Thermoaciduric *Clostridium pasteurianum* Spoilage of Shelf-Stable Apple Juice

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**ABSTRACT**

*Clostridium pasteurianum* BB, a saccharolytic and spore-forming obligate anaerobe, was isolated and identified from shelf-stable apple juice that was responsible for multiple large spoilage outbreaks. The growth and sporulation conditions of *C. pasteurianum* were atypical compared with those previously published. *C. pasteurianum* spores were heat resistant in apple juice at pH 3.80, with D-values at 80, 85, and 90°C being 34.4, 15.9, and 4.4 min, respectively, and a z-value of 11°C. The survival curves for thermal inactivation obeyed linear first-order kinetics. Apple juice with varying pH values was used to determine the effect of pH on germination capability of *C. pasteurianum* spores. The spores were found to be able to germinate at pH as low as 4.3 in pH-adjusted apple juice at low contamination levels. It was confirmed by PCR that *C. pasteurianum* isolated from spoiled apple juice did not contain the genes for botulinum toxins B and E, which were more commonly found in neurotoxigenic butyric clostridia. Control of finished-juice pH to below 4.0 in combination with mild heating was proposed to prevent potential spoilage of shelf-stable apple juice made with spore-contaminated apple juice concentrate.

*Clostridium pasteurianum* is an anaerobic, saccharolytic, spore-forming microorganism that causes spoilage in acid foods. *C. pasteurianum* produces hydrogen and carbon dioxide gas as a by-product of growth, which results in swollen packages of spoiled foods. It produces butyric acid, which gives an off-odor and off-flavor that is easily detected and unacceptable to consumers.

Spores of *C. pasteurianum* are widely distributed in soil (16). As a result, fruits and vegetables coming in direct contact with soil can be the source of the spores in the finished products, and consequent spoilage of processed food products becomes possible given anaerobic conditions, adequate pH conditions, and inadequate thermal processing. *C. pasteurianum* spoilage has been reported in canned tomatoes, pears, figs (3, 16), pineapples (14), and peaches (2). *C. pasteurianum* is an important spoilage organism in acid foods since it can tolerate high concentrations of sugar and salt at low pH levels (15). However, spoilage caused by *C. pasteurianum* in apple juice has not been reported.

Spoilage by *C. pasteurianum* in apple juice was identified from multiple large spoilage outbreaks of shelf-stable juice that exhibited severely swollen containers. The spoilage resulted in a voluntary recall of the product and a significant economic loss to the juice processor. The outgrowth of *C. pasteurianum* in apple juice resulted in severely swollen bottles and turbid juice with a strong butyric acid odor. The purpose of this study was to determine the decimal reduction time of *C. pasteurianum* spores at different temperatures in apple juice and the ability of spores to germinate at different pH values. The genotype for botulinum toxins that were reported in common saccharolytic butyric anaerobes was also studied. The source of the *C. pasteurianum* spores was found to originate in the imported apple concentrate that was used for the shelf-stable apple juice products.

**MATERIALS AND METHODS**

*Cultures and media.* *C. pasteurianum* BB isolated from spoiled apple juice was grown on potato dextrose agar (PDA; Difco, BD, Sparks, MD, and Criterion, Santa Maria, CA) in anaerobic jars (Difco, BD). The anaerobic jars containing the plates were incubated at 30°C for 3 days. GasPak EZ Anaerobe Container System and a catalyst (Difco, BD) were placed in the jars to produce anaerobic conditions, which were monitored by an anaerobic indicator (Oxoid, Hants, UK).

Isolation and identification of *C. pasteurianum*. *C. pasteurianum* was isolated from 1.89 liters of spoiled apple juice (showing turbidity and high levels of gas production). It was able to grow on saccharolytic media such as PDA under anaerobic conditions but was incapable of growing on media such as cooked meat medium (Difco, BD), anaerobic agar (Difco, BD), and Brewer anaerobic agar (Difco, BD). Phenotypic and genotypic identification of the isolate was performed. Phase-contrast microscopy (model BH-2, Olympus, Tokyo, Japan) was used to study the morphology of *C. pasteurianum* and its spores.

Genomic DNA of *C. pasteurianum* was extracted from cultures on PDA by a phenol-chloroform extraction method (9). The 16S rRNA gene of *C. pasteurianum* was amplified by PCR with primers 16S-F (5'-AGAGTTTGTATCCTACAGCAG-3') and
Potential genotypes for type B and E botulinum neurotoxin (BoNT) of *Clostridium pasteurianum* The primers used to target BoNT/B and BoNT/E. The pH of apple juice was adjusted to pH 3.5 to 9 Brix, titratable acidity equivalent of *C. pasteurianum* spores was added into each bottle by a sterile syringe via the genomic DNA. Primers targeting BoNT/B and BoNT/E were used as a menstruum to determine the decimal reduction time (D-value) of *C. pasteurianum* spores at three different temperatures. %|D|C. pasteurianum|9|Dm|C. pasteurianum|1|9|DC. pasteurianum|9|Dm|DC. pasteurianum (wt/vol) calcium carbonate after 1 month of incubation at 30 °C. The cultures from PDA under anaerobic conditions were sampled and subjected to homologous comparison by the BLAST program at NCBI (11) to determine the identity of the isolate.

Sporulation of *C. pasteurianum* and spore preparation. *C. pasteurianum* was grown on PDA with 0.75% (wt/vol) calcium carbonate (Mallinckrodt, Paris, KY) (2). Petri dishes were placed in anaerobic jars and incubated at 30 °C for 30 days. To harvest the spores, PDA plates were flooded and washed three times with 3 ml of sterile purified and deionized water (Millipore Co., Billerica, MA). The spore suspension was collected and centrifuged at 5,000 × g for 15 min (Sorvall RC-5B superspeed centrifuge, Du Pont Instruments, Newington, CT). Spores were washed in 100 ml of sterile water three times. The vegetative cells were inactivated by soaking the spores in 150 ml of 50% (vol/vol) ethanol at room temperature (22 ± 1 °C) for 60 min. The spores were then washed three additional times in 100 ml of sterile water and finally resuspended in 50 ml of sterile water and kept at 4 °C until use, within 1 month.

Thermal inactivation of *C. pasteurianum* spores. Commercial 100% apple juice (12° Brix, titratable acidity equivalent of 3.68 g of malic acid per liter) made from imported concentrate was used as a menstruum to determine the decimal reduction time (D-value) of *C. pasteurianum* spores at three different temperatures. The apple juice was adjusted to pH 3.80 with the addition of 1 M NaOH or 2 M malic acid. Harvested spores resuspended in sterile water were washed twice in pH-adjusted apple juice prior to performing thermal death kinetic studies. Apple juice inoculated with *C. pasteurianum* spores was transferred to a sterile 1-l syringe (Difco, BD), which was then mounted onto a PB-600 repeating dispenser (Hamilton Company, Reno, NV). The inoculated apple juice was dispensed into capillary tubes with an outside diameter of 1.5 to 1.8 mm, a length of 90 mm, and a thickness of 0.2 mm (Kimble Products, Vineland, NJ). A cumulative 200-μl volume of apple juice was dispensed into five tubes (40 μl per tube). The open ends of the tubes were flame sealed prior to heat treatment. All five tubes of apple juice were heated at 80 and 85 °C for 0 to 60 min with 10-min sampling intervals and at 90 °C for 0 to 18 min with 3-min sampling intervals. The heat treatments were performed in a water bath where the temperature was monitored by a calibrated thermometer. The controls were subjected to all the same treatments except the heating (0 min). After heating, capillary tubes were immediately placed in ice-chilled 70% ethanol and subsequently transferred to 20 ml of 0.1% sterile peptone water. The capillary tubes were crushed using a sterile glass pestle. The mixture was subjected to 10-fold serial dilutions, each of which was surface plated in duplicate onto PDA petri dishes without calcium carbonate. The PDA plates were incubated at 30 °C for 3 days prior to enumeration.

D-values were calculated as the absolute value of the reciprocal of the slope for the linear regression between the logarithm of surviving spores (in CFU per milliliter) and time (in minutes) required for heating at a specific temperature. The z-value was calculated as the absolute value of the reciprocal of the slope for the linear regression between the logarithm of D-value and temperature. To generate statistically sound D-values, heating was performed in independent triplicates for each temperature. Each D-value for a specific temperature was an average of three D-values from independent triplicates whose coefficients of determination were all ≥0.95.

Germination of *C. pasteurianum* spores in apple juice at different pH values. The pH of apple juice was adjusted to pH 3.5 to 4.5 as described above in increments of 0.2. The juice was heated to boiling while being sparged with oxygen-free CO₂. One-hundred-milliliter aliquots of juice were dispensed into 100-ml glass serum bottles, which were sparged with CO₂ and then sealed by rubber stoppers and aluminum crimp seals. Serum bottles with apple juice were autoclaved, and the pH of the apple juice was confirmed after autoclaving. Spores were heat activated at 80 °C for 10 min prior to inoculation. An inoculum of approximately 3 × 10⁵ spores was added into each bottle by a sterile syringe via the alcohol-sterilized septum. Inoculated apple juice was incubated at 25 °C for up to 2 months. The turbidity of the apple juice samples was monitored visually over the time course of the experiment as an indicator for germination and growth of *C. pasteurianum* spores. Germination studies for each pH were performed in triplicate.

Genotypic screening for botulinum neurotoxin genes by PCR. Potential genotypes for type B and E botulinum neurotoxin genes (BoNT/B and BoNT/E), which have been reported to be associated with saccharolytic clostridia (7), were analyzed in *C. pasteurianum* genomic DNA. Primers targeting BoNT/B and BoNT/E were used for PCR under conditions described by Franciosa et al. (5) and Wang et al. (17). The primers used to target BoNT/B gene are B1-a (5'-GATGGAACCTTGCTAG-3') and B2-d (5'-AACATCAATATTTCTCG-3'), B3 (5'-CAG-GAATGTATGGATGTT-3') and B4 (5'-AATACAGGAGACACT-3'), B5 (5'-TGTAAGAATACCTAAATAG-3') and B6 (5'-AAGCACTGACAATATG-3'), and JF-B1 (5'-ATGCCAGTTTACAAATTTTTATTC-3') and JF-B2 (5'-TTCAGCTCTCCTTCTCATTAGG-3'). The primers used for BoNT/E gene include GF-1 (5'-AAAGTCTATATTGATAA-3') and GF-3 (5'-GTTTGATGTACCACTTGGTTGG-3') and KAG165 (5'-CAAGATTACAATGTTGTTATGTTGATCTTTACATGA-3') and KAG166 (5'-CTAAGGCCCTTGGATTTATGACTTTACGCG-3').

RESULTS

Identification and sporulation. *C. pasteurianum* readily grew at 30 °C on saccharolytic media such as PDA. Colonies from 3-day incubation on PDA were large, white, opaque, and mucoid. The surface was shiny and smooth edged. Abundant amounts of gas (hydrogen and CO₂) were produced along with a strong odor of butyric acid. Sporulation was readily achieved on PDA with calcium carbonate after 1 month of incubation at 30 °C. The cultures from PDA under anaerobic conditions when examined with phase-contrast microscopy were found to be long rod-shaped and slightly curved or straight. Spores were oval, terminal, or subterminal, extending the cell terminus.
Under phase-contrast microscope, intracellular polysaccharide granules were observable, especially in vegetative cells. Sequencing of the 16S rRNA gene confirmed the identification of the isolate to be *C. pasteurianum*.

**D- and z-values of *C. pasteurianum* spores.** Although some of the semilogarithmic survival curves for bacterial spores have been reported to have a downward or upward concavity (12), thermal inactivation of *C. pasteurianum* spores at all temperatures appeared to obey first-order kinetics (Fig. 1A through 1C). Therefore, a first-order kinetic reaction was assumed in order to calculate the **D**- and **z**-values. **D**-values of *C. pasteurianum* spores at 80, 85, and 90°C in apple juice at pH 3.80 were determined to be 34.4, 15.9, and 4.4 min, respectively (Table 1). The z-value of *C. pasteurianum* spores was calculated to be 11°C (Fig. 1D). The coefficients of determination (*r*²) were all ≥0.95, indicating sound linear regressions.

**Germination ability of *C. pasteurianum* spores at different pHs.** To determine the germination ability of *C. pasteurianum* spores in apple juice, each pH level was repeated in triplicate. One bottle at pH 4.3 was spoiled after 1 month (Table 2). The pH was lowered to 3.9. At pH 4.5, *C. pasteurianum* spores spoiled two bottles within 1 month. The pH of the two bottles was lowered to 4.0. All spoiled bottles turned turbid with gas production and a butyric odor. However, spores were not able to germinate and grow at a pH range of 3.5 to 4.1.

**Botulinum neurotoxin genes by PCR.** Although neurotoxins have been reported in butyric clostridia, PCRs targeting BoNT/B and BoNT/E genes in the *C. pasteurianum* genome were all shown to be negative, suggesting that *C. pasteurianum* would not pose public health issues due to the presence of botulinum toxin production with cell growth in the juice samples.

**TABLE 1. D-values of *C. pasteurianum* spores at different temperatures**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>D-value (min)</th>
<th>Log D</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>34.44 ± 1.89</td>
<td>1.54</td>
</tr>
<tr>
<td>85</td>
<td>15.86 ± 2.49</td>
<td>1.20</td>
</tr>
<tr>
<td>90</td>
<td>4.36 ± 0.04</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* Each D-value was an average of three individual D-values whose coefficients of determination (*r*²) for log-linear regression were all ≥0.95.

**TABLE 2. Incidence of spore germination in apple juice at different pHs**

<table>
<thead>
<tr>
<th>pH</th>
<th>No. of bottles spoiled/ no. of bottles assayed</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.50</td>
<td>0/3</td>
<td>NA</td>
</tr>
<tr>
<td>3.70</td>
<td>0/3</td>
<td>NA</td>
</tr>
<tr>
<td>3.90</td>
<td>0/3</td>
<td>NA</td>
</tr>
<tr>
<td>4.10</td>
<td>0/3</td>
<td>NA</td>
</tr>
<tr>
<td>4.30</td>
<td>1/3</td>
<td>3.9</td>
</tr>
<tr>
<td>4.50</td>
<td>2/3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Approximately 3 × 10³ spores were inoculated into 100 ml of apple juice. Each assay was repeated in triplicate. NA, not applicable.
DISCUSSION

_C. pasteurianum_ was found to be the causative agent for a large spoilage incident that occurred with shelf-stable apple juice made from concentrate imported from a foreign country. This spoilage outbreak resulted in large economic losses due to the voluntary recall of the product and disposition costs of the finished product and concentrate. Based on the incidence of spoilage in the finished product, the concentrate contained approximately 1 spore per 2 liters. The source of _C. pasteurianum_ was not conclusively identified due to the foreign origin but may be associated with the holding of harvested apples in unprotected ground trenches used for storage prior to juicing and concentrate production. However, the buildup of spores in the facility due to use of spore-contaminated concentrate could be an additional inoculum source for further contamination of the concentrate that does not contain _C. pasteurianum_ spores.

Many semilogarithmic survival curves of bacterial spores have been found to be nonlinear (i.e., the survival curve has upward or downward concavity); therefore, the universal Weibull model was proposed to include nonlinear semilogarithmic survival patterns (12). In this study, inactivation of _C. pasteurianum_ spores indicated a first-order kinetics. Therefore, first-order kinetics was assumed for _C. pasteurianum_ spore inactivation, and the resultant D-values in apple juice at pH 3.8 were determined. The D-values suggest that _C. pasteurianum_ spores are heat resistant even in an acidic environment. Obviously, normal heat processing used to pasteurize apple juice is not sufficient to eliminate _C. pasteurianum_ spores. In addition, _C. pasteurianum_ spores were shown to be able to germinate in apple juice at pH as low as 4.3, suggesting that _C. pasteurianum_ spores are aciduric and can spoil apple juice at a relatively low pH. A pH of 3.8 was used to determine the heat resistance of _C. pasteurianum_ spores because it was the typical pH value of apple juice received from the manufacturer and concentrate distributor. However, the data for D- and z-values at pH 3.80 were a sufficient indicator of the heat resistance of _C. pasteurianum_ spores in apple juice at pH above 3.80.

Food spoilage by _C. pasteurianum_ has been reported previously for other fruit and vegetable juices, but heat resistance and decimal reduction time studies were scarce, partially due to the difficulty in sporulation of _C. pasteurianum_. Bocchi et al. (2) sporulated _C. pasteurianum_ in soil and determined the D-values of _C. pasteurianum_ spores in tomato juice at pH 4.2 and 4.5, in pear puree at pH 3.9, and in peach juice at pH 3.6. They found that D-values of _C. pasteurianum_ spores were relatively higher than those of other butyric clostridia. In addition, the D-values of _C. pasteurianum_ spores were higher than those reported in this study, although different temperatures and pHs were used for their inactivation study. The difference in heat resistance may be due to the different isolation source and sporulation conditions. _C. pasteurianum_ spores were able to germinate and grow in tomato juice, pear puree, and peach juice at pHs lower than 4.5 but rarely below 4.0. Germination at pH 4.0 occurred only at a higher inoculation level of $10^3$ spores per ml (1). This study established the lowest pH for germination of _C. pasteurianum_ spores in apple juice to be around 4.3, with a spore concentration of approximately 30 CFU/ml. This result conforms to the reports that confirmed that at low contamination levels _C. pasteurianum_ spores could not germinate and grow at pH below 4.2 in products other than apple juice (10, 13). Guerrero et al. (6) demonstrated that spoilage of banana puree with a high contamination level of _C. pasteurianum_ spores ($10^7$/ml) could be controlled by adjusting the pH to 3.4, but pH values ranging from 3.4 to 4.0 were not investigated.

Although no evidence has been found to support _C. pasteurianum_ as a neurotoxin producer, we screened our isolate for potential genotypes for neurotoxins. According to the results from PCR targeting for BoNT/B and BoNT/E genes, we conclude that _C. pasteurianum_ isolated from apple juice is not a pathogen that would pose public health concerns. However, spoilage by _C. pasteurianum_ cannot be overlooked since economic loss to industry could be potentially high.

In this study, sporulation of _C. pasteurianum_ was readily achieved on PDA with calcium carbonate. Other alternative media were also tried (data not shown). The liquid medium DM11, which was reported to allow sporulation (8), was not successful for our isolate, however. _C. pasteurianum_ cells did not sporulate well in potato dextrose broth with and without calcium carbonate, or in DM11 with and without calcium carbonate, after 2 months of incubation at 30°C. In general, it was shown that _C. pasteurianum_ spores sporulated better on solid agar media than in liquid media.

The results from this study indicate that _C. pasteurianum_ is a spoilage organism that is heat resistant in acidic environments and capable of growing in apple juice at a pH as low as 4.3. The most common pH for apple juice, however, is below 4.0, at which _C. pasteurianum_ spores may not be able to germinate and grow as shown in this study. The higher pH values of apple juice concentrate could be due to using low-acid varieties of apples and extremely mature fruit after extended storage. The associated spoilage outbreaks in apple juices were possibly due to the reason that the pH of finished apple juice product was close to or even above 4.3 occasionally. Since heat resistance would be even higher at such pH values, thermal processing of apple juice should not be considered as a sole intervention measure. Therefore, the key measure to prevent potential spoilage due to _C. pasteurianum_ in fruit juices is to control the pH of the finished product and maintain the pH below 4.0, provided the contamination level is low. Mild heating in combination with pH control should be performed, especially at higher _C. pasteurianum_ spore contamination levels, to prevent the remaining live spores from germinating.

However, the source of contamination is likely from the soil, occurring when apples are harvested or stored under poor conditions such as unlined dirt trenches. In the case of the _C. pasteurianum_ outbreaks, proactive measures by the concentrate manufacturer were not taken or were not implemented sufficiently to reduce the incidence of fresh
apples directly contacting the soil. Improper pre- and postharvest handling of apples may increase contamination levels of *C. pasteurianum* spores from the soil. Moreover, contamination of the processing facility in plants by contaminated apple juice concentrate and other related products could pose a worse situation wherein cross-contamination may happen since *C. pasteurianum* spores are resistant to heat and chemical sanitizers and are difficult to remove. Proactive measures such as good agricultural practices in the field would be an integral part of a farm-to-table strategy to minimize contamination by pathogens and spoilage organisms such as *C. pasteurianum*. With the increasing U.S. importation of apple juice concentrate and related apple products, global efforts are expected to incorporate good agricultural practices, good manufacturing practices, and hazard analysis critical control point plans by foreign exporting countries to guarantee safe, high-quality, stable finished-juice products.

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**REFERENCES**