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# Modelling the growth boundaries of *Staphylococcus aureus*: Effect of temperature, pH and water activity

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ABSTRACT

The microbial behaviour of five enterotoxigenic strains of *Staphylococcus aureus* was studied in the growth/no growth domain. A polynomial logistic regression equation was fitted using a stepwise method to study the interaction of temperature (8, 10, 13, 16 and 19 °C), pH (4.5; 5.0; 5.5; 6.0; 6.5 7.0 and 7.5) and water activity ($A_w$) (19 levels ranging from 0.867 to 0.999) on the probability of growth. Out of the 284 conditions tested, 146 were chosen for model data and 138 intermediate conditions for validation data. A growth/no growth transition was obtained by increasing the number of replicates per condition ($n=30$) in comparison to other published studies. The logistic regression model showed a good performance since 96.6% (141 out of 146 conditions) of the conditions for model data and 92.0% (127 out of 138 conditions) for validation data were correctly classified. The predictions indicated an abrupt growth/no growth interfaces occurred at low levels of temperature, pH and $A_w$. At 8 °C, *S. aureus* grew only at optimum levels of pH and $A_w$ while at temperatures above 13 °C, growth of *S. aureus* was observed at pH = 4.5 and $A_w=0.96$ (13 °C), 0.941 (16 °C) and 0.915 (19 °C). The optimal pH at which growth of *S. aureus* was detected earlier was 6.5. However, a slight decrease of the probability of growth was noticed in the pH interval of 7.0–7.5 at more stringent conditions. The ability of *S. aureus* to grow at low $A_w$ was shown since growth was detected at $A_w=0.867$ ($T=19$ °C; pH = 7.0). Finally, a comparison of model predictions with literature data on growth/no growth responses of *S. aureus* in culture media and cooked meat was made. Model predictions agreed with published data in 94% of growth cases and in 62% of no growth cases. The latter discordance is highly associated to other environmental factors (such as other preservatives, strains etc.) included in published models that did not match the ones included in our study. This study can help manufacturers in making decision on the most appropriate formulations for food products in order to prevent *S. aureus* growth and enterotoxin production along their shelf-life.



## 1. Introduction

*Staphylococcus aureus* has been recognized as an indicator of deficient hygiene of food and processing and a major cause of food gastroenteritis worldwide (Soriano et al., 2002). Food poisoning caused by staphylococcal intoxication is due to the ingestion of foods that contain thermotolerant staphylococcal enterotoxins (SE) (Scherrer et al., 2004). An enterotoxin dose of ≤1.0 μg in contaminated food produces symptoms of staphylococcal intoxication, but this toxin level is typically reached only when *S. aureus* populations exceed $10^5$ cfu/g (Notermans and Heuvelman, 1983; USDA, 2007). If environmental conditions during food storage and preparation allow the growth of *S. aureus* (i.e. time and temperature abuse) staphylococcal enterotoxins may be produced, being potentially harmful for consumers (Todd et al., 2008).

According to the most recent European Food Safety Authority report (EFSA, 2006) on zoonosis, 35 outbreaks associated to *S. aureus* toxins were reported in the European Union, which resulted in 777 cases, 14 hospitalizations and 1 death. In Spain, the number of cases reported to the Spanish Microbiological Information System caused by *S. aureus* ingestion, increased from 442 cases in 2001 to 649 cases in 2006. However, the true incidence of staphylococcal food poisoning is likely much higher because many cases are not reported to healthcare services.

There are several studies reporting the presence of *S. aureus* in Ready-To-Eat (RTE) and perishable foods, such as raw pork or smoked ham (Atanassova et al., 2001), poultry products (Pepe et al., 2006), milk (Fujikawa and Morozumi, 2006), fish (Simon and Sanjeev, 2007); or foods that are prepared in advance before consumption and stored after preparation without adequate refrigeration (Roberts, 1986). Staphylococcal intoxication can also occur through the consumption of contaminated fermented food products, cooked meat products or cheeses (Gilmour and Harvey, 1990).

The persistence of *S. aureus* in food preparation areas has been also widely demonstrated by several studies, reporting its presence on work surfaces and utensils in food services (Sneed et al., 2004; DeVita

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et al., 2007). Other studies report also its presence on worker hands since this microorganism is a common member of the transient and resident skin microflora (Sattar et al., 2001).

*S. aureus* is capable to grow in a wide range of temperatures, ranging from 7° to 48.5 °C with an optimum of 30 to 37 °C; (Schmitt et al., 1990), pH between 4.0 and 10.0, with an optimum of 6.0–7.0; (ICMSF, 1996) and sodium chloride with concentrations up to 25% (ICMSF, 1996). These characteristics enable *S. aureus* to grow and survive in a wide range of environmental conditions as well as to persist in stressful environments (e.g. dry surfaces) for long periods.

Based on both food safety and quality guarantee, growth/no growth models can help manufacturers to design alternative formulations in food products in order to inhibit microbial growth. This fact is especially interesting in foods that are subjected to minimal processing in the food industry and are distributed with a short shelf-life. Several growth/no growth models have been developed for pathogens (Koutsoumanis et al., 2004; Skandamis et al., 2007; Valero et al., 2007) and spoilage microorganisms (Masana and Baranyi, 2000).

Physico-chemical factors that influence *S. aureus* growth and enterotoxin production have been widely studied (Genigeorgis, 1989) and several kinetic predictive models can be found in the literature (Sutherland et al., 1994; Zurera-Cosano et al., 2004; Lindqvist, 2006). However the use of kinetic models without information of their limits may lead to wrong estimations because of extrapolation of the model beyond these limits (Le Marc et al., 2005). In this sense, growth/no growth models provide more detailed information about the microbial limits for growth. Lanciotti et al. (2001) studied the effect of temperature, pH, water activity ($A_w$) and ethanol concentration on the probability of growth of *S. aureus*, but only at growth temperatures >10 °C. Besides, in that study, $A_w$ levels were adjusted by using glycerol instead of NaCl as humectant. This can result in estimates of the minimal $A_w$ for growth that is biased towards higher values than could be expected in many foods and may possibly result in fail-dangerous predictions. Stewart et al. (2002) studied the effect of different humectants on the growth boundary of a five strain *S. aureus* cocktail, but this model was developed at optimal temperature (37 °C).

Also, these growth/no growth models generally predict an abrupt transition zone between growth and no growth conditions. This fact occurs because of the small number of replicates ($n$) in comparison to the number of conditions tested. In this situation, difficulties in the application of logistic regression models are found, especially when achieving convergence to a global optimum or to an appropriate set of conditions (Ratkowsky, 2002), unless alternative procedures are used (Geeraerd et al., 2004). By increasing the number of replicates ($n=30$), smoother transitions between growth and no growth zones can be achieved. With this methodology, probability of growth does not dramatically change along a narrow set of conditions since more reliable growth/no growth responses are provided.

In addition, there is a growing tendency to use a cocktail of different strains in order to predict a more realistic microbial behaviour in foods. In the case of growth/no growth models, as stated by Vermeulen et al. (2007), the use of different strains allows to obtain a broader growth/no growth domain, since at certain stressful conditions, growth is generally led by the most resistant strain. Besides, the importance to take into account the strain variability for predictive modelling and risk assessment purposes has been emphasized (Lindqvist, 2006).

In this study, the influence of temperature, pH and water activity on growth/no growth of a cocktail of five enterotoxigenic strains of *S. aureus* was evaluated. A polynomial logistic regression equation was built to study the interactions of these factors on the probability of growth of *S. aureus*. Subsequently, an internal validation of the model was performed by selecting a dataset within the interpolation region. Finally, a number of predictions obtained were compared and discussed with data from published studies.

## 2. Materials and methods

### 2.1. Experimental design and media preparation

A fractional factorial design was followed in order to know the growth limits of *S. aureus*. It was made by carefully choosing a subset (fraction) of the experimental runs of a full factorial design in order to reduce experimental time and resources. The selection was based on delimiting the levels of the environmental factors studied to the growth/no growth domain of *S. aureus*, as followed in other previous studies (Valero et al., 2007). Since no growth was detected at 7.5 °C or below (data not shown), data were collected at 8 (refrigeration temperature), 10, 13 (considered as temperature abuse), 16 and 19 °C (considered as extreme temperature abuse), at pH levels from 4.5 to 7.5 (0.5 intervals) and at 19 $A_w$ levels (from 0.856 to 0.999 at regular intervals). The experimental conditions investigated are summarized in Table 1. The initial dataset (287 conditions) was divided in two parts: model data (146 conditions covering the extreme domain of the model) and validation data (141 conditions within the interpolation region of the model). The purpose of this selection was to define a dataset for model data focused on the extreme regions of the growth/no growth domain, that actually represent the boundary zones. In this study, the number of replicates per condition ($n=30$) was increased in comparison to other studies to obtain the growth/no growth transition.

**Table 1**
Experimental conditions and levels of temperature, pH and $A_w$ considered for both model and validation datasets for *S. aureus*.

| $T$ (°C) | Model data | | Validation data | |
|---|---|---|---|---|
| | pH levels | $A_w$ | pH levels | $A_w$ |
| 8 | 4.5; 5.5; 6.5; 7.5 | 0.999 | 5.0; 6.0; 7.0 | 0.999 |
| | 5.0; 6.0; 7.0 | 0.995 | 4.5; 5.5; 6.5; 7.5 | 0.995 |
| | 4.5; 5.5; 6.5; 7.5 | 0.989 | 5.0; 6.0; 7.0 | 0.989 |
| | 5.0; 6.0; 7.0 | 0.983 | 4.5; 5.5; 6.5; 7.5 | 0.983 |
| | 4.5; 5.5; 6.5; 7.5 | 0.977 | 5.0; 6.0; 7.0 | 0.977 |
| | 5.0; 6.0; 7.0 | 0.970 | 4.5; 5.5; 6.5; 7.5 | 0.970 |
| | 4.5; 5.5; 6.5; 7.5 | 0.963 | 5.0; 6.0; 7.0 | 0.963 |
| 10 | 4.5; 5.5; 6.5; 7.5 | 0.989 | 5.0; 6.0; 7.0 | 0.989 |
| | 5.0; 6.0; 7.0 | 0.983 | 4.5; 5.5; 6.5; 7.5 | 0.983 |
| | 4.5; 5.5; 6.5; 7.5 | 0.977 | 5.0; 6.0; 7.0 | 0.977 |
| | 5.0; 6.0; 7.0 | 0.970 | 4.5; 5.5; 6.5; 7.5 | 0.970 |
| | 4.5; 5.5; 6.5; 7.5 | 0.963 | 5.0; 6.0; 7.0 | 0.963 |
| | 5.0; 6.0; 7.0 | 0.956 | 4.5; 5.5; 6.5; 7.5 | 0.956 |
| | 4.5; 5.5; 6.5; 7.5 | 0.949 | 5.0; 6.0; 7.0 | 0.949 |
| | 5.0; 6.0; 7.0 | 0.941 | 4.5; 5.5; 6.5; 7.5 | 0.941 |
| | 4.5; 5.5; 6.5; 7.5 | 0.933 | 5.0; 6.0; 7.0 | 0.933 |
| 13 | 4.5; 5.5; 6.5; 7.5 | 0.970 | 5.0; 6.0; 7.0 | 0.970 |
| | 5.0; 6.0; 7.0 | 0.963 | 4.5; 5.5; 6.5; 7.5 | 0.963 |
| | 4.5; 5.5; 6.5; 7.5 | 0.956 | 5.0; 6.0; 7.0 | 0.956 |
| | 5.0; 6.0; 7.0 | 0.949 | 4.5; 5.5; 6.5; 7.5 | 0.949 |
| | 4.5; 5.5; 6.5; 7.5 | 0.941 | 5.0; 6.0; 7.0 | 0.941 |
| | 5.0; 6.0; 7.0 | 0.933 | 4.5; 5.5; 6.5; 7.5 | 0.933 |
| | 4.5; 5.5; 6.5; 7.5 | 0.924 | 5.0; 6.0; 7.0 | 0.924 |
| | 5.0; 6.0; 7.0 | 0.915 | 4.5; 5.5; 6.5; 7.5 | 0.915 |
| | 4.5; 5.5; 6.5; 7.5 | 0.906 | 5.0; 6.0; 7.0 | 0.906 |
| 16 | 4.5; 5.5; 6.5; 7.5 | 0.941 | 5.0; 6.0; 7.0 | 0.941 |
| | 5.0; 6.0; 7.0 | 0.933 | 4.5; 5.5; 6.5; 7.5 | 0.933 |
| | 4.5; 5.5; 6.5; 7.5 | 0.924 | 5.0; 6.0; 7.0 | 0.924 |
| | 5.0; 6.0; 7.0 | 0.915 | 4.5; 5.5; 6.5; 7.5 | 0.915 |
| | 4.5; 5.5; 6.5; 7.5 | 0.906 | 5.0; 6.0; 7.0 | 0.906 |
| | 5.0; 6.0; 7.0 | 0.897 | 4.5; 5.5; 6.5; 7.5 | 0.897 |
| | 4.5; 5.5; 6.5; 7.5 | 0.887 | 5.0; 6.0; 7.0 | 0.887 |
| | 5.0; 6.0; 7.0 | 0.877 | 4.5; 5.5; 6.5; 7.5 | 0.877 |
| | 4.5; 5.5; 6.5; 7.5 | 0.867 | 5.0; 6.0; 7.0 | 0.867 |
| 19 | 4.5; 5.5; 6.5; 7.5 | 0.915 | 5.0; 6.0; 7.0 | 0.915 |
| | 5.0; 6.0; 7.0 | 0.906 | 4.5; 5.5; 6.5; 7.5 | 0.906 |
| | 4.5; 5.5; 6.5; 7.5 | 0.897 | 5.0; 6.0; 7.0 | 0.897 |
| | 5.0; 6.0; 7.0 | 0.887 | 4.5; 5.5; 6.5; 7.5 | 0.887 |
| | 4.5; 5.5; 6.5; 7.5 | 0.877 | 5.0; 6.0; 7.0 | 0.877 |
| | 5.0; 6.0; 7.0 | 0.867 | 4.5; 5.5; 6.5; 7.5 | 0.867 |
| | 4.5; 5.5; 6.5; 7.5 | 0.856 | 5.0; 6.0; 7.0 | 0.856 |

Modified media were prepared by adding the necessary quantities of sodium chloride (Panreac, 131659) to Tryptone Soya Broth (TSB, Oxoid, UK) 100 mL flasks. The sodium chloride percentage was calculated considering the salt content of the initial TSB (5.0 g/L). Water activity was subsequently measured with an Aqualab model 3TE (Decagon Devices, Inc., Pullman, Washington, USA). pH was adjusted with a 1 M HCl solution (Panreac, 181021), and 1 M NaOH solution (Panreac, 181691), when necessary, and pH values were measured with a pH/mv-meter digit 501 (Crison, Barcelona, Spain). Once modified, all media were sterilized and subsequently, water activity and pH values were verified.

### 2.2. Strains and culture conditions

Five enterotoxigenic strains of *S. aureus* (CCM 1484, ATCC 13565, CCTM La 2812, ATCC 19095, ATCC 23235) were obtained from the Spanish Type Culture Collection (CECT, Burjassot, Valencia). Strains were maintained at −18 °C in cryovials containing beads and cryopreservatives (MicrobankTM). All strains showed a typical growth on Baird–Parker agar (Oxoid, UK).

Three days before the experiment, stock cultures of each strain were transferred to a tube containing 10 mL of TSB Tryptone Soya Broth (TSB, Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h. From this, 1 mL was subcultured into a tube containing 10 mL of TSB and incubated at 37 °C for 24 h. One more time, *S. aureus* was subcultured in the same way until the early stationary phase was reached (18 h).

### 2.3. Cocktail preparation and inoculation procedure

A cocktail of strains was prepared in accordance to the protocol of Stewart et al. (2002). When the early stationary phase was reached, the inoculum concentration of the five cultures was individually adjusted to an optical density at 600 nm of 0.160 to 0.240 (Bioscreen C, Labsystems, Finland) with 0.1% peptone water (Oxoid) in order to reach a concentration of $10^8$ cfu/mL. Then, 10 mL of each diluted culture was transferred into a single sterile flask and shaken to obtain the *S. aureus* cocktail. To determine initial concentration of the working cocktail, an aliquot of 1 mL was serially diluted, surface plated onto Baird–Parker agar plates, incubated at 37 °C for 48 h, and colonies were counted.

Necessary dilutions of the obtained cocktail in 0.1% peptone water were performed to obtain a concentration of ca. $5\times10^5$ cfu/mL. Then, 30 replicate microtiter wells per condition were filled up with 200 µl of the modified media, and inoculated with 50 µl of the diluted cocktail, reaching a final concentration of ca. $10^5$ cfu/mL per well. 2 wells per condition studied served as controls (un-inoculated medium). Afterwards, microtiter plates were sealed with paraffin to avoid media dehydratation and refrigerated at the appropriate temperatures.

### 2.4. Growth/no growth evaluation

Growth was daily monitored by absorbance measurements at 600 nm in Bioscreen C (Labsystems, Finland) during 36 days at all conditions. The use of turbidimetric techniques is a widely used method for predicting microbial growth/no growth (Koutsoumanis et al., 2004; Valero et al., 2007; Vermeulen et al., 2007). The absorbance of un-inoculated medium, used as blank (around 0.10 U) was subtracted to the absorbance values of inoculated media. Previous calibration curves performed in Bioscreen C showed that optical density values reaching 0.350, corresponded to an increase of 0.5 log cfu/mL in the microbial population. Therefore, the methodology described in the study of Stewart et al. (2002) where growth was considered when OD values at 600 nm were above 0.350, was followed. For the samples under conditions near the boundary zone (with absorbance values between 0.1 and 0.350), the bacterial population in the well was determined by surface plating on Baird–Parker and compared with the initial inoculum size. Growth was confirmed when a difference of more than 0.5 log cfu/mL with the initial inoculum was detected.

### 2.5. Selection of data

When growth was confirmed, it was recorded as "1", and "0" if it was not. The classification criterion was carried out at a cut point of $p=0.5$, being $p$ = probability of growth. However, before fitting the logistic regression model to the data observed, an examination of growth results was performed in order to detect possible outliers (i.e. decrease of the probability of growth when environmental conditions are less severe, or viceversa). The procedure proposed by Gysemans et al. (2007) was followed. In that study, an anomalous condition was an unusual change of more than 10% in the observed probability of growth in comparison with the neighbouring data point. Three anomalies were detected in the validation dataset: (i) $T=8$ °C; pH = 7; $A_w=0.977$ ($p=0.567$); (ii) $T=13$ °C; pH = 7.5; $A_w=0.915$ ($p=0.867$); and (iii) $T=13$ °C; pH = 5.5; $A_w=0.915$ ($p=0.867$). The chi-square test used showed the goodness of fit of the logistic regression model with the whole dataset, and without these three anomalous data, the fitting was significantly better ($p<0.05$). Since these anomalies were eliminated from the original dataset, 284 combinations of temperature, pH and $A_w$ were selected for the fit of the logistic regression model: 146 for model data (growth/no growth = 82/64) and 138 (growth/no growth = 77/61) for model validation.

### 2.6. Data processing and model development

The whole dataset was implemented in an Excel spreadsheet and a polynomial logistic regression equation was fitted to the model data observed. Generally, this type of model contains a right-hand side term (which is a polynomial equation) and a left-hand side term, named "logit $p$", which is equal to $\ln(p/(1-p))$ (Agresti, 2002). The equation used in this study was a second-order linear logistic regression model, as follows:

$$\begin{aligned}\log it(p) = {} & a_0 + a_1\cdot T + a_2\cdot \mathrm{pH} + a_3\cdot b_w + a_4\cdot T\cdot \mathrm{pH} \\ & + a_5\cdot T\cdot b_w + a_6\cdot \mathrm{pH}\cdot b_w + a_7\cdot T^2 + a_8\cdot \mathrm{pH}^2 \\ & + a_9\cdot b_w^2\end{aligned} \quad (1)$$

where $p$ is the probability of growth, $a_0-a_9$ are the coefficients to be estimated, $T$ is temperature, and $b_w$ is equal to $\sqrt{(1-A_w)}$ (which is a transformation proposed by Gibson et al. (1994) to stabilize the variance and provide a better fit of the model).

This model was fitted in SPSS for Windows v15.0 (SPSS Inc., Chicago, Illinois, USA), by using a stepwise process. With this procedure a biologically consistent model was obtained, in accordance to data observed. For a better illustration of the adjustment of the developed model to data observed, predicted probabilities at 0.1, 0.5 and 0.9 were calculated maintaining constant the temperature, pH and $A_w$ terms, and then were plotted in contour graphs.

### 2.7. Evaluation of model performance

Once the model was obtained, its performance was evaluated by means of goodness of fit statistics and predictive performance indexes.

The determination coefficient ($R^2$-Cox–Snell; $R^2$-Nagelkerke) was calculated in SPSS v15.0 as goodness of fit statistic. It quantifies the proportion of variation explained by the logistic regression model. A better fit of the model entails higher values of $R^2$. $R^2$-Nagelkerke (Nagelkerke, 1991) is a modification of the Cox–Snell coefficient to assure that it can vary from 0 to 1.

In addition, predictive performance indexes were calculated as follows:

a) Hosmer–Lemeshow statistic: It indicates if the model fits the data adequately. This statistic divides the number of times growth occurred (observed events) into approximately ten groups (based on the predicted probabilities), and then, compares the observed and the expected number of events in the groups through a contingency table by using the $\chi^2$ Pearson coefficient. Lower values of the Hosmer–Lemeshow statistic (and consequently, high values of $p$) indicate a better fit.
b) The area under ROC (Receiver Operating Characteristic) curve, $c$, is a measure of discrimination, obtained from a plot sensitivity (the proportion of observed events that was correctly predicted to be events), against the complement of specificity (the proportion of observed non-events that was correctly predicted to be non-events). The closer the value of $c$ is to 1, the greater is the discrimination.
c) As the Hosmer–Lemeshow statistic does not give information about the nature of the lack of fit, the Pearson residuals were calculated. They measure the difference between observed and predicted events, taking into account the number of observations (Gysemans et al., 2007):

$$e_i = \frac{r_i - n_i \cdot p_i}{\sqrt{n_i \cdot p_i \cdot (1 - p_i)}} \quad (2)$$

where $r_i$ is the number of times growth occurred at the $i$th condition, $n_i$ is the number of replicates tested, and $p_i$ is the estimated probability of growth.

### 2.8. Comparison with other studies

The predictions obtained in our model were compared with 17 growth and 13 no growth responses of *S. aureus* in culture media and cooked meat products included in Combase (Baranyi and Tamplin, 2004) and other published studies. Growth behaviour at different levels of temperature, pH and $A_w$ was recorded. The main objective was to test the percentage of agreement/disagreement with other microbial responses observed. Experimental times from these published studies were also included to provide a better illustration about the potential of *S. aureus* to grow under different conditions. Growth was considered when the difference between the $\log_{10}$ counts at the end of the experimental period and the $\log_{10}$ of the initial concentration exceeds 0.5 $\log_{10}$ (ISO, 2006).

## 3. Results and discussion

### 3.1. Logistic regression model

Estimation of the coefficients of the logistic regression model together with their corresponding standard errors and $p$-values, is represented in Table 2. The quadratic terms of $T$, $b_w$ and the interaction of pH·$b_w$, were non significant ($p>0.05$).

**Table 2**
Estimation of the coefficients together with their corresponding standard errors (S.E.) and $p$-values of the logistic regression model for *S. aureus*.

| Coefficient | Estimate | S.E. | *df* | *p-value* |
|---|---|---|---|---|
| Constant ($a_0$) | −257.022 | 72.737 | 1 | <0.001 |
| $T$ ($a_1$) | 10.369 | 2.947 | 1 | <0.001 |
| pH ($a_2$) | 55.037 | 16.653 | 1 | <0.001 |
| $b_w$ ($a_3$) | 136.482 | 48.157 | 1 | 0.005 |
| $T$·pH ($a_4$) | 0.642 | 0.290 | 1 | 0.027 |
| $T$·$b_w$ ($a_5$) | −33.155 | 9.869 | 1 | <0.001 |
| pH$^2$ ($a_8$) | −4.756 | 1.436 | 1 | <0.001 |

**Table 3**
Classification table of observed *vs* predicted conditions of the model and validation datasets for *S. aureus*.

| | | Selected conditions | | | | | |
|---|---|---|---|---|---|---|---|
| | | Model data | | *c* (%) | Validation data | | *c* (%) |
| | | $p_{pred}$ | | | $p_{pred}$ | | |
| | | No growth | Growth | | No growth | Growth | |
| $p_{obs}$ | No growth | 62 | 2 | 96.9 | 55 | 6 | 90.2 |
| | Growth | 3 | 79 | 96.3 | 5 | 72 | 93.5 |
| Global percentage | | | | 96.6 | | | 92.0 |

The performance statistics obtained indicate a reasonable goodness of fit of the model obtained, mainly due to the high values of $R^2$-Nagelkerke (0.943) and $p$-value (0.851) of the Hosmer–Lemeshow statistic. These values are also in accordance with other studies (Masana and Baranyi, 2000; Koutsoumanis et al., 2004; Skandamis et al., 2007). Most of Pearson residuals for temperature, pH and $A_w$ were between −1 and 1, indicating that differences between observed and predicted probabilities were small considering the number of observations. Indeed the percentage of values between −1 and 1 were 77.3, 76.6 and 77.0% for temperature, pH and $A_w$ respectively.

Through the calculation of the area under ROC curve, the $c$ value was calculated for model and validation data. The classification percentage of observed *vs* predicted conditions is shown in Table 3. High $c$ values were obtained since 96.6% of model data and 92.0% of validation data, were correctly classified by the model. For model data, 5 conditions were misclassified (3 false-negative and 2 false-positive) while for validation data, there were 11 misclassified conditions (5 false-negative and 6 false-positive). However, given the high number of replicates included in this model ($n=30$), several boundary conditions (i.e. conditions in which not all replicates showed 100% growth or no growth) were obtained (Table 4). In this study a value of $p=0.5$ was taken as a cut point to determine growth or no growth. The interpretation of the results could be different

**Table 4**
Conditions in which boundary responses ($0<p$ observed$<1$) for *S. aureus* were obtained and their corresponding $p$ predicted for the model and validation datasets.

| Model data | | | | | Validation data | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $T$ (°C) | pH | $A_w$ | $p_{obs}$ | $p_{pred}$ | $T$ (°C) | pH | $A_w$ | $p_{obs}$ | $p_{pred}$ |
| 8 | 7.5 | 0.999 | 0.87 | 1.00 | 8 | 7.5 | 0.995 | 0.40 | 0.65 |
| 8 | 7.5 | 0.989 | 0.23 | 0.02 | 8 | 6.0 | 0.989 | 0.53 | 0.90 |
| 8 | 6.5 | 0.989 | 0.80 | 0.93 | 8 | 5.0 | 0.989 | 0.23 | 0.00 |
| 8 | 5.5 | 0.989 | 0.37 | 0.38 | 8 | 6.5 | 0.983 | 0.67 | 0.34 |
| 8 | 7.0 | 0.983 | 0.37 | 0.06 | 10 | 6.0 | 0.963 | 0.77 | 1.00 |
| 8 | 6.0 | 0.983 | 0.20 | 0.26 | 10 | 7.5 | 0.956 | 0.60 | 0.30 |
| 10 | 5.5 | 0.963 | 0.53 | 0.96 | 10 | 6.5 | 0.956 | 0.80 | 0.99 |
| 10 | 7.0 | 0.956 | 0.77 | 0.95 | 10 | 7.0 | 0.949 | 0.57 | 0.44 |
| 10 | 6.0 | 0.956 | 0.57 | 0.96 | 10 | 6.0 | 0.949 | 0.33 | 0.54 |
| 10 | 7.5 | 0.949 | 0.47 | 0.02 | 13 | 4.5 | 0.963 | 0.97 | 1.00 |
| 10 | 6.5 | 0.949 | 0.53 | 0.76 | 13 | 5.0 | 0.941 | 0.97 | 0.99 |
| 13 | 4.5 | 0.956 | 0.63 | 0.99 | 13 | 7.5 | 0.933 | 0.90 | 1.00 |
| 13 | 7.5 | 0.941 | 0.93 | 1.00 | 13 | 5.5 | 0.933 | 0.97 | 1.00 |
| 13 | 5.5 | 0.941 | 0.97 | 1.00 | 13 | 5.0 | 0.924 | 0.23 | 0.00 |
| 13 | 5.0 | 0.933 | 0.97 | 0.39 | 13 | 6.5 | 0.915 | 0.87 | 0.95 |
| 13 | 7.5 | 0.924 | 0.87 | 0.99 | 16 | 5.5 | 0.933 | 0.97 | 1.00 |
| 13 | 5.5 | 0.924 | 0.87 | 0.79 | 16 | 4.5 | 0.933 | 0.63 | 0.99 |
| 13 | 7.0 | 0.915 | 0.93 | 0.93 | 16 | 7.5 | 0.915 | 0.60 | 1.00 |
| 13 | 6.0 | 0.915 | 0.87 | 0.73 | 16 | 5.5 | 0.915 | 0.63 | 1.00 |
| 16 | 7.5 | 0.924 | 0.93 | 1.00 | 16 | 7.5 | 0.897 | 0.40 | 0.99 |
| 16 | 5.5 | 0.924 | 0.90 | 1.00 | 16 | 6.5 | 0.897 | 0.97 | 1.00 |
| 16 | 5.0 | 0.915 | 0.53 | 0.84 | 16 | 7.0 | 0.887 | 0.97 | 0.63 |
| 16 | 7.5 | 0.906 | 0.53 | 1.00 | 19 | 7.5 | 0.887 | 0.93 | 1.00 |
| 16 | 6.0 | 0.897 | 0.53 | 0.96 | 19 | 6.5 | 0.887 | 0.97 | 1.00 |
| 16 | 6.5 | 0.887 | 0.53 | 0.50 | 19 | 7.0 | 0.877 | 0.97 | 0.99 |
| 16 | 7.0 | 0.877 | 0.47 | 0.01 | | | | | |
| 19 | 7.5 | 0.877 | 0.77 | 0.98 | | | | | |
| 19 | 6.5 | 0.877 | 0.87 | 0.95 | | | | | |
| 19 | 7.0 | 0.867 | 0.23 | 0.09 | | | | | |

depending on the probability level assumed. For instance, at 10 °C; pH = 6 and $A_w$ = 0.949, 10 out of the 30 wells have grown ($p$ = 0.333) but this condition was considered as no growth. On the contrary, the model predicted a probability of growth of 0.54, i.e. growth was predicted. In order to apply this model for industrial purposes, it would be recommended to set an acceptable probability level of $p$ = 0.01, so the condition mentioned above would be considered as observed growth and consequently, correctly classified by the model.

### 3.2. Effect of environmental factors on S. aureus growth

The effect of temperature, pH and $A_w$ on the probability of growth of the *S. aureus* cocktail was examined through the contour plots. Contour lines were close to data observed in all cases, showing an appropriate goodness of fit of the logistic regression model.

Regarding the effects of the environmental factors on *S. aureus* growth, the lower the levels of temperature, pH and $A_w$, the more abrupt growth/no growth interfaces were obtained, though their influence on *S. aureus* growth differed among different factors, as will be described below.

#### 3.2.1. Influence of temperature

In this study, it was shown that at temperatures below 8 °C, the growth of *S. aureus* was inhibited, because at 7.5 °C, no growth was observed (data not shown), and at 8 °C *S. aureus* growth occurred only at optimum levels of pH and $A_w$. Other studies reported no growth of *S. aureus* at temperatures below 8 °C combined with low pHs (Normanno et al., 2005). As an example, at $A_w$ = 0.989 and pH<6.0, the microorganism grew at 8 °C in 50% of cases, while at lower $A_w$ levels (below 0.983), growth was only observed at neutral or close to neutral pHs (from 6.0 to 7.0).

Predicted probabilities ($p$ = 0.1, 0.5 and 0.9) as a function of pH and $A_w$ at 8, 10 and 13 °C are represented in Fig. 1a–c. As conditions became increasingly unfavourable for growth, the contour lines for predicted probabilities drew closer together, as shown by other authors (Presser et al., 1998; Stewart et al., 2002). In other words, at low temperatures and pHs, small changes in $A_w$ levels produced a large decrease in the probability of growth. At higher temperatures, this transition was smoother as it can be seen in Fig. 1b–c.

Regarding the interaction of temperature and pH on *S. aureus* growth, at optimal $A_w$ (0.999), no growth was observed at pH = 4.5 and 8 °C while the predicted probability of growth was lower than 0.5 at temperatures below 8.3 °C. However, for higher temperatures, pH alone did not inhibit *S. aureus* growth at optimal $A_w$ values (0.999).

#### 3.2.2. Influence of pH

Predicted probabilities at $p$ = 0.1, 0.5 and 0.9 as a function of temperature and $A_w$ at pH of 4.5, 5.5 and 6.5 are represented in Fig. 2a–c. Growth of *S. aureus* was observed at pH = 4.5, demonstrating the ability of *S. aureus* to grow under acidic pHs (Sutherland et al., 1994; Whiting et al., 1996; Lanciotti et al., 2001; Stewart et al., 2002). However, growth of *S. aureus* at low pH can be influenced by the acid used to adjust the pH (Sutherland et al., 1994).

On the other hand, the evolution of the probability of growth as a function of pH followed a decreasing trend in the pH interval of 7.0–7.5 at most temperatures and $A_w$ levels tested, though this effect was especially important in the boundary zone; i.e. the conditions in which the observed probability of growth was situated between 0 and 1 (because the number of wells that showed growth in the microtiter plates in those conditions were neither 0 nor 30). Observations obtained from the replicates tested showed that growth was higher at pH 7.0 than at pH 7.5. This fact is especially important when predicting the SE production, because the optimum pH and temperature for SE production are generally slightly higher than that for growth; 7.0–8.0 pH values (Genigeorgis, 1989) and above 10 °C (ICMSF, 1996).

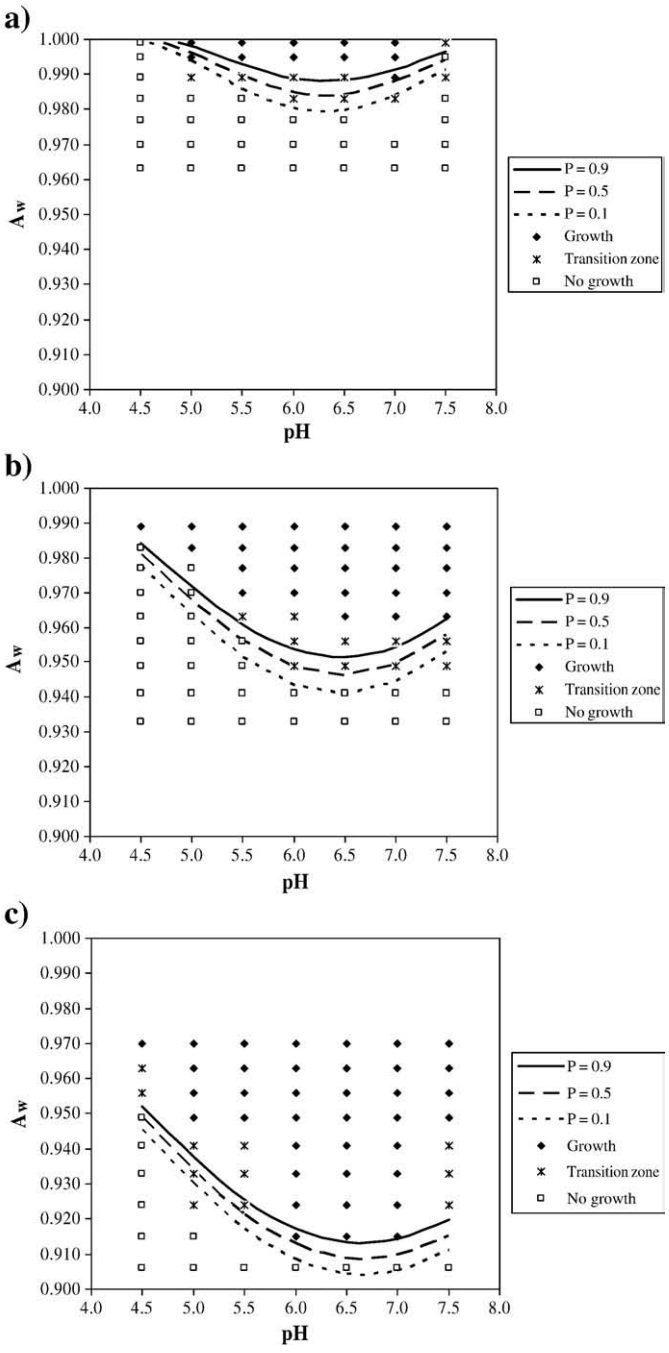


**Fig. 1.** Growth/no growth interfaces at 8 °C (a), 10 °C, (b) and 13 °C (c) for the predicted growth limits of *S. aureus* by the logistic regression model fixing the probabilities at 0.1, 0.5 and 0.9. Growth: $p$ = 1; transition zone: 0<$p$<1; no growth: $p$ = 0.

Possible reasons that can influence this change of the probability of growth between pH 7.0 and 7.5 are the use of a strain cocktail, that can produce a higher variability in the microbial response than when using a single control strain, the physiological state of the inoculum used, or the high number of replicates used in this study to evaluate the boundary zone. Other published growth models of *S. aureus*, reported a very similar microbial behaviour at both pHs (Buchanan et al., 1993; Zurera-Cosano et al., 2004), but none of the published boundary models of *S. aureus* included the level of pH 7.5, so results obtained in this study cannot be compared. Further research is necessary to study the microbial behaviour of *S. aureus* at pH levels slightly above neutrality, in order to obtain more reliable conclusions.

### 3.2.3. Influence of $A_w$

In this study, the resistance of *S. aureus* to low $A_w$ values was shown, since growth was detected at $A_w = 0.867$ ($T = 19$ °C; pH = 7.0). Similar minimum $A_w$ values for *S. aureus* growth are reported in scientific literature (Pepe et al., 2006), though growth/no growth interface is clearly affected by the type of humectants used, and the physical properties of the solutes. It seems that the use of other osmotic agents (e.g. glycerol, sucrose or fructose), different than NaCl, produce a more pronounced microbial growth inhibition (Stewart et al., 2002). These facts could account for the low $A_w$ levels allowing growth in this study. However, at low pH levels, the inhibitory effect of increasing NaCl concentration (i.e. low $A_w$), on microbial growth was higher. Fig. 3 represents the evolution of the probability of growth as a function of $A_w$ at different pH levels ($T = 10$ °C). It can be seen that at pH of 4.5, growth inhibition occurred at high $A_w$ values, as found by other authors (Martinez et al., 1986; Stewart et al., 2002).

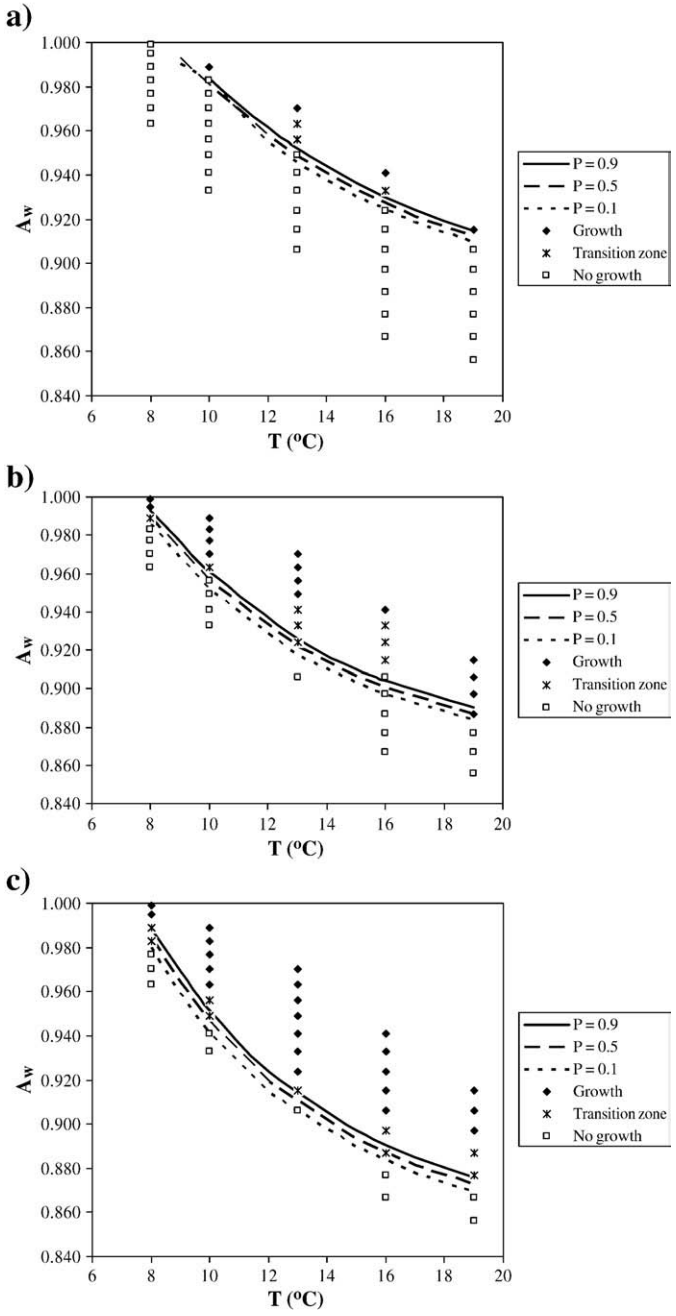


**Fig. 2.** Growth/no growth interfaces at pH = 4.5 (a), pH = 5.5, (b) and pH = 6.5 (c) for the predicted growth limits of *S. aureus* by the logistic regression model fixing the probabilities at 0.1, 0.5 and 0.9. Growth: $p = 1$; transition zone: $0 < p < 1$; no growth: $p = 0$.

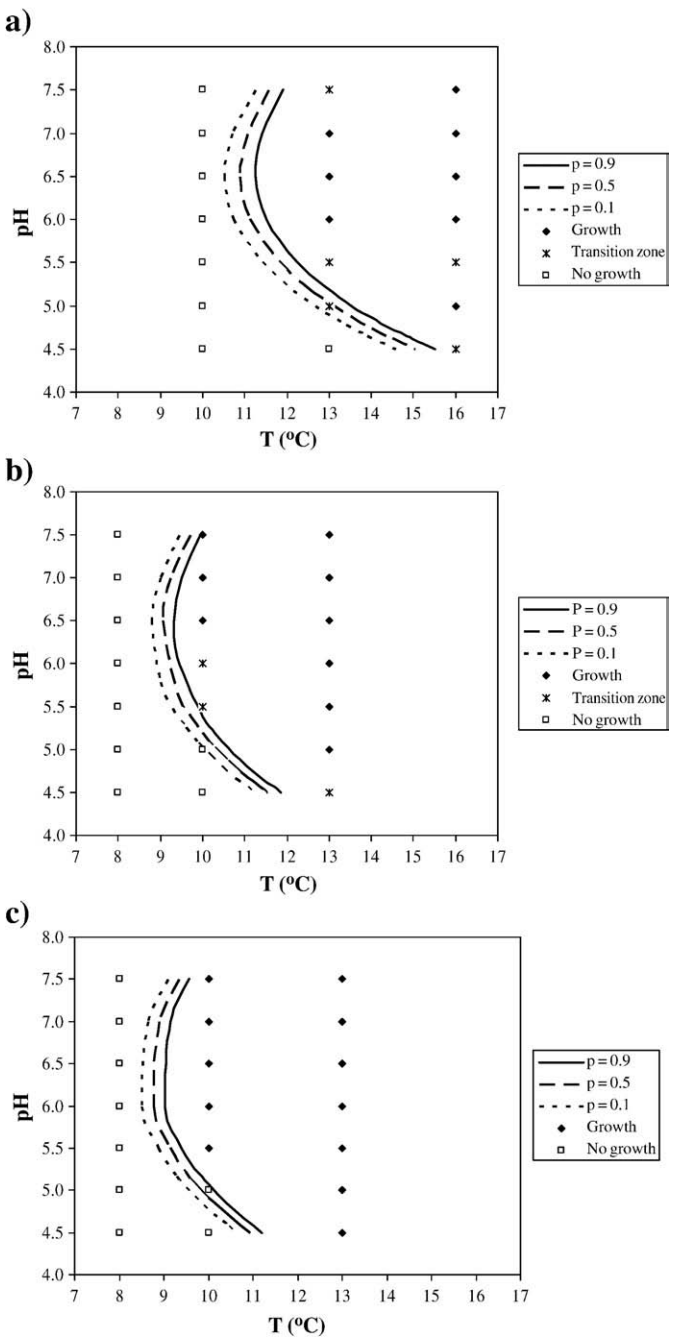


**Fig. 3.** Growth/no growth interfaces at $A_w = 0.933$ (a), $A_w = 0.963$, (b) and $A_w = 0.970$ (c) for the predicted growth limits of *S. aureus* by the logistic regression model fixing the probabilities at 0.1, 0.5 and 0.9. Growth: $p = 1$; transition zone: $0 < p < 1$; no growth: $p = 0$. *No transition zone is presented in (c).

### 3.3. Comparison with other published studies

Growth responses of *S. aureus* taken from published studies in both culture media and cooked meat products are shown in Table 5. The results of the comparison of our growth/no growth model with these observed cases are summarized in Table 6. It can be seen that, in 16 out of 17 observed growth cases (94%), the logistic regression model predicted growth ($p > 0.5$), while in 8 out of 13 no growth cases (62%), the model agreed with the observed cases ($p < 0.5$).

**Table 5**
Comparison of growth responses of *S. aureus* from the growth/no growth model and from published growth models performed in culture media and cooked meat products.

| Authors | $T$ (°C) | pH | $A_w$ | Growth | Obs time (h) | Experimental matrix | Especifications | Growth ($p_{pred}$) |
|---|---|---|---|---|---|---|---|---|
| Castillejo et al. (2002) | 10.0 | 6.22 | 0.971 | Yes | 400 | Cooked chicken breast | Vacuum | Yes (1.00) |
| Castillejo et al. (2002) | 13.5 | 6.22 | 0.971 | Yes | 400 | Cooked chicken breast | Vacuum | Yes (1.00) |
| Castillejo et al. (2002) | 17.7 | 6.22 | 0.971 | Yes | 400 | Cooked chicken breast | Vacuum | Yes (1.00) |
| Castillejo et al. (2002) | 10.0 | 6.31 | 0.971 | No | 340 | Cooked chicken breast | Vacuum | Yes (1.00) |
| Dempster and Kelly (1973) | 15.0 | 5.80 | 0.976 | Yes | <300 | Vacuum-packed bacon | NaCl = 4.1%; $NaNO_2$ = 51 ppm | Yes (1.00) |
| Dempster and Kelly (1973) | 15.0 | 6.20 | 0.976 | Yes | <300 | Vacuum-packed bacon | NaCl = 4.1%; $NaNO_2$ = 51 ppm | Yes (1.00) |
| Whiting et al. (1984) | 11.0 | 6.00 | 0.992 | Yes | <100 | Sausage (frankfurters) | NaCl = 1.5%; $NaNO_2$ = 150 ppm; AA = 430 ppm; vacuum | Yes (1.00) |
| Nielsen and Zeuthen (1985) | 8.0 | 6.60 | 0.978 | Yes | <300 | Cooked bologna-type sausage | NaCl = 3.8%; vacuum | No (0.04) |
| Nielsen and Zeuthen (1985) | 12.0 | 6.30 | 0.964 | Yes | <300 | Vacuum-packed minced meat | NaCl = 6%; $NaNO_2$ = 300 ppm; | Yes (1.00) |
| IFR[a] | 10.0 | 6.00 | 0.931 | No[b] | 335 | Culture medium | NaCl = 10.5% | No (0.00) |
| Sutherland et al. (1994) | 15.0 | 5.30 | 0.931 | Yes[b] | <200 | Culture medium | NaCl = 10.5% | Yes (1.00) |
| Landolo et al. (1964) | 16.0 | 7.50 | 0.950 | Yes[b] | <50 | Culture medium | NaCl = 8% | Yes (1.00) |
| Eifert et al. (1997) | 12.0 | 5.00 | 0.947 | No | 193 | Culture medium | NaCl = 8.5%; glucose = 1% | Yes (0.88) |
| Eifert et al. (1997) | 12.0 | 6.00 | 0.947 | Yes | 480 | Culture medium | NaCl = 8.5%; glucose = 1% | Yes (1.00) |
| Eifert et al. (1997) | 12.0 | 7.00 | 0.947 | Yes | 480 | Culture medium | NaCl = 8.5%; glucose = 1% | Yes (1.00) |
| Buchanan et al. (1993) | 19.0 | 5.50 | 0.880 | No | 192 | Culture medium | NaCl = 16.5%; $NaNO_2$ = 50 ppm; anaerobic | No (0.01) |
| Buchanan et al. (1993) | 19.0 | 7.50 | 0.915 | Yes | 720 | Culture medium | NaCl = 12.5%; $NaNO_2$ = 150 ppm | Yes (1.00) |
| Buchanan et al. (1993) | 12.0 | 6.50 | 0.950 | No | 696 | Culture medium | NaCl = 8%; $NaNO_2$ = 50 ppm | Yes (1.00) |
| Buchanan et al. (1993) | 12.0 | 7.50 | 0.950 | No | 696 | Culture medium | NaCl = 8%; $NaNO_2$ = 100 ppm | Yes (1.00) |
| Buchanan et al. (1993) | 19.0 | 7.50 | 0.870 | No | 576 | Culture medium | NaCl = 16.5%; $NaNO_2$ = 200 ppm | No (0.23) |
| Buchanan et al. (1993) | 19.0 | 5.90 | 0.915 | No | 576 | Culture medium | NaCl = 12.5%; $NaNO_2$ = 150 ppm | Yes (1.00) |
| Lee et al. (1977) | 12.0 | 6.20 | 0.915 | No | – | Sausage | NaCl = 12% | No (0.07) |
| Niskanen and Nurmi (1976) | 17.0 | 5.10 | 0.887 | No | – | Dry sausage | NaCl = 15% | No (0.00) |
| AFSCE[c] | 9.6 | 7.20 | 0.949 | No[b] | >1000 | Culture medium | NaCl = 8.16% | No (0.01) |
| AFSCE | 12.6 | 7.20 | 0.914 | No[b] | >1000 | Culture medium | NaCl = 12.66% | No (0.29) |
| Dengremont and Membré (1995) | 10.0 | 4.50 | 0.933 | No[b] | 168 | Culture medium | NaCl = 10% | No (0.00) |
| Dengremont and Membré (1995) | 14.0 | 5.50 | 0.933 | Yes[b] | 168 | Culture medium | NaCl = 10% | Yes (0.99) |
| Ingham et al. (2007) | 18.4 | 5.70 | 0.999 | Yes | <50 | Laboratory ground beef | NaCl = 0.06% | Yes (1.00) |
| Ingham et al. (2007) | 12.8 | 5.9 | 0.999 | Yes | <50 | Laboratory ground turkey | NaCl = 0.09% | Yes (0.99) |
| Lindqvist (2006) | 17 | 6.30 | 0.971 | Yes | 192 | Chicken broth | NaCl = 1.76%; $NaNO_2$ = 39 ppm | Yes (1.00) |

[a]Institute of Food Research (Norwich, UK).
[b]Published responses of *S. aureus* in which environmental conditions were the same as those included in the logistic regression model.
[c]Australian Food Safety Centre of Excellence (Tasmania, Australia).

When comparing the logistic regression model with data obtained in culture media, fail-safe predictions are provided, since all growth cases and 6 out of 10 no growth cases (60%) were correctly classified (Table 6). The latter lower percentage of correctly classified cases is likely associated with the inclusion of different environmental factors in other studies (e.g. the addition of organic acids, sodium nitrite, aerobic/anaerobic conditions, etc.). This fact was confirmed because all published responses under equal conditions to those tested in our model (i.e. performed in culture media, and taking exclusively temperature, pH and $A_w$ or % salt as environmental factors) agreed with the predictions of the logistic regression model (Table 5).

On the other hand, a good agreement was obtained with published data in cooked meat products (10 out of 11 growth cases and 2 out of 3 no growth cases agreed with model predictions). It is well known that the nature (liquid or solid) and the packaging atmosphere of the food should also be taken into account in the applicability of predictive models (Koutsoumanis et al., 2004; Cheng-An Hwang and Tamplin, 2005). Besides, microbial growth in foods is usually slower than that observed in culture broth (Te Giffel and Zwietering, 1999), and also *S. aureus* does not compete well with other microorganisms presented in foods, which leads to obtain even more conservative predictions (Walls et al., 1995).

These model predictions should be used with caution when evaluating its performance through the comparison with other studies.

**Table 6**
Number and percentage of correctly classified growth/no growth cases derived from Table 5 in culture media and cooked meat products.

| Cases | Growth | No growth | Total |
|---|---|---|---|
| Correctly classified (culture media) | 6/6 (100%) | 6/10 (60%) | 12/16 (75%) |
| Correctly classified (cooked meat products) | 10/11 (91%) | 2/3 (67%) | 12/14 (86%) |
| Total cases correctly classified | 16/17 (94%) | 8/13 (62%) | 24/30 (80%) |

An issue that should be considered is the experimental time (in our case, 864 h), which influence strongly the position of the growth/no growth boundary. The misclassified no growth cases shown above could be caused by this limitation, since only 2 out of the 30 records collected showed longer observation times than that of the present study.

However, this model can be clearly of great interest to food producers and food safety managers. An example of a model application in cooked meat products is given as follows. It is well known that these RTE products are subjected to recontamination after the heat treatment, especially if they are improperly handled (Reij et al., 2004). If contaminated, cooked meat products support the growth of *S. aureus* in the case of temperature abuse during storage (which may be

**Table 7**
Some predicted minimal values of temperature, pH and $A_w$ at which $p<0.01$.

| $T$ (°C) | pH | Minimal $A_w$ |
|---|---|---|
| 8.00 | 6.50 | 0.974 |
| 10.00 | 6.00 | 0.937 |
| 13.00 | 5.50 | 0.913 |
| 16.00 | 5.00 | 0.906 |
| 19.00 | 4.50 | 0.907 |
| $T$ (°C) | Minimal pH | $A_w$ |
| 8.00 | 4.75 | 0.995 |
| 10.00 | 4.63 | 0.970 |
| 13.00 | 4.51 | 0.941 |
| 16.00 | 4.67 | 0.915 |
| 19.00 | 4.51 | 0.906 |
| Minimal $T$ (°C) | pH | $A_w$ |
| 8.37 | 4.50 | 0.995 |
| 8.10 | 5.00 | 0.989 |
| 8.21 | 5.50 | 0.977 |
| 8.23 | 6.00 | 0.970 |
| 8.49 | 6.50 | 0.963 |

produced at retail establishments and domestic refrigerators). When applying model predictions, the manufacturer can know, a priori, the pH and $A_w$ conditions for producing *S. aureus* inhibition. For instance at 8 °C, a reduction of $A_w$ to 0.973 at neutral pH (6.2) can inhibit the growth of *S. aureus*, according to the model predictions. Likewise, alternative formulations can be evaluated; i.e. at higher $A_w$ (0.980), pH must be lowered to 5.53 to avoid the growth of *S. aureus*.

As suggested previously, an acceptable probability of growth = 0.01 should be desirable for stakeholders in the case of pathogens. Several conditions which showed a predicted probability of growth of *S. aureus* < 0.01, are presented in Table 7. These results have a practical implication for the identification of conditions that can be applied to food preservation in order to ensure no growth of *S. aureus*.

## 4. Conclusions

In this study, it was shown that different combinations of temperature, pH and $A_w$ can influence largely the growth *S. aureus* boundaries. Growth of *S. aureus* can be inhibited at refrigeration temperatures (8 °C) by appropriately lowering the pH and $A_w$ levels. The logistic regression model provided a quite reasonable adjustment to the data observed, obtaining a more straight cut-off between growth and no growth zones at stringent conditions. Especially interesting was the microbial behaviour in the pH transition from 7.0 to 7.5 in which a decrease in the probability of growth was observed, although this effect needs to be studied in depth.

In this study, difficulties were encountered when comparing results with other published works given the heterogeneity of the factors considered, though the misprediction of the no growth cases was safe in any case. To know those limiting factors for the growth of *S. aureus* is essential in order to avoid proliferation of the microorganism, and consequently, SE production. The logistic regression model presented in this paper can be a powerful tool to decide the most appropriate ranges of environmental factors in order to design safe formulations in RTE foods which are susceptible to be contaminated by *S. aureus*. However, further studies will be needed to evaluate the effect of individual strains on the whole *S. aureus* cocktail response at boundary conditions.

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