

Behavior of *Escherichia coli* O157:H7 during the Manufacture and Aging of Gouda and Stirred-Curd Cheddar Cheeses Manufactured from Raw Milk

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ABSTRACT

This study was conducted to examine the fate of *Escherichia coli* O157:H7 during the manufacture and aging of Gouda and stirred-curd Cheddar cheeses made from raw milk. Cheeses were manufactured from unpasteurized milk experimentally contaminated with one of three strains of *E. coli* O157:H7 at an approximate population level of 20 CFU/ml. Samples of milk, whey, curd, and cheese were collected for enumeration of bacteria throughout the manufacturing and aging process. Overall, bacterial counts in both cheese types increased almost 10-fold from initial inoculation levels in milk to approximately 145 CFU/g found in cheeses on day 1. From this point, counts dropped significantly over 60 days to mean levels of 25 and 5 CFU/g in Cheddar and Gouda, respectively. Levels of *E. coli* O157:H7 fell and stayed below 5 CFU/g after an average of 94 and 108 days in Gouda and Cheddar, respectively, yet remained detectable after selective enrichment for more than 270 days in both cheese types. Changes in pathogen levels observed throughout manufacture and aging did not significantly differ by cheese type. In agreement with results of previous studies, our results suggest that the 60-day aging requirement alone is insufficient to completely eliminate levels of viable *E. coli* O157:H7 in Gouda or stirred-curd Cheddar cheese manufactured from raw milk contaminated with low levels of this pathogen.

Foodborne outbreaks of *Escherichia coli* O157:H7 infection have been associated with a wide range of food products, including raw and pasteurized milk and milk products (2, 6–8, 13, 15, 17, 21, 26, 28, 37) such as cheese (5–8, 18, 36). In the late 1990s, both the U.S. Food and Drug Administration (FDA) and Health Canada proposed bans on the use of raw milk in cheesemaking. The FDA's proposal was supported in part by published reports detailing the survival of *E. coli* O157:H7 beyond 60 days of aging in Cheddar cheese (12, 32). Although outbreaks involving other pathogens have occurred, the only domestic outbreak of *E. coli* O157:H7 illness linked to the consumption of cheese occurred in 1998 in Wisconsin, where vats used to make raw milk Cheddar cheese were inadvertently used to make fresh cheese curds. These curds were incorrectly labeled and sold as "pasteurized" and eventually sickened 55 people (5). The first confirmed outbreak of *E. coli* O157:H7 infection in Canada associated with raw milk hard cheese occurred in 2002. Aged Gouda cheese was found to be contaminated with the pathogen 104 days after production despite having met regulated microbiological and aging requirements (18).

In addition to those serotypes identified in outbreaks, numerous serotypes of Shiga toxin-producing *E. coli*,

including O157:H7, have been isolated from a variety of cheese types manufactured from raw milk (4, 27, 35). *E. coli* O157:H7 can readily contaminate raw milk on the farm because dairy cattle are a known reservoir of Shiga toxin-producing *E. coli*, including enterohemorrhagic strains such as serotype O157:H7 (40). Variable contamination rates up to 10% have been reported for serotype O157:H7 in bulk tank milk samples collected throughout North America (11, 20, 29).

Given the potential for contamination and the severity of the resultant toxicoinfection, numerous researchers have examined the behavior of *E. coli* O157:H7 during the manufacture and aging of various cheese varieties, including Cheddar (32, 33). The applicability of previous research on the survival of this pathogen in Cheddar cheese has been questioned based on faults in experimental design and variations in cheese composition and starting pathogen populations (12). In these challenge studies, strain-to-strain variation in survival was not examined, and only one method of manufacture was evaluated. According to the U.S. Federal Standards of Identity (21 CFR §133.113), Cheddar cheese can be prepared by a traditional cheddaring process or by any other procedure that produces a finished cheese with the same physical and chemical properties. The procedure of choice is often a stirred-curd method. Both Cheddar and Gouda cheeses belong to the family of uncooked, pressed cheeses.

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The objectives of this study were (i) to determine the survival of *E. coli* O157:H7 during the manufacture and aging of stirred-curd Cheddar cheese manufactured from raw milk, (ii) to examine the variation in behavior among three *E. coli* O157:H7 strains, (iii) to examine the survival of a single outbreak strain of *E. coli* O157:H7 during the manufacture and aging of Gouda cheese manufactured from raw milk, and (iv) to determine whether milk quality and cheese composition are correlated with pathogen survival.

MATERIALS AND METHODS

Bacterial strains and milk preparation. Three strains of *E. coli* O157:H7 were utilized individually for Cheddar cheese-making trials: C7927, CWD EC1, and CWD EC2. Based on the results obtained in the Cheddar trials, a single strain (CWD EC2) was utilized in the Gouda trials. Strain C7927, a human isolate from an apple cider-associated outbreak, was obtained from the FDA Center for Food Safety and Applied Nutrition (College Park, MD). Strain CWD EC1 was isolated from raw goat's milk collected from a farmstead cheese operation in 2006 (10). Strain CWD EC2, associated with a 2002 outbreak linked to Gouda cheese (18), was kindly provided by the Provincial Health Laboratory of Alberta, Canada. Frozen stock cultures were grown in tryptic soy broth supplemented with 6% yeast extract (TSBYE; Difco, BD, Sparks, MD) for 24 h at $37 \pm 1^\circ\text{C}$. A loop ($\sim 10 \mu\text{l}$) was then used to inoculate 9 ml of TSBYE for two successive inoculations before the culture was used. Following regrowth, cultures were serially diluted and inoculated into approximately 9 liters of unpasteurized cow's milk (obtained from the Paul Miller Research Complex dairy farm, University of Vermont, Burlington) in sanitized 2-gallon (7.6-liter) plastic containers to achieve the target population of $\sim 20 \text{ CFU/ml}$. Inoculated raw milk was then stored overnight in a refrigerator at 4°C for use the following morning.

Cheddar cheese manufacture. Stirred-curd Cheddar cheese was manufactured from raw milk inoculated with *E. coli* O157:H7 on 12 different days. When possible, multiple trials using different strains of *E. coli* O157:H7 were conducted on a single day. A total of 19 experimental cheeses (6 with strain C7927, 7 with strain CWD EC1, and 6 with strain CWD EC2) were selected for use in the study. Temperature and acidification profiles during cheese manufacture were monitored and recorded using a temperature and pH data logger (model DO 9505, Delta Ohm, Padua, Italy).

For each trial, inoculated raw milk (pH 6.7 to 6.8, titratable acidity of 0.17 to 0.20%) was removed from the refrigerator and added to a sanitized 10-liter rectangular stainless steel jacketed cheese vat (model FT20, Armfield, Hampshire, UK). Milk was raised to 32°C and held at this temperature for at least 30 min after the addition of the freeze-dried direct set lactic acid starter (RA 021 or RA 022, Danisco A/S, Copenhagen, Denmark), which was added at 6 Danisco culture units (DCU) per 100 liters. These cultures both contain *Lactococcus lactis*, *Lactococcus cremoris*, and *Streptococcus thermophilus*. Calf rennet (16 ml/100 liters, strength 1:15,000; New England Cheesemaking Supply, Ashfield, MA) diluted 1:4 in sterile deionized water was then added, and the milk was stirred for 30 to 60 s. Cutting time was determined with the following equation: cutting time = time of flocculation + (time of flocculation $\times 1.5$). Once desired firmness was reached, the coagulum was cut into curds (0.5 by 0.5 cm) with sterile wire curd knives (16 by 11 cm). Curds were allowed to settle for 2 min and then stirred while the temperature was raised 1°C every 4 min until a temperature of $39 \pm 0.5^\circ\text{C}$ was reached. Stirring was then continued until a target pH of 5.8 ± 0.05 was reached, at which

point the whey was drained. The remaining curds were stirred until they reached a pH of 5.5 ± 0.05 , at which point they were dry salted using the formula described by Van Slyke and Price (38) for normal salting and mixed for 5 to 10 min to assure even distribution of salt. Curds were then transferred to a plastic hoop (10 by 14 cm; ARO-079, Tomme 1000, Fromagex, Rimouski, Quebec, Canada) lined with sanitized disposable cheese cloth (Sani-net, 057-4008, Nelson Jameson, Marshfield, WI), which was cut to fit. Curds were then pressed overnight in a cheese press (E28, New England Cheesemaking Supply) at approximately 236 g/cm^2 . Cheeses were removed from the hoop when the target pH of 5.2 ± 0.1 was reached. Cheeses were then vacuum sealed (Multivac C100, Wolfertschwend, Germany) in standard 3-mil-thick vacuum pouches (810VP-1, Butcher & Packer Supply Co., Detroit, MI) at $8 \pm 4 \text{ mbar}$ and stored at $9 \pm 1^\circ\text{C}$ in a refrigerator (model WC491BG, Avanti, Miami, FL).

Gouda cheese manufacture. Gouda cheese was manufactured from raw milk inoculated with *E. coli* O157:H7 strain CWD EC2 as previously described for Cheddar cheese with the following modifications on 3 successive days for a total of three trials yielding three cheeses. Milk was first standardized to a target protein:fat ratio of 1.07 through the addition of 1.1 liters of pasteurized skim milk to 7.6 liters of raw milk, with the assumption that the protein content of the raw and skim milks was similar and that differences would be negligible given the small proportion of skim milk added. Three liters of milk was then added to the vat, and 0.44 g of granular lysozyme HCl muramidase (Lysolac, Fonros S.A., Lugano, Switzerland) rehydrated 1:10 in sterile deionized water was added, followed by the addition of the remaining 5.7 liters of milk, for a final lysozyme concentration of 5 $\mu\text{g/ml}$. Lysozyme was added to control late blowing defects encountered in preliminary trials. With only three trials, no rotation of starters was employed, and only RA 021 and LH 100 (Danisco) were utilized at 6.25 and 0.75 DCU/100 liters, respectively. LH100 consists of *Lactobacillus helveticus* and *Lactobacillus lactis* subsp. *lactis*. After approximately 40 min of ripening at 30°C , undiluted microbial rennet was added (9.5 ml/100 liters, 1:30,000; Danisco). Cutting time was determined by multiplying the time of flocculation by 1: time of flocculation + (time of flocculation $\times 1$). The resulting coagulum was cut in the same manner as for Cheddar cheese. Curds were allowed to settle and rest for 1 min and then stirred for 30 min. After stirring, approximately 2.6 liters of whey was removed ($\sim 30\%$ of the initial milk volume), and approximately 1.8 liters of sterile tap water ($38 \pm 2^\circ\text{C}$) was added ($\sim 21\%$ of initial milk volume) to achieve a diluted whey titratable acidity of 0.08%. The temperature was then raised to $38 \pm 0.5^\circ\text{C}$, and stirring was continued for approximately 30 min at which point the curds were prepressed with light pressure under the whey for 10 min. The whey was then drained, and the matted curd was cut and placed in a plastic hoop and pressed overnight as previously described with a slightly lower pressure of approximately 177 g/cm^2 . Cheeses were removed from hoops when the target pH of 5.16 ± 0.05 was reached and transferred to a saturated NaCl solution adjusted to pH 5.2 ± 0.05 with acetic acid (5% acidity) and brined for 3.5 h/lb (7.7 h/kg). Cheeses were allowed to dry in a Biosafety cabinet and then vacuum sealed. Gouda cheeses were stored at $14 \pm 1^\circ\text{C}$ for the first 5 weeks and $9 \pm 1^\circ\text{C}$ for the remainder of the aging period.

Microbiological sampling. For Cheddar trials, milk and cheese samples were aseptically collected during the cheese manufacturing process at the following steps: raw milk before inoculation, inoculated milk after overnight refrigeration but before

use, milk before coagulation, curd after cutting, whey at the start of draining, curd at end of draining before salting, and cheese after overnight pressing (day 1). For Gouda trials, milk and cheese samples were taken at the following points: raw milk before inoculation, inoculated milk after overnight refrigeration but before use, milk before coagulation, curd after cutting, whey removed, whey after addition of wash water, curd before pressing, cheese before brining, and brined cheese (day 1). Whey inadvertently collected during removal of curd after cutting was poured off before addition of diluent as described below. Finished cheese samples were obtained by cutting each wheel in half with a sterile knife and collecting four wedge samples, which were placed together in a sterile Whirl-Pak bag (Nasco, Ft. Atkinson, WI). The 25-g analytical samples were taken from these bags. The remaining halves of the cheese wheels were vacuum sealed and stored as previously described. Two wedge samples from each half were removed and pooled at subsequent sampling points. After collection, samples were immediately analyzed for the presence of *E. coli* O157:H7, including the determination of population levels as described below. Cheese samples were collected during the aging period on days 7, 14, 21, 28, 35, 42, 60, 72, 90, 104, and 120 and then every 30 days until *E. coli* O157:H7 was no longer detected through direct plating of 100 μ l of enrichment culture, as described below.

Microbiological analysis. Milk standard plate counts (SPCs) and coliform counts (CCs) were determined on Petrifilm aerobic count (AC) plates and CC plates (3M Microbiology, St. Paul, MN) incubated at $32 \pm 1^\circ\text{C}$ for 48 and 24 h, respectively. Resulting milk counts from AC plates were rounded off to two significant figures at the time of conversion to SPC. For the detection and enumeration of *E. coli* O157:H7 in inoculated raw milk, milk before coagulation, and all whey samples, 1 ml of each sample type was plated in duplicate on four plates (250 μ l per plate) of CHROMagar O157:H7 (DRG International, Mountainside, NJ), which were then incubated for 24 h at 37°C . For the detection and enumeration of *E. coli* O157:H7 in curd and cheese samples, 25-g samples were placed in Whirl-Pak bags (Nasco), diluted 1:5 in 100 ml of room temperature enterohemorrhagic *E. coli* broth (EEB), and stomached in a Stomacher 400 circulator (Seward Limited, Worthington, UK) for 3 min and then by hand until sufficiently homogenized. One milliliter of this homogenate was plated in duplicate on four plates (250 μ l per plate) of CHROMagar O157:H7 and incubated as previously described. An additional 125 ml of EEB was then added to the homogenate to attain a 1:10 dilution, and this mixture was incubated for 24 h at 37°C for enrichment. To detect the presence of *E. coli* O157:H7 when no typical colonies were present for enumeration, 100 μ l of the enriched sample was streaked onto CHROMagar O157:H7 for isolation and incubated for 24 h at 37°C . *E. coli* O157:H7 levels in cheese were considered culturally undetectable when the pathogen could not be cultured after enrichment from samples collected at four successive sampling points, at which point sampling and analyses were terminated. Presumptive colonies enumerated or detected on CHROMagar O157:H7 were randomly purified on tryptic soy agar with 6% yeast extract (TSAYE; BD) and incubated for 48 h at 37°C for further confirmation. Purified colonies were subcultured in 9 ml of TSBYE that was incubated for 24 h at 37°C . Five-microliter aliquots of TSBYE culture were then analyzed by automated PCR using the *E. coli* O157:H7 assay for the BAX PCR system (DuPont Qualicon, Wilmington, DE) according to the manufacturer's instructions.

Physicochemical analysis of cheese. The following physicochemical analyses were conducted on the milk before cheese manufacture and on the cheese after dehooping in duplicate: fat

(Babcock method), pH (Accumet Research AR150 with a flat tip electrode, Accumet Reference 13-620-46, Fisher Scientific International, Hampton, NH), total solids (TS; after drying to constant weight at 102°C), and salt (Chloride Analyzer 926, Nelson Jameson, Marshfield, WI). Salt in moisture phase (SM), moisture nonfat substance (MNFS), and fat in dry matter (FDM) were determined using the following formulae:

$$\text{SM} = [\text{salt}/(100 - \text{TS})] \times 100$$

$$\text{MNFS} = [(100 - \text{TS})/(100 - \text{fat})] \times 100$$

$$\text{FDM} = (\text{fat}/\text{TS}) \times 100$$

Titrateable acidity (0.1 M NaOH, phenolphthalein indicator) was determined for the milk before and throughout manufacture. The targets for Cheddar cheese composition as suggested by Lawrence et al. (23) included pH of 5.1 to 5.3, 52 to 54% MNFS, 52 to 56% FDM, and 4.7 to 5.7% SM. The following composition was considered acceptable (23): pH of 5 to 5.4, 50 to 56% MNFS, 50 to 57% FDM, and 4 to 6% SM. The targets for Gouda cheese composition were pH of 5.1 to 5.4, 55 to 61% MNFS, 46 to 50% FDM, and 4 to 6% SM.

Statistical analysis. Enumeration data were log transformed for all analyses. The resulting data were analyzed using SPSS for Windows (version 17.0.1, SPSS Inc., Chicago, IL). Between-strain differences in the means of dependent variables, including bacterial counts and physicochemical parameters, were analyzed using the GLM procedure analysis of variance. Correlations between bacterial counts and physicochemical parameters were determined using Pearson's correlation. Where appropriate, the effects of strain, time, and strain over time and cheese type, time, and type over time on *E. coli* O157:H7 counts were determined using the GLM procedure for repeated measures. Regression analyses of counts over time during aging were calculated using log-transformed mean counts because of the presence of null values.

RESULTS

Cheddar and Gouda cheeses were manufactured from raw milk experimentally contaminated with *E. coli* O157:H7. Resulting Cheddar and Gouda cheeses ranged in weight from ~650 to 750 and ~775 to 800 g, respectively. *E. coli* O157:H7 was not detected in any milks used for cheese manufacture before inoculation. Mean log SPCs and log CCs did not differ significantly between Cheddar cheeses when grouped by strain of inoculated *E. coli* O157:H7. With the data from all three strains pooled, mean ($\pm$ standard deviation [SD]) SPC and CC were 2.6 ± 0.3 and 1.4 ± 0.4 log CFU/ml, respectively, for Cheddar cheese trials. Mean SPC and CC were 2.79 ± 0.09 and 2.09 ± 0.12 log CFU/ml, respectively, for Gouda trials. Although log SPC values did not differ significantly between cheese types, log CC values were significantly higher in the milk utilized in the manufacture of Gouda. Overall, levels of natural milk flora rarely correlated with counts of *E. coli* O157:H7 observed during the manufacture or aging periods. In Gouda cheese, log CCs were negatively correlated (0.999 , $P = 0.033$) with pathogen levels in fresh cheese (day 1) and log SPCs were positively correlated (0.999 , $P = 0.026$) with the mean duration of pathogen survival.

Cheese composition. According to U.S. Federal Standards of Identity (21 CFR §133.113), Cheddar cheese

TABLE 1. Percent moisture, moisture nonfat substance (MNFS), fat in dry matter (FDM), and salt in moisture phase (SM) of experimental cheeses^a

Characteristic	Cheddar	Gouda
Moisture (%)	30.82 ± 0.24 A	47.41 ± 1.22 B
MNFS (%)	52.10 ± 0.95 A	55.86 ± 0.71 B
FDM (%)	53.02 ± 0.82 A	47.86 ± 0.05 B
SM (%)	5.52 ± 0.30 A	4.79 ± 0.34 B

^a Values are mean ± SD. Within rows, means with different letters differ significantly at $P < 0.001$.

must contain at least 50% FDM and must be less than or equal to 39% moisture by weight. All experimental Cheddar cheeses met these requirements at dehooping (Table 1). Mean moisture, MNFS, FDM, and SM did not differ significantly between trials when analyzed separately by *E. coli* O157:H7 strain, so data from all three strains combined are displayed in Table 1. Although changes in natural milk flora did not appear to have any major effect on the physicochemical aspects of this cheese, log CC was positively correlated with MNFS (0.459, $P = 0.048$). As with other compositional parameters, pH at dehooping did not differ significantly between trials when grouped by strain with a mean of 5.23 ± 0.07 . Despite the large variance in log SPC, a strong negative correlation (-0.549 , $P = 0.015$) was observed between log SPC and pH of Cheddar cheese at dehooping. Overall four, four, and three trials using strains C7927, CWD EC1, and CWD EC2, respectively, were within our ideal target range for physicochemical characteristics. No differences in pathogen population levels in fresh or aged cheese were observed between trials within or outside this target range.

The FDA standard for Gouda cheese (21 CFR §133.142) states that the minimum FDM is 46% with a maximum moisture content of 45%. All experimental Gouda cheeses met the requirements for FDM after brining (Table 1). At this phase Gouda cheeses were slightly high in moisture but were within range by the theoretical time of sale and continued to decrease, reaching a mean of $39.75 \pm 0.66\%$ by day 290. Log CC was negatively correlated with moisture (-1 , $P = 0.014$) as was MNFS (-1 , $P = 0.006$). Mean pH at dehooping was 5.16 ± 0.03 .

Behavior of *E. coli* O157:H7 during cheese manufacture. A significant effect of time on *E. coli* O157:H7 counts from initial milk inoculation to fresh Cheddar cheese on day 1 was observed ($P = 0.003$). However, no significant effect of *E. coli* O157:H7 strain employed on counts observed was found at any sampling point, so the data for all three strains were pooled for further analysis. The results of all strains combined are displayed in Table 2. In 9 of the 19 Cheddar trials, *E. coli* O157:H7 colonies were not observed through direct plating of whey because of overgrowth by background flora. Overgrowth also was an issue in the direct plating of curd samples taken before salting in seven of those nine trials. These trials were not included in the analyses.

TABLE 2. *Escherichia coli* O157:H7 counts during the manufacture of stirred-curd Cheddar and Gouda cheeses^a

Sample	<i>E. coli</i> O157:H7 (CFU/ml or g)	
	Cheddar	Gouda
Milk	18 ± 2 A	23 ± 3 A
Milk before coagulation	22 ± 3 A	28 ± 4 A
Curd after cutting	44 ± 7 B	65 ± 19 AB
Whey removed ^b	NA	3 ± 1
Whey plus wash water ^b	NA	5 ± 2
Whey at draining ^b	20 ± 6	NA
Curd before salting	236 ± 55 C	NA
Curd before pressing	NA	382 ± 94 B
Cheese before brining	NA	988 ± 376 C
Cheese (day 1)	144 ± 47 C	145 ± 20 B

^a Values are mean (± SEM). Within columns, means with different letters differ significantly at $P < 0.05$. NA, not applicable.

^b Values obtained for whey samples were not included with those from curd and cheese in comparative analyses.

Levels of *E. coli* O157:H7 did not differ significantly between cheese types at any stage during manufacture. Similarly, there was no effect of cheese type on the change in *E. coli* O157:H7 levels over time during these manufacturing steps.

Behavior of *E. coli* O157:H7 during the aging of cheese. A significant effect of time on the change in mean (± standard error of the mean [SEM]) counts of *E. coli* O157:H7 in Cheddar cheese was observed ($P = <0.0001$) from day 1 to day 60 from 144 ± 47 to 25 ± 9 CFU/g for all strains combined (Fig. 1). Although the decrease in pathogen population levels during this time period did not differ significantly by strain, individual survival curves for each strain are displayed in Figure 2. Pathogen levels continued to decline during aging and dropped below the detection limit for direct plating (≈ 5 CFU/g) for all trials after day 180. This decrease in *E. coli* O157:H7 counts from day 60 to day 180 was significant ($P < 0.001$). No significant differences in mean counts between strains were found at each individual sampling point or during the 180-day aging period overall. On average, levels of *E. coli* O157:H7 fell and stayed below the culture detection limit

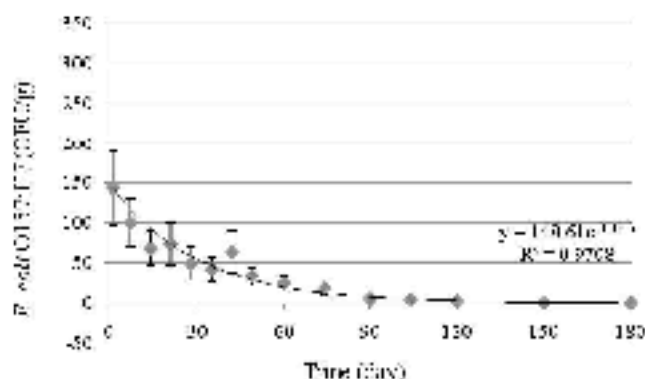


FIGURE 1. Counts (mean ± SEM) of *Escherichia coli* O157:H7 during the aging of stirred-curd Cheddar cheese (all strains combined).

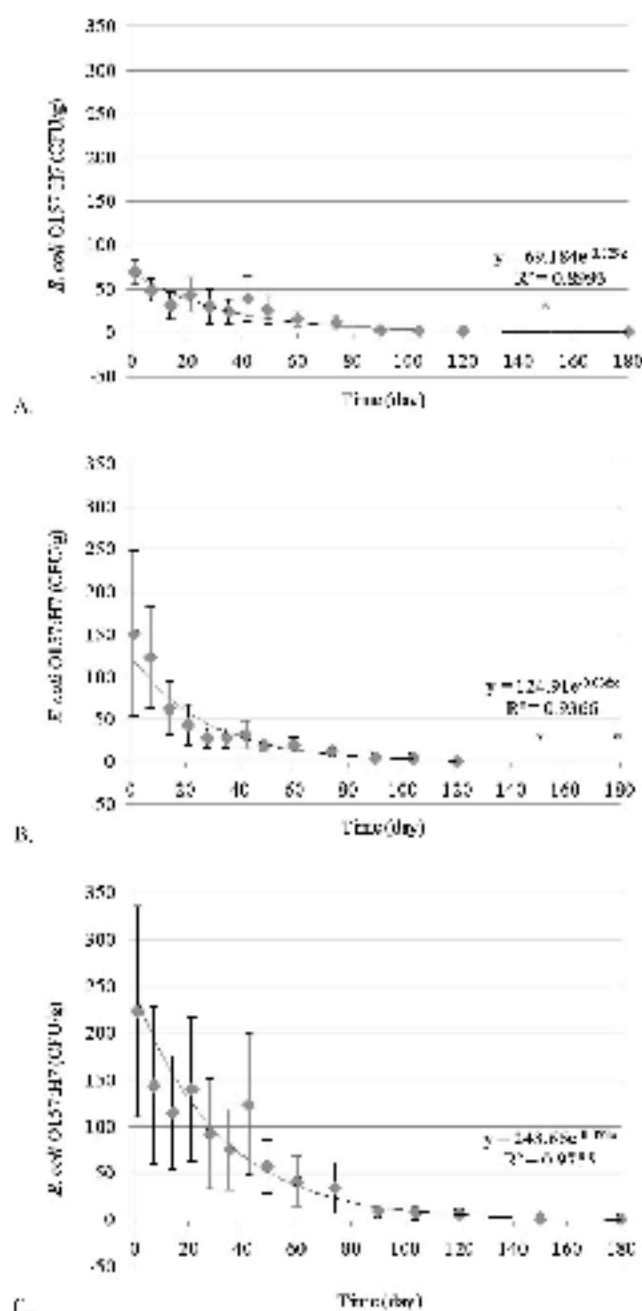


FIGURE 2. Counts (mean $\pm$ SEM) of *Escherichia coli* O157:H7 strains CWD EC1 (A), CWD EC2 (B), and C7927 (C) during the aging of stirred-curd Cheddar cheese. * Counts below enumeration limit but detectable after enrichment.

after 97 ± 53 , 112 ± 27 , and 116 ± 58 days for strains CWD EC1, CWD EC2, and C7927, respectively, or 108 ± 46 days for all three strains combined. With a culture detection limit of ≥ 5 CFU/ml, some cells may have remained in the cheese after this point, thus requiring enrichment for detection. After enrichment, *E. coli* O157:H7 was detectable by agar plating for a mean ($\pm$ SD) of 266 ± 56 , 337 ± 96 , and 295 ± 48 days for strains CWD EC1, CWD EC2, and C7927, respectively. The mean day at which *E. coli* O157:H7 became undetectable did not differ significantly between the three strains, with a survival time of 297 ± 72 days for all strains combined.

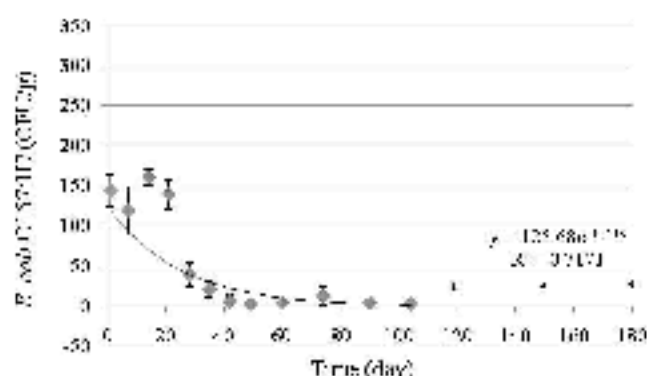


FIGURE 3. Counts (mean $\pm$ SEM) of *Escherichia coli* O157:H7 strain CWD EC2 during the aging of Gouda cheese. * Counts below enumeration limit but detectable after enrichment.

As observed in Cheddar cheese, mean ($\pm$ SEM) counts of *E. coli* O157:H7 in Gouda cheese dropped significantly ($P < 0.001$) from 145 ± 20 CFU/g on day 1 to 5 ± 3 CFU/g on day 60 (Fig. 3). Pathogen levels continued to decline during aging and dropped below the detection limit for direct plating after 120 days in all trials. The change in counts from day 60 to 120 was not significant. After falling below the detection limit, *E. coli* O157:H7 was detectable by enrichment and agar plating for a mean ($\pm$ SD) of 300 ± 30 days.

Levels of *E. coli* O157:H7 did not differ significantly between cheese types at any sampling point during aging through 180 days. Similarly there was no effect of cheese type on the change in *E. coli* O157:H7 levels during the 60-day aging period or through 180 days. Accordingly, the average duration of survival did not differ by cheese type.

Correlations. Because of limited data, strong correlations were not found between physicochemical measurements and pathogen levels in milk, curd, and finished cheese samples (day 1) for both cheese types. However, a strong positive correlation was found between MNFS and *E. coli* O157:H7 counts in Cheddar cheese on day 1 of sampling (0.537 , $P = 0.018$) and numerous subsequent sampling days up to day 150, as well as the mean duration of pathogen survival (0.487 , $P = 0.035$) in this cheese. Similarly, a strong positive correlation was found between dehooping pH and pathogen levels in the cheese on day 1 (0.561 , $P = 0.037$) but at no other sampling points. The pH measured at dehooping (0.505 , $P = 0.027$) was strongly correlated with the time at which *E. coli* O157:H7 was deemed undetectable. Similar to the situation in Cheddar cheese, a strong positive correlation was found between MNFS and *E. coli* O157:H7 counts in Gouda cheese on day 1 of sampling (0.999 , $P = 0.027$) and on numerous subsequent sampling days up to day 104.

DISCUSSION

In this study, the fate of *E. coli* O157:H7 was examined during the manufacture and aging of Gouda and stirred-curd Cheddar cheeses made from raw milk. Assuming that *E. coli* can contaminate raw milk at the time of collection, we opted to simulate the scenario of milk contamination followed by

bulk tank refrigeration before cheesemaking. This is an important consideration because *E. coli* O157:H7 can survive and may be able to grow in raw milk at low temperatures (25).

Challenge studies have revealed that *E. coli* O157:H7 can survive the processing conditions used for a variety of cheeses, including Cheddar (19, 24, 31–34). However, previous studies on Cheddar cheese have employed unrealistically high starting populations that in some cases were not in compliance with current FDA standards (12). The microbiological quality of the raw milk utilized was typically not reported and certainly questionable. For example, the raw milk used for the manufacture of experimental cheese by Schlessner and colleagues (33) contained various levels of naturally occurring *E. coli* O157:H7. The applicability of previous results obtained by Reitsma and Henning (32) for the survival of this pathogen in Cheddar cheese also has been questioned because the experimental design in that study failed to consider the potential safeguards provided by the use of unpasteurized milk and an adequate SM content (12). The SM ratio directly influences water activity (a_w) and thus the rate of microbial growth in cheese. For example, in an effort to minimize the development of off-flavors, the target SM for Cheddar cheese is 4.5 to 5.5% (23). In the present study, cheese SM levels were typically within suggested ranges with only minor variation, so it was not surprising that we did not find any significant correlations between SM and levels of *E. coli* O157:H7 during aging in both Gouda and stirred-curd Cheddar cheese. Schlessner et al. (33) investigated the survival of a five-strain cocktail of *E. coli* O157:H7 in Cheddar cheese manufactured from unpasteurized milk and concluded that the results of these studies confirmed those of previous reports and indicate that a 60-day aging period is not adequate for eliminating *E. coli* O157:H7 in cheese. However, in addition to SM, other critical and related parameters that must be controlled in the production of high-quality Cheddar cheese, including MNFS, FDM, and pH, varied considerably and at times were far out of the normal range. As observed in the present study, pH is positively correlated with levels of *E. coli* O157:H7 in fresh cheese. Schlessner et al. (33) stated that salt content was within typical range but were referring to total salt rather than SM. Although FDM is not necessarily a critical parameter, a high FDM is positively correlated with a high MNFS. Related to a_w , MNFS may be the most important parameter because it also is used as an indicator of the potential enzymatic and microbial activity during cheese ripening; in the present study, MNFS was positively correlated with pathogen levels and duration of survival. The manufacturing method employed in the present study differed from those used in previous studies of Cheddar cheese; the curds were stirred until desired acidity was attained rather than being “cheddared” (cut, turned, and stacked) and milled before salting and pressing.

Our results indicate that although counts of *E. coli* O157:H7 in milk did not change significantly through coagulation, population levels increased after expulsion of whey and contraction of the curd during heating in both

varieties. Increases can occur as a result of bacterial cell entrapment in the curd and population growth after a lag phase during the heating step of cheese manufacture (24). Increases in bacterial counts from initial levels in milk through ripening have been reported for numerous cheese varieties manufactured from raw and pasteurized milk, including Cheddar (1, 14, 16, 19, 24, 32, 33). *E. coli* O157:H7 levels increase during the manufacturing process (32, 33), with the majority of cells remaining entrapped in the curd and only minor losses to the whey (32). For Grana Padano, the heating stress incurred during the cooking of the curd affects *E. coli* O157:H7 viability (14). Despite a higher final temperature (62°C), the duration of heating employed in the manufacture of Indian cheese (Paneer) was not sufficient to inactivate *E. coli* O157:H7 (39). Heat inactivation is not likely to occur in Cheddar or Gouda because their manufacture is characterized by short heating times and low temperatures. Direct salting of the curds and pressing overnight did not appear to have an immediate effect on microbial counts in our experimental Cheddar cheese. Similarly, Reitsma and Henning (32) reported increases in *E. coli* O157:H7 populations after salting in pasteurized milk Cheddar with a low initial milk inoculation level (1 CFU/ml). In that study, pathogen levels in cheese increased after pressing in the high inoculation treatment (1,000 CFU/ml) but decreased in the low inoculation treatment. Overall, the levels reported by these investigators were similar in curd before salting and after pressing for both inoculation levels, in agreement with the observations in Cheddar in the present study. *E. coli* O157:H7 levels also remained unchanged through pressing in raw milk Cheddar inoculated at a level comparable to that employed in the present study (33 CFU/ml) (33). In contrast, reductions in levels observed in pasta filata cheeses after brining have been attributed to the increased susceptibility of *E. coli* O157:H7 to the antimicrobial effects of salt after heat-induced injury incurred during high temperature stretching (34).

Reductions in *E. coli* O157:H7 population levels observed during the aging (<30 days) of cheeses such as Colby and Romano are likely the result of prolonged exposure to low pH, the presence of starter culture, low temperature, and high salt concentration (19). Similar reductions from high initial milk inoculation levels to undetectable levels in cheese within 60 days of storage were observed in brined cheeses such as Feta and Telmes (16). However, in agreement with similar studies on Cheddar (32, 33), our results indicate that when present even at low levels in raw milk, this pathogen can survive a well-controlled cheese manufacturing process and remain viable in high-quality finished cheese during the aging period well beyond 60 days. Although levels fell below the detection limit in cheese with an initial milk inoculation of 1 CFU/ml, Reitsma and Henning (32) detected viable *E. coli* O157:H7 in Cheddar for up to 130 days (using an enrichment procedure) and for up to 158 days in cheese manufactured from milk inoculated at 1,000 CFU/ml. With an initial raw milk inoculation of ~1 CFU/ml, Schlessner and colleagues (33) detected *E. coli* O157:H7 after 210 days of aging, and

viable cells were found out to 240 days. Similarly, levels of *E. coli* O157:H7 remained above our detection limit (≥ 5 CFU/ml) for at most 180 days in the present study, as observed in Cheddar. We could still detect viable *E. coli* O157:H7 cells in cheese after more than 1 year of aging, as was reported by Schlessner and colleagues in their study of cheese with higher inoculation levels ($\sim 1,000$ to $\sim 100,000$ CFU/ml). Based on the mode of activity and the results of previous investigations (3, 30), the concentration of lysozyme utilized in the manufacture of Gouda cheese in the present study (50 $\mu\text{g/ml}$) would have no inhibitory effect on *E. coli* O157:H7.

Despite the incidence of *E. coli* O157:H7 in raw milk and its reported survival in cheese and the isolation from cheese at retail, it is not clear why the reported incidences of illness and outbreaks linked to raw milk cheeses are so low. It is possible that the incidence of *E. coli* O157:H7 in raw milk used for the manufacture of raw milk cheese is much lower than that reported for milk in general. In two surveys conducted in 2006 and 2008, a combined total *E. coli* O157:H7 incidence of 0.43% (1 of 234 samples) was found (9, 10). Similarly, the incidence of this pathogen in cheese may be lower than expected; in the FDA's domestic and imported cheese compliance program, only three cheese samples were positive for *E. coli* O157:H7 from a total of 3,360 samples tested between 1 January 2004 and 31 December 2006 (unpublished data). In the case of raw milk cheeses, this low incidence could be a function of a low incidence in milk used for cheesemaking or contamination levels that are sufficiently low to facilitate elimination during aging. Because of the variation in host susceptibility, a number of individuals exposed to cheeses contaminated with *E. coli* O157:H7 may develop mild forms of illness that go unreported. Human and bovine isolates of *E. coli* O157:H7 from the two distinct lineages that are disseminated within the United States may be differentially distributed, suggesting that one of these lineages may be less virulent for humans or may not be efficiently transmitted to humans from bovine sources (22). Alternatively, the expression of virulence within the cheese matrix may be altered over time by prolonged exposure to salt, acid, low temperatures, low moisture, and the presence of starter culture. This area of research needs further investigation.

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