Survival and Growth of *Escherichia coli* O157:H7 in Unpasteurized and Pasteurized Milk

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**ABSTRACT**

*Escherichia coli* O157:H7, which causes hemorrhagic colitis and hemolytic uremic syndrome, has been responsible for several outbreaks associated with consumption of unpasteurized and improperly processed pasteurized milk, and yogurt. Studies were conducted to determine the survival and growth characteristics of this pathogen in unpasteurized milk and pasteurized milk (3.5% fat, 2% fat, skim) at 5, 8, 15, and 22°C for up to 28 days. Two levels of inocula (10³ and 10⁵ CFU/ml) of a mixture of five nalidixic acid-resistant *E. coli* O157:H7 strains were used. *E. coli* O157:H7 did not grow at 5°C and decreased by 1.6 to 2.0 log CFU/ml in 28 days. Growth occurred at 8°C, with an approximately 1- to 2-log CFU/ml increase within the first 4 days. About a 3- to 5-log CFU/ml increase in *E. coli* O157:H7 populations was observed at 15°C within the first 3 days. In 3 pasteurized milk samples, *E. coli* O157:H7 continued to grow to populations of greater than 1.0 × 10⁸ CFU/ml at day 7 and remained constant during the remainder of the incubation period. At 22°C, the pH decreased rapidly to less than 4.0 within 4 days and *E. coli* O157:H7 decreased to undetectable populations within 14 days. *E. coli* O157:H7 grew more slowly (P < 0.01) in unpasteurized milk, which had a higher initial microbial population, than in pasteurized milks at 8, 15, or 22°C, likely because of antagonistic activity from preexisting bacteria. No significant differences (P > 0.05) in survival or growth of *E. coli* O157:H7 were observed among the pasteurized milk samples, regardless of fat concentration, at all temperatures throughout the study. The data indicate that temperature abuse during shipping and handling can result in significant growth of *E. coli* O157:H7. Holding milk at ≤5°C is recommended to prevent growth of this pathogen.

Key words: Milk, *Escherichia coli* O157:H7, enterohemorrhagic *E. coli"

Fluid milk is a highly perishable commodity. Milk has special importance as a principal component of the diets of the young and the elderly. The immune system of individuals in these groups often is not sufficiently responsive to prevent infection by pathogenic bacteria. For these reasons, greater emphasis has been placed upon the safety of milk than that of most other foods. The nutritional attributes of milk which make it an important part of the human diet are the same components that support the growth of many pathogenic bacteria that have been associated with milk and dairy products (2, 14). Contaminated milk and dairy products have been associated with foodborne illness caused by *Salmonella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, and most recently, *Escherichia coli* O157:H7 (15, 17). Thermally processed fluid milk has also been implicated as a source of human illness where inadequate pasteurization and pretreatment contamination were considered major contributing factors in many incidents (15).

*E. coli* O157:H7 has emerged with increasing frequency in the past decade as an important foodborne pathogen causing hemorrhagic colitis and hemolytic uremic syndrome (HUS) (7). Bovine products such as undercooked ground beef and unpasteurized milk have most often been associated with *E. coli* O157:H7 foodborne infections (6, 7, 8). Epidemiologic investigations have revealed that dairy cattle, especially young animals, are a principal reservoir of *E. coli* O157:H7 (1, 4, 9, 12, 19). Fecal contamination of milk is one likely route of transmitting *E. coli* O157:H7 to humans. Unpasteurized milk contaminated with *E. coli* O157:H7 has resulted in several milk-borne outbreaks of gastroenteritis, with several cases developing HUS (3, 11). Children in kindergarten visiting a dairy farm in southern Canada received fresh unpasteurized milk during a class outing and many of them were infected by this pathogen (16). An *E. coli* O157:H7 outbreak associated with consumption of pasteurized milk raised special concern about milk-borne transmission of this pathogen (16). Among more than 100 people infected in this outbreak, 46 were children under 15 years of age and 32 were under 5 years. Almost one-third of cases required hospital admission. Nine children from 9 months to 11 years of age developed HUS with 6 requiring dialysis, and one elderly woman developed thrombotic thrombocytopenic purpura. *E. coli* O157:H7 was isolated from a milk-handling pipe and the bottling machine in a local dairy plant, indicating that inadequate pasteurization or postpasteurization contamination was the likely factor responsible for the outbreak (16).

It is beneficial to know the survival and growth
characteristics of *E. coli* O157:H7 in milk to reduce its potential as a milk-borne disease agent. The purpose of this study was to determine the survival and growth characteristics of *E. coli* O157:H7 in unpasteurized and pasteurized milk at different temperatures.

**MATERIALS AND METHODS**

**Bacteria**

A five-strain mixture of nalidixic acid (50 μg/ml)-resistant *E. coli* O157:H7, including strain 932 (human isolate), E0122 (calf fecal isolate), C7927 (human isolate), E09 (meat isolate), and E0018 (calf fecal isolate) was used in this study. Each strain was grown separately in 150 ml of Trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 50 μg of nalidixic acid per ml (Sigma Chemical Co., St. Louis, MO) for 18 h at 37°C with agitation (150 rpm). The bacteria were sedimented by centrifugation (4,000 × g, 12 min), washed three times in 0.1 M phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Cells were adjusted with PBS to an optical density at 640 nm of 0.5 (approximately 10^8 CFU/ml). The five strains were combined at about equal concentrations. Cell counts of each individual strain and the five-strain mixture were determined by dilutions and subsequent enumeration on tryptic soy agar (TSA) (Difco). Two levels of inocula (10^3 and 10^5 CFU of *E. coli* O157:H7 per ml of milk for low and high inocula, respectively) were used.

**Milk**

Fresh unpasteurized milk was collected into sterile bags from the bulk tank of a local dairy farm. Pasteurized milk, including homogenized milk (3.5% fat), low-fat milk (2% fat), and skim milk (0% fat), was purchased from a local grocery store. The milk samples were held at 4°C with ice bags during transportation. Milk was used within 4 h after arrival at the laboratory. Before inoculation, each milk sample was tested for the presence of *E. coli* O157:H7 according to the procedure described by Zhao et al. (19). Aerobic plate counts (APC) of milk samples were determined by plating serial dilutions (1:10) of milk on plate count agar (PCA) (Difco) and incubating at 30°C for 48 h. Counts of bacteria in milk capable of on growing on sorbitol-MacConkey agar (SMAC) (Unipath, Oxoid Division, Ogdenburg, NY) with 0.1% 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma Chemical Co.) during incubation at 37°C for 24 h (SMAC bacteria counts) were determined. An inoculum (2 ml) of the *E. coli* O157:H7 five-strain mixture at the appropriate dilution was added to 198 ml of milk and mixed thoroughly in sterilized screw-cap bottles with agitation (150 rpm, 2 min) to obtain the desired bacterial populations.

**Incubation and sampling**

Inoculated milk samples in screw-cap bottles were held at 5, 8, 15, or 22°C. *E. coli* O157:H7 counts were determined at 0, 1, 2, 3, 4, and 7 days postinoculation and thereafter once every week until the pathogen could no longer be isolated after enrichment of samples in tryptic soy broth (TSB) (Difco). Milk samples (1 ml at each sampling) were serially diluted (1:10) in saline (0.85% NaCl) solution prepared from Millipore E-pure water and assayed for *E. coli* O157:H7 counts by directly plating 0.1 ml of each dilution in duplicate on SMAC-MUG with 50 μg of nalidixic acid. Plates were incubated at 37°C for 48 h. Sorbitol-negative, MUG-negative colonies were counted as presumptive *E. coli* O157:H7. Colonies typical of *E. coli* O157:H7 were randomly selected from plates (average five per plate) of the highest dilution and were confirmed by agglutination with an *E. coli* O157 antiseraum-coated latex test kit (Unipath) and H7 antiserum (Difco), and by biochemical tests using the API-20E miniaturized diagnostic kit (Analytab Products, Plainview, NY). When *E. coli* O157:H7 was not detectable by direct plating, samples were assayed for surviving cells by enrichment (18 h, 37°C, 150 rpm) in TSB with 50 μg of nalidixic acid per ml and subsequent plating on SMAC-MUG plates with the same concentration of nalidixic acid. The pH was measured by inserting an electrode of a pH meter (model 350, Corning Inc., Corning, NY) into 2 ml of milk sample at each sampling time. Milk samples were determined to be spoiled when visible coagulation and layer separation of milk occurred during incubation.

**Statistical analysis**

All microbiological assays were performed in duplicate and the entire study was duplicated. Mean populations of *E. coli* O157:H7 during the incubation period were subjected to an analysis of variance; the Duncan’s multiple range test (SAS Institute, Cary, NC) was used to determine statistical differences between treatments and among milk samples.

**RESULTS AND DISCUSSION**

The average initial APC of the unpasteurized and pasteurized milks were 4.7 × 10^3 CFU/ml and 2.3 × 10^1 to 2.6 × 10^1 CFU/ml, respectively. The average initial SMAC bacteria count was 9 CFU/ml in unpasteurized milk and <1 × 10^8 CFU/ml in the pasteurized milk samples. The average initial pH values of pasteurized and unpasteurized milks were 7.1 and 6.9, respectively. No *E. coli* O157:H7 was detected by using the enrichment method in any of the milk samples before inoculation.

At 5°C, *E. coli* O157:H7 did not grow and the population decreased about 1.5 to 2 log CFU/ml within 28 days of incubation (Figure 1). No significant differences (P > 0.05) in survival of *E. coli* O157:H7 were observed among any milk samples at this temperature. Spoilage of unpasteurized milk was observed within 21 days and no spoilage in 3 pasteurized milk samples occurred during 28 days at this incubation temperature. The observation that *E. coli* O157:H7 can not grow at 5°C in milk is consistent with our previous
studies (18). Growth of E. coli O157:H7 occurred at 8°C, with an approximate 1- to 2-log CFU/ml increase within the first 4 days and a 2- to 3-log CFU/ml increase within the first 7 days of storage (Figure 1). After day 7, E. coli O157:H7 populations in unpasteurized milk decreased, with about a 1.5-log CFU/ml reduction (Figure 1). In the 3 pasteurized milk samples at 8°C, E. coli O157:H7 populations ultimately increased to approximately $1.0 \times 10^3$ CFU/ml. E. coli O157:H7 populations decreased slowly after day 7, with about a 1-log CFU/ml decrease within the next 3 weeks. Spoilage was observed within 21 days in unpasteurized milk and no spoilage was observed in the 3 pasteurized milk samples.

About a 3- to 5-log CFU/ml increase in E. coli O157:H7 populations was observed at 15°C within the first 3 days (Figure 1). In 3 pasteurized milk samples, E. coli O157:H7 continued to grow to populations of greater than $1.0 \times 10^8$ CFU/ml at day 7 and remained constant during the remainder of the incubation period. Spoilage at 15°C was observed within 21 days for the 3 pasteurized milk samples and within 4 days for unpasteurized milk. There was no correlation between E. coli O157:H7 populations and the time of milk spoilage. No visible evidence of spoilage was observed between day 4 and day 14, although E. coli O157:H7 populations were very high ($>1.0 \times 10^8$ CFU/ml) in all 3 pasteurized milk samples (Figure 1). This finding suggests that E. coli O157:H7 may not produce overt signs of milk spoilage. No substantial changes (about 0.4 pH units) in pH were observed during incubation at 5 or 8°C (data not shown). At 15°C, the pH changed from 6.9 to 6.0 in pasteurized milks and 7.1 to 5.8 in unpasteurized milk within 28 days of incubation. At 22°C, E. coli O157:H7 populations increased rapidly during the first day of incubation (Figure 1). The pH decreased rapidly to less than 4.0 within 4 days, resulting in a decrease of E. coli O157:H7 to undetectable populations as determined by direct plating and enrichment) within 14 days for all milk samples (Figure 2). Spoilage at 22°C was observed within 1 and 4 days for unpasteurized and pasteurized milk samples, respectively (Figure 1).

The survival and growth characteristics of E. coli O157:H7 inoculated at $10^5$ CFU/ml in pasteurized low-fat milk and held at 5, 8, 15, or 22°C were very similar to those shown in Figure 1 for the $10^5$ CFU/ml inoculum (data not shown). Similar growth behaviors were observed in pasteurized homogenized and skim milk as well as unpasteurized milk. No significant differences ($P > 0.05$) in survival or growth of E. coli O157:H7 were observed among the 3 pasteurized milk samples, regardless of fat concentrations, at all temperatures throughout the study. E. coli O157:H7 grew at 8, 15, or 22°C more slowly ($P < 0.01$) in unpasteurized milk, which had a higher initial microbial count, than in pasteurized milks. It is likely that antagonistic activity from preexisting bacteria affected the survival and growth of E. coli O157:H7 in milk.

D’Aoust et al. (5) determined that heating unpasteurized milk at 72.0°C for 16.2 s inactivated a mixture of E. coli O157:H7, Yersinia enterocolitica, and Campylobacter spp. at populations of approximately $1.0 \times 10^8$ CFU/ml. Hence, E. coli O157:H7 will not survive high temperature–short time pasteurization treatment. Inadequate pasteurization or postpasteurization contamination are the likely explanations for E. coli O157:H7 infection acquired through consumption of pasteurized milk.

Results indicate that E. coli O157:H7 can grow in milk at 8°C, which is not an uncommon temperature for holding refrigerated milk at retail or in consumers’ homes. Palumbo et al. (13) determined that most E. coli O157:H7 isolates grow in brain heart infusion broth at 10°C and that some strains grow at 8°C. Three strains increased by 1,000-fold in 4 to 6 days at 10°C. E. coli O157:H7 strains used in our study could grow at 8°C, with populations increasing by 1- to 2-log CFU/ml within 4 days.

The most important control measures to ensure milk safety are proper pasteurization and avoiding postpasteurization contamination. However, storage temperature is also an important factor that influences the safety and quality of milk. Our data indicate that temperature abuse during shipping and handling can result in significant growth of E. coli O157:H7. Holding milk at ≤5°C is recommended to prevent growth of this pathogen.

REFERENCES


