Application of a Mathematical Model for the Inhibition of Enterobacteriaceae and Clostridia during a Sausage Curing Process

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ABSTRACT

The application of a mathematical model capable of providing expressions suitable for analysis of the population changes of Enterobacteriaceae and clostridia during the curing process of dry fermented sausage is proposed to facilitate better control of the safety of the product.

The mathematical model provides curing times that are crucial for control of the safety of the product: the beginning of the exponential phase of inhibition, the time during which the rate of inhibition is at a maximum, and the phase in which the rate of decline of the population of microorganisms decreases until they virtually disappear from the sausage.

For Enterobacteriaceae, growth inhibition and eventual disappearance are dependent on conditions during sausage drying; for clostridia the means of drying is not significant.

Under programmed controlled drying conditions, the maximum speed of inhibition and the disappearance of undesirable microorganisms are reached sooner than under natural climatic drying conditions.

A regression equation has been established that relates the cell number of Enterobacteriaceae to pH and a_w values, and the cell number of Lactobacillaceae, whose metabolic activities give rise to lactic and acetic acids. The significance of the drying conditions may verify the values reached by all these parameters during curing.

Key words: Dry fermented sausage, Enterobacteriaceae, mathematical model

Many undesirable microorganisms are incapable of growing under these conditions. During the initial stages of microbial fermentation, the number of Enterobacteriaceae usually remains constant, and as desiccation of the sausage progresses Enterobacteriaceae, including salmonellae, are slowly inactivated (25, 27, 29). Cases of salmonellosis as a result of ingestion of dry sausages are very rare, as salmonellae are inhibited by lactic acid bacteria (11).

Clostridia are also inhibited by the processes associated with curing the sausages. There is hardly any known epidemiological evidence that indicates the formation of botulinum toxins in dry fermented sausages; this is without doubt because growth of Clostridium botulinum is inhibited at characteristic low pH and water activity values and is also sensitive to nitrate, either added as such or formed by the reduction of nitrate (15, 23).

However, errors during the manufacture of such sausages can mean risks of foodborne infections and intoxications. Therefore, conditions such as an initial high a_w value and pH value, a small number of lactobacillae in the sausage mixture at the beginning of curing, high fermentation temperatures, etc. tend to favor the development of undesirable microorganisms (5, 17, 29).

Strict control over the conditions that govern the manufacture of these sausage products will be improved the more knowledge is gained of the changes in populations of these microorganisms and the factors that affect them. Over the past few years the application of various mathematical models, some based on the Gompertz equation, have been proposed to predict the growth of pure cultures of certain types of microorganisms (12, 13, 30). However, none of these models can be adjusted to the real population changes of microorganisms when these are found inside a sausage mixture undergoing a process of fermentation and desiccation.

For this reason, it is considered of interest to have a mathematical model that enables a deeper understanding of the response of certain microorganisms to the particular conditions of some foodstuffs and that enables better control of their safety. This study proposes and applies a mathemati-
MATERIALS AND METHODS

Preparation of dry sausage

The manufacture of Spanish dry fermented sausage (chorizo) took place in a semiindustrial pilot plant using the formula in Table 1.

The meat ingredients and the pork fat were chopped separately using a cutter and then minced separately using 25-mm and 12-mm plates respectively. Curing salts and seasonings were added and the mixture was kneaded for 10 min in a vacuum kneading machine to eliminate the occlusion of air. The mixture was then stuffed into 70-mm-diameter reconstituted collagen casings.

After this, the sausages were grouped into two batches, each batch being placed in a different chamber for the fermenting and drying processes. The first batch was subjected to natural drying conditions (NC): T: 12 to 24°C; R.H., 78 to 95%. The second batch was placed in a climatic chamber (CC) programmed so that the temperature could be maintained at or reduced from 22°C to 14°C, and with a variable relative humidity between 80% and 73%, so that water loss was suitable for the established program. In both cases, the study was considered complete after 17 days of curing, as the presence of undesirable microorganisms was no longer detected. Four sausages were taken from each batch for analysis at 0, 3, 10, and 17 days of curing. Samples were immediately taken to the laboratory for microbiological and chemical analysis.

The microbiological analyses that were carried out were as follows. Sausage (10 g) was homogenized in 90 ml of peptone water (sterile conditions) for 2 min using a stomacher. From this suspension decimal dilutions in peptone water were prepared and spread onto the following media: De Man Rogosa Sharpe agar (MRS)(Oxoid) plates for Lactobacillaceae (30°C, 72 h) in an anaerobic jar with a CO₂-enriched atmosphere (Gaspack, BBL); staphylococcus medium no. 110 (Oxoid) for Micrococcaceae; violet red bile agar (VRBA)(Oxoid) broth, with 1% added glucose (dextrose, Oxoid), incubated at 37°C for 48 h for Enterobacteriaceae (19); and iron sulfate broth for clostridia spore count. The spore count was determined after that, and covering the already-solid tubes with agar, with incubation at 37°C for 48 h. The results were expressed as CFU/g of sausage (3).

The pH was determined using a Potentiometer Orion Research microprocessor ion analyzer 901, with selective electrodes for solid samples. The water activity (a) was measured with an EELA-3 Novasina apparatus. Lactic acid was measured using the enzyme method according to Noll (24), with an enzyme test kit from Boehringer Marnheim GmbH. Acetic acid was measured by using the method described by Halvarson (16) and Duda et al. (8) for the isolation and separation of volatile fatty acids. Quantitative determination was carried out using a PerkinElmer autosystem gas chromatograph with FID and Nukol capillary column (30 m by 0.25 mm), according to Ceccon et al. (7): oven temperature, 172°C; detector temperature, 220°C; injector temperature 220°C; crotonic acid used as internal standard.

Mathematical and statistical analyses

The data included in the tables and figures correspond to the mean values of 12 analytical determinants. Statistical analyses were carried out using a Statgraphics program, version 5.1, in its application to the multifactorial analysis of variance with two factors of variation (curing days and means of controlling the climatic drying conditions) and the interaction between them.

For the study of changes in the populations of Enterobacteriaceae and clostridia during the curing of the dry sausage a mathematical function related to the Gompertz equation that adapts to the development of these microorganisms was proposed: \[ Y = Y_0 \cdot e^{-K(X-K_2)} \]
where \( Y_0 \) = value of the counts at the beginning of the experiment; \( X \) = mean curing time in days; \( K_2 \) = rate of relative depletion; and \( K_2 \) = curing time during which the maximum speed of depletion is reached. To interpret the characteristics of population changes, one determines the maximum and minimum points obtained by the first derivatives (rate of change in microbial counts) and second derivatives (acceleration) of the function.

RESULTS AND DISCUSSION

The changes in populations of Enterobacteriaceae and clostridia, undesirable microorganisms, were studied in the curing process of the dry fermented sausage chorizo, a traditional Spanish sausage. The sausage was manufactured using different drying conditions: in chambers of natural climatic drying conditions (NC) in chambers with programmed, controlled temperatures and relative humidity (CC).

Shown in Table 2 are the counts obtained for the fermentative microorganisms Lactobacillaceae and Micrococcaceae, as well as for Enterobacteriaceae and clostridia, at days 0, 3, 10, and 17 of curing. An analysis of variance highlights the fact that there were significant differences \((P < 0.005)\) between the counts obtained at each of the sample points. Furthermore, the multifactorial analysis of variance (Table 3) with two factors of variation (days of curing and means of drying) indicates significant differences \((P < 0.001)\) in the changes in populations of the two types of fermentative microorganisms and of Enterobacteriaceae, depending on the means of drying of the sausage (NC and CC). However, the type of drying did not significantly affect the changes of clostridia cell populations.

Fermentative microorganisms initially present in the sausage mixture undergo important growth during the first 3 days of curing, which slightly decreases as the mixture progressively dries out. In contrast to this, Enterobacteriaceae and clostridia are inhibited; there is an exponential fall in their counts, until they virtually disappear from the sausage mixture. This reduction of undesirable microorganisms presents...
TABLE 2. Changes in microbial populations (log CFU/g of sausage) during the ripening of dry fermented sausage under natural climatic drying conditions (NC) and controlled drying conditions (CC)

<table>
<thead>
<tr>
<th>Day</th>
<th>Lactobacilli (NC)</th>
<th>Lactobacilli (CC)</th>
<th>Microcilli (NC)</th>
<th>Microcilli (CC)</th>
<th>Enterobacteria (NC)</th>
<th>Enterobacteria (CC)</th>
<th>Clostridia (NC)</th>
<th>Clostridia (CC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.63 ± 0.03</td>
<td>6.70 ± 0.02</td>
<td>5.74 ± 0.02</td>
<td>5.79 ± 0.04</td>
<td>4.77 ± 0.03</td>
<td>4.59 ± 0.03</td>
<td>1.18 ± 0.03</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>8.35 ± 0.02</td>
<td>8.82 ± 0.04</td>
<td>6.55 ± 0.05</td>
<td>7.06 ± 0.06</td>
<td>3.68 ± 0.03</td>
<td>2.94 ± 0.04</td>
<td>0.61 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>8.28 ± 0.04</td>
<td>8.47 ± 0.03</td>
<td>6.48 ± 0.06</td>
<td>6.76 ± 0.05</td>
<td>1.29 ± 0.04</td>
<td>0.36 ± 0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>7.66 ± 0.03</td>
<td>7.85 ± 0.02</td>
<td>5.53 ± 0.03</td>
<td>5.56 ± 0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* n = 16.

TABLE 3. Analysis of variance with two factors of variation (days of curing and means of drying) applied to changes in microbial counts during the ripening of dry sausage in two climatic conditions (NC and CC)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Enterobacteria</th>
<th>Clostridia</th>
<th>Lactobacilli</th>
<th>Microcilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of Ripening</td>
<td>F  &lt;0.001</td>
<td>P &lt;0.001</td>
<td>F  137</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>Drying Conditions</td>
<td>135 &lt;0.001</td>
<td>0.9 NS</td>
<td>49 &lt;0.0011</td>
<td>9 &lt;0.001</td>
</tr>
<tr>
<td>Iteration</td>
<td>33 &lt;0.001</td>
<td>0.4 NS</td>
<td>4 &lt;0.001</td>
<td>9 &lt;0.001</td>
</tr>
</tbody>
</table>

* NS, not significant.

As the multifactorial analysis of variance had already established in this case a close similarity can be seen in the plots of the expressions for the changes of clostridia (Figure 2). The first and second derivatives result in values of $v'$, $a''$ and $A''$ that are very close under NC and CC conditions.

Similarly, the mathematical function has been applied to the counts obtained for clostridia, which also yields two expressions with highly significant regression coefficients.

(NC) clostridia = $1.26 \left[1-e^{-0.60(D-3.01)}\right]$, ($r^2 = 0.982$); (CC) clostridia = $1.22 \left[1-e^{-0.65(D-2.27)}\right]$, ($r^2 = 0.966$).

For the counts of Enterobacteriaceae two expressions are obtained that correspond to each of the drying conditions, resulting in each case in highly significant coefficients of regression.

(NC) Enterobacteria = $4.78 \left[1-e^{-0.25(D-4.89)}\right]$, ($r^2 = 0.993$); (CC) Enterobacteria = $4.68 \left[1-e^{-0.34(D-3.22)}\right]$, ($r^2 = 0.993$).

The plots of these mathematical expressions (Figure 1) highlight the differences in the changes of these microorganisms with curing, depending on the means of producing and controlling the desiccation of the product. The first derivative has a minimum value ($v'$) that indicates the curing time during which the rate of the fall in counts reaches a maximum value: 117 hours under NC conditions and 78 hours under CC conditions. This means that the manufacture of the sausage under strictly controlled programs of climatic control enables the maximum rate of reduction of enterobacteria to be advanced by 41 h. The second derivative produces two points essential to better understand the changes in populations of these microorganisms: a minimum ($a''$) and a maximum ($A''$). The first represents the moment at which the counts of the microorganisms initiate an exponential reduction (24 and 9 h), while the second represents the moment from which the marginal decline of these microorganisms is ever smaller, up to the point of their disappearance from the sausage mixture (210 and 144 h).
although it should be noted that under controlled drying conditions the points that characterize the reduction of clostridia are always reached 5 to 6 h earlier. Table 4 brings together the total curing times that characterize the particular changes of both types of undesirable microorganisms throughout the manufacture of the sausage.

Although the phase of exponential fall begins earlier with enterobacteria than with clostridia, the latter nevertheless reach the maximum rate of reduction and the phase of disappearance from the sausage mixture earlier. Thus it is the enterobacteria that may remain longer in the sausage; a more rigorous control is necessary of the parameters responsible for inhibiting their growth, i.e., water activity and pH (related to lactic and acetic acids).

During the manufacture of all dry sausage two fairly efficient systems of food preservation are brought together:

- desiccation and fermentation. The first gives rise to a reduction in water activity, while the second produces a fall in pH as a result of the metabolic activity of fermentative microorganisms, especially Lactobacillus. Raw sausage generally becomes a stable and safe product, having passed over several hurdles of that inhibit spoilage and food-poisoning bacteria. The pH and \( a_w \) values are very important hurdles determining product stability; the secret of microbiological safety of the dry-cured sausage is to make optimal use of the sequence of hurdles (21).

- Figures 3 and 4 are plots of the values found at each sample point for four parameters believed to be responsible for the reduction of enterobacteria and clostridia: \( a_w \), pH, lactic acid, and acetic acid. It is known that during the desiccation of sausage the enterobacteria slowly become inactivated (26, 29), but it is the lactic acid bacteria that represent a competitive flora for growth of these undesirable microorganisms by producing weak organic acids capable of penetrating into the cells and preventing growth (10), without considering other types of effects such as the production of bacteriocins by lactobacillae (21).

In view of previous results it could be thought that lactic acid is mainly responsible for the beginning of the exponential fall of the enterobacteria counts, because for the curing times indicated by the minimum \( a_w \), the pH and \( a_w \) values could be considered high.

Under NC conditions the maximum rate of fall in the counts is reached at day 4.9 when \( a_w \) is 0.947 and pH is 5.4; however under CC conditions the maximum rate is attained at day 3.2 when \( a_w \) is 0.943 and pH is 5.24. In both cases, the concentration of lactic and acetic acid is, nevertheless, similar: 3.8 and 0.8 g/kg of sausage respectively. At pH values less than 5.5, lactic acid is fairly efficient at inhibiting the growth of most undesirable microorganisms, because the percentage of nondissociated molecules favors its penetration into microbial cells, although the response varies according to the type of bacteria (1, 2, 6, 28). Lactic acid bacteria can produce small quantities of acetic acid (9), which somewhat limits antimicrobial activity (22), although in its nondissociated form it

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**TABLE 4. Curing times that characterize the changes in numbers of Enterobacteriaceae and clostridia calculated from the application of the mathematical model**

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Derivatives</th>
<th>Enterobacteria</th>
<th>Clostridia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( Y' ), ( Y'' )</td>
<td>NC</td>
<td>CC</td>
</tr>
<tr>
<td>Beginning exponential reduction</td>
<td>( a_w ) (minimum)</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Maximum rate decline</td>
<td>( v' ) (minimum)</td>
<td>117</td>
<td>78</td>
</tr>
<tr>
<td>Marginal decline</td>
<td>( A_w ) (maximum)</td>
<td>210</td>
<td>144</td>
</tr>
</tbody>
</table>

\( a_w \) NC, no climate control; C, climate controlled.
usually inhibits enterobacteria (4). There are, notwithstanding, references to a synergistic effect between acetic and lactic acid (1).

The phase leading to the total disappearance of enterobacteria always begins when the pH of the sausage falls below 5.0 and when the aw falls below 0.940. At this point the levels of lactic and acetic acid are high but these metabolites are still being produced due to the exponential phase of growth of the lactic acid bacteria.

All the parameters were subjected to a multiple regression statistical analysis which showed that the population changes of Enterobacteriaceae throughout the curing of the dry sausage in both NC and CC conditions is a function of the water activity and pH of the sausage mixture, as well as of the level of the lactic acid bacteria present. All parameters have a high level of significance (P < 0.001) in both conditions. The regression equations are as follows.

**Regression for drying with no climate control (NC):**

\[
\text{Enterobacteria (CFU)} = 12.11 \text{aw} + 3.98 \text{pH} + 0.07 \text{lactobacilli} - 30.71 \text{where } r^2 = 0.990, F (all parameters) = 378.3, \text{and } P < 0.001.
\]

**Regression for drying with climate control (CC):**

\[
\text{Enterobacteria (CFU)} = 30.89 \text{aw} + 3.77 \text{pH} + 0.56 \text{lactobacilli} - 50.79 \text{where } r^2 = 0.996, F (all parameters) = 9111.7, \text{and } P < 0.001.
\]

In conclusion, in the manufacture of dry fermented sausages control and programming of the drying process are shown to have a clear influence over the rate of inhibition of growth of Enterobacteriaceae. Similarly, the characteristics of the population changes can be studied using the mathematical development of a function related to the Gompertz equation. The particular characteristics shown in the development of the bacteria under specific conditions can be interpreted as a function of the parameters which for these undesirable microorganisms represents a sequence of hurdles: aw, pH, and production by lactic acid bacteria of lactic and acetic acids.

**REFERENCES**