Behavior of Salmonellae During Manufacture and Ripening of Manchego Cheese

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ABSTRACT

Six Salmonella strains were inoculated into 12 vats (2 vats/strain) of pasteurized sheep milk at a level of $10^6$ cells/ml, and Manchego cheese was manufactured by usual procedures, with 1% of a Streptococcus lactis culture as starter. Growth of Salmonella occurred during the first 6-9 h, with mean increases in log counts of 1.67, 1.49 and 1.71 respectively for Salmonella enteritidis, S. typhi and S. typhimurium; data inversely correlated to pH decrease. Mean numbers of Salmonella declined during the first week by 4.43, 1.18 and 3.97 log cycles for the three serotypes, respectively, with a significant correlation between decreases in pH and in Salmonella log counts. Salmonella survived for 4 weeks in 9 vats, for 6 weeks in 3 vats and was absent from all lots of 8-week Manchego cheese. Brilliant green agar yielded the highest productivity among five selective agars used for the enumeration of Salmonella by direct-plating procedures, while enrichment in selenite cystine broth followed by streaking to bismuth sulfite agar gave the highest Salmonella recovery of all eight broth-agar combinations tested.

Several recent food illness outbreaks in Spain have been attributed to cheese-borne salmonellosis. Manchego cheese, a domestic sheep milk variety, was incriminated in salmonellosis outbreaks. Manchego cheese was manufactured by usual procedures, with 1% of a Streptococcus lactis culture as starter. Growth of Salmonella occurred during the first 6-9 h, with mean increases in log counts of 1.67, 1.49 and 1.71 respectively for Salmonella enteritidis, S. typhi and S. typhimurium; data inversely correlated to pH decrease. Mean numbers of Salmonella declined during the first week by 4.43, 1.18 and 3.97 log cycles for the three serotypes, respectively, with a significant correlation between decreases in pH and in Salmonella log counts. Salmonella survived for 4 weeks in 9 vats, for 6 weeks in 3 vats and was absent from all lots of 8-week Manchego cheese. Brilliant green agar yielded the highest productivity among five selective agars used for the enumeration of Salmonella by direct-plating procedures, while enrichment in selenite cystine broth followed by streaking to bismuth sulfite agar gave the highest Salmonella recovery of all eight broth-agar combinations tested.

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sheep milk was pasteurized at 75°C for 15 s and cooled to 32°C. Vats containing 40 L of milk were inoculated with 400 ml of a *S. lactis* culture and 1 ml of a *Salmonella* culture in sterile skim milk, which yielded about 10^2 and 10^4 cfu/ml of vat milk, respectively. Rennet was added 30 min later. Curds were cut 75 min after inoculation, cooked at 37°C for 15 min and three cheeses, 20 cm in diameter and 8 cm high, were made from each vat. They were pressed for 7 h (time from inoculation to end of pressing), brine-salted for 39 h and ripened for 10 weeks at 10°C.

Cheeses were sampled during manufacture (3, 6 and 9 h after inoculation) and throughout their ripening period, as described by Law et al. (30). The pH of cheese was measured after homogenizing 10 g of cheese with 20 ml of distilled water at 70°C by means of a Stomacher 400 (Seward Laboratory, London, England).

**Enumeration of Salmonella by direct-plating procedures**

Ten grams of cheese were homogenized with 90 ml of a sterile 2% sodium citrate solution at 40°C. Decimal dilutions in sterile 0.1% peptone water were surface-plated (0.1-ml) on brilliant green (BG), bismuth sulfate (BS), Salmonella-Shigella (SS) and xylose lysine desoxycholate (XLD) agars. Agar plates were incubated 48 h at 37°C.

**Detection of Salmonella by enrichment procedures**

Thirty grams of cheese were homogenized with 270 ml of sterile 0.1% peptone water at 40°C and pH was adjusted to 6.8-7.0 with sterile 1 N NaOH. After adding 0.6 ml of 1% aqueous solution of brilliant green dye, 250, 10 and 1 ml of the homogenate were transferred to sterile flasks or tubes and incubated 24 h at 37°C. Following incubation, 1 ml portions of the pre-enriched samples were pipetted to 10 ml of selenite cystine (SC) and tetrathionate brilliant green (TBG) broths, which were incubated 24 h at 37 and 45°C, respectively (9). One loopful of each enrichment broth was streaked on plates of BG, BS, XLD and SS agars. Agar plates were examined for *Salmonella*-suspicious colonies after 24 and 48 h at 37°C. *Salmonella*-like colonies were picked to slants of Kligler iron (KI) and lysine iron (LI) agars and biochemically identified, to ensure that they were the serotypes added to cheese milk. Pasteurized milk before inoculation and rennet were tested throughout this study to confirm the absence of *Salmonella*.

**RESULTS AND DISCUSSION**

**Comparison of direct-plating procedures**

Detection of *Salmonella* by direct plating is not a common procedure in routine microbiological examination of foods, as enrichment techniques are usually recommended (2,17). A selective agar for direct enumeration of *Salmonella* has been developed (31), but the three layers of agar which must be poured make it a painstaking procedure. In the present study, the large inocula of *Salmonella* added to cheese milk made possible enumeration of this organism in cheese during the first week of ripening by direct plating on selective solid media. Starter streptococci did form colonies on any of these agars. No post-pasteurization contamination of milk or cheese by *Enterobacteriaceae*, which might have interfered with the results, was detected on any of the samples examined.

The mean log *Salmonella* counts of 56 cheese samples on five selective media were: 4.878 (BG agar), 4.688 (BS agar), 4.640 (TS agar), 3.180 (SS agar) and 3.054 (XLD agar). Yields on SS and XLD agars were significantly lower (P<0.01) than yields on the other three media, after the Student-Newman-Keuls test (35). BG was the most efficient agar when enumerating *S. typhi* and *S. typhimurium*, and TS agar when enumerating *S. enteritidis*. No significant differences (P<0.05) were recorded among BG, BS and TS agars for any of the three *Salmonella* serotypes.

Kroninger and Banwart (18) had already reported that BG agar gave higher counts than BS and SS agars, in a study on the growth of four *Salmonella* serotypes on ten selective solid media. Our conclusions may be useful for future studies on *Salmonella* survival in other cheese varieties.

**Comparison of enrichment procedures**

The efficiency of eight enrichment procedures for detection of *Salmonella* in cheese, after 1 week of ripening, has been determined and the results are shown in Table 1. The most productive broth-agar combination was a selective enrichment in SC broth followed by streaking to BS agar, with 93% of *Salmonella* isolations on positive samples. Analysis of variance did not show any significant (P<0.05) difference between SC-BS and SS-SC or SC-BG broth-agar combinations, which recovered 88% and 82% of the *Salmonella* isolates, respectively. When the combinations of each enrichment broth with all selective agars were considered, SC broth yielded the best average productivity (84%), which was not however significantly higher than the average productivity of TBG broth (72%).

Previous reports comparing the performance of SC and TBG or tetrathionate enrichment broths and the incubation temperatures of these broths have not shown agreement on this subject. Although some authors (34) have found significant differences between these broths for particular foods, most workers (1,3,10,11,23,40) base their choice of broth and incubation temperature on the product being examined or the serotype of *Salmonella* being recovered.

The A.O.A.C. (2) recommends BG, BS and SS as plating agars when streaking *Salmonella* from enrichment broths of dried yeast, dried egg products, dried milk and onion and garlic powders. Results obtained in the present

### Table 1. Recovery of *Salmonella* from cheese by enrichment techniques with different selective broths and plating agars.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Positive samples</th>
<th>SC</th>
<th>XLD</th>
<th>TBG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BG</td>
<td>BS</td>
<td>SS</td>
<td>XLD</td>
</tr>
<tr>
<td><em>S. enteritidis</em></td>
<td>33</td>
<td>67</td>
<td>88</td>
<td>76</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>33</td>
<td>79</td>
<td>94</td>
<td>91</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>35</td>
<td>100</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>101</td>
<td>82</td>
<td>93</td>
<td>88</td>
</tr>
</tbody>
</table>

*Expressed as percentage of *Salmonella* isolations on number of positive samples, based on all broth-agar combinations.*

*Enrichment broths used were: selenite cystine (SC) and tetrathionate brilliant green (TBG).*

*Plating agars used were: brilliant green (BG), bismuth sulfate (BS), Salmonella-Shigella (SS) and xylose lysine desoxycholate (XLD).*
work also show the convenience of using these three agars for examination of cheese samples. XLD agar gave the lowest recovery from both SC and TBG enrichment broths, though satisfactory results with this medium have been reported by some authors (41).

Behavior of Salmonella during manufacture and early ripening of Manchego cheese

Changes in the numbers of S. enteritidis, S. typhi and S. typhimurium and in the pH of Manchego cheeses during the first 7 days of ripening are shown in Fig. 1, 2 and 3, respectively. Mean increase in Salmonella counts due to entrapment of cells in the curd was lower than the 10-fold increase indicated for S. typhimurium during the manufacture of Cheddar cheese (14) and for enteropathogenic Escherichia coli in Camembert (28) and Brick (13) cheeses, as our average counts were only 0.77 log cycle higher in 3 h cheese than in milk (0 h). Maximum growth of S. typhimurium occurred after 6 h, with a mean increase in log counts of 1.71, very similar to the 1.67 log cycles reported for curd before hooping in Cheddar cheese (14), while S. enteritidis and S. typhi were able to continue growing for 9 h, with mean increases in log counts of 1.67 and 1.49, respectively.

Analysis of variance on the increase in Salmonella log counts during manufacture and pressing of Manchego cheese (Table 2) showed a significant (P<0.05) effect of the strain inoculated. Recent isolates of Salmonella are generally able to grow better in cheese than culture collection strains and give more valuable information about the behavior of Salmonella in cheese under real conditions at the dairy factory.

The mean pH of the cheese after pressing (9 h) was 5.34, 5.22 and 5.21 for vats inoculated with S. enteritidis, S. typhi and S. typhimurium. The decline in the pH value of cheese restrained growth of salmonellae during pressing. A significant (P<0.05) correlation with r = -0.602 was observed between the increase in log Salmonella counts and the decline in the pH value of cheese. The importance of pH and lactic acid production for the decrease in the number of Salmonella in cheese had already been

![Figure 1](http://meridian.allenpress.com/jfp/article-pdf/45/12/1091/1655329/0362-028x-45_12_1091.pdf)

**Figure 1.** Mean levels of S. enteritidis strains 23 and 9980 (2 vats/strain) and pH values in Manchego cheeses during the first week of ripening.

![Figure 2](http://meridian.allenpress.com/jfp/article-pdf/45/12/1091/1655329/0362-028x-45_12_1091.pdf)

**Figure 2.** Mean levels of S. typhi strains 54 and 16748 (2 vats/strain) and pH values in Manchego cheeses during the first week of ripening.

![Figure 3](http://meridian.allenpress.com/jfp/article-pdf/45/12/1091/1655329/0362-028x-45_12_1091.pdf)

**Figure 3.** Mean levels of S. typhimurium strains 55 and 14751 (2 vats/strain) and pH values in Manchego cheeses during the first week of ripening.

### TABLE 2. Analysis of variance for effects of serotype and strain on the increase in Salmonella log counts during manufacture and pressing of Manchego cheese.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotypes</td>
<td>2</td>
<td>0.085</td>
<td>0.043</td>
<td>0.142</td>
</tr>
<tr>
<td>Strains within serotypes</td>
<td>3</td>
<td>0.898</td>
<td>0.299</td>
<td>9.122</td>
</tr>
<tr>
<td>Within strains (error)</td>
<td>6</td>
<td>0.197</td>
<td>0.033</td>
<td></td>
</tr>
</tbody>
</table>

*Significant at the 5% level.

n.s. : not significant at the 5% level.

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TABLE 3. Analysis of variance for effects of serotype and strain on the decrease in Salmonella log counts during the first week of Manchego cheese ripening.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotypes</td>
<td>2</td>
<td>24.720</td>
<td>12.360</td>
<td>23.185*</td>
</tr>
<tr>
<td>Strains within serotypes</td>
<td>3</td>
<td>1.599</td>
<td>0.533</td>
<td>1.110 n.s.</td>
</tr>
<tr>
<td>Within strains (error)</td>
<td>6</td>
<td>2.882</td>
<td>0.480</td>
<td>1.110 n.s.</td>
</tr>
</tbody>
</table>

*Significant at the 5% level.

TABLE 4. Survival of Salmonella enteritidis, S. typhi and S. typhimurium during the ripening period of artificially contaminated Manchego cheeses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Weeks of ripening at 10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>S. enteritidis 23</td>
<td>+ +/++ + + +</td>
</tr>
<tr>
<td>S. enteritidis 9980</td>
<td>+ +/+ + + + +</td>
</tr>
<tr>
<td>S. typhi 54</td>
<td>+ +/+ + + + +</td>
</tr>
<tr>
<td>S. typhi 16748</td>
<td>+ +/+ + + + +</td>
</tr>
<tr>
<td>S. typhimurium 55</td>
<td>+ +/+ + + + +</td>
</tr>
<tr>
<td>S. typhimurium 14751</td>
<td>+ +/+ + + + +</td>
</tr>
</tbody>
</table>

*1st vat/2nd vat
+ + : positive 0.1-g samples; + + : positive 1-g samples; + : positive 25-g samples; - : negative 25-g samples.

Behavior of Salmonella after the first week of ripening in Manchego cheese

Data in Table 4 show the survival of Salmonella in cheese during the last stages of ripening. Nine of 12 vats of cheese yielded positive results in 25-g samples after 4 weeks of storage, with S. typhi persisting just in cheese of two vats. After 6 weeks we only detected S. enteritidis 9980 (2 vats) and S. typhi 16748 (1 vat), strains recently isolated from foodborne salmonellosis outbreaks. All cheeses were free from viable Salmonella cells after 8 and 10 weeks at 10°C, when examining 25-g samples.

The death rate of Salmonella in Manchego cheese held at 10°C was slower than the 10,000-fold reduction reported in Bleu d'Auvergne after 6 d (22). Conditions in the interior of Bleu d'Auvergne after 1 week (pH 4.6 and 3% NaCl) are less favorable than those of Manchego cheese at the same time (pH 5.0 and 0.6% NaCl). From our results, 4 weeks at 10°C may be estimated as the average ripening period of Manchego cheese necessary to ensure a 10,000-fold reduction in numbers of Salmonella. This 4-week period is shorter, however, than the 60 d reported for Samsoe cheese (5) or the 10-16 weeks for Cheddar cheese (14).

The survival periods of S. enteritidis, S. typhi and S. typhimurium in Manchego cheese are difficult to compare with data obtained for other cheeses, as inocula of Salmonella in milk vary considerably between different studies: from 25-100 cells/ml (14) up to 10^5-10^6 cells/ml (29). The 6-8 weeks necessary in our conditions for disappearance of Salmonella from Manchego cheese exceeded the survival periods of this microorganism in Hrudka cheese (3-7 d), Carré de l'Est cheese (7 d), Telemáche cheese (<10 d), Karish cheese (9-27 d) and Domiati cheese (15-36 d) found by different authors (4,24,25,26,29), but was less than the 7-10 months reported for Colby, Cheddar and Samsoe cheeses (5,14,16,27,38,39).

Since expected contamination levels of Salmonella at the dairy are well under the 10^4 cells/ml used in the present study, Manchego cheese manufactured with accidentally contaminated milk would be free from viable Salmonella cells after 1 month if starter growth and lactic acid production occur normally.
Lactic acid bacteria have been shown to inhibit *Salmonella* growth due to metabolic products such as acetic and lactic acids (32,36,37), and some organic acids usually present in cheese have also been reported to have inhibitory effects on *Salmonella* (15,19). Inhibition and decline in numbers of *Salmonella* during manufacture and early ripening of Manchego cheese may be attributed to low pH (mean pH value of 2-d cheeses in this study: 5.05) and to lactic acid concentration: 0.8-1.1% in 48 h cheese (Nuñez and Nuñez, unpublished data). The combined effect of these two factors, pH value and organic acids concentration, and disappearance of *Salmonella* have also been reported to have inhibited numbers of *Salmonella* present in cheese have also been reported to have inhibitory effects on *Salmonella* (15,19). Inhibition and decline in numbers of *Salmonella* during manufacture and early ripening of Manchego cheese may be attributed to low pH (mean pH value of 2-d cheeses in this study: 5.05) and to lactic acid concentration: 0.8-1.1% in 48 h cheese (Nuñez and Nuñez, unpublished data). The combined effect of these two factors, pH value and organic acids concentration, and disappearance of *Salmonella* during the final stages of Manchego cheese ripening may also be influenced by the production of volatile fatty acids during curing (14) and by salt diffusion, which yields a 2.5-3.0% NaCl concentration in the interior of 60-d Manchego cheese.

**ACKNOWLEDGMENTS**

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**REFERENCES**


7 h when the count was less than $10^6$ CFU/ml. In such an instance, it would not be possible to detect the individual enterotoxin as the quantity would be below the detection level of 1 ng/g. This would need to be taken into consideration in examining foods from which multiple enterotoxin producers were isolated.

ACKNOWLEDGMENTS

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