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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.

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).

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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. In addition, the conditions favourable 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 behaviour of a wide range of strains to account for the intraspecific variability. Besides, the use of cocktails of strains to forecast the behaviour of a species has been proposed by some authors (Hocking and Miscamble, 1995; Patriarca et al., 2001; Romero et al., 2007; Garcia 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, García et al., 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) 1987), 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 (European Commission 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. 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. 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 desirable 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/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

Twenty-five isolates of *A. flavus* were used 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