Evolutionary combined neural networks for modelling the growth boundaries for a five strain *Staphylococcus aureus* cocktail against temperature, pH and water activity

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Abstract

Staphylococcus aureus contamination of foods is one of the most prevalent causes of gastroenteritis worldwide, which is caused by ingestion of preformed toxins. Ready-to-eat foods without previous heat treatment before consumption are susceptible to be contaminated during processing, storage or handling at both retail points and domestic homes. In this study, a logistic regression based on product units neural networks (LRPU) was carried out to determine the probability of growth of a five strain *S. aureus* cocktail as a function of storage temperature (8-19ºC), pH (4.5-7.5) and water activity (a_w) (0.856-0.999). A good adjustment of the LRPU model was observed for both training and test datasets (91.10% and 89.36% cases correctly classified respectively). Results confirmed the tolerance to low levels of a_w of the working cocktail, since it was capable to grow at 19ºC up to a_w levels of 0.867 and at neutral pH (7.0). The storage temperature had a marked effect on *S. aureus* cocktail growth, since at 8ºC, it was only able to grow at a_w levels higher than 0.977. Regarding pH effect, at pH levels below 5.0, probability of growth was lower than 50 % in most cases at temperatures lower than 16ºC. This study provides a solid scientific basis that verifies the criteria established by food industries for the assurance of the microbiological safety of the product until the consumption phase, currently demanded by the European legislation.
Keywords: growth/no growth, *Staphylococcus aureus*, classification, product unit neural networks, food safety

Introduction

Food poisoning caused by staphylococcal contamination is one of the most prevalent causes of gastroenteritis worldwide, which is caused by the ingestion of food that contains preformed toxins (Jablonski and Bohach 2001). Specifically, *S. aureus* offen produces the most common types of food intoxication (Jablonski and Bohach 2001). The number of illnesses reported to the Spanish Microbiological Information System (SIM) caused by *S. aureus* ingestion, were increasing during the last five years, until reaching more than 550 annual cases (http://www.isciii.es/jsps/centros/epidemiologia/informacionMicrobiologica.jsp).
Ready-to-eat foods without a previous heat treatment are susceptible to be contaminated during processing, storage or handling in both retail points and domestic homes (Huang *et al.*, 2001). The key to controlling *S. aureus* is an understanding of the factors that influence on its growth in foods and the modification of these factors in order to limit potential risks (mainly temperature, pH and water activity).
Probability models are characterized by defining the growth/no growth limits of a specific microorganism in a medium as a function of some environmental factors, in a very limited range of conditions. There are several studies reported in the literature regarding the use of logistic equations to describe growth/no growth boundaries (Lanciotti *et al.*, 2001). However, when a strong interaction exists between the variables considered, the use of Product Unit Neural Network models (PUNN) is gaining attention, due to they are more effective in detecting those interactions and they have the ability to implement higher order functions (polynomial) as a particular case (Gurney 1992). The use of PUNN models integrated as

logistic regression techniques (LRPU) has been proposed as an alternative to determine bacterial growth/no growth limits (Valero *et al.*, 2007).
On the other hand, boundary models published in literature usually consider few replicates per condition selected, which leads to obtain a very narrow borderline between conditions that permit and not growth. This fact occurs because traditionally growth/no growth models were focused to define a growth/no growth interface as broader as possible that implies a minor number of replicates. However, a higher number of replicates may imply a more gradual transition between growth and no growth zones, providing more accurate estimations of microbial behaviour (Vermeulen *et al.*, 2007).
In this study, a non linear logistic regression procedure based on product units neural network models (LRPU) was performed to determine the probability of growth of a five strain *S. aureus* cocktail as a function of storage temperature (8-19ºC), pH (4.5-7.5) and water activity (a_w) (0.856-0.999).

Material and Methods

Source of data

A cocktail of five strains of *S. aureus* (CCM 1484, ATCC 13565, CCTM La 2812, ATCC 19095, and ATCC 23235) was chosen to consider the variability between strains at the different conditions performed (Stewart *et al.*, 2002). Growth/ no growth data of *S. aureus* were collected, considering as the main variables: temperature (8, 10, 13, 16 and 19ºC), pH (4.5; 5.0; 5.5; 6.0; 6.5; 7.0 and 7.5) and a_w (0.856; 0.867; 0.877; 0.887; 0.897; 0.906; 0.915; 0.924; 0.933; 0.941; 0.949; 0.956; 0.963; 0.970; 0.977; 0.983; 0.989; 0.995 and 0.999). Modified media at the different model conditions were inoculated with 10^5 cfu/ml of the working cocktail. Assessment of growth was performed by turbidity measurements in Bioscreen C (Labsystems, Finland) and checked by plating onto Baird Parker agar (Oxoid, UK) at regular time intervals during 40 days. 30 replicates per condition were made and for any combination of factors, growth was recorded as "*1*" if it occurred and "*0*" if did not. Each condition was considered as growth if more than 50% of the replicates grew (probability of growth (P) > 0.5).

Development of the LRPU model

For the model development, total data (287 conditions) were divided into two sets: one set for training (146 conditions that covered the extremes of domain) and another for testing (141 conditions). To start processing data, the input variables were scaled in the range [1, 2]. The new scaled variables were named T*, pH* and a_w *. For modelling purposes, a transformation of a_w into b_w ($(1-a_w)^{0.5)}$ was made.
Logistic regression is a widely used statistical modelling technique in which the probability (P) of the dichotomous output (0, 1) is related to a set of explanatory variables (x) in the form:

$$\text{logit } (P) = \ln\left(\frac{P}{1-P}\right) = f(x,\beta) \qquad (1)$$

where P is the probability of growth, $\boldsymbol{\beta} = (\beta_0, \beta_1, \beta_2, ..., \beta_k)$ are the model coefficients and $\mathbf{x} = (x_0, x_1, x_2, ..., x_k)$ the scaled variables (T*, pH* and b_w *).
We applied a new logistic regression model based on product-units by introducing a nonlinear term constructed with basis functions given by products of the inputs raised to real powers (w), which express the possible strong interactions among the factors (Hervás-Martínez and Martínez-Estudillo 2007). The general expression of $f(x,\beta)$ is given by:

$$f(x,\beta) = \beta_0 + \sum_{j=1}^{m} \beta_j \prod_{i=1}^{k} x_i^{w_{ji}} \qquad (2)$$

where the basis function is $\beta(x^w) = \left[\beta_1(x_1^{w_1}), \beta_2(x_2^{w_2}) ...., \beta_m(x_m^{w_m})\right]$. Estimation of the coefficients was based on the combination of an evolutionary algorithm and a local optimization procedure carried out by standard maximum likelihood optimization method

(Hervás-Martínez *et al.,* 2006). Model training was implemented in Java 1.6 (Sun Microsystems, Inc.).

Results and Discussion

Model adjustment

Out of the 287 conditions selected, 156 corresponded to microbial growth, while in 131 no growth occurred. Results obtained regarding the classification accuracy are expressed in Table 1. The optimal structure for the LRPU model was in the form 3:2:1 with three input nodes (corresponding to the variables introduced in the equation), two hidden nodes and one output layer that classified the results into two classes: growth and no growth. The final equation obtained was: $f(x,\beta)$ = 1.887 ($T^{*-1.554}$ $pH^{*-1.019}$ $b_w^{*\ 4.264}$) + 4.76 ($T^{*-6.404}$) - 7.328 For the training dataset, 91.10 % of the cases were correctly classified for the model. For the test dataset, a good generalization capacity of the model was obtained, since 89.36% of the cases were correctly predicted.

Table 1: classification of cases by the LRPU model

Training		Predicted probability	
		1	0
Observed probability	1	79	4
	0	9	54
Test		**Predicted probability**	
		1	0
Observed probability	1	77	5
	0	9	50

Furthermore, the LRPU model generated less false negative cases (i.e., no growth cases predicted while growth cases were observed) than the other models, which implies that it would be considered fail-safe. This fact is especially important in establishing new formulations for a food product which guarantee that no growth will occur.

Effects of environmental variables on probability of growth of S. aureus cocktail

Model predictions obtained indicated that temperature had a strong effect on the probability of growth of *S. aureus* cocktail, being the main factor that regulates its growth (Degremont and Membré 1995). At 8ºC, growth did not occur at pH 4.5; while values of P were lower than 0.5 at pH 6 and a_w = 0.990. As temperature increased, there was more resistance of *S. aureus* cocktail to lower pH and a_w values. These findings suggest that by designing different combinations of pH and a_w, *S. aureus* growth can be inhibited at refrigeration temperatures, which is the main factor to prevent staphylococcal intoxication. At 10ºC, growth was recorded at a_w levels of 0.963 and neutral pHs (>6.5). The minimum a_w value at which growth was observed decreased at higher temperatures until reaching 0.867 at 19ºC, confirming the resistance of the pathogen to low moisture levels and high salt concentrations (Mc Meekin *et al.,* 1987). This fact could be interesting for uncooked, refrigerated and shelf stable foods, in which the intrinsic properties of the food (pH and salt) and temperature must interact to prevent growth or preferably promote inactivation of the pathogen (Whiting *et al.,* 1996). Regarding pH, the optimal range was situated between 6.0 and 7.0, but a decrement of replicates that showed growth was obtained at pH 7.5. The optimum pH for growth is around 7.0 but it is influenced by other environmental conditions. Growth at a particular pH also depends on the acid used to adjust the pH. On the contrary, a higher sensitivity to low pH values was observed at 8 and 10ºC, since the pathogen did not grow at pHs<5 and a_w<0.97. This is in accordance to Lanciotti *et al.* (2001) who stated the influence on *S. aureus* growth at low pH values. The observed probabilities compared to the predicted ones at 0.9; 0.5 and 0.1 for temperature and a_w at a fixed pH of 5.5 are represented in Figure 1. It can be seen that a slighter transition between growth and no growth zones occurred at 13 and 16ºC rather than at 8, 10 and 19ºC, mainly favoured by the high number of replicates performed per condition. These models will allow product developers to visualize the “safe space” for the formulation of shelf-stable foods by employing these preservation factors. Regarding microbiologists, they can assess risks more effectively over a wide range of products, and finally, consumers will have greater assurance of food safety.

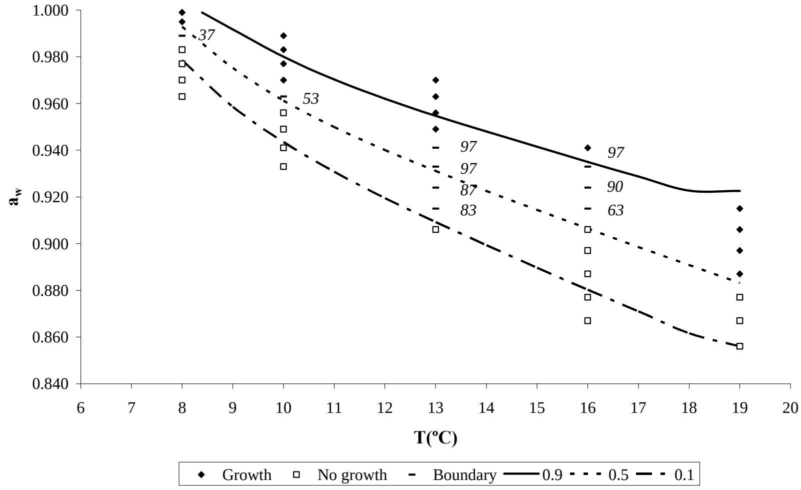

Figure 1: Growth/no growth interfaces for the predicted growth limits of *S. aureus* cocktail obtained by the LRPU model calculating the probabilities at 0.1, 0.5 and 0.9. pH = 5.5.

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