Survival of Enteropathogenic and Non-Pathogenic *Escherichia coli* During the Manufacture of Camembert Cheese

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**ABSTRACT**

Camembert cheese was manufactured from milk contaminated with pathogenic and nonpathogenic strains of *Escherichia coli*. *E. coli* was enumerated using Violet Red Bile Agar (VRB) pour plates, a most probable number method, and surface plating on Trypticase Soy Agar (TSA) plates followed by an overlay of VRB (TSA + VRB). Numbers of *E. coli* during cheese manufacture increased about two log cycles during the first 6 h, then decreased during the initial stages of ripening with some strains disappearing from the cheese within the first 2 weeks of ripening, and other strains surviving 4 to 6 weeks. The rate of inactivation of *E. coli* in the cheese decreased as the cheese ripened and the pH increased. No growth of *E. coli* was observed in ripe cheese at a pH of 6.7, however rapid growth of *E. coli* occurred on the surface of the cheese. The TSA + VRB method was acceptable for enumeration of *E. coli* in Camembert cheese.

Presence of *Escherichia coli* in dairy products has been used as an indicator of post-pasteurization contamination (15). Cheese manufacturers have for many years sought to control growth of *E. coli* and other coliforms during cheese manufacture to prevent gassy defects (7, 34). However, recently presence of *E. coli* in dairy products has taken on added significance since a strain (0124:B17) caused gastroenteritis in persons who consumed a certain lot of imported Camembert cheese (21). This was probably the first documented outbreak of *E. coli*-caused foodborne illness in the U.S. Outbreaks in other countries have also occurred (21).

The pathogenicity of certain strains of *E. coli* has been well documented (3, 25). These strains have been divided into two groups, those producing a shigella-like (invasive) and those producing a cholera-like (toxigenic) illness (25, 29). There appears to be some relationship between pathogenicity of an *E. coli* strain and its serotype (2).

Several investigators have surveyed raw foods (33) and dairy products (17, 23, 26, 30) for serotypes of *E. coli* associated with enteropathogenicity. It appears that wherever fecal coliforms occur there is also a chance that enteropathogenic *E. coli* (EEC) will be found, although the frequency of EEC occurring in foods contaminated with *E. coli* may be highly variable. Methods used in these surveys would not have isolated slow lactose fermenting EEC such as the strain isolated in the Camembert cheese outbreak (8, 22). Hall and Hauser (14) found that 6.4% of food handlers studied, many of them in good health, were carriers of EEC, thus demonstrating that a source of potential contamination exists.

Since the presence of EEC in dairy products and in particular in Camembert cheese is of public health significance, this study was undertaken as a continuation of the earlier study by Park et al. (27) to assess the fate of *E. coli* during manufacture and ripening of Camembert cheese, and to evaluate use of an enumeration method designed to recover injured cells from food products.

**MATERIALS AND METHODS**

**Cultures**

Sources of cultures used in this study were given by Frank and Marth (9). The *E. coli* cultures 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 100 viable cells/ml. The starter culture used was a commercial mixed strain lactic culture. It was incubated in sterile skim milk at 21°C for 20 to 22 h and used immediately for cheese manufacture. A 2.0% starter inoculum was used in milk for cheese making. The *Penicillium camemberti* culture (from K. B. Raper, Department of Bacteriology, University of Wisconsin, Madison, Wisconsin) was grown on Czapek Agar slants at 21°C for 7 to 9 days. The mold from one agar slant was blended with 50 ml of sterile water and this mixture was added to milk at the time of cheese manufacture.

**Manufacture of cheese**

Camembert cheese was manufactured from 18.1-kg lots of coliform-free pasteurized milk according to the procedure used by Park et al. (27). Milk was adjusted to 33°C; clotted milk was cut 50 min after addition of rennet, starter, mold, and *E. coli*; curd was dipped onto hoops and allowed to drain at room temperature overnight; and the 250-g (approximate) wheels of cheese were dry-salted the next day. Cheese was ripened for 1 week at 15.5°C in an incubator with high humidity to allow mold growth, then was wrapped in foil and stored at 10°C for up to 7 more weeks. The procedure just described is referred to as the standard manufacturing method. Samples analyzed for *E. coli* at time of inoculation and at intervals of 1.5, 1, 2, 3, 5, and 7 weeks.

An alternate method of manufacture was also used. This involved inoculating pasteurized milk at 30°C with 2.0% starter, mold, and *E. coli*.
coli; allowing 1.5 h of incubation; adding rennet; cutting 30 min later; draining hoops overnight at room temperature; brine-salting for 1 h; and allowing cheese to dry at 17 C for 1 day. Cheese was ripened at 12 C for 9 days, wrapped in foil, held at 12 C for 4 additional days, and then stored at 7 C for 5 weeks. This method is similar to that used by a manufacturing plant in France. All cheese manufacturing trials were done in duplicate and results are reported as average values. All cheese made complied with the Federal standard of 50% milkfat in dry matter. Cheese having slow acid production was made from fresh pasteurized milk, using 0.25% starter and the standard manufacturing method.

Surface inoculations were made when a mat of mycelia covered the cheese (5 days at 15.5 C). The surface on one side of a cheese wheel was marked off into areas of 20 cm² and each area was inoculated with 0.1 ml of a suspension of E. coli (approximately 500 cells). The inoculum was spread with a glass rod. No liquid remained on the surface of the cheese. At the time of analysis each section was cut deep enough to give 20 g of cheese which was blended with 180 ml of sterile 2.0% sodium citrate solution. Appropriate serial dilutions were made. Two samples were analyzed at each time interval and average values are reported. E. coli was enumerated using the surface plating technique of Speck et al. (J1).

Enumeration of E. coli

Three methods were used to enumerate E. coli. The first was a 3-tube Most Probable Number (MPN) method using incubation in nutrient broth for 24 h at 37 C followed by streaking of positive tubes on Eosin Methylene Blue Agar (Difco) to test for typical growth. Only tubes giving typical colonies as compared to a control were counted as positive. The second method employed Violet Red Bile Agar (VRB, Difco) with incubation at 37 C for 24 h. The third method was suggested by Speck et al. (J1) for enumeration of sublethally injured coliforms. It involves surface plating on Trypticase Soy Agar (TSA, Difco) and a 1-h incubation at room temperature followed by addition of an overlay of VRB agar. Incubation was then at 37 C for 24 h. This method is referred to as the TSA + VRB surface plating method. Cheese samples were diluted by adding 20 g of cheese to 180 ml of sterile 2.0% sodium citrate solution and blending at low speed for 3 min. Serial dilutions were made using 9 ml of sterile sodium citrate solution. Cheese was sampled by taking a pie-shaped cross-section of the wheel. Surface samples were taken by cutting 6.5 mm into the wheel, and center samples were taken from the unripened core of the wheel.

Survival of E. coli at various pH values

Survival curves for E. coli in Camembert cheese at a pH of 4.6 were obtained by thoroughly mixing E. coli with 4-day-old cheese and following the decline in numbers over a period of days at 15.5 C. Survival of E. coli at a pH of 5.4 was determined in a similar manner using cheese ripened for 4 weeks which was inoculated after the rind was removed. Incubation was at 10 C. Camembert ripened for 6 weeks was used to obtain a pH of 6.7 and incubation was at 5 and 10 C. The pH of all samples remained constant during the experiments. The TSA + VRB surface plating method was used to enumerate E. coli. These experiments were done in duplicate and results are reported as averages.

Measurement of pH, moisture, salt and fat

The pH of cheese, depending on degree of ripeness, was measured with either a Corning Model 10 pH meter having a miniature combination glass electrode or with a saturated calomel half-cell, gold electrode, and a Leeds and Northrup portable potentiometer. For moisture analysis cheese was dried for 16 h in a forced-draft air oven at 110 C. The Volhard method (16) was used to determine NaCl in the cheese. The percentage of milkfat was determined by the Babcock method. Chemical analyses were done in triplicate.

Serological typing

Serological typing of EEC isolates was done to confirm the absence of "wild" coliforms in the cheese and to help judge the effectiveness of our enumeration methods. A total of 120 isolates were serotyped by the FDA in Washington, D.C. These isolates were taken from four lots of 2-day-old cheese each contaminated with a different EEC strain. A minimum of 10 isolates was obtained from each enumeration method for each strain of EEC.

RESULTS

Composition of cheese

All cheese was compositionally acceptable (50-57% moisture, 1.6-2.0% salt, and 52-55% milkfat in dry matter) and ripened in a manner normal for Camembert cheese. Changes in pH of cheese made by the standard method are given in Fig. 1. Since Camembert ripens from the outside inward, values for ripening cheese shown in the figure were obtained from a cross section of the cheese wheel and indicate an average pH value.

Survival of E. coli during manufacture and ripening of the cheese

Figures 2 through 5 show growth and survival for seven strains of E. coli during manufacture and ripening of cheese. These figures also compare results obtained with three enumeration methods. Each strain of E. coli increased in numbers by about two log cycles during the initial stages of manufacture. Some of this increase resulted from concentration by entrapment of cells in curd. When largest numbers occurred, the pH of cheese was between 4.9 and 5.2 and decreasing rapidly. After overnight draining, the pH of cheese was close to its lowest point and the number of E. coli had decreased by about one log cycle, except for strains 4608 and 1624 which showed almost no change. After salting of cheese and incubation at 15.5 C for 1 week, a further decrease

Figure 1 Changes in pH occurring during the manufacture and ripening of Camembert cheese.

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occurred in numbers of all strains of *E. coli*, the rate of decrease being highly variable. Three pathogenic strains (1624, 4608, and H10407) and one nonpathogenic strain (K-12) did not survive in numbers greater than 10/g past 1 to 2 weeks after the date of cheese manufacture. At this point the cheese was not mature enough for consumption. One pathogenic strain (B2C) and two nonpathogenic strains (H-52 and B) survived in numbers ranging from 10 to 1000/g past 4 weeks, and thus could be present in the cheese at the time of consumption. Substantial increases in numbers did not occur in the ripened cheese.

The effect of inoculum size on survival of EEC B2C is shown by data in Fig. 6. With inocula of $10^2$, $10^3$, and $10^4$/ml, growth and survival curves were nearly parallel. As would be expected, the amount of contamination is an important factor in determining the length of survival and numbers of *E. coli* in the final product.

The effect of slow acid production by the starter culture on growth and survival of EEC 1624 and B2C is shown by data in Fig. 7. Use of 0.25% instead of 2.0% starter affected the pH of the cheese only in the initial hours of manufacture, it being lowered only to 6.4 instead of 5.0 after 6 h (Fig. 1 and 7). The cheese had a normal pH thereafter. The slower acid production allowed about 10 times more EEC 1624 to develop in the cheese than when acid production was normal. This strain was inactivated as rapidly as it was when acid production was normal (Fig. 2). Strain B2C increased in numbers by about four log cycles when acid production was slow, as compared to two log cycles during normal manufacture (Fig. 2).
During ripening, numbers of strain B2C decreased less when less starter was used than when acid production was normal. After 4 weeks of ripening, 10^5 cells/g remained in the low starter cheese compared to less than 100/g in the normal cheese.

Comparison of enumeration methods for E. coli

Figures 2 through 5 provide a comparison of three enumeration methods for seven strains of E. coli. The TSA + VRB surface plating method gave results comparable to those from the 3-tube MPN method in practically all instances. With two nonpathogenic strains (K-12 and H-52), the surface plating technique was more sensitive than the MPN method when low numbers were encountered. With three pathogenic strains (B2C, 1624, 4608), the VRB pour plate method produced results similar to those obtained with the other two methods. In contrast, strains K-12, H-52, and EEC H10407 were not recovered as well with VRB pour plates as with the other methods, especially after cells were exposed to acid conditions. E. coli B seemed especially sensitive to VRB during the first 2 days of cheese manufacture. Variability in testing samples from replicate vats of cheese also differed with enumeration method. The VRB pour plate method showed average variability of 46%, the MPN method had 50%, and the surface plating method had 40%. The enteropathogenic isolates recovered by these three methods were serotyped and 95 to 100% were found to match the inoculated strains. This indicates that only the inoculated E. coli strains were enumerated with each method and that the cheeses had not become contaminated with other coliforms.

Comparison of manufacturing procedures

Data presented in Fig. 8 deal with behavior of E. coli B2C and H-52 during the two manufacturing procedures having normal acid development. Slightly larger numbers were observed with the alternate rather than the normal manufacturing procedure during the initial stages of manufacture, possibly because milk was incubated 1.5 h before addition of rennet. Also, E. coli was inactivated sooner in the ripened cheese made with the alternate method, possibly because of the lower ripening and storage temperatures. Although pH changes were similar in cheese made with the two methods, the alternate method produced cheese which ripened more evenly and softened more slowly.

Survival of E. coli in different areas of the cheese wheel

Table 1 gives representative data on number of E. coli at the core and on and close to the surface of the wheel of cheese made from milk containing the coliform. Salting of cheese was associated with a reduction in numbers of
COLIFORMS IN CAMEMBERT CHEESE

Figure 8. Changes in numbers of E. coli H-52 and enteropathogenic E. coli B2C in Camembert cheese when made by two procedures. Enumeration was with the TSA + VRB surface plating method. Changes in pH of cheese manufactured with the alternate procedure are shown.

E. coli near the surface. Even though pH near the surface of the cheese rose quickly in the first weeks of ripening, E. coli near the surface did not survive as long as near the center of the cheese. However, when E. coli was inoculated onto the newly developed mycelial mat, rapid growth occurred, with increases of over three log cycles and subsequent survival of large numbers (Fig. 9). This demonstrates a major difference between behavior of E. coli near the surface and on the surface of ripened cheese.

Survival of E. coli in cheese at specific pH values

Because of the heterogenous nature of ripening Camembert cheese, an attempt was made to measure survival of EEC in a more homogeneous environment. During the first week of ripening at 15.5 C most of the cheese wheel is at a pH of 4.6 to 4.7. Survival of EEC in unripened cheese (inoculation after manufacture) at pH 4.6 is shown in Fig. 10. Strains 4608, B2C, and 1624 had approximate “D” values of 1 to 2 days. Erratic results were obtained with strain H10407. EEC inoculated into cheese at a pH of 5.4 had approximate “D” values of 8 to 10 days for strains H10407, 4608, and 1624, and over 14 days for strain B2C (Fig. 11). This longer survival for strain B2C coincides with its longer survival in ripening cheese (Fig. 2) and fermented milk (9). Data showing survival of EEC in cheese at pH 6.7 held at 5 and 10 C are in Table 2. Although there may be lengthy survival under these conditions, there does not appear to be growth. Variability of these data (Fig. 10, 11, and Table 2) was less than 20%.

TABLE 1. Populations of E. coli at the surface and in the core of Camembert cheese during its ripening.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample position</th>
<th>Age of cheese</th>
<th>pH</th>
<th>No. x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>1 wk</td>
<td>2 wk</td>
<td>4 wk</td>
</tr>
<tr>
<td>B</td>
<td>Surface</td>
<td>5.6</td>
<td>4.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Core</td>
<td>37</td>
<td>4.8</td>
<td>14</td>
</tr>
<tr>
<td>H-52</td>
<td>Surface</td>
<td>6.5</td>
<td>4.6</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Core</td>
<td>20</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1624</td>
<td>Surface</td>
<td>19</td>
<td>4.7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Core</td>
<td>31</td>
<td>4.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

^None detected
DISCUSSION

Enumeration of E. coli in cheese

Enumeration of coliforms in dairy products using the VRB pour plate method is recommended in Standard Methods (15). Although this method is useful for most dairy products, previous results have shown low recovery from fermented milks contaminated with certain strains of E. coli (10). Our data support the theory that some strains of E. coli may be stressed in the acidic environment of a fermented dairy product causing poor recovery on selective media. A comparison of E. coli counts in the cheese at 48 h shows the effect of acid exposure. Strains K-12, B, H-52, and H10407 all showed some sublethal injury. Susceptibility of E. coli to injury by an acidic environment has been demonstrated by Roth and Keenan (28). When compared to most other varieties of cheese, unripened Camembert has a low pH so injury is likely in this cheese but may not occur in others.

Advantages and disadvantages of MPN procedures are well-known (4, 6). Our data show that the TSA + VRB surface plating method has the sensitivity of the MPN method. With the inherently greater precision of a plate count method, the surface plating method may provide more useful results than does the MPN method. Even though selective agents in VRB agar are diluted in the overlay method, selectivity of this medium was sufficient in these experiments. To more accurately assess occurrence of acid-stressed coliforms in dairy products, studies with naturally contaminated products must be done.

Behavior of E. coli during cheese manufacture

The general pattern of the growth and survival curves for E. coli during manufacture of Camembert cheese are

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TABLE 2. Survival of enteropathogenic E. coli at pH 6.7 in ripened Camembert cheese.

<table>
<thead>
<tr>
<th>Strain of E. coli</th>
<th>Temp. (°C)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(log/g x 10^3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H10407</td>
<td>5</td>
<td>30</td>
<td>21</td>
<td>10</td>
<td>5.5</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51</td>
<td>13</td>
<td>7.5</td>
<td>7.0</td>
<td>5.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>4608</td>
<td>5</td>
<td>22</td>
<td>14</td>
<td>8.5</td>
<td>6.0</td>
<td>4.5</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37</td>
<td>16</td>
<td>20</td>
<td>22</td>
<td>16</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>B2C</td>
<td>5</td>
<td>24</td>
<td>16</td>
<td>17</td>
<td>15</td>
<td>11</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21</td>
<td>15</td>
<td>17</td>
<td>19</td>
<td>17</td>
<td>&lt;5.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>TSA + VRB enumeration method was used for all counts.
similar to those reported by Park et al. (27) who used different strains of EEC. The amount of growth observed also coincides with growth of these strains in cultured milk (9) when one substracts the nearly 10-fold increase in numbers caused by concentration in converting milk to cheese. Death of \( E. \) \( \text{coli} \) also occurs at similar pH values (4.6-5.0) in cheese and the fermented milks (9). The large numbers and long survival observed when the amount of starter was reduced (Fig. 7) are similar to results obtained by Park et al. (27) when they added penicillin to milk to inhibit acid production. Results of Nevot et al. (24) also emphasize the importance of acidification during the manufacture of Camembert cheese to achieve destruction of pathogens.

**Behavior of pathogenic \( E. \) \( \text{coli} \) in ripening cheese**

Knowledge of the physical, chemical, and microbiological aspect of the ripening process in Camembert cheese is helpful in understanding the behavior of \( E. \) \( \text{coli} \) in this product. Four structural zones can be distinguished in ripening Camembert. These include the surface, rind, ripe zone, and unripened core (18). Surface and rind characteristics are a result of rapid growth of yeasts and micrococci after salting (20) followed by growth of \( P. \) camemberti. The ripe zone results from the proteolytic action of enzymes diffusing from the mold on the surface into the cheese (18). After hydrolysis of casein into water-soluble nitrogenous compounds, proteolysis continues through action of starter streptococci, \( P. \) camemberti, and possibly \( Lactobacillus \) casei causing formation of low molecular weight nitrogenous compounds including amines and ammonia (5,14,32). During this ripening process, milkfat remains structurally unchanged (18) and lactic acid is degraded (1). As ripening proceeds, the unripened core becomes small and the amount of ammonia in the ripe zone and the rind increases (19). This results in the increase in pH shown in Fig. 1 and Table 1.

Our results show the effect of the ripening process on behavior of \( E. \) \( \text{coli} \) in cheese. The unripened core is bactericidal probably because of its low pH (Fig. 10). The ripe zone is initially bactericidal (Fig. 11) and then bacteriostatic for \( E. \) \( \text{coli} \) (Table 2). Inability of \( E. \) \( \text{coli} \) to grow in ripened Camembert cheese was also observed by Park et al. (27). The surface of the cheese will support rapid growth (Fig. 9). Thus, following the layers of cheese from the center outward, conditions become more favorable for survival and finally growth of \( E. \) \( \text{coli} \). Fantasia et al. (8) observed growth of EEC in naturally contaminated Camembert cheese stored at 4 C and room temperature. It was not determined if growth occurred throughout the cheese or only on the surface. It would be possible for \( E. \) \( \text{coli} \) surviving in the ripe zone of the cheese to contaminate the surface during packaging and handling, and then to initiate growth. The bacteriostatic effect of ripened Camembert cheese on \( E. \) \( \text{coli} \) might be related to the large number of lactic bacteria present (from \( 10^4 \) to \( 10^9 \) g) (20). Lactic streptococci have been observed to partially inhibit growth of staphylococci and salmonellae in milk maintained at a pH of 6.6 (12). Production of antibiotics by the internal microflora of the cheese is possible, but Grecz (13) did not find antibiotic activity in Camembert cheese. Ammonia in ripened cheese may be partially responsible for inhibition since it constitutes 4.9 to 7.9% of the total nitrogen in the ripened cheese (19).

Effective measures that Camembert cheese manufacturers can take to insure the safety of their product include use of an adequate amount of active starter, draining the curd below room temperature to slow growth of \( E. \) \( \text{coli} \) while allowing adequate acid production (9), and use of stringent sanitation measures during manufacture, especially during ripening and packaging of the cheese. Presence of even small numbers of \( E. \) \( \text{coli} \) in ripened Camembert cheese presents a potential health hazard because of their ability to grow when present on the cheese surface and past history of food poisoning associated with this product.

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