

Critical Factors Affecting the Destruction of *Escherichia coli* O157:H7 in Apple Cider Treated with Fumaric Acid and Sodium Benzoate

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ABSTRACT: Destruction of *Escherichia coli* O157:H7 in apple cider treated with fumaric acid and sodium benzoate (0.15% and 0.05% w/v, respectively) was determined under pH and storage temperatures that commonly occur in apple cider. At 5 °C storage, while destruction of *E. coli* O157:H7 in the presence of preservatives increased with time, there was little decline in *E. coli* O157:H7 populations in the absence of the preservatives. Increasing storage temperatures to 15 °C and 25 °C significantly increased the rate of destruction of *E. coli* O157:H7 in cider with the preservatives ($P < 0.05$). Increasing the pH of cider (from 3.2 to 4.7) decreased the rate of destruction of *E. coli* O157:H7.

Keywords: *Escherichia coli* O157:H7, apple cider, preservatives, fumaric acid, storage

Introduction

DU TO ITS ACID TOLERANCE AND LOW INFECTIOUS DOSE (10 TO 2000 CFU/g), *Escherichia coli* O157:H7 is a serious hazard (Bolton and others 1996; Lin and others 1996) that threatens the safety of unpasteurized fruit and vegetable beverages. Unpasteurized apple cider/juice contaminated with *E. coli* O157:H7 has been implicated in several outbreaks of foodborne illness (Besser and others 1993; CDC 1996; CDC 1997). The acid tolerance of *E. coli* O157:H7 enables it to be recovered in apple cider (pH 3.6 to 4.0) 3 wk after inoculation (Zhao and others 1993; Miller and Kaspar 1994). As a result of both the number and severity of outbreaks associated with *E. coli* O157:H7 in apple cider, the Food and Drug Administration (FDA) concluded that safety controls were necessary. In 2001, the FDA issued its final ruling, mandating the application of Hazard Analysis and Critical Control Point (HACCP) principles and sanitation standard operating procedures (SSOPs) to operators processing fruit and vegetable juices (FDA 2001). As part of the HACCP system, processors, regardless of operation size and capacity, must reduce a pertinent foodborne pathogen in the expressed juice by at least 100000-fold (5 log).

Previous study by Comes and Beelman (2002) demonstrated that fumaric acid in combination with sodium benzoate was an excellent preservative treatment that can increase the safety and prolong the shelf life of apple cider. The researchers demonstrated a 5-log reduction of *E. coli* O157:H7 in apple cider by adding a fumaric acid (0.15% w/v) and sodium benzoate (0.05% w/v) preservative mixture to the apple cider, followed by holding the cider at 25 °C for at least 6 h. When the holding temperature was increased to 35 °C, no *E. coli* O157:H7 populations were recovered after 3 h. In contrast, the control ciders (without fumaric acid and sodium benzoate) showed little reduction of *E. coli* O157:H7 populations even after 24 h at 25 °C. The authors also demonstrated that a fumaric acid (0.15% w/v)/sodium benzoate (0.05% w/v) mixture was more effective in reducing *E. coli* O157:H7 populations than a combination of fumaric acid (0.15% w/v) and potassium sorbate (0.05% w/v). Addition of sodium benzoate (0.05% w/v) and potassium sorbate (0.05% w/v), a common commercial formulation, reduced populations by only 1.7 logs after 4 h at 25 °C followed by further storage at 4 °C for 48

h. This reduction was significantly less ($P < 0.05$) than the reduction observed when fumaric acid (0.15% w/v) was added alone (2.2 logs). Based on the results of a consumer sensory panel (at least 70 panelists) evaluating apple cider with fumaric acid (0.15%) and sodium benzoate (0.05%), the authors (Comes and Beelman 2002) concluded that “panelists found the preservative-treated ciders to have an overall consumer rating between ‘like slightly’ and ‘like moderately’ (early season cider) and ‘neither like nor dislike’ and ‘like slightly’ (late season cider) on a 9-point hedonic scale. Comments of panelists seemed to indicate that some panelists reacted to the tartness imparted by fumaric acid (0.15% w/v) and sodium benzoate (0.05% w/v), which was probably more apparent when tasted in comparison to cider with no added preservatives than might be the case if tasted alone.”

Cider characteristics like °Brix and pH vary depending on the apple-growing location and apple varieties used. Since acid preservatives are normally less effective at higher pH values, it was deemed important to evaluate the fumaric acid/sodium benzoate treatment on ciders made from locations that produced less acidic, higher pH cider. Therefore, the first objective of this study was to validate the findings of Comes and Beelman (2002) with commercial apple ciders obtained from different locations in the United States. Though the fumaric acid/sodium benzoate preservative treatment has potential to be used in commercial formulations, the efficacy of this treatment was previously not studied at refrigeration temperatures. It must be noted that regulations require apple cider manufacturers to hold their cider at ≤ 3.33 °C (38 °F) at all stages of storage, transport, and retail. Therefore, the second objective was to study the destruction of *E. coli* O157:H7 in apple cider treated with 0.15% (w/v) fumaric acid and 0.05% (w/v) sodium benzoate under a range of storage temperatures and pH values that commonly occur in apple cider.

Materials and Methods

Apple cider

Fresh, unpasteurized, unpreserved apple cider was obtained from a commercial apple-cider producer (PA 1) in Pennsylvania. The cider was stored frozen (−18 °C) in 1-gallon plastic containers

until further use. One day prior to each experiment, 1-gallon containers were removed from the freezer and allowed to thaw at 22 °C for 24 h. Apple cider used in these experiments had an average pH of 3.6 ± 0.1 and an average °Brix of 11.7 ± 0.5 . The pH and °Brix of the commercial ciders used in the validation study are shown in Table 1.

Bacterial strain

Escherichia coli O157:H7 (SEA 13889), an isolate of the 1996 Odwalla apple juice outbreak, was obtained from The Pennsylvania State Univ., Dept. of Food Science Stock Culture Collection (University Park, Pa., U.S.A.). The culture was stored at 4 °C on nutrient agar (Difco Laboratories, Inc., Detroit, Mich., U.S.A.) slants. Fresh cultures were prepared every 2 mo from either lyophilized cultures (−60 °C; 50:50 glycerol:water) or from slant transfers.

Escherichia coli O157:H7 inoculum was prepared by streaking a pre-poured tryptic soy agar (Difco) with 0.6% yeast extract (Difco, TSAYE) plate from a nutrient agar slant, followed by incubation for 24 h at 37 °C. An isolated colony was transferred from the TSAYE plate into 10 mL of sterilized nutrient broth (Difco) prior to static incubation for 24 h at 37 °C. A 1.0-mL aliquot of inoculated and incubated broth was transferred into 99 mL of tryptic soy broth (Difco) with 0.6% yeast extract (Difco, TSBYE) and statically incubated for 20 h at 37 °C, producing stationary-phase populations. This suspension was centrifuged at $8000 \times g$ for 20 min and the pellet was resuspended in 10 mL of a 0.85% NaCl (Fisher Scientific, Inc., Pittsburgh, Penn., U.S.A.) solution to yield a final suspension containing approximately 5×10^9 CFU/mL. This suspension was serologically tested for *E. coli* O157 antibodies using Latex Agglutination (Oxoid, Ltd., Hampshire, England) prior to inoculation into the apple cider.

Validation of the destruction of *E. coli* O157:H7 in commercial apple ciders

Commercial apple cider was obtained from Tennessee, Georgia, Alabama (source: Dr. William Morris, Univ. of Tennessee, Knoxville) and 2 production sites in Central Pennsylvania (PA 1 and PA 2). The pH and °Brix of the cider samples ranged from 3.40 to 3.87 and 11.2 to 16.0, respectively (Table 1). Three hundred milliliters of each commercial cider was aseptically added into individual 1-L sterile flasks. Fumaric acid (0.15% [w/v]) (Mallinckrodt Baker, Inc., Paris, Ky., U.S.A.) and sodium benzoate (0.05% [w/v]) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were added to each commercial cider sample. The cider was magnetically stirred for 20 min to allow dissolution of the preservatives. In all cases, addition of the preservative mixture caused the pH of the cider to decrease by at least 0.2 pH units (Table 1). Aliquots (99 mL) of each cider sample were placed into individual 250-mL Erlenmeyer flasks. The flasks were immersed in a water bath set at 25 °C 30 min prior to inoculation, thereby allowing the cider to adjust to the temperature of the bath. The water level of the bath was at least 1.5 inches above the cider level in the flasks. One milliliter of the *E. coli* O157:H7 inoculum was added into each of the flasks and then magnetically stirred for 15 s for the inoculum to mix thoroughly before returning to the water bath. The flasks were magnetically stirred for 15 s prior to sampling for enumeration at the following times: 0, 2, 5, 9, 21, and 24 h. pH of the apple ciders was measured at the end of the holding time (24 h).

Effect of fumaric acid and sodium benzoate on the destruction of *E. coli* O157:H7 at 5 °C

Two 1-L batches of apple cider (source: PA 1) were aseptically added into two 3-L sterile flasks. The control flask contained no

Table 1—pH and ° Brix values of commercial apple cider samples obtained from five locations in the United States and pH of samples after addition of fumaric acid and sodium benzoate (0.15% and 0.05%) preservative treatment.

	TN ^a	GA ^b	AL ^c	PA 1 ^d	PA 2 ^e
pH ^f	3.87	3.40	3.81	3.56	3.52
° Brix	11.20	11.50	16.0	12.20	13.0
pH ^g	3.41	3.19	3.44	3.37	3.27

^aTennessee

^bGeorgia

^cAlabama

^dPennsylvania: Processor 1

^ePennsylvania: Processor 2

^fnatural cider-pH value

^gcider pH following the addition of fumaric acid/sodium benzoate (0.15%/0.05%, w/v)

added preservatives. The treatment flask contained 0.15% (w/v) fumaric acid (Mallinckrodt Baker) and 0.05% (w/v) sodium benzoate (Sigma). The apple cider was magnetically stirred for 20 min to allow complete dissolution of the preservatives. pH of the apple cider before and after the addition of the preservatives was 3.5 and 3.2, respectively. pH of the control (cider with no preservatives) was adjusted to 3.2 using a 5 M HCl solution (Fisher). Ninety-nine milliliter aliquots of control and treated cider were placed into 250-mL Erlenmeyer flasks. The samples were then placed at 5 °C in a programmable microprocessor-controlled temperature water bath (Model 221, Neslab Instruments, Portsmouth, N.H., U.S.A.) capable of having a temperature stability of ± 0.01 °C. The flasks were immersed in the water bath 30 min prior to inoculation, thereby allowing the cider to adjust to the temperature of the bath. The water level of the bath was at least 1.5 inches above the cider level in the flasks. One milliliter of the *E. coli* O157:H7 inoculum was added into each of the flasks and then magnetically stirred for 15 s for the inoculum to mix thoroughly before returning to the water bath. Flasks were stirred for 15 s prior to sampling for enumeration at the following times: 0, 2, 4, 5, 6, 8, 10, 24, 72, and 220 h. pH of the apple ciders was measured at the end of the holding time (220 h).

Effect of apple cider storage temperature (5 °C, 15 °C, 25 °C) on the destruction of *E. coli* O157:H7

One liter of apple cider (source: PA 1) containing 0.15% (w/v) fumaric acid (Mallinckrodt Baker) and 0.05% (w/v) sodium benzoate (Sigma) was prepared as previously described. Initial pH of the apple cider was 3.5. pH after addition of the preservatives was 3.2. Ninety-nine milliliter aliquots of cider were placed into 250-mL Erlenmeyer flasks. To precisely control the temperatures, the flasks were immersed in controlled temperature water baths previously described, set at 5 °C, 15 °C, or 25 °C. Cider inoculation and sampling were conducted as previously described. Sampling for enumeration of *E. coli* O157:H7 was performed at the following times: 0, 4, 8, 12, 24, 28, 32, 36, 52, and 60 h at 5 °C; 0, 2, 4, 8, 12, 24, 28 h at 15 °C; and 0, 1, 2, 4, 6, 8 and 12 h at 25 °C. pH of the apple ciders was measured at the end of the holding time in each case.

Effect of apple cider pH on the destruction of *E. coli* O157:H7

2.5 L of apple cider (source: PA 1) were aseptically added into a 3-L sterile flask with 0.15% (w/v) fumaric acid (Mallinckrodt Baker) and 0.05% (w/v) sodium benzoate (Sigma), and was magnetically stirred for 20 min to allow complete dissolution of the preservatives. Initial pH of the apple cider was 3.5. pH after addition of the preservatives was 3.2. Adjustment of cider pH was performed using a 10 M NaOH (Fisher) solution to pH 3.5, 3.8, 4.1, 4.4, and 4.7. Following pH adjustment, 25-mL aliquots of the cider samples were

Table 2—Survival of *E. coli* O157:H7 in commercial apple cider samples stored at 25 °C with or without addition of the fumaric acid/sodium benzoate (0.15% / 0.05%) preservative treatment.

Storage time (h)	<i>E. coli</i> O157:H7 population (log CFU/mL)									
	Tennessee		Georgia		Alabama		PA 1		PA 2	
	Ctrl*	F/B ^a	Ctrl*	F/B ^a	Ctrl*	F/B ^a	Ctrl*	F/B ^a	Ctrl*	F/B ^a
0	7.60	7.60	7.60	7.60	7.60	7.60	7.60	7.60	7.60	7.60
2	7.59	7.43	7.70	7.08	7.61	7.38	7.59	7.50	7.75	7.15
5	7.55	4.70	7.68	3.04	7.69	6.18	7.61	5.62	7.74	3.07
9	7.66	3.04	7.70	Nd ^b	7.66	Nd ^b	7.66	Nd ^b	7.72	Nd ^b
21	7.62	Nd ^b	7.68		7.59		7.50		7.65	

*Control samples.

^a0.15% fumaric acid/0.05% sodium benzoate.^bNd, not detectable (<1.4 log).

placed into wide-diameter test tubes (27 × 142 mm). The tubes were sealed with rubber stoppers and immersed in a water bath described above, set to 5 °C. The test tubes were placed in the water bath 30 min prior to inoculation, thereby allowing the cider to adjust to the temperature of the water bath. The water level of the bath was at least 1.5 inches above the cider level in the tubes. 0.25 mL of the *E. coli* O157:H7 inoculum was added into each of the tubes containing 25 mL of apple cider. The inoculated cider was stirred using a vortex mixer before being put back into the water bath. The tubes were vortex mixed for at least 5 s prior to sampling for enumeration. pH of the apple ciders was measured at the end of the holding time.

Enumeration of *E. coli* O157:H7. Inoculum levels for all experiments were determined by serially diluting the inoculation suspension in buffered peptone water (Difco). One-tenth milliliter aliquots were spread plated onto nonselective phenol red agar (Difco) plus 1.0% (w/v) sorbitol (Sigma) plates (PRSA), followed by incubation at 37 °C for 24 h. *Escherichia coli* O157:H7 colonies appeared pinkish-red with no zone of yellow clearing. Final *E. coli* O157:H7 counts were performed using the same method, with samples taken directly from apple cider prior to being serially diluted in buffered peptone water (Difco) and spread plated onto PRSA plates. Several colonies of presumptive *E. coli* O157:H7, as well as any questionable colonies, were serologically tested for *E. coli* O157 antibodies using latex agglutination (Oxoid, Ltd.) and Gram staining.

Statistical analysis

Each treatment was examined in triplicate. Analysis of variance and mean separation using Tukey's Honest Significant Differences were conducted on log reduction data of *E. coli* O157:H7 using Minitab (Minitab, Inc., State College, Penn., U.S.A.). Microsoft® Excel (Microsoft Corporation, Cambridge, Mass., U.S.A.) was used to generate trend lines and coefficients of determination when required.

Results and Discussion

IN THE EXPERIMENT CONDUCTED WITH COMMERCIAL CIDERS OBTAINED from different regions and manufacturers, reduction of *E. coli* O157:H7 was observed only in ciders treated with the fumaric acid/sodium benzoate preservative treatment (Table 2). Five log or greater reductions were observed in ciders from Georgia, Alabama, and the 2 Pennsylvania ciders when stored at 25 °C and sampled after 9 h, and in the Tennessee sample when sampled after 21 h.

Addition of 0.15% (w/v) fumaric acid and 0.05% (w/v) sodium benzoate treatment had a highly significant effect on the destruction of *E. coli* O157:H7 in apple cider stored at 5 °C (Figure 1). While there was a continuous decline in the population of *E. coli* O157:H7 in samples containing fumaric acid and sodium benzoate, the control samples (with no fumaric acid and sodium benzoate) showed

little or no decline in *E. coli* O157:H7 populations. The above results were consistent with the observations of Comes and Beelman (2002), demonstrating that the presence of the preservatives is critical for a significant reduction in *E. coli* O157:H7 populations in apple cider stored under refrigeration.

Increasing storage temperatures to 15 and 25 °C significantly increased the destruction of *E. coli* O157:H7 populations in apple cider containing the fumaric acid/sodium benzoate preservative mixture ($P < 0.05$) (Figure 2). These findings are consistent with studies that have demonstrated that the destruction of *E. coli* O157:H7 is enhanced when low pH foods like apple cider (Zhao and others 1993), salami (Faith and others 1998a), Lebanon bologna (Chikthimmah and Knabel 2001), commercial mayonnaise (Hathcox and others 1995), and pepperoni (Faith and others 1998b) are held at temperatures higher than refrigeration temperatures. Several studies have also concluded that the efficacy of preservatives decreased at refrigeration temperatures and that it may take several days (Zhao and others 1993) or weeks (Uljas and Ingham 1999) before a 5-log reduction in *E. coli* O157:H7 populations in cider is achieved. Comes and Beelman (2002) showed that in apple cider treated with the fumaric acid and sodium benzoate mixture and then held at 25 °C, *E. coli* O157:H7 populations were reduced by greater than 5 logs after 6 h of storage. When the temperature was increased to 35 °C, a 5-log reduction in *E. coli* O157:H7 was achieved after 3 h of storage. Higher temperatures may cause the fluidity of cellular membranes to increase, thereby allowing more rapid diffusion of preservatives into the cytoplasm, explaining the

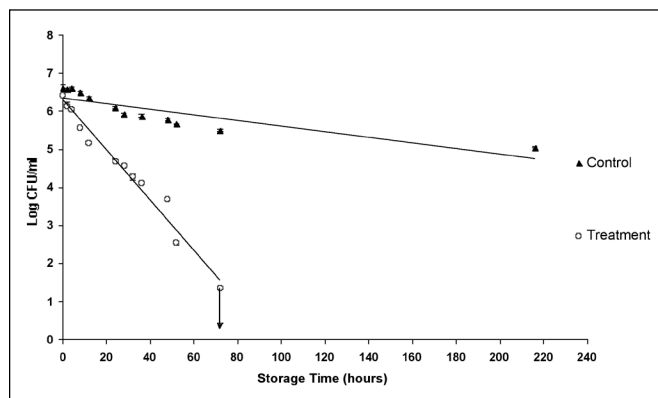


Figure 1—Survival of *E. coli* O157:H7 in untreated and preservative treated apple cider (fumaric acid, 0.15% / sodium benzoate, 0.05%, w/v) during storage at 5 °C. Initial pH of the apple cider was 3.5. pH after addition of the preservatives was 3.2. pH of the control was adjusted to 3.2 using 5 M HCl. Error bars represent standard errors of the means.

enhanced destruction of *E. coli* O157:H7 in apple cider stored at temperatures (15 °C and 25 °C) higher than refrigeration temperatures.

There was no significant change in the apple cider pH following the holding time in all experiments. Low pH is a critical hurdle that prevents foodborne pathogens from growing in acidic food products. According to a 3-y study conducted in New York, the pH of apple juice (clarified cider) ranged between 3.3 and 4.1 (Mattick and Moyer 1983). In the present study, where apple cider samples were

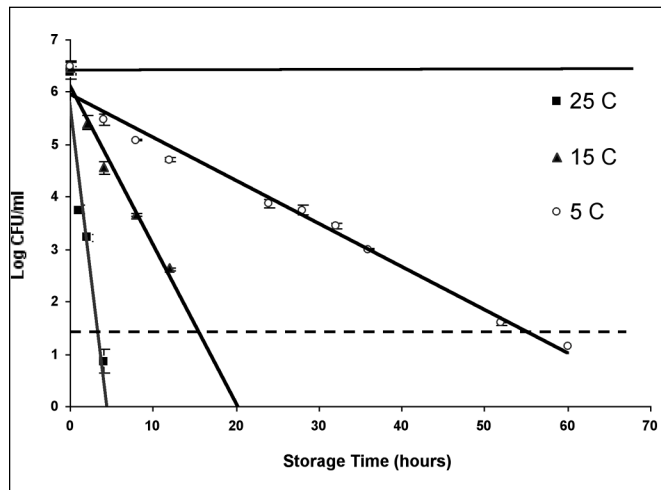


Figure 2—Effect of storage temperatures (5, 15, and 25 °C) on the survival of *E. coli* O157:H7 in preservative treated apple cider (fumaric acid, 0.15% / sodium benzoate, 0.05%, w/v). Initial pH of the apple cider was 3.5. pH after addition of the preservatives was 3.2. Solid line represents initial inoculum level (6.4 log CFU/mL); dashed line represents a 5 log CFU/mL reduction of *E. coli* O157:H7. Error bars represent standard errors of the means.

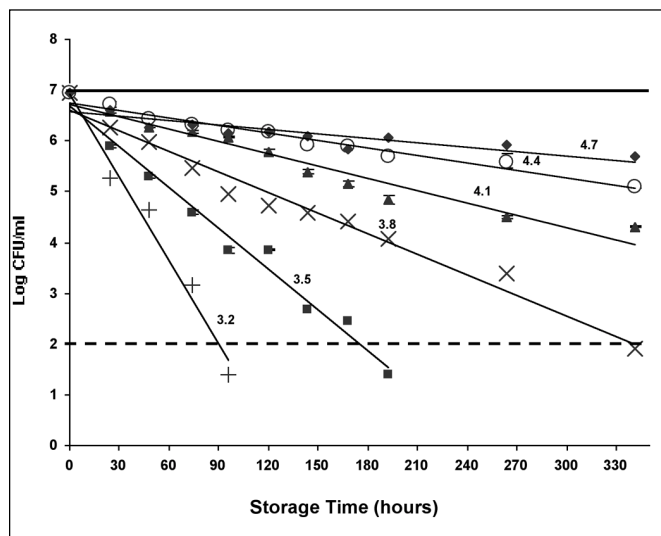


Figure 3—Effect of apple cider pH (3.2, 3.5, 3.8, 4.1, 4.4, and 4.7) on the survival of *E. coli* O157:H7 in preservative treated apple cider (fumaric acid, 0.15%/sodium benzoate, 0.05%, w/v) during storage at 5 °C. Initial pH of the apple cider was 3.5. pH after addition of the preservatives was 3.2. pH was adjusted to 3.5, 3.8, 4.1, 4.4, and 4.7 using a 10 M NaOH solution. Solid line represents initial inoculum level (7.0 log CFU/mL); dashed line represents 5 log reduction of *E. coli* O157:H7.

adjusted to pH values between 3.2 and 4.7 and held at 5 °C, destruction of *E. coli* O157:H7 significantly ($P < 0.05$) decreased with increasing pH value. Cider containing added fumaric acid and sodium benzoate (0.15% and 0.05%, w/v) and adjusted to pH 3.2 required 92 h to achieve a 5-log reduction. Increase in cider pH to 3.8 or above increased the required time to 342 h (14.3 d) or more (Figure 3). At low cider pH values, the concentration of the undissociated (more bactericidal) fumaric acid increases (Comes and Beelman 2002), which may explain the increased destruction of *E. coli* O157:H7 in low pH ciders with the preservatives added. Specific inhibition of a metabolic function by the undissociated acid and the acidification of the cytoplasm as H⁺ ions are released from the organic acids upon entering the cell may be responsible for the growth inhibition and subsequent destruction observed (Salmond and others 1984). There was a natural reduction in pH (ranging from 0.19 to 0.46 pH units) in all commercial apple cider samples, upon addition of the fumaric acid/sodium benzoate preservative treatment (Table 1). The importance of decreasing apple cider pH in contributing towards an enhanced destruction of *E. coli* O157:H7 has been demonstrated in this study (Figure 3) and the probable mechanism discussed previously. This reduction in pH due to the addition of the fumaric acid/sodium benzoate treatment contributes to pH as a hurdle factor in apple cider.

Conclusions

IT WAS DETERMINED THAT LOW APPLE-CIDER-PH VALUES (IN THE range of 3.2 to 3.8) were highly suitable for the fumaric acid and sodium benzoate preservative treatment to reduce *E. coli* O157:H7 populations. Natural reduction in pH resulting from addition of fumaric acid/sodium benzoate was consistent with all commercial ciders. When the pH is > 3.8 after the addition of the fumaric acid/sodium benzoate, this process may not be practical. Processors producing cider with high pH values (> 4.1) should explore alternative methods, like pasteurization, to ensure food safety.

This study clearly demonstrates that storage of apple cider above refrigeration temperatures actually enhances destruction of *E. coli* O157:H7. Regulatory agencies might want to consider appropriate studies in this area and provide storage recommendations for apple cider processors, retailers, and distributors. The study demonstrated that a 5 log reduction of *E. coli* O157:H7 in apple cider can be achieved without the need for pasteurization, a process that is likely to be economically unpractical for many small cider processors. The compounds used in this treatment are generally recognized as safe (GRAS) organic acid antimicrobials and were used within their legal limits. Critical limits for pH and temperature have been identified and these may be incorporated into HACCP programs that are currently mandated for fruit and vegetable juice processing. It is also important to note that if processors adopt this preservative treatment, a proper validation study needs to take place to ensure that a 5 log reduction of *E. coli* O157:H7 populations can be achieved under their conditions.

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