Growth of *Staphylococcus aureus* and Synthesis of Enterotoxin During Ripening of Experimental Manchego-Type Cheese

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ABSTRACT

To study the possible presence of staphylococcal enterotoxins in Manchego-type cheese, milk was inoculated with the enterotoxigenic *Staphylococcus aureus* collection strains FRI-100, S6, FRI-137, and FRI-472 to a final concentration of 10,000 to 25,000 cfu/ml. Cheese was prepared following the industrial specifications and ripened for 60 d. Batches were prepared with 1 and .1% lactic acid culture and labeled with the abbreviated name of the strain and the concentration of lactic acid culture. Mean staphylococcal counts in .1% lactic bacteria cheeses were usually more than 1 log higher than the corresponding 1% ones. Staphylococcal counts declined markedly after d 35 to 42, and, by the end of ripening, they had disappeared from some cheeses. Enterotoxins were present in five of the cheeses, three prepared with .1% and two with 1% lactic bacteria. Enterotoxins detected were A and D, the enterotoxins most commonly associated with human intoxication. The maximum level of enterotoxin A detected in cheese with strain FRI-100 and with the .1% culture was 222 ng/100 g of cheese; in cheese FRI-100 with 1%, 111 ng/100 g; in cheese S6 with .1%, 769 ng/100 g; and in cheese S6 with 1%, 33 ng/100 g. Maximum level of enterotoxin D detected in cheese FRI-472 with .1% was 38 ng/100 g.

INTRODUCTION

Staphylococcal enterotoxins are exoproteins that, when ingested, cause the development of a gastroenteric syndrome characterized by early onset, about 2 to 3 h after ingestion. Staphylococci can readily multiply in many foods, but, in Spain and other warm countries, dairy products are probably the type of foods most frequently implicated (3). Cheese has been repeatedly involved in staphylococcal outbreaks (3, 6, 8, 24, 27, 33).

Manchego cheese, with production of more than 30,000 tonnes/yr, is the main variety of cheese produced in Spain. It is made from ewe's milk, which may be pasteurized, but, in the rural areas, it is usually raw. Because of the high demand, a cheese that is basically similar to the traditional one, but with a mixture of milks from cow, ewe, and goat, is produced in the industry. To protect the identity of Manchego, it is called Manchego-type cheese or mixture milk cheese.

Enterotoxigenic staphylococci may be present in cheese, because it is commonly made from unpasteurized milk from cows with subclinical mastitis (8). However, milk may also be contaminated after pasteurization by staphylococci present in aerosols (8), in the equipment, on food handlers (8, 21), or in the starter culture (26). If there is a contaminating source,
Pasteurized milk is more suitable for *Staphylococcus aureus* growth and toxin production than raw milk (7). Although staphylococci are weak competitors and are readily outnumbered by the starter culture (27), the contamination of the starter by bacteriophages or by the presence of antibiotics in milk, which is frequent in subclinical mastitis, may arrest the effect of competition and allow growth of staphylococci (33). In these circumstances, staphylococci may multiply and produce enterotoxins throughout the ripening process, which in Manchego-type cheese lasts for at least 2 mo. Pasteurized, uncontaminated milk may contain staphylococcal enterotoxins synthesized by staphylococci prior to heat treatment because these proteins are thermostable (10, 15).

The effect of starter culture on staphylococci is mainly through the production of lactic acid, drop in the pH, production of H<sub>2</sub>S, lactoperoxidase system (16), competition for nutrients (22), and even the synthesis of antibiotic substances, such as nisin (22, 26). Salt (NaCl), which is either dispersed evenly in the cheese by addition to the curd or distributed along a gradient by immersing the cheese or rubbing its surface, is also detrimental to bacterial growth. In addition to its effect on the flavor and physical properties of the cheese, salt establishes a suitable environment to control microbiological activity in the cheese (19, 30). However, tolerance of staphylococci to salt has been stressed in studies (1, 11, 19) and salt even encourages their development in cheese.

The objective of the present work was to determine the growth of staphylococcal strains and production of enterotoxins during the ripening process of Manchego-type cheese prepared in the laboratory according to the industry procedure.

**MATERIALS AND METHODS**

**Cultures**

*Staphylococcus aureus* strains FRI-100, S6, FRI-137, FRI-361, and FRI-472 (kindly provided by M. S. Bergdoll, Food Research Institute, Madison, WI), which produce staphylococcal enterotoxins A (SEA), B (SEB) plus SEA, C<sub>1</sub> (SEC<sub>1</sub>), C<sub>2</sub> (SEC<sub>2</sub>), and D (SED), respectively, were used independently to inoculate milk prior to the manufacture of cheese. The strains were stored frozen in a cryopreservative medium composed of 10 g of tryptone, 20 g of skim milk powder, 80 ml of glycerol, and 320 ml of distilled water. These were revitalized prior to the beginning of the experiment by subculturing twice in brain-heart infusion broth (Micro=Adsa, Barcelona, Spain) plus 1% skim milk powder (1:1) in which they were incubated for 20 h at 37°C. The starter culture (lyophilized type CH-N 01, Chr. Hansen’s, Copenhagen, DK) included *Lactococcus lactis* (1 to 5%), *Lactococcus lactis* ssp. cremoris (75 to 80%), *Lactococcus lactis* ssp. diacetylactis (10 to 15%), and *Leuconostoc cremoris* (5 to 10%) and was prepared according to the manufacturer's instructions. It was divided into 50-ml aliquots and stored frozen at −20°C. Before use, an aliquot was thawed and added to 750 ml of pasteurized cow milk and grown at 22°C for 22 to 26 h.

**Manufacture of Cheeses**

Manchego-type cheese was prepared in the laboratory following industrial specifications; the procedure has been described previously (13). A volume of 35 L of a mixture of pasteurized milk from ewes (15%), goats (35%), and cows (50%) (kindly provided by Central Quesera S.A., Madrid, Spain) was warmed to 32 to 33°C and inoculated with 2 ml of the staphylococcal culture to render an initial concentration of approximately log<sub>10</sub> 4.2 to 4.5 cfu/ml. After the addition of CaCl<sub>2</sub> (13.5 g/100 L of milk), the starter culture was added into milk to a final concentration of 1% (normal) or .1% (subnormal) (vol/vol). Rennet (Chr. Hansen’s, DK; 2.5 g/100 L of milk) was added to milk, and, after coagulation, the curd was cut in 4- to 6-mm cubes. Curd was placed in cylindrical plastic molds of two sizes: 16 cm i.d. and 11 cm high (large) and 11 cm i.d. and 8 cm high (small). After pressing (9 g/cm<sup>2</sup>, 15 to 17°C) for 10 h, cheese pieces were placed in a brine solution (25°C) overnight. After brining, cheeses were placed in an incubation chamber at 15°C with high (85%) relative humidity and ripened for 60 d. Every 3 d, the cheeses were turned over. On d 14, cheeses were introduced into a bath of warm liquid paraffin. Two batches of cheese were prepared for each strain, each batch with one large and one small cheese piece. Samples were labeled.
TABLE 1. Enterotoxin concentration (nanograms of staphylococcal enterotoxin per 100 g of cheese) detected during ripening in experimental cheeses inoculated with *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Starter</th>
<th>SET</th>
<th>Days of ripening 16</th>
<th>24</th>
<th>31</th>
<th>38</th>
<th>45</th>
<th>49</th>
<th>58</th>
</tr>
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<tbody>
<tr>
<td>FRI-100</td>
<td>.1</td>
<td>A</td>
<td>NS</td>
<td>116</td>
<td>24.8</td>
<td>38</td>
<td>46</td>
<td>65.5</td>
<td>222</td>
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<tr>
<td>FRI-100</td>
<td>1</td>
<td>A</td>
<td>NS</td>
<td>17</td>
<td>71</td>
<td>111</td>
<td>66</td>
<td>69</td>
<td>86.5</td>
</tr>
<tr>
<td>S6</td>
<td>.1</td>
<td>B</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S6</td>
<td>1</td>
<td>A</td>
<td>NS</td>
<td>22</td>
<td>28.7</td>
<td>16</td>
<td>33</td>
<td>29</td>
<td>23.7</td>
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<tr>
<td>FRI-137</td>
<td>.1</td>
<td>C</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FRI-137</td>
<td>1</td>
<td>C</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FRI-361</td>
<td>.1</td>
<td>C</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FRI-361</td>
<td>1</td>
<td>C</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FRI-472</td>
<td>.1</td>
<td>D</td>
<td>NS</td>
<td>12.6</td>
<td>19.2</td>
<td>1.21</td>
<td>9.4</td>
<td>38</td>
<td>15.5</td>
</tr>
<tr>
<td>FRI-472</td>
<td>1</td>
<td>D</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SET contr</td>
<td>.1</td>
<td>A</td>
<td>1239</td>
<td>507</td>
<td>1307</td>
<td>536</td>
<td>1691</td>
<td>NS</td>
<td>934.8</td>
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<tr>
<td>SET contr</td>
<td>1</td>
<td>B</td>
<td>770</td>
<td>356</td>
<td>580</td>
<td>460</td>
<td>822</td>
<td>NS</td>
<td>731</td>
</tr>
</tbody>
</table>

**With the name of the strain used, followed by the concentration of the starter culture (e.g., S6-.1%). Additional batches included 1) 1% starter culture and no staphylococci and 2) 1.7 mg of SEA and SEB/20 L of milk.

**Bacterial Enumeration**

The cheese was sampled on d 1, 2, 3, 4, 7, 10, 14, 18, 21, 28, 35, 42, and 60 using a cheese sampler and refilling the hole with molten paraffin. Samples (10 g) were added to bottles containing 10 mL of 1% peptone water (Difco, Detroit, MI) plus .1% Tween 20 (Merck, Darmstadt, Germany) and frozen immediately, usually for less than 24 h. Before thawing, samples were mashed to a slurry with a sterile spatula. Serial 10-fold dilutions of test material were spread-plated in duplicate on Baird-Parker medium (Micro=Adsa) to determine staphylococcal counts and on plate count agar (Micro=Adsa) to determine aerobic total counts (APC) and incubated at 37°C for 48 h. The pH was measured with a pH meter (Crisson, type 414, Barcelona, Spain) on the 50% (wt/vol) cheese slurries just described.

**Enterotoxin Determination**

Periodically (Table 1), 100-g sectors were removed from the cheese pieces and examined for the presence of SEA, SEB, SEC, and SED by the ELISA method described by Freed et al. (9). Enterotoxins were extracted according to Hirooka et al. (14), but concentration was achieved by dialysis against 20 M polyethylene glycol (Fluka, Fuchs, Switzerland). In a batch, enterotoxins were added to milk prior to manufacture of cheese, and, after subtracting the losses in whey, the recovery rate was estimated to range between 35 and 42%. Recovery of enterotoxin added to uncontaminated commercial Manchego-type cheese slurries ranged between 45 and 70%, depending on the enterotoxin. The minimal enterotoxin concentration considered significant was 2 ng/100 g of cheese.

**RESULTS AND DISCUSSION**

The organoleptic characteristics of the contaminated cheeses were identical to those of control cheeses in odor, texture, and color.
Abnormal fermentations or unwanted contaminants were not observed. Before immersion in paraffin, all cheeses were externally contaminated by *Penicillium* spp. The mold was periodically withdrawn with a humid cloth.

Counts from particular samples before freezing and after two sequences of freezing and thawing demonstrated that microbial losses during this process were about 5 to 8%. Park and Humphrey (23) showed that freezing did not seem to affect the survival of staphylococci in goat milk, because 122% of the initial charge was recovered after freezing and thawing. These results agreed with the data of Berry et al. (2).

The initial concentration of staphylococci (log10 4.2 to 4.5 cfu) may seem higher than normal for postpasteurization contamination (16), but the concentrations are not considered high for cheeses manufactured from unpasteurized milk from cows with subclinical mastitis.

Small and large cheeses were sampled on different days in order to alter as little as possible their ripening conditions. Counts were expected to be different because of the NaCl and O2 gradients. However, staphylococcal numbers and APC did not differ markedly between the two sizes of cheese (nonsignificant difference), and curves could be plotted using the combined data.

An increase in the APC usually meant the decrease in staphylococci (Figure 1). The pH followed a curve parallel to the staphylococcal count. The mean staphylococci counts in batches made with .1% starter culture were more than 1 log higher than those in the cheeses made using 1% starter culture except in cheeses inoculated with FRI-100 and FRI-361. In the latter, staphylococcal counts were higher with the higher inoculation rate (Table 2). This agrees partially with the data of Koenig and Marth (19), who indicated that, after 8 wk of storage, cheese made with .5% starter culture had a significantly higher population of *S. aureus* than cheeses made with 1% starter culture. Maximum staphylococcal counts were usually higher in cheeses made with 1% starter, but the minimum counts did not follow any particular trend. Minimum staphylococcal
TABLE 2. Mean, maximum (max.), and minimum (min.) staphylococcal and aerobic total counts (log₁₀/ml) in experimental Manchego-type cheeses prepared with normal (1%) and subnormal (.1%) starter inoculation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Staphylococi</th>
<th>Aerobic total counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.1%</td>
<td>.1%</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Max.</td>
</tr>
<tr>
<td>FRI-100</td>
<td>6.00</td>
<td>6.6</td>
</tr>
<tr>
<td>S6</td>
<td>5.64</td>
<td>6.9</td>
</tr>
<tr>
<td>FRI-137</td>
<td>4.86</td>
<td>5.7</td>
</tr>
<tr>
<td>FRI-361</td>
<td>3.53</td>
<td>5.2</td>
</tr>
<tr>
<td>FRI-472</td>
<td>6.06</td>
<td>7.0</td>
</tr>
<tr>
<td>Control</td>
<td>6.95</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Counts were generally observed on the last day of ripening. The APC counts at the beginning were variable, and differences were not observed between APC of batches made with .1% and 1% starter, not even on the first days of ripening. At the end of ripening, APC counts were lower than in previous weeks but were seldom the lowest counts.

Montanaro et al. (20) reported that in Mozarella cheese staphylococcal numbers reached 10⁶ cfu/g during the first 48 h after addition to milk. We never detected staphylococcal concentrations this high, and maximum values were not obtained within 48 h. The highest concentration of *Staphylococcus* never occurred earlier than 7 d and was usually observed between 18 and 42 d. Generally, maximum counts occurred earlier in the batches made with .1% starter (Figure 1) and nearly always before maximum APC. Maximum staphylococcal counts ranged between 10⁵ and 10⁶ cfu/g.

Our results show that staphylococcal counts declined markedly after 35 to 42 d of ripening, and, by the last day of ripening (60 d), staphylococci were not detected in some cheeses. However, enterotoxins could be detected (Table 1). Tuckey et al. (28) reported staphylococcal growth up to 2 × 10⁸ cells/g of cheese during the first 2 or 3 wk of maturation, after which counts declined gradually. Our results agree with those of Tatini et al. (25), who indicated that no staphylococci could be recovered from cheese (Cheddar in their case) after 10 wk of ripening. However, Koenig and Marth (19) determined that, after 8 wk of storage of Cheddar cheese at 4 and 10°C, staphylococcal counts decreased only .5 log₁₀ cycles from the initial concentration.

The pH of Manchego cheese manufactured by industry initially decreases to 5.0, but, after 2 mo of ripening, it increases slightly, and the pH of the finished product will be between 5.1 and 5.3. These pH data are comparable with those for industrial cheese, although, in some cases, the pH in our cheese decreased below 5.0. The pH on 60 d was higher than 5.5 in five cheeses (100 with .1%, 100 with 1%, S6 with .1%, S6 with 1%, and 137 with 1%) (Figure 1). Todd et al. (26) reported that the pH of Swiss-type cheese with staphylococcal counts in excess of 10⁷ cfu/g was noticeably higher (5.92 to 6.2) than normal Swiss-type cheese matured for 4 to 6 mo (5, 7). In our experiment, although staphylococcal counts in cheese 100-1% were highest at 60 d, staphylococci were not detected in the other three cheeses at the end of ripening. The increase in pH that we measured could be due to cellular death, which would free amines, or to technological factors, because cheeses had been sampled repeatedly, and their size was significantly reduced by the end of the experiment. This modification of the physical characteristics may cause H⁺ to combine with certain substances and cause the pH to increase. Purta and Chandan (11) reported that, after 4 wk of ripening, water-soluble protein increased and was correlated with increased pH in goat cheese (pH increase = .83). This could also explain the increase in pH, although, in Manchego-type cheese, it could be delayed by the influence of milk from other species.

Enterotoxins were present in five of the cheeses produced (100 with .1%, 100 with 1%, S6 with .1%, S6 with 1%, and 472 with .1%) (Table 1). However, enterotoxin concentration was not constant, even in the cheeses with added SEA and SEB, but the enterotoxin never disappeared from cheese. Three to 4 wk after cheese manufacture, enterotoxin concentration
was minimal, and it then increased after 32 to
35 d. Neither APC nor pH could be associated
with these fluctuations. These data suggest the
degradation of enterotoxin by the presence of a
proteolytic enzyme, as had been proposed pre­
viously in connection to SEA (5, 7) or to
thermal nuclease (26), or it might be degraded
by proteases of staphylococci (32). Because
enterotoxins are very stable, the decreased con­
centration could also be due to the conjugation
with substances that diminish their immuno­
logical activity (although the biological activ­
ity could remain). These conjugated substances
might be released later because of the deple­
tion of specific nutrients from the cheese, and
the enterotoxins would be released and de­
tected by assay procedures.

Ibrahim and Baldock (17) suggested that
there was no correlation between growth of
Staphylococcus and enterotoxin production.
This was true in our experiments, because
better growth did not mean greater enterotoxin
production. It should be stressed that the only
enterotoxins detected in this study were SEA
and SED, which are those most frequently
involved in staphylococcal outbreaks. The pre­
domiance of these enterotoxins correlated
with the highest staphylococcal counts, espe­
cially in strains FRI-100 and FRI-472.

Wieneke (31), in an attempt to explain the
production of SEA and SED by staphylococcal
strains isolated from foods implicated in food
poisoning, suggested that factors other than the
presence of an enterotoxigenic strain of staphy­
lcocci in foods played important roles in the
development of enterotoxin in foods. Wieneke
(31) found that SEA was frequently produced
by strains from foods implicated in outbreaks,
whereas isolates from foods routinely sampled
produced SEA and SEC more frequently than
SEA. Bryant et al. (4) suggested that selective
enterotoxin production in suspect foods should
be considered when establishing a link be­
tween S. aureus isolates from food handlers
and the enterotoxin types produced in food
samples. Niskanen and Nurmi (22) studied the
effect of starter on the production of enterotox­
ins in sausage and reported that, in the absence
of starter, SEA could be detected, but SEB
could not be detected after 3 d with 10^6 cells/g.

van Schowenburg-van Foeken et al. (29) in­
duced starter failure and reported staphyloco­
cal counts in Gouda cheese of 3 to 4 x 10^6 cfu/g
after 24 h and the presence of .2 to .3 µg of
SEB/g or .05 to .2 µg of SEC/g. In addition,
SEC was detected when the S. aureus con­
centration was 8 x 10^7 cells/g; SEC disappeared
after 7 d. Active starter prevented the detection
of SEA but not SEC. We observed
staphylococcal enterotoxin when 8.5 to 10 x
10^5 cfu of staphylococci/g of cheese were pres­
ent.

The ratio of staphylococcal enterotoxin se­
creted in cheese prepared with .1% and 1%
starter is always higher in cheeses made with
.1% starter, which indicates better possibili­
ties for the synthesis of enterotoxins in these
cheeses compared with cheeses prepared with
1% starter, probably as a consequence of better
growth. Except in the case of SEA production
by S6, the mean production of enterotoxin was
higher in large cheeses than in small cheeses,
respect respectively, being 3 to 4 x 10^6 colony­
forming units per gram. In S6-.1%,
formed units per gram were higher in the
smaller cheese, coinciding with a higher
concentration of enterotoxin detected in the
smaller than in the larger.

CONCLUSIONS

Although the protocol was identical for all
the batches, results have shown high deviation.

However, staphylococcal enterotoxins may be present in Manchego-type cheese by the end of the ripening process and represent a health hazard when the initial staphylococcal contamination is high. This study demonstrates that staphylococci may survive the ripening process of Manchego-type cheese and that enterotoxins produced during this process may be present in the final product, posing a health problem to the consumer.

ACKNOWLEDGMENTS

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