Predictive mycology as a tool for controlling and preventing the aflatoxin risk in postharvest

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TESI DOCTORAL

Predictive mycology as a tool for controlling and preventing the aflatoxin risk in postharvest

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"Caminante no hay camino, se hace camino al andar"

Antonio Machado

"Es una verdad muy cierta que, cuando no esté a nuestro alcance determinar lo que es verdad, deberemos seguir lo que es más probable"

Descartes

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¡GRACIAS A TODOS!

A mi familia

Summary

Aflatoxins are potent carcinogens that pose a significant threat to human health. Incidence of these mycotoxins in foodstuffs is high, thus their control and prevention is mandatory in the food industry. The development of appropriate predictive models that allow us to predict fungal growth and mycotoxin production will be a valuable tool to monitor, predict and prevent the mycotoxin risk. To develop accurate predictive models it is important to account for the real conditions that we will encounter through the food chain. Such conditions include: suboptimal conditions for growth and mycotoxin production, even distribution of spores across the food matrix, presence of different strains of the same species or dynamic environmental conditions.

Given the scope and complexity of the problem there is a particular need to develop predictive tools that can help to manage mycotoxins in foodstuffs during transport and storage.

In the present work, it has been concluded that performance of predictive models may be compromised under marginal conditions for growth, where more variability on the results is expected. The use of probabilistic models under such conditions should account for spore's ability to initiate growth, since it was demonstrated that not all spores were able to initiate growth.

The presence of different strains of a species in a same niche is common. Predictive models developed for the different isolates studied revealed that growth and aflatoxin B1 (AFB1) production may differ among strains, overall under marginal conditions. *A. flavus* isolates showed much more variability in probability of AFB1 production compared to growth. The amount of AFB1 produced was highly variable among isolates in all the studies conducted, ranging from 9 up to 6000 ppb under the same conditions.

Great effect of inoculum size on fungal behavior has been proven throughout this thesis. Initiation of growth and toxin production was greatly delayed (up to 9 days, depending on the environmental condition) changing from a multispore inoculum (500 spores) to a single spore one. Besides, growth was stimulated by the presence of more spores in the inoculum. Nevertheless, AFB1 production was either stimulated or inhibited depending on the conditions and strains, without a clear trend.

In addition, three studies under dynamic environmental conditions were conducted.

Different approaches were envisaged including polynomial/probabilistic functions that included temperature and a_w profiles. Predictive models have been proven to properly represent the growth and AFB1 production in almost all cases, with concordances between 70-100% for nearly all profiles. Finally, validation of the dynamic predictive models on pistachio nuts and maize grains, showed the applicability of growth models generated at constant temperature either on agar media or food matrices, as long as a_w is included as a variable in the models.

The present work provides the basis for scientifically proven models, which can be applied in the food industry in order to improve postharvest control of commodities.

Resumen

Las aflatoxinas son potentes carcinógenos que representan una amenaza significativa para la salud humana. La incidencia de estas micotoxinas en los alimentos es alta, por lo que su control y prevención es obligatoria en la industria alimentaria. El desarrollo de modelos predictivos apropiados que nos permitan predecir el crecimiento fúngico y la producción de micotoxinas es de gran utilidad como herramienta para controlar, predecir y prevenir el riesgo de micotoxinas en alimentos. Es importante que los modelos predictivos sean capaces de explicar las condiciones ambientales que se encuentran a lo largo de la cadena alimentaria. Entre tales condiciones encontramos: condiciones subóptimas para el crecimiento y producción de micotoxinas, distribución aleatoria de esporas fúngicas en el alimento, presencia de diferentes cepas de la misma especie o condiciones ambientales dinámicas.

Dado el alcance y la complejidad del problema, existe una necesidad de desarrollar herramientas predictivas que puedan ayudar a controlar las micotoxinas en los alimentos, y que sirvan de ayuda durante el transporte y el almacenamiento.

En este trabajo se ha demostrado que la capacidad predictiva de los modelos puede verse comprometida cuando las condiciones experimentales son marginales, tanto para el crecimiento como para la producción de micotoxinas, ya que en estas condiciones se espera mayor variabilidad en los resultados. Además, los modelos probabilísticos deben ser capaces de explicar la habilidad de iniciar el crecimiento de las esporas presentes en el sustrato, ya que ha quedado demostrado que no todas las esporas inoculadas fueron capaces de dar lugar a colonias en condiciones subóptimas.

La presencia de diferentes cepas de una especie en un mismo nicho es común. Los modelos predictivos desarrollados para las diferentes cepas estudiadas revelaron que el crecimiento y la producción de AFB1 pueden variar entre las cepas, sobre todo a medida que las condiciones ambientales se vuelven más marginales. Se ha observado una mayor variabilidad entre cepas para la probabilidad de producción de aflatoxina B1 (AFB1) que para el crecimiento. La cantidad de AFB1 producida fue muy variable entre las distintas cepas, oscilando entre 9 y 6000 ppb para las mismas condiciones.

En esta tesis ha quedado demostrado el gran efecto que tiene la concentración de esporas del inóculo inicial tanto en el crecimiento como en la producción de AFB1. Así el inicio del

crecimiento como el de la producción de AFB1 se vio retrasado hasta 9 días cuando se pasó de un inóculo concentrado (500 esporas) a una sola espora. En cuanto a la cantidad de AFB1 producida, no se encontró un patrón claro relacionado con la concentración de inóculo, ya que en ocasiones se estimuló la producción y en otras se vio inhibida.

Además, se realizaron tres estudios bajo condiciones ambientales dinámicas. Se llevaron a cabo varios enfoques incluyendo funciones polinómicas/probabilísticas donde se introdujeron diferentes perfiles de temperatura y a_w. Los modelos predictivos obtenidos demostraron ser capaces de predecir tanto el crecimiento como la producción de toxina, con niveles de concordancia entre 70-100% para casi todos los perfiles. Finamente, la validación de dichos modelos predictivos dinámicos en pistachos y granos de maíz, mostró la aplicabilidad de los mismos para predecir el crecimiento y la producción de AFB1, siempre que se incluya la a_w como variable en el modelo.

El presente trabajo proporciona una base para el desarrollo de modelos científicamente probados, que pueden ser aplicados por la industria alimentaria para mejorar el control de micotoxinas en postcosecha.

Resum

Les aflatoxines són potents carcinògens que representen una amenaça significativa per a la salut humana. La incidència d'aquestes micotoxines en els aliments és alta, de manera que el seu control i prevenció són necessaris en la indústria alimentària. El desenvolupament de models predictius apropiats que ens permetin predir el creixement fúngic i la producció de micotoxines és de gran utilitat com a eina per controlar, predir i prevenir el risc de micotoxines en aliments. És important que els models predictius siguin capaços d'explicar les condicions ambientals que es troben al llarg de la cadena alimentària. Entre aquestes condicions trobem: condicions subòptimes per al creixement i producció de micotoxines, distribució aleatòria d'espores en l'aliment, presència de diferents soques de la mateixa espècie o condicions ambientals canviants.

Donat l'abast i la complexitat del problema, hi ha una necessitat de desenvolupar eines predictives que puguin ajudar a controlar les micotoxines en els aliments, i que serveixin d'ajuda durant el transport i l'emmagatzematge.

En aquest treball s'ha demostrat que la capacitat predictiva dels models es pot veure compromesa quan les condicions experimentals són marginals, tant per al creixement com per a la producció de micotoxines; en aquestes condicions s'espera major variabilitat en els resultats. A més els models probabilístics han de ser capaços d'explicar la capacitat d'iniciar el creixement de les espores fúngiques presents en el substrat, ja que ha quedat demostrat que no totes les espores inoculades van ser capaces de donar lloc a colònies en condicions subòptimes.

La presència de diferents soques d'una espècie en un mateix nínxol és un tret comú. Els models predictius desenvolupats per a les diferents soques estudiades van revelar que el creixement i la producció de aflatoxina B1 (AFB1) poden variar entre les soques, sobretot a mesura que les condicions ambientals es tornen més marginals. S'ha observat una major variabilitat entre soques per a la probabilitat de producció de AFB1 que per al creixement. La quantitat de AFB1 produïda va ser molt variable entre les diferents soques, oscil·lant entre 9 i 6000 ppb per a unes mateixes condicions.

En aquesta tesi ha quedat demostrat el gran efecte que té la concentració d'espores de l'inòcul tant en el creixement com en la producció de AFB1. Tant l'inici del creixement com l'inici de producció de AFB1 es va veure retardat fins a 9 dies quan es va passar d'un inòcul

concentrat (500 espores) a una sola espora. Pel que fa a la quantitat de AFB1 produïda, no es va trobar un patró clar relacionat amb la concentració d'inòcul, ja que en ocasions es va estimular la producció i en altres es va veure inhibida.

A més, es van realitzar tres estudis sota condicions ambientals canviants. Es van dur a terme diversos enfocaments incloent funcions polinòmiques / probabilístiques on es van introduir diferents perfils de temperatura i a_w. Els models predictius obtinguts van demostrar ser capaços de predir tant el creixement com la producció de toxina, amb nivells de concordança entre 70-100% per a gairebé tots els perfils. Finament, la validació d'aquests models predictius dinàmics en pistatxos i grans de blat de moro, va mostrar l'aplicabilitat dels mateixos per predir el creixement i la producció de AFB1, sempre que s'inclogui l'a_w com a variable en el model.

El present treball proporciona una base per al desenvolupament de models científicament provats, que poden ser aplicats per la indústria alimentària per millorar el control en postcollita.

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LIST OF ABBREVIATIONS

μ, maximum growth rate

λ, estimated apparent lag time for growth

AFB1, aflatoxin B1

a_w, water activity.

P_G, probability of growth

P_{AF}, probability of AFB1 production initiation

R_0, initial radius

R_t, radius at time t

T, temperature (°C)

t, time

t10, estimated time to reach 0.10 probability

t50, estimated time to reach 0.50 probability

t90, estimated time to reach 0.90 probability

t100, estimated time to reach 1 probability

$t_{g,}$ Geometrical germination time

tvg, time to visible growth

Introduction

1. Mycotoxins in agricultural products: an overview

The contamination of agricultural products by fungi is often an additive process, which begins in the field and increases during harvest, drying and storage (CAST, 2003). Fungal infection entails many disadvantages such as grain yield losses or decreasing the processing and nutritional quality of the grain (Christensen and Kaufmann, 1969). However, the losses incurred as a result of fungal growth are not only economic but also are of public and animal health concern due to the possible production of mycotoxins.

Mycotoxins are secondary metabolites produced naturally by about 200 recognized filamentous fungi growing under a wide range of climatic conditions on different substrates (Atanda et al., 2011; Binder et al., 2007). Pitt (1996) defined mycotoxins as "fungal metabolites that when ingested, inhaled or absorbed through the skin cause illness to human and animal death". Contamination of foodstuffs with mycotoxins is of concern both pre- and post-harvest and can represent a great risk for human and animal health. Up to now, it has been documented that approximately 400 secondary metabolites with toxicity potential are produced by more than 100 moulds. Main fungal genera producing mycotoxins are *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps*. Key mycotoxins which are highly prevalent in contaminated agricultural products are aflatoxins (AFs), ochratoxin A (OTA), trichothecenes (deoxynivalenol (DON) and T-2 toxin), zearalenone (ZEA), fumonisins (FBs) and patulin (PAT). It is important to highlight that the same fungal species can produce different mycotoxins, for example, *Aspergillus flavus* produces mainly AFs but can also produce cyclopiazonic acid (CPA). Human exposure can occur through contaminated foods such as cereal grains, ground nuts, milk, meat, eggs, etc., or more unusually by the inhalation of polluted air and dust (Bryden, 2007). When ingested by humans or animals above a certain concentration, mycotoxins will cause a toxic response referred to as mycotoxicosis.

Toxicological effects in humans comprise: carcinogenic (AFs, OTA and FBs), mutagenic (AFs and sterigmatocystin), teratogenic (ochratoxins), estrogenic (ZEA), hemorrhagic (trichothecenes), immunotoxic (AFs and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (AFs), dermotoxic (trichothecenes) and neurotoxic (ergotoxins) effects (Marín et al., 2013; Steyn, 1995). As mentioned previously, besides their health impacts, mycotoxins

also affect the agricultural trade among countries through decreasing livestock and crop yield production. According to FAO surveys during the last decades, up to a 25% of world agriculture products are contaminated with mycotoxins. A summary of the main mycotoxins, the producing fungi, with description of their health effects and commodities affected is listed in Table 1.

Modern mycotoxicology began with the discovery of AFs after the death of a large number of turkeys in 1961 in England which was attributed to consumption of peanut meal incorporated in the diets (Blount, 1961). Since then, many human and animal diseases have been associated with mycotoxins spoiling food and feed. Ergotism (Saint Anthony's fire) is the oldest known mycotoxicosis caused by the toxic mould *Claviceps purpurea* that contaminate rye flour. Other examples are the yellow rice disease, caused by the consumption of rice contaminated with citreoviridin in Japan; the alimentary toxic aleukia (ATA) in Russia is believed to be caused by consumption of grains contaminated with T-2 and HT-2 toxins produced by *Fusarium sporotrichioides* and *F. poae*. The ingestion of AFs is related to human primary liver cancer, in Africa and South East Asia.
Since the late 1960's, regulations for mycotoxins have been established in food and feed in many countries to protect consumers from mycotoxins. International and government authorities in many countries have been investing in mycotoxins research, elaborating legislation and implementing regulatory measures for the control of mycotoxins. The current European legislation CE n. 1881/2006 and subsequent amendments establishes the maximum levels of mycotoxins allowed in human food and Directive 2003/100/EC amending Directive 2002/32 EC, for animal feed.

Due to all of these reasons, mycotoxins control through the food chain is of paramount importance, and developing tools that allow their prevention, control and prediction are highly needed. Predictive mycology is one of these tools, since the prediction of fungal growth and mycotoxin production seems to be a promising approach and could play a role in improving the quality and safety of food (Dantigny and Panagou, 2013; Dantigny et al., 2005). This tool may help for adequate decision making purposes, risk assessment and in the implementation of mitigation strategies (see section 3).

Table 1. (part 1 of 2) Main mycotoxins, producing fungi, with description of their health effects and commodities affected.

Mycotoxin	Mould	Commodity	Possible toxic effects
Aflatoxins	*Aspergillus* section *Flavi*	Corn, peanut, cotton, nuts, soya beans, spices, dairy products	Liver diseases (hepatotoxic, hepatocarcinogenic), carcinogenic and teratogenic effects, hemorrhages, reduced growth rate, immune suppression
Ochratoxins	*Aspergillus* section *Circumdati*, *Aspergillus* section *Nigri*, *Penicillium verrucosum*, *Penicillium nordicum*	Wheat, barley, corn, beans, grapes, wine, spices, cheeses, nuts	Carcinogenic, nephrotoxic, mild liver damage, teratogenic effects, poor feed conversion, reduced growth rate, immune suppression
Fumonisins	*Fusarium* section *Liseola*	Corn, soya beans	Equine leukoencephalomalacia nephrotoxic and hepatotoxic, immune suppression, pulmonary edema
Alternariol and tenuazoic acid	*Alternaria* spp.	Tomatoes, sunflower, barley, apples	Mutagenic, fetotoxic and teratogenic
Trichothecenes	*Fusarium acuminatum, F. poae, F. sporotrichioides, F. langsethiae* *F. graminearum, F. culmorum, F. cerealis*	Corn, wheat, soya beans, animal feeds	Digestive disorders, reduced weight gain; hemorrhages, edema, oral lesions, dermatitis, blood disorders, infertility, degeneration of bone marrow, reduced growth rate, immune suppression

Adapted from (Bennett et al., 2003; CAST, 2003; García-Cela et al., 2013).

Table 1. (part 2 of 2) Main mycotoxins, producing fungi, with description of their health effects and commodities affected.

Mycotoxin	Mould	Commodity	Possible toxic effects
Zearalenone	*Fusarium graminearum, F. culmorum, F. equiseti, F. cerealis, F. verticillioides, F. incarnatum*	Corn, wheat, soya beans, animal feeds	Estrogenic effects, edema of vulva, prolapse of vagina, enlargement of uterus, atrophy of testicles, atrophy of ovaries, enlargement of mammary glands, infertility, abortion
Ergot alkaloids	*Claviceps purpurea, C. fusiformis, C. africana, Neotyphodium* spp.	Rye, wheat	Nervous or gangrenous syndromes, digestive disorders, reduced weight gain, convulsion, abortion
Cyclopiazonic acid	*Penicillium* and *Aspergillus* species	Peanut, sunflower, corn	Necrotic effects (liver, gastrointestinal tissue, kidneys, skeletal muscles), carcinogenic, neurotoxic
Citrinin	*Penicillium* and *Aspergillus* species	Wheat, barley, corn, rice	Nephrotoxic, teratogenic, hepatotoxic
Roquefortine	*Penicillium roqueforti, P. camemberti (P. caseicola)*		Neurotoxic
Patulin	*Penicillim expansum, Bysochlamis nívea, Aspergillus clavatus*	Apples and by-products	Mutagenic, genotoxic, neurotoxic, immune suppression

Adapted from (Bennett et al., 2003; CAST, 2003; García-Cela et al., 2013).

1.1. Aflatoxins

Amongst mycotoxins, AFs are a highly important group of toxins of greatest concern from a global perspective due to their unavoidable presence in a great variety of foods and feeds and to their high toxicity. As mentioned in the previous section, they were discovered in England as a consequence to the death of 100.000 turkeys ('Turkey X disease'). AFs are primarily produced by *Aspergillus flavus* and *A. parasiticus*, and rarely by *A. nomius*. More than 20 different types of AFs structures have been identified, but the most prevalent and toxic forms are AFB1, AFB2, AFG1 and AFG2 (Pittet, 1998).

Major aflatoxins have been classified into B and G series due to their fluorescence, being blue and green in UV, respectively. The B series (AFB1 and AFB2) are chemically known as difurocoumarocyclopentenones and the G series (AFG1 and AFG2) are difurocoumarolactone series. Structurally the dihydrofuran moiety, containing a doublebond, and the constituents linked to the coumarin moiety are of importance in producing biological effects. For the B series, cyclopentenone was reported to be responsible for the major toxicity observed (Figure 1).

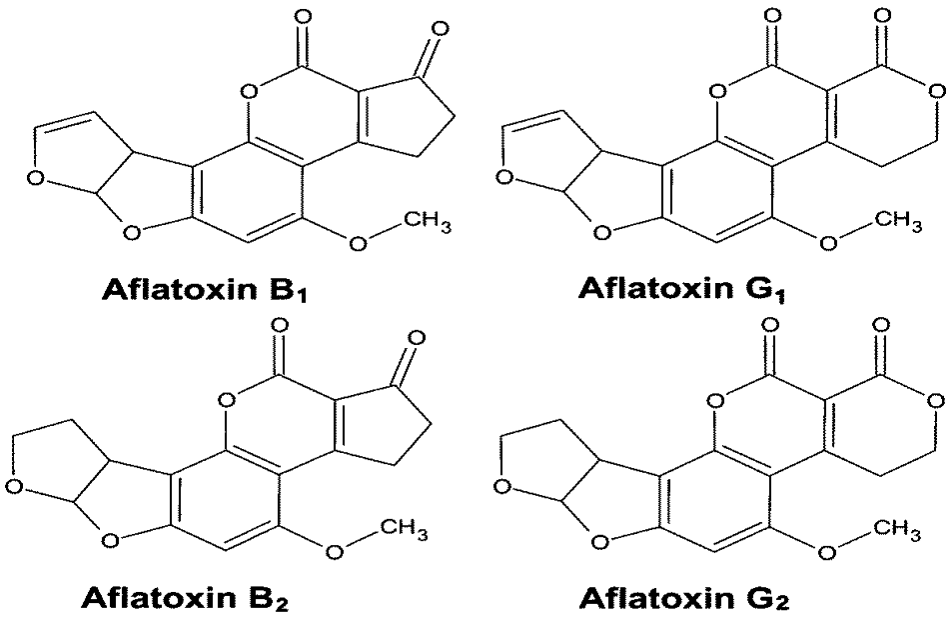

Figure 1. Structure of aflatoxins (AFB1, AFB2, AFG1 and AFG2) (Cole et al., 2003).

Foods most commonly contaminated by AFs include maize, rice, peanuts, pecans, almonds, hazelnuts, Brazil nuts, pistachio nuts, and walnuts (Barkai-Golan, 2008; Bui-Klimke et al., 2014; Carvalho et al., 2016; Chauhan et al., 2010; Chhotaray et al., 2015; Fernane et al., 2010; Ferre, 2016; Wu, 2015).

AFB1, the most abundant and most toxic aflatoxin, is often referred to as the most potent naturally occurring carcinogen. It is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993).

The incidence of AFs in food and feed is relatively high in tropical and subtropical regions where the warm and humid climate provides optimal conditions for the growth of these moulds (Klich, 2007).

Figure 2 illustrates the general time course trend of AFs (including production and diminution of content), using as a food example pistachio nuts (Figure 2a) and maize (Figure 2b). The figure shows in a qualitative way the increase/decrease in AFs levels in these two agricultural products from the field to final products. From this figure it is concluded that postharvest stages, including transport and storage, are steps of particular risk for AFs accumulation.

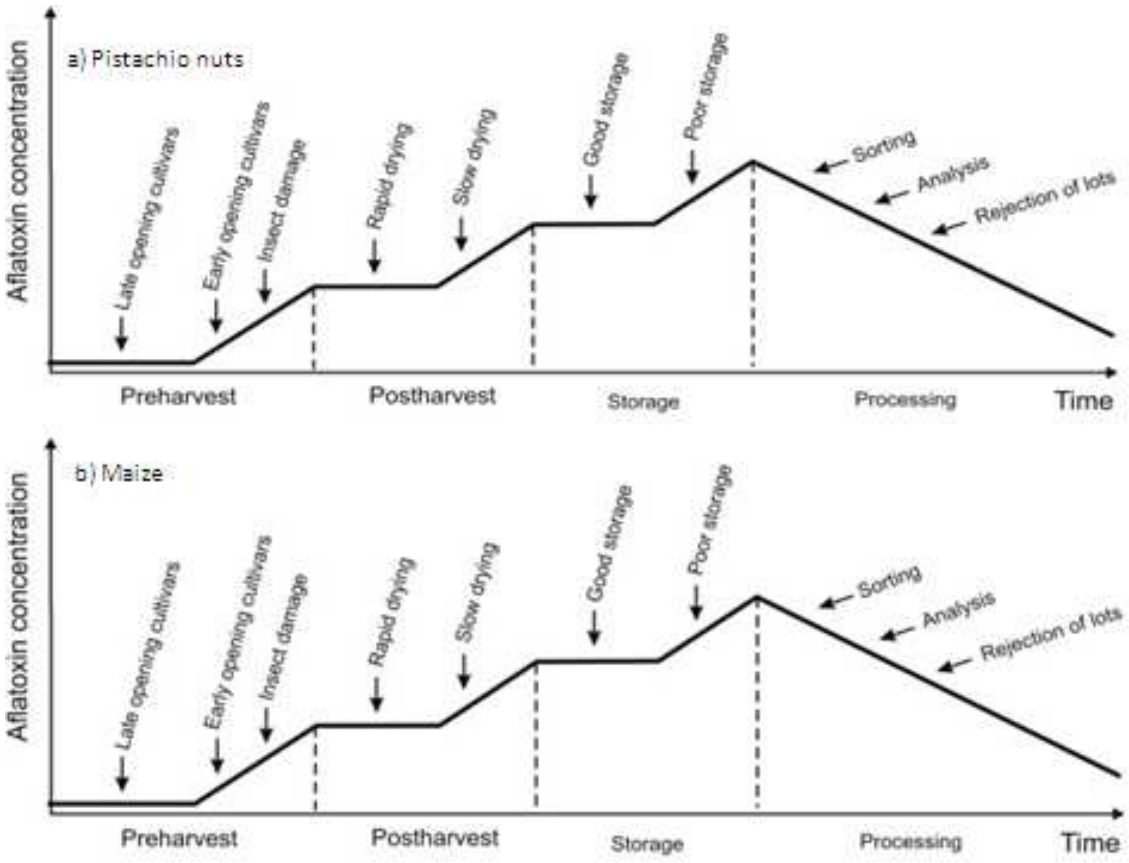

Figure 2. The time course of aflatoxin formation and reduction in a) pistachio nuts and b) maize. From Pitt et al. (2013).

Due to the climate change, a global increase in temperature and changes in the atmosphere composition (gases) and humidity will occur. As a result, the pattern of mycotoxins distribution in the different regions of the world will change (Magan et al., 2011). Battilani et al (2016) using a model for climate change, forecasted that aflatoxin may become an even major issue in the agricultural industry, especially in a scenario where temperatures rise >2 °C.

Considering the above points, greater consideration must be taken to reduce and prevent *Aspergillus* sp. in food products all over the food chain in order to protect human and animal health.

2. Mycotoxins prevention through the food chain: Predictive mycology

Mycotoxins can contaminate a product all over the food chain, in the field as well as during storage, or at later points (Figure 3). Approaches to prevent mycotoxins in foods and feeds include pre- and post-harvest strategies. Regarding aflatoxins, *A. flavus* comes in contact with crops before harvest, and then the fungi can remain associated with the crop through harvest and storage (Lillehoj, 1987), and the agricultural product becomes contaminated with aflatoxins, both before and after harvest. However, aflatoxin contamination is more likely to occur in the post-harvest stage if the product is not handled properly to minimize the thriving of the fungi.

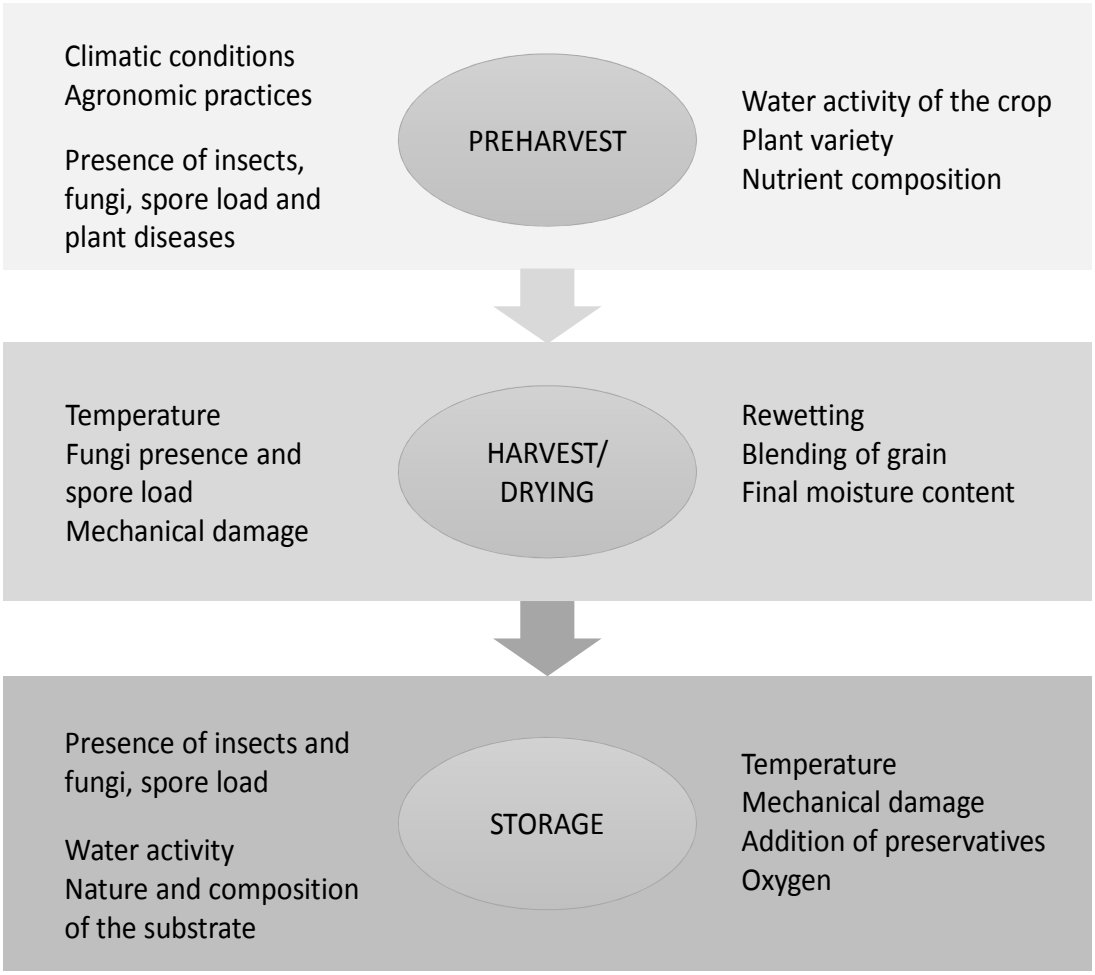

Figure 3. Brief description of the food chain and the main factors influencing fungal growth and mycotoxin production. Modified from Magan et al. (2004).

The best way to prevent fungal contamination in an agricultural product would be the prevention of mycotoxin formation in the field (preharvest stages), which is supported by proper crop rotation and fungicide administration at the right time and in overall Good Agricultural Practices (GAP). However, control of field factors is known to be difficult and can be affected by many factors such as plant varieties, environmental conditions or climate change. The implementation of Good Manufacturing Practices (GMP) during handling, storage, processing and distribution represents an important line of defense in controlling the postharvest contamination of commodities by mycotoxins. Post-harvest control measures are often categorized into physical, chemical and biological methods (Jouany, 2007). The most common strategy to decrease mould contamination after harvest is rapid drying of the agricultural product in order to reduce its water activity (a_w). Removal or detoxification of mycotoxins has been studied using physical (Liu et al., 2011; Mann et al., 1967; Paster et al., 1985), chemical (Dombrink-Kurtzman et al., 2000; Jalili et al., 2011; Prudente and King, 2002) or biological (Aiko and Mehta, 2013; Das and Mishra, 2000; Shantha, 1999; Singh et al., 1994) methods. Efficient degradation of mycotoxins is a challenge since most mycotoxins are heat-stable and form toxic degradation products (Bullerman and Bianchini, 2007). Although several detoxification methods have been developed, only a few have been accepted for practical use.

Beyond these strategies, predictive mycology can provide valuable information allowing prediction of fungal development and mycotoxins production under certain conditions and substrates. Predictive mycology is a subfield of predictive microbiology which is devoted to predict the occurrence of food-borne pathogens, through mathematical equations, taking into account mould specificities.

2.1. Predictive mycology, a subfield of predictive microbiology

Predictive models are mathematical tools that can be used to assess product shelf-life and safety. Models can also be used for products development, to identify areas where challenge testing should be undertaken or as a tool for HACCP plan and risk assessment development.

Predictive models are a very quick, efficient and cost effective way of assessing the potential for growth of microorganisms under specific conditions without needing practical studies. Predictive microbiology enables quantitative prediction based on the sequence of events in the whole history of the product, and can be used to determine what may happen in many different scenarios.

For over 20 years, predictive microbiology has been developed for predicting the occurrence of food-borne pathogens, however it has been focused mainly on bacterial behavior. The inherent differences between bacterial and mould growth imply that such tools take into account for mould specificities, thus the name “predictive mycology” was coined to differentiate the modeling of fungal growth and mycotoxin production from that of bacteria (Dantigny et al., 2005).

In the last years a growing number of studies are available in the literature dealing with predicting fungal growth (Astoreca et al., 2012; Battilani and Leggieri, 2015; Garcia et al., 2013, 2010; Ioannidis et al., 2015; Marín et al., 2008; Pardo et al., 2005; Samapundo et al., 2007b, 2005, 2007a; Tassou et al., 2007; Yogendrarajah et al., 2016) highlighting the importance of this field for the food and feed industry.

Membré and Lambert (2008) presented some applications of predictive microbiology in industrial contexts, categorizing them into three groups related to food safety, namely “product innovation”, “operational support” and “incident support”. Predictive models are split up into two categories regarding their objectives: kinetic and probabilistic models.

Kinetic models

Whiting and Buchanan (1993) classified predictive models as primary, secondary and tertiary models.

a) Primary models describe how population density changes with time in a specified environment. These types of models represent the basis for developing strategies in the food industry. Regarding mycological studies the main primary models employed are: Baranyi model (Baranyi and Roberts, 1994) and the modified Gompertz model.

b) Secondary models are the mathematical models that describe the responses of one or more parameters of a primary model to one or more environmental conditions. The most used secondary models in predictive mycology include the polynomial, cardinal, and logistic models.

c) Tertiary models are application tools such as computer software or expert systems that utilize the primary and secondary models to predict the fate of microorganisms in foods. The primary and secondary models are the foundation for building a tertiary model.

Dagnas and Membré (2013) and Garcia et al. (2009) reviewed the existing models regarding mould growth and mycotoxins production and gave a comprehensive description of them.

Probabilistic models

In predictive mycology, probabilistic models are used to predict the probability of growth or mycotoxin production of a microorganism under different conditions (Tienungoon et al., 2000). Probabilistic models are useful tools where the objective is to determine whether or not an event, such as fungal growth or mycotoxin production, will occur. Besides, prediction of boundaries between growth and no growth of foodborne pathogens may be achieved with probabilistic models.

The logistic equation relates the independent variable (X) to the probability of an event occurring (y), often linked to a polynomial model.

$$logit(P) = ln\ \frac{P(x)}{1 - P(x)} = b_0 + \sum b_i x_i$$

Where P is the probability of the event occurring (range from 0 to 1), the coefficients b_i are the estimated parameters, and the x_i values are the independent factors (e.g., aw, temperature, and pH).

Logistic regression can be a useful method for modeling boundaries between growth and no growth or presence and absence of mycotoxin production by fungi (Astoreca et al., 2012; Garcia et al., 2011a; García-Cela et al., 2014; Koutsoumanis and Sofos, 2005; Marín et al., 2009, 2008; Tassou et al., 2009; Vermeulen et al., 2012).

3. Relevant variables to take into account for developing predictive models

In order to make the prediction obtained through the predictive models as real and accurate as possible, some factors should be considered under the experimental conditions defined to generate the data which will be used to build the models.

Among these factors we can find marginal environmental conditions, intraspecies variability, inoculum size, fluctuation of environmental conditions, etc.

3.1. Marginal environmental conditions

Contamination may occur in the field as well as during (improper) storage and is largely dependent on environmental factors, these environmental conditions play a key role in mould development and determine how the fungi will behave. In general, foods and feeds are stored under marginal conditions for mould growth and mycotoxin production. Thus, in order to correctly extrapolate results from the experiments to real contamination scenarios experimental design should include such conditions.

Lillehoj (1987) highlighted the importance of taking into account marginal conditions when setting the conditions of mycological experiments. They worked with AFs production in maize and pointed that some of the literature on aflatoxin may not be useful or realistic in helping to determine the likelihood of aflatoxin production because of the experimental conditions used.

Some studies have also demonstrated the impact that marginal environmental factors may have on mould growth and mycotoxin production (Garcia et al., 2011c, 2010; Marin et al., 1998; Pose et al., 2010; Romero et al., 2007; Tassou et al., 2007).

3.2. Strain variability

Strain variability is defined as an inherent characteristic of microorganisms that cannot be reduced when strains are identically treated under the same set of conditions (Whiting and

Golden, 2002) .

Fungal populations in agricultural products and foods are complex communities that contain many different strains of a specie. Their growth and mycotoxin production potential are known to vary among them (Abbas et al., 2004; Adhikari et al., 2016; Singh et al., 2015; Yogendrarajah et al., 2016; Yousefi et al., 2009). There are many studies reporting the variability among isolates in terms of growth and mycotoxin production (Abbas et al., 2005; Astoreca et al., 2007; Bellí et al., 2004; Garcia et al., 2011a, 2011b, 2011c; Lahouar et al., 2016; Mitchell et al., 2004; Pardo et al., 2004; Romero et al., 2007; Singh et al., 2015; Yogendrarajah et al., 2016). For example, Garcia et al. (2011a) studied the growth and ochratoxin A (OTA) production of thirty isolates of *A. carbonarius*. Their results showed a wide dispersion in both growth rate and mycotoxin production, especially under marginal conditions. Thus, accounting for intraspecies variability in mycological studies is required to give more realistic predictions.

3.3. Inoculum size

The vast majority of studies in predictive mycology were carried out with large spore inoculum sizes (Char, 2005; Garcia et al., 2013; Koutsoumanis et al., 2010; Romero et al., 2010). However, in practice most foods are contaminated by low numbers of fungal spores (Burgain and Dantigny, 2016; Burgain et al., 2013; Gougouli et al., 2011). It is known that growth results may differ following inoculation by single spores compared with a higher concentration of spores (Baert et al., 2008; Burgain et al., 2013; Chulze et al., 1999; Garcia et al., 2010; Gougouli and Koutsoumanis, 2013; Morales et al., 2008; Sautour et al., 2003).

Sautour et al. (2003) reported that there is a need for standardizing spore preparation in predictive mycology, and thus it should be considered in the design of challenge tests and in quantitative risk assessment. Few studies have investigated the effect of inoculum size on mycotoxin production (Chulze et al., 1999; Morales et al., 2008).

3.4. Dynamic environmental conditions

Most of the available information on fungal growth and mycotoxin production has been

developed and validated based on data from constant conditions. However, environmental factors can fluctuate during transport, storage, distribution, retail and domestic storage of food. Such fluctuations should be taken into account, otherwise the model outcome may lead to wrong decisions. Only few studies have dealt with this issue (Garcia et al., 2012; Gougouli and Koutsoumanis, 2012, 2010; Ryu and Bullerman, 1999), thus there is a need for studying and modeling fungal behavior in real situations, such as dynamic temperature conditions.

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Objectives and work plan

Moulds can grow and produce mycotoxins during storage of raw products, and subsequent transport, additional storage periods, and sale causing considerable economic losses for food manufacturers and consumers. Predictive mycology, by providing tools allowing for the prediction of fungal growth and mycotoxin production, can play a very important role in improving the quality and safety of food. Among fungi, *Aspergillus flavus* is a worldwide widespread fungus producer of aflatoxins, which contaminates many agricultural products and it is responsible for quantitative and qualitative yield losses. In the last few years, probability models have been proposed as a useful alternative to predict and prevent from mycotoxin accumulation in raw materials. Before such models can be applied to real situations, a number of issues must be addressed. In order to develop realistic and accurate predictive models, the experimental data used to generate the models should take into account many factors which may affect the predictions. Such factors are strain variability, inoculum size, fluctuating environment and marginal conditions for growth. The present dissertation study was set-up and carried out to address some of the issues highlighted above, focusing in:

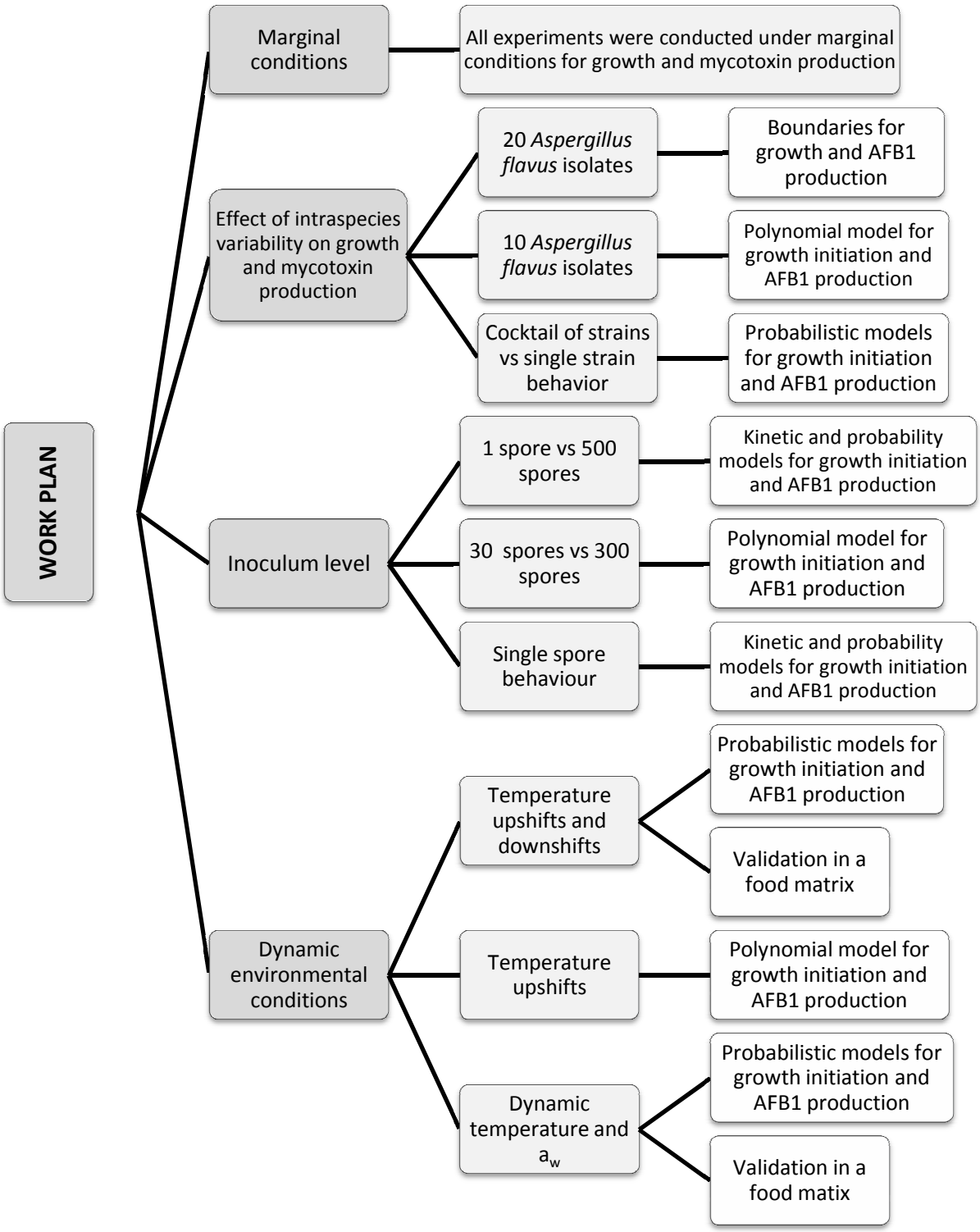
a) To assess the effect of marginal environmental conditions on fungal behaviour and probability models development.

b) To identify and study the differences among isolates in a species under different environments.

c) To evaluate the effect of inoculum size in the outcome of predictive models.

d) To determine the influence of dynamic environments on *Aspergillus flavus* grow and aflatoxin production, and to try to develop probability predictive models that account for such environments.

To achieve these goals, the following work plan was proposed:



Results

Chapter I

Single vs multiple-spore inoculum effect on growth kinetic parameters and modeled probabilities of growth and aflatoxin B1 production of Aspergillus flavus on pistachio extract agar

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Abstract

The objective of the present study was to assess the differences in modeled growth/AFB1 production probability and kinetic growth parameters for *Aspergillus flavus* inoculated as single spores or in a concentrated inoculation point (~500 spores). The experiment was carried out at 25 °C and at two water activities (0.85 and 0.87) on pistachio extract agar (3%). Binary data obtained from growth and AFB1 studies were modeled using linear logistic regression analysis. The radial growth curve for each colony was fitted to a linear model for the estimation of the lag phase for growth and the mycelial growth rate. In general, radial growth rate and lag phase for growth were not normally distributed and both of them were affected by the inoculation type, with lag phase for growth more affected. Changing from the multiple spore to the single spore inoculation led to a delay of approximately 3-5 days on the lag phase and higher growth rates for the multiple spore experiment were found. The same trend was observed on the probability models, with lower predicted probabilities when colonies came up from single spores, for both growth and AFB1 production probabilities. Comparing both types of models, it was concluded that a clear overestimation of the lag phase for growth occurred using the linear model, but only in the multiple spore experiment. Multiple spore inoculum gave very similar estimated time to reach some set probabilities (t10, t50 and t100) for growth or AFB1 production due to the abruptness of the logistic curve developed. The observed differences suggest that inoculum concentration greatly affects the outcome of the predictive models, being the estimated times to growth/ AFB1 production much earlier for the concentrated inoculum than for a single spore colony (up to 9 days). Thus the number of spores used to generate data in predictive mycology experiments should be carefully controlled in order to predict as accurately as possible the fungal behaviour in a foodstuff.

Keywords inoculum, predictive models, probability, logistic regression, mould growth, aflatoxin, *Aspergillus*

1. Introduction

Considering that airborne fungi are ubiquitous, mould food contamination is very likely. Once contamination occurs, fungal growth and hence mycelium appearance on its surface within the product shelf life may occur. Knowledge of environmental conditions promoting fungal growth and mycotoxin biosynthesis seems to be a crucial step towards minimising mycotoxin formation in stored products, and preventing their deleterious effects on animals and humans. A helpful approach to mycotoxin prevention is to minimize its production both in the field and during storage (Bruns, 2003), by monitoring and controlling the environment. Predictive mycology is a useful tool to model fungal responses such as growth and mycotoxin production in food products and a valuable tool in controlling parameters in the food industry environment. Due to the high variability in mycotoxigenic potential of different fungal strains, modeling toxin formation may be challenging (Marín et al., 2008). Thus the use of either growth or mycotoxin probability models to forecast the mycotoxin presence may be a good alternative.

The vast majority of studies in predictive mycology were carried out with large spore inoculum size (Char, 2005; Garcia et al., 2013; Gougouli and Koutsoumanis, 2010; Parra and Magan, 2004; Patriarca et al., 2001; Romero et al., 2010). However, in practice most foods are contaminated by low numbers of fungal spores (Burgain et al., 2013; Burgain and Dantigny, 2016; Gougouli et al., 2011). It is known that growth results may differ following inoculation by single spores compared to a higher concentration of spores (Baert et al., 2008; Burgain et al., 2013; Chulze et al., 1999; Garcia et al., 2010; Gougouli and Koutsoumanis, 2013; Morales et al., 2008; Sautour et al., 2003). Sautour et al. (2003) reported that there is a need for standardizing spore preparation in predictive mycology, and thus it should be considered in the design of challenge tests and in quantitative risk assessment. Few studies have investigated the effect of inoculum size on mycotoxin production (Chulze et al., 1999; Morales et al., 2008).

Aspergillus flavus is worldwide distributed with a high frequency of occurrence in warm climates (Pitt and Hocking, 2009). *A. flavus* produces aflatoxin B_1 (AFB1), a potent carcinogen which is listed in group I by the International Agency for Research of Cancer (IARC, 1993). Knowing and predicting its production is of paramount importance for the food and feed industries.

The objective of the present study was a) to model the probability of growth and AFB1 production using a single spore and a concentrated inoculum, as well as b) examine the distribution of the kinetic growth parameters and probabilities of growth and AFB1 production due to the different inoculum levels. *A. flavus* was chosen as a model mycotoxigenic microorganism, and was grown in pistachio extract agar (3%) under marginal water availability conditions.

2. Material and methods

2.1. Fungal isolate and inoculum preparation

The isolate used in the study, *A. flavus* (UdL-TA 3.267), was taken from the Food Technology Department Collection of the University of Lleida, and had been previously isolated from pistachio nuts. The isolate was sub-cultured on potato dextrose agar (PDA) medium and incubated at 25 °C for 7 days to enable significant sporulation. After incubation, spores were collected by scraping the surface of the plates, diluting them in sterile water adjusted to a_w values of 0.85 and 0.87 with glycerol containing Tween 80 (0.05% v/v), and filtered through sterile glass wool into a tube. . Immediately total spore concentrations were determined using a Thoma counting chamber and decimal dilutions (prepared in sterile water adjusted to a_w values of 0.85 and 0.87 with glycerol containing Tween 80 (0.05% v/v)), were prepared to 10^2 spores/mL for the single spore studies and to 10^5 spores/mL for the multiple spore inoculation studies.

2.2 Medium

The medium used was 3% (w/v) pistachio extract agar (PEA) that was made by boiling 60 g of raw dehulled ground pistachio in 1 L distilled water for 30 min. After that, the extract was filtered and the amount of evaporated water was made up to adjust it to 6% of pistachio. Water activity of the media was adjusted by addition of certain amounts of glycerol for a final a_w of 0.85 and 0.87 and 3% of pistachio. Then, 12 g of agar were added per L of medium (for each a_w) and they were autoclaved and poured into 90 mm sterile Petri dishes which were prepared under aseptic conditions. A total of 25 plates for the growth study and another 25 for the AFB1

production assessment, for each a_w, were prepared for the multiple spore inoculum experiment. For the single spore experiment, 40 additional plates, per a_w, were prepared.

2.3 Inoculation and incubation

2.3.1. Single spore inoculum growth and AFB1 production studies

A 0.1 mL aliquot of 10^2 spores/mL suspensions were surface plated onto PEA (3%) and spread with a sterile bent glass rod, in order to obtain ca. 5-10 spores per Petri dish. Ten plates were prepared for each a_w and study (growth or AFB1 production). Thus, each Petri plate would contain 5 to 10 colonies, each one originating from one spore. Petri dishes with the same a_w were enclosed in polyethylene boxes together with a glycerol–water solution at the same a_w to maintain the relative humidity inside the boxes. Plates were incubated at 25 °C, and each Petri dish was checked daily (about 250 total colonies).

2.3.2 Multiple spore inoculum growth and AFB1 production studies

The inoculation was performed by pipetting 5 μL of the spore suspension (10^5 spores/mL), onto four equidistant points on each of 25 Petri plates (=100 colonies). Thus, in each Petri plate there were 4 colonies, each one originating from approximately 500 spores. Petri dishes with the same a_w were enclosed in polyethylene boxes and incubated at 25 °C, during which the growth of the colony was monitored. Growth was assessed by measuring the perpendicular colony diameters in millimetres periodically, without opening the dishes. The experiment was repeated for both a_w (0.85 and 0.87) and for the AFB1 production study (200 further colonies).

2.4. AFB1 determination

AFB1 presence was determined from the first sign of growth till the end of the study (39 days). For the multiple spore inoculum study, the size of the inoculation drop was about 3 mm, then the radii of the colonies from which AFB1 presence was assessed varied from 3 mm up to 25 mm. In the case of the single spore inoculum, colony size ranged from 0.5 to 7.5 mm radius (due to the presence of other colonies in the plates). Nevertheless, this range was enough to obtain

the required data. A 5-mm agar plug was taken from the centre of a colony at appropriate time intervals, 4 agar plugs were collected at each sampling time for the multiple spore experiment and from 3 to 6 agar plugs (depending on the number of colonies on the plate) were collected at each sampling time for the single spore inoculum experiment. After sampling, the plates were taken back to incubation, for latter assessment of the other colonies present in the Petri plates. Plugs were weighed and vortexed for approximately 5 seconds in 1mL of methanol and left stationary. After 1 hour, extracts were vortexed again and filtered (Millex^{R} SLHV 013NK, Millipore, Bedford, MA, USA). Extracts were dried in a nitrogen stream and stored at 4 ^{o}C until HPLC analysis. The analysis was carried out using a previously described high performance liquid chromatography (HPLC) method (Aldars-García et al., 2015). For the HPLC analysis all extracts were resuspended with 0.5 mL of methanol:water (50:50 v/v) and 100 µL was injected in the HPLC system (Waters, Milford, MA, USA). The detection limit of the analysis was 0.1 ng/g of AFB1, based on a signal-to- noise ratio of 3:1.

2.5. Model fitting

2.5.1. Growth study through kinetic models

During incubation, Petri plates were examined every day and the perpendicular diameters of each colony were measured. Radii of colonies from the multiples spores experiment were corrected by subtracting 1.5 mm (radius of the inoculation drop) from the diameter registered of each colony. Radii of growing colonies were plotted against time, and a linear model (1) was fitted to the growth curves to estimate maximum radial growth rate (µ, mm/day) and lag time for growth (λ, day) for each condition, using R statistical software (R Development Core Team, www.R-project.org, v 2.14.1).

$$R_{(t)} = R_{(0)} + \mu\,(t-\lambda) \quad (1)$$

where $R_{(t)}$ is the radius at time t, $R_{(0)}$ is 0 for both the single spore experiment and the multiple spore one, and t is the incubation time (d).

The goodness of fit was evaluated by the r^2 and the root mean square error (RMSE). Moreover, the distribution of the estimated parameters was also assessed.

2.5.2. Probability of growth and AFB1 production

Logistic regression was used to model the probability of growth (Eq. 2) and AFB1 production (Eq. 3) as a function of time, using R statistical software with the glm function. The binary values along time (0=no visible growth/no AFB1 production; 1= growth/AFB1 production) were adjusted by linear logistic regression, in order to obtain the four different probability models; for each type of inoculum (single spore and concentrated inoculum) two models were developed: one for growth and another one for AFB1 production probability. From each regression curve, the time to reach 0.10 (t10), 0.50 (t50) and 0.90 (t90) probability was estimated by inverse prediction.

$$logit(P_G) = ln\ \frac{P_G(x)}{1-P_G(x)} = b_0 + b_1 t \quad (2)$$

$$logit(P_{AF}) = ln\ \frac{P_{AF}(x)}{1-P_{AF}(x)} = b_0 + b_1 t \quad (3)$$

Where P_G and P_{AF} are the probability of growth or AFB1 production (in the range of 0–1), t is the time, and b_i are the coefficients to be estimated.

The percentage of concordance between observed and predicted values with a cut off of 0.5 and the area under the curve (AUC) were calculated to measure the goodness-of-fit of the developed logistic models. The fitted linear logistic models described satisfactorily the probability of growth and AFB1 production over time for all the experiments, with concordances between observed and predicted values (cut off 0.5) between 85-100%. None of the Hosmer-Lemeshow tests were significant (confidence level 95%) which demonstrated the good fitting of the model to the observations. The Hosmer-Lemeshow goodness-of-fit statistic involves grouping objects into a contingency table and calculates a Pearson ratio chi-squared statistic at the 95% confidence interval. When this statistic is not significant then the model fits the data well (Hosmer and Lemeshow, 1989). AUC values were higher than 0.93, where the target value is one.

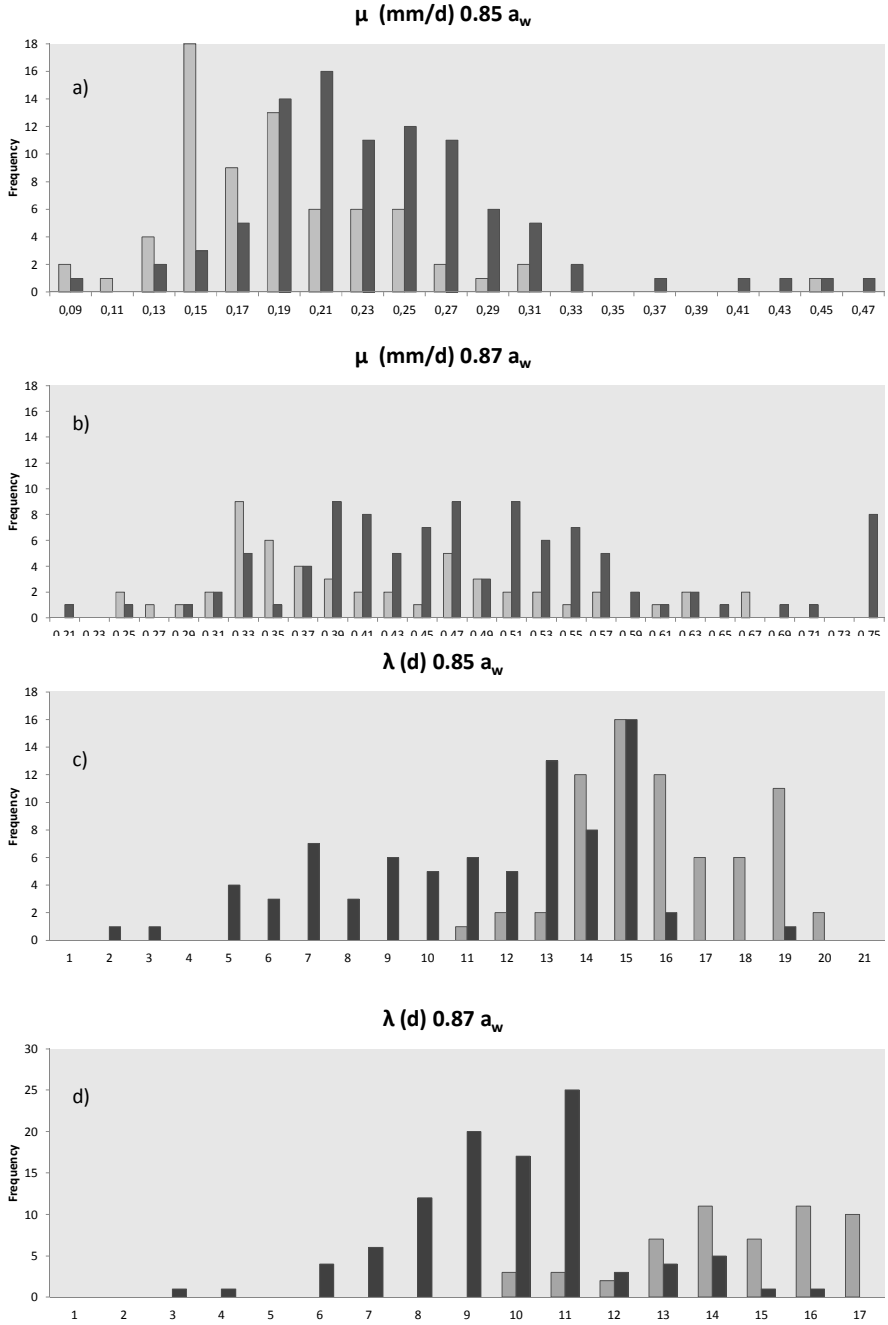

Figure 1. Distribution of mycelium growth rates (a and b) and lag phases (c and d) for growth for single spore (light grey columns) and multiple spore (dark grey columns) inoculum at both a_w.

3. Results

3.1. Growth kinetic parameters (λ and μ)

The colonies were monitored, and the radius vs time was adjusted through the linear model as described in Eq. (1). The fitted models showed acceptable goodness of fit (r^2 > 0.83, RMSE< 0.06). Growth was not detected (experimental data) until days 16-17 in single spore studies, but was detected after 8-10 days in the multiple spore inoculum ones. Significant differences (P< 0.05) among the four estimated μ were found, with higher μ for the concentrated inoculum and at higher a_w. Longer λ were estimated for the single spore experiments. It must be noted that, under these low a_w levels, the 0.02 difference between the a_w levels tested had a higher effect on μ than the type of inoculum (Table 1). On the other hand, λ were increased in 3-5 days with the change of inoculum type. No correlation (neither lineal nor non-lineal) between λ and μ was detected.

Table 1. Summary statistics for the apparent lag time for growth (λ) and the mycelium growth rate (μ) for both a_w.

	0.85 a_w		**0.87 a_w**	
	λ (d)	**μ (mm/d)**	**λ (d)**	**μ (mm/d)**
Mean	16.63	0.23	14.85	0.41
Median	16.39	0.17	15.27	0.37
Standard deviation	2.64	0.10	2.13	0.10
Variation coefficient	0.16	0.33	0.14	0.31
Stnd. skewness	3.51	4.56	-1.90	2.96
Stnd. kurtosis	8.80	7.35	-0.60	2.67
No. colonies	72	72	55	55

The effect of a_w and type of inoculum on the distribution of μ and λ is illustrated by the histograms in Fig. 1. From the graphs it can be depicted that only λ at 0.87 a_w and for the multiple spore inoculum was normally distributed. The μ histograms were skewed to the left indicating that the majority of the single spores germinated and gave rise to slow growing colonies. This trend was much clearer for the single spore experiments, which inform on the real distribution of spores; when the multiple spore inoculum is used the probability to include a fast growing spore increases, thus the distribution moves to the right, obtaining higher μ. Regarding λ, due to the more significant effect of the inoculation treatment applied, the distributions did no overlap as much as the μ distributions. Moreover, the values range was wider for the multiple spore inoculum, with more density of data on the right (close to the single-spore experiment data). By contrast, the single spore inoculum comprised a smaller range, but also the distribution was skewed to the right, which means that most of the spores have long λ.

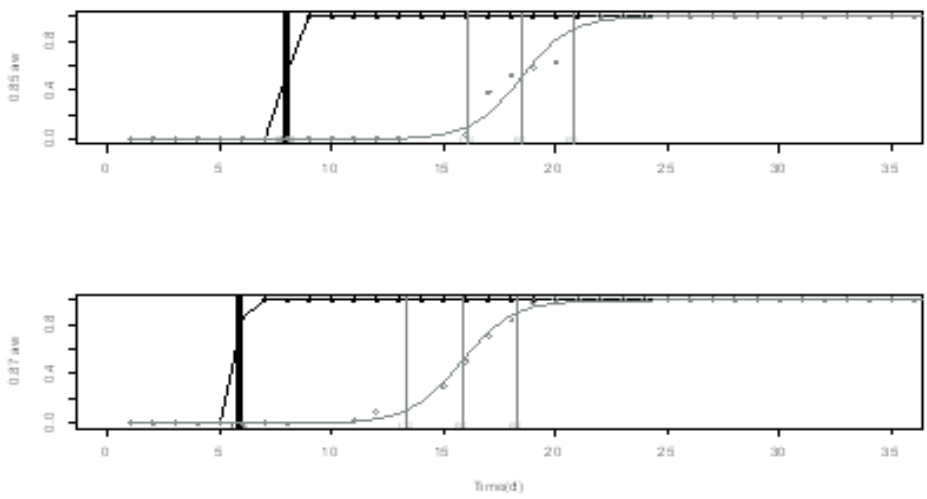

Figure 2. Probability of growth models obtained through logistic regression for the four different conditions studied and their time estimates for probabilities 0.10, 0.50 and 0.90 (t10, t50 and t90). Single spore (light grey lines) and multiple spore (dark grey lines) inocula at both a_w.

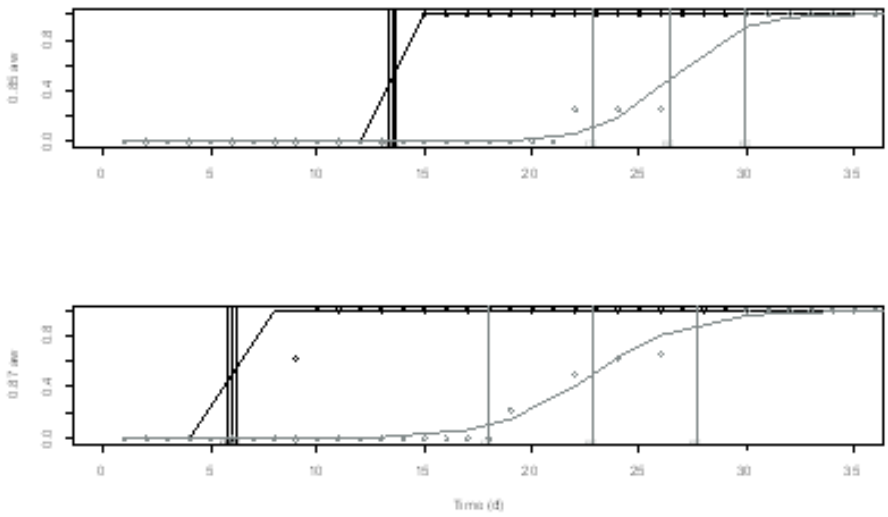

Figure 3. Probability of AFB1 production models obtained through logistic regression for the four different conditions studied and their time estimates for probabilities 0.10, 0.50 and 0.90 (t10, t50 and t90). Single spore (light grey lines) and multiple spore (dark grey lines) inocula at both a_w.

3.2. Probability models

Probability of growth raised earlier for the multiple spore inoculum at both a_w (Fig.2). The results showed significant growth differences depending on the type of inoculation, as the initiation of growth was delayed about 8 days for colonies arising from single spore inocula. Moreover, the slopes of the probability curves were smoother in the case of the single spores. In fact, the two curves did not clearly overlap, which may mean that when spores are together there could be some kind of stimulation among them. Statistical analysis showed that there were significant differences between the two probability curves (inoculum factor) at the same a_w, for the two scenarios tested.

Plots of probability of AFB1 production obtained through the logistic regression are presented in Fig.3. For the multiple spore inoculum, the probability of AFB1 production quickly increased from 0 to 100% within a period of 2-3 days (from day 12 until day 14 for 0.85 a_w and from day 4 to 7 for 0.87 a_w). For the single spores, initiation of AFB1 production was considerably delayed at both a_w (following the initiation of growth pattern) in comparison with the multiple spore inoculum. Moreover, the AFB1 probability curves arising from single and multiple spore inoculums did not overlap, which suggests again that stimulation among spores may occur. The effect of a_w, was more relevant for the AFB1 models, with about a 7 days delay in the multiple-spore experiment compared to the 2-3 days delay in growth (Fig. 2 and 3). Despite the small difference between the two a_w studied, it is interesting that in the case of the concentrated inoculum this difference made an important gap, which means that besides the delayed growth, enzymatic mechanism required for AFB1 biosynthesis are further affected by a_w in the environment.

3.3 Relationship between estimated λ and estimated probabilities of growth and AFB1 production

The link among λ and the different times to reach the estimated probabilities for both growth and AFB1 production is presented in Fig. 4. A clear progression of the 5 events studied It can be graphically depicted in the case of the single spore experiment. When using a single spore inoculum, the estimated end of lag phase came first ($P_G \approx 0.2$), in 15.27 and 16.38 days for 0.87 and 0.85 a_w respectively. This means that when there is 0.50 probability of visible mycelium detection (t50 growth), the lag phase has concluded. Then 0.50 probability of AFB1 production (t50 AFB1) was reached (22.80 and 26.38 days for 0.87 and 0.85 a_w respectively), before all spores have led to visible colonies (t100 growth) in 38.92 and 42.87 days for 0.87 and 0.85 a_w respectively.

On the other hand, the multiple spore experiment gave different results, since in both cases the estimated λ values were longer than the estimated times to reach 50% visible growth (t50 growth) or even 100% (t100 growth). While the results obtained from the probability models agreed with the observed results, this did not occur with the estimated λ values which were

overestimated in almost all the cases, as can be seen in the distribution graphs where there is a high density of data in the right side, due to shorter λ (Figs. 1c and 1d). The possible interaction among spores may be the cause of such overestimation of λ. This interaction could be due to substances secreted by the germinating spores or some other phenomena that requires in depth study. In any case, stimulation among spores should be taken into account if data generated from highly concentrated inoculums are used to estimate growth parameters and develop predictive models.

4. Discussion

Studies on mould growth have been traditionally carried out with high inoculum levels of spores, when in fact infection of food occurs with a low number of spores, and colonies most likely originate from single spores. In this study, the aim was to assess the effects of inoculum size using two different levels (colonies arising from a single spore or colonies from 500 spores). Working with single spores of *A. flavus* and *Fusarium verticillioides*, Samapundo et al. (2007a) showed that when more limiting conditions were assessed, wider distributions of the growth parameters were obtained and greater degree of overlapping was observed between the histograms of the colony growth rates compared to those for the lag phase for growth. These findings are consistent with our results (see Fig. 1). The significant effect of the inoculum size on the fungal growth parameters has been confirmed by several authors, who have used inoculum sizes ranging from 1 to 10^6 spores (Baert et al., 2008; Burgain et al., 2013; Dagnas et al., 2015; Garcia et al., 2010; González et al., 1987; Gougouli et al., 2011; Morales et al., 2008; Samapundo et al., 2007b) (Table 2). Such investigations have shown that changes in the inoculum size affected the lag phase but not the growth rate (Baert et al., 2008; González et al., 1987; Gougouli et al., 2011). Baert et al. (2008) studied *Penicillium expansum* in apples and they observed that using high inoculum levels resulted in smaller λ than using lower inoculum levels, but no significant difference was observed for the growth rate. Similar results were obtained by González et al. (1987) when they studied the influence of inoculum size on growth rate and lag phase of seven different fungi isolated from Argentinean corn. Garcia et al. (2010) reported no significant differences in the rate of growth of two tested moulds at 0-1, 1-10 and 10-100 spores. On the other hand, Morales et al. (2008) found significant differences in the growth rate

of fungi depending on the inoculum size, as we have found in the present study. They reported that colonies from conidial suspensions of 10^6 spores/mL had faster growth rates than those from the 10^4 spores/mL suspensions. They worked under marginal conditions, with apples at 1 or 20 °C, as did we with different marginal conditions, 0.85/0.87 a_w and 25 °C. From these data we may suggest that growth rate could be affected by the inoculum size when stressful conditions and very different inoculation levels are tested. Thus, it seems that in these cases, as the histograms in Fig 1 and Fig 2 do overlap, either some single spores dominated the behavior of the multiple spore inoculum or stimulation between spores took place. Similarly, working with bacteria, Robinson et al. (2001) envisaged two types of inoculum size effect on population lag time: (a) cooperative or inhibitory effects of high cell concentrations or (b) statistical effects at low cell concentrations arising from the variability in individual lag times. Working with *Listeria monocytogenes* they obtained that under optimal conditions the lag times were little affected by inoculum size. However, under stressing conditions, the lag time increased as the inoculum size became smaller.

In particular, lag phase prior growth, seems to be affected by inoculum size. Our results showed that λ decreased when changing from a single spore to a multiple spore inoculum. Lag time encompasses time for germination plus the beginning of hyphal elongation (Gougouli and Koutsoumanis, 2013), thus studying the germination process would help in the understanding of lag phase variability. Several studies have investigated the variability in germination time (Chitarra et al., 2004; Chitarra and Dijksterhuis, 2007) suggesting that it may be due to biological sources such as self-inhibitors or auto-stimulators, which might prevent from premature germination of all the spores at the same time. Another study showed that inoculum concentration affected the rate of germination of some species of *Aspergillus* (Araujo and Rodrigues, 2004), being the germination inhibited when spores were present in high densities (1.4×10^5 to 5×10^5 conidia/mL, in RPMI 1640 medium). If germination time is altered so would happen to the lag phase. Gougouli and Koutsoumanis (2013) studied the effect of storage temperature on the kinetic behavior of *Aspergillus niger* and *P. expansum* individual spores, demonstrating that the lag time variability of single spores was mainly due to the germination variability. In our case, histograms in Fig 1c and 1d did not overlap much, thus it seems that the hypothesis that some single spores (fast ones) may dominate the multiple –spore inoculum is unlikely. The wider λvariability of the multiple spore inoculum compared to the single spore λ

obtained in the present study, may be explained by the interaction between spores within the multiple inoculum.

The legal limit for AFB1 presence in food and feed is very low, also low amounts of this toxin pose a risk for human health. Moreover, there is a high variability in the amount of AFB1 produced among isolates of the same species. Then in the present study we focused 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). Probability models are of particular interest in managing the safety of foods which may be contaminated with pathogenic microorganisms and for which subsequent growth would increase the risk of food-borne illness. As far as we know, only one study have dealt with the effects of inoculum size on probability models applied to moulds (Garcia et al., 2010). They investigated different environmental conditions (optimal and suboptimal) and three inoculum levels (see table 2) and concluded that probabilistic models were not much affected by the inoculum size when the probabilities are low. By contrast, in the present study, both the probability of growth and the probability of AFB1 production were affected by the inoculum type. However, it should be taken into account that conditions used in this study were more restrictive and the change of inoculum level wider.

Table 2. Some publications studying the effect of different inoculation levels of spores on fungal growth parameters.

Reference	Fungi	Medium	a_w/T	Spore level (amount at inoculation point)	Significantly affected by spore level	
					μ	λ
González et al., (1987)	*Aspergillus* spp. *Penicillium* spp. *Fusarium spp.*	Malt extract agar (MEA)	0.95/30 °C 0.99/30 °C 0.99/30 °C	10^4, 10^3, 10^2, 10^1		[a]
Sautour et al., (2003)	*Penicillium chrysogenum*	Potato Dextrose Agar (PDA)		0-1, 1-10^1, 10^1-10^2, 10^2-10^3	[a]	[a]
Baert et al., (2008)	*Penicillium expansum*	Apples	25, 12 and 4 °C	2 x 10^6, 2 x 10^5, 2 x 10^4, 2x10^3, 2 x 10^2, 2x 10^1		[a]
Morales et al., (2008)	*Penicillium expansum*	Apples	1 and 20 °C	2 x 10^4, 2 x 10^2	[a]	[a]
Garcia et al., (2010)	*Aspergillus carbonarius* *Penicillium expansum*	MEA	0.98/15 °C and 0.90/25 °C 0.98/15 °C and 0.92/25 °C	0-1, 1-10^1, 10^1-10^2		[a]
Gougouli et al., (2011)	*Penicillium* spp. *Aspergillus* spp. *Fusarium* spp. *Mucor circineloides* *Rhizopus oryzae* *Cladosporium cladosporioides*	Yogurt	0, 5, 10, 15, 20, 25, 27.5, 30, 33, 35 and 40 °C	10^5, 10^4, 10^3, 10^2, 10^1		*

[a] indicates that the fungal parameter is significantly affected by the inoculum size assayed in the study.

The shift from no growth/no AFB1 production to 100% probability of growth/AFB1 production is much sharper for the multiple spore inoculum. This abrupt shift can also be easily observed through the times estimates for probabilities 0.10, 0.50 and 0.90, which are almost obtained at the same time in the four multiple spore inoculum scenarios (both a_w levels, growth/AFB1 production), whilst for the single spore experiment these estimates are quite separated over time. The differences between models suggest that the use of a concentrated inoculum leads to significant underestimation of times for growth and toxin production compared to what happens in single spores. For example, if we estimate the probability of AFB1 production after 20 days of incubation at 25 °C from the multiple spore inoculum model we will obtain a 100% probability of AFB1 presence, when the situation from one spore would be a 5-20% probability (depending on the a_w). The above results are in agreement with a previous study, which reported the significant effect of the inoculum size on the toxin production (Chulze et al., 1999). They studied the effect of inoculum size on irradiated corn kernels inoculated with different spore concentrations (10, 10^2, 10^3, 10^5, and 10^6 spores/mL) of *Fusarium moniliforme*, obtaining a direct relationship between the level of fumonisins produced and inoculum size.

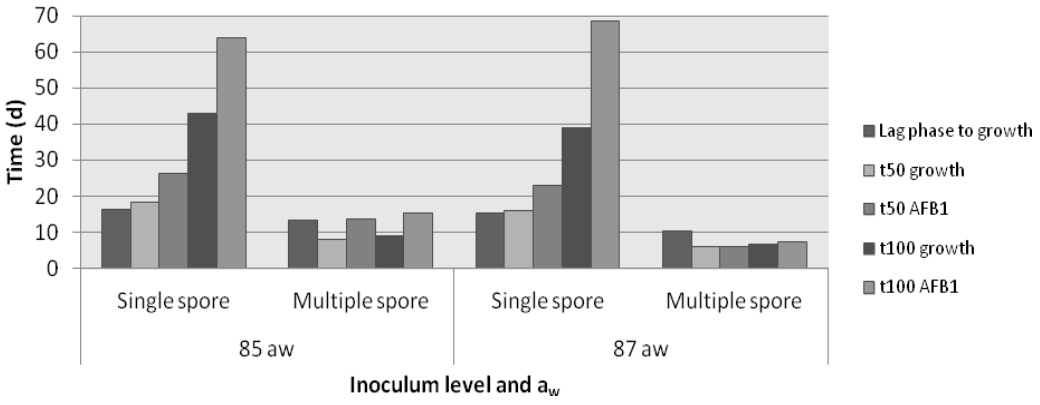

Figure 4. Estimated λ and times for 0.5 and 1 probability of growth (t50 growth and t100 growth) and AFB1 production (t50 AFB1 and t100 AFB1) for all the conditions assayed.

Probabilistic models are built with growth/no-growth data, thus these models should reflect the trends observed for lag phases. λ were calculated by extrapolating the regression line to $R_{(t)}=0$ thereof, and the estimated values did not agree with the probability curves obtained for the multiple spore inoculum (Fig. 4). While according to the probability curves 100% of plates showed growth, according to λ distribution, in most of the plates the lag phase had not ended. A possible explanation to this issue could be found in Gougouli and Koutsoumanis (2013), who studied the relation between germination time and lag time of mycelium growth of individual fungal spores of *P. expansum* and *A. niger* under isothermal conditions ranging from 0 to 30 °C and 10 to 41.5 °C, respectively. They observed an important delay between germination time and lag phase; this was due to the existence of two phases of hyphal development: first an early hyphal development which increased exponentially and then a linear growth. When estimating λ by extrapolating the regression line to $R_{(t)}=0$, the linear part of the growth process is taken into account, bypassing the exponential one which may had an important significance on estimation of the duration of the lag phase. Therefore, the linear model could not be suitable to estimate this growth parameter under marginal conditions and relatively concentrated (5×10^2 spores) inoculum, because it does not take into consideration the initial exponential growth stage.

In conclusion, despite that the inoculum level was not exhaustively assessed in the present study, it has been shown that it can significantly affect growth kinetic parameters and probabilities of growth/AFB1 production. Sautour et al. (2003) studied the growth of *Penicillium chrysogenum* under different inoculation levels, observing a decrease in the lag phase duration as the inoculum size increased. They inferred that the lag phase was highly dependent on the inoculum size, thereby the inoculum level should be standardised in fungal experiments in order to correctly estimate the shelf life of foods. If we estimate the shelf life (being the 0.05 probability of growth the bound for rejecting the product) of a certain commodity from the single spore growth model at 0.87 a_w it would result in 12.54 days but if we do the same with the multiple spore growth model we get 5.76 days, under the conditions studied. Thus there is about 7 days of difference between the two estimations. From the food safety point of view developing fail safe models is the most desirable approach, even though being too conservative may lead to economic losses for the food industry, due to rejection of safe goods. On the other hand, the use of single spore experiments for developing predictive models may not be the

safest approach to consumer's health risk, even though the majority of food contaminations occur with one or very few spores. Thus, a balance between the use of highly concentrated inoculums, as is usual in predictive mycology, and the use of very low inoculum size, such as only one spore, should be the best option for both producers and consumers. Thus, taking consumer safety into account, the findings provided herein have shown that the number of spores used to generate data for predictive models should be controlled carefully in order to simulate real fungal food contamination scenarios.

5. Acknowledgments

The authors are grateful to the Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya (AGAUR, grant 2014FI_B 00045), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2014-55379-P) for funding this work.

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Chapter II

Time-course of germination, initiation of mycelium proliferation and probability of visible growth and detectable AFB1 production of an isolate of Aspergillus flavus on pistachio extract agar

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Food Microbiology (2017), 64, 104-111

Abstract

The aim of this work was to assess the temporal relationship among germination, mycelial growth and aflatoxin B_1 (AFB1) production from colonies coming from single spores, in order to predict as accurately as possible the presence of AFB1 at the early stages of contamination. Germination, mycelial growth, probability of growth and probability of AFB1 production of an isolate of *Aspergillus flavu*s were determined at 25 °C and two water activities (0.85 and 0.87) on 3% Pistachio Extract Agar (PEA). The percentage of germinated spores versus time was fitted to the modified Gompertz equation for the estimation of the germination parameters (geometrical germination time and germination rate). The radial growth curve for each colony was fitted to a linear model for the estimation of the apparent lag time for growth and the growth rate, and besides the time to visible growth was estimated. Binary data obtained from growth and AFB1 studies were modeled using logistic regression analysis. Both water activities led to a similar fungal growth and AFB1 production. In this study, given the suboptimal set conditions, it has been observed that germination is a stage far from the AFB1 production process. Once the probability of growth started to increase it took 6 days to produce AFB1, and when probability of growth was 100%, only a 40-57% probability of detection of AFB1 production was predicted. Moreover, colony sizes with a radius of 1-2 mm could be a helpful indicator of the possible AFB1 contamination in the commodity. Despite growth models may overestimate the presence of AFB1, their use would be a helpful tool for producers and manufacturers; from our data 5% probability of AFB1 production (initiation of production) would occur when 60% probability of growth is observed. Legal restrictions are quite severe for these toxins, thus their control from the early stages of contamination throughout the food chain is of paramount importance.

Keywords

Germination, growth, mycelium, aflatoxin probability, predictive mycology

Brief description of variable abbreviations used throughout the manuscript.

Variable	Variable Abbreviation	Meaning
Geometrical germination time	t_g	Is the intercept in the time axis of the tangent through the inflection point of the germination curve
Maximum growth rate	μ	In the linear growth model, is the slope of the regression line
Estimated apparent lag time for growth	λ	In the linear growth model, is the intercept of the linear part of the graph to a zero increase in radius
Time to visible growth	t_{vg}	The time to reach 3 mm diameter colonies, calculated through the linear model
Probability of growth	P_G	In the logistic model, percentage of colonies which were visible at a given time
Probability of AFB1 production initiation	P_{AF}	In the logistic model, percentage of colonies with detectable AFB1 at a given time
Estimated times to reach 0.10 (t10), 0.50 (t50) and 0.90 (t90) probabilities	t10, t50, t90	In the logistic model, necessary time to reach the given probability (either for growth of AFB1 production)

1. Introduction

Fungi have a worldwide distribution and grow in a wide range of habitats. Food mould spoilage occurs when a product is contaminated with fungal spores that germinate and form a visible mycelium before the end of the shelf life. As a result of the metabolic activity of moulds in a substrate, a number of desirable or undesirable consequences may occur. One of these undesirable consequences is the production of mycotoxins, which are secondary metabolites that are toxic to humans and animals.

Among all mycotoxins, there is a great concern about the risk derived from consumption of food and feed contaminated by aflatoxin B1 (AFB1), the most potent natural hepatocarcinogen. AFB1 is a toxic metabolite produced by some *Aspergillus* species (Hedayati et al., 2007).

Predictive mycology has dealt mainly with germination, growth and inactivation of fungi (Dantigny et al., 2005), while mycotoxin production has been less studied (Garcia et al., 2009). Studies that deal with the relationship between growth and mycotoxin production (Baert et al., 2007b; Garcia et al., 2013; Magan and Lacey, 1984; Marín et al., 2006; Samapundo et al., 2005) reveal that mycotoxins, as secondary metabolites, are still produced when growth of the colony has already stopped (in most of the cases due to limitation of growth by the size of Petri plates). Few reports deal with the relation among germination, growth and toxin production of fungi. Spore germination, mycelium proliferation and mycotoxin production are successive and not independent events: knowledge of the relationship among these events is essential for the assessment of food safety. Furthermore the link between the colony size and the probability of AFB1 production should be also studied, to determine if there is any correlation. Some studies have dealt with this issue (Baert et al., 2007a; Garcia et al., 2013; Marín et al., 2006; Samapundo et al., 2005), proving the relation among radius/diameter/surface/weight of the colonies along with the concentration of mycotoxins.

In general, fungal behavior has been studied using high inoculum levels. However, real contamination of food products involves single or few spores. Up until now, studies of single spores are still scarce. The objective of the present study was (a) to model the germination (population level), mycelial growth and aflatoxin B_1 (AFB1) production under a single spore contamination scenario by *Aspergillus flavus*, and (b) to assess the time link among the three events which may be useful for predicting safe storage conditions for foodstuffs.

2. Material and methods

2.1. Single spore inoculum preparation

This study was performed using one aflatoxigenic strain of *Aspergillus flavus* (UdL-TA 3.267) isolated from pistachio nuts purchased from a wholesaler in Lleida, Spain, and deposited in the Food Technology Department Collection of the University of Lleida. Briefly, samples of pistachio were plated on DRBC, and the isolated colonies were identified according to the taxonomical descriptions of Pitt and Hocking (2009). *A. flavus* was grown on potato dextrose agar (PDA) medium at 25 °C for 7 days. Spores were collected by scraping the surface of the plates and

diluting them in sterile water adjusted to 0.85 a_w and 0.87 a_w with glycerol containing Tween 80 (0.05% v/v). These a_w were selected in order to simulate fungal growth due to an inadequate control of the environment during transport/storage. After counting the spores on a Thoma chamber, the spore suspensions were then serially diluted to a concentration of 10^7 spores/mL for the germination study and 10^2 spores/mL for the single spore growth and AFB1 production studies.

2.2 Medium

The medium used was 3% (w/v) pistachio extract agar (PEA), prepared as follows: 60 g of raw dehulled ground pistachio in 1 L distilled water were boiled for 30 minutes. After that, the extract was filtered and the amount of evaporated water re-added to adjust it to 6% of pistachio. The water activity of the medium was adjusted by addition of certain amounts of glycerol and water for a final a_w of 0.85 and 0.87 and 3% of pistachio content. 12 g of agar were added per L of medium (for each a_w) and bottles were autoclaved and media poured into 90 mm sterile Petri dishes. All the experiment was prepared under aseptic conditions. 3 Petri dishes for the germination study per a_w (0.85 and 0.87) were prepared. 10 Petri dishes for the growth study and further 10 plates for AFB1 production study, per a_w, were prepared.

2.3 Inoculation, incubation and measurement

2.3.1 Germination study

A 0.1 mL aliquot of the spore suspension (10^7 spores/mL) was inoculated onto PEA (3%) 0.85 and 0.87 a_w and spread on the surface of the medium with a sterile bent glass rod as quickly as possible. 3 Petri dishes per a_w were incubated at 25 °C.

Periodically three agar discs (5 mm diameter) were aseptically removed from the Petri dish using a cork borer, and transferred to a microscopic slide. The slides were then placed under a microscope (Leica DM2000, Barcelona, Spain) for examination of individual spores. 50 single spores per disc were observed (150 spores). Thus for each condition 450 spores were counted along time, until all the spores were germinated.

2.3.2. Growth and AFB1 production studies

A 0.1 mL aliquot of 10^2 spores/mL suspensions were pipetted onto PEA (3%) and spread on the surface of the agar medium with a sterile bent glass rod, aiming to have ca. 5-10 spores per Petri dish. Ten plates were prepared for each a_w. Petri dishes with the same a_w were enclosed in polyethylene boxes together with a glycerol–water solution at the same a_w to maintain the relative humidity inside the boxes. They were incubated at 25 °C. Two parallel experiments were conducted for the growth and AFB1 production studies for both a_w (10x2x2, a total of 40 plates and about 250 colonies).

2.3.3. AFB1 determination

AFB1 production was determined from the first sign of growth up until the end of the incubation time (39 days for all the experiments), in different size colonies (from 0.5 to 7.5 mm radius), using a previously described high performance liquid chromatography (HPLC) method (Aldars-García et al., 2015). A 5-mm diameter agar plug from the centre of each colony was weighed and introduced into 3-mL vials. After sampling, the plates were taken back to incubation, for the assessment of the other colonies present in the Petri plates which were not sampled. 1 mL of methanol was added to vials and vortexed for 5 seconds. After being left stationary for 60 minutes, the extracts were shaken again, filtered (Millex^R SLHV 013NK, Millipore, Bedford, MA, USA), dried in a nitrogen stream and stored at 4 °C until HPLC analysis. All extracts were resuspended with 0.5 mL of methanol:water (50:50 v/v) and a volume of 100 µL was injected into the HPLC system (Waters, Milford, MA, USA). The detection limit of the analysis was 0.1 ng/g of AFB1, based on a signal-to-noise ratio of 3:1.

2.4. Model fitting

2.4.1. Germination

Petri dishes were examined regularly to determine the percentage of germinated spores. Spores were considered to have germinated when the length of the germ tube was greater than or

equal to the diameter of the greatest dimension of the swollen spore (Marín et al., 1998). The percentage of germinated spores was calculated as follows:

$$P(\%)=(N_{germinated\ spores} / N_{total\ spores})*100 \quad (1)$$

The time at which spores were suspended in the solution was defined as the initial time. For each condition, data of P(%) over time were fitted to the modified Gompertz equation [2] (Zwietering et al., 1990):

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

where t (h) is the time, P(%) is the percentage of germinated spores at time t, P_{max} (%) is the asymptotic P(%) value at $t \rightarrow +\infty$, μ_g (1/h) is the slope term of the tangent line through the inflection point (germination rate), and t_g (h) is the geometrical germination time (t-axis intercept of the tangent through the inflection point). By replacing t with t_g in Eq. (2) it can be demonstrated that the geometrical germination time corresponds to the time at which 6.6% of spores have germinated.

Nonlinear regressions were made by using R statistical software (R Development Core Team, www.R-project.org, v 2.14.1). The goodness of fit of the developed models was evaluated graphically and by the coefficient of determination r^2 and the root mean square error (RMSE).

2.4.2. Growth study

During incubation, Petri dishes were examined every day and the radii of the colonies were measured. Radii of growing colonies were plotted against time, and a lineal model with breakthrough (Dantigny et al., 2005) was fitted to the growth data of the colonies to estimate maximum radial growth rate (μ, mm/day) and apparent lag time for growth (λ, day) for each a_w.

$$R_{(t)} = \mu\,(t-\lambda) \quad (3)$$

where t is time (d), $R_{(t)}$ is the radius at time t, μ is the slope of the regression line (maximum growth rate) and λ is the estimated apparent lag time for growth.

The time to visible growth (t_{vg}) (4) (Gougouli and Koutsoumanis, 2013) was defined as the time to reach 3 mm diameter colonies, based on the possibility to see them in a food product. t_{vg} was calculated as:

$$t_{vg} = \lambda + \frac{1.5}{\mu} \quad (4)$$

λ and μ values were estimated through linear regression using R statistical software. The goodness of fit was evaluated by the r^2 and the root mean square error (RMSE). Moreover, the distribution of the estimated parameters was also assessed (with Statgraphics Plus 5.1) in order to generate suitable information for stochastic assessments. Two statistics, standardized skewness and standardized kurtosis, were used to check whether or not the data came from a normal distribution.

$$Skewness = \frac{n}{(n-1)(n-2)} \sum \frac{(X_i - \bar{X})^3}{s^3} = \frac{n}{s^3(n-1)(n-2)} (S_{above} - S_{below}) \quad (5)$$

$$S_{above} = |\sum (X_i - \bar{X})^3| \text{ if } X_i \text{ is above the average}$$

$$S_{below} = |\sum (X_i - \bar{X})^3| \text{ if } X_i \text{ is below the average}$$

S_{above} is the "size" of the deviations from average when X_i is above the average, and S_{below} is the "size" of the deviations from average when X_i is below the average.

$$Kurtosis = \frac{n(n+1)}{(n-1)(n-2)(n-3))} \sum \frac{(X_i - \bar{X})^4}{s^4} - \frac{3(n-1)^2}{(n-2)(n-3)} \quad (6)$$

where n is the sample size, X_i is the i^{th} X value, X is the average and s is the sample standard deviation.

2.4.3. Probability of growth and AFB1 production

Taking into account the total final number of colonies reached in each plate at the end of the experiment, every incubation day a 1 value was assigned to each new visible colony, while 0

values were allocated to the still non detectable colonies. AFB1 was considered to be present from values higher than 0.1 ppb (limit of detection of the HPLC equipment). A logistic regression was applied to binary data in order to model the probability of growth and AFB1 production as a function of time, and AFB1 production as a function of the radius of the colony of *A. flavus*, using R statistical software, with the glm function. Thus the models developed in the present study are not based on any biological and/or conceptual assumption.

$$logit(P_G) = ln\ \frac{P_G(x)}{1-P_G(x)} = b_0 + b_1 t \quad (7)$$

where P_G is the percentage of colonies which were visible at a given time. P_G=detectable colonies/total colonies at the end of the growth experiment.

$$logit(P_{AF}) = ln\ \frac{P_{AF}(x)}{1-P_{AF}(x)} = b_0 + b_1 t \quad (8)$$

Where P_{AF} is the percentage of colonies with detectable AFB1 at a given time. P_{AF}= AFB1 positive colonies /total colonies at the end of the AFB1 production experiment.

Where logit (P_G or P_{AF}) represents ln[P_G or P_{AF} /(1- P_G or P_{AF})], ln is the natural logarithm, P_G and P_{AF} are the probability of growth or AFB1 production initiation (in the range of 0–1), t is the time, and b_i are the coefficients to be estimated.

The percentage of concordance (%C) between observed and predicted values with a cut off of 0.5 was calculated to measure the goodness-of-fit of the developed logistic models.

$$\%C = \frac{Number\ of\ correct\ predicitions}{Number\ of\ total\ predicions} * 100 \quad (9)$$

3. Results

3.1. Kinetic parameters from the germination and growth studies

Germination curves (population level) were generated based on the cumulative frequency of the germinated spores over time. All spores developed germ tubes. Germination occurred earlier at 0.87 than at 0.85 a_w (Fig. 1), t_g were 56.70 hours for the 0.85 a_w experiment and 45.19 hours for

the 0.87 a_w experiment. However there was not a significant difference between the two a_w for the t_g neither for the μ_g (p-value >0.05). These results indicated that ability of the individual spores to germinate on the PEA was not significantly affected by the 0.02a_w difference tested in this study.

Table 1. Summary statistics for the apparent lag time for growth (λ) and the mycelium growth rate (μ) for both a_w.

	0.85 a_w		**0.87 a_w**	
	λ (d)	**μ (mm/d)**	**λ (d)**	**μ (mm/d)**
Mean	16.63	0.18	14.85	0.41
Median	16.39	0.17	15.27	0.37
Standard deviation	2.64	0.10	2.13	0.10
Variation coefficient	0.16	0.33	0.14	0.31
Stnd. skewness	3.51	4.56	-1.90	2.96
Stnd. kurtosis	8.80	7.35	-0.60	2.67
No. colonies	72	72	55	55

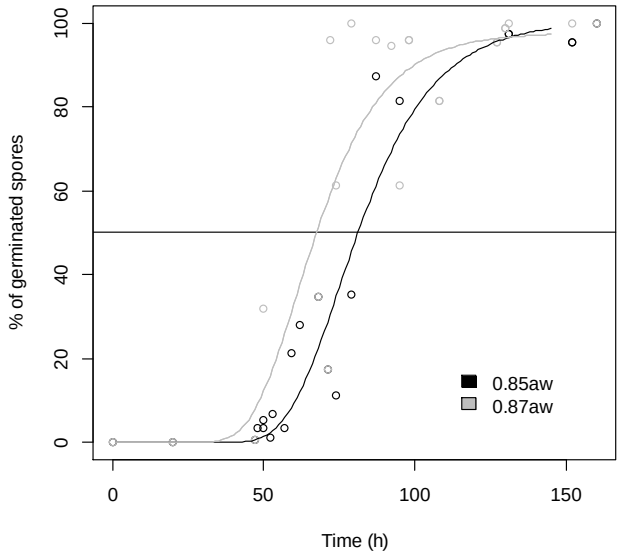

Figure 1. Percentage of germinated spores of *A. flavus* on PEA (3%) at 25 °C at 0.85 and 0.87 a_w. The lines depict the fitting of the Gompertz model to the germination data. Points (○) represent observed values of the percentage of germinated spores.

The Gompertz model described satisfactorily the percentage of germinated spores over time for both tested conditions with $r^2 > 0.82$ and RMSE < 0.06, for 0.85 and 0.87 a_w (all pooled replicates together).

The suboptimal conditions for growth set for these experiments, led to a colony radius increase which followed a two step growth function. All growth curves showed linear fungal growth after an initial lag period, then a linear model was fitted to the data (Eq. (3)). The λ and the μ were estimated using the linear model as described in Eq. (3). No linear correlation was found between μ and 1/ λ. ($R^2_{0.85=}$ 0.13, $R^2_{0.87}$=0.04).

For 0.85 a_w the λ values ranged from 9.48 ± 0.043 to 20.54 ± 0.15 days (r^2 = 0.613–0.998, RMSE = 0.004–0.03) (Table 1). At 0.87 a_w, λ ranged from 9.75 ± 0.012 to 17.9 ± 0.004 days (r^2 = 0.750–0.991, RMSE = 0.005–0.06). The λ occurred after the completion of the germination process, i.e. 100% germinated spores, shown above.

The distribution of λ and μ of the individual spores of *A. flavus* at both a_w is illustrated by the histograms in Fig. 2. The standardized skewness and standardized kurtosis (table 1) determine whether the sample comes from a normal distribution; values of these statistics outside the range of -2 to +2 indicate significant deviation from normality, thus considering this, only the λ at 0.87 a_w followed a normal distribution (Fig 2.) The positive skewness values for μ for both conditions (Table 1) indicated that the distribution was not centered, and more density of data was found on the left side of the distribution, suggesting that only a small subpopulation of the inoculated spores led to fast growing colonies, this being more pronounced in 0.87 a_w experiments (Fig. 2)**.**

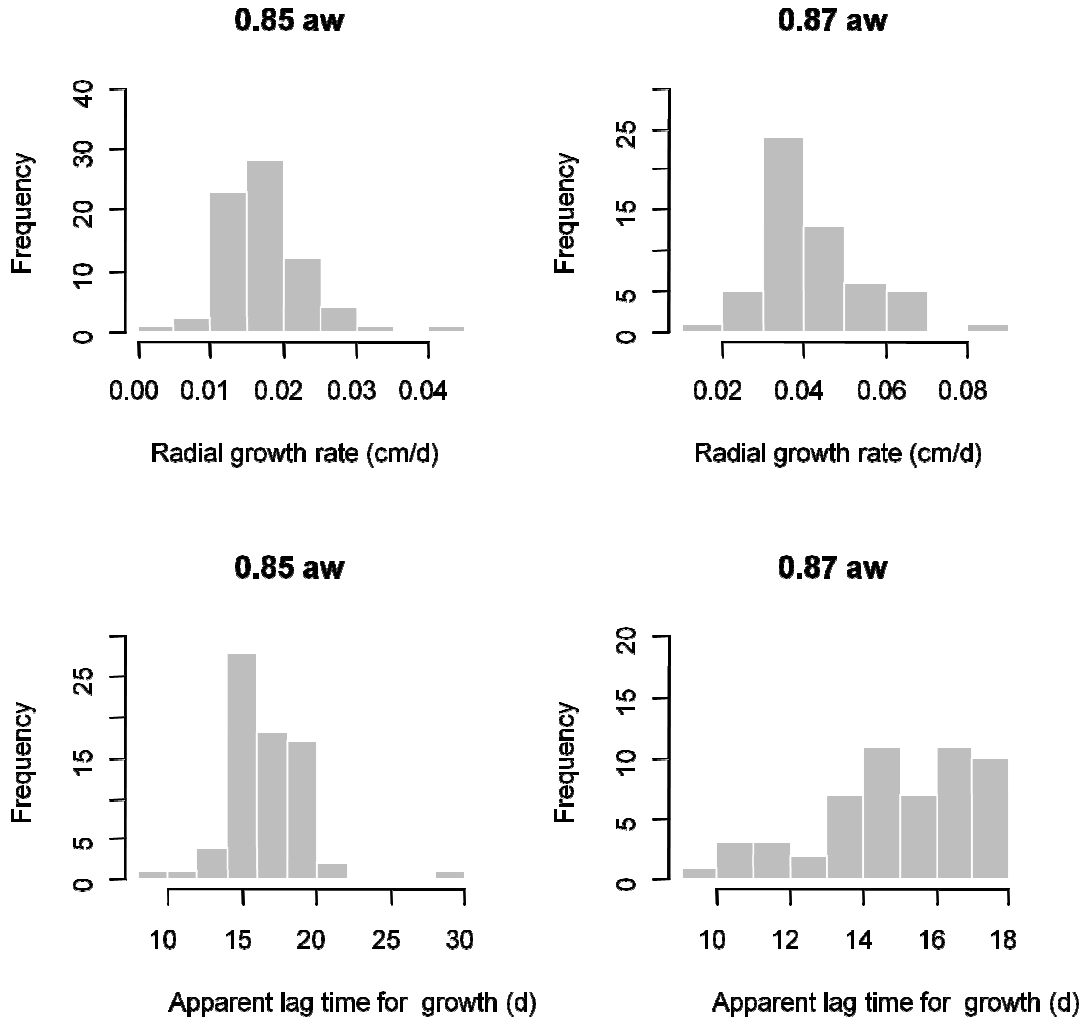

Figure 2. Distribution of the apparent lag time for growth and mycelium growth rates of individual spores of *A. flavus* at 25 °C at 0.85 and 0.87 a_w.

Results of mycelial growth indicated that μ were more widely distributed than the λ (variation coefficients in table 1). For the 0.87 a_w experiment the apparent lag time values were within a narrower range than that of lag time at 0.85 a_w experiment, with more spread frequencies in the later case. This infers that when conditions become more stressful, more variability of the

results will be found. In the case of the growth rates, distributions were similar, but higher rates were found at 0.87 a_w.

3.2. Probability of growth and AFB1 production over time

The probability curves for growth and AFB1 production over time are shown in Fig. 3. The results showed that the logistic curves for each a_w had almost the same shape but shifted to earlier values for the 0.87 a_w experiment. The same trend was also reported in the AFB1 production probability. Concordance index, which indicates the percentage of correctly predicted values with a cut off of 0.5, was used to assess goodness of fit of the developed probabilistic models. They were 95.78% and 96.55%, for 0.85 and 0.87 a_w, for the growth models, and 93.33% and 85%, for 0.85 and 0.87 a_w, for AFB1 production models.

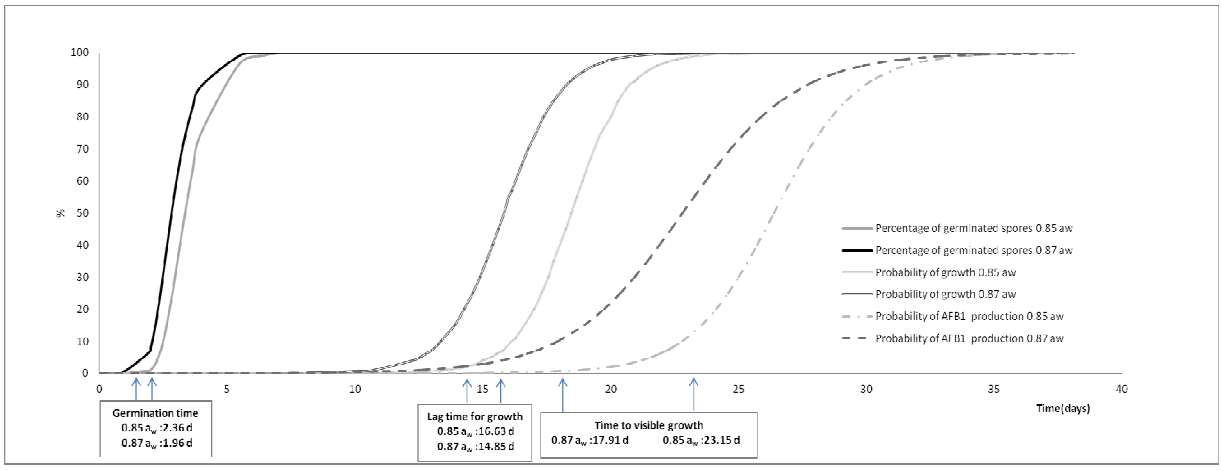

Figure 3. Variation of the percentage of germinated spores, probability of growth and probability of AFB1 production over time for the conditions studied.

Table 2. Mean radius and mean aflatoxin B1 concentration of *A. flavus* colonies at 25 °C. For shorter times, no AFB1 was detected.

Incubation time (days)	0.85 a_w experiment			0.87 a_w experiment		
	n	Radius (mm)	Aflatoxin B1 (ng/g PEA)	n	Radius (mm)	Aflatoxin B1 (ng/g PEA)
17	2	0.05 ± 0.01	-	6	0.09 ± 0.06	-
19	3	0.05 ± 0.00	-	9	0.14 ± 0.06	0.18 ± 0.43
22	4	0.11 ± .006	0.35 ± 0.69	4	0.18 ± 0.05	0.63 ± 0.91
24	4	0.12 ± 0.04	0.23 ± 0.44	8	0.25 ± 0.07	0.89 ± 1.06
26	6	0.14 ± 0.04	0.16 ± 0.39	6	0.28 ± 0.06	1.12 ± 1.09
30	5	0.19 ± 0.04	2.99 ± 1.71	7	0.32 ± 0.13	3.63 ± 2.95
32	5	0.17 ± 0.02	2.13 ± 0.83	5	0.34 ± 0.07	5.63 ± 2.87
36	5	0.25 ± 0.03	11.17 ± 1.52	4	0.48 ± 0.15	7.26 ± 3.58
39	3	0.27 ± 0.05	3.44 ±1.73	3	0.64 ± 0.21	5.09 ± 2.41

n: number of colonies collected at each time point. Different number of colonies were collected depending on the total number of colonies arisen on the Petri plates for each condition.

Values are means ± standard deviation (n).

For this experiment, based on colonies from single spores, at both a_w, no chance of predicted AFB1 production was obtained until almost 50% of growth probability had been reached, i.e. once growth reached the 100% probability, there was a probability of 40 and 57% of detecting AFB1 in the sample. Indeed, at 0.85 a_w experimental AFB1 data showed a time delay after 100% of the colonies had appeared (Fig 4). From figure 4 it can be depicted that the larger the colony size, the higher the probability of having AFB1 production. In general, colonies smaller than 2mm of radius contained less than 4ng AFB1/g agar (Table 2), while bigger colonies contained up to 14 ng AFB1/g agar (Table 2). Besides a radius threshold could be established for a certain AFB1 production probability, for example, at least 1 mm of colony radius was necessary to have any chance of AFB1 production, and colonies larger than 1.5 mm of diameter have approximately 20% or more probability of AFB1 production, under the studied conditions.

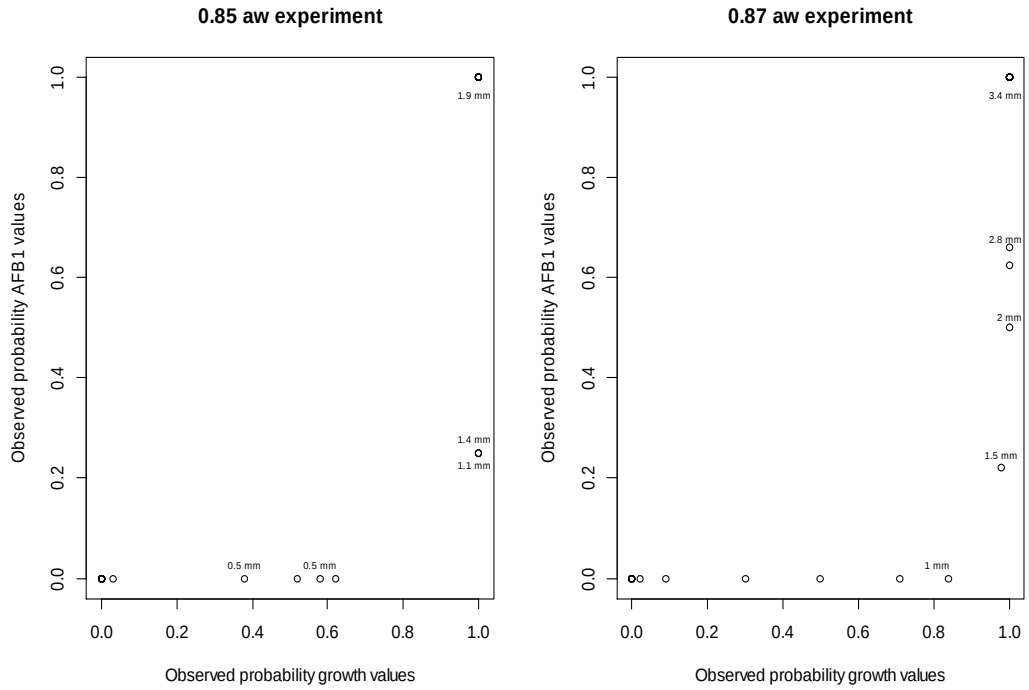

Figure 4. Correlation between observed probability of growth and observed probability of AFB1 production. Mean radii (mm) of the colonies for the given probabilities are shown in the chart.

Table 3. Estimated time probabilities (t10, t50 and t90 in days) of growth and AFB1 production at 25 °C for both a_w

		0.85 a_w	**0.87 a_w**
Probability of growth	**t10**	16.12	13.37
	t50	18.45	15.82
	t90	20.78	18.27
Probability of AFB1 production	**t10**	22.80	17.94
	t50	26.38	22.87
	t90	29.96	27.67

Estimated time to reach 0.10 (t10), 0.50 (t50) and 0.90 (t90) probabilty values are shown in Table 3. In 4-5 days probabilities of growth shifted from 10% to 90% (Fig. 3). This suggests that although germination takes place in a brief lapse of time once contamination occurs (see section 3.1.), visible growth is a slower and progressive phenomenom. Similarly, the AFB1 production curves showed that aproximately 9 days were required from 10% to 90% probability of AF production. Probability of AFB1 production curves paralleled those for growth, but they were less steep, thereby slightly longer times were observed amongst the three estimated times (t10, t50, t90).

3.3. Time-course of germination, growth and AFB1 studies

The germination time, apparent lag time for growth and time to visible growth were compared (Table 4). Germination time is the time to have 6.6% of spores germinated (see Gompertz model in section 2.4.1). Considering that the apparent lag time is estimated by the extrapolation of the mycelium radial growth curve to radius=0 (Horner and Anagnostopoulos, 1973), it would be expected to follow the germination time in a short period. In our case the results showed a significant delay of the λ compared to the t_g, the differences were 14.27 and 12.89 days for 0.85 and 0.87 a_w respectively; even more, the λ occurred 9.63 and 8.40 days after 100% of the spores had germinated, for 0.85 and 0.87 a_w respectively. These delays are explained by the fact that water activities of the experiment were suboptimal for mould growth. Once λ had been reached, another 6.52 days were needed to see the mycelium with a naked eye at 0.85 a_w and half that time was needed (3.06 days) for the 0.87 a_w experiment (Fig. 3).

The estimated λ took place at a 0.15 probability of growth for 0.85 a_w and 0.29 for 0.87 a_w, which may be expectable, as λ is calculated as the departure from colony size equal to zero, and probabilities are calculated on the basis of visible colonies. On the other hand, the times to visible growth coincided with 0.99 and 0.87 probabilities of growth for 0.85 and 0.87 a_w.

In the case of AFB1 production probability, λ finished with only 0.06 and 0.03 probability of AFB1 production, while probabilities were 0.11 and 0.10, at 0.85 and 0.87 a_w respectively, when visible growth was observed.

Table 4. Comparison among germination time (t_g), apparent lag times for growth and time to visible growth (t_{vg}) for *A. flavus* at 25 °C, at 0.85 and 0.87 a_w.

	t_g (days)		λ (days)		t_{vg} (days)	
a_w	Mean	SD	Mean	SD	Mean	SD
0.85	2.36	0.32	16.63	2.64	23.15	3.67
0.87	1.96	0.35	14.85	2.13	17.91	2.57

The time relation of the studied events is shown in Fig. 3. In the event of one spore contaminating a foodstuff, a long period since germination would be needed to have a significant chance for AFB1 detection. Once germination had been completed, a period of approximately 16 and 14 days, for 0.85 and 0.87 a_w respectively, was necessary for initiation of fungal growth, i.e. 0.01 probability of growth. Time to visible growth happened when almost 100% probability of growth had been reached. Values were 98.82 and 86.71% probabilities of growth for 0.85 and 0.87 a_w respectively. Once reached the t_{vg} there was about 10% probability of AFB1 production at both a_w. After growth had begun, the probability of AFB1 production arose, however at the beginning the probability values were low, and increased slowly, thus until day 27 and 23 for 0.85 and 0.87 a_w, respectively, the 50% probability of AFB1 production was not reached (Fig. 3).

4. Discussion

The data presented in this study can provide information regarding the timing of the biological events involved in fungal development, i.e., germination, mycelial proliferation and mycotoxin production under marginal conditions. Although the behavior of the fungi may vary depending on the food matrix and other interacting factors, experiments were carried out on PEA (3%) at 0.85 and 0.87 a_w at 25 °C, illustrating a possible contamination scenario during transport/storage of pistachio due to an inadequate control of the humidity of the environment, although not much differences were observed between these a_w levels.

Spore germination is a stage prior mycelial growth, which involves spore swelling and germ tube formation (d´Enfert, 1997), thus its prevention may be the main step to prevent the contamination of a foodstuff with aflatoxins. In our study t_g represented 14.19% and 13.20%, for 0.85 and 0.87 a_w respectively, of the λ, and 10.19% and 10.94%, for 0.85 and 0.87 a_w respectively, of the t_{vg}. The issue that emerges here is why the apparent lag times of single spores are that much higher than the t_g, even if the later is a population parameter. Burgain et al. (2013) obtained similar results when they studied the relation between the germination time and the time to visible growth of *Penicillium chrysogenum* on potato dextrose agar (PDA) at room temperature (18 °C to 25 °C) with different inoculum levels (10^1–10^5 spores at inoculation point). Their t_g accounted for 18.1 to 24.3% of the t_{vg}, being far from the λ, but as their a_w was less limiting (0.93-0.99) than ours, their difference between the parameters was narrower. Gougouli and Koutsoumanis (2013) reported that estimating the apparent lag time for fungal growth through the linear model may lead to some overestimation of λ, thus its distance from the t_g would be wider. According to Trinci (1969), elongation of the germ tube from the swollen spore is exponential for a certain period, then it becomes linear. Thus, in our case, the hypothesis that prevention of germination may be a way to prevent toxin production may be too conservative.

Distribution graphs for λ showed that few spores resulted in fast growing colonies and most of the spores took more time to growth. When working with a large inoculum instead of a single spore inoculum, a small number of spores may lead to a high probability of germination and growth. Morales et al. (2008) reported that higher inoculum size led to shorter apparent lag times when working with 10^4 and 10^6 spores/mL inocula of *Penicillium expansum* in apples. Similar results were obtained by Baert et al. (2008) when they tested the effect of inoculum size on the growth of *P. expansum* in apples obtaining that the inoculum size influences the estimated growth parameters and that using a low inoculum level will also result in a larger variability of the estimated apparent lag time.

Our results also showed a delay between growth and AFB1 production. Garcia et al. (2013) determined that aflatoxin production may follow a mixed-growth associated model suggesting that toxin formation does not present a clear delay in relation to growth under certain conditions. They worked with maize agar medium and maize grain at 0.90 and 0.99 aw at 25 °C

with a central inoculation of 5 µL of a 10^4 spores/mL suspension. This apparent contradiction could be explained by the fact that working under a less restrictive environment and a higher inoculum size allows the global inoculum to show simultaneous growth and aflatoxin production.

The t_{vg} parameter is particularly interesting as it corresponds approximately to the t10 estimated probability of AFB1 production for both a_w. A product is considered spoiled as soon as visible mycelium is present, then t_{vg} not only could be used as an spoilage indicator but also as an AFB1 presence indicator. Colony size in early stages of growth (from 1 to 2 mm of radius), could be a helpful indicator of the possible AFB1 contamination in the commodity, however, the detection of such small size colonies could be challenging in most foodstuffs. It has been shown, also in pistachio medium, that the detection of AFB1 in colonies is more dependent on their size than on the age of such colonies (Aldars-García et al., 2016)

Our results showed a significant delay between growth and AFB1 production models, taking approximately 6 days to produce AFB1 once the probability of growth started to increase. Several authors have reported that mild stress conditions seem to induce mycotoxin production, but when increasing the stress conditions an inhibitory effect is produced (Baert et al., 2007a; Jurado et al., 2008). The a_w herein reported is rather stressful for *A. flavus*, thus AFB1 may not be inhibited but delayed. These findings may suggest that due to the limiting a_w, toxin production would not start until all the mechanisms of growth have been launched, even when temperature is not a restrictive parameter. As far as AFB1 presence in the commodity is concerned, under the suboptimal environment set for these experiments (similar to those encountered during transport/storage of pistachio nuts), forecasting AFB1 presence from growth probability models may overestimate its presence representing a worst case scenario of food contamination. A safe period of 12-18 days, once germination has occurred, and 5-7 days from the apparent lag time (probability of growth of 0.01) is predicted before AFB1 production detection, if water activity does not surpass 0.87.In terms of probability of growth, a minimum of 0.6 would have to be reached to attain a 0.05 probability of AFB1 production in the commodity (in our less restrictive condition).

It is important to note that estimations of AFB1 production may depend on the established conditions to carry out the experiment, and on the limit of detection (LOD) determined in the HPLC method.

In conclusion, the generated results enabled to follow the time progression of *A. flavus* development on a pistachio extract under marginal conditions. Single spore assays are a good approach to model the real situation in fungal contamination of commodities. Keeping in view the interests of the food industry, setting a maximum growth probability which may lead to, e.g. 0.05 probability of AFB1 production is an interesting enforcement, since very small amount of this toxin is legally allowed.

Developing these tools to predict the probability of presence of aflatoxins in food and feedstuffs, from early stages, will help to devise control strategies to shut down aflatoxin production through manipulation of environmental conditions.

5. Acknowledgments

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Chapter III

Probability models for growth and aflatoxin B1 production as affected by intraspecies variability in *Aspergillus flavus*

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Food Microbiology (Submitted)

Abstract

The probability of growth and AFB1 production of 20 isolates of *Aspergillus flavus* was studied using a full factorial design with eight water activity levels (0.84 to 0.98) and six temperature levels (15 to 40 °C). Binary data obtained from growth studies were modelled using linear logistic regression analysis as a function of temperature, water activity and time, for each isolate. In parallel, aflatoxin B1 was extracted at different times from newly formed colonies (up to 20mm of diameter). Although a total of 950 AFB1 values along time for all conditions studied were recorded, they were not considered to be enough to build probability models along time, and only models at 30 days were built. Confidence intervals of the regression coefficients of probability of growth models showed some differences among the 20 growth models. Further, to assess the growth/no growth and AFB1/no-AFB1 production boundaries, 0.05 and 0.5 probabilities were plotted at 30 days for all the strains. Boundaries for growth and AFB1 showed that in general the conditions for growth were wider than those for AFB1 production. Probability of growth and AFB1 production initiation seemed to be less variable among strains than AFB1 accumulation is. Apart from AFB1 production probability models, using growth probability models for AFB1 probability prediction could be, although conservative, a suitable alternative. Predictive mycology should include a number of strains to generate data to build predictive models and take into account the genetic diversity of the species and thus make predictions as similar as possible to real fungal food contamination.

Keywords intraspecies variability; predictive mycology; probability models; Aspergillus; Aflatoxin;

1. Introduction

Mould spoilage and mycotoxin contamination of food products cause large economic losses (Dantigny et al., 2005; Mitchell et al., 2016) and pose a serious risk to public health (Marín et al., 2013). *Aspergillus* spp. is recognized as one of the most widely distributed fungal genera in nature. The two most agriculturally important species are *Aspergillus flavus* and *A. parasiticus*, which are found all over the world, being present in both the soil and the air (Hedayati et al., 2007; Horn and Dorner, 1998; Wicklow et al., 1998). *A. flavus* is the major causal agent of food and feed contamination with aflatoxins (AFs) (Klich, 2007). AFs (B1,B2,G1,and G2) are a group of toxic, mutagenic, carcinogenic and teratogenic secondary metabolites which are health hazards to humans and animals (Bottalico, 1999). Aflatoxin B1 (AFB1) is reported as the most toxic natural compound and is classified by the International Agency for Research on Cancer (IARC) as a class 1 toxin (IARC, 2002) due to its demonstrated carcinogenicity to humans.

Both fungal growth and mycotoxin production can be influenced by different factors such as temperature, water activity (a_w), inoculum concentration, isolate, microbial interactions, physiological state of mould, genotype, etc., and these factors may affect in a different way growth and mycotoxin production (Garcia et al., 2009).

Populations of *A. flavus* in agricultural products and foods are complex communities that may contain many different strains. Their growth and AFB1 production potential are known to vary (Abbas et al., 2004; Adhikari et al., 2016; Singh et al., 2015; Yogendrarajah et al., 2016; Yousefi et al., 2009). There are many studies reporting the variability among isolates in terms of growth and mycotoxin production (Abbas et al., 2005; Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Pardo et al., 2005, 2004; Parra and Magan, 2004; Romero et al., 2007; Singh et al., 2015; Yogendrarajah et al., 2016). Garcia et al. (2011a) studied the growth and ochratoxin A (OTA) production of thirty isolates *A. carbonarius*. Their results showed a wide dispersion in both growth rate and mycotoxin production, especially under marginal conditions. Foods are generally stored under marginal conditions, of either a_w or temperature, for fungal growth and subsequent mycotoxin production. In these cases growth is compromised but still occurs, thus knowing the growth and AFs production boundaries (growth/no growth and toxin/no-toxin interface) of microorganisms becomes primordial for the food safety.

Predictive models are helpful tools to estimate the safety and shelf-life of foods. Within these predictive models, probabilistic models are used to predict the probability of growth or mycotoxin production of a microorganism under different conditions (Tienungoon et al., 2000). Logistic regression is a useful method for modelling boundaries between growth and no growth or mycotoxin production and no mycotoxin production of fungi (Aldars-García et al., 2016a, 2015; Astoreca et al., 2012; Garcia et al., 2011c; García-Cela et al., 2014; Marín et al., 2012, 2009; Tassou et al., 2009). An important aspect of predictive model development is ensuring that predictions made by the models are applicable to real situations. Thus, predictive models should take into account suboptimal conditions (the usual storage food environment) and strain variability, because in natural ecosystems different strain can occupy the same niche. The objective of the present work was to check if the intraspecies variability detected for growth and toxin production in kinetic models can be overcome by the use of probability models.

The particular aim of the present work was to develop probabilistic models for 20 isolates of *A. flavus* isolated from foodstuffs on maize grain extract medium as a function of temperature and a_w in order to explore the possibility of using models built on one strain to predict the behaviour of the others in the same species.

2. Materials and methods

2.1. Strains

Twenty isolates of *A. flavus* isolated from maize grains, pistachio nuts, and chilli were tested in this study. A previous study was conducted in order to characterize the isolates in terms of their ability to produce AFB1 on Potato Dextrose Agar (PDA) at 25 °C, after 7 incubation days. Isolates were categorized as “low AF producer” if AFB1 levels were between LOD and 100 ng/g agar and “high AF producer” for isolates that produced concentrations higher than 100 ng/g agar. Results of this previous study and more details of the isolates studied are shown in Table 1.

Table 1. Description of the isolates used in the present study.

Isolate code*	Origin	AFB1 production in 7 days on PDA at 25 °C (ng/g agar)	Category
UdL-TA 3.268	Pistachio nuts	471.2	High producer
UdL-TA 3.270	Pistachio nuts	114.8	High producer
UdL-TA 3.318	Maize grains	1189.3	High producer
UdL-TA 3.321	Maize grains	748.3	High producer
UdL-TA 3.322	Maize grains	698.1	High producer
UdL-TA 3.327	Maize grains	178.5	High producer
UdL-TA 3.328	Maize grains	243.6	High producer
UdL-TA 3.329	Maize grains	109.3	High producer
UdL-TA 3.331	Maize grains	547.2	High producer
UdL-TA 3.332	Maize grains	2114.6	High producer
UdL-TA 3.244	Chilli	20.5	Low producer
UdL-TA 3.267	Pistachio nuts	25.9	Low producer
UdL-TA 3.269	Pistachio nuts	28.3	Low producer
UdL-TA 3.319	Maize grains	39.7	Low producer
UdL-TA 3.320	Maize grains	1.9	Low producer
UdL-TA 3.323	Maize grains	3.9	Low producer
UdL-TA 3.324	Maize grains	5.4	Low producer
UdL-TA 3.325	Maize grains	1.5	Low producer
UdL-TA 3.326	Maize grains	52.3	Low producer
UdL-TA 3.330	Maize grains	37.2	Low producer

*The isolate names are the codes of cultures held in the Food Technology Department Culture Collection of University of Lleida, Spain.

2.2. Inoculum and media preparation

The twenty isolates were sub-cultured on PDA plates and incubated at 25 °C for 7 days to obtain heavily sporulating cultures. After incubation, spores were collected by scraping the surface of the plates, diluting them in sterile water adjusted to a_w values 0.84, 0.86, 0.88, 0.90, 0.92, 0.94, 0.96 and 0.98 with glycerol containing Tween 80 (0.05% v/v), and filtered through sterile glass wool into a tube. Immediately total spore concentrations were determined using a Thoma counting chamber and decimal dilutions (in sterile water adjusted to the correspondent a_w value with glycerol, containing Tween 80 (0.05% v/v)), were prepared to adjust the final concentration to 10^2 spores/mL for each a_w and strain.

The basic medium used in this study was maize extract agar (MEA) adjusted to the 8 different a_w. The medium was made by boiling 40 g of raw ground dry maize grains in 1 L distilled water for 30 min. After that, the extract was filtered and the amount of evaporated water was made up to adjust it to 4% of maize extract. Water activity of the media was adjusted by addition of certain amounts of glycerol-water to obtain the a_w of each treatment and 2% of maize grain in the medium. Then, 12 g of agar were added per L of medium (for each a_w) and they were autoclaved and poured into 90 mm sterile Petri dishes which were prepared under aseptic conditions. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003.

2.3. Experimental design

A full factorial design with 8 a_w (0.84, 0.86, 0.88, 0.90, 0.92, 0.94, 0.96 and 0.98) and 6 temperature (15, 20, 25, 30, 35 and 40 °C) levels was built to study the growth and AFB1 production of several *A. flavus* isolates on maize extract agar (MEA) during 39 days. To carry out the experiment not all the combinations within the a_w and temperature range were studied. We aimed to study those conditions were growth (and then also mycotoxin production) were compromised. The T-a_w combinations studied are shown in Figure 1, a total of 32 T-a_w conditions.

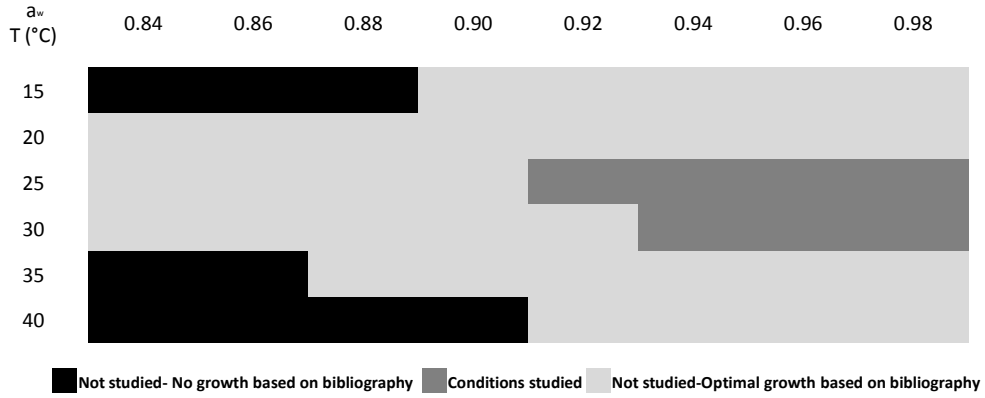

Figure 1. Temperature and water activity combinations studied in the present study.

2.4. Inoculation and incubation

The growth andAFB1 production by *A. flavus* grown from single spores were assessed in the present study. A 0.2 mL aliquot of 10^2 spores/mL suspensions was surface plated onto MEA (2%) and spread with a sterile bent glass rod, in order to obtain ca. 20 spores per Petri dish (then ca. 20 colonies originating from one spore each). Petri dishes with the same a_w were enclosed in polyethylene boxes together with a glycerol–water solutions at the same a_w to maintain the relative humidity inside the boxes. Plates were incubated at the required temperature and each Petri dish was checked daily (one Petri dish per isolate and T-a_w combination, a total of 640 Petri plates).

2.5. Growth assessment and AFB1 determination

Growth initiation was assessed periodically, daily or as required. Growth was considered to have initiated when colony diameters were greater than 2 mm.

AFB1 presence was determined at certain time intervals depending on how many colonies had arisen on each Petri dish. Colony size of colonies taken for AFB1 analysis ranged from 2 to 30 mm diameter. Growth was assessed by measuring the perpendicular colony diameters in millimetres. A 5-mm agar plug was taken from the centre of a colony at appropriate time

intervals. After sampling, the plates were taken back to incubation, for latter assessment of the other colonies present in the Petri plates. Plugs were weighed and vortexed for approximately 5 seconds in 1mL of methanol and left stationary. After 1 hour, extracts were vortexed again and filtered (Millex^R SLHV 013NK, Millipore, Bedford, MA, USA). Extracts were dried in a nitrogen stream and stored at 4 °C until HPLC analysis. The analysis was carried out using a previously described high performance liquid chromatography (HPLC) method (Aldars-García et al., 2015). For the HPLC analysis all extracts were resuspended with 0.5 mL of methanol:water (50:50 v/v) and 100 µL was injected in the HPLC system (Waters, Milford, MA, USA). 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. Logistic models development

Logistic regression was used to calculate the probabilities of growth (Eq.1)) as a function of time, temperature and a_w and AFB1 production (Eq. 2) as a function of temperature and a_w. Logistic regression describes the log odds of the event, which is the natural logarithm of the probability of the event occurring (P) divided by the probability of the event not occurring (1 − P).

The binary values (0=no visible growth/no AFB1 detection; 1= growth/AFB1 detection) were adjusted by linear logistic regression, in order to obtain all the probability models; one for probability of growth and one for AFB1 production probability for each strain (a total of forty probability models).

$$logit(P_G) = ln\ \frac{P_G(x)}{1-P_G(x)} = b_0 + b_1 t + b_2 T + b_3 aw + b_4 t^2 + b_5 tT + b_6 t\ aw + b_7 T^2 + b_8 t\ aw + b_9 aw^2 \quad (1)$$

$$logit(P_{AF}) = ln\ \frac{P_{AF}(x)}{1-P_{AF}(x)} = b_0 + b_1 T + b_2 aw + b_3 T^2 + b_4 aw^2 + b_5 T\ aw \quad (2)$$

Where P_G and P_{AF} are the probability of growth or AFB1 production (in the range of 0–1), t is the time, T is the temperature in °C, aw is the water activity and b_i are the coefficients to be estimated.

The goodness of fit of the forty logistic models was assessed by means of the percentage of concordance (%C).

As we aimed to make predictions in real scenarios were conditions are usually restrictive for growth and mycotoxin production, most of the conditions set in the present study were highly compromising for the events aforementioned. Thus, under most of the conditions there were a number of spores which never germinated and developed colonies. Consequently, for each case, P was calculated as follows:

$$P = \frac{n}{n_T}(P_G \; or \; P_{AF} \; from \; logistic \; models) + \frac{1-n}{n_T}(0)$$

$$\text{Then } P = \frac{n}{n_T}(P_G \; or \; P_{AF})$$

Where n is the number of growing colonies and n_T is the potential number of colonies which could have grown according to the number of inoculated spores, calculated as 16 or 21 in the two different runs in which the experiments were performed.

3. Results

The total number of conditions studied in the present work were 32 (Fig. 1), for each strain. The large number of isolates included in this study permitted a comprehensive investigation of the relationships among growth, AFB1 and producing strains. All probability models developed described satisfactorily the phenomena studied. %C of the models for each strain are shown in Table 2. The probability models included square and interaction terms. Adding an interaction term to a model changes the interpretation of all of the coefficients. For example, if there were no interaction term, b_2 in eq. 2 would be interpreted as the unique effect of temperature on the probability of growth. But the interactions mean that the effect of temperature on the probability of growth is different for example for different values of a_w. So the unique effect of

temperature on the probability of growth is not limited to b_2 (in eq. (2)), but also depends on the values of other regression coefficients.

Table2. Percentage of concordance (%C) of the 40 logistic models developed.

Isolate name	Probability growth models	ProbabilityAFB1 production models
	%C	%C
UdL-TA 3.268	91.08	84.91
UdL-TA 3.270	96.23	90.00
UdL-TA 3.318	96.63	87.04
UdL-TA 3.321	97.80	83.33
UdL-TA 3.322	97.38	80.36
UdL-TA 3.327	97.83	92.10
UdL-TA 3.328	97.95	75.00
UdL-TA 3.329	98.32	83.02
UdL-TA 3.331	96.68	88.89
UdL-TA 3.332	97.70	88.89
UdL-TA 3.244	97.47	88.00
UdL-TA 3.267	97.86	82.50
UdL-TA 3.269	96.60	87.18
UdL-TA 3.319	96.68	78.95
UdL-TA 3.320	96.71	82.00
UdL-TA 3.323	90.03	90.52
UdL-TA 3.324	97.95	76,60
UdL-TA 3.325	95.31	91.67
UdL-TA 3.326	94.56	91.11
UdL-TA 3.330	97.72	90.00

Effect of water activity and temperature on intraspecific differences in growth

Firstly, n/n_T was calculated for the different conditions and strains, it varied from 0 under no growth conditions to 1 under the most suitable conditions. A polynomial model was fitted to n/n_T values for each strain, including only 0 values surrounding the positive growth conditions.

Figure 2 shows a graphical example of one of these models. Regarding this fitting, in several cases there was a certain disagreement between observed and predicted values at 40 °C, where observed data were 0 under certain a_w levels (usually 0.92) and 1 when a_w increased just in 0.02 units (usually to 0.94), in such cases predicted values at 0.94-0.98 were lower than the observed ones.

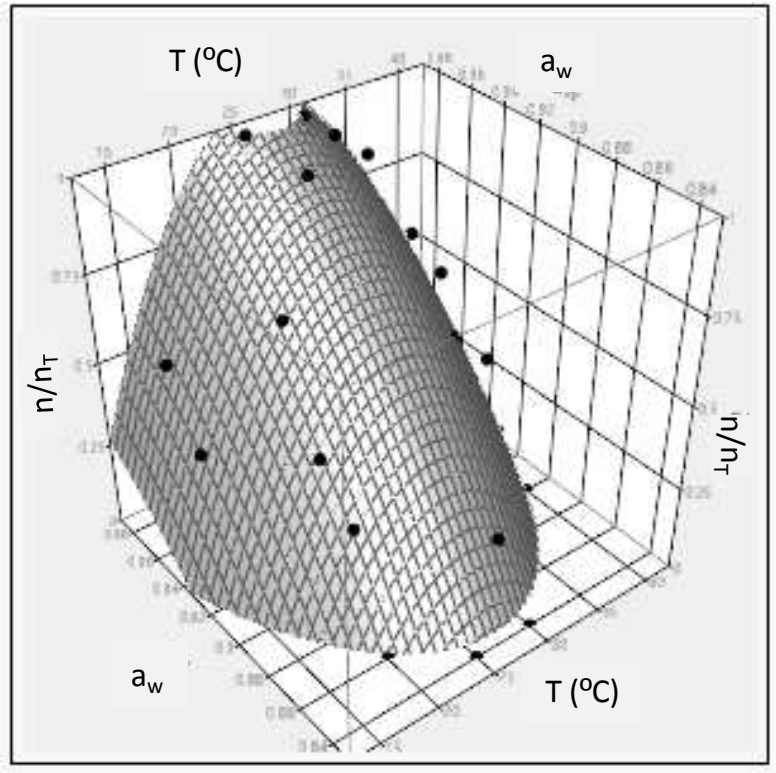

Figure 2. Graphical example of a polynomial model fitted to n/n_T values for isolate UdL-TA 3.269. Dots represent the observed points.

As an example, Table 3 shows the percentage of spores which led to growing colonies (n/n_T,) at 20 °C and 0.88 a_w. The maximum percentage of spores which developed to colonies tells us about the ability of each strain to initiate growth under a stressing environment. Almost all 20 isolates found it hard to start growing under this set marginal condition, the maximum n/n_T was

up to ca. 30% except for isolates UdL-TA 3.370 and UdL-TA 3.318 and in to a lesser extent isolate UdL-TA 3.325. Notwithstanding this percentage, similar times to reach the maximum number of colonies under this marginal condition were obtained for the 20 isolates, most of them ranged between 4.3 and 6 days; suggesting a possible maximum period of time of adaptation after which the spores are not able to initiate growth regardless the total % of germinated spores.

Table 3. Maximum proportion of spores which initiated growth n/n_T) at 20 °C - 0.88 a_w for the 20 studied isolates and the incubation time (days) required to it.

Isolate name	n/n_T	Time (d)	Isolate name	n/n_T	Time (d)
UdL-TA 3.268	0.22	3.90	UdL-TA 3.244	0.31	5.29
UdL-TA 3.270	1.00	12.23	UdL-TA 3.267	0.31	6.17
UdL-TA 3.318	0.81	5.98	UdL-TA 3.269	0.29	5.60
UdL-TA 3.321	0.38	5.55	UdL-TA 3.319	0.29	6.15
UdL-TA 3.322	0.28	4.77	UdL-TA 3.320	0.23	4.32
UdL-TA 3.327	0.28	5.10	UdL-TA 3.323	0.12	2.83
UdL-TA 3.328	0.24	4.39	UdL-TA 3.324	0.19	5.17
UdL-TA 3.329	0.19	5.44	UdL-TA 3.325	0.56	5.98
UdL-TA 3.331	0.19	5.49	UdL-TA 3.326	0.31	5.81
UdL-TA 3.332	0.37	5.24	UdL-TA 3.330	0.34	6.21

Secondly, linear logistic regressions were carried out. Tables S1 and S2 (supplementary material) show the regression coefficients of all models developed. Direct comparison of those coefficients among strains indicate existing significant differences among the models built for the different strains. In particular, coefficients for the T x a_w terms were clearly different across strains. This can be confirmed from Fig. 3, where a comparison among the 20 growth models is shown. 0.05 and 0.5 probabilities were plotted at 30 days for all the strains, as isopleths. 0.5 isopleth represents the points where 50% of probability exists for growth detection, while 0.05 isopleth describes the conditions under which growth is hardly unlikely to occur. These plots are based in total probability, once both n/n_T and logistic models have been merged.

The growth/no growth boundaries varied among the tested *A. flavus* strains. Fig. 3 shows some differences in the curvatures of the contour lines for the 20 plots of growth. In general strains grew under a wider range of a_w when temperature was between 25-35 °C. Greater differences among the 20 isolates occurred when temperature is <25 °C and >35 °C. Only isolate UdL-TA

3.318, showed different behaviour, its two isopleths showed a very different curvature at 17-22 °C from the other strains, indicating a lower optimal temperature for growth.

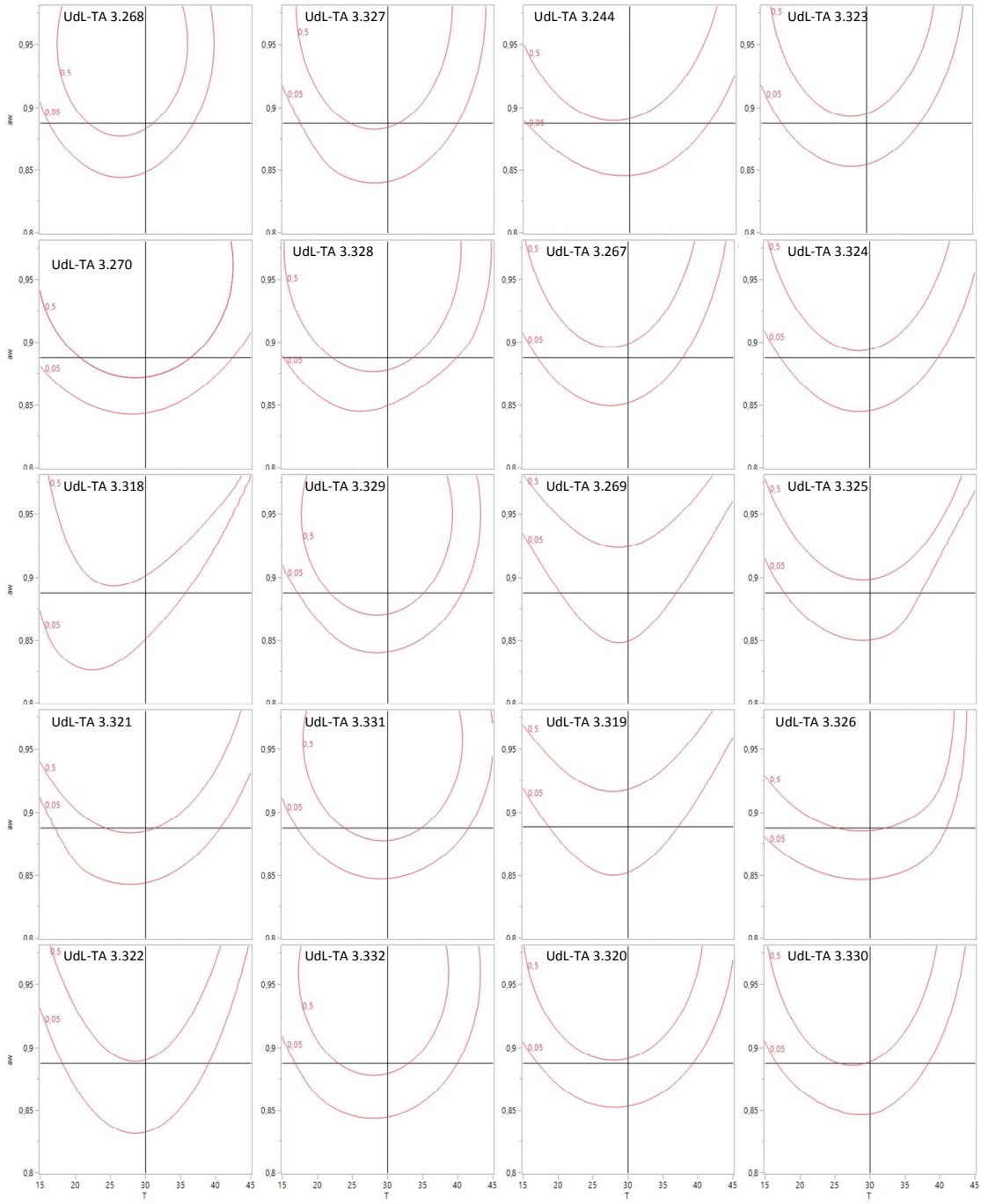

Figure 3. The predicted growth/no growth boundaries after 30 days with respect to a_w and temperature at probabilities of 0.05 and 0.5 for the 20 *A. flavus* isolates studied.

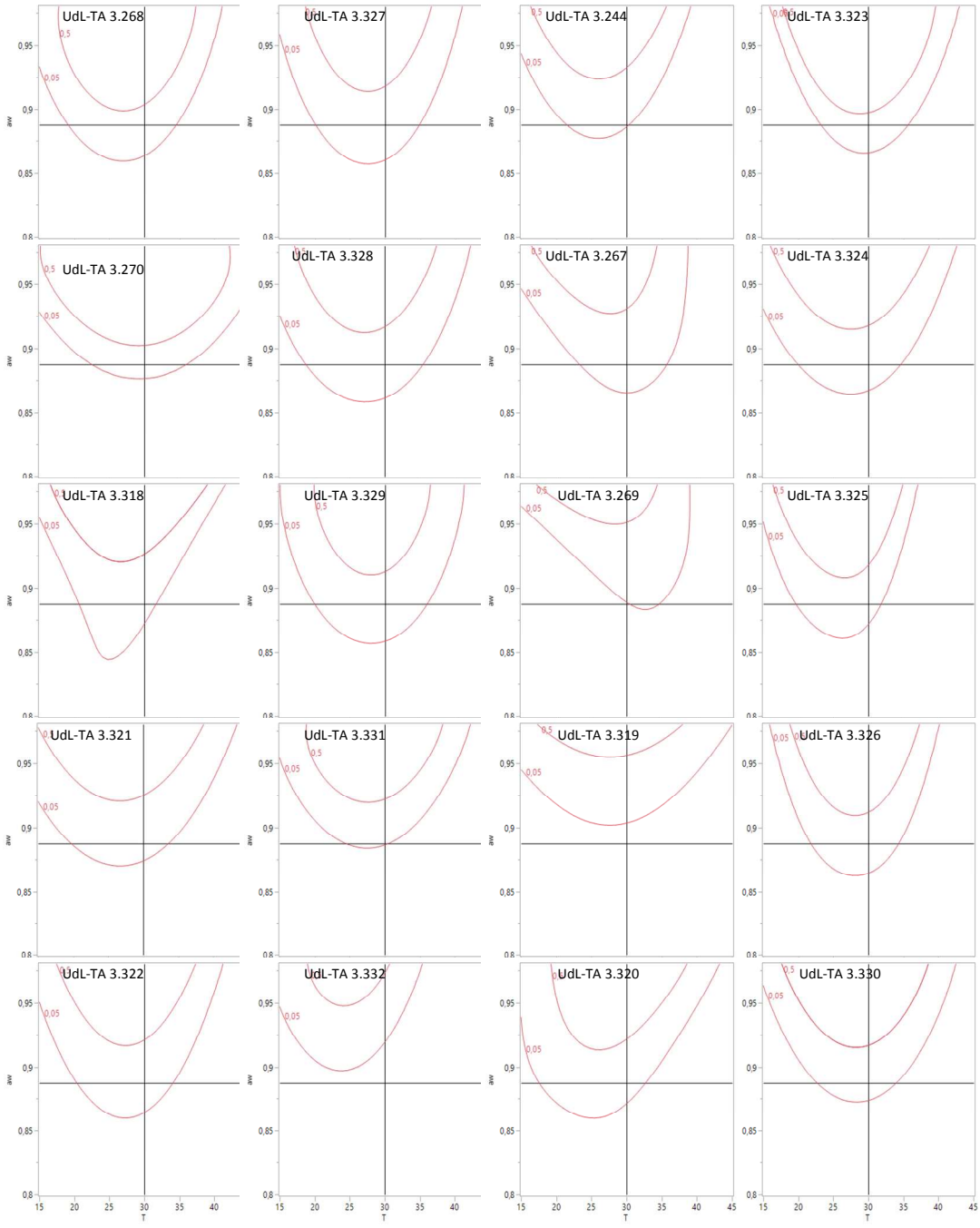

Figure 4. The predicted AFB1/no AFB1 production boundaries after 30 days with respect to a_w and temperature at probabilities of 0.05 and 0.5 for the 20 *A. flavus* isolates studied.

Effect of water activity and temperature on intraspecific differences in AFB1 production

Observed probability of AFB1 production sometimes decayed in the long period, which could be due to the possible degradation of the AFB1 along time. Thus, only AFB1 production probability models at 30 days were developed.

The predicted AFB1 production at probabilities of 5 and 50% for all strains after 30 days is shown in Figure 4. Some differences among strains can be extracted from this figure. Generally, temperature bellow 30 °C encompasses a wider range of a_w for AFB1 production, only 2 isolates, namely UdL-TA 3.267 and UdL-TA 3.269 had a greater a_w range for AFB1 production at higher temperatures. The different curvatures of the isopleths illustrate the variability in the a_w and temperature tolerance of the different strains for AFB1 production. Both for the high and low AF producing strains (see Table 1) the shape of the isopleths was very similar for growth and AFB1 production, however the conditions for AFB1 production were narrower than those for growth.

Table 4. Predicted probability of AFB1 production and experimental amount of AFB1 (ng/g) detected in maize extract agar at two different temperature and a_w combinations at day 30.

Temp (°C)	a_w	Isolate name	AF probability	AFB1 (ng/g agar)	Temp (°C)	a_w	Isolate name	AF probability	AFB1 (ng/g agar)
25	0.9	UdL-TA 3.268	0.49	2.92	20	0.92	UdL-TA 3.268	0.42	nd
25	0.9	UdL-TA 3.270	0.33	nd	20	0.92	UdL-TA 3.270	0.38	nd
25	0.9	UdL-TA 3.318	0.24	nd	20	0.92	UdL-TA 3.318	0.16	nd
25	0.9	UdL-TA 3.321	0.24	9.34	20	0.92	UdL-TA 3.321	0.28	nd
25	0.9	UdL-TA 3.322	0.29	nd	20	0.92	UdL-TA 3.322	0.22	92.81
25	0.9	UdL-TA 3.327	0.32	nd	20	0.92	UdL-TA 3.327	0.21	nd
25	0.9	UdL-TA 3.328	0.34	25.37	20	0.92	UdL-TA 3.328	0.31	nd
25	0.9	UdL-TA 3.329	0.33	nd	20	0.92	UdL-TA 3.329	0.21	nd
25	0.9	UdL-TA 3.331	0.15	nd	20	0.92	UdL-TA 3.331	0.15	nd
25	0.9	UdL-TA 3.332	0.06	nd	20	0.92	UdL-TA 3.332	0.12	nd
25	0.9	UdL-TA 3.244	0.20	nd	20	0.92	UdL-TA 3.244	0.21	nd
25	0.9	UdL-TA 3.267	0.17	nd	20	0.92	UdL-TA 3.267	0.11	nd
25	0.9	UdL-TA 3.269	0.02	nd	20	0.92	UdL-TA 3.269	0.01	nd
25	0.9	UdL-TA 3.319	0.04	nd	20	0.92	UdL-TA 3.319	0.06	nd
25	0.9	UdL-TA 3.320	0.37	27.78	20	0.92	UdL-TA 3.320	0.33	1.79
25	0.9	UdL-TA 3.323	0.42	nd	20	0.92	UdL-TA 3.323	0.05	nd
25	0.9	UdL-TA 3.324	0.31	3.99	20	0.92	UdL-TA 3.324	0.29	10.73
25	0.9	UdL-TA 3.325	0.40	2.12	20	0.92	UdL-TA 3.325	0.35	5.56
25	0.9	UdL-TA 3.326	0.30	nd	20	0.92	UdL-TA 3.326	0.10	nd
25	0.9	UdL-TA 3.330	0.21	nd	20	0.92	UdL-TA 3.330	0.13	nd

From Table 4, at 20 °C -0.92 a_w, 4 strains out of 20 produced AFB1, the amount of AFB1 produced ranged between 1.79 and 92.81 ppb (probability 0.22-0.35 in these cases). In the same way, at 25 °C -0.90 a_w 6 strains out of 20 were able to produce AFB1, ranging between 2.12-27.78 ppb (probability 0.24-0.49 in these cases). No correlation was found between probability values and AFB1 concentrations, confirming that the amount of toxin produced is highly strain dependent (Table 4).

These results show the high variability in the amount of AFB1 produced, while one strain cannot produce the toxin at all, another strain under the same condition is able to produce up to 93 ppb. From the data we can infer that predicting kinetics of the amount of AFB1 produced will be

highly variable among strains. On the other hand, probability models give a more common trend, although still differences exist. Below a probability value of 0.22 no AFB1 was detected; thus we can use this value as a maximum limit below which AFB1 production is very unlike to occur across the 20 strains.

4. Discussion

Despite the known differences in growth and mycotoxin production by individual strains, this field has not yet been studied in detail for mycotoxigenic fungal species. Many sources can be the cause of this intraspecies variability, e. g. molecular characteristics, geographical origin, environmental conditions, etc. The present study compared probabilities of growth and AFB1 production of 20 isolates of *A. flavus* using a wide range of T-a_w combinations. In this work no effect was observed due to the isolation source of the strains.

It is important to highlight that the results of growth and AFB1 production in this work correspond to the behaviour of colonies arising from single spores. Aldars-García et al. (2016b) modelled the probability of growth and AFB1 production using single spores and a concentrated inocula in order to assess the differences between them. The effect of inoculum concentration greatly affected the outcome of the predictive models, growth/AFB1 production occurred much earlier for the concentrated inoculum than for colony arising from a single spore (up to 9 days). That study demonstrated that the number of spores used to generate data in predictive mycology experiments should be carefully controlled in order to predict as accurately as possible the fungal behaviour in a foodstuff. Although the aim of the present work was to assess the intraspecies variability, we tried to get predictions in conditions as similar as possible to real food storage conditions.

Regarding the 20 probability growth models, the differences arises when the combination of variables takes place, since the regression coefficients for the interaction factors are quite variable among strain models (Table S1, supplementary material). This means for example that the use of a restrictive a_w and temperature together have a greater effect on the fungal behaviour than restricting only one of these variables, and that the adaptation and response is strain dependent. Other studies also suggest that the combination of factors can be restrictive

on growth of *A. ochraceus* and OTA production affecting in a different way depending on the isolate (Pardo et al., 2006). Furthermore, regression coefficients for the AFB1 production probability models were less different among the 20 isolates (Table S2, supplementary material) than the growth ones, this was in general due to wider confidence intervals in the AFB1 models due to the lower number of observations, and also to the fact that time was not included as a variable in the AFB1 models. When the twenty 0.05 probability lines were superposed (Fig. 5) it was clear that initiation of AFB1 production was an event much more dependent on the strain involved than growth was. Lines in Figure 5a are closer than lines in Figure 5b. A study conducted with 8 strains of *Penicillium expansum* in apples to study fungal growth and patulin production showed this strain dependence of mycotoxin production (Baert et al., 2007a). In that study the combination of stress factors, such as temperature and O_2 level, gave different results of patulin production depending on the strain.

Boundaries for growth/no-growth or AFB1/no-AFB1 production were variable among strains. Regions out of the temperature range 20-35 °C, showed the highest variability. These regions correspond to marginal conditions. As it has been reported several times, the differences among isolates are more marked under marginal than under optimal conditions (Astoreca et al., 2007; Baert et al., 2007b; Garcia et al., 2011b; Romero et al., 2010). As an example of these studies, Garcia et al. (2011b) studied the impact of suboptimal environmental conditions on the intraspecific variability of *A. carbonarius* growth and OTA production using 30 isolates and found higher intraspecies variability under marginal conditions of growth.

Under almost all T-a_w combinations and for all isolates, the separation between the two isopleths (0.05 and 0.5) is quite broad (Fig. 3 and 4). This breadth is related to the slopes of the probability curves, which are smaller due to the wider distributions obtained when working with colonies arisen from only one spore.

In general, growth of *A. flavus* is unlikely for a_w values under 0.85, regardless of the temperature level. Certain combinations of T-a_w, especially those combinations which imposed stress on the fungus resulted in a significant diminution on the probability of growth and AFB1 production. For example, 15 °C -0.90 a_w or 35 °C -0.85 a_w are almost not supportive for growth. At temperatures beyond ca. 40 °C very small probability of AFB1 production was obtained.

However in this sense, high differences among isolates were found; the ability of producing AFB1 at high temperatures seemed to be very strain dependent.

The 20 predicted boundaries for 0.05 probability were plot together with literature data for both growth (Fig. 5a) and AFB1 production (Fig. 5b). The two thicker lines in Figure 5a and 5b represent the general behaviour of the isolates that grew/produced AFB1 under the wider T-a_w combinations and the narrower combinations. However it proved difficult to find appropriate literature data to compare our logistic models as no similar approach has been employed so far for *A. flavus* colonies originating from single spores. Due to this drawback some literature data points in Fig. 5 showed a high probability of growth or toxin production out and around the boundaries that we predicted. For growth, less agreement with literature data was found at higher temperatures (above 25 °C). Literature data were obtained experimentally using inoculum sizes higher than 50 spores per inoculation point, except for 4 data points extracted from Aldars-García et al. (2016b), which were at 25° C and 0.85 and 0.87 a_w, with ca. 100% probability of growth at 30 days for colonies originating from single spores. The same conclusion was given by (Garcia et al., 2010), while working with *A. carbonarius* and *P. expansum* at suboptimal conditions and different inoculum levels. Their results showed that as conditions become limiting and the inoculum size decreases, more variability on the growth probability is obtained. Then we would expect a quite variable fungal behaviour under marginal conditions when one spore leads the growth. Regarding AFB1 production, more variability was found in the literature data, as is reflected in Figure 5b (points), were for example some researchers detected AFB1 at 30° C-0.82 a_w but others did not a 30° C -0.84 a_w. The 20 isopleths for AFB1 production probability were more widely distributed than those for growth. Besides, Figure 5 clearly shows the narrower T-a_w combinations that allow for AFB1 production compared to those that allow growth. In general, comparison with literature data showed that, despite these differences, growth boundaries are much more similar among strains than those for AFB1 production are.

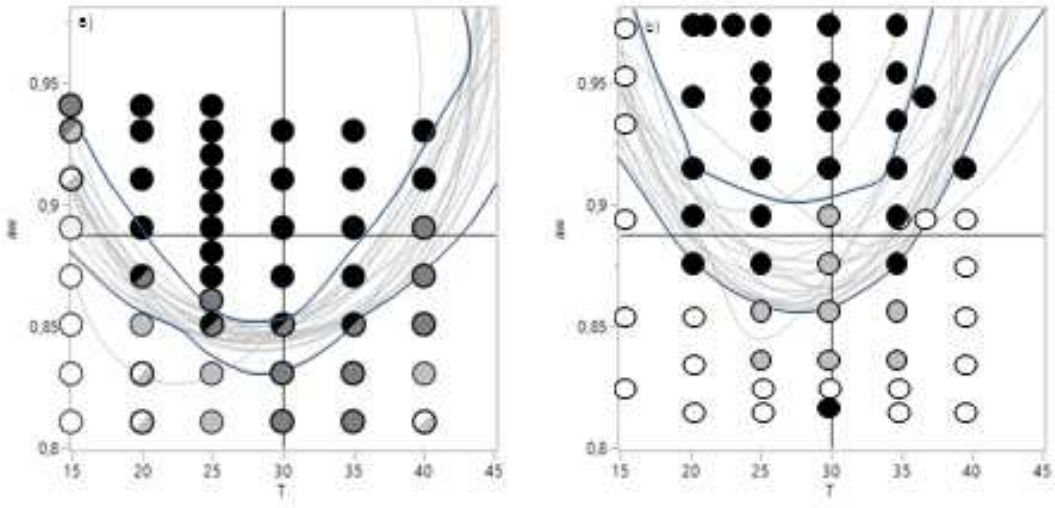

Figure 5a. The predicted growth/no-growth boundaries at 0.05 probability of the twenty isolates at day 30 were plot together with literature data (28-30 days). Points represent the literature data;●, 0.9 < Probability < 1; ● 0.2 < Probability < 0.9; ●, 0.05 < Probability < 0.2; ○, P=0. Data extracted from: Aldars-García et al. (2016b), Astoreca et al. (2012), Marín et al. (2012, 2009).

Figure 5b. The predicted AFB1 production/no AFB1 production boundaries at 0.05 probability of the twenty isolates at day 30 were plot together with literature data (days between 21 and 30). Points represent the literature data; ●, detected AFB1 in all studies; ●, AFB1 detected in some studies and not detected in other; ○, AFB1 not detected. Data extracted from: (Astoreca et al., 2014; Lahouar et al., 2016; Mohale et al., 2013; Mousa et al., 2013)

The amount of mycotoxins produced by fungi has been demonstrated to be highly variable among isolates (Garcia et al., 2011b; Yogendrarajah et al., 2016). On the contrary, boundaries for toxin/no-toxin seemed to be variable, but much less than the quantity of toxin produced. Thus, taking into account the high variability in the amount of AFB1 produced by different strains, modelling of the probability of toxin production seems a suitable alternative. Boundaries for AFB1 production were narrower than those for growth, thus the possibility of using growth/no-growth models for predicting AFB1 production, may lead to unnecessary rejection

measures. However, the food industry needs to appropriately deal with the mycotoxin risk and anyway this will be a “fail-safe” scenario.

Results from Figures 3 and 4 show the possibility of encountering some strains with a better ability to grow and produce AFB1 than others. Thus, when developing the predictive models several strains should be taken into account in order to overcome this issue. Working separately with a large number of strains may be tedious, and some authors had investigated the possibility of using a cocktail of strains inoculum (Aldars-García et al., 2015; Garcia et al., 2014). Garcia et al. (2014) compared the growth among 25 isolates separately and an inoculum with the 25 strains together, and they concluded that the best adapted isolated led the behaviour of the pooled inoculum, thus it would be equivalent to work with the fastest strain, in a worst scenario situation. This approach would not be useful for quantitative risk assessment.

In conclusion, the results of this work showed that under marginal conditions, the combined effect of temperature and a_w had different effect on the 20 isolates of *A. flavus* tested, in terms of growth and AFB1 production responses. Contour plots for each isolate satisfactorily predicted the response studied. However, to build better models, more information accounting for a wider range of strains from different climatic conditions is required.

5. Acknowledgements

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Table S1. (Part 1 of 2). Regression coefficients of the 20 probability growth models.

Parameter	UdL-TA 3.244		UdL-TA 3.267		UdL-TA 3.269		UdL-TA 3.319		UdL-TA 3.320	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	558.19	49.57	1200.52	54.59	168.13	46.08	137.21	49.16	654.72	33.41
t	-0.59	0.03	-1.37	0.03	-0.48	0.02	-0.48	0.02	-0.39	0.01
T	ns	ns	-6.66	0.33	-5.41	0.33	-3.99	0.33	-1.82	0.19
a_w	-1291.86	103.46	-2413.71	112.88	-293.48	94.15	-281.95	99.76	-1447.58	68.91
t^2	-1.00E-05	2.82E-06	6.96E-05	4.63E-06	-1.13E-05	1.22E-06	-1.28E-05	1.25E-06	-1.90E-05	9.18E-07
t*T	1.00E-03	1.11E-04	0.01	1.48E-04	2.87E-03	1.10E-04	3.09E-03	1.09E-04	7.79E-04	4.98E-05
t*a_w	0.69	0.03	1.41	0.04	0.50	0.02	0.50	0.02	0.45	1.31E-02
T^2	-0.08	2.51E-03	-0.09	2.71E-03	-0.07	2.06E-03	-0.06	1.82E-03	-0.08	1.51E-03
T*a_w	5.51	0.32	12.60	0.43	9.60	0.38	7.87	0.36	6.58	0.22
a_w^2	661.21	53.43	1125.73	57.44	ns	ns	90.17	50.83	726.58	35.05
	UdL-TA 3.323		**UdL-TA 3.324**		**UdL-TA 3.325**		**UdL-TA 3.326**		**UdL-TA 3.330**	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	151.58	27.97	42.57	62.52	661.86	28.23	-563.40	25.57	92.08	63.18
t	0.01	4.34E-03	-0.53	0.03	-0.03	4.07E-03	0.11	4.88E-03	-0.84	0.03
T	ns	ns	2.16	0.44	3.85	0.16	4.29	0.17	-4.51	0.39
a_w	-374.58	59.10	ns	ns	-1707.37	61.13	969.77	52.84	-169.50	127.72
t^2	-1.60E-05	5.57E-07	-2.98E-05	5.82E-06	-3.97E-05	7.60E-07	-2.10E-05	7.92E-07	ns	ns
t*T	-4.24E-04	2.15E-05	2.10E-03	1.70E-04	-7.07E-04	1.61E-05	-7.28E-04	3.43E-05	2.46E-03	1.32E-04
t*a_w	0.03	4.84E-03	0.61	0.03	0.11	4.79E-03	-0.06	5.21E-03	0.93	0.03
T^2	-0.02	7.69E-04	-0.10	3.38E-03	-0.10	1.65E-03	-0.05	1.29E-03	-0.12	3.55E-03
T*a_w	1.40	0.15	3.57	0.44	2.15	0.15	-1.17	0.14	11.98	0.47
a_w^2	204.10	31.31	157.07	62.72	969.74	32.79	-448.88	28.09	ns	ns

ns, not significant

Table S1. (Part 2 of 2). Regression coefficients of the 20 probability growth models.

Parameter	UdL-TA 3.268		UdL-TA 3.270		UdL-TA 3.318		UdL-TA 3.321		UdL-TA 3.322	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	291.12	15.57	-40.29	33.42	97.43	25.16	31.98	38.03	83.52	37.5638
t	-0.02	2.59E-03	-0.17	0.02	-0.11	0.01	-0.33	0.02	-0.29	0.02
T	0.72	0.09	1.77	0.19	6.87	0.20	3.12	0.30	1.69	0.29
a_w	-717.49	33.56	ns	ns	-561.68	54.36	-302.30	75.88	-344.55	75.44
t^2	-1.70E-05	3.21E-07	-2.34E-05	1.33E-06	-2.87E-05	8.03E-07	-4.03E-05	3.44E-06	-3.69E-05	2.31E-06
t*T	-3.92E-04	1.26E-05	-2.66E-04	6.27E-05	-2.23E-03	5.12E-05	3.78E-03	1.28E-04	2.65E-03	1.24E-04
t*a_w	0.05	2.94E-03	0.25	0.02	0.24	0.01	0.37	0.02	0.33	0.02
T^2	-0.02	5.97E-04	-0.06	1.47E-03	-0.09	1.67E-03	-0.06	1.87E-03	-0.06	1.85E-03
T*a_w	0.82	0.09	1.85	0.19	-1.27	0.16	ns	ns	2.17	0.28
a_w^2	406.43	18.02	51.61	35.76	387.75	29.58	220.68	38.22	207.41	38.25
	UdL-TA 3.327		**UdL-TA 3.328**		**UdL-TA 3.329**		**UdL-TA 3.331**		**UdL-TA 3.332**	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	449.04	53.87	501.32	40.16	-246.44	61.39	141.94	41.36	25.27	35.94
t	-0.45	0.03	-0.53	0.02	-0.53	0.03	-0.36	0.02	-0.24	0.02
T	-2.55	0.43	1.27	0.24	ns	ns	1.47	0.24	2.55	0.29
a_w	-1015.10	107.62	-1210.20	84.41	382.24	121.04	-438.38	84.69	-234.16	71.77
t^2	-3.42E-05	3.03E-06	-1.51E-05	1.77005E-06	-5.40E-05	3.89E-06	-7.00E-06	2.54E-06	-4.48E-05	2.25E-06
t*T	4.37E-03	1.91E-04	-1.53E-03	9.98E-05	3.66E-03	1.56E-04	-1.70E-04	7.74E-05	2.05E-03	1.24E-04
t*a_w	0.47	0.03	0.70	0.03	0.61	0.03	0.45	0.02	0.29	0.02
T^2	-0.05	2.33E-03	-0.08	2.19E-03	-0.10	3.02E-03	-0.07	1.70E-03	-0.06	1.85E-03
T*a_w	5.46	0.41	4.34	0.24	7.06	0.40	2.80	0.24	0.98	0.28
a_w^2	522.21	53.99	621.42	43.77	-228.59	60.31	232.97	43.24	155.11	36.10

ns, not significant

Table S2. (Part 1 of 2). Regression coefficients of the 20 probability AFB1 production models.

Parameter	UdL-TA 3.244		UdL-TA 3.267		UdL-TA 3.269		UdL-TA 3.319		UdL-TA 3.320	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	-75.19	21.78	-147.35	64.85	-229.43	101.67	1.07	20.61	40.77	44.46
T	1.83	0.62	4.80	2.50	7.33	3.58	0.53	0.39	-1.93	1.78
a_w	56.85	17.60	139.36	64.93	221.47	100.34	-64.15	46.06	-60.72	47.57
T^2	-0.04	0.01	-0.02	0.01	-0.03	0.01	-0.01	0.01	-0.03	0.01
a_w^2	ns	ns	ns	ns	ns	ns	58.49	28.93	ns	ns
$T*a_w$	ns	ns	-3.72	2.38	-6.23	3.30	ns	ns	3.48	1.97
	UdL-TA 3.323		**UdL-TA 3.324**		**UdL-TA 3.325**		**UdL-TA 3.326**		**UdL-TA 3.330**	
	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error	Estimate	Standard error
CONSTANT	-179.00	73.00	-59.79	19.06	-89.95	30.55	-77.26	25.12	-79.02	25.22
T	4.77	2.42	1.35	0.48	3.21	1.01	2.61	0.92	1.72	0.58
a_w	123.30	47.74	47.47	15.41	57.27	22.68	45.71	16.64	60.20	21.04
T^2	-0.08	0.04	-0.02	0.01	-0.06	0.02	-0.05	0.02	-0.03	0.01
a_w^2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$T*a_w$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns, not significant

Table S2. (Part 2 of 2). Regression coefficients of the 20 probability AFB1 production models.

	UdL-TA 3.268		UdL-TA 3.270		UdL-TA 3.318		UdL-TA 3.321		UdL-TA 3.322	
	Estimate	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**
CONSTANT	-79.96	23.17	-117.64	36.75	365.88	297.11	-52.25	15.16	-56.75	16.18
T	1.86	0.59	1.50	0.70	2.15	0.72	0.92	0.38	1.38	0.45
a_w	62.77	18.36	106.39	31.67	-917.30	663.46	44.15	12.89	43.18	13.03
T^2	-0.04	0.01	-0.03	0.01	-0.04	0.01	-0.02	0.01	-0.03	0.01
a_w^2	ns	ns	ns	ns	532.30	364.86	ns	ns	ns	ns
$T*a_w$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	UdL-TA 3.327		**UdL-TA 3.328**		**UdL-TA 3.329**		**UdL-TA 3.331**		**UdL-TA 3.332**	
	Estimate	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**	**Estimate**	**Standard error**
CONSTANT	-38.42	10.71	-30.00	9.01	-50.28	14.51	-88.52	26.87	-67.04	26.59
T	1.55	0.48	1.04	0.39	1.24	0.42	1.52	0.59	1.37	0.65
a_w	-0.03	0.01	-0.02	0.01	36.65	11.63	74.30	22.54	54.61	21.65
T^2	21.63	6.88	19.95	6.12	-0.02	0.01	-0.03	0.01	-0.03	0.01
a_w^2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$T*a_w$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns, not significant

Chapter IV

Assessment of intraspecies variability in fungal growth initiation of *Aspergillus flavus* and aflatoxin B_1 production under static and changing temperature levels using different initial conidial inoculum levels

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Abstract

Intraspecies variability in fungal growth and mycotoxin production has important implications for food safety. Using the Bioscreen C we have examined spectrophotometrically intraspecies variability of *A. flavus* using 10 isolates under different environments, including temperature shifts in terms of growth and aflatoxin B_1 (AFB_1) production. Among the isolates tested, there were five high and five low AFB_1 producers. The study was conducted at 5 isothermal conditions (from 15 to 37 °C) and 4 dynamic scenarios (between 15 and 30 °C). The experiments were carried out in a semisolid YES medium at 0.92 a_w and two inoculum levels, 10^2 and 10^3 spores/mL. The Time to Detection (TTD) of growth initiation was determined and modelled as a function of temperature through a polynomial equation and the model was used to predict TTD under fluctuating conditions using a novel approach. The results obtained in this study have shown that a model can be developed to describe the effect of temperature fluctuations on the TTD for all the studied isolates and inoculum levels. Isolate variability increased as the growth conditions became more stressful and with a lower inoculum level. Inoculum level affected the intraspecies variability but not the repeatability of the experiments. In dynamic conditions, isolate responses depended both on the temperature shift and, predominantly, the final temperature level. AFB_1 production was highly variable among the isolates and depended on the inoculum level. This suggests that, from an ecological point of view, the potential isolate variability and interaction with dynamic conditions should be taken into account in developing strategies to control growth and predicting mycotoxin risks by mycotoxigenic fungi. This type of study could also be useful practically in predicting relative risks in colonisation and contamination with AFB_1 in staple stored food commodities.

Keywords intraspecies variability; *Aspergillus flavus*; growth; aflatoxin; dynamic temperature; inoculum level

1. Introduction

Fungal growth and mycotoxin contamination of food products represent an important food safety issue for the food industry. *Aspergillus* species are particularly important because of they are xerophilic and able to colonise a range of food matrices, resulting in spoilage problems and mycotoxin contamination, causing significant economic losses of staple food crops. Many factors can influence fungal growth in food products including nutritional composition, temperature, pH, water activity (a_w), atmospheric composition, presence and concentration of preservatives, different fungal communities, as well as storage times. Inter- and intra-species differences have been shown to be an important source of variability in terms of fungal growth and mycotoxin production (Abbas et al., 2005; Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Romero et al., 2007; Santos et al., 2002; Singh et al., 2015; Yogendrarajah et al., 2016).

Usually, spoilage by filamentous fungi is visible in the form of colonies on the surface of food products, especially bakery goods. In general, spoilage has been evaluated by physically measuring the rate of colonisation on the food surface. However, the assessment of filamentous fungal activity is complex because they grow in three dimensions and are able to colonise a greater substrate surface area than yeasts or bacteria (Dantigny et al., 2005). The measurement of hyphal extension rates, usually reported as radial growth rate, is probably the simplest and most direct method to measure fungal growth. Nevertheless, as stated by Medina et al. (2012), these measurements do not account for the true representation of the three-dimensional nature of fungal growth, although there is a relationship between radial growth and fungal biomass (Trinci, 1971). In addition, the whole process is time consuming and requires significant inputs of time and consumables. Methods based on spectrophotometry (turbidimetric measurements) have been widely used for bacterial growth but used in only a small number of fungal growth studies. Spectrophotometric assays provide fast results that are expressed in Optical Density (O.D.) units. Only a few authors have used this kind of approach for mycological studies (Medina et al., 2012; Mohale et al., 2013; Rossi-Rodrigues et al., 2009; Samsudin et al., 2016). The use of a semi-solid agar medium has been effectively utilised to examine relative growth in relation to environmental factors and also in relation to different anti-fungal compounds (Medina et al., 2012).

There is interest in understanding the relationship between initial inoculum size and how this affects the relative growth rate in relation to environmental conditions (Aldars-García et al., 2016; Baert et al., 2008; Barberis et al., 2012; Burgain et al., 2013; Garcia et al., 2010; Gougouli et al., 2011; Morales et al., 2008). In addition, how does initial inoculum size affects the capacity for mycotoxin production. This could be important as the initial levels of fungal contamination of a food matrix may influence, or indeed determine, how much mycotoxin is produced. In this study, we have used *Aspergillus flavus* as the target mycotoxigenic pathogen because it colonises a range of cereals, nuts and also spices and contaminates them with aflatoxins, especially aflatoxin B_1 (AFB_1) which is a class 1A carcinogen (IARC, 1993). Thus, it is an important model fungal species to utilise for ecophysiological studies of a mycotoxigenic species. While usually the fungal community consists of a range of species and isolates of the same species, we have focused here on different inoculum sizes of different isolates of the same species only.

Thus, the objectives of this study were to (i) compare the impact of different steady state temperatures (15-35 °C) on relative initial growth of 10 isolates of *A. flavus* at two initial inoculum levels (log2, log3); (ii) examine the effect of four temperature shifts (between 15-30 °C) and inoculum size on rates of growth using the Bioscreen C; (iii) evaluate the effect of initial inoculum size and steady state and temperature shifts on AFB_1 production and (iv) examine what impact these environmental conditions/shifts have on within-isolate variation using a secondary metabolite conducive medium. The ten tested isolates were divided into two groups according to their AFB_1 production ability: one high AFB_1 producer and another low AFB_1 producer.

2. Material and methods

2.1. *A. flavus* isolates

Ten isolates of *A. flavus* isolated from chilli powder, maize grains and pistachio nuts were used in this study (table 1). There were five high and five low AFB_1 producers.

Table 1. Description of the isolates used in the present study

Isolate code*	Origin	AFB_1 production on PDA at 25 °C for 7 days (ng/g)
UdL-TA 3.244	Chilli	20.5
UdL-TA 3.267	Pistachio nuts	25.9
UdL-TA 3.269	Pistachio nuts	28.3
UdL-TA 3.324	Maize grains	5.4
UdL-TA 3.325	Maize grains	1.5
UdL-TA 3.268	Pistachio nuts	471.2
UdL-TA 3.270	Pistachio nuts	114.8
UdL-TA 3.327	Maize grains	178.5
UdL-TA 3.331	Maize grains	547.2
UdL-TA 3.332	Maize grains	2114.6

*Isolate codes are the names of the cultures held in the Food Technology Department Culture Collection of University of Lleida, Spain.

2.2. Inoculum preparation, culture medium and inoculation

Isolates were sub-cultured on Malt Extract Agar (Sigma-Aldrich, Dorset, UK) at 25 °C for 7 days to obtain heavily sporulating cultures. After incubation, spores were collected by scraping the surface of the plates, diluting them in sterile water adjusted to 0.92 a_w with glycerol containing Tween 80 (0.05% v/v), and filtered through sterile glass wool into a tube. Immediately total spore concentrations were determined and decimal dilutions (in sterile water adjusted to 0.92 a_w with glycerol, containing Tween 80 (0.05% v/v)), were prepared to obtain the two different spore concentrations: 10^5 and 10^4 spores/mL for each isolate. The basic medium used in this study was a semisolid Yeast Extract Sucrose (YES) agar, whose protocol of preparation was previously optimised by Medina et al. (2012) and adjusted to 0.92 a_w. Spore suspensions were used to inoculate semi-solid YES medium. 100 µL of the spore suspension was pipetted into 9.9 mL of semi-solid YES agar, for each spore concentration, thus two final concentrations of 10^3 and 10^2 spore/mL in the semisolid YES agar were prepared for each isolate.

2.3. Growth assessment

Growth was studied at 15, 20, 25, 30, 35, 37 °C and four changing profiles from 15 to 25 °C (F1), 15 to 30 °C (F2), 20 to 25 °C (F3) and 20 to 30 °C (F4), after 48 hours at the lowest temperature. Optical densities, which are directly related to the fungal biomass of *A. flavus* (Medina et al., 2012) were recorded using a Bioscreen C Microbiological Growth Analyser (Labsystems, Helsinki, Finland). 100-well microtitre plates specifically manufactured for this machine were loaded with the 10^3 and 10^2 spore/mL semisolid YES agar of each isolate; one plate per spore concentration was used. The wells of the microplate were filled with 300 µL of the inoculated medium, thus ca. 300 and 30 spores were inoculated in each well for the 10^3 and 10^2 spores/ mL inocula, respectively. For each temperature condition two inoculum levels were set with 9 replicates (well) per isolate (10 isolates x 9 replicates=90 wells plus 10 empty wells, this means one plate per inoculum level and temperature condition).

The O.D. was recorded every 30 min using the 600nm filter over a 7 days period, except for 20 °C and 15 to 25 °C (F1), where 14 and 9 days respectively, were needed to reach the growth threshold set for the experiments. Data were recorded using the software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer and then exported to a Microsoft® Excel® Professional 2010 (14.0.4756.1000) (Microsoft Corporation, Redmond, Washington, USA) sheet for further analysis.

2.4. Aflatoxin B_1 assessment

Following incubation, wells' content was collected in order to analyse the mycotoxin concentration. AFB_1 extraction was carried out as follows: the content of 3 wells was collected for each isolate and temperature condition, in triplicate. AFB_1 was extracted with 0.8 mL of chloroform, shaken for 1 min and left stationary for 20 min. The chloroform phase was separated and the aqueous phase was re- extracted twice with 0.8 mL of chloroform. The organic extracts were combined and evaporated to dryness. The residues were derivatized using trifluoroacetic acid as described by the AOAC (2000) and transferred to a HPLC vial. All derivatized samples were analysed by HPLC (Agilent 1200 series HPLC (Agilent, Berkshire, UK)). Chromatographic separations were performed on a stainless steel, C18 column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 µm). Methanol: water: acetonitrile (30:60:10) was used as the

mobile phase at a flowrate of 1mL/min. AFB_1 derivative fluorescence was recorded at excitation and emission wavelengths of 360 and 440 nm respectively. Standard curves were constructed with different levels of AFB_1. Aflatoxin recovery assays were performed to ensure the analytical quality of the results, obtaining for all the concentrations tested >87% of recovery.

2.5. Data analysis

2.5.1. TTD for static temperatures

Raw datasets obtained from the Bioscreen C were subjected to further analysis. Before analyses, the average of the measurements for each well during the first 60 min was calculated and automatically subtracted from all subsequent measurements in order to remove the different signal backgrounds. Then, the TTD for an O.D. of 0.1 was obtained using a Microsoft® Excel® template (kindly provided by Dr. R. Lambert), which used linear interpolation between successive O.D. readings.

In order to stabilise the variance, for TTD comparison, a square root transformation was used. However, raw data are presented in tables and graphs. The Kruskal–Wallis test was used to establish the differences among median TTD values of the 10 isolates under the different levels at $p<0.05$.

Finally, based on the TTD, a polynomial model that described the TTD as a function of the temperature was fitted. The general expression of the polynomial model is:

$$TTD = a_0 + a_1 T + a_2 T^2 \quad (1)$$

a_i are the constants to be estimated, T is the independent variable (temperature) and TTD is the response variable. This model was chosen as only temperature levels in the range 15-37 °C were tested, which were not enough to fit models considering the whole range of growth temperatures.

Statistical analysis was carried out with Statgraphics® Centurion XVI.I (Statpoint, Inc., Maryland, USA).

2.5.2. Prediction of TTD under fluctuating temperatures

The TTDs obtained under the static temperatures were used to design the experiments for estimating the TTDs under fluctuating temperature conditions. The time-temperature scenarios studied included a single abrupt shift from a low to an upper temperature. The TTD under

fluctuating temperature was predicted through the model fitted at constant temperature, and compared to the experimental results generated under the temperature changing scenarios (experimental TTD). These experiments were carried out in the same way as for the static conditions.

The TTD of each isolate at changing temperature (total TTD=TTDT) with a single abrupt temperature change from an initial temperature (TI) to a final temperature (TF) at a time ts (Fig. 1) was calculated by the following equation:

$$TTDT = \begin{cases} TTDI & \text{if ts} > TTDI \\ TTDF + (TTDI - TTDF) * \left(\frac{TF-TI}{TF}\right) * \left(\frac{ts}{TTDF}\right) & \text{if ts} < TTDI \end{cases} \quad (2)$$

Where TTDI and TTDF were calculated by substituting the corresponding temperature in the polynomial equation (Eq. (1)). That is, when the temperature shift occurs before the end of TTDI, after TTDF is consumed, the remaining TTD is a percentage of the interval (TTDI-TTDF), which was assumed to be proportional to i) the temperature shift, and ii) the timing of the temperature shift.

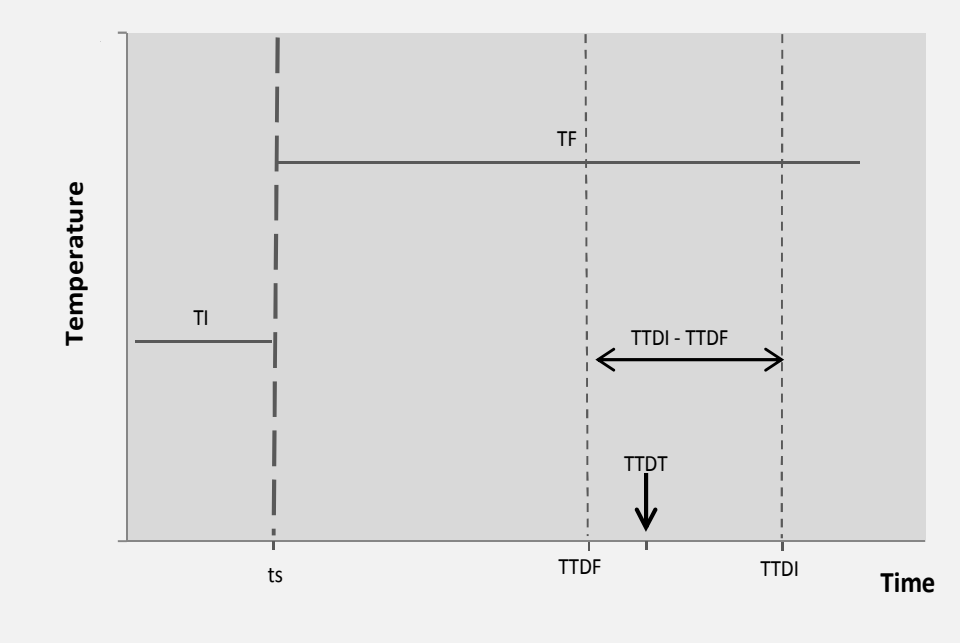

Figure 1. Schematic representation of TTD as affected by the temperature shift.

3. Results

3.1. TTD at constant temperature conditions: isolate variability and inoculum size effect

In the first part of the study, the objective was to determine the influence of temperature on the relative initial growth of the 10 *A. flavus* isolates and address their differences. Thus, TTDs of 0.1, indicating initial growth of the spores, were calculated. In this study, all experiments were carried out at constant temperature conditions. TTDs were further fitted to a second order polynomial equation (Eq. (1)) in order to predict the TTD as a function of the temperature for each isolate. The developed polynomial models accurately predicted the influence of temperature on the TTD, with goodness of fit (r^2) ranging from 0.980 to 0.999. Both temperature and its quadratic term had significant effects on TTD (Table 2).

Table 2. Parameter estimation of the polynomial models for TTDs of the 10 *A. flavus* isolates at both inoculum levels.

Inoculum level	Variable	Coefficient estimated value [a]									
		UdL-TA 3.244	UdL-TA 3.267	UdL-TA 3.269	UdL-TA 3.324	UdL-TA 3.325	UdL-TA 3.268	UdL-TA 3.270	UdL-TA 3.327	UdL-TA 3.331	UdL-TA 3.332
10^2 spores/ml	Constant	77434.80 ± 5282.23	66100.10 ± 2336.32	75437.91 ± 3320.37	61534.18 ± 2424.18	65000.17 ± 2643.21	70988.46 ± 3070.81	64260.80 ± 3259.81	59395.60 ± 1958.30	60100.50 ± 4172.01	67532.80 ± 3684.03
	T	-4329.09 ± 374.55	-3643.88 ± 168.53	-4348.05 ± 240.76	-3372.45 ± 175.77	-3569.84 ± 191.66	-4096.93 ± 222.66	-3532.86 ± 236.37	-3383.57 ± 141.99	-3292.88 ± 302.51	-3777.97 ± 260.32
	T^2	63.48 ± 6.43	52.89 ± 2.92	65.29 ± 4.19	48.99 ± 3.05	51.57 ± 3.34	61.71 ± 3.87	51.25 ± 4.11	50.97 ± 2.47	47.72 ± 5.26	55.74 ± 4.45
	r^2	93.58	98.22	96.67	97.77	97.63	96.54	96.25	98.00	93.20	95.32
10^3 spores/ml	Constant	62490.8 ± 3171.44	51181.3 ± 2514.44	60660.88 ±2836.99	51837.57 ± 1772.97	52029.60 ± 2182.80	59022.71 ± 2360.32	54020.90 ± 2557.70	45936.10 ± 1254.28	48765.90 ± 2865.00	64245.00 ± 4101.45
	T	-3470.54 ± 228.74	-2774.71 ± 182.32	-3488.48 ± 205.71	-2842.81 ± 128.56	-2835.64 ± 158.27	-3398.32 ±171.15	-2964.15 ± 185.46	-2571.03 ± 90.95	-2643.11 ± 207.74	-3646.82 ± 295.85
	T^2	50.592 ± 3.97	39.8439 ± 3.17	52.43 ± 3.58	41.3474 ± 2.24	40.76 ± 2.75	51.17 ± 2.98	42.96 ± 3.23	38.40 ± 1.58	38.04 ± 3.62	54.18 ± 5.13
	r^2	96.19	96.65	96.03	98.22	97.54	97.16	96.87	98.68	95.42	93.62

[a] mean values ± sd

*All coefficient estimates were significant at $P < 0.05$

All *A. flavus* isolates grew under all set temperature profiles tested, except for 15 °C. According to the Kruskal-Wallis test, there were significant differences ($p<0.05$) in TTD among isolates, however these differences depended on the temperature profile tested. TTD values followed the time sequence 37 <35 <30 < 25 <20 °C in all cases. This pattern is shown in Fig. 2. In general, all isolates showed similar TTD values at the same temperature and inoculum level. The TTDs at 30, 35 and 37 °C did not revealed much significant differences between isolates, at the 95% confidence level.

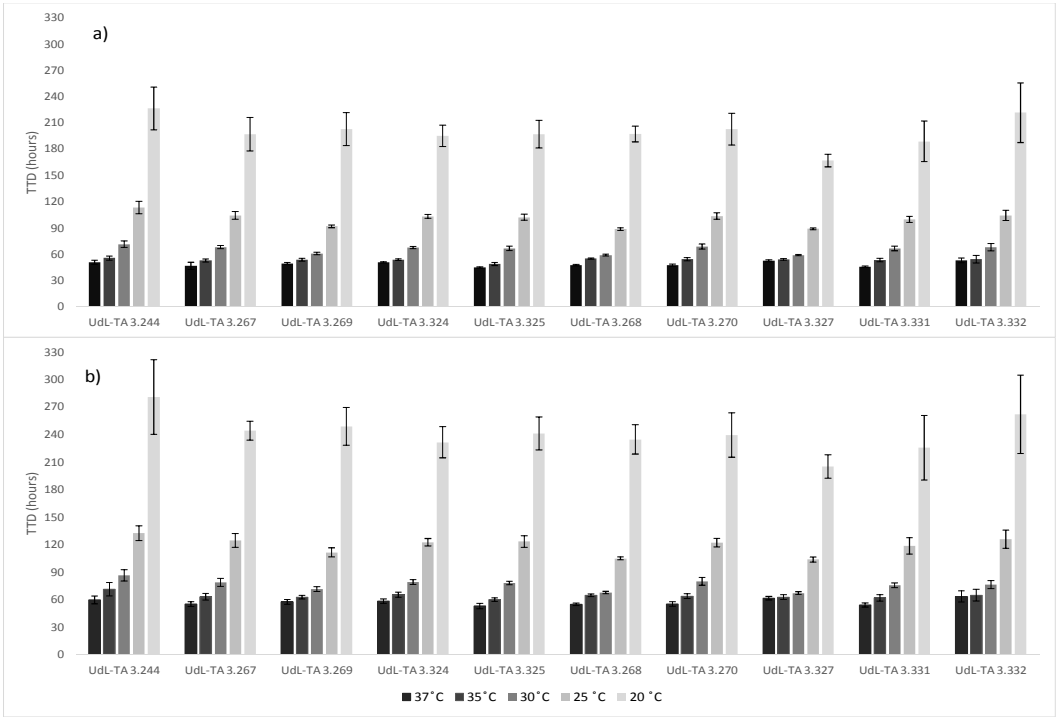

Figure 2. TTDs at 37, 35, 30, 25 and 20 °C for the ten isolates studied at a)10^3 and b)10^2 spores/mL.

At 25 °C, differences between the 10 isolates increased (higher coefficient of variation (CV %)) which could suggest more within-isolate variability as temperature became more marginal for growth. Isolate response at 20 °C was highly variable, not only among isolates but also within replicates of the same isolate. Furthermore at 20 °C in some replicates, conidia failed to initiate grow at all. At the five constant temperatures, the isolate UdL-TA 3.327 showed the lowest

TTDs, and isolates UdL-TA 3.244 and UdL-TA 3.332 the highest. No differences in growth pattern were found between the low AFB_1 producer and the high AFB_1 producer groups.

Statistical analysis showed a clear difference between the TTD for the two inoculum levels for all temperature profiles (Fig. 2). In addition, differences among isolates' TTD increased for the lower initial inoculum size (10^2 spores /mL), i.e., the difference in time was greater at the lower inoculum level. The differences between the 2 inoculum levels were greater at 20 °C. For example, for isolates UdL-TA 3.270 and UdL-TA 3.332, the differences at 30 °C were 0.72 and 3.54 hours for the high and the low inoculum level treatments respectively. At 20 °C these differences were 18.74 and 22.48 hours, respectively, for the low and the high inoculum levels. From Figure 2 it was also observed that the TTD was significantly affected by the spore concentration, and these differences were more marked as conditions became more marginal for conidial germination. In general, within-isolate variability was more affected by the marginal conditions (20 °C) than by the inoculum level. Increasing the conidial inoculum size from 10^2 to 10^3 spores/mL, when temperature was 20 °C (a realistic practical storage temperature for agricultural products), had a profound effect on the ability *of A. flavus* to initiate growth. At this temperature, a difference in the prediction of growth initiation of more than 2 days occurred depending on the inoculum level.

3.2. Prediction of relative initial growth times (TTD) as affected by temperature shifts

Generally, under ambient transport conditions temperature is not a fixed value and fluctuates during distribution and the length of the food chain. Thus, it is important to better understand the effect of such fluctuations on fungal growth due to temperature or indeed inoculum size impacts on relative level of risk from growth or indeed toxin contamination. Thus, an approach to predict the effect of temperature shifts on the ability of fungi to grow (in our case by means of the TTD) was developed taking into account the results from the static temperature studies.

In order to evaluate the suitability of the model to predict the TTD of *A. flavus* conidial germination and growth under changing temperature scenarios, the predicted TTDs derived from equation 2 were compared to experimental TTDs obtained by carrying out the experiment at set shifting temperatures profiles.

The four temperature shifts were imposed prior to the TTD. Agreement between model predictions and experimental data results was assessed by plotting predicted TTD versus observed TTD (Fig. 3). The observed response values agreed well with the predicted response values except for the temperature shift from 15 to 30 °C where a clear overestimation was obtained (approx. 50 hours).

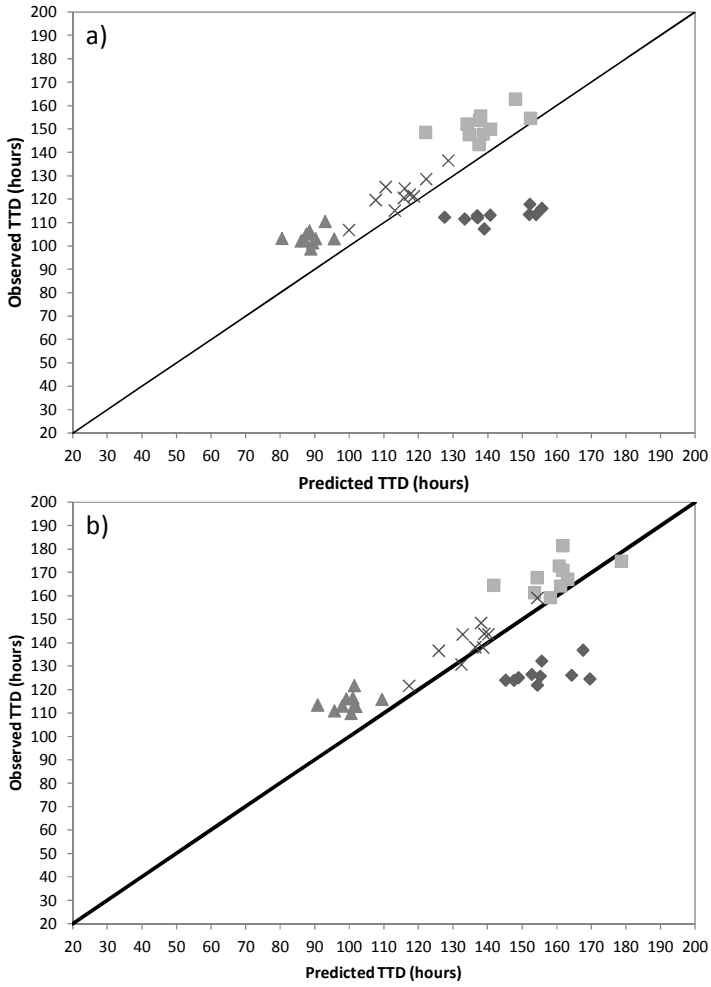

Figure 3. Predicted TTD versus observed TTD at the four changing temperature for the ten isolates studied at a)10^3 and b)10^2 spores/mL and the four dynamic temperature profiles: 15 to 25 °C (■), 20 to 25 °C (×), 15 to 30 °C (◆) and 20 to 30 °C (▲).

Nonetheless, accurate predictions were possible under the two profiles with 25 °C as the final temperature, with a mean underestimation of only 4 hours made. Also, good agreement between the experimental and predicted TTD for an initial temperature of 20 °C and a final temperature of 30 °C (Fig. 3) was obtained, with a mean underestimation of 9 hours.

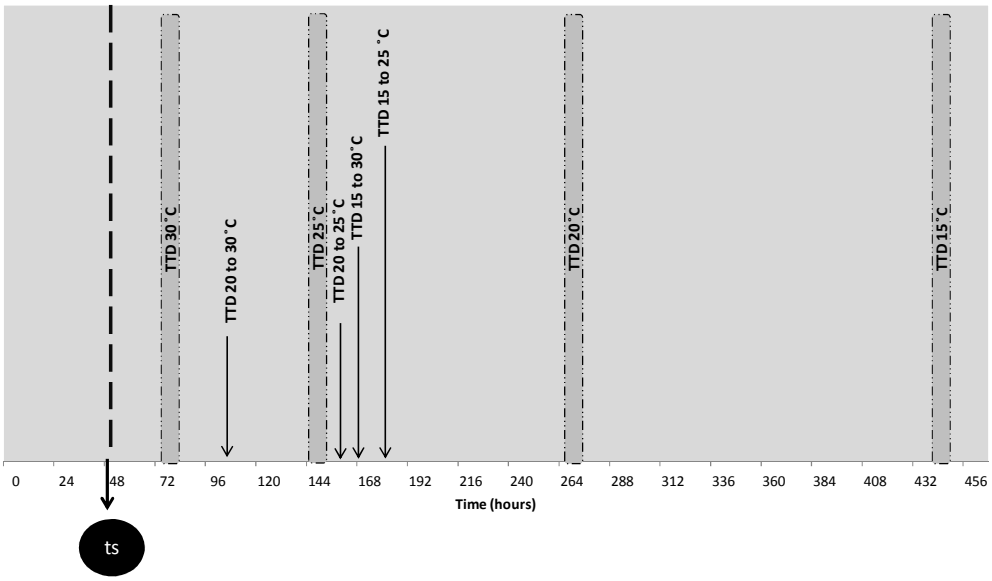

Figure 4. Comparison among the calculated TTD under both static and dynamic temperature profiles for isolate UdL-TA 3.244 at 10^2 spores/ml.

Results for isolate UdL-TA 3.244 at 10^2 spores/mL are summarised in Figure 4 which represents the TTD for the four shifting temperature scenarios and TTD of the static temperatures involved in the dynamic profiles. At the same temperature profile and inoculum concentration level the 10 tested isolates did not differ much in their TTDs. TTDs followed the time sequence F4<F2<F3<F1 except for isolate UdL-TA 3.327 which was F4<F3<F2<F1 for both inoculum levels (Fig. 5). The highest difference among isolates was observed in the 20 to 25 °C temperature regimes. The harshest temperature shift to initiate growth was from 15 to 25 °C, which took approximately 167.67 and 141.67 hours to reach the TTD, for the low (ca. 30 spores) and higher inoculum (300 spores) concentration, respectively. However, the differences observed in this

temperature profile (F1) were more marked within isolate than among isolates (see large error bars). The standard deviation was always greater for those profiles where the initial temperature was 15 °C.

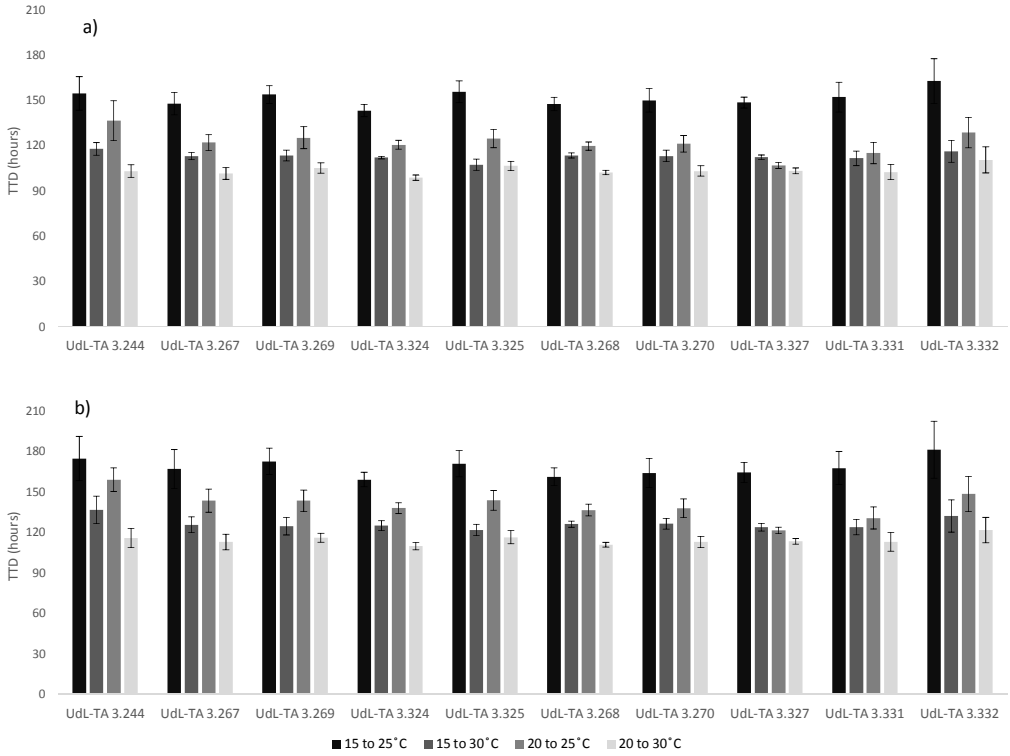

Figure 5. Experimental TTD obtained under the dynamic temperature profiles: 15 to 25 °C (black bars), 20 to 25 °C (dark grey bars), 15 to 30 °C (white bars) and 20 to 30 °C (light grey bars) for the a) 10^3 spores/mL inoculum and the b) 10^2 spores/mL inoculum for the 10 isolates tested.

3.3. Effect of inoculum level, temperature profiles and intra-species variability on aflatoxin B_1 production

The amount of AFB_1 produced after 7 days of growth (except for 20 °C and 15 to 25 °C which were 14 and 9 days respectively due to the slow growth rates) was determined for all isolates

and both inoculum levels (Fig. 6). First of all, AFB_1 was not detected at 15 °C, when tested as a single constant temperature treatment. The amount of AFB_1 production depended on the temperature, and followed the profile 35>30>25>37>20 °C, for the higher inoculum level and the profile 35>30>25>20>37 °C, for the lower inoculum level. For some isolates the optimum production was at 30 °C, for some others at 35 °C.

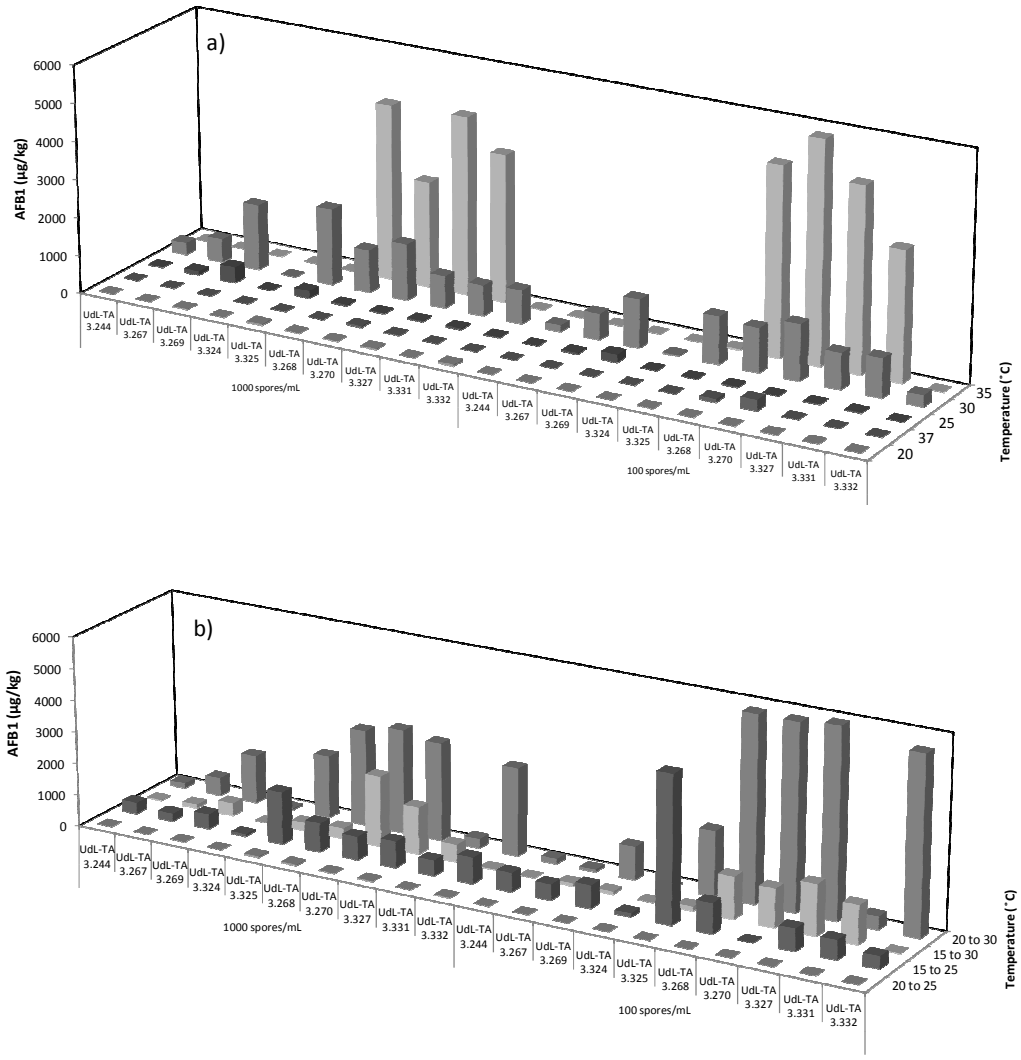

Figure 6. Aflatoxin B1 production (µg/kg) of the 10 *A. flavus* isolates under a) five constant temperature levels and b) four dynamic scenarios at both inoculum levels.

The inoculum level had different effects on the AFB_1 production depending on the temperature. AFB_1 production was in general higher at the higher inoculum, except for the 37 °C steady state treatment, and the temperature shift from 20 to 30 °C. This pattern was more marked for the high AFB_1 producer isolates. For example, at 37 °C, isolate UdL-TA 3.270 produced 282.97 and 40.45 µg/kg for the 10^2 and 10^3 spores/mL inocula, respectively or to a lesser extent isolate UdL-TA 3.268 which produced 86.45 and 1.44 µg/kg for the 10^2 and 10^3 spores/mL inocula respectively. The same pattern was found in the temperature shift from 20 to 30 °C (detailed later). At 35, 30 and 25 °C, no differences were observed among inocula. Only at the constant temperature of 20 °C was more AFB_1 produced at the higher inoculum level for all isolates. However, some isolates were not able to produce the toxin at this temperature.

Looking at the low producing isolates, no AFB_1 was produced at 35 °C and very low amount at 37 °C. At 25 °C some isolates of this group produced more AFB_1 than the ones in the high producer group, namely isolates UdL-TA 3.267, UdL-TA 3.269 and UdL-TA 3.325 at both inoculum levels. A similar behaviour was found at 20 °C where isolates UdL-TA 3.325, and UdL-TA 3.269 and UdL-TA 3.325 for the 10^2 and 10^3 spores/mL inocula respectively, produced more AFB_1 than some isolates of the high producer group at the same inoculum level.

In order to determine the effect of temperature shifts, the theoretical amount of AFB_1 produced taking into account the time periods at each temperature was calculated as follows:

$$Calculated\ AFB_{1TI} = \frac{(AFB_{1TI} * ts) + (AFB_{1TF} * tf)}{ts + tf} \quad (3)$$

Where AFB_{1TI} and AFB_{1TF} correspond to the amount of toxin produced at the initial and final temperature respectively under the single steady state scenarios, and ts and tf are the time periods for the initial and final temperature respectively for each shifting temperature profile. Table 3 shows both theoretical and experimental AFB_1 concentrations.

Table 3. Experimental amount of AFB1 and the calculated AFB1 produced (in µg/kg in YES medium) for the four dynamic scenarios, both inoculum levels and the ten strains.

Dynamic temperature		15 to 25 °C		15 to 30 °C		20 to 25 °C		20 to 30 °C	
		Calculated AFB1	Experimental AFB1	Calculated AFB1	Experimental AFB1	Calculated AFB1	Experimental AFB1	Calculated AFB1	Experimental AFB1
1000 spores/ml	UdL-TA 3.244	10.05	348.41	224.57	21.71	9.23	1.20	224.57	154.89
	UdL-TA 3.267	79.01	246.21	437.89	117.92	72.75	0.73	438.08	560.09
	UdL-TA 3.269	317.75	461.84	1227.65	394.23	296.00	6.13	1231.83	1506.65
	UdL-TA 3.324	0.46	71.83	3.75	0.93	0.69	0.14	4.02	52.95
	UdL-TA 3.325	147.43	1639.31	1434.11	242.69	145.93	41.92	1444.65	1967.94
	UdL-TA 3.268	27.34	904.74	797.56	320.56	27.82	32.25	800.27	3003.27
	UdL-TA 3.270	14.02	728.72	1052.71	2197.34	15.84	12.53	1055.67	3253.55
	UdL-TA 3.327	21.04	824.82	598.63	1467.84	26.83	21.62	606.13	3069.58
	UdL-TA 3.331	31.16	484.30	571.24	541.71	28.65	1.79	571.27	297.59
	UdL-TA 3.332	14.46	818.21	625.43	19.07	26.19	16.98	638.34	2759.85
100 spores/ml	UdL-TA 3.244	0.22	594.31	121.42	2.79	0.20	0.00	121.42	164.94
	UdL-TA 3.267	27.81	492.69	487.17	81.80	25.54	0.00	487.17	82.73
	UdL-TA 3.269	159.54	720.58	904.19	81.78	146.59	0.00	904.27	1041.60
	UdL-TA 3.324	3.39	117.15	19.65	3.41	3.11	0.00	19.65	11.68
	UdL-TA 3.325	5.30	4606.23	885.59	131.34	6.39	0.00	887.11	2023.25
	UdL-TA 3.268	20.17	944.26	826.20	1304.90	18.68	0.12	826.36	5820.10
	UdL-TA 3.270	4.47	0.00	1045.16	1195.52	4.16	0.00	1045.21	5811.20
	UdL-TA 3.327	13.55	696.70	672.71	1587.49	14.16	2.86	674.42	5930.62
	UdL-TA 3.331	18.02	612.17	727.45	1209.34	16.59	0.00	727.49	416.03
	UdL-TA 3.332	0.82	389.94	214.84	11.30	0.80	0.00	214.89	5571.19

No clear pattern was found where the shifting temperature treatments were used. For the changing scenario F1 (15 to 25 °C) a dramatic increase in AFB_1 production was detected, compared to the low levels detected at 15 and 25 °C, under steady state conditions. The same trend was observed for the shift from 20 to 30 °C, where in general an increase of 3-4 folds on AFB_1 production in the experimental treatment was observed, when compared to the calculated AFB_1. In contrast, the shift from 20 to 25 °C resulted in a lower AFB_1 production regarding the amount found in the steady state at the same two temperatures. More variability was found for the shift from 15 to 30 °C where for some isolates there was an overestimation and for others an underestimation of AFB_1 production.

4. Discussion

4.1. Intraspecies variability for growth and AFB_1 production

Generally , food products are stored at suboptimal conditions to minimise mould growth and this may influence the intraspecies variability in both germination and initial colonisation and potential for mycotoxin production (Astoreca et al., 2007; Bellí et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Mohale et al., 2013; Mugrabi de Kuppler et al., 2011; Pardo et al., 2004; Parra and Magan, 2004; Romero et al., 2007; Tassou et al., 2009; Tauk-Tornisielo et al., 2007; Yogendrarajah et al., 2016).

This study has utilised a suboptimal a_w level, representing environmental stress, to examine and quantify effects of steady state and shifting temperatures on growth of groups of high and low AFB_1 producing *A. flavus* isolates for the first time. The parameter studied was the TTD, which is the time in which mould growth is detected at a certain biomass level as demonstrated previously by Medina et al. (2012). These values are a very good approximation to understand the fungal growth of fungal colonies in a 3D space and at very low biomass levels. The data were based on spectrophotometric measurements, thus if a full model was developed and applied to realistic food products, thorough validation would be necessary. The present study has shown that it is possible to predict TTD under steady state and some shifting temperatures. The results obtained in the first part of the study (steady state temperatures) showed that as temperature became more marginal for conidial germination and mycelial growth, intraspecies variability increased. This trend has been observed using other criteria by some authors for other fungi.

For example, Romero et al. (2007) evaluated the effects of a_w (0.80-0.95) and temperature (15-35 °C) on lag phase of four *A. carbonarius* isolates, and found the greatest difference at limiting conditions. Garcia et al. (2011a) working with 79 isolates of *Penicillium expansum* reported coefficients of variation for the lag phase of 12.7 and 14.3% at 20 and 1 °C, respectively. This suggests that intraspecies variability is dependent on the environmental conditions, and is higher when conditions are closer to the boundaries for activity. In the present study, we have focused on steady state temperatures and shifting temperatures but under a fixed water stress condition. In this situation, under marginal conditions isolate variability was found to be higher.

For shifting temperature scenarios, intraspecies variability did not appear to be significant, with only few isolates, among the 10 studied, behaving differently from the others. The final temperature had the major effect on intraspecies variability. Within isolate variability was more affected by the initial temperature than by the final temperature, since %CV was higher for those profiles which were set initially at 15 °C.

Longer TTDs were observed when low inoculum levels were used. Conceptually TTD should parallel lag phases prior to growth. Of course, the latter parameter has been studied many times under different inoculum levels (Aldars-García et al., 2016; Baert et al., 2008; Burgain et al., 2013; Chulze et al., 1999; Morales et al., 2008; Sautour et al., 2003). Such studies have shown that changes in the inoculum size affected and increased the length of the lag phases prior to growth when the inoculum size decreased. Moreover, the inoculum level was also found to be a critical factor in TTD intraspecies variability: as inoculum decreased, intraspecies variability increased.

Considering the effect of temperature on AFB_1 production, the amount produced was highly variable among the 10 isolates. Santos et al. (2002) studied the production of patulin and citrinin by 10 isolates of *P. expansum*, and showed that patulin production was isolate dependent. Aldars-García et al. (2015) predicted the probability of growth and AFB_1 production of *A. flavus* using a cocktail inoculum of 25 isolates and an inoculum with a single isolate. Different results in terms of growth behaviour were obtained for both inocula but not for AFB_1 production which gave very similar probabilities, highlighting the possibility of a homogeneous boundary of AFB_1 production among isolates, although the amount produced by isolates was different. The

variability in the amount of mycotoxin produced will be influenced by nutritional substrate, interacting environmental conditions, source, age and whether wild or sub-cultured on rich artificial laboratory media (Garcia et al., 2011a; Romero et al., 2010; Yogendrarajah et al., 2016). Of course, prevention is better than cure. However, the present work perhaps provides some insight into ways to minimise mycotoxin production by reducing inoculum load or controlling germination of conidia of such fungi.

4.2. Predicting relative initial growth (TTD) and AFB_1 production under shifting temperature scenarios

In order to measure the effect of a temperature shift on the time needed to initiate growth, *A. flavus* isolates were subjected to sudden temperature upshifts. Adaptation to environmental stresses is usually explained by biological mechanisms in the cell, which requires a certain amount of time depending on the cells physiological state and the new environment conditions (Brooks et al., 2011; Swinnen et al., 2005). Many studies have been carried out under fluctuating temperature for bacterial pathogens (Bovill et al., 2000, 2001; Kim et al., 2008; Koseki and Nonaka, 2012; Muñoz-Cuevas et al., 2010; Zwietering et al., 1994) and into a lesser extent for fungi (Aldars-García et al., 2015; Gougouli and Koutsoumanis, 2012, 2010). In some of them when models included germination or growth rates, instantaneously adaptation to the new environment was assumed for these rates (Gougouli and Koutsoumanis, 2012) and in other cases, when primary observations were modelled, for example, visible growth, inclusion of a 'memory parameter' in the models was required for acceptable predictions (Aldars-García et al., 2015).

Muñoz-Cuevas et al. (2010) developed a dynamic growth model for a *Listeria monocytogenes* isolate. They found that growth behaviour depended not only on the magnitude of the change between the previous and current environmental conditions but also on the current growth conditions. Similarly, we found that TTD under dynamic temperature depended mainly on the final temperature and into a lesser extent on the magnitude of the change and initial temperature. Figure 4 illustrates an example of this dependence for isolate UdL-TA 3.244, at 10^2 spores/mL, in which all TTD for the changing temperature scenarios are around the TTD at 25 °C. This behaviour may suggest that 48 hours at a restrictive temperature could not be enough to

slow cell's metabolism to a point which prevents them from quickly adaptation to better growth conditions.

AFB_1 production under dynamic temperature conditions was enhanced under some scenarios and inhibited under others. Several authors have described that abiotic stress is involved in the activation of mycotoxin biosynthetic genes (Jurado et al., 2008; Kohut et al., 2009; Schmidt-Heydt et al., 2009). Then, the stress induced by the temperature shift may have a similar effect, triggering AFB_1 production. Nevertheless, in some cases, the temperature shift appeared to inhibit AFB_1 production.

Garcia et al. (2012) studied mycotoxin production by *Fusarium* spp. under 3 changing temperature scenarios (15 to 20 °C, 15 to 25 °C and 25 to 30 °C). They also found that for some profiles the mycotoxin production was enhanced and for other was inhibited. Furthermore, this pattern was different depending on the mycotoxin studied. Ryu and Bullerman (1999) studied the production of deoxynivalenol and zearalenone on rice with cycling temperatures, finding that mycotoxin production was stimulated under the temperature shifts.

Studies on the effect of inoculum revealed different outcomes on mycotoxin production; direct relationship between the amount of mycotoxin produced and the inoculum size was reported by Aldars-García et al. (2016) and Chulze et al. (1999). On the other hand, Morales et al. (2008) reported that colonies from conidial suspensions of 10^6 spores/mL produced lower amount of patulin (in apples) than those from the 10^4 spores/mL suspensions. These results may suggest a possible inhibition of germination, and thus mycotoxin production, when spore concentration is too high. This certainly occurs in soil fungi where fungistasis can limit the number of fungal spores germinating to ensure survival under stress conditions (e.g. *Fusarium* species). Thus, mycotoxin production may be enhanced as inoculum size decreases. A possible explanation of such behaviour is that when fewer spores colonise a niche, there is more nutrient availability for the fungus, and then more energy to be utilised for secondary metabolite production. Further research on this area is required to understand how inoculum size affects the mycotoxin production.

As conclusion, taking into account isolate variability and inoculum size in mycological studies would give more realistic results, since in a real scenario contamination we may encounter

different isolates in a food product. Furthermore, it becomes evident that temperature shifts have an important effect on fungal behaviour, and that there is potential for modelling and predicting toxigenic mould behaviour under steady state and fluctuating temperatures. The data generated in the present study is useful for a better understanding of the behaviour of isolates under dynamic temperature scenarios, in order to improve our understanding of mycotoxin contamination of food matrices, and thus help in the development of approaches to improve shelf-life of products prone to fungal spoilage and improve food safety.

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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).

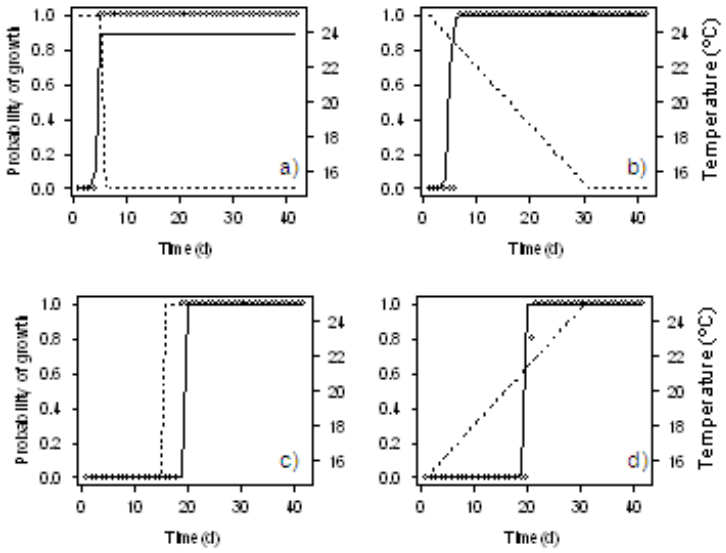

Figure 1. Observed growth probability of *A. flavus* TA-3.267 in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR

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.

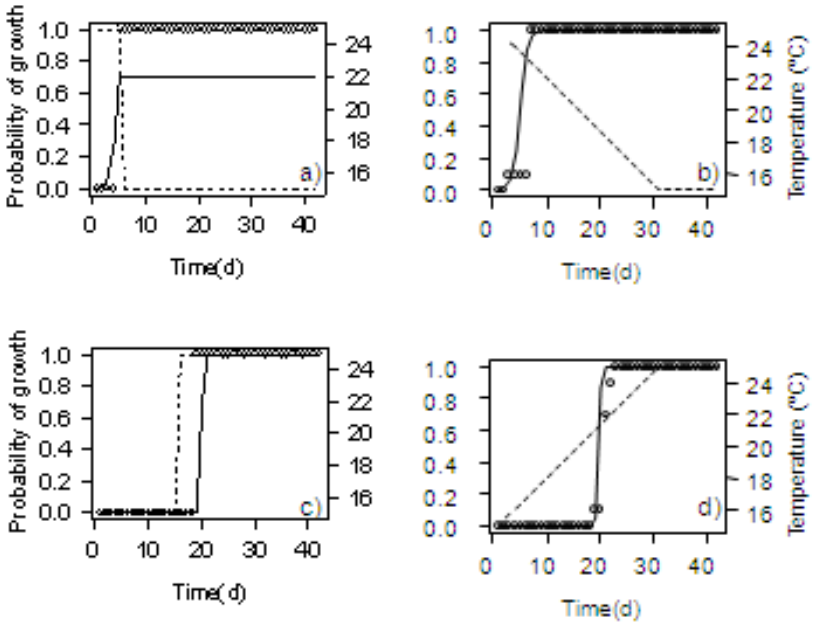

Fig.ure 2. Observed growth probability of *A. flavus* mixed inoculum in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

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.

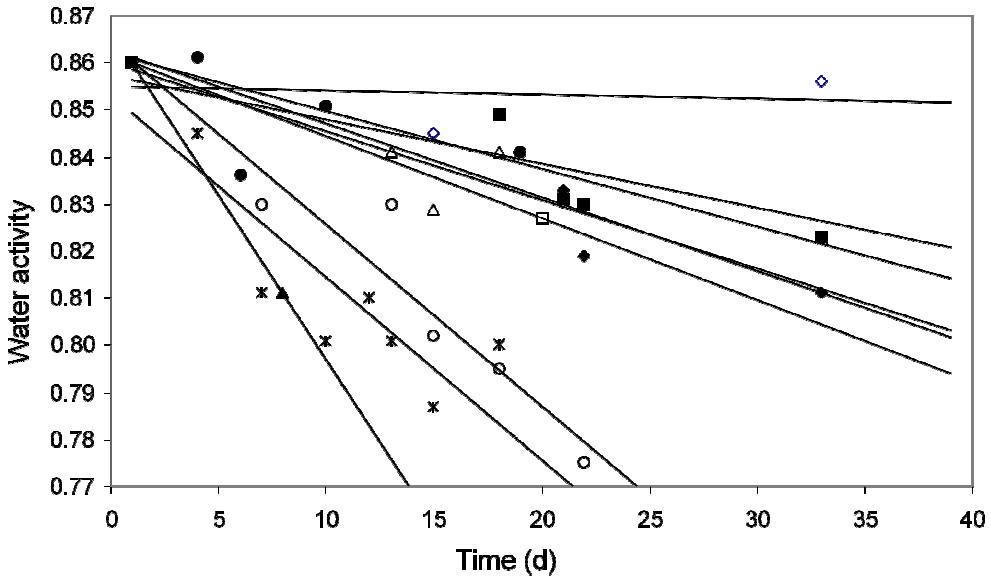

Figure 3. Checking of a_w values during incubation of the different treatments in pistachio nuts.

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%).

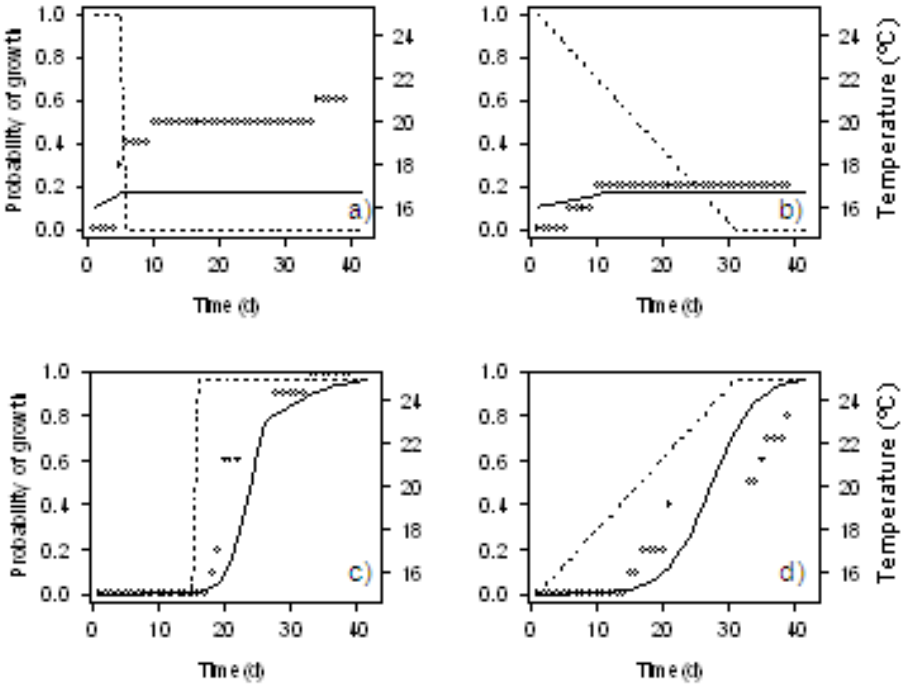

Figure 4. Observed growth probability of *A. flavus* TA-3.267 in pistachio nuts under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

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.

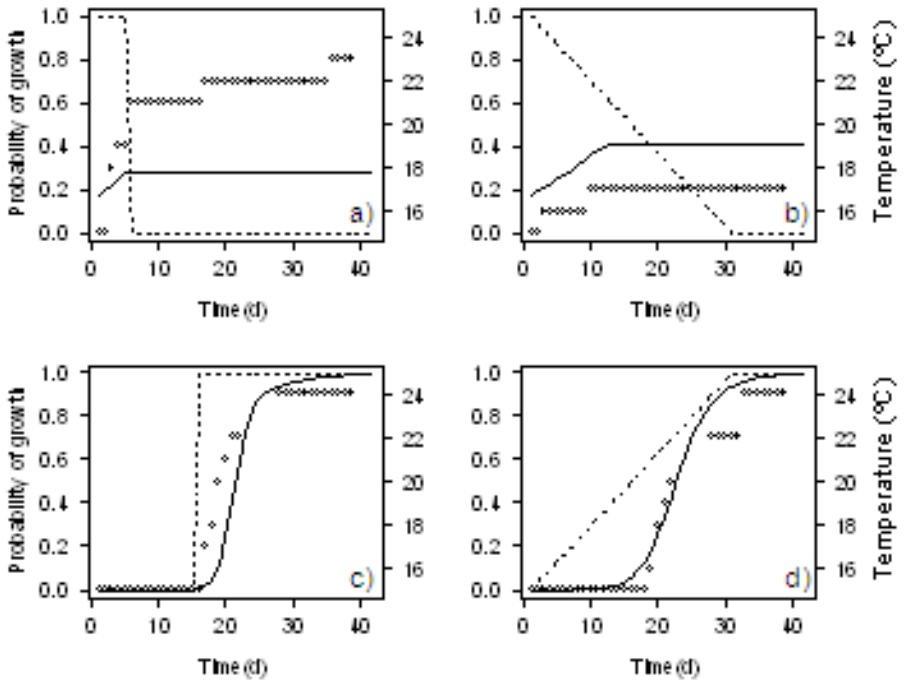

Figure. 5. Observed growth probability of *A. flavus* mixed inoculum in pistachio nuts under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

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).

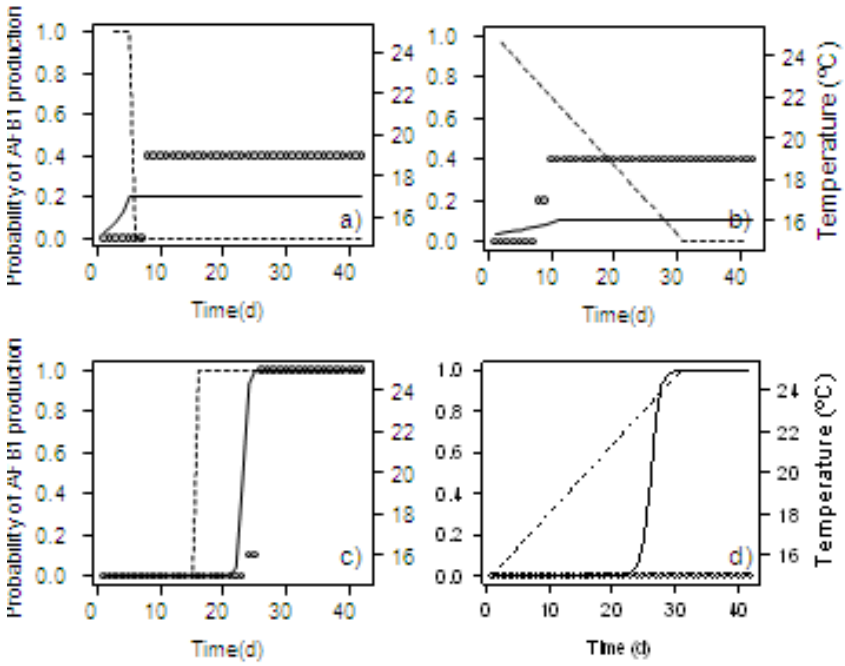

Figure. 6. Observed AFB1 production probability of *A. flavus* TA-3.267 in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

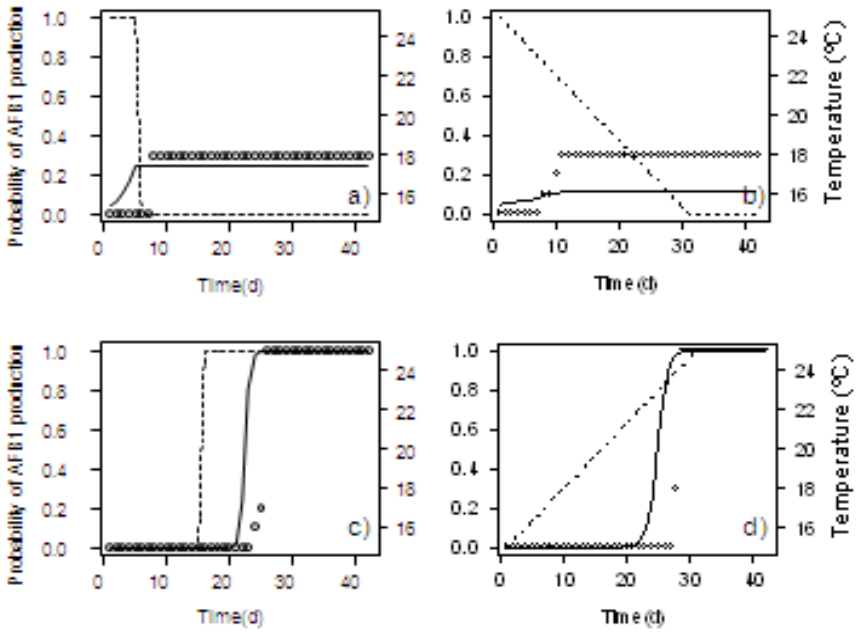

Figure. 7. Observed AFB1 production probability of *A. flavus* mixed inoculum in pistachio extract agar (PEA) under non-isothermal conditions (o) and predicted values (-). a) DS; b) DR; c) US; d) UR.

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.

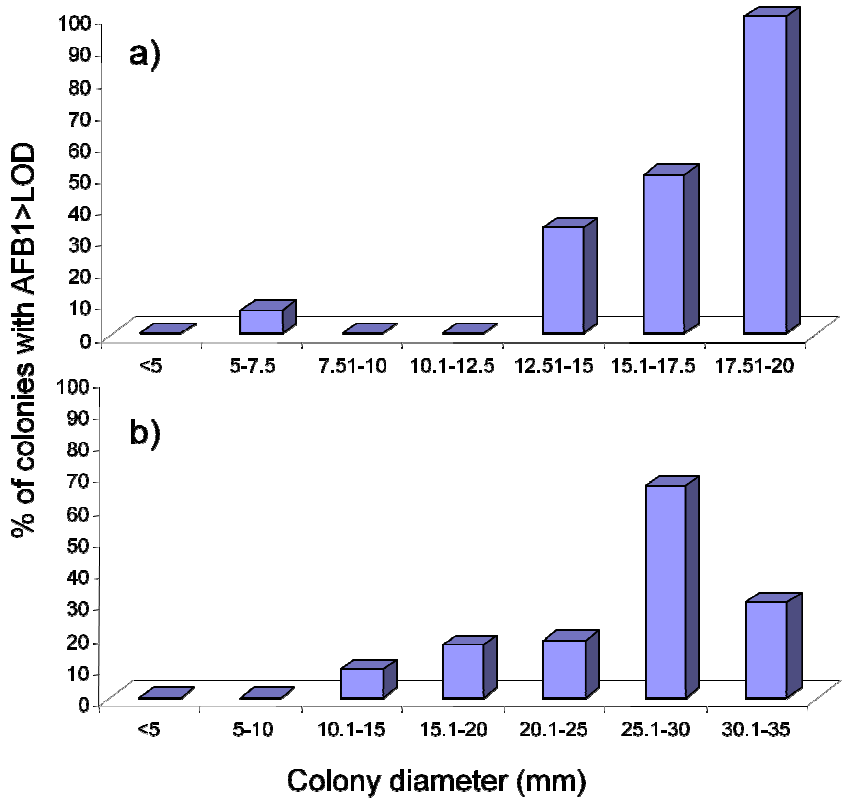

Figure. 8. Percentage of AFB1 positive *A. flavus* colonies as affected by colony size. a) Isolate TA-3.267; b) cocktail inoculum.

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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Chapter VI

Predictive models to describe the behaviour of *Aspergillus flavus* in maize under dynamic temperature and water activity environments

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Food Microbiology (Submitted)

Abstract

This study developed predictive models to describe the behaviour of *A. flavus* in maize extract agar and maize grains under a dynamic environment. Growth and AFB1 production was recorded along time under static (20, 25, 30 and 35 °C) temperatures and different water activities. Afterwards, logistic models, with temperature, a_w and time as explanatory variables, were fitted to binary data of growth and AFB1 production probability under static conditions. In addition, independent data were generated in maize extract agar and maize grain under two changing temperature profiles. During incubation air relative humidity (RH) was recorded in order to model a_w as a function of the initial a_w value, RH and time, and including it is input a_w in probability predictions.

Predictions of growth and AFB1 production under dynamic conditions showed different levels of concordance depending on the temperature profile and substrate, ranging from 66 to 100%, in almost all cases. For AFB1 lower concordances were obtained. In addition, maize grains were more conducive to AFB1 production than maize extract medium. Probability growth models in maize grains can be used to properly predict AFB1 in maize grains. These results indicate that the mathematical models developed can be a useful in describing fungal behaviour as a function of temperature and a_w, however, further refining of such models is required.

Keywords *Aspergillus flavus*, aflatoxin, predictive mycology, probability, changing temperature; water activity

1. Introduction

Fungi are distributed worldwide, may grow on a number of food commodities, and due to their capacity to utilize a variety of substrates and their relative tolerance to low water activity, temperature and pH they can be found in various foods and feedstuffs from almost every part of the world (Atanda et al., 2011). They can produce mycotoxins, toxic secondary metabolites structurally diverse, which can infect food, feed and agricultural commodities, during pre-harvest and post-harvest stages (Bryden, 2007). Overall, water activity and temperature are important criteria for the evaluation and control of food safety and quality.

Aspergillus flavus is a ubiquitous fungus in the soil where its growth is promoted by heat and humid environmental conditions (Klich, 2007). *A. flavus* produces aflatoxins, known to be carcinogenic. Maize is an agricultural product considered to be highly susceptible to fungal colonization and mycotoxins contamination worldwide (Barug et al., 2004), including *A. flavus* growth and aflatoxins production. Improper harvesting, storage or processing practices can lead to the occurrence of high levels of aflatoxins in maize used as human food or animal feed.

In order to improve the control and management of food safety and quality, there is a need to predict microbial responses in foods using mathematical models (Dantigny et al., 2005). Predictive models may provide important data about fungal 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. Prediction and prevention of mould spoilage is important for the food industry, it can be accomplished by limiting the contamination in the whole production chain (Dagnas and Membré, 2013; Georgiadou et al., 2012; Huang, 2014).

Most of the studies in predictive mycology focus on the effect of environmental factors on fungal growth and mycotoxins production under static conditions. However, dynamic environmental conditions occur along the food chain. Unfortunately, less attention has been paid on such fluctuations. Only few mycological studies were developed under dynamic conditions (Aldars-García et al., 2015; Garcia et al., 2012; Gougouli and Koutsoumanis, 2010; Kalai et al., 2014; Palacios-Cabrera et al., 2004; Peleg and Normand, 2013). Palacios-Cabrera et al. (2004) pointed out that changing the temperature conditions may stimulate

growth/mycotoxin production under certain circumstances. Then it is important to take into account these fluctuations during the developing and validation of models, otherwise their applicability is compromised. Prediction of mycotoxin production has been shown to be complex under static temperature levels (Garcia et al., 2013, 2011a; Marín et al., 2009), thus prediction under changing temperature is challenging.

The ability to grow and the amount of mycotoxin which a mould produces in the food depends completely on the ecological and processing parameters of the particular foodstuff (Filtenborg et al., 1996), as well as on its genetic ability to synthesise the toxin. Besides, fungal growth does not imply necessarily the presence of mycotoxins, this is explained by the fact that not all the strains in a mycotoxigenic species are able to produce mycotoxins, and in addition, the conditions favourable to growth may not be conducive to mycotoxins production. Also it is important to consider that the absence of fungi in a food product does not mean the absence of mycotoxins, because these molecules are highly resistant and may bear most industrial food processes. Moreover, growth is a parameter which presents less intraspecific variability, and its kinetics is more known, than mycotoxins production. Then, the prevention of fungal growth in all the steps of the food production and processing, lead to the prevention of the mycotoxins presence in the final food product (Marín et al., 2008). Considering all this information, we focussed on probabilistic models, which predict the probability of a given event occurring, such as fungal growth or toxin production.

In a previous work (Aldars-García et al. 2015) in pistachio nuts, it was evidenced that for correct prediction of growth and toxin probabilities in real food substrates, there was a need to include in the model a_w as a variable as function of temperature and time. The objective of this study was to develop a predictive model to assess the effect of variable temperature and a_w on the growth and aflatoxin production of *A. flavus* at dynamic temperature conditions.

2. Material and methods

2.1. Fungal isolate

Aspergillus flavus UdL-TA 3.327 was used in these experiments. This strain was isolated from maize grains purchased from a wholesaler in Lleida, Catalonia, Spain. Briefly, maize grains were plated on DRBC, and the isolated colonies were identified according to the taxonomical descriptions of (Pitt and Hocking, 2009). Aflatoxigenic capacity was assayed on Potato Dextrose

Agar at 25 °C, after 7 incubation days and determined by high performance liquid chromatography (HPLC).

2.2. Media preparation

Maize Extract Agar (MEA): Maize extract was prepared by boiling 40 g of raw ground dry maize grains in 1 L distilled water for 30 min. After that, the extract was filtered and the amount of evaporated water was made up to adjust it to 4% of maize extract. Water activity of the media was adjusted by addition of certain amounts of glycerol-water to obtain the desired a_w of each treatment and 2% of maize grain in the medium. Then, 12 g of agar were added per L of medium (for each a_w) and they were autoclaved and poured into 90 mm sterile Petri dishes which were prepared under aseptic conditions. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003.

Maize grains: An initial analysis showed that AFB1 concentration in the grain was under the LOD. Maize grains were autoclaved (15 min at 121 °C) in 1-L bottles filled with 300 g of maize grains. Once sterilized, the a_w was adjusted by aseptically adding the correspondent amount of distilled water to the maize grains to 0.87 a_w. The bottles were cooled down to approximately 4 °C for 48 h with periodic hand-shaking during this period. After that, maize grains were placed in 90 mm Petri dishes under aseptic conditions.

2.3. Experimental design

In order to build a model under static conditions, the growth and AFB1 production of *A. flavus* was studied using a full factorial design, where factors involved where temperature and a_w. Four temperature levels were studied: 20, 25, 30 and 35 °C. Regarding a_w, four levels were included for the static experiments: 0.84, 0.86, 0.88, and 0.90. Maize extract agar 2% (MEA) was used as medium. For the static conditions, four plates were prepared for inoculation per T-a_w combination (4 x 4 x4, a total of 64 plates) and two plates per T-a_w combination were prepared (2 x 4 x4, a total of 32 plates) for controlling the a_w throughout the study.

On the other hand, experiments under changing conditions of temperature were carried out both in MEA and maize grains. In both cases, two temperature profiles were tested (Fig.3). Regarding a_w, one level (0.89 a_w) was set for the MEA experiments. Maize grain experiments were set at 0.87 a_w. In this case, ten MEA plates at 0.89 a_w and ten maize plates at 0.87 a_w were

inoculated. Additionally, four MEA plates per condition and ten maize plates were prepared for a_w determination throughout the study.

2.4. Inoculum preparation and inoculation

A. flavus UdL-TA 3.327 was grown on potato dextrose agar (PDA) medium at 25 °C for 7 days. Spores were collected by scraping the surface of the plates and diluting them in sterile water adjusted to the correspondent a_w value with glycerol, containing Tween 80 (0.05% v/v)) and filtered through sterile glass wool into a tube. After counting the spores on a Thoma chamber, the spore suspensions were then serially diluted to a concentration of 10^5 spores/mL.

Petri dishes (both MEA and maize grains) were inoculated with 5 µL of the spore suspension (10^5 spores/mL), onto four equidistant points (4 points per plate, ca. 500 spores per point).
For the experiments at static temperature conditions, inoculated plates of the same a_w were sealed with Parafilm M®, in order to keep a_w as constant as possible, and placed in plastic containers and incubated at the corresponding temperature condition for 24 days.

For the experiments at dynamic temperature conditions (both in MEA and maize grain), after inoculation, plates were not sealed with Parafilm M®, and were placed in sealed containers for incubation at the different temperature profiles for 24 days. In this case, as Parafilm M® was not used, a variation in a_w was expected.
Air relative humidity sensors were placed inside the containers both under static and dynamic conditions in order to measure its variation with temperature. The relative humidity of the air inside the Petri plates was not measured. Instead a_w measurements were taken along time.

2.5. Growth assessment

For both MEA and maize grains, fungal colony radii were determined from the first sign of growth till the end of the study (24 days), with the aid of a binocular magnifier.

2.6. AFB1 analysis

AFB1 production was determined from the first sign of growth up until the end of the incubation time (24 days) in different size colonies (from 3 to 18 mm radius), using a previously described

high performance liquid chromatography (HPLC) method (Aldars-García et al., 2015). A 5-mm diameter agar plug from the centre of each colony was weighed and introduced into 3-mL vials. After sampling, the plates were taken back to incubation, for the assessment of the other colonies present in the Petri plates which were not sampled. 1 mL of methanol was added to vials and vortexed for 5 s. After being left stationary for 60 min, the extracts were shaken again, filtered (MillexR SLHV 013NK, Millipore, Bedford, MA, USA), dried in a nitrogen stream and stored at 4 °C until HPLC analysis. All extracts were resuspended in 0.5 mL of methanol:water (50:50 v/v) and a volume of 100 mL was injected into the HPLC system (Waters, Milford, MA, USA). The detection limit of the analysis was 0.1 ng/g of AFB1, based on a signal-to-noise ratio of 3:1

2.7. Model fitting: Probability of growth and AFB1 production

Logistic regression was used to model the probability of growth (Eq. (1)) and AFB1 production (Eq. (2)) as a function of a_w, temperature and time, using R statistical software with the glm function. Using the data generated under static temperature conditions, the binary values along time (0=no visible growth/no AFB1 detection; 1=growth/AFB1 detection) were adjusted by linear logistic regression. Thus the models developed in the present study are not based on any biological and/or conceptual assumption.

$$logit(P_G) = ln\ \frac{P_G}{1-P_G} = \sum b_0 + b_1 * T + b_2 * a_w + b_3 * t + b_4 * T^2 + b_5 * {a_w}^2 + b_6 * T * a_w + b_7 * t * a_w + b_8 * t * T \quad (1)$$

$$logit(P_{AF}) = ln\ \frac{P_{AF}}{1-P_{AF}} = \sum b_0 + b_1 * T + b_2 * a_w + b_3 * t + b_4 * T^2 + b_5 * {a_w}^2 + b_6 * t^2 + b_7 * T * a_w + b_8 * t * a_w + b_9 * t * T \quad (2)$$

logit(P) represents ln[P/(1_P)], ln is the natural logarithm, P_G or P_{AF} are the probability of growth initiation or AFB1 detection (in the range of 0-1), T is the temperature (°C), t is the time of incubation (d), a_w is the water activity 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.

Based on these previously generated models, predictions were performed for the non-isothermal conditions. 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 determined the predicted probability in a certain time point. However, as suggested by Aldars-García et al. (2015), the R algorithm included a 'memory' correction. Thus the mean temperature in the ten preceding days was used as input temperature in the model. Water activity values were estimated at each time point under the variable temperature profiles as a function of initial water activity and measured relative humidity (Eq. (3),(4), (5) and (6)) measured by the sensors. Moreover, the algorithm was made somewhat cumulative, as predictions lower than those of the preceding time point were not allowed.

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.

3. Results and Discussion

3.1. Dynamics of water activity and relative humidity under non-isothermal profiles

In the experiment at constant temperature conditions, a_w measurements along time revealed no significant differences in a_w values, due to the use of parafilm to seal the plates. This allowed to consider both temperature and a_w constant along time, consequently the probability models generated based on these data could subsequently be used for prediction of probabilities at variable profiles of both temperature and a_w.

Two different dynamic temperature profiles were tested in this work (dotted lines in Fig. 3). Through the incubation period, air relative humidity (RH) was recorded continuously; while four a_w measurements were taken along time both in agar and maize plates. RH values recorded are presented in Fig. 1; for the first profile, RH varied between 60 and 75%, with a slight decrease along time, while for the second profile, RH remained quite constant between 75 and 85%.

Regarding a_w, it did not vary significantly along time in the agar plates (thus it was not represented in Fig. 1), while it decreased significantly in maize plates. For prediction purposes, a_w was predicted from RH profiles. Although the variation of a_w in agar was not significant under our particular temperature conditions, to obtain a general procedure, a_w was proposed to be a function of initial a_w and the decrease in air RH (although in this case coefficients were mostly insignificant, a_w was equivalent to initial a_w):

First profile:

$$a_w = initial\ a_w - 0.000181845 * (initial\ RH - RH) + 0.00000194 * (initial\ RH - RH)^2 \quad (3)$$

Second profile:

$$a_w = initial\ a_w - 0.00000723 * (initial\ RH - RH) + 0.00000745 * (initial\ RH - RH)^2 \quad (4)$$

For predictions in maize grain, a_w was proposed to be a function of initial a_w, decrease in RH and time (both functions and experimental points are shown in Fig 1):

First profile:

$$a_w = initial\ a_w - 0.0006547815 * (initial\ RH - RH) + 0.000163769 * (initial\ RH - RH)^2 - 0.005039122 * time \quad (5)$$

Second profile:

$$a_w = initial\ a_w - 0.031737683 * (initial\ RH - RH) + 0.000874214 * (initial\ RH - RH)^2 - 0.005039122 * time \quad (6)$$

Predicted a_w values obtained from RH correctly agreed with the experimental a_w measured experimentally (Fig. 1). Using these models for a_w would allow to know a_w evolution in stored cereals in which initial a_w is known and only air relative humidity would be required to be measured, which is much easier to be recorded, and in addition air RH meters are not expensive.

The knowledge and understanding of the evolution of a_w in low and intermediate moisture stored foodstuffs is highly important in the food industry for the design and optimization of drying equipment, design of packages, predictions of quality, stability and shelf-life. Several researchers have investigated the temperature dependence of moisture or a_w in such food products, for example Arena et al. (2013) showed a significant decrease in moisture content of pistachio nuts, during postharvest storage, after several days of storage at 30°C.

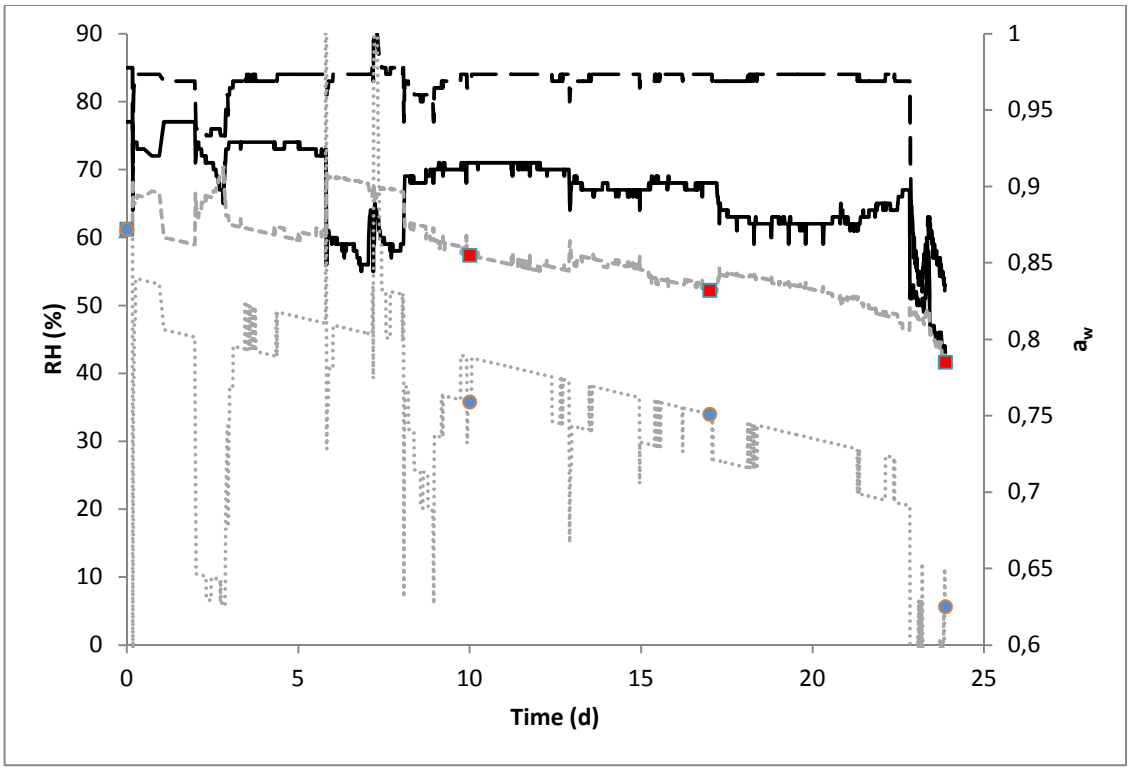

Figure 1. Air relative humidity (RH) recorded continuously throughout the experimental time (RH profile 1 (—), RH profile 2 (– – –)), a_w predicted from RH (a_w profile 1 (----), and a_w profile 2 (·····)) of maize experiments. Four a_w data points measured along experimental time in maize plates: ■ Experimental a_w for maize under profile 1 and ● experimental a_w for maize under profile 2.

3.2. Prediction of *A. flavus* behaviour under dynamic conditions

3.2.1. Growth prediction

Linear logistic regression on isothermal data led to the following model:

$$logit\ (P) = 3834.8786014 - 18.3764129 * T - 8538.4207020 * a_w - 74.4936756 * t - 0.1569008 * T^2 + 4549.0688443 * {a_w}^2 + 31.3329125 * T * a_w + 80.9958871 * a_w * t + 0.3735236 * T * t \quad (7)$$

The index of concordance of the developed model was 99%. Figure 2 shows probability lines at 0.84 and 0.88 a_w, at 20, 22, 25 and 30 °C. Growth was delayed about 5 days at 0.88 a_w when temperature decreased from 30 to 20 °C, while at 0.84 a_w, growth was delayed about 10 days when temperature decreased from 30 to 22 °C, and no growth was detected at 20 °C after 24 days. When growth occurred, the increase from 0 to 1 probability was sharp. This abrupt increase in growth has been reported by several authors (Aldars-García et al., 2015; Astoreca et al., 2012; Marín et al., 2012), and usually occurs when multispore inocula are used (Aldars-García et al., 2016).

When this model was used to predict growth probability under the dynamic temperature profiles, with variable a_w coming from the above equations, there was a certain deviation, which differed when predictions were made in agar or maize. Predictions in MEA are shown in Fig. 3. For the first profile, observed growth was delayed two days compared to the predicted one (93% concordance). Even though the predictions were carried out taking into account the mean temperature in the 10 preceding days (as suggested in Aldars-García et al. (2015)), and initiation of growth occurred while temperature increased, growth was still predicted earlier than observed. A possible explanation of this small overestimation may be due to the corrected values for a_w and temperature (Fig. 4) (taking into account the conditions of the preceding days).

Looking at the corrected values, 21-24 °C and 0.89 a_w were the conditions used for prediction in the first days, compatible with efficient growth and responsible of the slightly earlier growth prediction. For the second profile, there was a 100% concordance between observed and

predicted data in agar. The corrected a_w and temperature values properly defined the experimental conditions. In general good agreement was obtained for both dynamic profiles in MEA, and no concordance between observed and predicted values corresponded to “fail-safe” scenarios in which growth is predicted but not experimentally observed.

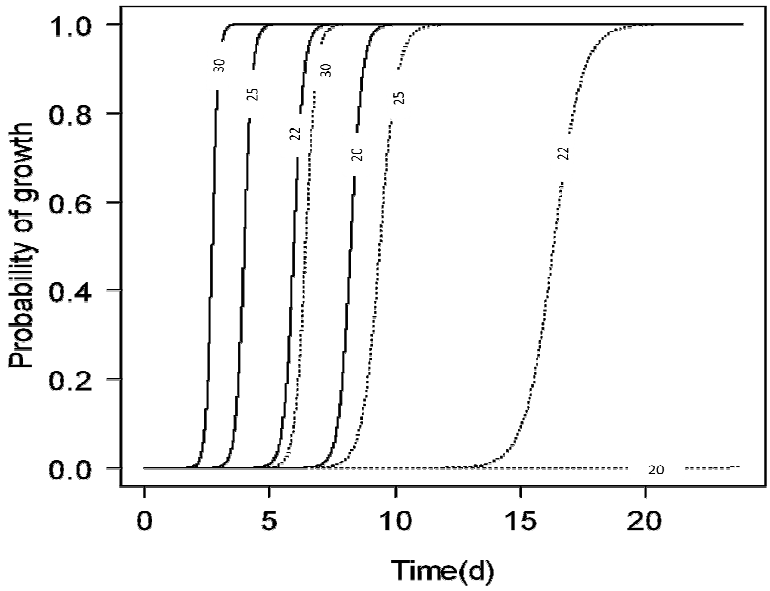

Figure 2. Probability growth lines at 0.84 (dotted lines) and 0.88 a_w, (continuous lines) at 20, 22, 25 and 30 °C.

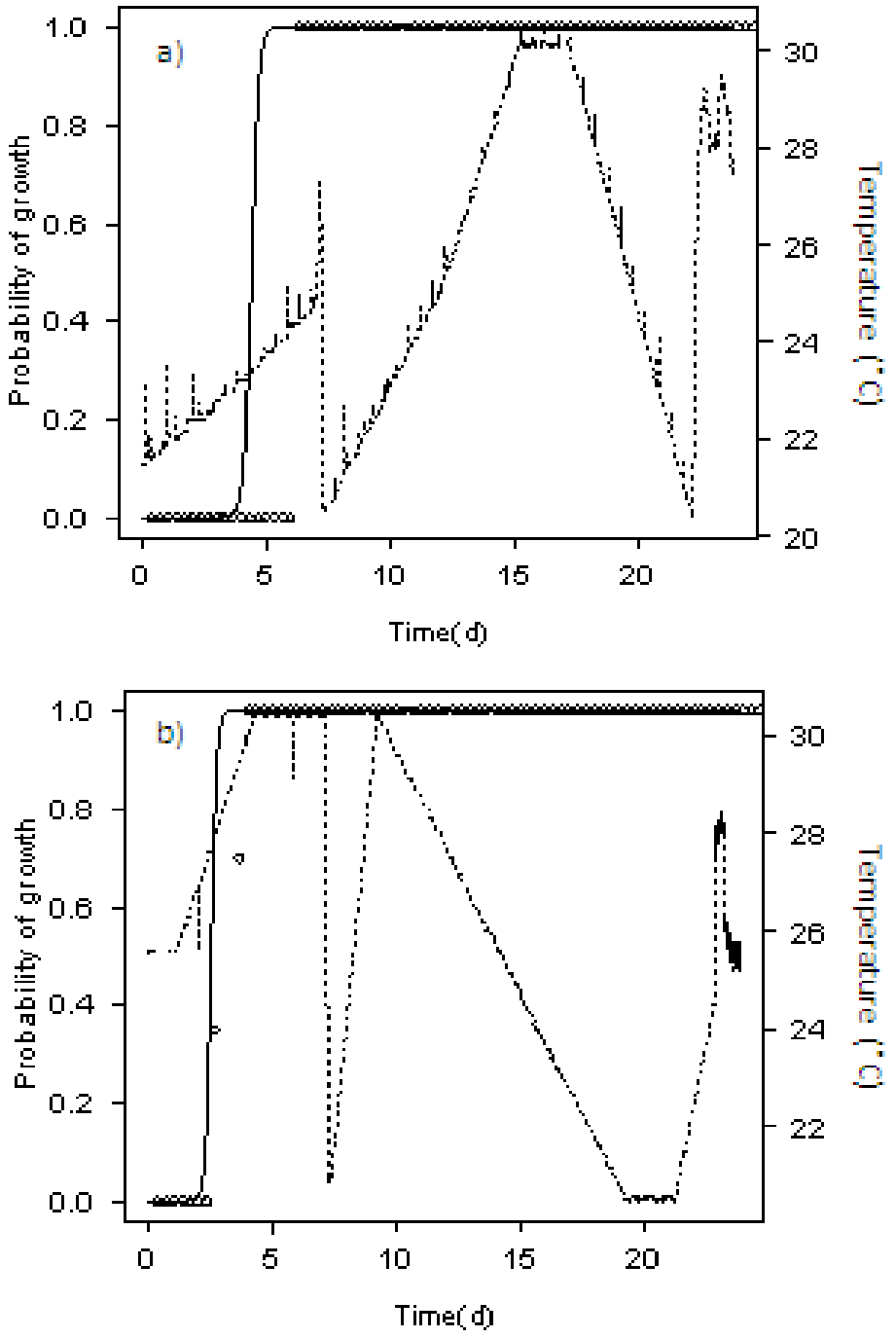

Figure 3. Observed growth probability (o) and predicted values (-) of *A. flavus* in MEA under dynamic conditions of a) profile 1 and b) profile 2. Dotted line represents the temperature along time

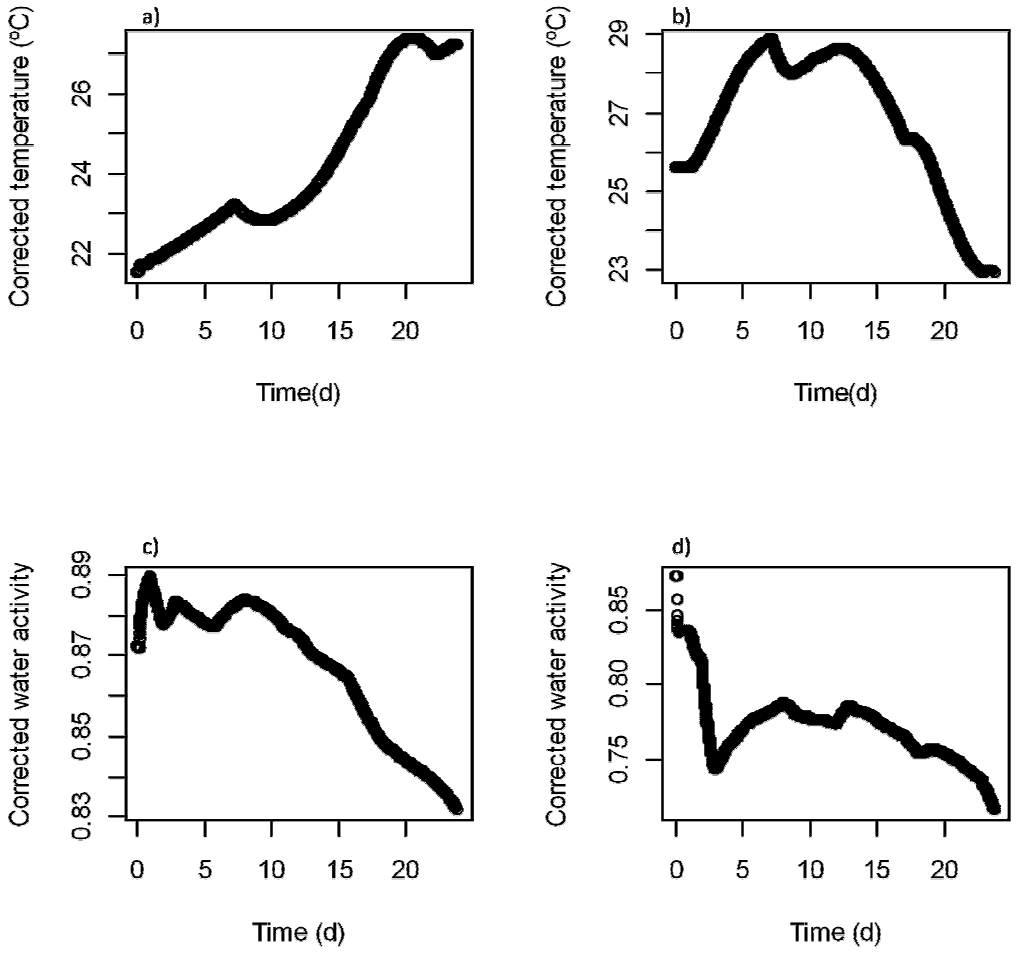

Figure 4. Corrected values for temperature and a_w (accounting for the 10 preceding days) used to predict growth under dynamic temperature along time.

Considering the mean temperature of the 10 preceding days for the dynamic predictions suggests a "memory effect" on fungal behaviour due to the past conditions, as suggested in Aldars-García et al. (2015). Also, for bacterial growth, Juneja et al. (2009) included a "memory parameter" on the secondary modelling of growth obtaining accurate estimations. These results suggest that the inclusion of a "memory parameter" will improve such predictions and then is required for the proper development of the non-isothermal models.

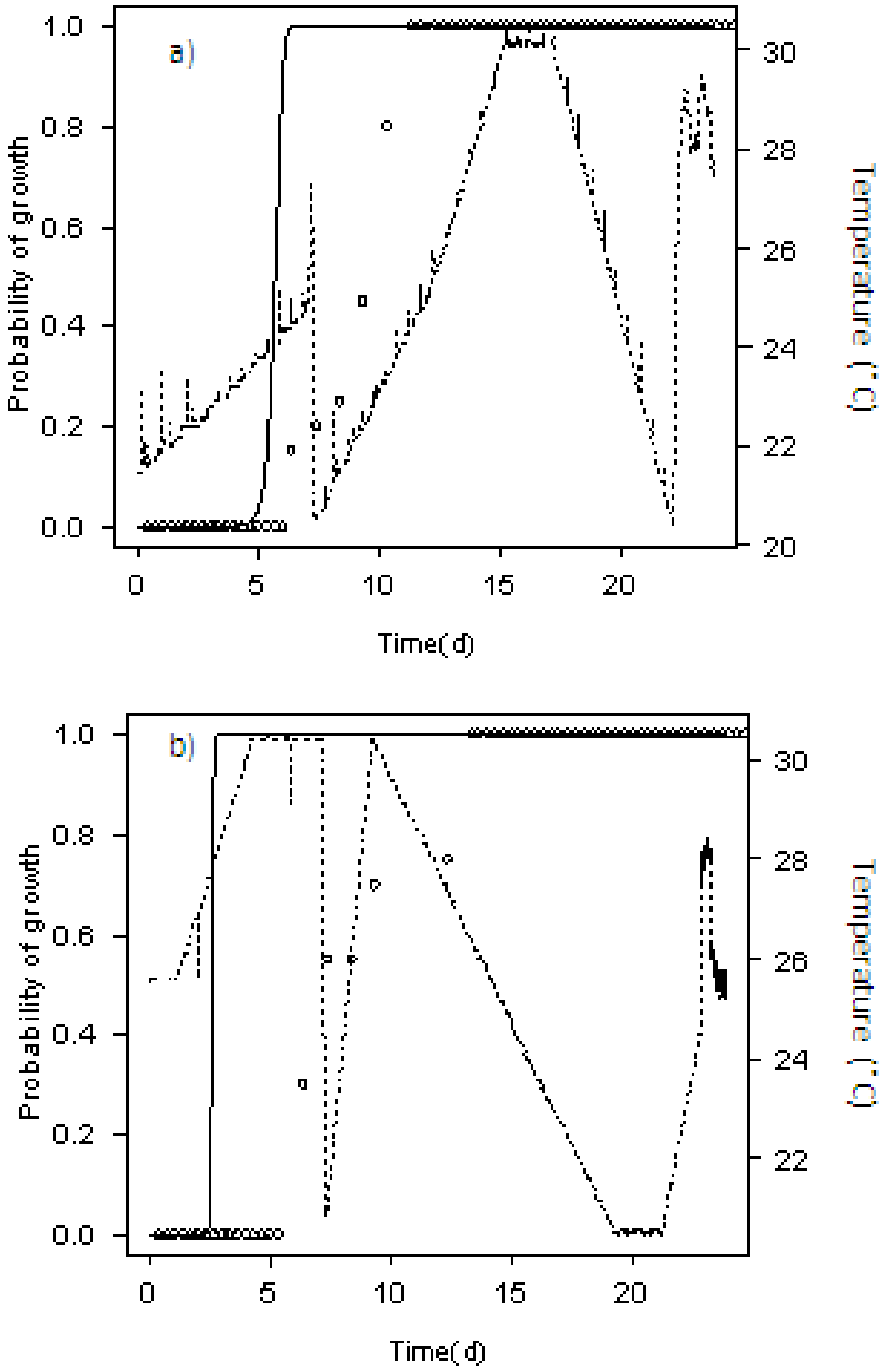

Figure 5. Observed growth probability (o) and predicted values (-) of *A. flavus* in maize grains under dynamic conditions of a) profile 1 and b) profile 2. Dotted line represents the temperature along time.

Validation was performed on maize grains under both dynamic temperature profiles. For the first profile, there was a delay in 4 days in observed growth, compared to the predicted one, and in this case the index of concordance was reduced to 84% (Fig. 5). On the 6th day observed probability was under 0.2 while predicted one had already reached almost 1. Looking at the corrected values for a_w and temperature (Fig. 4) (taking into account the conditions of the preceding days), 21-24 °C and 0.87-0.89 a_w were the conditions used for prediction in the first days, compatible with efficient growth in agar (Fig. 2), but not so much in maize according to results.

For the second profile, a delay in 5 days occurred between observed and predicted data, on days 3 to 5 observed probability was 0 while predicted one was 1, in this case the index of concordance was 75%. Looking at the corrected values for a_w and temperature (Fig. 4), temperature was highly conducive to growth (25-29 °C) but a_w decreased from 0.85 to 0.75 before the 3 day were the conditions used for prediction in the first days (Fig. 4), however, before it decreased there was a chance for growth to initiate, and it was predicted as such, while in real maize growth only occurred later, when a_w newly increased to almost 0.80.

The overestimation of growth initiation in maize grains, apart from the slight deviance that may be attributed to the corrected a_w values used for predictions, must be due to the ability to utilize the nutrients present in the maize compared to agar medium. It has been reported several times that the availability of nutrients may affect the chances of growth, specially at marginal a_w values (Mousa et al., 2013; Pardo et al., 2004; Yogendrarajah et al., 2016). Kapetanakou et al. (2011) reported that the substrate structure has an important effect on growth. Including the viscosity of the substrate in their experimental design, they modelled the effect of temperature, a_w and (gel) structure on OTA production by *A. carbonarius* on malt extract broth and food matrices with different viscosities. Their results showed that growth and OTA production decreased as medium viscosity increased. Thus, herein when we develop the predictive models on agar, and extrapolate them to a food matrix, the difference in substrate structure should be taken into account. The same conclusion was given by Garcia et al. (2012) studying the effect of cycling temperatures on growth and mycotoxin production by *Fusarium graminearum* and *F. verticillioides* on soybean agar extract and soybean seeds. They determined the growth rate and the amount of zearalenone (ZEA), deoxynivalenol (DON) and fumonisins (FBs) produced in both

substrates. The extrapolation of growth on agar to soybeans led to overestimated values. In fact, this difference was already observed in predictions under non-isothermal conditions in pistachio nuts in Aldars-Garcia et al. (2015), however, as in that case a_w was not controlled or modelled, the different behaviour was mainly attributed to the different a_w levels and not to substrate composition or structure. Also, Garcia et al.(2011b) modelled the growth of *A. parasiticus* and *A. ochraceus* under static marginal conditions, and validated the models in maize grains, peanuts and coffee beans. In general, their predictions gave an overestimation of times to growth when used for prediction in the food matrices. To overcome these problems with validation, either a correction factor from agar to real foods could be applied (to apply an additional percentage on the predicted times) or just assume that the predictions are in the 'safe' side).

3.2.2. AFB1 production prediction under dynamic conditions

Linear logistic regression led to the following model:

$$logit\ (P) = -77.33557 - 1.049601 * T + 196.8969 * a_w - 2.166014 * t - 0.01580680 * T^2 - 147.4760 * {a_w}^2 + 0.005724380 * t^2 + 2.368521 * t * a_w + 2.420820 * a_w * t + 0.001692884 * T * t \quad (8)$$

The index of concordance of the AFB1 production probability model developed was 82%. Figure 6 shows AFB1 production probability lines at 0.84 and 0.88 a_w, at 20, 22, 25 and 30 °C. In any case the probability reached 1 during the 24 days. At 0.84 a_w the probability never reached the 0.5 value, while at 0.88 a_w this value was reached after 15, 19 and 23 days at 30, 25 and 22 °C, respectively. When toxin production was detected, the departure from the 0 value was smooth. Probabilistic models reporting mycotoxin production are scarce, mainly due to the high amount of work involved. Marín et al. (2012) developed probabilistic models for growth and AFB1 production by *A. flavus* on pistachio nuts as a function of time, temperature and moisture content. They reported a similar behaviour, under static conditions, with decreased AFB1 production with lower a_w and temperature, also reporting the smooth increase in the probability lines.

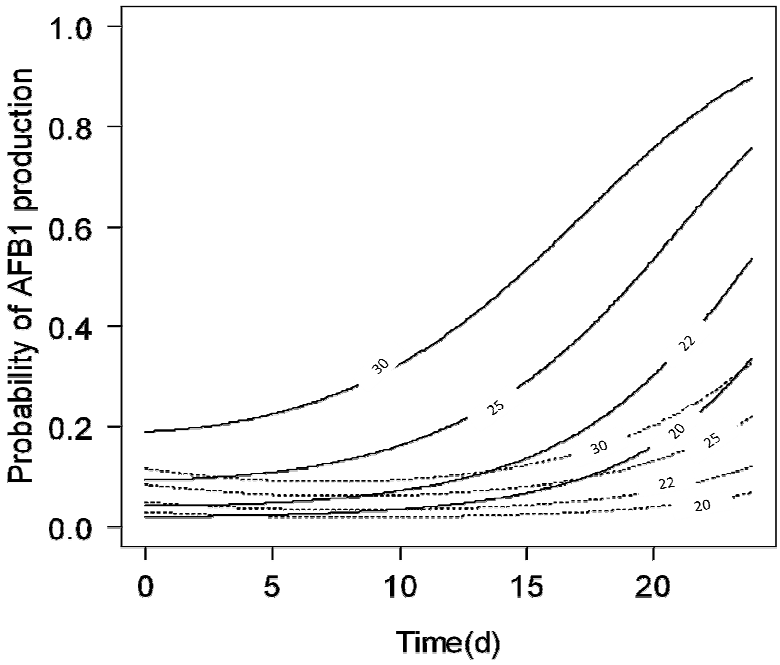

Figure 6. AFB1 production probability lines at 0.84 (dotted lines) and 0.88 a_w (continuous lines) at 20, 22, 25 and 30 °C.

When this model was used to predict AFB1 probability under the dynamic temperature profiles, with variable a_w coming from the above equations, there was a certain deviation, which differed when predictions were made in agar or maize.

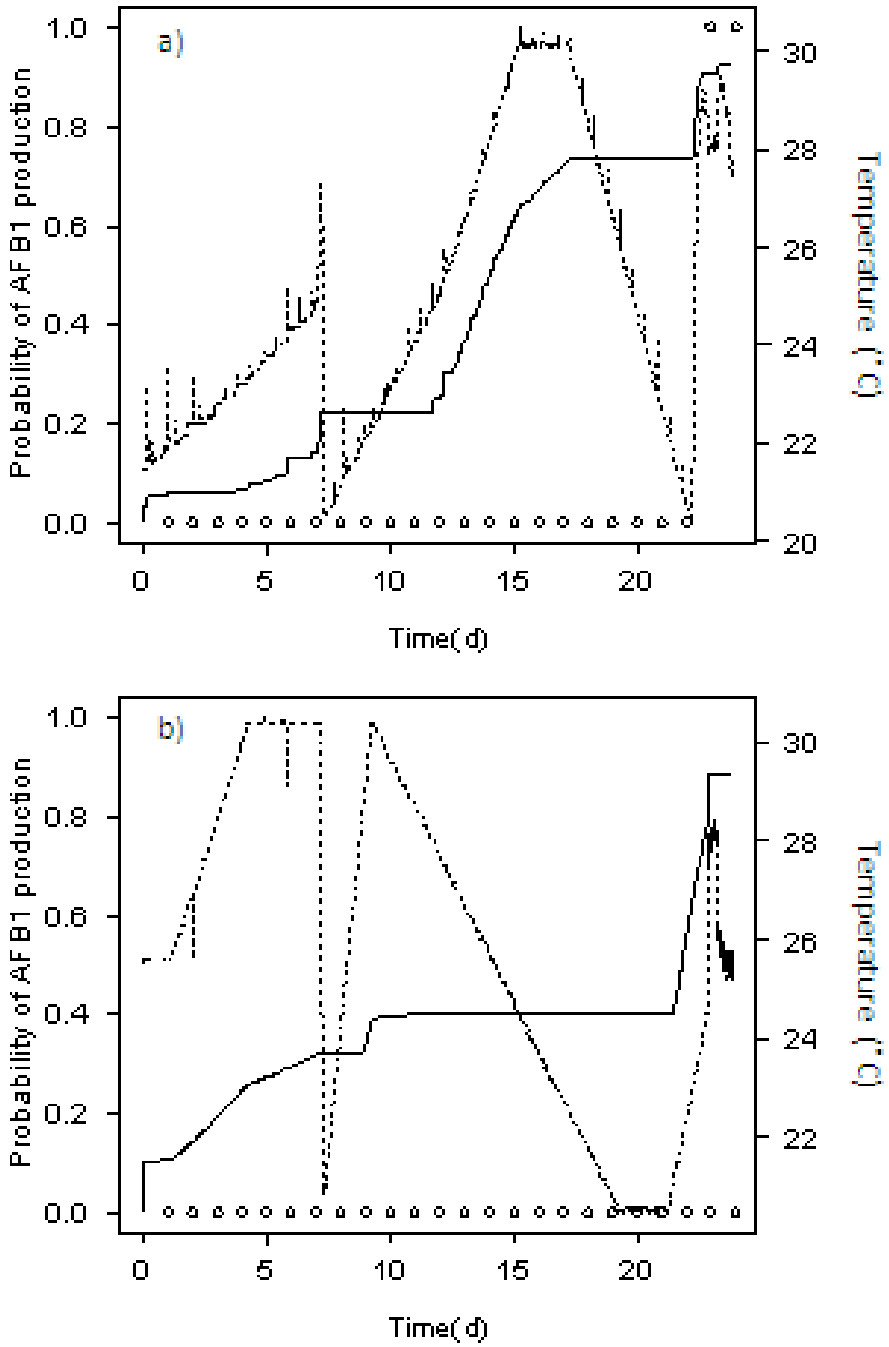

Figure 7. Observed AFB1 production probability (o) and predicted values (-) of *A. flavus* in MEA under dynamic conditions of a) profile 1 and b) profile 2. Dotted line represents the temperature along time.

Predictions in MEA are shown in Fig. 7. For the first profile, observed AFB1 production was delayed nine days compared to the predicted one (only 66% concordance was observed in this case). Looking at the corrected values for a_w and temperature, predicted AFB1 production took place at about 0.86 a_w and 25 °C, while experimentally occurred after this point, at a_w 0.83-0.84 and 27 °C, but with a longer adaptation period. For the second profile, AFB1 was not detected experimentally in 24 days, and according to the model probability of 0.5 was only reached in the 22nd day (87% concordance).In general, overestimation of AFB1 production probability was clearer than that in the case of growth probability. Obviously the dynamic conditions impose some kind of stress in the fungus, which is difficult to be reflected in the predictive model. In the present study we developed the predictive models that accounted for the mean temperature value of the 10 preceding days and so for a_w. If such modification is not included in the model more disagreement between observed and predicted values will be obtained. Nevertheless, much more effort should be done in developing predictive models under dynamic environments, as this is what happens in a real context.

Validation in maize, for both profiles, showed low agreement, as low probabilities of AFB1 production were predicted. Surprisingly, AFB1 was detected experimentally at day 8 for both profiles, and observed probability reached 100% after 21 days in the first profile and after 10 days in the second one (Fig. 8). Looking at the corrected values for a_w and temperature, experimental AFB1 production occurred at the higher temperature in the first profile, although this coincided with the lower a_w levels. For the second profile, observed AFB1 production occurred at about 28 °C even though a_w was about 0.77. Consequently, AFB1 production took place at the optimal temperature level despite of the low a_w levels, under which the model would not predict any production. Thus, as models were based on data generated in agar, it seems clear that even if temperature fluctuations may limit the potential for AFB1 biosynthesis, maize grain may trigger it, compared to agar.

These results, both in MEA and maize, highlight that mycotoxin production is a complex event, where many factors are involved. In our study, a_w was included as a monitored, uncontrolled variable in the model predictions, thus results can be fully interpreted using a_w values. In maize, AFB1 was detected under low a_w conditions, following conditions of more suitable values, which may suggest that after this period an imposed stress may trigger toxin biosynthesis.

Schmidt-Heydt et al. (2009) studied the aflatoxin biosynthesis gene cluster under several environmental conditions. They reported that under certain T-a_w combinations which imposed stress on the fungus results in a reduction of the growth rate and an induction of the aforementioned cluster. Studies on patulin (Baert et al., 2007), also showed that abiotic stress such as reduction of oxygen or low temperature can induce patulin production. Furthermore substrate may play an important role in mycotoxin production, since sometimes AFB1 was produced in maize grains but not in agar medium under the same conditions (profile 2). The previously mentioned study conducted by Garcia et al. (2012), reported a similar trend. Under certain environmental conditions *F. graminearum* produced DON in soybeans but not in agar medium.

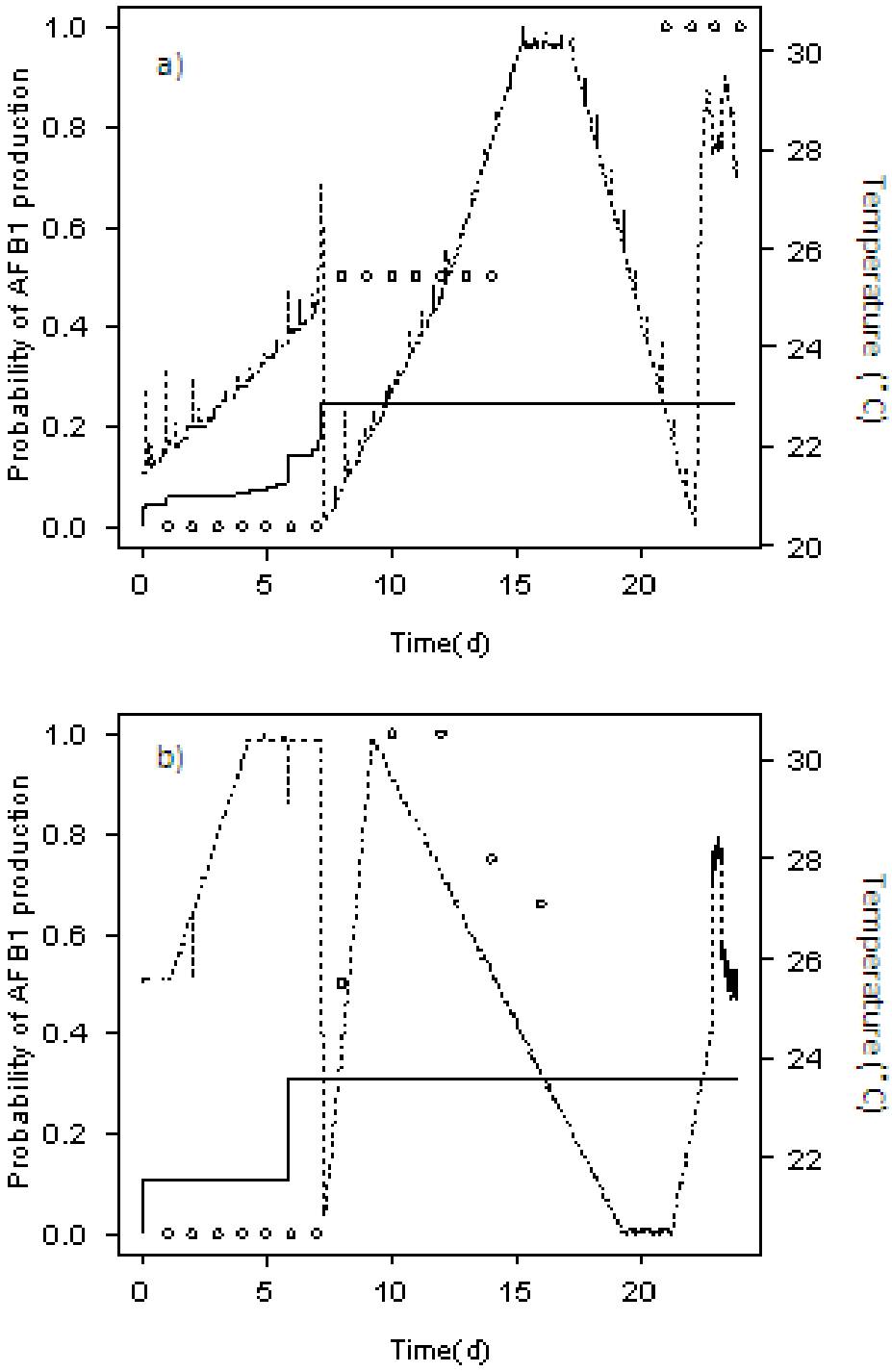

Figure 8. Observed AFB1 production probability (o) and predicted values (-) of *A. flavus* in maize grains under dynamic conditions of a) profile 1 and b) profile 2. Dotted line represents the temperature along time.

3.3. Relationship between observed growth and AFB1 production

For the first profile, observed growth occurred after 6 days in MEA and 9 days in maize, while AFB1 production was delayed till 23 days in MEA and 21 days in maize, such later observation was different than expected as production in MEA was expected to occur earlier than in maize. The model predicted AFB1 production after 14 days in MEA and no production was predicted in maize. For the second profile, observed growth occurred after 2 days in MEA and 7 days in maize, while AFB1 production was not detected in MEA and was delayed till the 10^{th} day in maize. Again, AFB1 production in MEA was poorer than in maize; the model predicted AFB1 production in day 22 in MEA and no production was predicted in maize. The low prediction in maize compared to MEA was due to the inclusion of the variable a_w level in the model, which accounted for the more marked decrease in a_w in maize than in agar through the experiments. Thus, it seems that the real impact of the decreasing a_w in maize is not so limiting for AFB1 production; probably once growth had occurred in the kernels, the decrease in a_w instead of preventing from toxin production, triggered it. Mycotoxin production as secondary metabolism is expected to parallel growth but delayed in time. Thus, in our experiments, once growth occurred, in spite of the dramatic decrease of a_w, AFB1 was synthesized. Besides, under the same conditions, once growth occurred, it took less time to produce AFB1 in maize grains than in agar medium. For the first profile, 17 and 12 days lasted between growth initiation and AFB1 production, in MEA and maize grains, respectively. For profile 2, no AFB1 was detected in MEA and it was detected after 3 days of growth initiation, in maize grains. As far as we know, no similar observation has been reported before in the literature, mainly due to the scarce number of studies that determine fungal growth and mycotoxin production on both agar medium and a food matrix.

The higher temperature levels occurring in the first part of the second profile led to more conducive conditions for growth and AFB1 production. As conclusion, temperature levels (ranging from 20 to 30 °C) were more determinant than a_w variations (almost constant in MEA and ranging from 0.60 to 0.90 in maize).

Due to the complexity and variability in mycotoxins production, developing models with AFB1 data led to wrong predictions, including an underestimation in maize grains. If we compare the

observed growth probabilities for maize grains (Fig. 5) and experimental data of AFB1 under fluctuating conditions in maize (Fig. 8), good agreement was observed. Then if suitable models could be built for prediction of growth probability in maize, they could be applied to AFB1 production prediction in maize. Similarly, working on pistachio nuts, Aldars-García et al. (2015) concluded that probability of AFB1 could be correctly predicted from either growth models generated on pistachio nuts or by AFB1 production models generated on pistachio extract agar.

In conclusion, in the present work, a_w was included in a probability model as a function of air RH and time, for the prediction of growth and toxin production under non-isothermal conditions, for the first time. This led to acceptable growth predictions in agar, however, there is still a gap between growth and toxin production in agar media and those in real food substrates. In particular, in the temperature profiles assayed poor correlation was found between toxin production on agar and maize, thus future work in this area should be focussed on prediction of toxin accumulation on foods from growth data generated in such foods.

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Discussion

Aflatoxin B_1, mainly produced by *A. flavus*, *A. parasiticus* and less often by *A. nomius*, is the most common mycotoxin found in food and also the most toxic. The ability of aflatoxin-producing fungi to grow on a wide range of food commodities and the stability of aflatoxins in foods make them a major issue for the food industry. The food industry is responsible for supplying safe food to consumers, thus controlling these toxins becomes imperative.

Control measures have been designed to prevent the contamination of crops in the field and during storage, or to detect and remove the contaminated material from the food supply chain. Besides, the application of predictive mycology as a tool allowing for the prediction of fungal growth and mycotoxin production through the food chain can further support decision making, crop management or risk management. In order to be as realistic and accurate as possible predictive mycological studies should account for many factors affecting fungal growth. Such factors include marginal conditions for growth, strain variability, inoculum size, and fluctuating environmental conditions.

Due to the high variability in growth and mycotoxigenic potential of different fungal strains, kinetic modeling of growth and toxin formation may be challenging and very dependent on the strains and conditions studied. Thus the use of either growth or mycotoxin probability models to forecast the growth and mycotoxin presence/absence may be a good alternative. Besides, in terms of food safety, the objective is not to quantify the amount of growth, but to ensure that toxins are not produced.

A particular discussion of the results found in this work has been provided in detail in the discussion section of each of the manuscripts included in this thesis.

This section of the Thesis has been divided into four subsections in order to address the different issues studied in the thesis.

- Effect of marginal conditions on fungal behavior and model development

- Predictive models outcome as affected by inoculum size

- Effect of intraspecies variability on fungal growth and mycotoxin production

- Prediction of fungal behavior under dynamic conditions of temperature

1. Effect of marginal conditions on fungal behaviour and model development

All over the food chain, environmental conditions encountered are nearly always marginal for microbial growth, especially during postharvest stages, where such marginal conditions (eg. low temperature or water activity levels) are imposed to prevent from food spoilage. Thus, developing predictive models under such conditions is of paramount importance for an agreement of the predictive models to real situations. For this reason, all experiments carried out in the present work were conducted under limiting conditions for growth and mycotoxin production. Few works in the literature focus on such conditions; it is known that the biological responses under adverse conditions become more unpredictable.

Garcia et al. (2010) reported that the performance of predictive models may be compromised under marginal conditions for fungal growth. However, they developed kinetic and probability models under such conditions, and found that this poor goodness of fit was more evident for kinetic models. Probability models may be a helpful predictive approach and may not be as affected by marginal conditions.

The main outcomes in fungal behavior under marginal conditions found in this work are:

1.1. Spore`s ability to initiate growth

Not all the spores are able to initiate growth under marginal conditions. The development of a predictive model generally involves the following steps (figure 1):

Figure 1. Main steps for developing a predictive model.

Before data generation, we need to design the experimental conditions among which the amount of spores to be inoculated is set. In the three steps shown in Figure 1, we assume that the results obtained account for all the spores we have inoculated. Thus in the case of a probabilistic model, in which we obtain the probability of growth under certain conditions, with

multiple spore inocula, we use to take for granted that all spores present in the inoculum contributed to the growing colonies, and directly we obtain the prediction under certain conditions. However, with small size inocula (or single spore ones), as we saw in chapter III, there is a need to develop a correction factor which takes into account the ability of spores to initiate growth under different environmental conditions, otherwise we overestimate the potential for growth. If such factor is not applied probability models may lead to inaccurate predictions and compromise their applicability.

Table 1 shows an example of the ability of the inoculated spores to initiate growth under several marginal conditions for five of the isolates tested in the present work.

Table 1. Ability of spores to initiate growth (%) under different T-a_w combinations for isolates UdL-TA 3.324, UdL-TA 3.325, UdL-TA 3.326, UdL-TA 3.330 and UdL-TA 3.268.

Isolate name	Temperature (ºC)	a_w							
		0.84	**0.86**	**0.88**	**0.9**	**0.92**	**0.94**	**0.96**	**0.98**
UdL-TA 3.324	**15**	[a]	[a]	[a]	0.00	0.00	25.00	37.50	37.50
	20	0.00	0.00	18.75	25.00	87.50	37.50	100.00	50.00
	25	0.00	25.00	31.25	31.25	[b]	[b]	[b]	[b]
	30	0.00	12.50	31.25	56.25	56.25	[b]	[b]	[b]
	35	[a]	[a]	0.00	0.00	0.00	56.25	62.50	100.00
	40	[a]	[a]	[a]	31.25	31.25	37.50	75.00	62.50
UdL-TA 3.325	**15**	[a]	[a]	[a]	0.00	12.50	12.50	50.00	56.25
	20	0.00	0.00	56.25	25.00	31.25	43.75	81.25	31.25
	25	0.00	0.00	12.50	25.00	[b]	[b]	[b]	[b]
	30	0.00	12.50	25.00	68.75	87.50	[b]	[b]	[b]
	35	[a]	[a]	0.00	31.25	18.75	37.50	100.00	100.00
	40	[a]	[a]	[a]	0.00	0.00	100.00	75.00	75.00
UdL-TA 3.326	**15**	[a]	[a]	[a]	0.00	56.25	56.25	68.75	93.75
	20	0.00	0.00	31.25	37.50	62.50	87.50	100.00	87.50
	25	0.00	43.75	43.75	56.25	[b]	[b]	[b]	[b]
	30	0.00	43.75	43.75	62.50	100.00	[b]	[b]	[b]
	35	[a]	[a]	25.00	0.00	100.00	100.00	100.00	100.00
	40	[a]	[a]	[a]	43.75	43.75	100.00	100.00	100.00
UdL-TA 3.330	**15**	[a]	[a]	[a]	0.00	0.00	37.50	31.25	43.75
	20	0.00	0.00	37.50	37.50	37.50	100.00	62.50	93.75
	25	0.00	12.50	43.75	43.75	[b]	[b]	[b]	[b]
	30	0.00	12.50	37.50	100.00	87.50	[b]	[b]	[b]
	35	[a]	[a]	0.00	0.00	37.50	31.25	50.00	100.00
	40	[a]	[a]	[a]	12.50	12.50	43.75	43.75	50.00
UdL-TA 3.268	**15**	[a]	[a]	[a]	100.00	100.00	100.00	100.00	100.00
	20	0.00	0.00	23.81	47.62	90.48	100.00	71.43	100.00
	25	0.00	33.33	38.10	100.00	[b]	[b]	[b]	[b]
	30	0.00	33.33	100.00	76.19	100.00	[b]	[b]	[b]
	35	[a]	[a]	0.00	76.19	100.00	100.00	100.00	100.00
	40	[a]	[a]	[a]	0.00	0.00	90.48	100.00	100.00

[a] Not studied, expected to be near 0%

[b] Not studied, expected to be near 100%.

From table 1 it can be extracted that as conditions become marginal, the ability of fungal spores to initiate growth decreases. Besides, this ability is strain dependent (see section 4.1.2. of the discussion), which means that for certain strains tested most of the spores had similar potential for growth, and that for others more variability among spores existed.

Other researchers, using different sizes of multispore inocula, also reported this issue. For example Garcia et al. (2010) observed that *P. expansum* and *A. carbonarius* led to lower percentages of growth under suboptimal conditions compared to more optimal ones that they tested.

In chapter IV, where we measured growth by a turbidimetric method in a microtitre plate, under 5 temperature levels (15, 20, 25, 30, 35 and 37 °C), the most restrictive one (20 °C) showed no growth in some wells in the plates. In general, the existence of no-growth observations is common in all the studies conducted under marginal conditions. Thus, one important point is to consider the effect of limiting conditions on the spore's behaviour, and to find a way to manage the no-growth observations.

1.2. Increase of the lag phase and decrease of the growth rate under marginal conditions

Furthermore, the increase of the lag phase duration and decline of the growth rate under marginal conditions is obvious.

All the experiments conducted throughout this thesis were at marginal conditions, thus a comparison of growth parameters between marginal and optimal conditions using only our data cannot be done. However, in chapter II, we worked at 25 °C and two a_w, 0.85 and 0.87. Despite this low difference between the two a_w tested, this decrease in a_w had a profound effect on the growth of *A. flavus, s*ince time to visible growth was delayed about 5 days. All the parameters studied in this chapter pointed out to a slower growth at 0.85 a_w and also a delay in the initiation of AFB1 production. These results stand out that even a small change in the environmental conditions (making them only a bit more marginal), has an important effect on both growth and AFB1 production.

In order to clearly reflect this delay in growth, in Table 2 some results extracted from the literature together with our data of lag phases and growth rates under different environmental conditions are presented. Inoculum is also included in the table, as we will see later that it has also an important effect on lag phase and AFB1 production.

Table 2. (part 1 of 2) Literature data and data from this thesis, accounting for lag phase to growth and colony radial growth rate under different environmental conditions.

Model	Fungal species	No. of isolates	Temperature (°C)	a_w	Inoculum size (at inoculation point)	Lag (day)	Growth rate (mm/day)	References
Baranyi and Roberts	*P. expansum*	1	25	0.99	10^6	0.20	3.60	(Basak and Guha, 2015)
Baranyi and Roberts	*P. expansum*	1	1	Apples(0.98)	10^4	49	0.98	(Morales et al., 2008)
Baranyi and Roberts	*P. expansum*	1	20	Apples(0.98)	10^4	4.20	4.30	(Morales et al., 2008)
Baranyi and Roberts	*P. expansum*	1	1	Apples(0.98)	10^6	32	0.85	(Morales et al., 2008)
Baranyi and Roberts	*P. expansum*	1	20	Apples(0.98)	10^6	3.10	3.50	(Morales et al., 2008)
Baranyi and Roberts	*P. expansum*	79	20	0.98	$10–10^2$	2.52	4.50	(Garcia et al., 2011a)
Baranyi and Roberts	*P. expansum*	79	1	0.98	$10–10^2$	13.61	0.98	(Garcia et al., 2011a)

Table 2. (part 2 of 2) Literature data and data from this thesis, accounting for lag phase to growth and colony radial growth rate under different environmental conditions.

Model	Fungal species	No. of isolates	Temperature (°C)	a_w	Inoculum size (at inoculation point)	Lag (day)	Growth rate (mm/day)	References
Linear	*A. flavus*	1	20	0.88	1	2.98	2.70	(Samapundo et al., 2007)
Linear	*A. flavus*	1	20	0.98	1	2.65	4.60	(Samapundo et al., 2007)
Linear	*A. flavus*	1	30	0.88	1	1.31	3.95	(Samapundo et al., 2007)
Linear	*A. flavus*	1	30	0.98	1	0.69	5.51	(Samapundo et al., 2007)
Linear	*F. verticillioides*	1	20	0.92	1	4.95	1.75	(Samapundo et al., 2007)
Linear	*F. verticillioides*	1	20	0.98	1	3.44	3.33	(Samapundo et al., 2007)
Linear	*F. verticillioides*	1	30	0.92	1	1.62	3.40	(Samapundo et al., 2007)
Linear	*F. verticillioides*	1	30	0.98	1	1.45	4.35	(Samapundo et al., 2007)
Linear	*A. flavus*	1	25	0.87	1	14.85	0.41	Chapter I
Linear	*A. flavus*	1	25	0.85	1	16.63	0.18	Chapter I
Linear	*A. flavus*	1	25	0.87	500	10.32	0.50	Chapter I
Linear	*A. flavus*	1	25	0.85	500	12.34	0.23	Chapter I

1.3. Marginal conditions promote intraspecies variability

As it has been reported several times, the differences among isolates are more marked under marginal than under optimal conditions (Astoreca et al., 2007; Baert et al., 2007; Garcia et al., 2011b; Romero et al., 2010). As an example of these studies, Garcia et al. (2011b) studied the impact of suboptimal environmental conditions on the intraspecific variability of *A. carbonarius* growth and OTA production using 30 isolates.They tested the effect of one optimal (0.98a_w-25 °C) and two suboptimal conditions (0.90a_w-25 °C and 0.98a_w-37 °C). Coefficient of variation of the growth rate was more than 4-fold higher at 0.98a_w-37 °C compared to 0.98a_w-25 °C, and lag phase was widely distributed at 0.98a_w-37 °C compared to the other 2 conditions.

Marginal conditions highly affected isolates behavior, as the difference among isolates increased as conditions became more limiting for growth. For example in chapter IV, temperatures 37, 35 and 30 °C did not lead to much differences among strains but when 25 and 20 °C were set, the intraspecies variability started to increase. The different ability to grow under marginal conditions could be attributed to the isolate origin, genetic variability, etc. Contour plots presented in chapter III, showed that above and below the temperature range 25-35 °C, the a_w range for growth and AFB1 production is smaller and the differences in probability among the 20 isolates are more marked. However these differences are not as marked as the quantitative growth or mycotoxin produced differences found throughout this work and in the literature.

Considering the effect of marginal conditions on AF production, the amount produced was highly variable among strains (see chapter IV) and the boundaries for AFB1 production at 30 days (see chapter III) were also quite variable at limiting conditions, but far less than the amount produced.

When it comes to developing predictive models, as marginal conditions are most realistic situation, the inclusion of several strains should be required in order to account for the distribution of the behavior of all the possible strains present in a foodstuff.

2. Mould growth and mycotoxin production as affected by inoculum size

Another issue to consider is the inoculum size, as contamination of most foods occurs with a low number of fungal spores (Gougouli et al., 2011; Lahlali et al., 2005), or even if contamination is high, single spores are expected to be deposited in different positions in a food matrix. It is known that growth results may differ following inoculation by single spores compared with a higher concentration of spores (Baert et al., 2008; Burgain et al., 2013; Chulze et al., 1999; Garcia et al., 2010; Gougouli and Koutsoumanis, 2013; Morales et al., 2008; Sautour et al., 2003). Sautour et al. (2003) reported that there is a need for standardizing spore preparation in predictive mycology, and thus it should be considered in the design of challenge tests and in quantitative risk assessment.

Some authors have studied the impact of different inoculum sizes on growth and mycotoxin production, and the main results are summarized in Table 2, together with the results of the present thesis.

Results from Table 2 of this section, and Table 2 from chapter I and chapter IV, clearly demonstrate that inoculum size has a great effect on growth initiation. In all cases, the quantitative comparison of the impact of inoculum size on the lag time for growth, TTD or times to reach certain probabilities of growth, showed longer times for a smaller inoculum concentration. This is a very important issue since lag phase/growth initiation is the main focus in food safety and for estimating products shelf life.

Regarding mycotoxin production, the inoculum level highly affected the AFB1 production initiation. In chapter I, we developed AFB1 production probability models under 25 °C, two a_w (0.85 and 0.87) and two inoculum levels (a single spore and 500 spores). The inoculum level highly affected the AFB1 production initiation, as a difference of ca. 9 days in the estimated times to initiate AFB1 production (t10) was obtained through the different probability models. Besides, the logistic curves for the higher inocula were much more abrupt (fast increase from 0 to 1 probability) than those for the lower ones.
However, further research is required, in order to understand the inhibition/enhancement of mycotoxin production depending on the inoculum concentration. Results obtained at two inoculum levels and different temperatures (chapter IV), showed that depending on the inoculum size we can find a different pattern in mycotoxin production depending on the temperature, sometimes enhanced and in other cases inhibited, without an easy explanation.

Finally, the relationship among lag phase to growth and 50 and 100% probability of growth and AFB1 production is shown in chapter I, Figure 4. The succession of the aforementioned events occurs almost at the same time when a 500 spores inoculum is set instead of a single spore inoculum. Such demeanor shows us the great impact of inoculum concentration on the prediction. Then, chapter II was conducted so as to build a better understanding of single spore´s behavior. We found that germination, initiation of growth and initiation of AFB1 production, are quite separated events when we work with only one spore, under suboptimal conditions. Once the probability of growth started to increase it took 6 days till the initiation of

AFB1 production, and when probability of growth was 100%, only a 40-57% probability of detection of AFB1 production was predicted Given these results, standardization of the inoculum size, taking into account the most realistic spore´s contamination, in mycological studies should be done, to neither overestimate nor underestimate the mycotoxin risk and to properly develop the predictive modeling.

Results from chapter I showed that there was a difference of approximately 8-9 days in the time to reach 10% probability of growth when the colonies arose from one spore or from 500 spores. On the other hand, the fastest growing isolate and the slowest one (chapter III) under the same environmental condition (25 °C -0.92 a_w), need 18 and 78 hours to reach the same probability (10% probability of growth), which means a difference of 2.5 days. From these results we can infer that inoculum size has a greater effect on the results that intraspecies variability. Anyway, as we will see in the next section, intraspecies variability also affects (but into a lesser extent) the predictive outcome.

3. Effect of intraspecies variability on fungal growth and mycotoxin production

Differences in growth and mycotoxin production by individual strains are well known (Abbas et al., 2005; Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Pardo et al., 2005, 2004; Parra and Magan, 2004; Romero et al., 2007; Singh et al., 2015; Yogendrarajah et al., 2016). However, this field has not yet been studied in detail for fungal species. Many sources can be the cause of this intraspecies variability, e.g. molecular characteristics, geographical origin, environmental conditions, etc.

Populations of *A. flavus* in agricultural products and foods are complex communities that contain many different strains.

Regarding intraspecies variability we conducted two different studies (chapter III and chapter IV) in order to address the differences among isolates under different experimental conditions (including different environments and two inoculum sizes).

In terms of growth, the differences among strains were dependent on the environmental conditions, as we saw in section 1.3 for marginal conditions. In chapters III and IV we observed that divergence among strains' growth start to arise when conditions become limiting.

In these chapters we compared the growth initiation (TTD) and the probability of growth. In both cases, intraspecies differences were found; for example, Figure 2 in chapter IV shows the different TTD of the 10 isolates tested under 5 temperatures (20, 25, 30, 35, 37 °C) and at 0.92 a_w. Differences in TTD of around 30 hours (1.25 days) were found at 25-37 °C among the 10 strains, however, at 20°C the difference was almost 3 days. In chapter III, regression coefficients of growth models developed indicate existing significant differences among the models built for the different strains, especially, for the T x a_w terms (Table S1 supplementary material in chapter III).

AFB1 production was variable in both studies, and did not follow a clear pattern. In chapter IV, we found that the amount of AFB1 produced did not follow a pattern related to the environmental conditions, and furthermore some strains were more able to produce AFB1 than others. Under the same condition (30 °C- 0.92 a_w and 7 days of incubation) one strain can produce as much as 3000 ppb of AFB1, while others produce around 1000, 200 ppb or cannot produce at all. Besides AFB1 production boundaries in chapter III revealed that AFB1 production is highly strain dependent. For example, at 25 °C- 0.90 a_w 6 strains out of 20 tested in that study, were able to produce AFB1, and the AFB1 probability for these 6 strains ranged between 0.24-0.49, while for the 20 strains ranged between 0.02-0.49.

As mentioned several times throughout this thesis, AFB1 legal limits in food are very strict, and due to the highly variable demeanor, the focus during postharvest should be on avoiding mycotoxin production. Then, the use of probabilistic models may be a good approach to properly perform this assessment. The amount of mycotoxins produced by fungi has been demonstrated to be highly variable among isolates (Garcia et al., 2011b; Yogendrarajah et al., 2016). However, results in chapter III showed that boundaries for AFB1/no-AFB1 seemed to be variable, but much less than the quantity of toxin produced (as reported in chapter IV). Thus, taking into account the high variability in the amount of AFB1 produced by different strains, modeling of the probability of toxin production seems a suitable alternative.

We should develop predictive models that account for the behavior of several strains. However, 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 experiments has been proposed by several authors

(Hocking and Miscamble, 1995; Patriarca et al., 2001; Romero et al., 2007) in order to represent the given species. In chapter V, we compared the growth and AFB1 production behavior of a single isolate and a cocktail of 25 isolates. Some authors used a cocktail inoculum to minimize the possible variability among isolates and then account for the behavior of all of them in a single experiment. In all conditions tested in that chapter, growth occurred earlier for the cocktail inoculum. For AFB1 production, a different trend was observed, where both type of inocula gave similar results with no significant differences between them. From these results we can conclude that the cocktail inoculum is dominated by the fastest growing isolate. Then, the use of a cocktail inoculum will represent the "worst scenario" contamination in which the growth is led by a fast growing isolate. However, using a cocktail inoculum we miss the information on the variables distribution for the different isolates.

4. Predicting growth and mycotoxin production under dynamic environmental conditions

One of the main aims of this thesis was to determine the effect of dynamic environments on growth and aflatoxin production, and try to model it. It should be considered that steady-state is a very poor assumption in the environment of a food chain where non constant conditions prevail (Dantigny and Nanguy, 2009). Variation of the environmental conditions, during production and distribution chain of a food product is a common situation, therefore it is essential to model the microorganisms behavior as a function of some variables under fluctuating conditions (Giannakourou et al., 2005; Gougouli and Koutsoumanis, 2012; Koutsoumanis et al., 2010; Neumeyer et al., 1997). Generating mycotoxin data for model fitting in real food matrices is costly, thus the alternatives considered were either generating data in agar media or generating growth data in the food matrix, then fit the models and try to make predictions of mycotoxin production probability in food matrices.

Predictive models developed in chapters IV, V and VI were based on predictive models corresponding to static conditions which were modified in order to consider the effect of temperature or a_w changes. In chapter V, four different fluctuating temperature scenarios were proposed, including upshifts and downshifts, by gradual or abrupt changes. The development of predictive models, for both growth and AFB1 production, under dynamic temperature showed the need to take into account the previous temperature history. In this chapter, the

temperature used in the R algorithm was the mean temperature of the preceding 10 days. If only the temperature of each time point was used, bad agreement between predicted and observed data would be obtained under some temperature scenarios, especially for toxin production. These modifications entail a memory effect of fungal behavior. Another important conclusion of this chapter is that temperature fluctuations may result in a_w fluctuation. Thus, in chapter VI we developed predictive models accounting for the fluctuation of both temperature and a_w. Following the same approach as in chapter V the algorithm constructed accounted for the mean temperature of 10 days and in this case also for a_w. The models obtained were used to make predictions of growth and AFB1 production under dynamic conditions in maize extract agar and maize grains. Besides, this study highlighted the effect of the food matrix in mycotoxin production. Since AFB1 was produced in maize grains despite of the a_w decrease along time due to the already present mycelia in the maize grains, this behaviour was not reported in the maize extract agar.

Furthermore, two approaches were developed in chapter IV for estimating the TTD and AFB1 production under dynamic temperature scenarios. In both approaches the two temperatures included in the dynamic scenario and their duration were taken into account. Envisaged approaches allowed predicting TTD and amount of AFB1 under the temperature shifts studied.
On the whole, the results of these three chapters indicate that even a small temperature upshift, such as from 20 to 25 °C, may induce a reduction of about 5 days in the initiation of growth.

In general, the inclusion of the past environmental conditions in the models was necessary to accurately predict the events studied. Agreement between predictions and observed data under dynamic scenarios was found in almost all scenarios. Thus, the mathematical approaches developed in these 3 chapters could be satisfactorily employed to describe the phenomena studied under a dynamic environment.

5. Validation of predictive models

Furthermore predictive microbiological models must undergo validation before they are used to support food safety decisions. Validation procedures involve comparing model predictions to the same experimental observations used to build the model (internal validation) and with data not used in model development (external validation). Validation is one of the most important aspects of model development in order to ensure that predictions made by the model are applicable to real situations.

For this reasons, in the present study, two chapters were conducted in both culture media and food matrices (pistachio nuts and maize grains).

In chapter V, predictive models obtained under fluctuating temperature were validated on pistachio nuts. Growth probability models obtained in pistachios and pistachio extract agar and AFB1 production models obtained in pistachio extract agar were used to validate AFB1 production on pistachio nuts. Table 3 shows the agreement between the 3 different models and the data obtained in pistachio nuts.

Table 3. Percentage of agreement between AFB1 data obtained in pistachio nuts and the three different probability models developed in chapter V.

Type of model	Type of inoculum	%Agreement
Growth probability model in pistachio extract agar	Single strain	15%
	Cocktail of strains	40%
AFB1 production probability model in pistachio extract agar	Single strain	81%
	Cocktail of strains	67%
Growth probability model in pistachio nuts	Single strain	81%
	Cocktail of strains	70%

Results in Table 3 showed that prediction of AFB1 in pistachio nuts could be performed using AFB1 models in agar or growth models in pistachio nuts. Such models are much easier to be generated and cheaper, than AFB1 models generated on data from pistachio nuts.

Predictive models under dynamic temperature and a_w were validated on maize grains in chapter VI. In general, poor agreement was found for AFB1 production models, both in agar and maize grains. However, accounting for growth conditions, the developed growth model for maize grain could better predict AFB1 in maize grains, even though data used to generate that model were initially from growth in agar.

As a conclusion, both food matrices showed that studying growth may help for assessing AFB1 presence through probabilistic models, as presented in chapters V and VI. Thus the possibility of using growth models to predict mycotoxin production is proposed as a valuable tool in the assessment of mycotoxin risk, although more effort should be done to consider as many factors affecting fungal behaviour as possible.

These works were carried out with low inoculum levels (but not single spores) and with an only strain; looking at the results presented in the present thesis, there is a need to include corrections for both factors in newly generated models. In the past, high inoculum sizes have been used to take the worst scenario, which according to the present work is too unrealistic. Strain variability has not been taken into account in most studies, according to the present work, the use of a cocktail inoculum would be unrealistic, for the same reason as high inoculum. As the intraspecies difference effect was not so big, an additional confidence interval could be applied to model estimations to take into account such variability.

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Conclusions

The conclusions drawn in this Thesis are detailed below:

- It is required to develop models under real conditions encountered in the agrifood systems (usually marginal for fungal growth). The presence of no growth situations under such conditions can be efficiently managed by the use of probability models, instead of kinetic models. It has been demonstrated that such conditions make model development more complex due to:
  - More variable growth and toxin responses under such conditions, and also more intraspecies variability, which means worst fittings.
  - All inoculated spores are not capable to initiate growth, thus the models built only represent the behavior of the germinating ones which led to colonies. As consequence, there is a need to model both percentage of germinated spores and growth/toxin production by the germinating ones, and merge both models.

- Fungal growth was clearly linked to inoculum size, which had a great impact on the outcome of predictive models. It has been shown that growth kinetic parameters, and probability of growth and AFB1 production attain greater values with time when a single instead of a multiple spore inoculum is used. The amount of AFB1 produced was affected by inoculum size but sometimes it was stimulated and sometimes inhibited, without a clear trend. Inoculum size should be chosen according to the most realistic situation to simulate a possible food contamination.

- Although smaller than that attributed to inoculum size, there is certain variability in the probability models (growth or AFB1 production) developed for different *A. flavus* strains. Such variability should be taken into account in future models in order to represent the species.

- Dynamic conditions affected growth and mycotoxin production. Growth pattern could properly be predicted by the developed models, however AFB1 production was more difficult to predict (AFB1 biosynthesis showed much less repeatability and predictable dependence with environmental factors). When growth and AFB1 production under dynamic environmental conditions were predicted from static environmental conditions, a correction

factor accounting for the past conditions was required. Moreover, the resulting probability models should 'accumulate' probability, at least in the case of growth, and lead to an increasing function with time.

- Probability of AFB1 production under non-isothermal profiles can be predicted from growth models generated at constant temperature either on agar media or food matrices, as long as a_w is included as a variable in the models. Besides, there is a need to further improve these models by the inclusion of the impact of inoculum size and intraspecies variability.

- In terms of food safety, the legal limits of AFB1 presence in a foodstuff are very low. It is proposed therefore to focus on predicting those conditions where low or no probability of neither growth nor AFB1 production occurs in postharvest.

Annex

Review

Modeling postharvest mycotoxins in foods: recent research

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Abstract

Available information on the prediction of postharvest production of mycotoxins in recent years is reviewed. Predictive mycology has been focused mainly on fungal growth whereas studies on prediction of mycotoxins in foods are scarce. Modeling mycotoxin production is challenging due to the high variability in mycotoxigenic potential among species and isolates. Besides mycotoxin biosynthesis pathways and factors influencing them are still poorly understood. Baranyi and Luedeking-Piret models have been recently used as primary models for mycotoxin prediction, while for secondary modeling, polynomial approaches have been used. Furthermore, probability models can be a different alternative. In any case, media for data generation, intraspecies variability, and microbial interactions should not be disregarded before model application in food safety management systems.

Introduction

Food industry aims to obtain good quality and safe products and to maintain this throughout their shelf-life. Nevertheless, mycotoxins, as natural contaminants are not easy to control for both producers and exporters. Mycotoxins are secondary metabolites, toxic to human and animal health, produced by a wide range of fungi. Mycotoxins contamination represents a worldwide problem in terms of human/animal health and furthermore can pose a heavy economic burden to the industry. Mycotoxins can contaminate a product all over the food chain, in the field as well as during storage, or at later points (figure 1). Herein we will focus on the postharvest stage, where many factors are involved in the production of each particular mycotoxin; (a) intrinsic nutritional factors, (b) extrinsic factors, (c), processing factors and (d) implicit microbial factors [1].

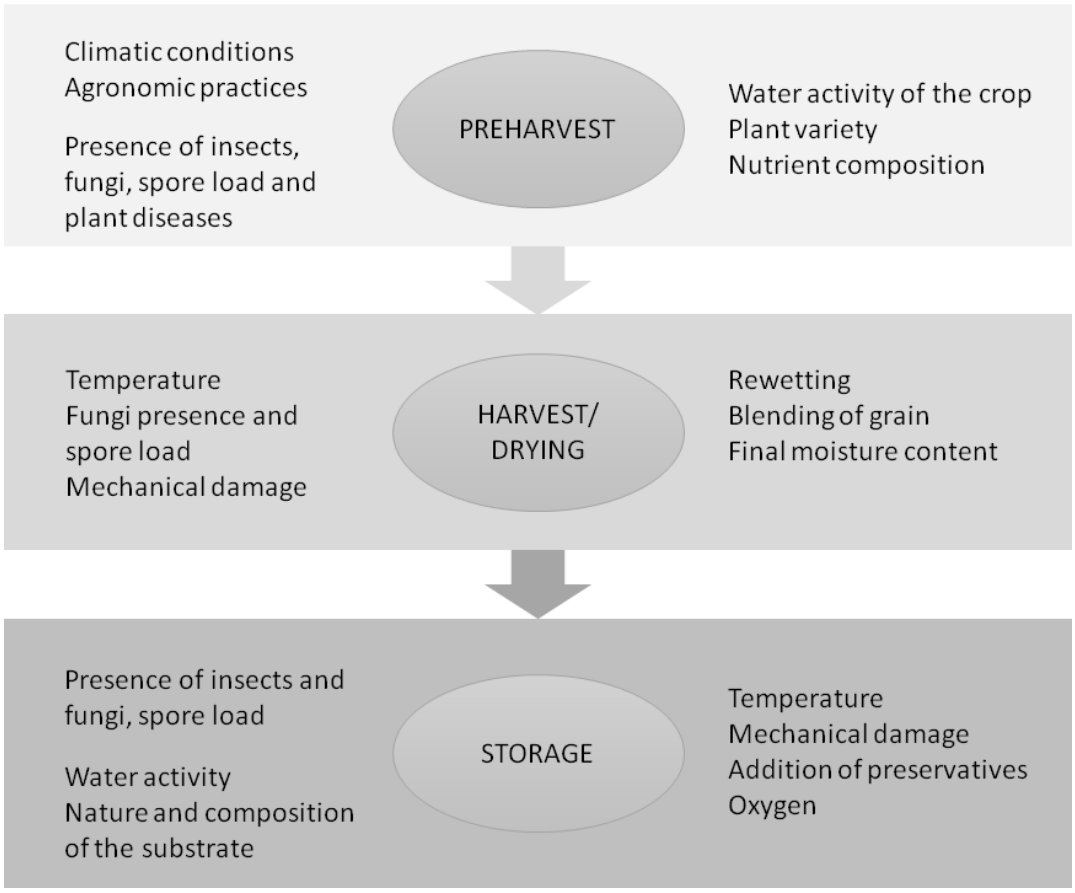

Figure 1. Brief description of the food chain and the main factors influencing fungal growth and mycotoxin production (Modified from Magan et al., [27]).

As a result of inadequate handling/logistic structures, fungal growth and subsequent mycotoxin production are allowed. While the complete elimination of mycotoxin in contaminated foodstuffs is not achievable at this time, the aim is to focus on minimizing the occurrence of these toxins throughout the food chain. The implementation of good manufacturing practices (GMP) during handling, storage, processing and distribution represents an important line of defense in controlling the postharvest contamination of commodities by mycotoxins. To date, several postharvest strategies to prevent/reduce growth and mycotoxin production have been proposed. It is clear for the industry that drying of cereals and nuts, and temperature and moisture control during storage are factors of great importance, and other techniques including the application of compounds with antifungal effects such as synthetic antioxidants, essential oils [2,3], salts [4], natural phenolic compounds [5], or the use of modified atmospheres [6] have been used. In the past few years, there have been an increasing number of studies dealing with the use of bacteria, yeasts and moulds to control mycotoxigenic moulds in foods [7,8]. Beyond this, predictive mycology, providing tools for the prediction of fungal growth and mycotoxin production [9,10*], seems to be a promising approach and could play a role in improving the quality and safety of food. This tool may help for adequate decision making purposes, risk assessment and in the implementation of mitigation strategies.

Postharvest mycotoxins

Many fungi can invade and cause damage to grains, seeds, raw materials and different foods and feeds during transport and storage steps, either before or after drying. Particular postharvest practices which may be conducive to toxin accumulation and need further control and prevention strategies are slow drying of fruits in certain areas [11] or postharvest ensilage of dairy cow feed materials [12]. *Aspergillus* and *Penicillium* are the major mycotoxigenic postharvest fungi. The minimal necessary water activity (a_w) for most *Aspergillus* and *Penicillium* species is 0.75–0.85, but they can grow optimally at a_w 0.93–0.98. These fungi can grow at temperatures between 25-40 °C [13,14]. Typical postharvest mycotoxins are ochratoxin A (OTA), aflatoxins (AFs) (also typical in preharvest) and, in to lesser extent, deoxynivalenol (DON) [15]. A special reference must be made to patulin, which is an exclusively postharvest mycotoxin which affects fruits, mainly apples. This review deals, however, with OTA and AFs in which more attention has been focused lately.

Recent research on predictive modeling of postharvest mycotoxins production

Postharvest modeling tries to simulate the conditions to which food would be exposed in order to forecast the microbial behavior and then to optimize postharvest management. Detailed predictive studies on mycotoxin production under various storage conditions are limited, but there is a wealth of information aiming to predict the growth of mycotoxigenic fungi and the influence of environmental factors on it. During the past decade several publications have dealt with the production of different mycotoxins over time, nevertheless, these studies rarely took into account the possibility to model such production. From the food safety point of view the target to be modeled are the mycotoxins, however modeling mycotoxin concentration could be an unpractical approach due to the high variability in mycotoxin potential among species and even more, among strains [16**]. The mycotoxins modeled and the models used in the existing studies presented in the following sections are listed in table 1.

Table 1. Recent predictive mycotoxin models used in food mycology.

Reference	Type of model			Mycotoxin
	Primary	Secondary	Probabilistic	
García et al. [18]	Luedeking-Piret	-	-	AF
Lee et al. [17*]	Baranyi	Gaussian and polynomial	-	
Medina et al. [21**]	Luedeking-Piret	-	-	
Marín et al. [19]	-	-	Logistic	
Aldars- García et al. [20**]	-	-	Logistic	
Kapetanakou et al. [25]	Baranyi	Polynomial	-	OTA
Ioannidis et al. [26*]	Linear	Polynomial	-	
Lappa et al. [16**]	Luedeking-Piret	-	-	

Modeling aflatoxins

Although AFs are a common problem at harvest, the situation may worsen during postharvest when foodstuffs are stored under conditions that promote the growth of the specific microorganisms that produce them, *Aspergillus flavus* and *A. parasiticus,* which primarily contaminate food crops such as maize, peanuts, and tree nuts in tropical and subtropical climates.

Regarding AFs, several studies are available which model the effect of a_w and temperature on synthetic media [17*] and on real food matrices like maize grain [18] or pistachio nuts [19,20**]. Recently, Baranyi and Luedeking-Piret models have been the two primary models used to predict aflatoxin (AF) production over time. Garcia et al. [18] modeled the kinetics of aflatoxin B_1 (AFB1) production by *A. flavus* using the general mixed-growth associated Luedeking-Piret model for product formation under the assumption that both no-growth-associated and growth-associated toxin production existed. They considered three possibilities to estimate AFs formation, namely through colony radius, colony surface or biomass dry weight, demonstrating that AFs were produced during active growth of the fungus and when the growth had stopped, therefore AF biosynthesis did not present a clear delay in relation to growth. In such approach, parameters α and β are estimated, where α is the growth-associated coefficient for toxin production (g toxin /g biomass) and β is the non-growth-associated coefficient for toxin production (g toxin/g biomass per unit of time). This kind of modeling allows for some understanding of the global physiology of fungi, and it would be of interest to know the variation of these parameters as a function of environmental conditions. Later, Lee et al. [17*] estimated the maximum AFs production rate (ng/day), and the lag phase duration for AFs (day) by fitting the primary model of Baranyi to the production of AFs with respect to time. In this approach, toxin production is modeled independently of the coexisting fungal growth, thus the estimated rate accounts for both the increase in toxin linked to growth plus the increase due to already existing biomass, but no information is given by the model on each contribution.

Secondary modeling of AFs has been carried out by polynomial approaches. Lee et al. [17*] employed Gaussian and polynomial models to fit the maximum specific AFs production rate and the lag phase duration for AFs production, respectively, to describe the effects of a_w and temperature on these kinetic parameters. Interestingly, Medina et al. [21**] linked AFs production with gene expression by developing a modified Luedeking-Piret model including gene expression of AFB1 production, temperature, a_w and growth rate. This new approach gives

a helpful understanding on the relationship between environmental stressing factors and the genes involved in the biosynthetic pathways of mycotoxins production, and may allow for refining of the existing models, through tuning of the potential for toxin production depending on gene activation.

Probability models have rarely been used to model mycotoxin production. Due to the high variability of toxin production (concentration) among strains, the predicted concentration from a model developed with one/various strains may not be applicable to other strains existing in nature, thus an alternative might be probability models, if they are proved not to be strain-dependent. Marín et al. [19] obtained probabilistic models (toxin/no-toxin) to predict AFB1 production by *A. flavus* including % moisture content, temperature and time. They converted the AFB1 experimental data into probabilities of AFB1 contamination by assigning 1 to samples with AFB1 presence and 0 to those without AFB1 (threshold of presence was established by the limit of detection of the equipment). Afterwards linear logistic regression was applied to obtain the probabilistic model for AFB1 production. In this case, instead of predicting the toxin concentration produced over time, the probability of toxin production is obtained, thus, for example to avoid the risk of toxin accumulation in the storage a probability under 0.5 or 0.10 should be achieved through temperature and humidity control of the storage.

For application of these models to food and feed safety management in postharvest operations, there is a need to go a step further and work on predictions under variable temperature/water activity scenarios. In a preliminary study, Aldars-García et al. [20**] attempted to predict AFB1 formation under a changing temperature environment, using probabilistic models too, for the prediction of AFB1 presence in pistachio nuts. They developed predictive models that could predict the presence of AFB1 in pistachio nuts under a changing profile of temperatures with 67-81 % of concordance between observed and predicted data, depending on the profiles.

Modeling ochratoxin A

OTA is a mycotoxin of major concern which can be produced by several species of *Aspergillus* and *Penicillium* species [22], and it is a common natural postharvest contaminant in cereals, nuts, dried fruits, spices, etc. Few studies modeled the production of this toxin, as most of them just quantified the toxin, either at various time points or at a single incubation time, and related it to the modeled growth of the mould [23,24].

Kapetanakou et al. [25], including the viscosity of the substrate in their experimental design, modeled the effect of temperature, a_w and (gel) structure on OTA production on malt extract broth and food matrices with different viscosities. The Baranyi model was applied to estimate the OTA production kinetic parameters, namely OTA production rate (ppm/day) and total toxin accumulation (ppm), showing good fitting to the experimental data. The Luedeking-Piret model was applied by Lappa et al. [16**] to assess the differences in OTA production among ten different strains of *A. carbonarius* isolated from Greek vineyards. As Garcia et al. [18] did, they firstly determined fungal growth parameters and correlated them with OTA, and finally took into consideration those growth parameters with the highest correlation with OTA, i.e. colony diameter, colony area and biomass dry weight, excluding colony density. Further they used them to model the amount of OTA produced in relation to incubation time, concluding that OTA was a mixed-growth associated metabolite of *A. carbonarius*; this would support its early accumulation in fungal cultures. Besides, OTA production revealed a wide dispersion among isolates, pointing out the importance of taking into account the intraspecies variability in the predictive models.

Ioannidis et al. [26*] studied the effect of sodium metabisulphite (NaMBS) as a control technique in grapes during postharvest. OTA production over time was modeled with linear primary model to estimate the OTA production rate. To fit the model, they plotted the OTA concentrations against sampling times (3, 7, 10, 14, 17 days). However, in most of the cases a decrease in OTA amount was detected in the last two OTA sampling points, thus these points were excluded from the regression, to take into account only the linear part. Existing studies on most mycotoxins have shown that toxin concentration in open solid systems usually increases with time till a plateau is reached and sometimes a decrease is observed; however there are no concluding works on how and why degradation takes place. It is a pity that some of the latest studies on primarily modeling of toxins did not include the plots of their raw data over time, as

there is a lack of availability of such data in order to decide on which primary model should be used.

Finally, as for AFs, polynomial models are the main mathematical tools used for secondary modeling of OTA formation under different environmental conditions. Kapetanakou et al. [25] modeled the square root of the OTA production rate using a polynomial model and a cardinal model. Nonetheless, the latter model showed poor adjustment possibly due to the narrow range of temperatures and a_w assessed in the experiment. Using a quadratic polynomial model, Ioannidis et al. [26*] described the effect of temperature, a_w, NaMBS concentration on the OTA production rate by *A. carbonarius* on grape juice based medium. The statistical indices used to assess the goodness of fit of the models displayed the difficulty of predicting the toxin formation in comparison with growth parameters.

Conclusions

Postharvest mycotoxins pose a threat for the safety of food products during transport, storage and distribution. Despite the high cost of data generation, and the challenging variability of mycotoxin data, a significant effort for developing predictive models for estimating mycotoxin contamination has been made during the past years and it is still in progress. There are a number of points which still need to be addressed:

(i) Most models include a_w and temperature as the most critical factors which determine mycotoxin production. It is known that pH plays a minor role in most food and feed materials, however, it would be of interest to take into account in the models the impact of microbial interactions, which may be the main source of biased predictions.

(ii) Mycotoxin production in agar systems is quite different from that in real food and feed matrices, there is an urgent need for validation of the developed models in real substrates; or even to generate the data directly in foods and feeds.

(iii) Intraspecific variability in mycotoxin production is still a challenge, if even probability models result to be strain-dependent, the last resource would be to use growth models to predict growth boundaries and apply them to prevent toxin production, in a worst scenario approach.

Overall, the prediction of the accumulation of mycotoxins in foods and feeds is a challenging task due to the variety of factors influencing their production such as temperature, a_w, inhibitors, fungal strains, accompanying microbiota, etc., and the need to understand the mycotoxin biosynthesis more deeply.

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