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Application predictive modelling of *Penicillium roqueforti* germination in environmental conditions in cake

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Abstract: This study aimed to predict the germination of *Penicillium roqueforti* as one of the most important moulds in cake in certain environmental conditions that lead to cake spoilage. The germination rate of *P. roqueforti* was evaluated by culturing in the Yeast Extract Glucose Chloramphenicol Agar medium with different pH, water activity (a_w), and inoculum size at three levels of temperature (15, 25, 30 °C) during 60 days by the factorial experiment. The results of analysis of variance (ANOVA) proved that environmental conditions affect germination significantly ($P < 0.05$). Predictive modelling illustrated that the temperature did not affect germination significantly, while no germination was seen at a_w = 0.65. The minimum lag phase of germination was observed at a_w = 0.9, pH = 6.5, and inoculum size of 1 000 spores per mL. The logistic model was found to be more precise for fitting the data of *P. roqueforti* in cakes.

Keywords: food safety; mould germination; modelling; NeuroSolutions

Food safety has always been considered a crucial aspect of food science and nutrition, affecting human health and economics. Food spoilage leads to capital losses in the food industry, from farm to fork. Bakery products are regarded as a favourable habitat for microorganisms, including moulds, when among all, *Penicillium*, *Fusarium*, and *Aspergillus* are encountered as the critical ones (Van Long et al. 2017; Arena et al. 2020). Fungal development on food ingredients or the final product causes significant unsafety in foods and essential losses, which should also be considered through some strategies such as Hazard Analysis and Critical Control Points (HACCP). Nowadays, the global demands for food safety are increasing day by day, highlighting the mathematical methods for predicting and quantifying microbial behaviour (Garcia et al. 2010; Santos et al. 2020). Moulds are also the primary source of mycotoxin production, which are a vital danger for human and animal health as well. Some studies proved the decontamination ability of some novel technologies, including cold plasma on cake ingredients such as almonds, walnuts, and pistachio, which are considered appropriate nutrition for moulds, followed by controlling the mycotoxin development (Shirani et al. 2020; Makari et al. 2021). Although the direct correlation between mould growth and mycotoxin production has not been found yet, prevention of fungal development leads to the prevention of mycotoxin accumulation efficiently (Garcia et al. 2010). Determination of microbial responses concerning time and environmental conditions can be provided by kinetic models that can estimate growth parameters: lag phase and growth rate.

Fungal contamination is defined by fungal spore germination, which is the critical aspect to result in food spoilage (Dijksterhuis 2019). The fungal spore germination has been recognised by several methods, including

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establishing cell polarity, isotropic growth (swelling), and development of a germ tube (polarised growth) (Dijksterhuis 2019). Germination is described as a time when the germ tube length is equal to or greater than the most significant dimension of the swollen spore. Also, the time of visibility of 50% of spores is regarded as germination time (τ) (Kalai et al. 2017; Van Long et al. 2017). After completing germination, the following steps include extension and branching of the germ tube while the mycelia form apparent colonies. It is worth noting that the appearance of the mycelium may occur immediately after germination, while evaluation of this step should be considered important as well when the target is to promote the mycological stability of food (Santos et al. 2020).

The variability and heterogeneity in spore germination or vegetative cells might be related to their biological mode of action as self-inhibitors or auto-stimulators, or in the other way, it is related to the variety of environmental conditions (Gougouli and Koutsoumanis 2012). Provided that probability distributions should investigate the evaluation of certain variability and obscurity of spores. Most of the documented models consider several environmental parameters and their interaction such as temperature, water activity, and pH; nevertheless, the studies also demand to consider the inoculum size. To the best of our knowledge, no study has been performed that can fulfil the demands by covering all of the variables. Indeed, the inoculum size that is regarded as the regulating spore preparation influences the growth parameter in predictive mycology in the following steps (Garcia et al. 2010).

Primary models are kinetic and reveal how biological responses vary during time. However, secondary models define how primary model parameters depend on environmental and biological factors (Dantigny 2007).

In this study, moulds were isolated from the microbial cultivation of a cake sample, followed by recognition of the most dominant one (*Penicillium roqueforti*) by molecular and microscopic methods. Then, firstly, several environmental parameters and their interaction were evaluated on the germination of *P. roqueforti*. Secondly, the germination rate of *P. roqueforti* spores was simulated by predictive modelling.

MATERIAL AND METHODS

Culture medium preparation. Yeast Extract Glucose Chloramphenicol Agar (Germany) was used as a culture medium; then, the agar was dissolved in distilled water with different amounts of glycerol in order to control the water activity (a_w). The a_w was evaluated by an a_w meter (Novasina, Switzerland) (Hocking and Pitt 1980; Marín et al. 2000). Sodium bicarbonate (1, 5, and 10% wt aqueous solutions) was used to adjust the pH of the culture medium. The pH meter was used with an accuracy of 0.01 (Labtron, Czech Republic). The relationship between glycerol content and 1% bicarbonate solution was plotted as a function of the a_w of the culture medium in a standard curve.

The bottled culture medium was autoclaved at 121 °C for 15 min. Finally, 20 mL of culture medium was poured into sterile plates (8 cm in diameter), and then they were used for the inoculation stage. After inoculation and during incubation time, to confirm the stability of a_w, the control plates were incubated at the same time as the inoculated plates and analysed at regular intervals.

Preparation of spores and inoculation suspension. The *P. roqueforti* spores isolated from the cake were cultured on PDA (Potato Dextrose Agar) at 25 °C for seven days in order to provide the full-spored medium. The culture medium was then removed, and the spores were added to sterile physiological saline containing 0.5% (v/v) of Tween 80. The final suspension was passed through a sterile four-layer cloth, and finally the spore concentration was assessed using a neo bar counter (HGB, Germany) (Gougouli and Koutsoumanis 2010). The dispersion of spores was evaluated from haemocytometer slides and 10× magnification by a microscope. One mL of the prepared suspension was spread on the PDA medium in order to confirm the viability and germination capacity of the spores. The spore germination spores was explored again at three levels of 10, 100, and 1 000 spores per mL.

Inoculation and incubation conditions. An amount of 0.1 mL of the suspension for inoculation (10^7 spores per mL) was spread on the surface of the culture medium with a glass rod to evaluate the spore germination or nongermination (Gougouli et al. 2011).

Next, the plates were sealed with parafilm and kept in a plastic bag for the prevention of air interference. A large volume of glycerol solution (with a_w equal to the culture medium) was placed in a plastic container with the culture medium plates to balance the relative humidity of the medium during incubation. The lid of this container was tightly sealed with parafilm to keep the culture media constant (0.01 a_w). Subsequently, the container was transferred to incubators at 15, 25, and 30 °C under isothermal conditions (±2 °C) for incubation.

Fitting the model to explore the germination kinetics. Microscopic changes of plates were evaluated with a 40× magnification at regular intervals to investigate germination, nongermination, or percentage of germination (Gougouli and Koutsoumanis 2010).

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Germination evaluation. Once the first germination was observed, all spores and germinated spores were counted and this continued until the end of the incubation period (60 days). The percentage of germinated spores was calculated from Equation 1:

$$P(\%) = \frac{N_1}{N_2} \times 100 \quad (1)$$

where: N_1 – germinated spores; N_2 – total spores.

P data for each treatment were fitted over time according to the logistic model and Gompertz model (Equations 2 and 3). The time of germination beginning (t_i) (when 6.6% of spores have germinated) for the obtained parameters of the Gompertz model was calculated by Equations 2–4, and $P(t_i)$ was compared with $P(\tau)$ (the turning point of the logistic equation).

$$P = \frac{P_{max}}{1 + \exp\left[k(-t)\right]} \quad (2)$$

$$P(\%) = P_{max} \exp\left\{-\exp\left[\frac{m_g \exp(1)}{P_{max}}(-t) + 1\right]\right\} \quad (3)$$

$$t_i = +\left[\frac{P_{max}}{m_g \times \exp(1)}\right] \quad (4)$$

where: t_i – time of germination beginning; k – growth rate constant; m_g – maximum growth rate.

Relationships between the parameters obtained from logistic models (k and τ) and Gompertz models (μ and λ) were evaluated by the studied variables using multivariate linear regression and considering x1, x2, x3, x4 and their interaction as independent variables. A step-by-step regression test was performed by SigmaStat software (version 0.4) to eliminate the dependent factors to check the independence of the variables (Gougouli and Koutsoumanis 2012). The turning point of the Gompertz equation (ti) was calculated based on Equations 2–4 and compared with the turning point of the logistic equation (τ).

Statistical analysis. To investigate the effect of temperature, water activity, pH, inoculation level, and their interaction on the germination time of *P. roqueforti* as well as the parameters obtained from the models, analysis of variance (ANOVA) by Minitab (version 13.2, USA) was performed, using a completely randomised design in the form of factorial experiments.

Various parameters such as correlation coefficient (R^2), mean square error (MSE), Akaike information criterion (*AICC*), bias factor (B_f), and accuracy factor (A_f) were used to calculate the accuracy and compare the fitted models. B_f and A_f were considered by Equation 5.

$$A_f = 10^{\left[\Sigma\left|\log\left(\frac{X_p}{X_o}\right)\right| \times n\right]} \quad (5)$$

$$B_f = 10^{\left[\Sigma\log\left(\frac{X_p}{X_o}\right) \times n\right]} \quad (6)$$

where: X_p – predicted response; X_o – observed response; n – number of observations.

Germination modelling. Mould germination on the yeast extract medium under different conditions (pH, a_w, temperature, and inoculation level) was evaluated by the factorial method (162 treatments) over 60 days, and then data were used to design a model for optimum germination conditions. In this study, the data includes determining the germination time and subsequently the radial growth rate of moulds isolated from cake on a culture medium. The major parameters affecting mould growth, including water activity, pH, and temperature, have been considered within the real ranges of the cake (water activity: 0.65–0.90, pH: 6.5–7.5, and temperature: 15–30 °C). The cake used in the experiment is a product of an Iranian factory under the brand name Babana, with a daily production capacity of one million pieces. The formulation of this cake is similar to that of a muffin and contains liquid oil. The production, cooling, and packaging conditions in the factory were non-sterile and carried out under normal conditions. The moulds were also isolated from the same type of cake.

RESULTS

ANOVA of the data revealed that all of the treatments and their interaction caused significant changes in the germination of both moulds ($P < 0.05$) provided that the ability of *P. roqueforti* spores to germinate on the yeast culture medium is affected by pH, a_w, temperature, inoculation level, and time.

The germination period was completed by 52 treatments of *P. roqueforti*. These treatments (with 3 phases: lag phase, exponential or logarithmic phase, and stationary phase) were fitted by logistic model and Gompertz model (Equations 2 and 3). Germination curves were obtained based on the cumulative frequency of germinated spores over time. Having the status documented from previous studies, the effect of the studied treatments on the germination rate of *P. roqueforti* follows an expo-

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nential function (Pardo et al. 2004; Schubert et al. 2010; Aldars-García et al. 2017). Finally, the relationship between the parameters and the studied treatments was obtained by multivariate nonlinear regression.

Kinetic parameters k and τ were obtained from the logistic model. The effects of the variables influenced the k value insignificantly. On the other hand, the effects of the variables on the τ value were insignificant except for the temperature ($P < 0.05$). According to the logistic model, the effect of the variables on μ_g was indicated insignificant as well. Furthermore, the impacts of the variables on λ_g were not significant except for the temperature ($P < 0.05$). The shortest lag phase of germination of *P. roqueforti* spores was observed at pH = 6.5, a_w = 0.9, and 1 000 spores per mL inoculation level. The temperature had no effect on germination rate or lag phase duration. However, the shortest lag phase of germination of *P. roqueforti* spores was observed at 15 °C. The impact of conditions on model parameters is clearly illustrated in Figure 1.

The lowest germination percentage of *P. roqueforti* spores was observed at the end of the logarithmic phase at pH = 0.7, temperature 30 °C, a_w = 0.85, and inoculation level of 10 spores per mL (51.78% according to logistic modelling and 49.18% according to Gompertz modelling); pH= 6.5, temperature 15 °C, a_w = 0.75 and inoculation level of 100 spores per mL (52.21% according to logistic modelling and 52.28% was observed according to Gompertz modelling). According to the results, *P. roqueforti* shows no germination at a_w = 0.65, while germination increased significantly when intensifying a_w to 0.90. Regardless of the other parameters, minimum a_w = 0.75 was indicated for spore germination. However, pH = 6.5 was the best for germination, and then after that, germination decreased through the pH increase. The best tem-

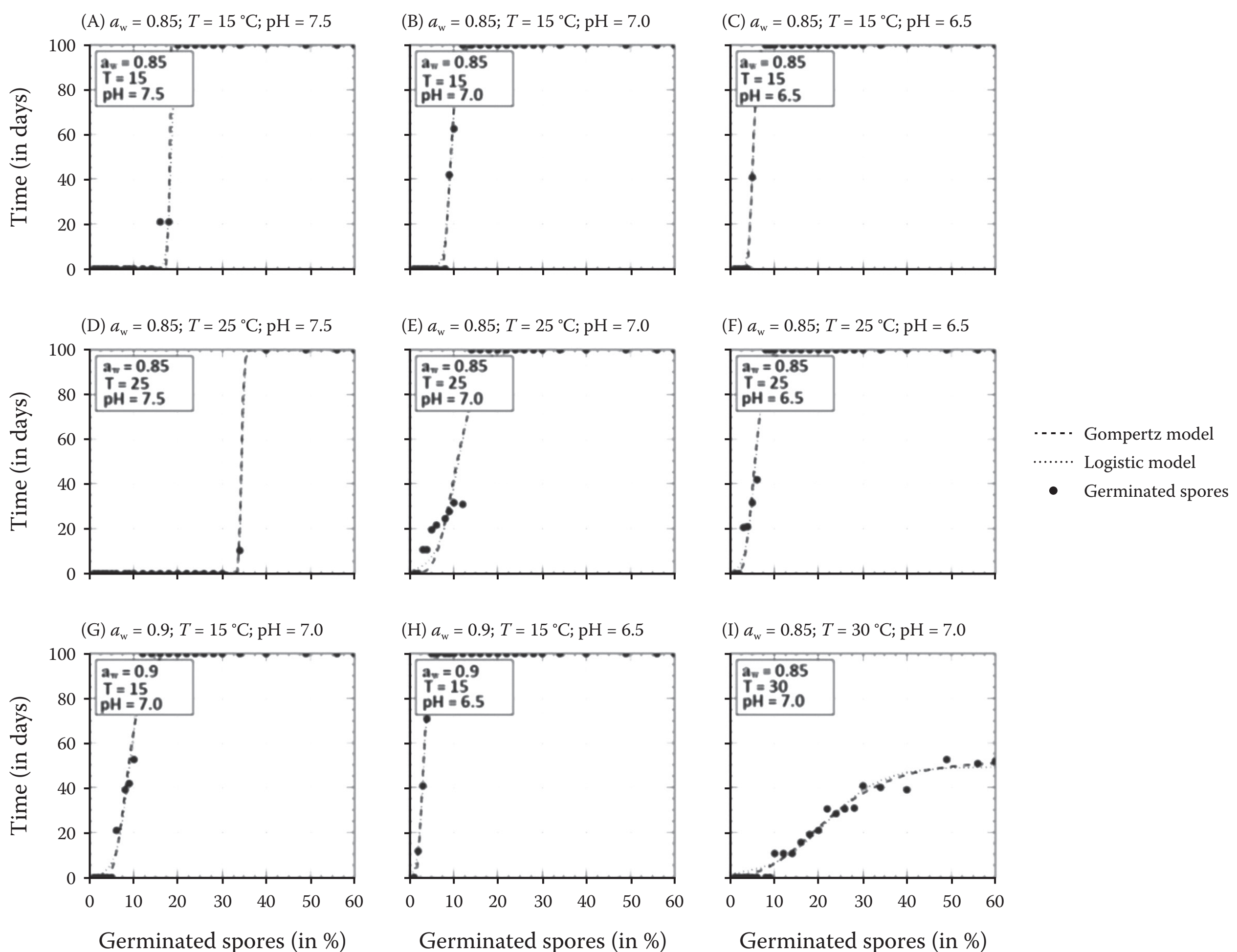

Figure 1. Percentage of germinated spores of *Penicillium roqueforti* on the yeast extract culture with inoculation rate of 10 spores per mL

a_w – water activity; T – temperature

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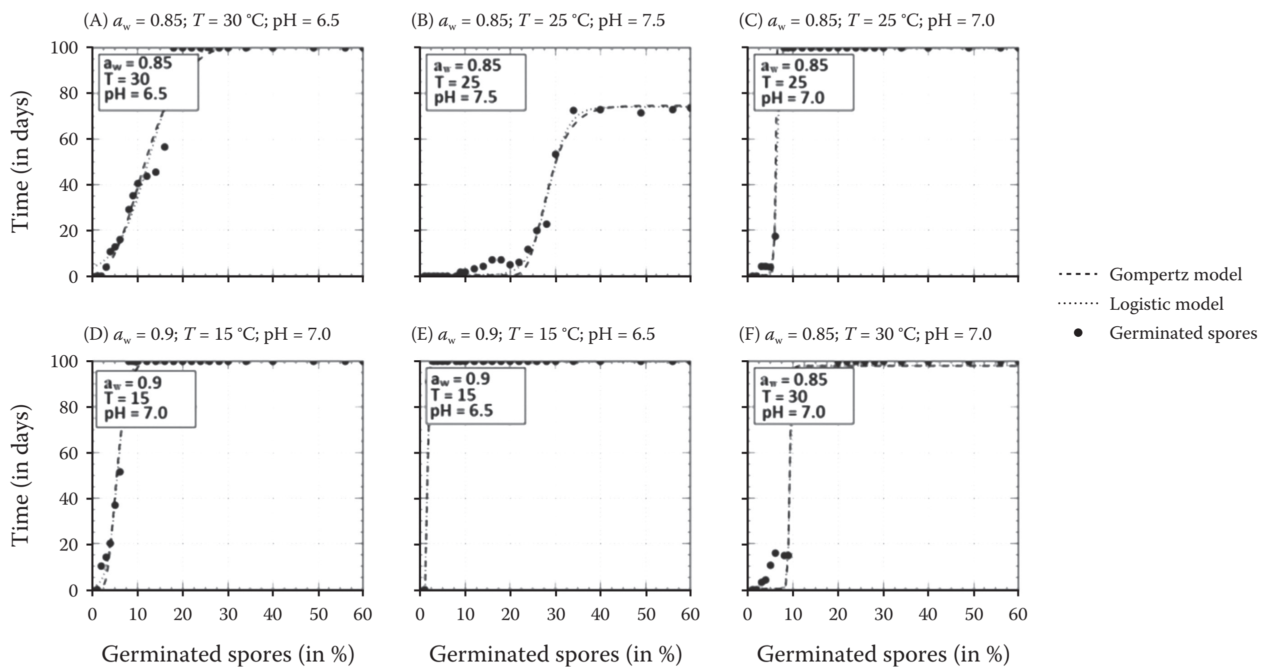

Figure 2. Percentage of germinated spores of *Penicillium roqueforti* on the yeast extract culture with inoculation rate of 100 spores per mL

a_w – water activity; T – temperature

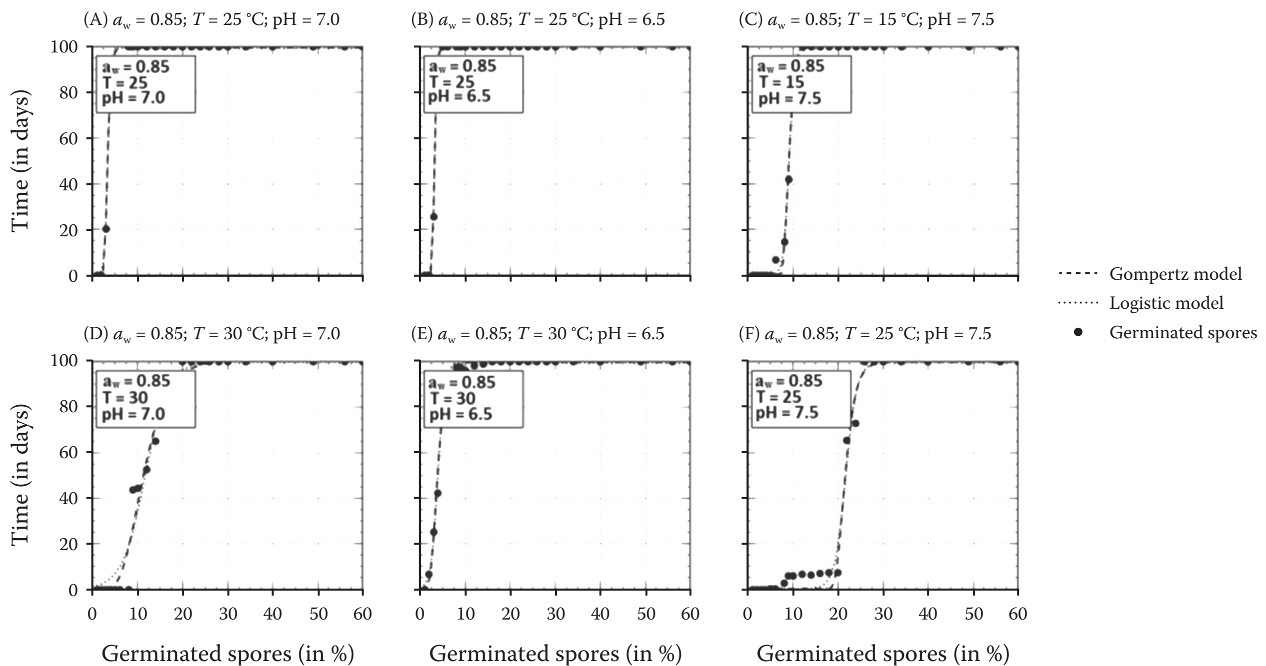

Figure 3. Percentage of germinated spores of *Penicillium roqueforti* on the yeast extract culture with inoculation rate of 1 000 spores per mL

a_w – water activity; T – temperature

perature was 15 °C, and the minimum germination was seen at 30 °C. Also, the germination rate increased with the growth in inoculation rate. Germination rates were 36–37% and 48–50% for the Gompertz model and the logistic model, respectively, when all spores were visible [(t_i) P and (τ) P] (Figures 1–3).

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DISCUSSION

In *P. roqueforti*, none of the variables influenced the value of k significantly, and in the case of τ the effect of all variables except the temperature was significant ($P < 0.05$). In general, the results showed that none of the spores of *P. roqueforti* was able to germinate at a_w = 0.65, while germination increased significantly during intensifying a_w to 0.90. Therefore, it was concluded that pH, $a_{w,}$ and inoculation level at the points studied in this study did not impact the germination rate of *P. roqueforti* spores in the yeast culture medium but they affected the lag phase of germination time significantly.

Pardo et al. (2006) achieved similar results by inoculating *P. verrucosum* mould on barley meal extract agar (Pardo et al. 2006). They concluded that no germination of this mould occurred at $a_w < 0.75$. Also, they stated that germination at $a_w = 0.80$ was seen at 20 °C. As the consequences of this study show, it is noticeable that the best conditional aspects that improved the germination capability include pH = 6.5, 15 °C, $a_w = 0.9$, and inoculation rate of 1 000 spores per mL (Figure 3). Compelling, *P. roqueforti* is considered a mesophilic mould whose optimum germination conditions are in the range of 15–25 °C. Therefore, in winter, when the temperature is less than 15 degrees, germination may not occur, or it might occur slowly. Similarly, no germination was observed at $a_w = 0.65$, and germination at $a_w = 0.70$ occurred only at the optimum pH and storage temperature. It happened because decreasing a_w causes water stress to fungal spores. However, in some cases, water stress may be affected by the microstructure of the food (Huang et al. 2010), meaning that the physical microstructure has an inhibitory effect on fungal growth (Huang et al. 2009).

Germination fitting models were measured by R^2, root mean square error (RMSE), *AICC*, A_f, and B_f parameters. The proficiency of logistic and Gompertz equations to describe the germination of mould spores was proved previously (Pardo et al. 2004; Pardo et al. 2006; Schubert et al. 2010; Aldars-García et al. 2017).

In the logistic model, R^2 ranged from 0.954 to 1.000, RMSE from 0.00 to 9.45, and *AICC* from 10.37 to 66.189. In the Gompertz model, R^2 ranged from 0.937 to 1 000, RMSE from 0.00 to 11.09, and *AICC* from –636.76 to 116.85 for *P. roqueforti*. Hence, it can be concluded that both models have acceptably fitted the percentage of germinated spores over time.

The average of R^2, RMSE, *AICC* was assigned –2.68, 3.07, and 0.990 in the logistic model, and –18.88, 3.27, and 0.989 were attained from the Gompertz model, provided that the logistic model was conducted as the best model compared to the Gompertz model with higher R^2 and lower RMSE. Based on the studies that have been performed on *Aspergillus ochraceus, Fusarium verticillioides,* and *P. verrucosum*, the logistic model was exclusive to the Gompertz method to present the effect of a_w on the germination of these moulds under optimal conditions (Dantigny et al. 2007). In addition, a model must provide an empirical estimate of the model parameters. *A* and P_{max} parameters are valuable parameters for predicting mould germination, but parameters of lag phase evaluation and germination time are considered more crucial (Dantigni et al. 2007), which must be estimated with precise accuracy. However, the smaller the 95% confidence interval, the higher the fit accuracy of the models was achieved, which was lower in the logistic model, except for the k parameter, than in the Gompertz model.

Germination modelling of *P. roqueforti* has been performed by NeuroSolutions software (version 7.1.1.0), and actual data without fitting, including germination percentage, were applied. As shown in Table 1, at different levels of the factors, germination or nongermination was labelled with the letters A (nongermination) and B (germination) during 60 days. After

Table 1. Standard deviation and performance metrics for validation of nongermination (A) and germination (B) models

Performance	Nongermination A (%)	Germination B (%)
MSE	0.016844903	0.016368322
NMSE	0.104718414	0.103137604
MAE	0.061217743	0.060588624
Minimum absolute error	3.01097E–05	5.4036E–05
Maximum absolute error	1.034119386	1.004794985
r	0.947808063	0.948383790
Percent correct	87.387964150	2.380952381

MSE – mean square error; NMSE – normalized mean square error; MAE – mean absolute error; *r* – correlation coefficient

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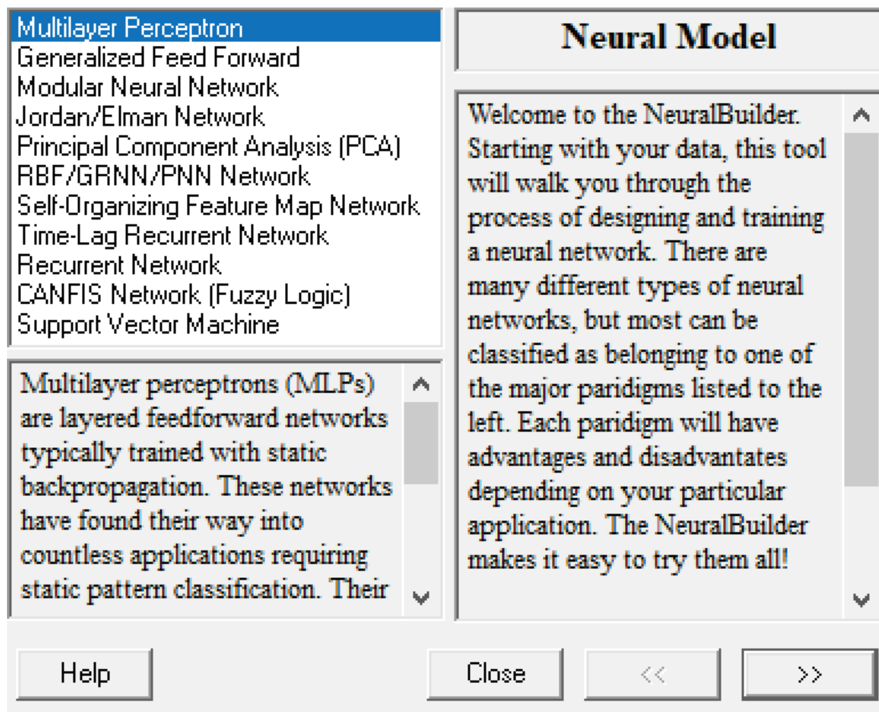

Figure 4. Multilayer Perceptron (MLP network)

randomising the rows, 60% of data were allocated for training, 15% for validation, and 25% for testing. Then, the germination column was labelled as the first result. Compelling, the germination percentage above 95% was named as label B and less than 95% was labelled as A. If it is labelled A, it will not be visible; however, if it is label B, the NeuroSolutions could estimate when the mould appears (shelf-life). NeuroSolutions networks include Multilayer Perceptron, Generalized Feedforward, Support Vector Machine, Modular Neural Network, hidden layers = 2, process elements = 50, and the Learning Rules of Momentum and Transfer Functions, the hyperbolic tangent was selected for both layers. In the last step, the number of performances that lead to error reduction was considered 1 000.

As presented in Figure 4, the best result was obtained in the Multilayer Perceptron network, among the different networks. Furthermore, Figure 5 presents the final network created by the NeuroSolutions software.

According to Figure 6, two hidden layers that were in the first layer with 50 process elements, the hyperbolic tangent transfer function and the Learning Rules of Momentum, and in the second layer, the number of process elements was 29, the hyperbolic tangent transfer function and the Learning Rules of Momentum were set.

According to Figure 7, the minimum standard deviation is reduced through the different operations that are repeated up to 1 000. The validity coefficient was 0.947 for nongermination and 0.948 for germination.

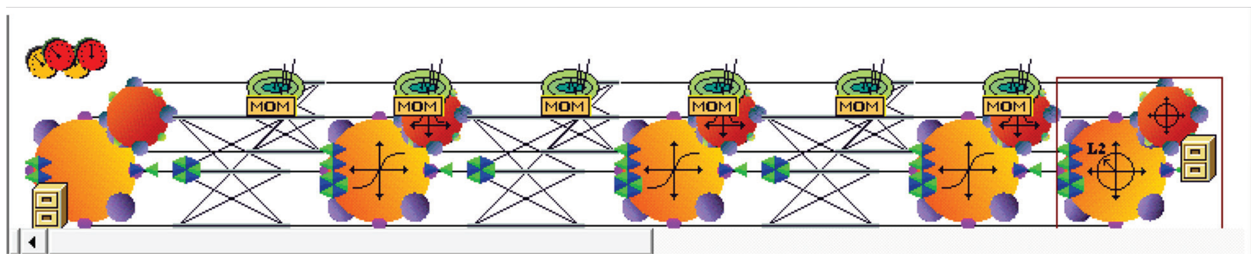

Figure 5. The network created by the NeuroSolutions software (version 7.1.1.0)

MOM – method of moments

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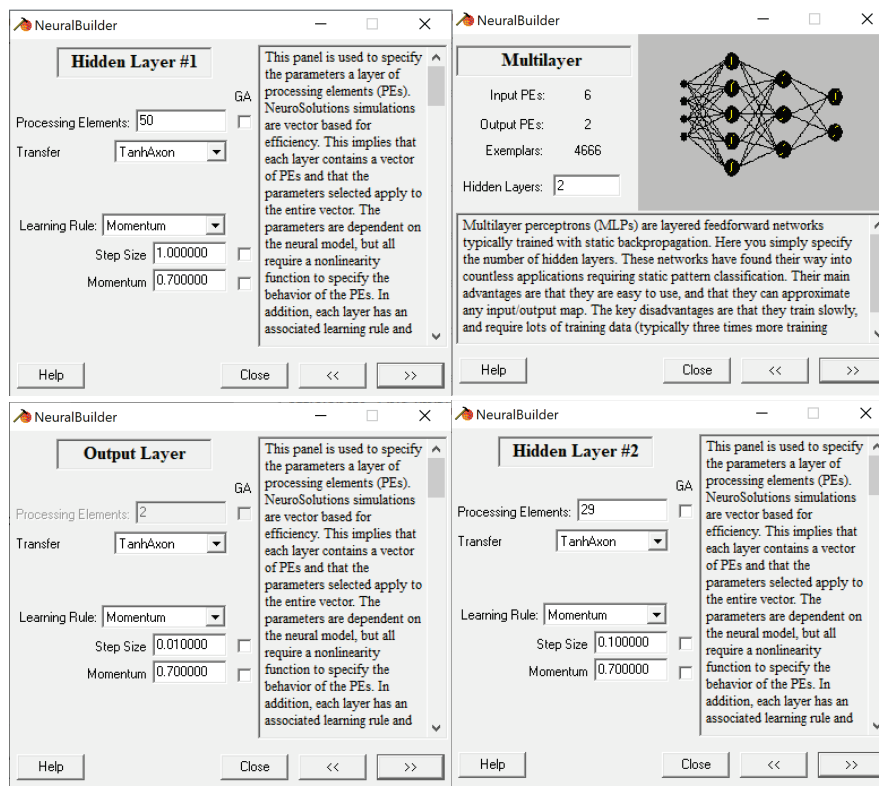

Figure 6. Hidden layers

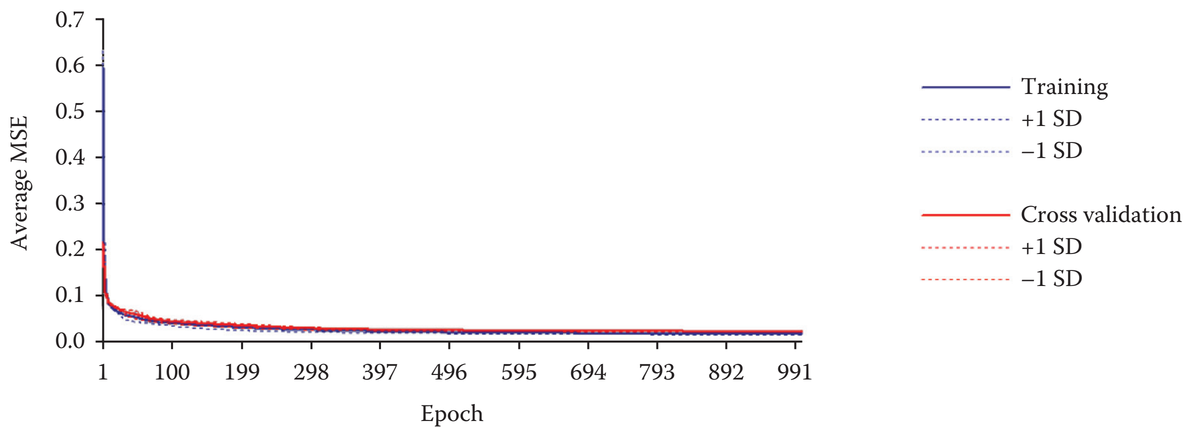

Figure 7. Standard deviation for validation – Average MSE with SD boundaries for 2 runs

MSE – mean square error; SD – standard deviation

CONCLUSION

Bakery products are considered a crucial part of the diet of people around the world. The cake has a particular position among consumers due to its organoleptic properties and nutritional value. According to the critical aspects of food safety, increasing the shelf life of cakes has been a significant issue in the bakery industry. The germination and growth of moulds, especially the growth of *Aspergillus* and *Penicillium* species on the surface of bakery products has been reported as the principal problem of manufacturers. In general, environmental factors such as temperature, pH, food moisture, etc., are considered effective parameters for the shelf-life limitation of the product. In the present study, *P. roqueforti,* a critical mould that develops on the cake, was cultured on a yeast extract medium in several environmental conditions (pH, temperature, inoculation rate, and a_w). Then, the best germination conditions were described as pH = 6.5, a_w = 0.90, 15 °C, and greatest inoculation

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rate. It appears that the culture medium used for the moulds is more favourable than the actual cake, thus it can be stated that the moulds will have a more challenging time of germinating and growing in the cake compared to the test conditions. The results of the present study could be used for extension of the mycological shelf life of cakes.

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