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ScienceDirect

Procedia Food Science 7 (2016) 76 – 79

Procedia Food Science

9th International Conference on Predictive Modelling in Food

Modelling the probability of growth and aflatoxin B_1 production of *Aspergillus flavus* under changing temperature conditions in pistachio nuts

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Abstract

The aim of this work was to use probability models for the prediction of growth and aflatoxin production by *Aspergillus flavus* as a strategy to mitigate the aflatoxin presence in pistachio nuts during postharvest. Logistic models, with temperature and time as explanatory variables, were fitted to the probability of growth and aflatoxin B_1 (AFB1) production under constant temperature levels, afterwards they were used to predict probabilities under non-isothermal scenarios. The models obtained showed levels of concordance from 80 to 100% in most of the cases. Moreover, the presence of AFB1 in pistachio nuts could be correctly predicted through AFB1 models developed in agar medium or through growth models in pistachio nuts. These findings can support decision making, at transport and storage level, and could be used by producers and processors to predict the time for AFB1 production by *A. flavus* in pistachio nuts in postharvest.

Peer-review under responsibility of Department of Food Science, Faculty of Food Engineering, University of Campinas.

Keywords: Predictive mycology; *Aspergillus flavus*; food safety; pistachio; temperature; non-isothermal conditions; probability model

1. Introduction

Pistachio nut (*Pistacia vera* L.) is one of the most popular tree nuts in the world, and is subjected to infection by a variety of microorganisms that can cause foodborne illness, spoilage or toxic effect on human[1]. Within these microorganisms, *Aspergillus flavus* and *Aspergillus parasiticus*, weak opportunistic plant pathogenic fungi[2], are the

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Peer-review under responsibility of Department of Food Science, Faculty of Food Engineering, University of Campinas.
doi:10.1016/j.profoo.2016.02.091

most relevant species. Both species can produce aflatoxins (AFs), secondary metabolites produced by various strains[3]. AFs are the most important mycotoxins, and the AFB1 is listed as a carcinogen of group I by the International Agency for Research of Cancer, and due to their hepatocarcinogenic potential, AFs are highly regulated (European Commission Regulation 165/2010). The maximum limits for AFB1 are 12 ㎍/kg for pistachios to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs, and 8 ㎍/kg for pistachios intended for direct human consumption or use as an ingredient in foodstuffs. According to the RASFF (EU Rapid Alert System for Food and Feed) in 2014 there have been 125 notifications related with AFs in nuts, nut products and seeds from Iran, China and Turkey. From the food safety point of view, only mycotoxins entail a hazard, while yeast and moulds themselves may cause food spoilage but are not harmful to humans.

Nut infections may occur along all the food chain, but are more common to occur during preharvest; nevertheless it might occur in the subsequent steps (storage, manufacturing, transport and packaging), if minimum preventive measures are not established. During postharvest, fungal growth should not occur if the freshly harvested nuts are dried as soon as possible to 6% of moisture content and then cool stored. However, shipping of nuts is not always carried out under cool conditions, as this is economically costly. It is noticeable that the temperature fluctuations during transport and retail storage can affect the quality and food safety. Increases in temperature and humidity within the bulk of pistachio nuts during transport and storage may allow fungal growth and mycotoxin production. In this way, it is important to control temperature and humidity during transport and do not allow the pistachio bulk to reach a temperature which jeopardizes the safety of the product.

The specific objectives of the present study were to: i) model the probability of growth/AF production of *A. flavus* under non-isothermal conditions; ii) validate the derived models on AFB1 data generated directly in pistachio nuts under non-isothermal conditions.

2. Material and methods

2.1. Experimental design and data generation

A full factorial design was developed, where factors involved were temperature and medium. Regarding medium, the whole experiment was carried out in both pistachio extract agar (PEA) and pistachio nuts. Regarding temperature, nine profiles were tested: five static temperatures (15, 17.5, 20, 22.5 and 25 ºC), plus four different scenarios of dynamic temperature levels (upward shift (US), downward shift (DS), upward ramp (UR) and downward ramp (DR)). These temperature levels were chosen based on the levels which may be encountered during shipping of pistachios at room temperature. Both the static and changing temperatures were kept for a 42 days period. a_w was initially adjusted to 0.87, corresponding to about 15% moisture content. The experiments were carried out with a minimum of ten replicates per treatment.

A. flavus suspensions were point-inoculated on the center of each Petri dish, on both PEA and pistachio nuts, under aseptic conditions, and incubated in computer controlled incubators set at the conditions designed for this study. PEA and nuts Petri dishes were daily checked for visible growth, using a binocular magnifier for easy viewing in the case of pistachios nuts.

Once positive growth had been recorded, 10/12 existing Petri plates per treatment were taken from incubation at different time points for AFB1 analysis, always when colonies were in the range 4-20 mm diameter. Extraction of the AFs from the agar was carried out with methanol and filtered. For pistachio nuts, the moldy ones were extracted with acetonitrile:water. Diluted extracts were passed through immunoaffinity columns (Easi-extract Aflatoxin immunoaffinity columns, R-Biopharm Rhône) and the eluate was dried in a nitrogen stream. All extracts were resuspended and injected in the HPLC system (Waters, Milford, MA, USA).

2.2. Model fitting

A logistic model was used to model the probability of growth and AFB1 production of *A. flavus* as a function of time under static conditions, using R statistical software (R Development Core Team, www.R-project.org, v 2.14.1), with the glm function. The percentage of plates with growth was calculated as P_G = plates with growth/total plates.

For each condition, data of PG over time was modelled. Thus the models developed in the present study are not based on any biological and/or conceptual assumption.

$$logit(P_G) = ln\frac{P_G(x)}{1-P_G(x)} = \sum b_0 + b_1T + b_2T^2 + b_3t$$

The percentage of plates with AFB1 was calculated as P_{AF} = plates with detected AFB1/total plates. For each condition, data of P_{AF} over time was modelled.

$$logit(P_{AF}) = ln\frac{P_{AF}(x)}{1-P_{AF}(x)} = \sum b_0 + b_1T + b_2T^2 + b_3t$$

where logit(P) represents ln[P/(1-P)], ln is the natural logarithm, P_G or P_{AF} is the probability of growth initiation or AFB1 production (in the range of 0-1), T is the temperature (ºC), t is the time of incubation (d) and b_i are the coefficients to be estimated. The goodness of fit of the models was determined through the calculated % concordance between observed and predicted values with a cut off of 0.5 probability.

For the non-isothermal prediction, the approach of Koseki and Nonaka[4] (2012) was used; in particular, they estimated the probability of the end of lag time for *Bacillus cereus*, but the same methodology could be applied here. Briefly, an R algorithm was built that for each time point in the variable temperature profiles it took the estimation for the previously built logistic model using the constant temperature profiles, taking as initial assumption that the previous temperature levels in the profile did not affect the prediction at a certain time point. This simple data-driven empirical modelling procedure using logistic regression offers the possibility of considering the intermediate lag time as a change in the probability of the end of lag time[5].

The goodness of prediction under non-isothermal conditions was also determined through the calculated % concordance between observed and predicted values with a cut off of 0.5 probability.

Growth models in PEA and pistachios and AFB1 model in PEA were validated on AFB1 data obtained from the pistachio experiment. The aim was to assess the goodness of prediction of AFB1 production probability in pistachio nuts of the different models. For validation, colonies of size 5-20 mm of diameter grown in pistachios were taken at different times from incubation an analysed for AFB1 presence; these colonies should be in the boundary of AFB1 presence/absence. The results were compared with the predicted probability through growth models in agar and nuts, and AFB1 model in agar.

3. Results and discussion

Growth data obtained under non-isothermal conditions in PEA showed that the initiation of growth occurred at a similar time point to that in pistachio nuts; however, in pistachio nuts a longer time was taken for a significant amount of plates to show growth and, most of the times the probability did not reach 1. Consequently, the predicted probability lines showed smaller slopes in pistachio nuts. If the agar models were used to predict growth in pistachio nuts, either at isothermal or non-isothermal regimes, the predictions would fail in the long term, due to overestimation of growth. When predicting *A. flavus* growth probability in pistachio nuts under non-isothermal conditions from the model developed at constant temperature level, the concordance was 98 and 100% for the gradual profiles, and 98% for the US profile, while the prediction at the step descending profile failed because low probability was predicted while 0.8 probability was attained in the observed data (Fig. 1).

AFB1 production in PEA under decreasing temperature profiles was only detected in a reduced number of plates in the first days (Fig. 2). After that, growth of colonies stopped and so did the toxin production, thus no additional AFB1 positive plates were recorded. For the step increase profile, no positive plate was detected till day 23, but in the 26th day probability of 1 was reached; by contrast, the shift from 0 to 1 probability of growth occurred after 18-19 days. Finally, in the continuously increasing profile, AFB1 production reached probability 1 after 29 days; however, the shift for growth occurred between 19 and 23 days. In this case, the same assumption made for the growth models, as well as the 'memory' correction were used. Without such correction, estimated probability lower than observed in decreasing temperature profiles was predicted, which suggests that the metabolic adaptation to toxin accumulation occurred in the preceding days under suitable temperatures. On the other hand, in the increasing

temperature profiles the prediction of toxin production was in much earlier days that in fact occurred, suggesting in this case a delay in cells predisposition to secondary metabolism due to lower past temperature levels. Using the modified algorithm the levels of concordance were 100, 100, 92.9 and 90.5% for DS, DR, US, and UR, respectively, with a cut off of 0.5.

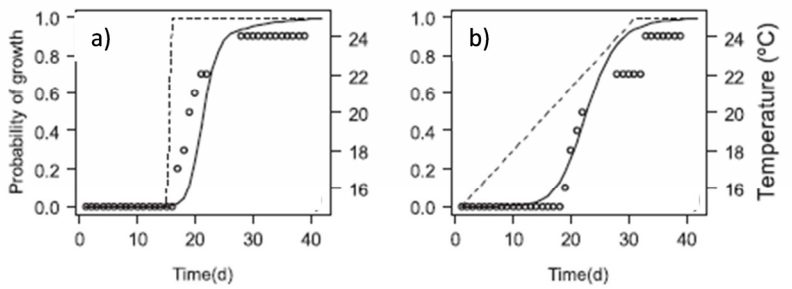

Fig.1. Observed growth probability of *A. flavus* in pistachio nuts under non-isothermal conditions (o) and predicted values (-). a) US; upward shift; b) UR, upward ramp.

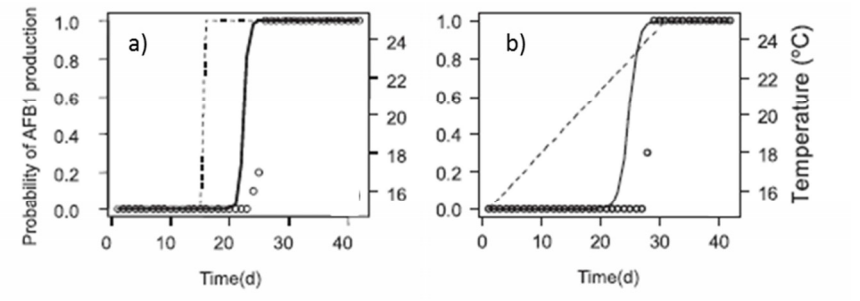

Fig. 2. Observed AFB1 production probability of *A. flavus* in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) US; upward shift; b) UR, upward ramp.

Validation of the obtained models for prediction of AFB1 data obtained from pistachio nuts showed that comparing the conditions in which toxin was detected in nuts with those in which probability of growth in nuts was over 0.50, there was a 70% of concordant cases. Moreover, the concordance between probabilities predicted for AFB1 presence in pistachio agar and observations in pistachio nuts was of 67%. Thus the development of models for prediction of AFB1 presence in nuts could be based on either AFB1 experiments on agar or growth experiments in pistachio nuts. Still, the prediction was not accurate; however, even in the event of development of models from AFB1 data in situ in pistachio nuts the accuracy would not probably be higher. This is illustrated by the fact that, for example, the observed data in UR in the single inoculum where toxin was detected after 18 days but not after 21 and 34 days; when checking the colony diameters they were 11.5, 5.5/8.5 and 8 mm in the colonies analysed at the 18th, 21st and 34th days. This suggests that colony diameters in pistachio nuts are quite variable, and a good correlation with time may not be possible.

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