Chapter V

An attempt to model the probability of growth and aflatoxin B1 production of *Aspergillus flavus* under non-isothermal conditions in pistachio nuts

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ABSTRACT

Human exposure to aflatoxins in foods is of great concern. The aim of this work was to use predictive mycology as a strategy to mitigate the aflatoxin burden in pistachio nuts postharvest. The probability of growth and aflatoxin B_1 (AFB1) production of aflatoxigenic *Aspergillus flavus*, isolated from pistachio nuts, under static and non-isothermal conditions was studied. Four theoretical temperature scenarios, including temperature levels observed in pistachio nuts during shipping and storage, were used. Two types of inoculum were included: a cocktail of 25 *A. flavus* isolates and a single isolate inoculum. Initial water activity was adjusted to 0.87. Logistic models, with temperature and time as explanatory variables, were fitted to the probability of growth and AFB1 production under a constant temperature. Subsequently, they were used to predict probabilities under non-isothermal scenarios, with levels of concordance from 90 to 100% in most of the cases. Furthermore, the presence of AFB1 in pistachio nuts could be correctly predicted in 70-81 % of the cases from a growth model developed in pistachio nuts, and in 67-81% of the cases from an AFB1 model developed in pistachio agar. The information obtained in the present work could be used by producers and processors to predict the time for AFB1 production by *A. flavus* on pistachio nuts during transport and storage.

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

1. Introduction

Predictive models may provide important data about the probability of mycotoxin contamination of foods during shipping and storage, and enable manufacturers to reduce the amount of tests and ensure the quality and safety of products and establish an adequate shelf-life. It is known that sampling and analysis of mycotoxins in nuts is not always an efficient control measure, due to the heterogeneous distribution of mycotoxins, in particular aflatoxins (AFs)(García-Cela et al., 2013).

Fungal colonization and /or mycotoxin production are generally influenced by a variety of factors such as water activity (a_w), temperature (T), substrate or pH. However, it has been demonstrated that water availability is the most important environmental factor affecting germination and growth of moulds (Holmquist et al., 1983). Most of food commodities prone to mycotoxin presence rely on low a_w for their safe postharvest life, thus studies in such commodities are required including low water availability levels. Moreover, most of the studies in predictive mycology focus on the effect of environmental factors, on fungal growth and mycotoxins production under static conditions. But in fact, the environmental conditions during the food chain change, especially storage temperature can fluctuate. Then it is important to take into account these fluctuations during the developing and validation of models, otherwise their applicability is compromised. Unfortunately very little information on the modelling of fungal germination and growth or mycotoxins production under fluctuating conditions is available (Dantigny and Nanguy, 2009; Gougouli and Koutsoumanis, 2012, 2010; Kalai et al., 2014; Peleg and Normand, 2013). On the other hand, prediction of bacterial growth under non-isothermal conditions has been studied during the past decade, where it has been demonstrated that the instantaneous specific growth rate adapts to the changing temperature practically immediately, except in extreme cases, when the temperature change is abrupt and close to the boundary of growth (Bovill et al., 2000).

Detection of fungal growth does not imply necessarily the presence of mycotoxins, as not all the strains of a mycotoxigenic species are able to produce mycotoxins and, in addition, the conditions favorable to growth may not be conducive to mycotoxin production. Moreover, growth is a parameter which presents less intraspecific variability, and its kinetics are more known, than those of mycotoxin production (Garcia et al., 2009). It is important that the models

developed to predict how the microorganism will behave under certain conditions account for the behavior of a wide range of strains to account for the intraspecific variability. Besides, the use of cocktails of strains to forecast the behavior of a species has been proposed by some authors (Hocking and Miscamble, 1995; Patriarca et al., 2001; Romero et al., 2007; García et al., 2014). As working with a bunch of strains is time consuming and costly, the use of a mixed inoculum with a variety of the strains to develop the experiment has been studied. Using a mixed inoculum, no significant differences between the growth rates of the mean of the single strains and the growth rate of cocktail inoculum were found, however a delay in the time to growth was observed for the mean of the single inocula, a difference which is even more evident when the environment conditions of the experiment are suboptimal (Baert et al., 2007; Garcia et al., 2011, 2012, 2014; Romero et al., 2010). Four strains of *Aspergillus carbonarius* differed in maximum ochratoxin A yield, and the toxin accumulation by the mixed inoculum showed intermediate levels (Romero et al., 2010).

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 (Al-Moghazy et al., 2014). Within these microorganisms, *Aspergillus flavus* and *Aspergillus parasiticus*, weak opportunistic plant pathogenic fungi (Mojtahedi et al., 1979), are the most relevant species. Both species can produce AFs, secondary metabolites produced by various strains (Georgiadou et al., 2012). AFs are the most important mycotoxins (World Health Organization (WHO) 1998), and the aflatoxin B_1 (AFB1) is listed as a carcinogen of group I by the International Agency for Research of Cancer (IARC, 1993), and due to their hepatocarcinogenic potential, AFs are highly regulated (EC Regulation 165/2010). The maximum limits for AFB1 are 12 μg/kg for pistachios to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs, and 8 μg/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 2013 there have been 341 notifications related with AFs. 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. High temperature and humidity within the bulk of pistachio nuts during transport and storage can provide good conditions for fungal growth and mycotoxin production. In this way, it is important to have a good control of the temperatures and humidity during transport and do not allow the pistachio bulk to reach a temperature which jeopardizes the safety of the product. For this reason it is advisable to install vent pipes in solid-sided trailers or transport them in vented pallet bins (Thompson et al., 1997). Moreover, air flow induced by transport or by fans can be used for cooling (Brusewitz, 1973; Kader et al., 1978).

For many years, AFs have been reported in pistachios (Abdulkadar et al., 2000; Ariño et al., 2009; Cheraghali et al., 2007; Dini et al., 2013; Fernane et al., 2010a, 2010b), and many batches have to be rejected (Bui-Klimke et al., 2014).Developing a model capable of predicting the presence of AFs in pistachio nuts may be highly suitable for the pistachio production and trade.
Therefore the general objective of the present research was to develop a predictive model to assess the effect of temperature on the growth rate/aflatoxin production of *A. flavus* under non-isothermal conditions, taking into account the intra- species variability. Predictive models in food microbiology can be splitted, according to their aim, into two main categories: kinetic and probability models. In the present study we will focus on probabilistic models, which determine whether or not growth or toxin production can occur or exceed a certain level under specific conditions (Lindblad et al. 2004; Marín et al. 2012). Given the above, the specific objectives of the present study were to: i) study the role of temperature on the growth of *A. flavus*; ii) model the probability of growth/AF production of *A. flavus* under non-isothermal conditions; iii) investigate the effect of the growth medium (pistachio agar and pistachio nuts) on such models; iv) compare the probability of growth and AF production of a single and a mixed inoculum of *A.*

flavus; v) validate the derived models on AFB1 data generated directly in pistachio nuts under non-isothermal conditions.

2. Materials and methods

2.1. Selection of aflatoxigenic isolates

We used twenty-five *A. flavus* isolates in the cocktail taking into account the studies developed by García et al. (2012). All of them were isolated from Iranian pistachio nuts purchased from a wholesaler in Lleida, Catalonia, Spain. Briefly, samples of pistachio were plated on DRBC, and the isolated colonies were identified according to the taxonomical descriptions of Pitt and Hocking (2009). Twenty-five of the isolates found to produce AFs in coconut agar medium (CAM), were selected for the trials conducted in the present study.

2.2. Experimental design

A full factorial design was developed, where factors involved were: temperature, medium and inoculum. The inoculum factor included two levels: single inoculum of isolate TA-3.267 (taken at random from the 25) and mixed inoculum of 25 isolates. Regarding medium, the whole experiment was carried out in both pistachio agar and pistachio nuts (preparation described later). Regarding temperature, nine profiles were tested: five static temperatures (15, 17.5, 20, 22.5 and 25 °C), plus four different scenarios of dynamic temperature levels (upward shift (US), downward shift (DS), upward ramp (UR) and downward ramp (DR) (Fig. 2, dotted lines). 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, this value was chosen to simulate a postharvest product which was not safely dried, although still it was far from the optimal for fungal growth. The experiments were carried out with a minimum of ten replicates per treatment.

2.3. Preparation of media

Pistachio extract Agar (3%) (PEA): Pistachio extract was prepared by boiling 60g of ground pistachio in 1L distilled water for 30 min. After that, the extract was filtered and the amount of

evaporated water re-added. This concentrated extract was diluted to 3% by addition of water+glycerol for a final a_w of 0.87. 20g of agar were added per L of medium and it was autoclaved and poured into 90 mm sterile Petri dishes under aseptic conditions. A total of 12 plates per condition and type of inoculum (9x2x12, a total of 216 plates) were prepared.

Pistachio nuts: Iranian shelled pistachios were purchased from a wholesaler in Lleida, Catalonia, Spain. An initial analysis showed that AFB1 concentration was under the LOD. Pistachios were autoclaved (15 minutes at 121°C) in 1-L bottles filled with 300 g of pistachios. Once sterilized, the a_w was adjusted to 0.87, by aseptically adding 1mL/10g of distilled water (Marín et al., 2008) to the pistachios. The bottles were cooled down to approximately 4 °C for 48 h with periodic hand-shaking during this period. After that, pistachios were placed in Petri dishes (55 mm diameter; 10g in each Petri dish) under aseptic conditions. A total of 10 plates per condition and type of inoculum (9x2x10, a total of 180 plates) were prepared.

a_w values in PEA and pistachio nuts were determined using an Aqualab CX2T (Decagon Devices, Pullman, WA, USA).

2.4. Preparation of spore suspensions, inoculation and incubation

The 25 aflatoxigenic isolates were grown on potato dextrose agar (PDA) medium at 30 °C for 7 days, to enable significant sporulation, and spores were collected by scraping the colony with a sterile spatula and then suspended in sterile distilled water containing Tween 80 (0.1% v/v). After counting the spores on a Thoma chamber, the spore suspensions were adjusted to 10^4 spores/mL. Two types of inocula were prepared: a cocktail inoculum with all 25 isolates at a final concentration of 10^4 spores/mL and a single inoculum of isolate 3.267, at the same concentration.

5 µL of the spore suspensions were point-inoculated on the center of each Petri-dish, on both PEA and pistachio nuts, under aseptic conditions, having then about 50 spores in each Petri plate. PEA and pistachio Petri-dishes were placed separately in sets of temperature inside plastic containers together with beakers containing distilled water in order to avoid media dehydration and allow moisture absorption from the environment. The containers were kept in computer controlled incubators (Memmert ICP-600, United Kingdom) set at the conditions designed for this study (see experimental design) for 42 days.

PEA and nuts Petri dishes were daily checked for visible growth, using a binocular magnifier (ZEISS, Stemi DV4) for easy viewing in the case of pistachios nuts.

For AF analysis, a preliminary trial was performed in order to determine which range of colony diameters were going to be analysed in order to save time and costs. This preliminary experiment was carried out with strain 3.267 in pistachio nuts following the same methodology as described above but at 3 constant temperature levels (15, 22 and 30 °C). In this case pistachios were at 0.92 a_w. From this experiment a relationship between colony diameter and AF presence was established (see section 3.1) and used to take the decision on the Petri plates that would undergo AF analysis in each particular day in both PEA and pistachio nuts. Consequently, once positive growth had been recorded, 10/12 existing Petri plates per treatment were taken from incubation at different time points, always when colonies were in the range 4-20 mm diameter (see section 3.1). While a significant number of PEA plates were analysed, only a few (57) colonies grown on pistachio were analysed, which were used for validation purposes (section 2.7).

2.5. Detection and quantification of AFs by HPLC

Extraction of the AFs from the agar was carried out by removing a 5-mm agar plug from the centre of each colony. Plugs were weighed and introduced into 3-mL vials. Methanol (1 mL) was added, and the vials were shaken for 5 s (Autovortex SA6, Surrey, UK). After being left stationary for 60 min, the extracts were shaken again, filtered (MillexR SLHV 013NK, Millipore, Bedford, MA, USA) and dried in a nitrogen stream.

For pistachio nuts, the moldy ones were weighed and ground. Each ground sample was extracted (1+4 w/v) with 60% acetonitrile in water by blending for 20 min. Extracts were filtered and the filtrate was diluted 1:24 in phosphate-buffered saline (PBS) pH 7.4. Diluted extracts were passed through immunoaffinity columns (Easi-extract Aflatoxin immunoaffinity columns, R-Biopharm Rhône) at a flow rate of 2–3mL/min. Later, the columns were washed with 20 mL of PBS at a flow rate of 5 mL/min. Desorption was carried out with 3 mL of methanol slowly passed through the column and the eluate was finally dried in a nitrogen stream.

All extracts were resuspended with 0.5 mL of methanol + water (50+50 v/v) and a volume of 100μL was injected in the HPLC system (Waters, Milford, MA, USA). The presence of AFs was

detected and quantified by HPLC with fluorescence detection (λ_{exc} 330 nm; λ_{em} 460 nm) (Waters 474), using a C18 column (5 µm Waters Spherisorb, 4.6 x 250 mm ODS2). The mobile phase (water: acetonitrile: methanol, 70: 17: 17) was pumped at 1.2 mL/min. Both AFB1 and AFB2 were detected in the chromatograms, the former in much higher amount, and in some cases AFB1 was present but AFB2 was not detected. Thus, for the present study only AFB1, the most common in food, was taken into account. The detection limit of the analysis was 0.1 ng/g of AFB1, based on a signal-to- noise ratio of 3:1.

2.6. 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 P_G 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 (°C), 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 (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 modeling 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 (Koseki and Nonaka, 2012).

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.

Finally, we worked on the assumption that no degradation of AFB1 took place.

2.7. Validation

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 3 different models. For validation, colonies of size 5-20mm 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

3.1. Assessing the colony sizes leading to AFB1 presence

The preliminary study on the relationship between colony diameter and AFB1 production for strain 3.267 in pistachio nuts at 15, 22 and 30 °C and 0.92 a_w revealed that colonies with mean diameter smaller than 4 mm did not contain AFB1, while colonies with diameters over 12 mm always contained AFB1 regardless of the temperature level (Table 1, supplementary material). However, colonies between 4 and 12 mm of diameter presented different results. Consequently, for the present study, to save laboratory work and expenses, it was decided to specifically analyze colonies in the range 4-20 mm, assuming that smaller colonies do not contain detectable levels of toxin, while bigger colonies were always scored as positive for AFB1 presence in section 3.6.

3.2. Modelling of *A. flavus* growth probability in pistachio agar under static temperature conditions

No growth was observed at 15 °C in any case after 42 days, thus the models were built without this temperature level.

Table 1. Coefficients ± standard errors for models developed at constant temperature levels.

	Growth model in PEA		**AFB1 model in PEA**		**Growth model in nuts**	
Inoculum type	**Single**	**Cocktail**	**Single**	**Cocktail**	**Single**	**Cocktail**
b_0	-1214.1±255.8	-552.6±69.5	-60.9±4.8	-61.2±4.9	-14.6±0.8	-40.0±6.1
B_1	94.8±20.1	43.5±5.5	2.3±0.2	2.3±0.2	0.5±0.0	2.9±0.6
B_2	-1.9±0.4	-0.9±0.1	ns	ns	ns	-0.1±0.0
B_3	4.1±0.8	1.7±0.2	0.5±0.0	0.5±0.0	0.1±0.0	0.2±0.0
Residual deviance	47.5	127.0	331.9	331.5	1121.3	1036.2
Null deviance	2002.7	2104.5	1992.9	1999.2	1790.5	1839.1

ns, not significant at p=0.05

3.2.1. Single isolate of A. flavus

All factors included in the probability model were significant (T, T^2, t, $p<0.01$, Table 1), with 99.6% concordance between observed and predicted values with a cut off of 0.5. The model shows an increasing delay in growth initiation with decreasing temperature, from about 5 days at 24-26 °C to about 34 days at 17 °C, although the increase in probability was similarly sharp at 17-24 °C (Fig. 1a, supplementary material). No growth was predicted before 40 days at 16 °C.

3.2.2 Cocktail inoculum

Similarly, when working with the 25 strains-based cocktail inoculum, all factors were significant (T, T^2, t, $p<0.01$, Table1), with 98.8% concordance between observed and predicted values with

a cut off of 0.5. Looking at the coefficients of both models (single and mixed inocula), they were significantly different at p=0.05. This second model showed slightly shorter delays in growth, mainly at the higher temperature levels, however, the time at which all plates exhibited growth (P=1) was similar, leading to probability curves with slightly smaller slopes. This may be due to the presence in the inoculum of faster growing isolates than our single one. No growth was predicted before 40 days at 16 °C (Fig. 1b, supplementary material).

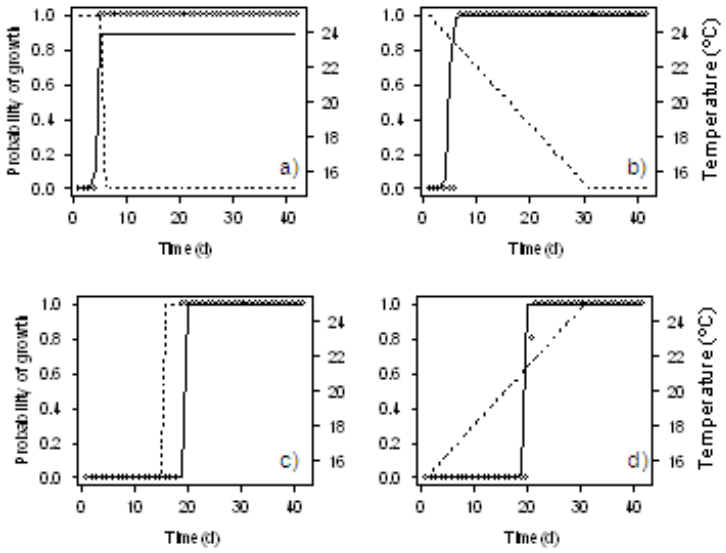

Figure 1. Observed growth probability of *A. flavus* TA-3.267 in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR

3.3. Modelling of *A. flavus* growth probability in pistachio agar under non-isothermal conditions

Probability of growth was calculated for non-isothermal profiles based on modeled probabilities at isothermal conditions, assuming no past accumulated temperature effect, as assumed in Koseki and Nonaka (2012) for *B. cereus* lag time. However, for increasing temperature profiles (US and UR), the model predicted growth 3-5 days before it was observed in non-isothermal experiments (data not shown). This suggests that a memory effect occurred. As an alternative, the R algorithm was modified and, instead of using the point prediction for the actual

temperature in the variable temperature profile, the mean temperature in the preceding 10 days was used for the prediction. On the other hand, under decreasing temperature profiles, decreasing probabilities were estimated over time as a result of decreasing temperatures and consequent no-growth prediction. To overcome this issue, and in order to obtain a model suitable to be applied to real situations, we forced the R algorithm to maintain the predicted value over time at the higher probability value reached. Taking this modification into account, the percentage of concordance was 100% for DS and UR profiles, and 98% for DR and US profiles (Fig.1). Interestingly, when the change of temperature was slow and held constant, the initiation of growth occurred sharply, in a range of 1-2 days, as it was with a sudden change in temperature.

Very similar results were observed for a cocktail inoculum. Although the observed values were slightly different, the initiation of growth occurred in the same days under non-isothermal conditions, and lasted for the same periods of time, thus the levels of concordance with the predicted values through the model developed under isothermal conditions were almost the same (100, 95, 98 and 98% for DS, DR, US and UR, respectively) (Fig.2).

3.4. Modelling of *A. flavus* growth probability in pistachio nuts under static temperature conditions

3.4.1. Single isolate of *A. flavus*

The logistic regression applied to binary data obtained in pistachio nuts showed that T and t were significant, but not T^2, thus this term was omitted from the model (Table 1). The resulting model showed 81% concordance between observed and predicted data with a cut off level of 0.5. The concordance is clearly lower than in agar as a result of a much more heterogeneous growth in pistachio nuts, and lower repeatability. When comparing this model for isolate 3.267 with that in agar, a higher variability in the initiation of growth was observed, evidenced by the smaller slopes in the Figure 2a (supplementary material), and by the fact that probability of 1 was rarely reached. On the other hand the fitted model overestimated the probability of growth during the first days, as growth was not observed till 6^{th}, 9^{th} and 18^{th} day at 25, 22.5 and 20 °C, while the model estimated probabilities of growth of 0.05-0.15 before these days.

3.4.2. Cocktail inoculum

When a cocktail inoculum was used, all factors were significant (Table 1), with a percentage of concordance of 83%. When comparing the confidence intervals of the estimated coefficients for the two inocula, it was clear that both models were different, thus the inclusion of more strains in the inoculum led to a different overall behavior. In this case higher slopes in the probability curves were observed compared to the single inoculum (Fig.2b, supplementary material), with higher probabilities of growth from the beginning, suggesting that some faster isolates among the 25 might led the behavior of the combined inoculum.

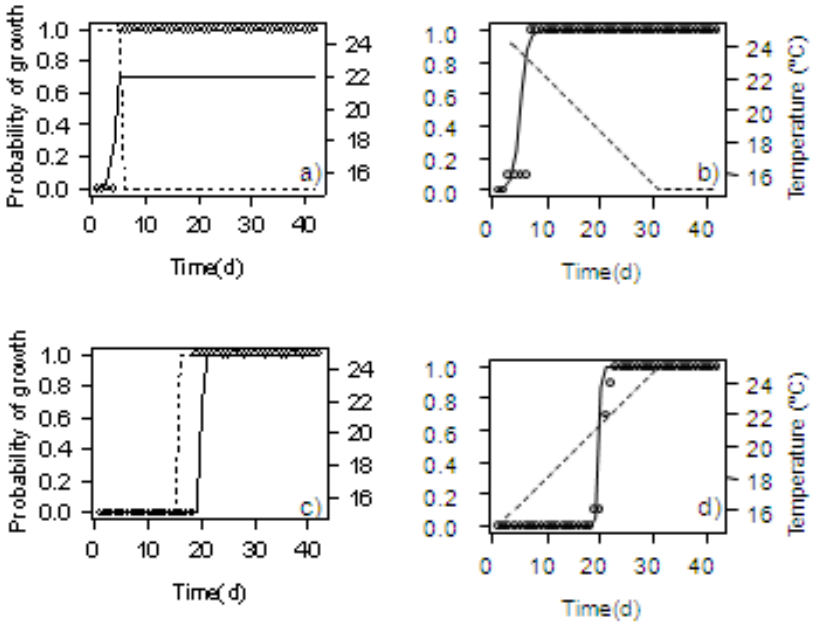

Fig.ure 2. Observed growth probability of *A. flavus* mixed inoculum in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

In conclusion, in spite of the overestimated predicted probability in the first days for pistachio nuts, the probability of growth was clearly lower in pistachios than in agar. The reason was likely the dramatic decrease in a_w in some of the treatments. While a_w in the agar plates was periodically checked and it was nearly constant, for the pistachio nuts a marked decrease

occurred both under constant and variable temperature profiles, except at 15 °C (Fig. 3). Previous studies used the same experimental design but placing glycerol-water solutions in the beakers instead of water; thus in the present work the conditions were less favorable to dehydration. However, the low initial a_w value chosen here, 0.87 a_w, evidenced the limitations of the experimental set up to maintain the a_w value at low levels. According to the sorption curve of pistachio nuts published in Marin et al. (2008), while a decrease in moisture content from 50 to 18% involves a decrease in a_w from 0.99 to 0.90 a_w, a loss of moisture content as small as 8% (from 18 to 10%) implies a decrease of a_w from 0.90 to 0.80. Thus the shape of the sorption curve determines the higher degree of dehydration, due to warm incubation temperature, when the initial a_w is under 0.90 a_w.

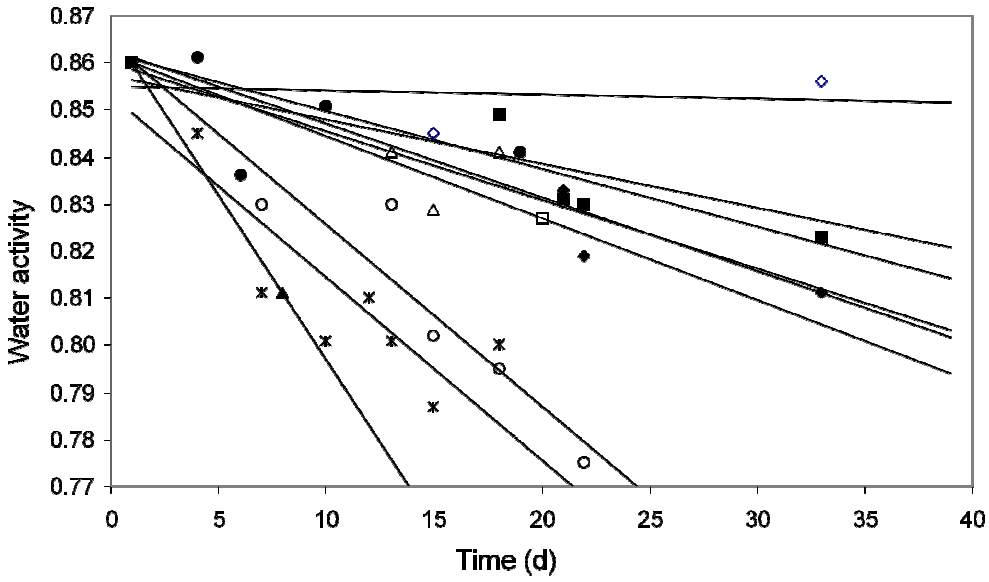

Figure 3. Checking of a_w values during incubation of the different treatments in pistachio nuts.

3.5. Modelling of *A .flavus* growth probability in pistachio nuts under non-isothermal conditions

The same assumptions than for non-isothermal predictions in agar were applied here. For the single inoculum, under ascending temperature profiles a good prediction was observed (93% and 100% concordance for US and UR, respectively) (Fig. 4). For descending temperature profiles, the predicted probabilities of growth were always under 0.2, while observed values for

continuously decreasing temperature were always under 0.3 (100% concordance). However, in the step descending profile, the observed probability reached values over 0.5 after 35 days, leading to a decreased concordance level (81%).

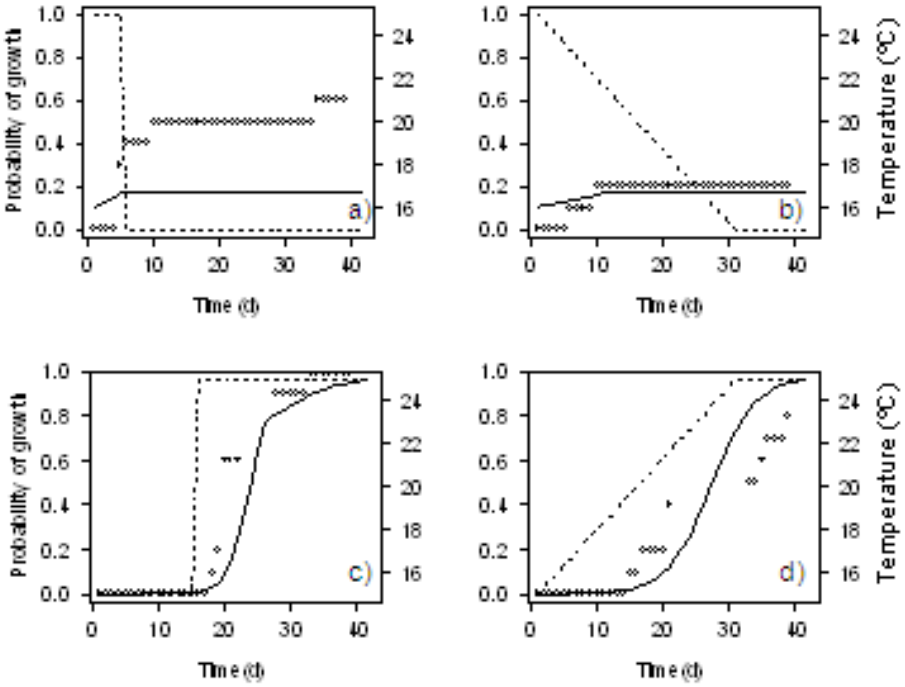

Figure 4. Observed growth probability of *A. flavus* TA-3.267 in pistachio nuts under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

For the cocktail inoculum, the concordance was similar, 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. 5). In both inoculum types a lower slope of the probability curve was observed under increasing temperature levels when the increase was slow.

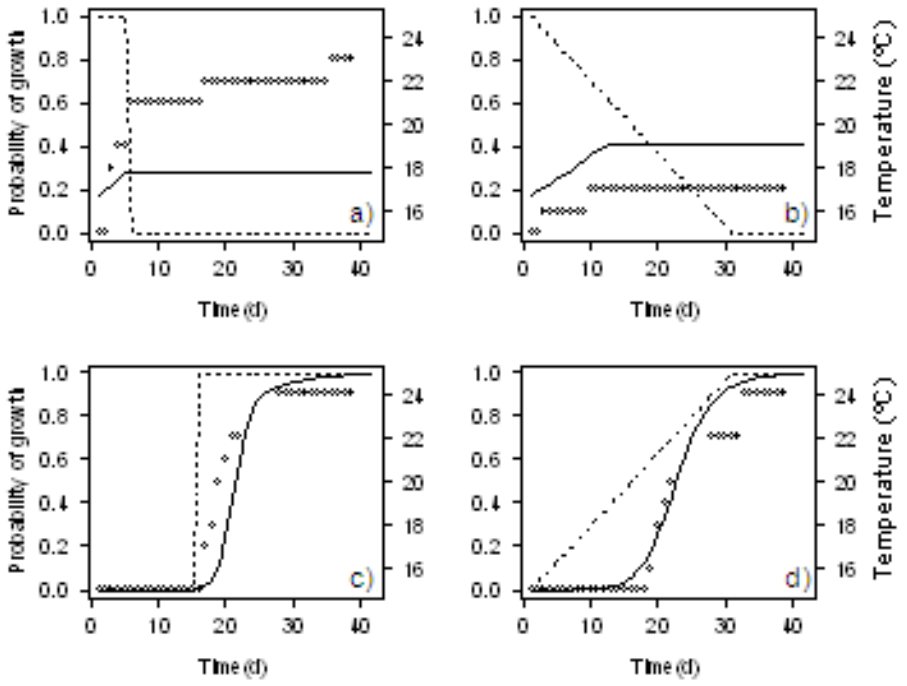

Figure. 5. Observed growth probability of *A. flavus* mixed inoculum in pistachio nuts under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

When comparing with non-isothermal agar data, it was observed that the initiation of growth occurred at a similar time point; 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.

3.6. Modelling of *A. flavus* AFB1 production probability in pistachio agar under static temperature conditions

3.6.1. Single isolate of A. flavus

The squared term for temperature was not significant according to the logistic regression model (Table 1). The logistic model for prediction of toxin accumulation showed that less than 0.2 probability of AFB1 production would be expected at <18 °C for 40 days. While AFB1 production was probably overestimated in the first days at 26 °C, it would start as early as about 2 days at 24 °C, with probability over 0.5 at this temperature before 15 days. The probability curves at the different temperatures were quite parallel, suggesting that although the initiation of production was delayed by decreasing temperatures, the shift from 0 to 1 probability occurred in about 20 days, regardless of the temperature level (Fig. 3, supplementary material). In this case the concordance between observed and predicted values was of 98.6%; the discrepancies occurred at 22.5 and 25 °C during the 4-6 days around the transition from non-production to production.

3.6.2. Cocktail inoculum

For the cocktail inoculum, T^2 was neither significant (Table 1) and a 95.6% concordance between observed and predicted values was obtained. The non-concordant values occurred at 22.5 and 25 °C during the days around the transition from 0% production to 100% production. The predicted probabilities were very similar to those for the single inoculum, and looking at the confidence intervals of the coefficients of both models, they were not significantly different (Fig. 3, supplementary material).

3.7. Modelling of *A.flavus* AFB1 production probability in pistachio agar under non-isothermal conditions

AFB1 production under decreasing temperature profiles was only detected in a reduced number of plates in the first days. After that, growth of colonies stopped and so did the toxin production, thus no additional AFB1 positive plates were recorded. In these profiles, the AFB1 positive cases were delayed compared to growth-positive ones, and the attained probability was lower. For the step increase profile, no positive plate was detected till day 23, but in the 26^{th} day probability of 1 was reached; by contrast, the shift from 0 to 1 probability of growth occurred after 18-19 days. Finally, different situations were observed in the continuously increasing

profile, where AFB1 was not detected with the single inoculum, but with the cocktail inoculum reached probability 1 after 29 days; however, the growth profiles were similar in both cases: the shift occurred between 19-23 days in the cocktail inoculum and from 21-22 days in the single one (Fig. 3, supplementary material).

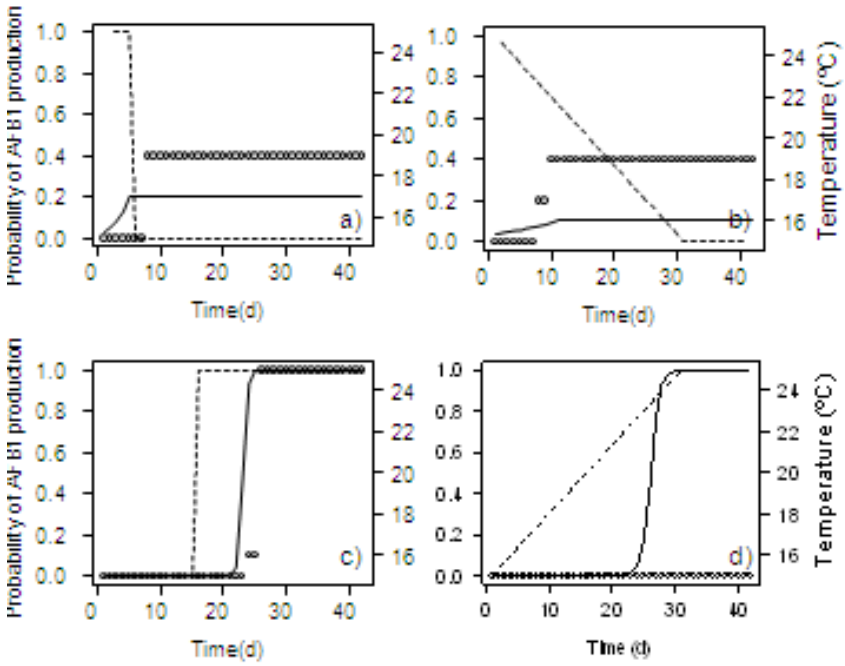

Figure. 6. Observed AFB1 production probability of *A. flavus* TA-3.267 in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

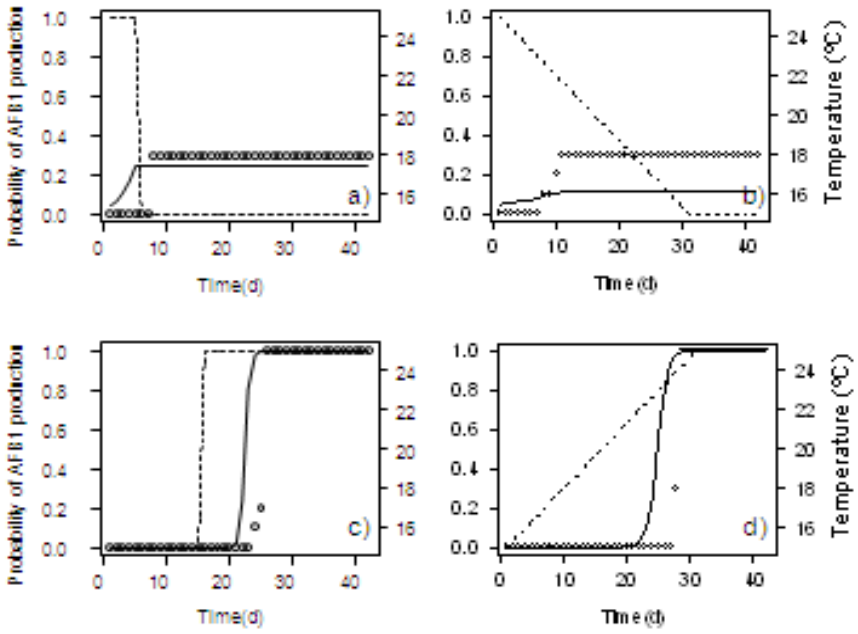

Figure. 7. Observed AFB1 production probability of *A. flavus* mixed inoculum in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

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.

Once the correction was included in the algorithm, there was not a clear improvement for the prediction under decreasing temperature profiles, while an improvement was observed under increasing temperatures (Fig. 6 and 7), in particular at the step increase for which the level of concordance increased from 76 to 95%, using the single inoculum. Using the modified algorithm

the levels of concordance for the cocktail inoculum were 100, 100, 92.9 and 90.5% for DS, DR, US, and UR, respectively, with a cut off of 0.5.

Table 2. Observed detected AFB1 presence (>LOD) in pistachio nuts under different time/temperature conditions and predicted probability values through growth models in pistachio agar and nuts, and AFB1 model in agar under the same conditions. Experiments carried out using a single inoculum of *A. flavus* 3.267.

Condition	Mean colony diameter	AFB1 presence (>LOD)	Predicted P from growth model in agar	Predicted P from growth model in pistachio	Predicted P from AFB1 model in agar	Predicted P from AFB1 model in agar (cocktail)
6d/DS	5.5	-	0.89	0.17	0.20	0.25
6d/25 °C	5.5	-	1.00	0.19	0.29	0.35
6d/25 °C	14	-	1.00	0.19	0.29	0.35
6d/CD	5	-	0.99	0.13	0.06	0.07
6d/DS	4.5	-	0.89	0.17	0.20	0.25
6d/DS	5.5	-	0.89	0.17	0.20	0.25
6d/DS	7	-	0.89	0.17	0.20	0.25
7d/25 °C	6	-	1.00	0.21	0.40	0.47
8d/22.5 °C	5	-	0.68	0.08	0.00	0.00
13d/25 °C	11	-	1.00	0.37	0.93	0.94
13d/22.5 °C	5.5	-	1.00	0.15	0.04	0.05
18d/20 °C	5	-	0.99	0.09	0.00	0.00
18d/UR	11.5	+	0.00	0.06	0.00	0.00
21d/22.5 °C	6	+	1.00	0.33	0.70	0.73
21d/US	8.5	-	1.00	0.15	0.02	0.02
21d/US	5	-	1.00	0.15	0.02	0.02
21d/US	9	-	1.00	0.15	0.02	0.02
21d/US	12	-	1.00	0.15	0.02	0.02
21d/UR	5.5	-	1.00	0.13	0.01	0.01
21d/UR	8.5	-	1.00	0.13	0.01	0.01
28d/US	15	+	1.00	0.81	1.00	1.00
32d/25 °C	14	-	1.00	0.88	1.00	1.00
33d/17.5 °C	7.5	-	1.00	0.17	0.01	0.01
33d/20 °C	11	-	1.00	0.42	0.74	0.75
33d/20 °C	17	+	1.00	0.42	0.74	0.75
33d/US	20	+	1.00	0.89	1.00	1.00
34d/UR	8	-	1.00	0.86	1.00	1.00
Concordance observed/predicted			15%	81%	81%	81%

Table 3. Observed detected AFB1 presence (>LOD) in pistachio nuts under different time/temperature conditions and predicted probability values through growth models in pistachio agar and nuts, and AFB1 model in agar under the same conditions. Experiments carried out using a single a mixed inoculum of 25 isolates.

Condition	Mean colony diameter	AFB1 presence (>LOD)	Predicted P from growth model in agar	Predicted P from growth model in pistachio	Predicted P from AFB1 model in agar
4d/25 °C	5.5	-	0.29	0.25	0.17
4d/DS	3.5	-	0.29	0.25	0.17
4d/DS	7	-	0.29	0.25	0.17
6d/25 °C	5	-	0.93	0.32	0.35
6d/DS	7	-	0.70	0.28	0.25
8d/DR	5	-	0.99	0.32	0.09
9d/25 °C	10.5	-	1.00	0.42	0.70
11d/DS	7.5	+	0.70	0.28	0.25
13d/20 °C	6.5	-	0.00	0.13	0.00
13d/20 °C	7	-	0.00	0.13	0.00
13d/20 °C	5	-	0.00	0.13	0.00
13d/22.5 °C	6.5	-	1.00	0.39	0.05
13d/22.5 °C	7.7	-	1.00	0.39	0.05
13d/25 °C	6	-	1.00	0.58	0.94
13d/25 °C	10	-	1.00	0.58	0.94
15d/22.5 °C	5	-	1.00	0.46	0.12
18d/22.5 °C	10	-	1.00	0.58	0.38
18d/25 °C	12	-	1.00	0.75	0.99
19d/US	10.5	-	0.00	0.11	0.00
19d/US	5	-	0.00	0.11	0.00
21d/US	12	-	1.00	0.82	0.02
21d/US	15.5	-	1.00	0.41	0.02
21d/UR	8	-	1.00	0.41	0.01
22d/US	17	+	1.00	0.34	0.02
28d/UR	14	+	1.00	0.59	0.98
28d/UR	14.5	-	1.00	0.86	0.98
33d/20 °C	13.5	-	1.00	0.86	0.75
33d/25 °C	20.5	+	1.00	0.77	1.00
33d/US	17	-	1.00	0.97	1.00
34d/UR	5.5	-	1.00	0.97	1.00
Concordance observed/predicted			40%	70%	67%

3.8. Validation of the obtained models for prediction of AFB1 data obtained from pistachio nuts

The results showed that the prediction of growth in pistachio agar differed from the detected toxin, which were only concordant in 15/40% of the cases for single and mixed inoculum, respectively (mostly, false positives) (Tables 2 and 3). Moreover, 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 81 and 70% of concordant cases in the single and cocktail inoculum, respectively (although both false negatives and false positives were observed, in the mixed inoculum most of them were false positives, in concordance with a narrower set of conditions allowing AFB1 production than growth). Finally, the concordance between probabilities predicted for AFB1 presence in pistachio agar and observations in pistachio nuts was of 81 and 67%, for single and cocktail inoculum, respectively. 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 18^{th}, 21^{st} and 34^{th} days. This suggests that colony diameters in pistachio nuts are quite variable, and a good correlation with time may not be possible. As a result, the prediction of AFB1 along time may also be inaccurate. As an alternative, both time and colony sizes could be included as model terms.

Moreover, looking at the prediction of the observed toxin production by the single inoculum in nuts, using the model for AFB1 production developed in agar with the cocktail inoculum, the level of concordance was the same (81%) as when the model was developed for the single inoculum. This suggests that the cocktail inoculum would represent the behavior of this particular single isolate.

4. Discussion

According to the Transport Information Service of the Federation of the German Insurance Association (2014), the travel temperature of 0 °C is the ideal temperature for achieving the longest possible storage life, but higher travel temperatures (5-25 °C) are feasible (depending upon the duration of the voyage), so this product need not necessarily be carried as chilled goods, as long as ventilated containers are used. This German Federation recommends initial moisture content (mc) of 4-6% for safe travel, however, in the present work mc was initially adjusted to a somewhat risky value of 13% mc, equivalent to 0.87 a_w, which would allow *A. flavus* development but far away from its optimum. Focusing just in this single low a_w level, led as to realize that, while the classical methodological approach of initially adjusting a_w values and consider them constant for the whole duration of experiments was good for the agar experiments, it was not for nut ones where although water beakers were included in the closed containers, a_w decreased with time at temperature regimes >15 °C. Unfortunately, this decrease in a_w does not probably occur during real bulk transport, although constant a_w values are neither expected. As fluctuations in the a_w levels are expected as a result of temperature fluctuations, for further development of models it would be important to characterize the a_w variation as a function of temperature in bulk pistachio nuts. Previous models have been published on *A. flavus* growth, mostly kinetic models, including in general a_w levels in the range 0.80-0.99, where data were produced in agar media, except for some works in maize (Samapundo et al., 2007; Yue et al., 2013) and rice (Mousa et al., 2013, 2011)), and the minimum a_w for growth has been reported around 0.82. Similarly, minimum a_w for AF production has been reported at 0.82-0.86 in rice (Mousa et al., 2013, 2011). AF production has been rarely included in such models, due to the complexity and cost of building primary models. There are no additional existing works on the single effect of temperature at a constant a_w level.

4.1. Model building under isothermal conditions

Our results on growth probabilities were concordant in general with other studies performed on mycelial growth of *A. flavus* (Astoreca et al., 2012; Marín et al., 2012; Moghadam and Hokmabadi, 2010; Mousa et al., 2013). Probabilistic models reporting mould growth or mycotoxin production are scarce, both under constant and dynamic conditions. In 2001, the first one was published, using the logistic regression to develop predictive model to predict the

probability of growth of *Aspergillus niger* and *Penicillium spinulosum* in response to different factors (Battey et al., 2001). Subsequently, other authors applied them to *A. flavus* (Astoreca et al., 2012; Marín et al., 2009), but none included dynamic conditions. An observation made from our data is that due to the symmetrical shape of the logistic model, when conditions are less conducive to growth, and thus the slope of the probability curve is smaller, there is an overestimation of the probability of growth during the earlier days of incubation, as in those days no growth was observed, but the predicted probability did not overtake a 0.20 value.

4.2. Impact of single/mixed inocula in models

The work was designed to predict the behavior of *A. flavus* in a representative manner through the use of a cocktail inoculum including 25 isolates. Additionally, a single inoculum with an isolate taken at random was included in order to have an additional repetition of the temperature experiment and, at the same time to get some confirmation of the conclusions in Garcia et al. (2014). Certainly, the results showed an earlier initiation of growth in the mixed inoculum, although both inocula reached probability 1 in the same time period in agar, while in nuts the single inoculum showed delayed probability curves from the beginning to the end of the incubation period. Thus the growth probability models were significantly different for the two inocula but, interestingly, there was no significant difference among the AFB1 probability models. This point must be highlighted as this could imply that although the impact of intraspecific differences is known to be much higher in the level of AF produced than on growth, the T boundary for toxin production may be more repeatable along individual strains. No previous knowledge exists regarding this point. On the other hand, the observed growth/AFB1 production probabilities for both inocula under non-isothermal conditions were very similar.

4.3. Predicting *A. flavus* growth and AFB1 production under non-isothermal conditions

Many studies have been carried out under fluctuating temperature for bacterial pathogens. Gompertz, logistic and Baranyi models have been used considering that under non-isothermal conditions the momentary growth rate is the isothermal growth rate at the momentary temperature at a time that corresponds to its instantaneous population size (Corradini and Peleg, 2005). As a result, besides temperature, the parameters become also a function of time. Consequently, the integral in the growth equation cannot be solved analytically, but numerically

(Runge-Kutta 4th order method) to produce the growth curve. Instead of integrating conventional models continuously, in the case of alternating constant temperatures, the models can be applied piecemeal (Koutsoumanis, 2001). It is assumed that the bacterial growth rate instantaneously takes the corresponding value for the changing temperature levels. While the past history of the population since its introduction in the growth medium was considered irrelevant by Corradini and Peleg (2005), Juneja et al. (2009) working with *Clostridium perfringens* required the inclusion of a 'memory parameter' in their standard model for acceptable predictions in cooked ground chicken. In our case, when the models were applied piecemeal to the non-isothermal situation, delayed predicted values were observed under decreasing temperature profiles, while earlier growth was predicted under increasing temperature profiles. The issue was solved by assigning to each temperature level in the non-isothermal profiles the mean of that temperature and those in the 9 preceding days. Memory effect was much more important for toxin production, suggesting that it requires more complex metabolic adaptation than growth does.

Four different hypothetic temperature profiles were proposed as a starting point for this research, including increasing and decreasing temperature situations, and shift and ramp temperature variations. In fact, it is traditional procedure in process engineering to use shifts or ramps to identify model parameters such as induced dead, or lag times of first order processes. Temperatures in the range 15-25 °C were included, which may be consistent with the levels that may occur during unrefrigerated shipping for an extended period of time. The final aim is to provide a tool which, for any fluctuating temperature profile derived from a temperature data logger located in a silo, storage room or container, provides a prediction on the risk probability. The results showed a good agreement between the observed values and the predicted ones based on the isothermal model (93-100%), with the exception of the DS profile in the model developed in pistachio nuts for which low probability of growth was predicted, while growth in fact occurred. As this occurred in nuts but not in agar, one possible reason could be that at the initial temperature in the profile, a clear dehydration would be expected, and then little increase in probability is expected in the long term from the isothermal model. However, under the variable profile, the temperature shifted to 15 °C in the 5th day, preventing partially from

dehydration (Fig. 5), and allowing for a further increase in probability in the spores that probably germinated during the 5 days at 25 °C (note that no growth was observed at isothermal 15 °C).
On the other hand, the slopes of the probability curves observed with abrupt temperature changes were slightly higher than those observed when the temperature changes were smooth. Moreover, in the real situations smooth temperature changes, where prediction performance seems to be better, are expected rather than abrupt ones from growth to no growth conditions.

Pioneer studies on modeling germination and growth of *P. expansum* and *A. niger* under fluctuating temperature conditions have been recently published by Gougouli et al (2010, 2012). The assumptions were: a temperature shift does not result in an additional lag, after a shift the germination and growth rates adapt instantaneously to the new temperature. Although a memory factor was not applied in any case, the germination function was recalculated taking into account the remaining %germination to reach 100%, thus a new germination rate was calculated which took into account the preceding situation. Probability of growth, as modeled in our study, is affected by germination kinetics and reflects mainly the end of the germination step at the population level, as once the %germination in a population of spores approaches 100%, the first signs of hyphal growth become visible.
Gougouli et al. (2010) indicated that during storage at a temperature below the minimum temperature for growth no lag time was consumed. This point was confirmed in our work in the US profile when memory effect was not taken into account, where even the observed initiation of growth was delayed compared to the predicted one. Once memory correction was applied such delay disappeared, confirming Gougouli et al. (2010) hypothesis: instead of consuming lag time, the time under no-growth conditions delayed the initiation of growth once conditions conducive for growth were achieved.

4.4. The impact of the media and variables used for data generation in model validation

Generating data from agar experiments can be much easier and cheaper, and also growth measurements are less costly than AF analysis. It can be inferred that as soon as fungal growth becomes visible there is some probability of finding mycotoxins in the foodstuff. In fact, from our preliminary experiment it was shown that colonies as small as 5 mm of diameter may contain <LOD-20.5 μg/kg of AFB1 depending on the condition. The European Union has

determined the maximum residue limit of AFB1 to be 8 µg/kg in pistachios (EC Regulation 165/ 2010), thus there is not much room to allow for fungal growth till risky AF levels are reached.

Rather to generate data for model building in pistachio nuts, two alternatives were envisaged: the first one, generating AF data in pistachio agar medium, the second, generating growth data in nuts (instead of AF data, much simple) and then assume that the conditions which prevent growth also prevent toxin accumulation. The first option should lead to a narrower set of conditions. Looking at tables 2 and 3, however, similar agreement was observed in both cases. The agreement with the model developed for AFB1 data in agar confirms that, similarly to what reported in Marin et al. (2012), the boundaries for growth and AF production are similar, although this point contrasts with the general agreement that toxin production conditions are narrower than those for growth. The difference might be the long duration of our experiments, leading to accounting for delayed toxin production. In this case, no deviations are expected derived from methodological issues, as the decreased a_w levels occurred in both cases as both data were obtained from the same experiment in pistachio nuts. When using AFB1 data in agar to predict AFB1 probability in nuts, the non-concordant values were, in general, due to overestimated probability, thus the model was fail-safe. Such overestimation can be tentatively attributed to the different a_w levels in both cases; while the initial level was the same, in pistachio nuts it decreased over time, but not in agar.

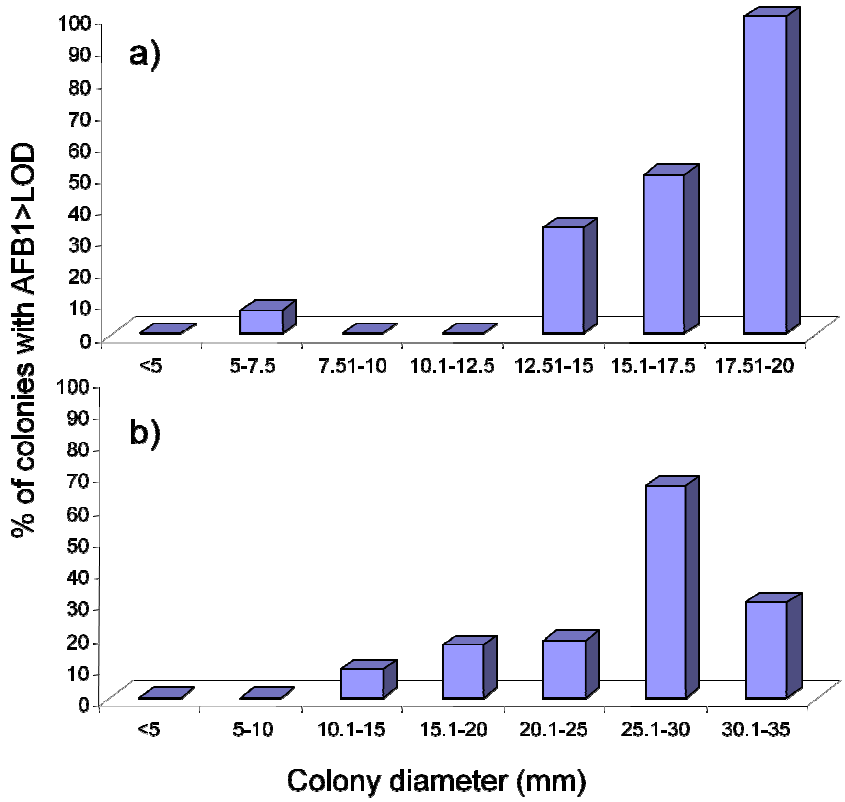

Figure. 8. Percentage of AFB1 positive *A. flavus* colonies as affected by colony size. a) Isolate TA-3.267; b) cocktail inoculum.

From the 57 single AFB1 data obtained for validation in pistachio nuts, it was clearly observed that, although there was a rough relationship between toxin presence and colony size, the relationship between time and toxin was weak (Fig. 11), as depending on the temperature conditions long time periods were required to attain significant colony sizes, likely to accumulate AFB1. For this reason, after 25 days, there were still a number of small size colonies which were AFB1-negative (more than 33%). This suggests that, besides time and environmental factors, including in mycotoxin models a parameter related to colony size would help. Mixed-growth associated models have been recently applied to mycotoxin production (Abdel-hadi et al., 2012; Garcia et al., 2013; Medina et al., 2007). Similarly, Baert et al. (2007) previously developed a model for patulin accumulation including colony surface of *P. expansum* as a term of the model.

4.5 Conclusions

In this work we have generated an R-script that for any temperature profile in an spreadsheet file or text file that is loaded, produces the probability plot for AFB1 along the given time period (also numerically). Obviously, at this moment it can only be applied to lots with initial a_w of 0.87, which is unrealistic, if they are correctly dried, and no condensation due to changes in temperature occur. On the other hand, the use of a cocktail inoculum for data generation seems sound. There is a need to refine it, in particular, solving the variable a_w issue; the objective may not be predicting probabilities at a constant level of a_w, but taking into account its fluctuation along time as a function of the initial a_w itself and of temperature variation that may occur in bulk pistachios.

The application of this tool would allow support decision, at storage level, on the timing for ventilation or use of stored raw materials, or even on the final use given to them. At the transport level, it would enable to decide whether refrigerated transport is required or not, depending on the international routes, as well as complement (or substitute) the control analyses at the destination ports. It is well known that sampling plans for control of heterogeneously distributed contaminants, such as mycotoxins, are costly and the results obtained are not always totally reliable (García-Cela et al., 2013), thus a prediction based on data loggers inserted in the containers would give an additional information on the safety of the shipping operation (assuming that there is no unacceptable contamination from origin).

Finally, two assumptions are implicit in our approach: the presence of aflatoxigenic strains in stored/transported batches (this is highly expectable, thus the prediction should not be much affected), and the absence of insects and other pests which may interact with AF producers (if this is the case the predictions may be compromised).

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