *C. botulinum* spores. The trays were packed under O_2/N_2 (65:35) or N_2, and the packages were sealed by a Koch KATS 400 packaging unit. Another two trays of each type of meat (inoculated with *C. botulinum* spores) were vacuum packaged. All packages (trays and vacuum packs) were held at 12 °C for 2 weeks before sampling. Headspace gas composition of each package was determined using an O_2/CO_2 headspace gas analyzer (Model 6600, Illinois Instruments, Johnsburg, Illinois), and the pH

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