Comparison of Methods for Determining Coliform and *Escherichia coli* Levels in Apple Cider†

TODD M. SILK, ELLIOT T. RYSER, and CATHERINE W. DONNELLY*

Department of Animal and Food Sciences, University of Vermont, Burlington, Vermont 05405, USA

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**ABSTRACT**

The main objective of this research was to determine the easiest and most reliable media for enumerating coliform bacteria and *Escherichia coli* levels in apple cider. During the autumn of 1994 a total of 59 apple cider samples were collected directly from 12 cider producers and were assessed for bacterial levels and pH. Plate count agar was used to determine heterotrophic bacteria levels. Coliform levels were determined using three different media: violet red bile agar (VRBA), Petrifilm High Sensitivity Coliform Count Plates (PHSCCP), and Trypticase soy agar with a VRBA overlay (TSA/VRBA) for attempted recovery of coliforms injured by the low pH of the apple cider. Eosin methylene blue agar (EMBA) and Petrifilm *E. coli* Count Plates were used to screen cider samples for *E. coli*. Apple cider had an average pH of 3.34 ± 0.08. Heterotrophic bacterial levels ranged from 2.30 to 7.11 log CFU/ml. All cider samples contained coliform bacteria with levels varying greatly; on the different media, we found the following: on VRBA, <1.00 to 4.37 log CFU/ml; on TSA/VRBA, 1.20 to 4.40 log CFU/ml; and on PHSCCP, <1.00 to 4.56 log CFU/ml. Coliform levels were most easily determined in apple cider by using PHSCCP. However TSA/VRBA proved to be more reliable; coliform detection was significantly (P < 0.05) increased. EMBA was ineffective for screening apple cider for *E. coli*, with the low pH of the cider producing many false-positive results. *E. coli* was only recovered by using Petrifilm *E. coli* Count Plates with one of the 59 samples positive for *E. coli* (non-O157:H7) at a level of 10 CFU/ml.

Key words: Apple cider, coliform detection

Concerns over the microbiological safety of unpasteurized apple cider have been expressed for more than 20 years. During 1974 apple cider was implicated in a large outbreak of salmonellosis in New Jersey, in which 296 people became ill (2). Six years later, an outbreak of hemolytic uremic syndrome (HUS) that affected 14 children was linked to apple cider consumption in Pickering, Ontario, Canada. Although an infectious agent was not found, researchers believed *Escherichia coli* O157:H7 was involved because of the development of HUS (9). During 1991 28 people were infected with *E. coli* O157:H7 in an outbreak that was epidemiologically linked to fresh apple cider produced in Southeastern Massachusetts. Symptoms of disease included diarrhea and HUS (1). Outbreaks of *Cryptosporidium* spp. have also been linked to apple cider consumption. One outbreak occurred in Maine during 1993 in which over 160 people became ill; a second outbreak occurred in New York during 1996 (4, 7). During October, 1996, two outbreaks of *E. coli* O157:H7 were linked to the consumption of apple cider. The first of these outbreaks occurred in the Western U.S. and involved 66 cases of illness, including one death (3). The second outbreak affected 8 individuals in Connecticut (4). In response to these recent outbreaks, the U.S. Food and Drug Administration (FDA) is now considering changing the federal regulations that govern unpasteurized juices. Regulations will likely require the development of good manufacturing programs and possibly hazard analysis critical control point (HACCP) programs along with mandatory labeling of unpasteurized products. While pasteurization of apple cider may also be required by the FDA to enhance product safety, such a mandate for pasteurization would not be economically feasible for most small cider producers, with production of fresh apple cider likely ceasing.

Low pH (<4.0), a characteristic of apple cider, was at one time considered a safeguard against the survival and growth of pathogens in foods. However, research has shown that *E. coli* O157:H7 can survive in apple cider (pH 3.7) stored at 8°C for up to 31 days and that levels of this pathogen are likely to remain relatively constant for the first 2 to 3 weeks of storage (10). Certain strains of *E. coli* O157:H7 can also reportedly survive 21 days in cider that contains 0.1% sodium benzoate as a preservative (8). Research done in response to the 1974 Salmonella typhimurium outbreak that was linked to apple cider found that some serotypes of Salmonella could also survive at a pH of 4.0 for up to 30 days at 4°C (5).

Coliform levels will give some indication of how likely apple cider is to contain enteric pathogens. The main objective of this study was to determine coliform levels in apple cider.
Materials and Methods

A total of 59 0- to 7-day-old apple cider samples (500 ml) were collected at 12 cider mills during the autumn of 1994. Samples were collected, transported in a cooler with ice, refrigerated upon arrival in the laboratory, and then analyzed within 24 h for pH and levels of heterotrophic bacteria, coliforms, and Escherichia coli.

Bacteriological analysis

Heterotrophic bacteria levels were determined by surface plating samples appropriately diluted in Butterfield’s phosphate buffer on plate count agar (PCA) (Difco Laboratories, Detroit, MI). CFU were enumerated after 24 h of incubation at 35 ± 2°C.

Coliform levels were determined using three different media: violet red bile agar (VRBA) (from Difco Laboratories, Detroit, MI); Petrifilm High Sensitivity Coliform Count Plates (PHSCCP) (from 3M, St. Paul, MN) used as recommended by the manufacturer; and Trypticase soy agar (TSA) (Becton Dickinson, Cockeysville, MD) with a VRBA overlay (TSA/VRBA). Samples were surface plated on TSA and incubated for 2 h at room temperature before being overlayed with a double-strength concentration of VRBA. TSA/VRBA was used to recover coliforms injured by the low pH of the apple cider. All samples were appropriately diluted in Butterfield’s phosphate buffer before being plated.

Minimum coliform detection levels were 10 (1.00 log) CFU/ml. Coliform-like colonies were enumerated after 24 h of incubation at 32 ± 2°C. Two to five coliform-like colonies from selected plates were inoculated into brilliant green bile lactose broth (BGBLB) (Difco); colonies were confirmed by the observation of gas production in cultures at 35°C within 48 h (6).

E. coli screening was performed by using eosin methylene blue agar (EMBA) (Difco) and Petrifilm E. coli Count Plates (3M) as recommended by the manufacturer. Samples were serially diluted in Butterfield’s phosphate buffer and plated on the above media. EMBA plates were incubated at 35°C for 24 h. Petrifilm E. coli Count Plates were incubated at 32 to 35°C for 24 to 48 h; then colonies resembling those of E. coli were counted. Presumptive E. coli colonies were confirmed using API 20E test strips as recommended by the manufacturer (BioMerieux, Hazelwood, MO).

pH determination

The pH of the apple cider was determined at room temperature using a digital ion analyzer pH meter equipped with a standard combination electrode (from Orion Research Inc., Boston, MA).

Producer surveys

The production of apple cider involves four major steps: harvesting apples from the tree or from the ground, washing the apples with a high-pressure water spray or high-pressure water spray with brushing, chopping the apples, and pressing the chopped apples. Because the methods for harvesting and washing vary, a survey of producers was conducted to determine what method was used by each cider maker. The survey also examined equipment-washing practices.

Statistical analysis

A total of 59 apple cider samples were collected from 12 different apple cider producers over several weeks. Mean coliform values were statistically analyzed using Scheffe’s multiple comparison for means (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA) with a significance level of 0.05.

Results

The apple cider samples had a mean pH of 3.34 ± 0.08, which is typical of a good-quality apple cider. Heterotrophic bacterial levels ranged from 2.30 to 7.11 log CFU/ml, with the mean being 4.64 ± 1.02 log CFU/ml.

All coliform media yielded high levels of false-positive results, with only 23.2, 41.3, and 56.3% of presumptive coliforms confirmed on VRBA, TSA/VRBA and PHSCCP, respectively. These percentages were used as correction factors and applied to all coliform levels obtained. Coliform levels ranged from <1.00 to 4.37 log CFU/ml on VRBA, <1.00 to 4.56 log CFU/ml on PHSCCP, and 1.20 to 4.40 log CFU/ml on TSA/VRBA. PHSCCP were easiest to use, taking the shortest time and distinguishing presumptive coliforms that were subsequently confirmed to be coliforms. When coliform CFU, standard deviations (SD), and standard errors (SE) for each media were compared, (Table 1) the mean coliform count was higher and more consistent on TSA/VRBA; using these media includes a resuscitation step. Based on Scheffe’s multiple comparison method for means, coliform counts were significantly higher (P ≤ 0.05) from TSA/VRBA, whereas no significant differences (P ≥ 0.05) were noted when comparing mean coliform counts obtained from VRBA and PHSCCP.

Cider producer surveys which identified the type of apple-harvesting methods, apple-washing procedures, and equipment-cleaning practices indicated that there was variation between cider producers, and that some individual producers varied practices with regard to apple harvesting methods. Out of the 8 producers that participated in the survey, more than half used apples harvested from the ground (dropped apples) to produce cider (producers A, E, F, and J (Figure 1)). The majority of producers used high-pressure water and mechanical brushes to clean apples. Producer E only used a high-pressure water rinse to clean apples. All apple cider producers surveyed washed equipment with detergent and sanitized with chlorine.

Mean heterotrophic and coliform CFU obtained on each medium, with respect to the cider producer, clearly show great variation from sample to sample within a producer and variation from producer to producer (Table 2). Apple cider producer F, who used dropped apples, had the greatest mean

<table>
<thead>
<tr>
<th>TABLE 1. Comparative coliform counts in apple cider determined by using violet red bile agar, Trypticase soy agar overlaid with violet red bile agar, and Petrifilm high-sensitivity coliform count plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium*</td>
</tr>
<tr>
<td>VRBA</td>
</tr>
<tr>
<td>TSA/VRBA</td>
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<tr>
<td>PHSCCP</td>
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</tbody>
</table>

* VRBA, violet red bile agar; TSA, Trypticase soy agar; PHSCCP, Petrifilm high-sensitivity coliform count plates.
TABLE 2. Variation of heterotrophic and adjusted coliform levels in apple cider as a function of evaluation media and cider producer.

<table>
<thead>
<tr>
<th>Producer (no. of samples)</th>
<th>Heterotrophs and coliforms, log CFU/ml, mean (SD) on medium*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA</td>
<td>VRBA</td>
</tr>
<tr>
<td>A (6)</td>
<td>4.78 (0.29)</td>
<td>1.55 (0.79)</td>
</tr>
<tr>
<td>B (9)</td>
<td>4.75 (1.05)</td>
<td>1.02 (0.62)</td>
</tr>
<tr>
<td>C (1)</td>
<td>5.96 (0.00)</td>
<td>&lt;1.00</td>
</tr>
<tr>
<td>D (3)</td>
<td>4.85 (0.36)</td>
<td>1.46 (0.88)</td>
</tr>
<tr>
<td>E (5)</td>
<td>4.49 (1.06)</td>
<td>1.17 (1.55)</td>
</tr>
<tr>
<td>F (3)</td>
<td>6.12 (0.84)</td>
<td>3.65 (0.65)</td>
</tr>
<tr>
<td>G (6)</td>
<td>5.20 (0.54)</td>
<td>2.45 (0.69)</td>
</tr>
<tr>
<td>H (8)</td>
<td>3.75 (0.57)</td>
<td>1.27 (0.85)</td>
</tr>
<tr>
<td>I (4)</td>
<td>5.74 (1.15)</td>
<td>1.68 (1.17)</td>
</tr>
<tr>
<td>J (5)</td>
<td>3.80 (0.57)</td>
<td>2.38 (0.57)</td>
</tr>
<tr>
<td>K (7)</td>
<td>3.97 (0.77)</td>
<td>1.84 (0.93)</td>
</tr>
<tr>
<td>L (2)</td>
<td>5.19 (0.06)</td>
<td>1.04 (0.32)</td>
</tr>
</tbody>
</table>

*PCA, plate count agar; VRBA, violet red bile agar; TSA/VRBA, Trypticase soy agar/VRBA; PHSCCP, Petrifilm high sensitivity coliform count plates.

coliform CFU on all coliform media. Producer F also had the highest mean heterotrophic bacterial population. However, no other correlations could be made when comparing population levels of heterotrophic bacteria with mean CFU of coliforms. Grouping mean coliform CFU obtained on VRBA, TSA/VRBA, and PHSCCP also indicates differences in coliform population levels when comparing apple cider producers (Fig. 1). Producers who did not use dropped apples (B, C, and D) generally had lower mean coliform populations in their cider. No other correlations could be made comparing mean coliform CFU with survey results regarding apple-washing practices or equipment-washing procedures.

E. coli detection by using EMBA proved extremely difficult. The low pH of the cider, even when diluted in Butterfield’s phosphate buffer, caused a color change in the medium that produced false-positive results. The majority of colonies that grew on EMBA had a green metallic sheen characteristic of E. coli. Limited confirmation studies indicated that the bacteria present in many of those instances were not even coliform bacteria. Petrifilm E. coli Count Plates were effective at screening apple cider samples for E. coli. Blue colonies producing gas were easily distinguished from non-E. coli bacteria. Using Petrifilm E. coli Count Plates, one sample from producer H, who used dropped apples for cider production, contained E. coli (non-O157: H7) at a concentration of 10 CFU/ml. However, cider from this producer had the lowest mean heterotrophic bacteria population and a low mean coliform population.

DISCUSSION

Coliform populations in apple cider were most easily determined using PHSCCP with these organisms being readily identified by the presence of visible gas from the fermentation of lactose. PHSCCP also yielded the highest percentage of confirmed coliforms.

There was no significant ($P > 0.05$) difference when comparing mean coliform CFU obtained on PHSCCP and VRBA. Distinguishing coliform bacteria from noncoliform bacteria on VRBA proved more difficult and time-consuming than on PHSCCP. VRBA also yielded the lowest confirmation percentage value.

Although PHSCCP required no prior preparation and were easy to use, coliform detection significantly increased ($P < 0.05$) when resuscitation was used. Coliform detection increased by 1.19 and 1.06 log units when mean coliform CFU obtained on PHSCCP and VRBA, respectively, were compared. Increased detection may be attributed to recovery of acid-injured coliforms or to the diffusion of acid from the apple cider into the nonselective medium limiting interference with the selective medium. TSA/VRBA yielded the smallest values for standard deviation and standard error. The fact that coliform levels were most consistent suggests that apple cider may contain appreciable numbers of injured coliforms. Therefore, TSA/VRBA was most reliable for detecting coliforms in apple cider, and use of this resuscitation method should be considered when determining coliform levels in other high acid foods.

No correlation was observed between population levels of heterotrophic and coliform bacteria. Cider samples with high heterotrophic bacterial counts did not necessarily have the highest coliform levels. Therefore, heterotrophic bacterial levels should not be used to estimate coliform contamination in apple cider.

E. coli screening was most easily performed using Petrifilm E. coli count plates with presumptive E. coli colonies easily distinguished from other coliforms by their blue color, indicating glucuronidase activity. Petrifilm E.
coli count plates also required no preparation before use. EMBA yielded poor results when screening apple cider samples for E. coli. The acidity of the cider changed the color of the medium, resulting in growth of many noncoliform colonies that had typical E. coli characteristics. Neutralization of acidic samples should be considered when such a selective medium is to be used. No attempts were made to recover potentially injured E. coli using EMBA or Petrifilm E. coli count plates.

Clearly coliform bacteria are present in apple cider and their detection can be increased by allowing recovery of injured cells. Further research should be done to increase detection of injured coliform bacteria and E. coli.

Our methods for evaluating the quality of apple cider have shown that coliform bacteria are present in apple cider and that considerable variation in coliform counts exists between different cider samples and cider producers. Variation may be attributed to several factors, including methods of apple harvesting (picking from the tree or from the ground), apple washing practices (general rinse or scrubbing brush wash), and equipment cleaning practices (general rinse or wash with sanitizing detergents). Data obtained revealed that the highest mean coliform CFU obtained was from a producer who used dropped apples in cider, and E. coli was isolated from cider from a different producer that also used dropped apples for cider production.

As a result of disease outbreaks the FDA is now considering changing the federal regulations that govern unpasteurized juices. Regulations may require pasteurization or the development of HACCP programs as well as good manufacturing practices to ensure the safety of such a product. Further research needs to focus on safe production of apple cider and alternatives to pasteurization which will be more economically feasible for small cider producers.

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REFERENCES