Production of Italian Dry Salami. I. Initiation of Staphylococcal Growth in Salami Under Commercial Manufacturing Conditions

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ABSTRACT

Three strains of Staphylococcus aureus (S-6, 137 and 472) were inoculated, in duplicate, into Italian-style dry salami made with finished product as starter and processed under commercial manufacturing conditions. Five levels of S. aureus ranging from 2.2 x 10⁴ - 1.8 x 10⁶ cells/g were used. A fourth strain (264) was inoculated at a level of 10⁵ cells/g. All strains of S. aureus grew at every level of inoculation, but the amount of growth was dependent on inoculum size. Strains S-6 and 472 increased in number by 1.2 - 2.9 logs (mean 2.14) at inoculum levels of 3.7 x 10⁶ - 6.6 x 10⁶ cells/g. Strain 137 was very sensitive to salami environment and only increased by 0.47 - 1.86 logs (mean 1.23) even at the greatest inoculum level. Strain 264 increased in numbers by 1.5 logs in the presence of 5 x 10⁴ inoculated lactobacilli/g and by 2.5 logs in the presence of 6 x 10⁴ naturally occurring lactic acid bacteria. Staphylococci occurring naturally in salami mix were unable to grow to levels greater than 2 x 10⁴ cells at any time during processing of experimental sausages. Thermonuclease was detected only in salamis inoculated with strains S-6 and 472 at initial levels of greater than 3.7 x 10⁶ cells/g and only when growth reached levels greater than 10⁷ cells/g. No enterotoxin was detected in any of the inoculated samples. Development of regression equations allowed description of the growth of inoculated S. aureus in the salami during manufacturing as affected by a number of variables.

Staphylococcal intoxication is one of the three most common types of food poisoning in the United States (10). Although cured meat products, especially ham, frequently are implicated, food poisoning outbreaks involving fermented meat products like Genoa and Italian-style dry salami have been reported only recently (4-7). Because of these recent outbreaks, the safety of fermented meat products is being reevaluated.

Italian-style dry salami, manufactured primarily on the West Coast of the United States, is a naturally fermented meat product made of pork, beef, various spices and other nonmeat ingredients. Pork meat, the higher percentage of meat, consists of frozen shoulders and jowls while beef is mainly fresh, whole-carcass bull meat.

Use of commercial lactic starter culture in the fermentation of Italian style salami, was introduced only during the last 3 years. Before that time some companies added a natural "sausage starter" made from chopped finished salami to the sausage mix and most used no starter of any kind. Since many of these sausages do not receive any heat treatment, a successful fermentation is required not only to yield a desirable product, but also to prevent growth of food-borne pathogens, particularly staphylococci (8,11,12,14,24).

Limited information is available on the effect of various chemical, physical and microbiological factors on growth or enterotoxin production by S. aureus in fermented sausage manufactured under commercial processing conditions (8,21,24,25,29). This is especially true with regard to the processing of Italian-style dry salami (12). The initial staphylococcal contamination level required to overcome the inhibitory effect of the various processing parameters and create a safety problem is not known for this type of sausage. This investigation was undertaken to evaluate the effect of commercial processing of Italian-style dry salami on the growth and enterotoxigenesis of S. aureus.

MATERIALS AND METHODS

S. aureus and starter culture inocula

S. aureus strains 264, S-6, 137 and 472, producing enterotoxins A, A-B, C and D, respectively, were obtained from the culture collection of Dr. C. Genigeorgis. Lyophilized stock cultures were inoculated into brain heart infusion (BHI) broth (Difco) and incubated overnight at 37°C. Using the overnight culture, BHI broth and Plate Count Agar (PCA, Difco) were inoculated and incubated for 24 h at 37°C. Twenty-five ml of sterile BHI broth in a 250-ml Erlenmeyer flask were inoculated with S. aureus cells from the second BHI tube and the flask was incubated overnight on a reciprocal shaker at 37°C. The cells were harvested by centrifugation, suspended in 30 ml of fresh BHI broth and kept in ice until used to inoculate the sausages, 3 to 4 h later. The number of viable cells in the inoculum was determined by plating on PCA agar. Five ten-fold increasing levels of S. aureus strains S-6, 137

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and 472 ranging from 2.2 × 10^5 to 1.8 × 10^6 cells/g were used as inocula. The experiments were replicated once. For strain 264, only one level of 10^6 cells/g of sausage was used.

Two types of starter culture were used. A “natural” starter consisting of a sufficient amount of ground finished salami to attain approximately 1 × 10^5 Lactobacillus sp/g of sausage mix was used as inoculum in experiments involving S. aureus strains S-6, 137 and 472. For strain 264, a Lactobacillus sp., which is the major contributor to commercial Italian-style dry salami fermentation, was used at two levels (0 and 10^6 g). The Lactobacillus sp. had been used successfully as an experimental starter culture (Genigeorgis et al., unpublished).

 Cultures, prepared in APT broth (Difco), were kept lyophilized on porcelain beads. To regenerate the stock cultures, the beads were transferred into 10 ml of sterile APT broth and the broth was incubated at 30 °C for 48 h. Purity of the culture was tested by plating on APT agar. The 48-h-old subculture was used to inoculate 200 ml of APT broth in a 500-ml Erlenmeyer flask. The flask was incubated at 30 °C for 48 h. Cells were harvested by centrifugation and were resuspended in 30 ml of APT broth in the proper concentration to achieve a final level of 10^6 cells/g of sausage mix. The tubes containing the cultures were kept in ice until sausages were inoculated, usually within 3 to 4 h.

**Sausage formulation and processing**

The average composition of the commercial sausage mix used in these experiments was: frozen pork shoulders, 65.3%; frozen pork jowls, 10.3%; refrigerated fresh beef, 13.8% and non-meat ingredients, 10.6% (skim milk, 3.5%; NaCl, 3.2%; spices, 0.8%; NaNO_2, 140 ppm; and NaNO_3, 101 ppm). The average fat content in the sausage mix was 24.3%.

The sausage was prepared in the following manner. Frozen pork shoulders and jowls (temperatures -17 to 20 °C) were passed through a hydroflaker and conveyed by a screw-type conveyor to a large mixer. After thorough mixing in the chopper, the non-meat ingredients were separated from the production area of the plant. After addition of appropriate inoculum, each batch was ground twice in an electric grinder and stuffed into fibrous-material casings of 5.97-cm diameter to make standard 13-oz (369 g) sausages, using a manually operated stuffer.

The fermentation was carried out in the following manner. Strings of sausages were stored in a fermentation room at 16 °C and 88-90% relative humidity (RH) for 24 h and then at 24-26 °C and 75-80% RH for 2 days. Ripening was completed at 15-18 °C and 75-80% RH for 17 days. Individual sausages were taken from each batch at 0, 1, 2, 3, 7, 14 and 21 days after preparation. The samples were placed in cartons and shipped from the plant to the laboratory in the afternoon (a 2-h trip). Upon being received the samples were placed in the refrigerator overnight and were then analyzed for microbiological and chemical characteristics. Leftover and duplicate samples were held at -20 °C to be used within 1 to 2 months for enterotoxin assay.

**Microbiological assay**

The sausages were sliced into two approximately equal parts and samples were taken from cross sections on each side of the center of the sausage. Twenty-five grams of sausage were weighed aseptically into sterile Mason jars, 225 ml of sterile water was added and the mixture was blended for 3 min. The resulting slurry (1:10 dilution) and suitable dilutions were used for microbiological analyses. Total number of aerobes was determined on APT Agar (Difco) incubated at 25-28 °C for 2 days; total number of lactic acid bacteria on Rogosa SL (RA) Agar (Difco) at 25-28 °C for 3 days; mannitol fermenting, salt-tolerant bacteria, mainly cocci, on Mannitol Salt Agar (MS) (Difco) at 37 °C for 2 days; and staphylococci on Plate Count Agar (PCA) (Difco) at 37 °C for 24 h. After incubation for 24 h, PCA plates were overlayed with toluidine blue-DA agar (TBA) and incubated at 37 °C for 4 h. Nuclease-positive colonies were detected according to the method described by Lachica et al. (19).

**Chemical assays**

Moisture and nitrite were measured using standard AOAC (18) procedures. The pH was measured in the slurry (1:10 dilution) used for bacteriological analyses and in a mixture of 10 g of sausage and 20 ml of deionized distilled water (1:1 dilution). Reported pH was that of the 1:3 dilution.

Salt content of sausage was determined by means of Quantab chloride titrator strips, as recommended by AOAC (18). From values for water and salt, percentages of brine were computed (14).

Water activity (a_w) was determined using a Sina water activity meter (Model SJT-B, Sina, Switzerland).

**Assay for thermonuclease and enterotoxin**

Production and yield of S. aureus thermonuclease was determined according to the method of Lachica et al. (19). A homogenate of 10 g of sausage in 10 ml of distilled water was adjusted to pH 7.0 and boiled for 15 min.

Enterotoxin B or C was extracted from 100-g samples of periphery of sausages by affinity chromatography, as described by Genigeorgis and Kuo (13). Presence of detectable amounts of enterotoxin A or D was determined in 100-g samples of the periphery of sausages, using the extraction and assay procedures of Casman and Bennett (2,3).

**Statistical methods**

For statistical evaluation the biomedical computer program BDM03R (9) for multiple regression analysis was used.

**RESULTS AND DISCUSSION**

Chemical and microbiological changes occurring during the fermentation of four commercially processed lots of Italian-style dry salami are presented in Fig. 1 and Fig. 2.

![Figure 1](https://example.com/figure1.png)

**Figure 1. Changes in moisture, nitrite, brine and a_w during the fermentation of Italian-style dry salami (based on four commercial lots).**

No significant change in a_w was observed during the first 3 days. The a_w of the finished product (21 days) was approximately 0.89. The initial moisture of the sausage was approximately 55% and the finished product moisture about 40%. A brine concentration of approximately 10% was reached by the seventh day of fermentation. Residual nitrite decreased rapidly from 140 ppm initially to less than 10 ppm in the finished product.

Lactic acid bacteria reached a maximum level (10^3/g) at about the third day of fermentation and stayed almost constant during the ripening period (Fig. 2). The pH decreased with growth of lactic acid bacteria. Naturally
Figure 2. Changes in the growth of lactic acid bacteria (circle), salt tolerant cocc (square) and S. aureus (hexagon), and in pH (triangle) during the fermentation of Italian-style dry salami (based on four commercial lots).

occurring S. aureus in the salami mixes ranged from less than 1 x 10^2 - 1.9 x 10^3/g. At these initial levels staphylococci grew to a maximum of 3 x 10^2 - 2 x 10^4/g at about the second day of fermentation and then decreased in numbers with time. Total plate counts and lactic acid bacterial counts were very similar in numbers after the second day, indicating that the major flora of the sausage was lactic acid bacteria. There were no significant microbiological or chemical differences between sausages with or without inoculated S. aureus.

Growth of S. aureus strains S-6, 472 or 137 inoculated into salami mixes at five different levels is shown in Fig. 3. Growth occurred at every level of inoculation, but the magnitude differed with strain.

Figure 3. Effect of initial inoculum level on the growth of three S. aureus strains in commercially manufactured Italian-style dry salami.

In 16/20 inoculation studies, strains S-6 and 472 reached maximum growth by the third day of fermentation and in 20/20 inoculations by the seventh day. The same strains increased in numbers by 1.2 - 2.9 logs (mean 2.14) at the inoculum levels of 2.2 x 10^9/g - 2.5 x 10^9/g, by 2.1 - 3.2 logs (mean 2.66) at the inoculum levels of 3.7 x 10^9/g - 6.6 x 10^9/g and by 1 - 1.8 logs (mean 1.48) at the inoculum levels of 3.9 x 10^9/g - 1.8 x 10^7/g.

Strain 137 was very sensitive to the salami environment. Growth to maximum levels was slower than strains S-6 or 472. In 5/10 inoculations, maximum counts were reached by the third day and in 9/10 by the fourteenth day. In all ten studies, inocula of 3.0 x 10^9/g - 6.6 x 10^9/g increased during fermentation by a maximum of 0.47 - 1.86 logs (mean 1.23).

Growth of S. aureus strain 264 in salami is presented in Fig. 4. In the presence of 5.0 x 10^9 lactic acid/g of salami initially, 1 x 10^3 cells/g of strain 264 increased by 1.5 logs during fermentation. In the presence of a natural starter of 6 x 10^4 lactic acid bacteria/g salami, staphylococci grew well and increased by a maximum of 2.5 logs. This demonstrated the importance of the initial level and type of lactic acid bacteria in controlling staphylococcal growth.

This study indicated that fresh S. aureus cultures present in salami mixes at levels less than 1 x 10^9/g were unable to grow to levels greater than 1 x 10^5/g of salami under the commercial formulation and manufacturing conditions used. Heavier initial contamination levels grew and reached numbers which have been associated with enterotoxin production (10,14,23). Increased probability of initiation of growth and attainment of high levels of various foodborne bacteria with increasing levels of initial contamination of food has been established previously (14,15,30). Naturally occurring staphylococci in salami mixes may be debilitated and may not behave in the same manner as fresh cultures. Consequently, heavy initial contamination will be needed to result in growth to dangerous levels.

Recent studies (12) of salami ingredients and mixes used by two major companies demonstrated a S. aureus prevalence of less than 14.3% and 51.4%. The highest count recorded in mixes over a period of 2 years was 7.1 x 10^5/g. Maximum count during fermentation was 3.7 x 10^4/g.

The Italian-style dry salami implicated in recent staphylococcal food poisoning outbreaks (6,7) was found to have millions of staphylococci/g and to contain...
enterotoxins. Data on the initial staphylococcal contamination level of the sausage mix were not reported. It is apparent that formulation and processing conditions of these salamis were unable to prevent growth of staphylococci present initially in the mixes.

Using stepwise multiple regression analysis, we attempted to derive equations which described the observed behavior of the individual staphylococcal strains, and the combined behavior of strains S-6 and 472 as influenced by a number of parameters. The model used for the equations was:

$$Y_i = a + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + \ldots$$ Where $Y_i = \log_{10} S. aureus$ counts/g salami at a given day of fermentation, $a = \text{intercept}$, $b_1$, $b_2$, $b_3$, $b_4 = \text{regression coefficients}$ and $X_1$, $X_2$, $X_3$, $X_4 = \text{variables affecting growth of S. aureus}$. Regression coefficients of the variables and other pertinent information for each inoculum type are presented in Table 1. They can be used to estimate the level of $S. aureus$ for any particular day of fermentation. Application of the model equation indicated relatively close values for estimated and observed $S. aureus$ levels in the salami. Efforts to describe mathematically the behavior of naturally occurring staphylococci in four lots of salami were not satisfactory. This is due to limited sampling and the fact that the exact number of $S. aureus$ present in salamis with < 100 cells/g was not known.

### TABLE 1. Summary of intercepts (a), variables, regression coefficients, standard error (S.E.) and multiple correlation coefficients (M.C.C.) of the derived prediction for $S. aureus$ growth in Italian dry salami.

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>137</th>
<th>S-6</th>
<th>472</th>
<th>S-6 + 472</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.821</td>
<td>2.188</td>
<td>2.092</td>
<td>2.150</td>
</tr>
<tr>
<td>Staph</td>
<td>0.867</td>
<td>2.086*</td>
<td>0.901</td>
<td>0.871</td>
</tr>
<tr>
<td>i</td>
<td>-0.052</td>
<td>-0.307*</td>
<td>-0.157</td>
<td>-0.232</td>
</tr>
<tr>
<td>e/i</td>
<td>-0.693</td>
<td>-1.304*</td>
<td>-0.840</td>
<td>-1.071</td>
</tr>
<tr>
<td>it/1000</td>
<td>-0.002</td>
<td>0.011*</td>
<td>-0.004</td>
<td>0.008</td>
</tr>
<tr>
<td>M.C.C.</td>
<td>0.938</td>
<td>0.863</td>
<td>0.890</td>
<td>0.868</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.470</td>
<td>0.842</td>
<td>0.746</td>
<td>0.804</td>
</tr>
</tbody>
</table>

* Regression coefficients.

* Day of fermentation.

None of the salami samples taken during the processing or ripening period in this study contained detectable enterotoxins, even when staphylococci were greater than $10^7$ g of salami. However, when staphylococci were greater than $10^7$ g of salami. It has been reported that $S. aureus$ concentrations of over $1 \times 10^6$ cells/g are required before enterotoxin is produced in quantities detectable in 100-g samples of foods $$(10,11,23,24).$$. Tatini et al. (29), however, were unable to detect enterotoxin production in pepperoni inoculated with $10^4$ or $10^6 S. aureus$/g even when the numbers of staphylococci reached levels of $10^7$ cells/g. Similarly, other investigators working with culture media and various foods observed staphylococcal growth to levels greater than $10^9$ g without enterotoxin production. This was especially true for enterotoxin B producing strains $$(10,21,23,24).$$

The inability of lactobacilli and curing agents used in sausage formulation to prevent staphylococcal growth at all times is in agreement with reported data $$(1,14,16,17,23,26).$$ The changes in the pH from 6.1 to 5.2 or the increase of brine concentration from 6% to 8% observed during the first 3 days of fermentation were not expected to stop either staphylococcal growth or enterotoxin production. The lack of enterotoxin production at detectable levels, however, might be due to the combined effect of processing conditions (pH, brine, NaN0 2, aw) and the effect of lactic acid bacteria. Barber and Deibel $$(1)$$ have shown that growth and enterotoxin production by staphylococci are dependent on available oxygen, pH and presence or absence of lactic acid starter culture in the sausage. Haines and Harmon $$(16)$$ indicated that, although lactobacilli alone may not inhibit growth of $S. aureus$, they can exert an inhibitory effect on enterotoxin production. Niskanen and Nurmi $$(24)$$ reported that the starter culture used in their study (lactobacilli and micrococcii) had an inhibitory effect on $S. aureus$ only when the staphylococcal inoculum was smaller than the starter inoculum and that the starter culture used prevented formation of detectable levels of enterotoxin.

In the absence of any starter culture and thus effective competition, Lee et al. $$(21)$$ were able to demonstrate enterotoxin A but not B production in laboratory-made Genoa sausage inoculated with $10^6$ to $10^7 S. aureus$/g. No enterotoxin A production was detected in salami inoculated with $10^7$ cells/g, even when there was growth to $10^9$ cells/g. Raccach and Baker $$(25)$$ reported that the lactic acid producing organisms should outnumber $S. aureus$ population by a range of $10^5$ to $10^4$ to suppress the pathogen. Raccach et al. $$(26)$$ reported that the repressive effect of lactic acid bacteria upon $S. aureus$ decreased with increasing temperature from 15 C to 35 C.

Data on the threoninuclease production and growth of $S. aureus$ strains S-6 and 472 during salami manufacturing are presented in Fig. 5. Threoninuclease was detected in sausages inoculated only with strains S-6 and 472 and at initial levels greater than 3.7 $\times 10^4$ cells/g. Data in Fig. 5 indicate that with increased levels of inoculation both initial detection and maximum yield of threoninuclease were observed when $S. aureus$ growth reached numbers greater than $10^8$ cells/g of sausage. Maximum yield of threoninuclease for strain 472, at $10^6$ g of sausage, was nearly four times higher than at the other two levels of inoculation. Similar maximum yields of threoninuclease were obtained at all of the three highest inoculation levels of strain S-6; however, less threoninuclease was produced by strain S-6 than by strain 472.

A significant effect of process on production of threoninuclease was observed. Strains 137 and 264 produced no detectable threoninuclease, while strain 472 appears to be the least affected by the process. The tolerance to heating, prolonged storage and bacterial proliferation exhibited by staphylococcal threoninuclease has been studied. $$(20,22,24,25).$$ Lachica et al. $$(20)$$ found that, of seven bacterial species studied, only Bacillus...
The fact that a decreased thermonuclease activity was observed after the seventh day of fermentation does not reduce the utility of the thermonuclease test as a means of evaluating staphylococcal growth in Italian-style dry sausage. Since brine concentration in the salamis is greater than 10% by the seventh day of fermentation and the pH is below 5.0, it is highly improbable that growth of *S. aureus* after the seventh day will result in enterotoxin production (11,14). Thus a test for nuclease on or before the seventh day of processing will be of great value in determining staphylococcal growth and, indirectly, possible enterotoxin production during the early phase of fermentation.

The present data emphasize the importance of keeping the staphylococcal contamination of sausage ingredients at the lowest possible level. Lack of enterotoxin production observed in these studies, even at levels of greater than 10⁶ *S. aureus*/g of sausage, can be attributed to the successful fermentation attained by this particular processor (Fig. 1). When there are low initial levels of lactic acid bacteria and a rapid fermentation is not attained, growth of *S. aureus* and enterotoxin production becomes possible. In the food poisoning outbreak involving Italian-style dry salami (6), no starter culture, either commercial or natural, was used. Thus initially present staphylococci were able to grow to dangerous levels in the absence of effective competition.

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