APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2526–2530
0099-2240/93/082526-05$02.00/0

Vol. 59, No. 8

Fate of Enterohemorrhagic *Escherichia coli* O157:H7 in Apple Cider with and without Preservatives

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Received 11 January 1993/Accepted 8 June 1993

A strain of enterohemorrhagic *Escherichia coli* serotype O157:H7 isolated from a patient in an apple cider-related outbreak was used to study the fate of *E. coli* O157:H7 in six different lots of unpasteurized apple cider. In addition, the efficacy of two preservatives, 0.1% sodium benzoate and 0.1% potassium sorbate, used separately and in combination was evaluated for antimicrobial effects on the bacterium. Studies were done at 8 or 25°C with ciders having pH values of 3.6 to 4.0. The results revealed that *E. coli* O157:H7 populations increased slightly (ca. 1 $\log_{10}$ CFU/ml) and then remained stable for approximately 12 days in lots inoculated with an initial population of 10^5 *E. coli* O157:H7 organisms per ml and held at 8°C. The bacterium survived from 10 to 31 days or 2 to 3 days at 8 or 25°C, respectively, depending on the lot. Potassium sorbate had minimal effect on *E. coli* O157:H7 populations, with survivors detected for 15 to 20 days or 1 to 3 days at 8 or 25°C, respectively. In contrast, survivors in cider containing sodium benzoate were detected for only 2 to 10 days or less than 1 to 2 days at 8 or 25°C, respectively. The highest rates of inactivation occurred in the presence of a combination of 0.1% sodium benzoate and 0.1% potassium sorbate. The use of 0.1% sodium benzoate, an approved preservative used by some cider processors, will substantially increase the safety of apple cider in terms of *E. coli* O157:H7, in addition to suppressing the growth of yeasts and molds.

Enterohemorrhagic *Escherichia coli* O157:H7, first recognized as a human pathogen in 1982, is now known as an important cause of hemorrhagic colitis and hemolytic uremic syndrome (7, 12). Many food-associated outbreaks caused by enterohemorrhagic *E. coli* have occurred during the past decade, with undercooked ground beef being the principal vehicle (7, 12). In the fall of 1991, an outbreak of hemorrhagic colitis associated with drinking apple cider occurred in Massachusetts (1).

Apple cider also was identified as the vehicle of two previous outbreaks. One outbreak, caused by *Salmonella typhimurium*, occurred in New Jersey in 1974 (2). The cider was made from apples collected under trees fertilized with cow manure. A second outbreak, with 14 cases of hemolytic uremic syndrome, occurred in 1980 in Canada (13). *E. coli* O157:H7 was likely the causative agent because patients had bloody diarrhea and cramps before developing hemolytic uremic syndrome.

The principal difference between apple cider and apple juice is that cider is unpasteurized, whereas apple juice is pasteurized by heating, usually at 68°C for 20 to 30 min or 82°C for 20 to 30 s (4). Hence, any pathogens on apples before pressing are likely to be present and viable in apple cider, whereas pathogens in apple juice could be killed by pasteurization. The purpose of this study was to determine the fate of *E. coli* O157:H7 in unpasteurized apple cider both with and without preservatives.

MATERIALS AND METHODS

Bacterial inoculum. *E. coli* O157:H7 (strain 7927, isolated from a patient involved in the U.S. apple cider-associated outbreak) was grown in 50 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.) for 16 h at 37°C with agitation (150 rpm). Cells were sedimented three times by centrifugation at 10,000 × *g* for 3 min and washed with 0.1 M phosphate-buffered saline (PBS) (pH 7.2). Bacteria were adjusted with PBS to an optical density at 640 nm of 0.5 (approximately 10^8 CFU/ml). The population of bacteria was verified by plate counts on tryptic soy agar (Difco Laboratories) incubated for 16 to 18 h at 37°C. Cells were appropriately diluted in PBS, and 0.5 ml was inoculated into 50-ml samples of apple cider in 100-ml Erlenmeyer flasks for a final population of approximately 1.0×10^2 or 1.0×10^5 *E. coli* O157:H7 organisms per ml.

Incubation and sampling of apple cider. Six different lots of apple cider were used. These included five lots of previously frozen cider (no. 2 to 6) processed by a producer in Massachusetts whose product was associated with an outbreak of *E. coli* O157:H7 infection (1). One lot (no. 1, control) was produced in Missouri by a different producer. All lots were tested for *E. coli* O157:H7 according to the procedure of Padhye and Doyle (11) before inoculation. Inoculated samples were incubated at 8 or 25°C for up to 5 weeks or 8 days, respectively. Before inoculation, cider from each lot was assayed for sugar concentration according to the method described by Joslyn (8), pH, *E. coli* O157:H7, aerobic plate counts (APC), and mold and yeast counts. APC were determined on plate count agar inoculated at 35°C for 2 days. Molds and yeasts were enumerated on dichloran-rose bengal-chloramphenicol agar (9) (30°C, 4 days). At each sampling time, cider was assayed for pH and *E. coli* O157:H7 counts, which were done in duplicate on Sorbitol-MacConkey agar no. 3 (Unipath Co., Oxoid Division, Ogdensburg, N.Y.) with 0.01% 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma Chemical Co., St. Louis, Mo.) (37°C, 16 to 18 h). Sorbitol-negative, MUG-negative colonies were counted as presumptive *E. coli* O157:H7. Colonies typical of *E. coli*

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TABLE 1. Sugar concentration, pH, and mold, yeast and APC of apple cider before inoculation with *E. coli* O157:H7[a]

Lot no.	Sugar concn (%)	pH	CFU/ml		
			Mold	Yeast	APC
1	11.6	4.0	4.5×10^2	7.0×10^3	1.1×10^2
2	11.2	3.9	7.5×10^1	4.0×10^1	3.0×10^1
3	12.0	3.8	2.5×10^2	3.2×10^1	1.0×10^1
4	11.0	3.7	8.0×10^0	1.5×10^1	2.0×10^1
5	11.0	3.9	3.5×10^1	6.0×10^3	4.0×10^1
6	10.2	3.6	1.0×10^1	2.0×10^4	8.5×10^1

[a] *E. coli* O157:H7 was not detected in any lots assayed by enrichment (11).

O157:H7 were selected randomly from plates (five per plate) of the highest dilution and confirmed as *E. coli* O157:H7 by agglutination with O157 antibody-coated beads (Unipath Co.), by an enzyme-linked immunosorbent assay using monoclonal antibody 4E8 C12, which is specific for enterohemorrhagic *E. coli* serotypes O157:H7 and O26:H11 (10), and by biochemical characterization with the API 20E miniaturized diagnostic test (Analytab Products, Division of Sherwood Medical, Plainview, N.Y.). Each *E. coli* O157:H7 count was the average of duplicate determinations. Duplicate samples were assayed from a single flask of each lot or treatment at each sampling time, and the results reported were the averages of these data.

Statistical analysis. An analysis of variance was performed on data to determine significant differences among lots and treatments (3).

RESULTS

Analyses of apple cider before inoculation with *E. coli* O157:H7 revealed that none of the lots contained detectable *E. coli* O157:H7, the pH ranged from 3.6 to 4.0, the sugar content ranged from 10.2 to 12%, the mold count ranged from 8 to 450 CFU/ml, the yeast count ranged from 15 to 2×10^4 CFU/ml, and the APC ranged from 1.0×10^1 to 1.1×10^2 CFU/ml (Table 1). The pH of all samples decreased during incubation and ranged from 3.1 to 3.7 when held at 25°C for 3 days and from 3.4 to 3.7 when held at 8°C for 15 days (data not shown). Adding 0.1% sodium benzoate or 0.1% potassium sorbate increased the initial pH of apple cider 0.1 to 0.2 or 0.2 to 0.3 pH units, respectively.

The fate of two different initial populations (10^2 or 10^5 CFU/ml) of *E. coli* O157:H7 was evaluated for five or six different lots of apple cider, respectively, at 8°C. A slight increase (ca. 1 $\log_{10}$ CFU/ml) in populations occurred at 8°C within 2 to 4 days in cider inoculated with 10^5 *E. coli* O157:H7 organisms per ml (Fig. 1), whereas populations did not increase in samples inoculated with 10^2 CFU/ml (Fig. 2). For lots inoculated with 10^5 CFU/ml, viable *E. coli* O157:H7 organisms were detected for up to 31 days in one lot and were not detectable (<5 CFU/ml) within 15 days in another lot. Visibly detectable mold growth was observed at 10 days after inoculation in one lot (no. 1), at 15 days in three lots, and at 20 days in two lots. The pH of the cider decreased 0.1 to 0.2 pH units within 10 days and up to 0.5 pH units within 28 days. For lots of cider inoculated with 10^2 CFU/ml, *E. coli* O157:H7 was not detectable within 11 to 14 days.

Differences in survival of *E. coli* O157:H7 in different lots of cider were statistically significant (<0.05) at levels of both 10^2 and 10^5 CFU of inoculum per ml.

Studies at 25°C were done only with an initial population of 10^5 *E. coli* O157:H7 organisms per ml (Fig. 3). Survivors were detected at 2 to 3 days but not at 6 days postinoculation. The pH of cider decreased from 0.3 to 0.8 units within 3 days, at which time visible mold growth was detectable on samples of two lots.

The effect of potassium sorbate on survival of *E. coli* O157:H7 at 8°C was not statistically significant ($P > 0.05$), with organisms detectable (decrease of 3 $\log_{10}$ to 4 $\log_{10}$ CFU/ml) in four of six lots of cider at 20 days (Table 2). *E. coli* O157:H7 survived longer in the presence of 0.1% potassium sorbate than in control samples in three lots of cider and for less time in the other three lots. Similar results

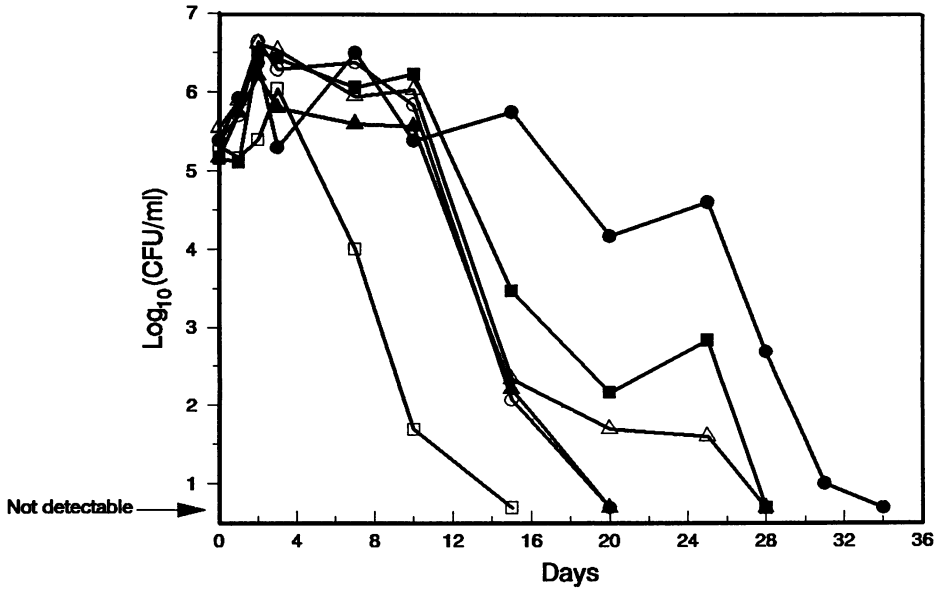

FIG. 1. Fate of *E. coli* O157:H7 at 8°C in six lots of apple cider inoculated with 10^5 *E. coli* O157:H7 cells. Lots: no. 1, □; no. 2, △; no. 3, ○; no. 4, ●; no. 5, ■; no. 6, ▲.

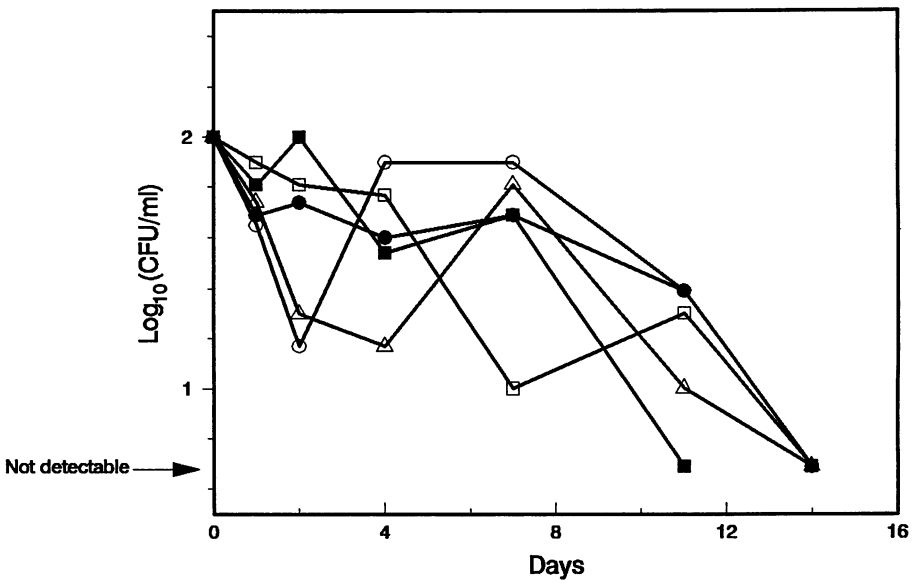

FIG. 2. Fate of *E. coli* O157:H7 at 8°C in apple cider inoculated with 10^2 *E. coli* O157:H7 cells per ml. See the legend to Fig. 1 for a description of the symbols.

were obtained for samples held at 25°C, with survivors detected (decrease of 1 $\log_{10}$ to 3 $\log_{10}$ CFU/ml) at 3 days but not at 6 days postinoculation in four or three lots treated with or without potassium sorbate, respectively.

In contrast, 0.1% sodium benzoate was an antimicrobial agent to *E. coli* O157:H7 at 8°C ($P < 0.01$), reducing the number of organisms to undetectable populations (reduction of >4 $\log_{10}$ CFU/ml) within 7 days in five of six lots and between 9 to 15 days in one lot (Table 2). At 25°C in sodium benzoate, the number of organisms decreased to undetectable populations within 2 days in five of six lots and within 3 days in one lot. Death of the organism to undetectable populations in untreated lots ranged from 3 days (for three lots) to 6 days (for three lots) postinoculation (Fig. 3).

When used in combination, 0.1% potassium sorbate plus 0.1% sodium benzoate had enhanced antimicrobial activity at 8°C, with survival times being reduced by approximately 50% (Table 2) compared with those with treatment with 0.1% sodium benzoate only (Table 2). In contrast, the results of the treatment with a combination of potassium sorbate and sodium benzoate at 25°C were approximately the same as those of the treatment with sodium benzoate alone (data not shown), with a reduction of the number of *E. coli* O157:H7

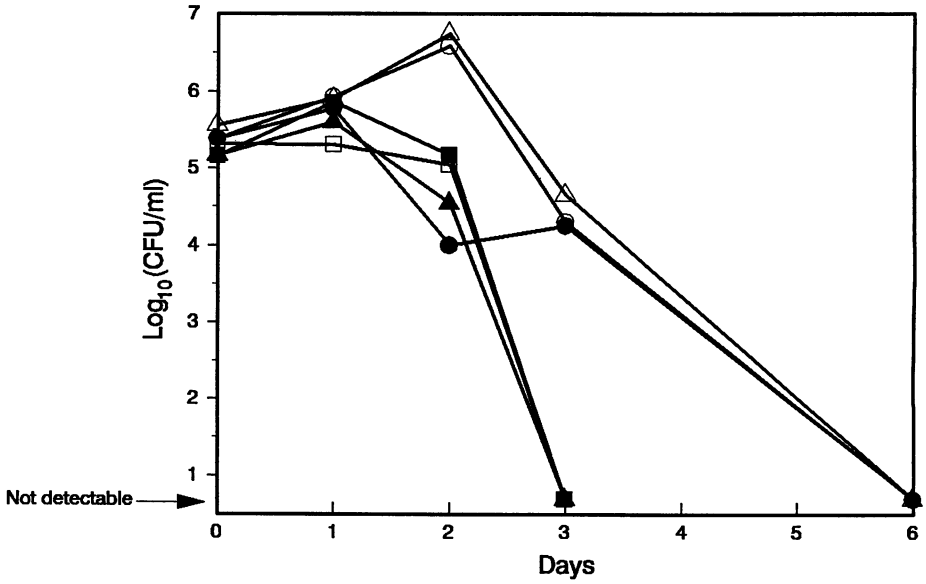

FIG. 3. Fate of *E. coli* O157:H7 at 25°C in apple cider inoculated with 10^5 *E. coli* O157:H7 cells per ml. See the legend to Fig. 1 for a description of the symbols.

TABLE 2. Fate of *E. coli* O157:H7 (10^5 CFU/ml) in different lots of apple cider with and without preservative and held at 8°C

Lot no.	No. of *E. coli* O157:H7/ml in:											
	Apple cider only			Cider with potassium sorbate[a]			Cider with sodium benzoate[b]			Cider with potassium sorbate + sodium benzoate[c]		
	Day 15	Day 20	Day 28	Day 25	Day 20	Day 25	Day 3	Day 7	Day 15	Day 1	Day 3	Day 7
1	ND[d]			4.6	1.7	ND	5.7	2.0	ND	5.8	4.8	ND
2	2.3	1.7	ND	3.3	1.7	ND	5.3	ND		5.5	3.2	ND
3	2.1	ND		3.3	1.7	ND	2.5	ND		3.5	ND	
4	5.8	4.2	2.7	3.0	ND		2.9	ND		3.0	ND	
5	3.5	2.2	ND	3.1	ND		3.5	ND		3.0	ND	
6	2.2	ND		2.0	1.7	ND	ND			ND		

[a] No significant difference ($P > 0.05$) between groups of cider treated with and without 0.1% potassium sorbate.
[b] Significant difference ($P < 0.01$) between groups of cider treated with and without 0.1% sodium benzoate.
[c] Significant difference ($P < 0.001$) between groups of cider treated with and without 0.1% potassium sorbate plus 0.1% sodium benzoate.
[d] ND, not detectable; the minimum detection level was 0.7 $\log_{10}$ *E. coli* O157:H7 cells per ml.

cells to undetectable populations (decrease of >4 $\log_{10}$ CFU/ml) occurring within 1 to 3 days.

DISCUSSION

Freshly pressed, unpasteurized apple cider is a documented vehicle of food-borne illness (1, 2, 13). Apple cider has a pH which is normally below 4.0 and would not be considered a medium conducive to the survival and growth of food-borne pathogens. However, *S. typhimurium* has been isolated from cider samples that were associated with an outbreak of gastroenteritis (2). These samples had pH values of 3.4 to 3.9. In subsequent studies of the survival of *S. typhimurium* in apple juice, the bacteria grew in some juices depending on the variety of apple and pH (6). An increase (ca. 3 $\log_{10}$ CFU/ml) in growth at 22°C occurred in juices at pH 3.7 to 4.0 but not at pH 2.9 to 3.6. Salmonellae survived at 4°C for more than 30 days in apple juice with a pH of 3.6.

E. coli O157:H7, when initially present at 10^5 CFU/ml, survived for up to 31 days in one lot of apple cider with a pH of 3.7 held at 8°C. A slight increase (ca. 1 $\log_{10}$ CFU/ml) in population within 2 days was observed in all lots inoculated with 10^5 *E. coli* O157:H7 organisms per ml. Studies of the fate of *E. coli* O157:H7 in Trypticase soy broth adjusted to different pH values with HCl or lactic acid revealed that the minimum pH for growth at 37°C was between 4.0 and 4.5 or 4.6, respectively (5). Hence, the acidity of apple cider at pH ≤4.0 would likely suppress the growth of *E. coli* O157:H7, and small increases in detectable populations are more likely the result of resuscitation of injured cells rather than growth. Populations of *E. coli* O157:H7 at 8°C remained relatively constant for 7 to 12 days, depending on the initial population of cells inoculated. Since the expected refrigerated shelf life of apple cider is typically less than 2 to 3 weeks, the pathogen is likely to survive during most of the time that the cider would be consumed, at numbers close to the size of the initial population that was introduced into the product.

Interestingly, *E. coli* O157:H7 can tolerate the acidity (ca. pH 4.5) of fermented, dry sausage, further indicating the organism's endurance in acid environments (5). Glass et al. (5) determined that when initially inoculated into commercial sausage batter at 4.8×10^4 *E. coli* O157:H7 organisms per g, the organism survived during fermentation, drying, and subsequent storage at 4°C, decreasing by about 2 $\log_{10}$ CFU/g by the end of 8 weeks of refrigerated storage.

Mold growth in cider may influence inactivation of *E. coli* O157:H7. Visible mold growth covering the entire surface was evident by 10 days in lot no. 1 at 8°C, but not in equivalent samples treated with 0.1% potassium sorbate or 0.1% sodium benzoate. It is likely that metabolites produced by molds in untreated cider of lot no. 1 reduced the number *E. coli* O157:H7 cells to undetectable populations within 15 days, whereas repression of mold growth by potassium sorbate or sodium benzoate resulted in greater survival of *E. coli* O157:H7 in this lot. Mold growth is likely to influence survival of *E. coli* O157:H7; however, it also causes spoilage of the product. Hence, it is not a practical approach to eliminating the pathogen from cider.

The past identification of apple cider as a vehicle of outbreaks of gastroenteritis and hemolytic uremic syndrome indicates that the acidity of apple juice is inadequate to kill certain pathogens. The results of this study support these findings. *E. coli* O157:H7 can survive in apple juice, with minimal death occurring during most of the expected shelf life of cider. Considering that most apple cider receives no treatment to eliminate food-borne pathogens, cider processors are at considerable risk if soil- or manure-borne pathogens are present on fallen apples. Using 0.1% sodium benzoate is an option processors have to substantially increase the safety of apple cider.

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