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Use of predictive modelling as tool for prevention of fungal spoilage at different points of the food chain

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Moulds cause severe economic losses at different points of plant food commodities production, from the field to the final foodstuffs. Predictive modelling is an increasingly used tool applied to solve different issues in food production. In this opinion, we have dealt, in one hand, with the latest publications on predictive mycology used for early prediction of fungal spoilage of foods, as well as for assessing efficacy of antimicrobials in foods. Moreover, prediction models have been applied to assess the impact that climate change may have in the near future in terms of geographic fungal distribution and impact on mycotoxin occurrence. Finally, there is a growing interest on analysing fungal growth and mycotoxin contamination in cereals and nuts using infrared spectrometry models. All these cases exemplify the increasing interest of predictive modelling to assist decision making in different points of the food chain.

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Introduction

Molds infect most of agricultural commodities and cause severe economic losses. Another major concern related to the fungal contamination of agricultural commodities is the production of mycotoxins, which are derived from the fungal secondary metabolism. Mycotoxins may have diverse deleterious effects on human's and animal's health with impacts on public health and farm productivity. Crop production, picking, drying, handling, packaging, storage and transportation comprise steps in which fungi may grow and release mycotoxins. Fungal growth may also take place due to physical damage of crops and insect infestation. Fungal growth is dependent on environmental factors: nutrient availability and structural condition of the substrate, microbial competition, water activity, temperature, pH, light intensity, relative humidity, application of pesticides and insect damage. Nonetheless, the impact of these factors on mycotoxin production may not be the same as observed on fungal growth. Besides, it is not possible to describe a single set of conditions that are favorable to growth and mycotoxin production by fungi due to the large variety of species involved [1,2]. However, as fungal growth will further result in the release of mycotoxins in the substrate, controling/preventing fungal growth comprises a fundamental measure to safeguard public and animal health as well as to avoid economic losses.

Predictive modelling aims to support decision making in food quality and safety management. In particular, mycotoxins management in the field may be assisted by climate-based field models. In the food industries, food quality and safety management is based on raw materials selection and sorting, controlled storage and safe dehydration processes. While predictive mycology may be helpful during raw materials storage, dehydration and during food products shelf life, predictive modelling can be applied to most operations in the food industry, in particular, in raw materials diversion and sorting. Main applications of predictive modelling related to fungal spoilage are summarised in the following sections of this opinion.

Predictive mycology for early prediction of fungal spoilage

In the last few years, several works have been published on the application of predictive mycology in the early prediction of fungal spoilage. As mouldy appearance is, in general, to be avoided in foods, modelling of lag time before colony emergence or its probability are of particular interest. And a key event for mycelia to be visible to naked eyes is germination, which is a key event in the whole process of studying fungal spoilage of foods [3]. Several of these studies have employed the growth/no-growth approach, in which regression equations are employed to predict growth and no-growth boundaries as affected by several factors. Through this approach, the use of lag time or the time for visible colonies to appear seem to comprise the best choice as they also clearly

relate to practical world, that is, it is known that fungal colonies are visible to naked eyes when reaching $\geq$2 mm diameter [4]. This shift of predictive mycology works from growth rate measurements towards lag time or probability of visible colonies to appear in certain conditions is a crucial point and development in this field. For instance, Debonne *et al.* [5] assessed the impact of temperature, pH, a_w and essential oil (thyme) on *Penicillium paneum in-vitro* development, for which growth/no-growth models have been generated. In another work, growth/no-growth modelling was used to validate the *in-vitro* antifungal activity due to the production of acetic and lactic acids during bread fermentation [6]. Besides, growth/no-growth models were used to evaluate the robustness of wholemeal breads as impacted by temperature, moisture, pH and calcium propionate towards two fungi (*Paecilomyces variotii* LMQA-001 and *Penicillium paneum* LMQA-002) [7•].

This shift to prediction of fungal spoilage at the earlier stages makes more rellevant the phisiological state of the fungal spores used for data generation to be subsequently modelled. Optimum cultivation conditions, including substrate properties and storage temperature, are frequently employed to obtain a suspension of fungal spores. This is mostly done because the high yield of conidia is key for the conduction of inactivation or growth/germination studies (i.e. suspensions with high concentration of conidia are needed for food inoculation, for instance). However, by targeting yield during sporulation, the germination properties of conidia may be overwhelmed which will likely impact on their responses during growth/inactivation conditions. For instance, it has been found that low a_w and reduced temperature used to produce *Penicillium roqueforti* conidia led to up to 45 hours longer germination times when the incubation for sporulation was done at 20°C compared to the incubation done at 5°C [8]. Given these findings, it becomes very clear that the conditions the conidia are produced and their impacts on conidial properties during growth/inactivation conditions seems to be different from those responses seen in bacterial spores. More studies targeting to assess the impact of physiological state of fungal conidia should be conducted and these aspects should also be considered when designing experiments dealing with fungal growth and inactivation.

In addition, another major characteristic of recent predictive mycology works comprises the validation of models on real food matrices. Some years ago, some studies just showed the calibration models or included validations in agar media. In general, discrepancy has been observed between growth in agar medium and real foods. In foods with continuous surface, colonies spread generally slower than in agar media, while in particulate foods, such as cereals, colonies spread faster, filling the gaps amongst grains. In agar media, solutes have been used to depress a_w. While glycerol has been the preferred solute as a_w depressor in most of the existing studies, recently, the use of NaCl and glycerol were compared for estimation of radial growth and/or germination of conidia as affected by a_w. Nonetheless, data suggested that NaCl presented restricted influence on the behaviour of the fungal species and conditions studied when used as a_w reduction agent [9].

Moreover, recent predictive mycology works comprise not only the validation of models on real food matrices, but also the development of the models per se on food matrices. Developing predictive mycology models on food matrices may be challenging in several aspects, with particular impact on the number, range and size of experiments. However, on the other hand, it is an approach that may result in less uncertainty regarding the model's usability because factors such as food structure, amongst others, are implied in the predictions. Examples of recent works that used real food matrices for validation purposes include Debonne *et al.* [5,6], while dos Santos *et al.* [7•] comprises an example of work in which models were calibrated and validated on real food matrices.

Despite the above, still validation of cardinal models with inflection (CMI) was mainly carried out on growth rates instead of lag times in recent years, both in pear and lime fruits [10,11]. In fruits this may make sense, as they are a particular case where usually fungal spoilage initiates in a single point and the colony is readily visible. Moreover, predicting the size of the rotten area may have an interest in case fruits are processed into derived products, such as juices. The use of CMI for growth rate as a function of different factors is widespread in predictive mycology as the estimated parameters have biological meaning; however they show better fitting to growth rate data than to the reverse lag time. For example, validation of CMI for *Aspergillus niger* in limes was carried out; whereas the accuracy (A_f) and the bias (B_f) factors for growth rate of 1.5 and 1.3 were observed, respectively. Despite this, a great discrepancy was observed for lag time [11]. A second category of food products where predictive mycology is important for quality management are bakery products. CMI and gamma concepts were recently applied to characterise the influence of a_w and temperature the appearance of bread spoilage fungi on agar medium. Then, a validation was carried out considering a_w (0.91–0.97), pH (4.6–6.8) and temperature (15–25°C) of different bread formulations. It has been found that the growth rates for some species on bread were about 2 fold that obtained on agar medium. A_f were, in general, close to 1, while dispersion of data led to B_f different from 1 [12].

Generalisation of CMI has led to generation of data in the full growth domain, including suboptimal growth conditions. Also, no growth conditions have been explored

through the use of probability models. For instance, a time for appearance of *Aspergillus flavus* colony was employed by Kosegarten *et al.* [13], and Astoreca *et al.* [14] built a probabilistic model with the aim to evaluate safe storage conditions for one month. Later, germination, mycelial development and the resulting increase in probability of fungal development and release of aflatoxin B_1 (AFB1) were analysed in parallel in a single experiment conducted at 25°C and 0.85−0.87 a_w employing 3% Pistachio Extract Agar inoculated with a strain of *A. flavus*. The germination time, apparent lag phase (intercept of lineal growth equation with diameter = 0) and time to visible growth (observed colonies of 3 mm diameter) were estimated as early indicators. It has been reported a substantial postponement of the apparent lag phase when contrasted to the germination time (more than 10 days) and even with 100% of spores completed the germination period, the apparent lag time occurred about 9 days later. These lengthy periods are related to the low a_w of the trial, which were known as non-optimal for mould development. After apparent lag time had been reached, 3–6 days more were needed to observe visible colonies. Probability of growth was calculated as the percentage of visible colonies from the total for each time observation. The estimated apparent lag time took place at a 0.1-0.3 probability of growth. Regarding AFB1, it was concluded that a long period since a spore contaminates a foodstuff is required for AFB1 to be potentially detected. By the time that fungal colony was detectable to naked eyes corresponded to a AFB1 production probability of 10%. This probability increased slowly, thus a AFB1 production probability of 50% was not attained before 23 days [15••].

Regarding modelling of mycotoxin production, complete factorial scheme with six temperature points (15−40°C) and eight a_w values (0.84−0.98 a_w) was employed to estimate the development and production of AFB1 probabilities by a set of 20 *A. flavus* strains. Then, linear logistic regression was employed to model the binary data explained by time, temperature and a_w obtained from the growth experiments for each of the 20 strains tested. Besides, once colonies were freshly formed (maximum diameter = 20 mm), extraction of AFB1 took place at diverse periods. Variability amongst the probability of growth model estimations from the 20 strains were observed; however, the accumulation of AFB1 was found to be more variable than probability of AFB1 production and growth. Overall, it has been found that the conditions for production of AFB1 are more restrict than the boundaries for fungal development. Therefore, it is clear that works dealing with the development of models to predict fungi behaviour and mycotoxin production ought to embrace a set of strains that encompass the diversity within and amongst the species to allow models outputs to be as real as possible from real world scenarios [16].

As an alternative, Versicolorin A (Ver A), which is an AFB1 precursor can be identified even when AF is undetectable and could then work as an indicator of early presence of AFs. An interesting approach was presented by Jiang *et al.* [17••]. The risk of AF presence in maize during storage was determined based on Ver A amounts and a logistic regression, whereas the maximum safe storage period was not estimated. Experiments were conducted to define the counts of aflatoxin-producing fungi, humidity content, early and highest Ver A concentrations at diverse time periods, which were then used as inputs in the model. The storage experiments lasted for 9 months. The validations (external and internal) showed good outcomes, as high as 93.3% and 96.4%, respectively. The period elapsed between AFB1 concentration was $\geq$5 μg/kg and largest production of Ver A was deemed as the maximum safe storage period. The multiple correlation coefficient was 0.801, suggesting a high correlation between explanatory variables and safe storage time. Moreover, the model was found to present adequate fitting, with an adjusted R^2 of 0.569. This means that Ver A level responds to the environmental conditions, and may be useful for determining the production of AF underneath a lengthy storage period. Including Ver A as an explanatory variable has a first significant advantage over other existing models which are based on constant conditions. Besides, another advantage comprises the fact that aflatoxigenic fungi metabolism is reflected by the *in situ* data [17••].

In summary, probability models have been increasingly used in the latest years, as they are useful to describe the transition from the growth to the no-growth region which, in general is the region of interest in food quality and safety management. Moreover, due to the intraspecific differences in mycotoxin production, predicting the boundary from production to non-production seems more suitable than predicting the actual mycotoxin concentration produced. Models built to forecast the storage time until the food or crop can be considered safe (mycotoxin was not produced), could comprise a valuable tool to assist management throughout the food and feed chains.

Assessing efficacy of antimicrobials in foods through predictive mycology

Predictive mycology has a specific application in determining the effectiveness of antimicrobials and the suitable doses to be used depending on other intrinsic and extrinsic factors. Experimental designs can be protocolised in order to develop suitable models for easy estimation of MIC (minimum inhibitory concentration) avoiding a high number of experimental runs. First, primary models are developed under different antimicrobial doses, subsequently, growth rates or lag time estimated from the primary models are plotted versus antimicrobial doses, and MIC estimated from the intercept of the fitted model with growth rate equal to 0 or at infinite lag time. For

example, models assessing the antifungal effect of a microemulsion containing essential oil of betel leaf (BLEO) towards *A. flavus* development in tomato paste and *Penicillium expansum* in apple juice agar were developed. In the work, growth kinetic parameters such as growth rate and lag phase were obtained by inoculating the fungi in different media [potato dextrose agar (PDA), apple juice agar and tomato paste agar]. Subsequently, it has been modelled the influence of varied concentrations of BLEO towards the growth kinetic parameters of fungi. The minimum concentration of BLEO capable of preventing fungal growth (E_{max}) and the minimum concentration of BLEO that led to an infinite lag time (MIC) were estimated to be much lower in tomato paste challenged with *A. flavus* than for apple juice agar challenged with *P. expansum* [18,19]. Similarly, Rosegarten *et al.* [16] modelled the effects on *A. flavus* of a_w, temperature, and concentration of cinnamon essential oil using both kinetic and probability models.

Moreover, the primary-secondary modelling approach was applied to assess *Aspergillus carbonarius* growth and production of OTA using a culture media containing grape juice. The phenomena mentioned above were studied as a function of range of sodium metabisulphite (NaMBS) (0−200 mg/L), a_w (0.88−0.98) and temperature (15−38°C). The model of Baranyi was used for primary modelling of colony diameters and a CMI for modelling of NaMBS, a_w and temperature on growth rate and lag phase. Moreover, OTA production was determined during fungal development and a quadratic polynomial equation was employed to model the impact of three parameters on the constant rate of mycotoxin production. It has been found that the antifungal agent stimulated OTA production, including conditions in which a delay in fungal growth was reported. Similar observations have been done in the past when using low levels of antifungal agents. Validation was done in this case using the same medium but a different strain. The validation statistical parameters allowed the conclusion that growth rate would be slightly underestimated ($B_f < 1$), with the same trend seen for lag time ($B_f > 1$, in this case). On the other hand, the deviance amongst observations and model predictions (A_f, accuracy factor) obtained for growth rate was 22% and 42% for lag time, which is consistent with the comments in the first section [20].

Another recent study modelled the impacts of organic acids, a_w, temperature and pH on the germination and development of *P. roqueforti* and *Penicillium camemberti*. Besides, their MIC were also determined. The conditions under which no germination takes place, above which no germination takes place and in which the germination time is optimal (i.e. cardinal values) were not dependent on the species. *P. camemberti* had a lower MIC than *P. roqueforti* for propionic acid, while these two fungi were capable of germinating in the maximum lactic acid concentrations tested (1 M). This fact, prevented the determination of MIC for these fungi towards lactic acid [21].

Shelf-life extension can also be attained through the modification of atmosphere within packaging (MAP). In this way, the maximum germination fraction and the median germination interval of five fungal species (*P. roqueforti*, *Paecilomyces niveus*, *Penicillium brevicompactum*, *P. expansum*, and *Mucor lanceolatus* was estimated as affected by CO_2 (0.03–70%) and O_2 (0–21%) [22]. It has been found that the germination of conidia was not completely prevented when the partial pressures of O_2 or CO_2 varied from <1% and 70%, respectively. Nonetheless, an effect on maximum germination fraction (increase or decrease) was observed and it has been found to be dependent on the species of fungi tested when levels of CO_2 and O_2 were >20% and <1%, respectively [22].

Finally, recently the impact of calcium propionate, pH, and a_w on *P. paneum* single spores germination have been reported [23]. The use of single spores modelling in the field of predictive mycology is still much less developed when compared with the studies of bacterial single cells modelling. Nonetheless, the development of more studies towards the characterization of individual heterogeneity of fungal growth will be crucial in the understanding of fungal behaviour in the context of clean label formulations as well as when considering the large scale application of emerging food processing technologies. Further single spore's studies are required and will likely allow to gain insights on basic phenomena involved in fungal lag time and time for visible colonies to appear. These data will then be useful for the design of more robust formulations and more effective processes towards controlling early fungal spoilage.

Predictive modelling for climate change impact prediction on mycotoxins

Model projections for the next years have been created to assess how climate change could influence on fungal development and on the ability to release mycotoxins from available data bases. Meteorological data such as precipitation regimes, variations in temperature and sunlight patterns have been included [24]. However, most of these studies are performed in culture medium and require validation in crops.

Smarter mechanistic models have been built based on the life cycle of infection of fungal pathogens; such models are fed with climatic variables, crop phenology, as well as specific models for fungal sporulation, germination, survival, development and mycotoxin release. Such models have the aim of giving an early warning on mycotoxin risk and support decisions on either fungicide treatments or early harvesting date. However, they have also been applied to estimate the risk of mycotoxin presence and

concentration as influenced by climate change. For example, a model generated to assess the aflatoxin presence in maize in Europe, after a 2 and 5°C rise in temperature, shows that a larger production region could be exposed to maize contamination by *A. flavus* and consequent aflatoxin production [25]. There is a great potential for application of GIS (Geographical Information System) to improve such mechanistic models.

Different regions might have different impacts with increasing or reducing the fungal presence and the mycotoxins production. For instance, a minimization of *Alternaria* occurrence and alternariol, tenuazonic acid and altenuene production may be observed in tomatoes in Spain. On the other hand, in Poland an increase may be seen [26].

Although in some countries the contamination and production of mycotoxins may remain similar or reduce, in other regions there may be increased contamination and even a change in species of higher incidence in food and a change in the main mycotoxins occurring. Therefore, the growth and production of mycotoxins by mycotoxigenic fungi could be managed and prevented through the use of predictive models, which could work as support tools for decision-making.

Predictive modelling for fungal growth and mycotoxin estimation using infrared spectrometry

While predictive mycology has mainly focused in aflatoxins production postharvest, in the case of *Fusarium* toxins, which are mostly produced in the field, apart from predictive modelling of preharvest toxin accumulation in crops linked to climate [25], as explained in the previous section, an additional tool is analysing raw materials before processing and rejecting contaminated batches. However, HPLC-FLD, HPLC-UV or HPLC-MS cannot be applied to routinely analysis at raw materials reception, and usually rapid screening immunoassays are used. Currently infrared spectroscopy methods have been assessed as potential replacements for detection of mycotoxins in cereals. These methods would present as advantages features such as simple execution/interpretation of results, quickness in obtaining results and the fact that sample destruction is not needed. Nonetheless, recent studies reported that infrared spectroscopy methods with quantification intents seem not accurate enough to fulfil requirements for their application to be supported by national regulations and specifications. Nonetheless, serving for rejection purposes (pass/no-pass a certain criteria) seems to comprise the highest potential for an extensive application of predictive models based on infrared spectroscopy methods [27].

The principle of infrared spectroscopy relies on the absorption of particular light frequencies (800–25 000 nm) associated with a molecular structure (bonds) of a compound. Thus, reflectance or transmission spectra of samples allow to assess the energy linked to the profile of molecular surfaces and vibrations of bonds of compounds in that specific sample. Data on the bonds in the molecules are then obtained based on the energy involved in the vibrations. Interpreting NIR spectra is complex, due to light scattering and superpositions of spectral peaks. The information from chemical data are extracted as much as possible through the use of statistical tools, in particular chemometrics [28].

Data of the target mycotoxin obtained using a reference instrumental method is necessary for spectrometer calibration and further construction of the predictive model. The calibration set should comprise samples that present the broad of characteristics of the samples that are expected to be further examined. Either modes of spectroscopy (transmission or reflection) can be employed for obtaining references' samples infrared spectra. Finally, a model is built based on the relationship amongst spectral data (preprocessed) and reference data, and validated on an independent set of samples [27].

Most of the existing studies target aflatoxins or deoxynivalenol (DON) and *Fusarium* head blight. Models for quantification or for classification of consignments based on the amount of mycotoxins have been developed. Equations for quantification of DON levels are poor at predicting a specific level, because DON is a minor chemical in food. However, grain batches could be classified through the use of models, especially with respect to regulatory levels. The indirect estimation of DON levels is based on detecting either physical alterations of the grains or changes in carbohydrates and proteins due to fungal growth. For DON, published quantification models have variable performances [29–31]. This was mainly due to the use of either inoculated or naturally contaminated cereals, crushed or whole grains, highly variable levels of toxin. Correlation coefficients in quantification works have been shown in the range 0.46−0.91, while in classification works 75–90% of correct classifications were reported.

Finally, cereal sorting using near infrared hyperspectral images analysis (HSI-NIR) is starting to show promising results, and may be a suitable tool to be applied in the coming years. Recently, a study has suggested that HSI-NIR can be used for non-destructive and rapid detection of wheat loads presenting high levels of DON [32]. HSI-NIR could be rather used as a screening approach due to the error of prediction found (~501 μg/kg) and as a classification method due to an accuracy of >90%. As such, this tool would serve for rejection of batches of wheat presenting high levels of DON [33•].

As conclusion, predictive modelling is a suitable tool for assisting decision making in food quality and safety

management related to fungi, at different points of the food chain, from primary production to finished products.

Declarations of interest

None.

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