Behavior of Enteropathogenic *Escherichia coli* During Manufacture and Ripening of Brick Cheese

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**ABSTRACT**

The ability of enteropathogenic *Escherichia coli* (EEC) to grow and survive during the manufacture and ripening of brick cheese was determined. Pasteurized milk artificially contaminated with EEC was used to make cheese by the washed-curd method. EEC was enumerated by surface plating samples on Trypticase Soy Agar (TSA) with an overlay of Violet Red Bile Agar (VRB), and by using VRB Agar pour plates. EEC increased 1000-fold during the manufacturing process and numbers decreased slowly during ripening while the smear developed and during refrigerated storage of cheese. Seven weeks after manufacture, numbers of EEC in cheese ranged from 700 to 20,000/g with initial contamination of milk at about 500/ml. Limited growth of EEC on the surface of brick cheese occurred during ripening and these bacteria were inactivated slowly during storage. Counts of EEC obtained with VRB Agar pour plates were 6-59% as large as counts obtained using TSA surface plating with VRB Agar overlay.

Foodborne illness associated with *Escherichia coli* has recently become a public health concern (12,13), although enteropathogenic *E. coli* (EEC) has been most often associated with outbreaks of infantile diarrhea in nurseries (20) and with outbreaks of travelers' diarrhea (3). There are two types of enteropathogenic *E. coli* capable of causing foodborne disease. Enterotoxin producing strains cause a cholera-like illness, and invasive strains cause a *Shigella*-like illness (dysentery). The incidence of EEC foodborne illness is difficult to estimate because of problems in diagnosis of the disease (21). Also, presence of EEC in our food supply has not been adequately determined because of problems in isolating the organisms (14) and in determining pathogenicity of isolates (13).

Presence of EEC in some dairy products can be of public health significance as evidenced by an outbreak in 1971 of gastroenteritis associated with Camembert cheese that contained EEC (12). The ability of EEC to grow during initial stages of Camembert cheese manufacture, and subsequent survival of the bacteria in the ripening cheese have been reported by Fantasia et al. (5), Frank et al. (8), and Park et al. (16). Growth of EEC on the surface of ripening Camembert cheese has also been observed (8). Behavior of *E. coli* in skim milk fermented by lactic acid bacteria has been described by Frank and Marth (6,7). The combination of high starter concentration and low incubation temperature was most effective of the variables tested in controlling growth of *E. coli*.

Although there are no documented outbreaks of foodborne illness caused by EEC and associated with surface-ripened semisoft cheese, Olson (15) has noted that these varieties of cheese have some characteristics which are cause for concern. Included are slow acid development during manufacturing, initial low salt concentrations at the interior of the cheese, and relatively high ripening temperatures. "Sweet curd" brick cheese was chosen for this study as representative of semisoft cheeses because it has all the characteristics just described. By manufacturing brick cheese with pasteurized milk artificially contaminated with EEC, we hoped to help evaluate the safety of the brick cheese manufacturing process.

**MATERIALS AND METHODS**

** Cultures**

EEC cultures used in this study were an enterotoxigenic strain, B2C, and two invasive strains, 1624 and 4608. Lactose is fermented slowly by strain 1624. These strains were obtained from the FDA, Washington, D.C. Cultures of *E. coli* were grown in nutrient broth at 37 C for 24 h before use as an inoculum. Enough culture was added to coliform-free pasteurized milk to provide about 500 CFU/ml. The starter culture used was *Streptococcus lactis* 4175 from Marschall Div., Miles Lab., Madison, Wisconsin. The starter culture was incubated at 21 C for 20-22 h before cheesemaking; the skim milk was coagulated under these conditions. A 0.25% inoculum of starter was used.

**Manufacture of cheese**

Brick cheese was manufactured by the washed-curd method described in detail by Price and Buyens (17) and Olson (15). Starter, rennet, and coliform were added simultaneously to 21 kg of pasteurized whole milk tempered at 30 C. Approximately 30 min later, coagulated milk was cut using 6-mm knives. Ten minutes later, curd was slowly heated to 36.5 C (this took 25 min). After 15 min at 36.5 C, whey was drained to 50% of the original volume of milk, and the same amount of water was mixed with the curd. This mixture was stirred for 30 min at 36.5 C. Then the whey-water mixture was drained and curd was put into one hoop. The hoop was turned periodically for 6 h with a 2.3-kg weight placed on top for a 2-h period. Eight hours after the start of manufacture, cheese was placed in a salt (22%) brine at 15.5 C for 24 h. Cheese was removed from the brine and ripened quiescently at 15.5 C for 2 weeks in an incubator with high humidity. The block was turned and the surface was rubbed with 0.5% salt solution each day of...
After ripening, the surface smear was gently washed from the block. Cheese was allowed to dry and then was cut into six pieces. Each piece was wrapped in Saran, heat sealed, and stored at 7°C for up to 5 weeks. Duplicate trials were made with each strain of E. coli. Results are reported as average values.

**Surface inoculations**

E. coli was inoculated on the surface of blocks of coliform-free cheese ripened for 1 week. The surface of each block was marked into areas of 20 cm² each and each area was inoculated with 0.1 ml of water containing approximately 5000 CFU. Excess water on the surface of the block from the inoculum was allowed to dry before cheese was returned to the ripening chamber. Thereafter, cheese was treated normally. After one additional week of ripening, the surface smear was washed from cheese and before packaging, inoculated areas were reincubated with the same strain and amount of E. coli as used initially. At each sampling time, analyses were done on two 20-cm² areas. The average of the two is reported. To enumerate E. coli, each 20-cm² area was cut from the block deep enough to give 20 g of cheese. This was blended with 180 ml of sterile 2.0% sodium citrate solution. Appropriate serial dilutions were made in 2.0% sodium citrate.

**Enumeration of E. coli**

Two methods were used to enumerate E. coli. The first was that suggested by Speck et al. (22) to enumerate sublethally injured coliforms. It involves surface plating the sample on Trypticase Soy Agar (TSA, Difco) and a 1-h incubation at room temperature followed by adding an overlay of Violet Red Bile Agar (VRB, Difco). Incubation was then at 37°C for 24 h. This method is referred to as the TSA + VRB surface plating method, and was used to enumerate E. coli in all experiments. The second method used VRB Agar pour plates with incubation at 37°C for 24 h. This method was used only with trials that involved inoculated milk.

Cheese samples for enumeration of E. coli were diluted by adding 20 g of cheese to 180 ml of sterile 2.0% sodium citrate solution and blending with a Waring Blender operating at low speed for 3 min. Serial dilutions were made using 9.0 ml of sterile sodium citrate solution. Cheese was sampled by taking a rectangular shaped cross-section of the cheese block to include portions both at the surface and the interior of the block.

**Measurement of pH and moisture**

These analyses were done with the same methods and equipment as described in a previous paper (8). Cheese contained 41.7-43.8% moisture and developed an apparently normal surface smear during ripening.

**RESULTS AND DISCUSSION**

**Growth of EEC during manufacture of cheese**

Both strains 1624 and 4608 increased approximately 1000-fold in numbers during the first 7 h of cheese manufacture (Fig. 1). The day after manufacture numbers of these strains began a slow decline. Strain B2C had a different pattern of growth, increasing 100-fold in the first 7 h, but then increasing further after 24 h before numbers began to decline. The pH value for cheese manufactured during one of the trials with strain B2C was higher than normal after 7 h (S.9). The increase in numbers occurring in this trial was greater than when the pH of the cheese was normal. This accounts for some of the growth between 7 and 24 h which did not occur with the other strains.

The 2- to 3-log increase in numbers of E. coli during the initial hours of cheese manufacture is partially the result of concentration by entrapment of cells in curd. However, entrapment would account for only a 10-fold increase in numbers. The pH of cheese after 48 h in nearly all instances are approximately 5.2. Behavior of these strains of EEC during fermentation of skim milk at 32 and 21°C, and during manufacture of Camembert cheese has been previously reported (6,8). The amount of growth occurring during the initial hours of brick cheese manufacture was about 10 times greater with strains 4608 and 1624 than occurred during the initial hours of Camembert cheese manufacture. This additional growth may have occurred because of the higher temperature and less rapid decline in pH during brick cheese manufacture than during manufacture of Camembert cheese. Data in Fig. 1 show that inhibition of EEC occurred at pH 5.2-5.5, whereas during Camembert cheese manufacture these same strains were inhibited at pH values of 5.0-5.2. It has been previously demonstrated that inhibition of pathogens by lactic acid bacteria is not solely dependent on acid production and pH (1,18).

**Survival of EEC during ripening and storage of brick cheese**

Numbers of EEC strains 1624 and 4608 decreased by 90-95% during the 2-week ripening period (Fig. 2). Strain B2C decreased by only 50% during the same time. This decrease may not be significant since the coefficient of variation of these data is 45%. Upon refrigeration of the cheese after ripening (data from 2-7 weeks, Fig. 2), each strain decreased only slightly in numbers. These strains...
of EEC were inactivated more rapidly in Camembert than in brick cheese (8). This probably is related to the lower pH of unripened Camembert cheese. When brick cheese samples ranged in pH from 5.15 to 5.3, little difference was found in survival of the coliform. Most brick cheese is at a pH of 5.3 after 2 weeks of ripening (15).

Populations of EEC in brick cheese after 7 weeks were 20,000, 2,000, and 700/g for strains B2C, 4608, and 1624, respectively. The number of bacteria necessary to cause illness, as estimated through feeding studies, is $10^6$ to $10^8$ for invasive EEC and $10^8$ to $10^9$ for enterotoxigenic EEC (13). If one assumes ingestion of 100 g of cheese, brick cheese made from milk contaminated with $10^3$ to $10^4$ CFU/ml of strains B2C or 4608 could cause illness. There is also the possibility of toxigenic strains of EEC producing enterotoxins during growth, then declining in numbers and leaving the enterotoxin behind to cause illness. This possibility is yet to be investigated.

Survival of EEC on the surface of brick cheese

Strain B2C was able to grow during the second week of ripening when inoculated onto the surface of cheese (the first week after inoculation, Fig. 3). Subsequently, there was a general decrease in numbers and after 7 weeks the population of strain B2C was about the same as the initial inoculum. Growth of strain 4608 was more limited than that of strain B2C, and after 6 weeks strain 4608 was at about 10% of the initial inoculum. The behavior of these strains of E. coli on the surface of brick cheese contrasts with their ability to grow by 3 to 4 log cycles on the surface of Camembert cheese (8). The major difference in the environment at the surface of these cheese varieties is the presence of Penicillium camembertii on Camembert cheese. Yeasts and micrococci predominate on the surface of brick cheese during the early stage of ripening, causing the pH at the surface to increase to 5.4-5.5 (11). The pH at the surface of Camembert cheese is much higher, thus probably providing a more favorable environment for growth of the coliform. Also, the microflora of the brick cheese surface may inhibit growth of E. coli through competitive inhibition (23) or by production of inhibitory substances (9).

Recovery of EEC from brick cheese using VRB pour plates

Recovery of EEC on VRB Agar pour plates relative to VRB + TSA surface plating decreased during the first hours of cheese manufacture and varied from 6 to 59% of the VRB + TSA count throughout the ripening and aging of the cheese (Table 1). Results from the VRB + TSA surface plating method have been previously shown to compare favorably with those obtained with TSA pour plates and MPN enumeration when these strains of EEC were in Camembert cheese or fermented skimmilk (7,8). The poor recovery on VRB Agar of the strains listed in Table 1 is similar in degree to that reported for refrigerated skimmilk which had been inoculated with these strains of E. coli and fermented with lactic starter cultures for 15 h at 21°C (7). A similar

Figure 2. Survival of enteropathogenic E. coli during ripening and refrigerated storage of brick cheese.
magnitude of decreased recovery of these strains on VRB Agar was also observed during ripening of Camembert cheese (8). These comparisons are interesting because both fermented skimmilk and Camembert cheese exposed E. coli to a pH of 4.6-4.7, considerably lower than the pH of brick cheese. Yet there was no increased recovery on VRB Agar, as would be expected if there was less sublethal acid injury to the cells. It has been demonstrated that lactic acid can cause sublethal injury to E. coli cells (11). Exposure of the cells to the heat of molten agar could also be a factor in the decreased recovery of these strains with VRB Agar pour plates (22).

These results do not imply that the VRB Agar pour plate method for estimating coliforms in cheese, as recommended in Standard Methods (10), is inaccurate to the extent shown with these strains of E. coli. The strains used in this study are not typical of coliforms that naturally contaminate dairy products. Elliott and Millard (4) have shown that the VRB pour plate procedure is reliable to estimate the amount of coliform contamination in commercial cheese.

The amount of growth and subsequent survival of EEC during brick cheese manufacture and ripening indicate the need for the manufacturer to strictly control the amount of coliform contamination in this product. This can best be done through strict sanitation measures. Results of this study show that relatively high levels of coliform contamination would be necessary to produce cheese likely to cause EEC foodborne illness. Collins-Thompson et al. (2) found in a survey of Canadian cheeses that 13.6% of semisoft and 18.1% of soft cheeses contained more than 1600 coliforms/g. Also, the numbers of fecal coliforms exceeded 1600/g in 0.8% of semisoft and 2.1% of soft cheese. Although this does not necessarily indicate a level of contamination capable of causing illness if EEC were present, it indicates the occurrence of unnecessary coliform contamination during the manufacture of these cheese varieties. The disease-causing potential of large numbers of E. coli in dairy products should not be ignored.

REFERENCES

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