Efficacy of Chlorine Dioxide Gas Sachets for Enhancing the Microbiological Quality and Safety of Blueberries

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ABSTRACT

In response to increasingly stringent microbial specifications being imposed by purchasers of frozen blueberries, chlorine dioxide (ClO2) gas generated by a dry chemical sachet was assessed for inactivation of Listeria monocytogenes, Salmonella spp., and Escherichia coli O157:H7 as well as five yeasts and molds known for blueberry spoilage. Fresh blueberry samples (100 g) were separately inoculated with cocktails of L. monocytogenes, Salmonella, E. coli O157:H7 (three strains each), or yeasts and molds (five strains each) to contain ~10⁶ CFU/g and exposed to ClO2 (4 mg/liter, 0.16 mg/g) for 12 h in a sealed 20-liter container (99.9% relative humidity) at ~22°C. After gassing, 25 g of blueberries was added to 225 ml of neutralizing buffer, pulsed for 1 min, and plated using standard procedures to quantify survivors. This treatment yielded reductions of 3.94, 3.62, 4.25, 3.10, and 3.17 log CFU/g for L. monocytogenes, Salmonella, E. coli O157:H7, yeasts, and molds, respectively. Thereafter, 30 lugs of uninoculated blueberries (~9.1 kg per lug) were stacked on 1.2 by 1.2-m pallets (5 lugs per level × six levels), tarped, and exposed to ClO2 (18 mg/liter, 0.13 mg/g) for 12 h. After gassing, significant (P < 0.05) reductions of 2.33, 1.47, 0.52, 1.63, and 0.48 log CFU/g were seen for mesophilic aerobic bacteria, coliforms, E. coli, yeasts, and molds, respectively, compared with non-gassed controls. No significant differences (P > 0.05) in microbial inactivation were seen between lug levels and, with one exception (mesophilic aerobic bacteria), between the bottom and top surface of individual lugs. Based on these findings, ClO2 sachets may provide a simple, economical, and effective means of enhancing the microbial shelf life and safety of blueberries.

Blueberry processing typically begins within 12 to 24 h of harvest with dumping of the berries onto a conveyor belt, followed by removal of leaves, sticks, and other debris; passage through a chlorinated water tank to decrease microbial load and remove any unripened fruit; de-stemming; mechanical inspection and sorting; and boxing for the freezer (24). Like other types of fresh produce, blueberries are prone to microbial contamination during growing, harvesting, and processing (20). Furthermore, current use of 50 to 100 ppm chlorine in the water tank in the blueberry industry does not effectively reduce the microbial load, with substantial growth of survivors occurring during batch freezing.

Microbial safety of fresh produce is an increasing concern with any recall or outbreak negatively affecting the entire industry. In 1984, fresh blueberries were linked to a possible outbreak of listeriosis in Connecticut (17), with an undetermined quantity of frozen blueberries recalled 14 years later from California, Illinois, and Australia, without incident due to contamination with L. monocytogenes (21). In response to these events and a more recent outbreak of hepatitis A in New Zealand in which blueberries were likely contaminated from infected food handlers or fecally contaminated groundwater (2), buyers of frozen blueberries are now beginning to test for foodborne pathogens including L. monocytogenes, E. coli O157:H7, and Salmonella.

Most of the Michigan blueberry crop is processed and frozen for later use in pies and other products rather than marketed as fresh fruit (21). Use of frozen blueberries with high levels of yeast often results in pies that do not “set up” due to enzymatic breakdown of starch and other stiffening agents. In one Michigan study, bacteria, yeasts, and molds isolated from fresh blueberries frequently produced an amylase that facilitated starch hydrolysis, with some isolates also producing pectinase and cellulase. While many buyers of frozen blueberries have now established arbitrary limits for mesophilic aerobic bacteria, E. coli, coliforms, yeasts, and molds, such microbial limits are often difficult to meet since contamination levels vary widely between fields and seasons, depending on rainfall, temperature, insect levels, plant health, and harvest management practices, with microbial populations peaking at the end of the harvest season.

One particularly promising microbial reduction strategy is the use of chlorine dioxide (ClO2) gas that has been shown to effectively reduce foodborne pathogens on inoculated apples (4, 5), green peppers (7, 9, 11), lettuce (14), tomatoes, cabbage, carrots, and peaches (19) as well as blueberries, raspberries (18), and strawberries (10, 18), under laboratory conditions. Hence, two different studies were conducted to assess the efficacy of ClO2 gas for microbial reductions on blueberries: (i) a pilot study in which fresh blueberries were inoculated to contain various foodborne pathogens, spoilage yeasts, or molds and then exposed to
ClO₂ gas for 12 h in sealed 20-liter buckets; and (ii) a pallet study in which 30 lugs of blueberries (~272 kg) were placed on a pallet and exposed to ClO₂ gas for 12 h under a plastic tarp.

**MATERIAL AND METHODS**

**Blueberries.** In the pilot study, fresh blueberries (*Vaccinium corymbosum*) variety Bluecrop were obtained from a local retailer and stored at 4°C for a maximum 2 days, with the fruit tempered for 1 to 2 h at 22°C before inoculation. In the pallet study, fresh mechanically harvested blueberries were obtained through the Michigan Blueberry Growers Association (Grand Junction, Mich.) from the same grower at different field locations. Thirty lugs of uninoculated blueberries (~9.1 kg per lug) were immediately exposed to ClO₂ after stacking on wooden pallets (1.2 by 1.2 m; ~272 kg of fruit per pallet) at a blueberry processing facility to obtain five lugs per level and six levels per pallet.

**Microbial cultures.** Bacterial pathogens included three strains of *E. coli O157:H7* (AR, AD305, AD317), and *L. monocytogenes* (CWD 95, CWD102, CWD184) previously obtained from C. W. Donnelly (Department of Nutrition and Food Sciences, University of Vermont, Burlington) as well as three *Salmonella* serovars (*Salmonella* Typhimurium H 3380, *Salmonella* Heidelberg FS038 BG1, and *Salmonella* Enteritidis H3502) previously obtained from V. K. Juneja (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pa.). All strains were maintained at ~80°C in Trypticase soy broth (TSB; Difuco, Becton Dickinson, Sparks, Md.) containing 10% (vol/vol) glycerol. Individual strains were separately activated by transferring a loop of frozen stock culture into 9 ml of sterile TSB containing 0.6% (wt/vol) yeast extract (TSBYE; Difuco, Becton Dickinson), followed by 18 to 24 h of incubation at 35°C, and then subjected to an identical transfer in 20 ml of TSBYE before use.

Five spoilage molds (*Colletotrichum sp.*, *Epicroccum sp.*, *Cladosporium sp.*, *Phoma sp.*, and *Alternaria sp.*) and yeasts (*Aureobasidium sp.*, *Bullera sp.*, *Cryptococcus sp.*, *Sporidiobolus sp.*, and *Filobasidium sp.*) originally isolated from blueberry fields in Michigan were obtained from A. C. Schilder (Department of Plant Pathology, Michigan State University, Lansing). All yeasts and molds were maintained at ~80°C in TSB containing 10% (vol/vol) glycerol, and separately activated by transferring a loop of frozen stock culture onto duplicate plates of potato dextrose agar (PDA; Difuco, Becton Dickinson) containing 20 ppm streptomycin (Sigma-Aldrich Co., St. Louis, Mo.) and 50 ppm ampicillin (Sigma-Aldrich) (PDA-SA), with the yeasts and molds incubated 3 to 4 and 10 to 12 days at 26°C, respectively, before use.

**Preparation of inocula.** Cultures of *E. coli O157:H7*, *L. monocytogenes*, and *Salmonella* were harvested by centrifugation (Sorvall Super T21, Newtown, Conn.) at 7,000 x g for 10 min at 4°C and resuspended in equal volumes of sterile phosphate-buffered saline (PBS). Suspensions of each strain containing approximately equal populations (~10⁶ CFU/ml) were combined to produce three separate three-strain cocktails (~60 ml each) of *E. coli O157:H7*, *L. monocytogenes*, and *Salmonella*. Populations in these cocktails were determined by plating appropriate PBS dilutions on sorbitol MacConkey Agar (SMAC; Difuco, Becton Dickinson), modified oxford agar (MOX; Difuco, Becton Dickinson), and MacConkey agar (MAC; Difuco, Becton Dickinson), respectively. Two separate five-strain cocktails of yeasts and molds containing approximately equal populations (~10⁶ CFU/ml) were obtained by washing each of the five previous yeast or mold PDA-SA plates with 20 ml of PBS, and then combining the rinsates to obtain two 100-ml suspensions. Yeast and mold populations in these cocktails were determined by surface plating appropriate serial dilutions on PDA-SA, followed by 3 to 4 and 10 to 12 days of incubation at 26°C, respectively.

**Inoculation of blueberries.** For the pilot study, five 125-g blueberry samples were placed in separate sterile polyethylene bags (25 by 20 cm; Whirl-Pak, Fisher Scientific, Pittsburgh, Pa.) containing each of the five previously prepared microbial suspensions at ~22°C, and then shaken 20 min at 100 rpm on a G2 Gyromatic Shaker platform (New Brunswick Scientific, Edison, N.J.). These inoculated blueberries containing ~10⁶ CFU/g of *E. coli O157:H7*, *L. monocytogenes*, *Salmonella*, yeasts, or molds were then air dried under laminar flow in a biosafety cabinet for 2 h, stored overnight at 4°C, and re-dried under laminar flow for 2 h before use.

**ClO₂ exposure.** In the pilot study, five separate half-pint plastic clamshell containers each containing 100 g of inoculated blueberries were placed on a metal rack inside a sealed 20-liter bucket and exposed to ClO₂ gas (4 mg/liter, 0.16 mg/g berries) for 12 h at ~22°C and 99.9% relative humidity (RH). ClO₂ gas was generated by placing a 20-g commercial ClO₂ sachet (ICA TriNova, LLC Forest Park, Ga.) below the rack on the bottom of the bucket with a brushless 12 VDC fan (5 by 5 by 1 cm; model D50SM, RadioShack, Fort Worth, Tex.) attached to the underside of the bucket lid for gas circulation. An RH of 99.9% at 22°C was maintained by placing a petri dish containing 20 ml of sterile distilled water on the bottom of the bucket with the temperature and RH continuously monitored, using a thermohygrometer (Model Traceable, Fisher Scientific) that was sealed into the bucket lid. All gassing experiments were conducted in triplicate, using blueberries that were purchased on different days.

In each of six pallet experiments, 30 lugs of fresh uninoculated blueberries (~9.1 kg of fruit per lug) were stacked on each of two pallets measuring 1.2 by 1.2 m (5 lugs per level × six levels). Three 3-kg ClO₂ generating sachets (ICA TriNova) were then placed on the top lugs of one pallet, with two 2-m-long flexible hoses (10-cm diameter) containing ventilating fans that were installed along opposite sides of the pallet from top to bottom for gas circulation. Immediately after activating the ClO₂ sachets, both pallets were tarped with a plastic sheet, sealed at the bottom, and held for 12 h at ~12 to 14°C, with the second pallet serving as the ungassed control. According to the manufacturer, the three 3-kg sachets generated a total of 36,000 mg of ClO₂ gas in each 2,000-liter pallet containing ~273 kg of blueberries, giving an estimated final ClO₂ concentration of 18 mg/liter or 0.13 mg/g of fruit.

**Sample collection and microbial analyses.** In the pallet study, blueberry samples were obtained from each of the 30 lugs per pallet before and after 12 h of gassing. Five samples (~20 g each) were collected from the top and bottom of each of the five lugs per level: one sample from the center and one sample from each of the four corners (Fig. 1). These 25 top and 25 bottom samples per level were then composited to obtain one 100-g top and one 100-g bottom sample for each of the six pallet levels. All samples were plated in clamshell containers that were individually bagged, transported to the laboratory on ice, and analyzed within 24 h of collection.

Blueberry samples from the pilot (25 g) and pallet studies (100 g) were respectively placed in sterile 20 by 10-cm or 25 by 20-cm Whirl-Pak bags containing 100 or 200 ml of neutralizing buffer (Difuco, Becton Dickinson). The sealed bags were shaken.
horizontally for 20 min at 100 rpm on a G2 Gyroratory Shaker platform, and then pulsed for 1 min using a pulsifier (Filtaflex Ltd., Almonte, Ontario, Canada). In the pilot study, 1-ml aliquots were serially diluted in PBS and spiral plated (Autoplate 4000, Spiral Biotech, Exotech, Inc., Gaithersburg, Md.) in duplicate on SMAC, MOX, MAC, and PDA-SA to enumerate *E. coli* O157: H7, *L. monocytogenes, Salmonella*, and yeasts and molds, respectively. SMAC, MOX, and MAC plates were counted after 48 h of incubation at 37°C, whereas PDA-SA plates were counted after 72 to 96 h of ambient incubation at ~22°C. In the pallet study, appropriate dilutions in PBS were spiral plated in duplicate on Trypticase soy agar (Difco, Becton Dickinson) containing 0.6% (wt/wt) yeast extract and 100 ppm cyclohexamide (TSAYE-C; Sigma-Aldrich) for enumeration of mesophilic aerobic bacteria (MAB), and PDA-SA for enumeration of yeasts and molds. Additional 1-ml aliquots were plated on Petrifilm *E. coli*Coliform Count (EC) Plates (3M Corp., St. Paul, Minn.) for quantitation of coliforms and *E. coli*. TSAYE-C and EC plates were counted after 48 h of incubation at 37°C, whereas PDA-SA plates were counted after 72 to 96 h of ambient incubation at 22°C.

**Sensory analysis.** Uninoculated blueberries (variety Bluecrop) from Grand Junction, Mich., were exposed to 0.19 mg of ClO₂ gas per g of fruit for 12 h at 22°C and 99% RH as in the pilot study. Gassed and ungassed (control) blueberries were rinsed in tap water and air dried for 1 h, after which 15 to 20 berries were dispensed into individual sample cups with lids. Following overnight storage at 4°C, sets of coded samples were evaluated by 110 untrained panelists for appearance, aroma, texture, flavor, and overall acceptability using a 9-point hedonic scale that ranged from “like extremely” (9) to “dislike extremely” (1).

**Statistical analysis.** All microbial count data from the pilot and pallet studies were analyzed by analysis of variance, using the Statistical Analysis System (SAS, version 8, SAS Institute, Inc., Cary, N.C.). Data are means from replicate experiments with significance between means determined using the least-significant difference test at the 95% confidence level (*P* = 0.05). All sensory data were analyzed with a SIMS 2000 computer software program at a significance level of *P* < 0.05.

**RESULTS AND DISCUSSION**

Blueberries were surface inoculated with the target organisms by dipping to more closely mimic contamination during mechanical harvesting and processing. In preliminary work, pulsification by ultrasound after surface washing yielded greater recovery than did stomaching, without rupturing the fruit and decreasing the pH to <4.0, which could be detrimental to any cells injured during ClO₂ exposure. When Sy et al. (18) compared washing and stomaching for recovery of *Salmonella* from inoculated blueberries, populations were 10-fold lower after stomaching. However, several years earlier the same laboratory (1) reported comparable recovery of *Salmonella* from surface-inoculated blueberries by shaking, stomaching, and homogenizing in a mechanical blender.

In the pilot study, initial populations of *L. monocytogenes, Salmonella, E. coli* O157:H7, yeasts, and molds on inoculated blueberries before treatment were 6.46, 6.38, 6.35, 5.99, and 6.22 log CFU/g, respectively. Significant (*P* < 0.05) reductions of 3.94, 3.62, 4.25, 3.10, and 3.17 log CFU/g were achieved for *L. monocytogenes, Salmonella, E. coli* O157:H7, yeasts, and molds, respectively, after exposure the inoculated fruit to 4 mg/liter (0.16 mg/g fruit) ClO₂ gas in a sealed bucket for 12 h at 22°C and 99.9% RH (Fig. 2), with high RH maintained to enhance the efficacy of ClO₂ gas as also reported by Han et al. (8). According to an earlier study by Sy et al. (18), exposing *Salmonella*-inoculated blueberries to 4.1 to 8 mg/liter ClO₂ gas and 75 to 95% RH for 30 to 120 min reduced *Salmonella* populations up to 3.7 log, with inherent yeasts and molds decreasing as much as 2.5 log.

Efficacy of ClO₂ gas has been examined for other types of fresh produce, with *L. monocytogenes* and *E. coli* O157:H7 populations reportedly decreasing 3.5 and 6.4 log on the surface of injured green peppers after a 30-min exposure to 3 and 1.2 mg/liter ClO₂ gas, respectively (7, 9, 11). When the skin, calyx, and stem scar areas of apples were surface inoculated with *L. monocytogenes* and exposed to 4 mg/liter ClO₂ gas for 10 min at 21°C and 90% RH, Du et al. (4) obtained higher reductions of 5.5, 3.2, and 3.6 log, respectively. Variation in the surface structure and microbial attachment sites between green peppers, apples, and blueberries likely account for the observed differences in biocidal efficacy. Waxes on the smooth surface of apples contain alcohols, morpholine, and surfactants that may enhance the penetration of sanitizers resulting in higher microbial reductions (13). However, β-diketone—the dominant naturally occurring wax on the blueberry surface—produces a dense network of interlocking branched rodlets

![FIGURE 1. Blueberry pallet with six levels and five lugs per level (left) and lug (right). Every lug was sampled at five points on the top (shown) and bottom (not shown).](image)

![FIGURE 2. Populations of L. monocytogenes, Salmonella, E. coli O157:H7, yeasts, and molds (mean ± SD, n = 3) on inoculated blueberries before and after exposure to 4 mg/liter, 0.16 mg/g ClO₂ gas in a sealed 20-liter bucket for 12 h at 22°C and 99.9% RH. Values with different letters within the same microbial category are significantly different (P < 0.05).](image)
FIGURE 3. Populations of MAB, yeasts, molds, coliforms, and E. coli (mean ± SD, n = 6) recovered from blueberries initially (0 h) and from gassed and ungassed fruit after 12 h of storage. Blueberry pallets containing fruit harvested from the same grower were stored under the same conditions for 12 h at 12 to 14°C. Gassed pallets were exposed to 18 mg/liter (0.13 mg/g) ClO2 gas. The limits of detection were 1.78 (MAB, yeast, mold) and 0.48 (coliforms, E. coli) log CFU/g. Values with different letters within the same microbial category are significantly different (P < 0.05).

or closed, tubelike structures (6) that may lead to increased microbial attachment, with the hydrophobicity of β-diketone also hindering penetration of aqueous-based sanitizers, leading to decreased efficacy.

Blueberry quality and safety are greatly impacted by the methods of harvesting, storage, and processing with populations of bacteria, yeasts, and filamentous fungi increasing considerably from the first to the second harvest. According to NeSmith et al. (15), machine harvesting caused the greatest loss in fruit firmness (20 to 30%), followed by grading and sorting, with 24 h of postharvest ambient storage decreasing blueberry firmness by only 3 to 8% as compared with cooling the fruit immediately. Loss of firmness leading to blueberry decay during postharvest holding remains one of the industry’s biggest concerns, with postharvest fungi capable of invading the berries and increasing the pH from 3.5 to levels that permit the growth of bacterial pathogens and spoilage organisms. When Jackson et al. (12) assessed the quality of blueberries during storage, microbial populations increased ~1 log when either processing was delayed for up to 45 h or when the prepacking temperature was increased to 26°C, as is typical for pallets of blueberries. In most blueberry-processing operations, the sole microbial reduction step involves brief passage of the berries through a tank of chlorinated sanitizer solution, with a 5-min exposure to 100 ppm chlorine reportedly reducing populations of bacteria, yeast, and mold on blueberries by only 0.83, 0.77, and 0.61 log CFU/g, respectively (3). Hence, given these concerns the efficacy of ClO2 was assessed as a microbial reduction strategy for pallets of blueberries waiting to be processed.

In the pallet study, initial mean populations of MAB, coliforms, E. coli, yeasts, and molds on naturally contaminated untreated blueberries were 4.03, 1.12, 0.51, 4.32, and 4.57 log CFU/g, respectively (Fig. 3). After 12 h of storage, significant (P < 0.05) microbial growth was observed in the ungassed pallets with populations of MAB, yeasts, molds, coliforms, and E. coli increasing 0.69, 0.36, 0.11, 1.00, and 0.50 log, respectively. However, after 12 h of storage under the same conditions, significant (P < 0.05) reductions of 2.33, 1.63, 0.48, 1.47, and 0.52 log CFU/g, respectively, were seen between the gassed and ungassed pallets. Overall, reductions for bacteria, yeasts, and molds were 1 to almost 3 log higher on inoculated as compared with naturally contaminated fruit. Reasons for the observed decrease in ClO2 efficacy may be related to the use of uninoculated blueberries in the pallet study with microorganisms more heavily imbedded in the wax layer and the lower ClO2 concentration in the pallet (0.13 mg/g) as opposed to the pilot study (0.16 mg/g). Populations of naturally occurring yeasts and molds were less susceptible to ClO2 than were the bacteria. These findings agree with those of Rodgers et al. (16), who reported reductions of up to 5 log CFU/g for L. monocytogenes and E. coli O157:H7 as compared with 1.5 log CFU/g for yeasts and molds when apples were treated with aqueous solutions of 3 and 5 ppm ClO2. The very low initial populations can explain the lower reductions for coliforms and E. coli on gassed uninoculated blueberries, with numbers decreasing below the limit of detection (0.48 log CFU/g) after gassing.

Uniformity in the reduction of MAB, yeasts, molds, coliforms, and E. coli on palletized blueberries at different pallet levels (Fig. 4) and sample locations within the lugs (Fig. 5), using ClO2 gas was also evaluated. After gassing, no significant differences (P > 0.05) in microbial load were seen between pallet levels 1 to 6 and, except for MAB, between the top and bottom surface of the lugs. Furthermore, no interactive effects were observed between different pallet levels and locations within the lugs, thus demonstrating uniform dispersion and penetration of ClO2 gas throughout the pallets. Regarding quality of the gassed blueberry pallets, no visible changes in the fruit were evident; however, some bleaching of the blueberry leaves was observed.

Based on results from sensory analyses obtained after overnight storage at 4°C, no significant (P < 0.05) differences in appearance, aroma, texture, flavor, or overall acceptability were observed between blueberries exposed to...
FIGURE 5. Populations of MAB, yeasts, molds, coliforms, and E. coli (mean ± SD, n = 6) recovered from the top and bottom surface of lugs from the same pallet level (B). Blueberry pallets were stored under the same conditions at 12 to 14°C, exposed to 18 mg/liter (0.13 mg/g) ClO2 gas, tarped, and sealed for 12 h. The limits of detection were 1.78 (MAB, yeast, mold) and 0.48 (coliforms, E. coli) log CFU/g. Values with different letters within the same microbial category are significantly different (P < 0.05).

0.19 mg/g ClO2 gas for 12 h and ungassed fruit (results not shown). Sy et al. (18) also reported no significant changes in sensory attributes including appearance, color, aroma, and overall quality between blueberries exposed to 4.1 mg/liter ClO2 gas and ungassed blueberries after 0 and 3 days of storage, with the treated samples ranked significantly higher for overall quality and aroma after 7 and 10 days of storage, respectively.

Since the terrorist events of 2001, ClO2 has received increased attention as a decontamination method for buildings with the U.S. Food and Drug Administration also having since approved the use of several ClO2-generating packaging materials for fresh fruits and vegetables (22). Hence, gassing pallets of blueberries that will not be processed until the following day appears to be a useful means for enhancing the microbial quality of blueberries and better meeting the increasingly stringent microbial standards being imposed by buyers of frozen berries, with ClO2 sachets and the newly marketed ClO2-generating packaging films also likely amenable for fresh marketed blueberries.

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