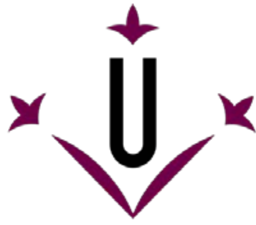


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1 **Probability models for growth and aflatoxin $B_1$ production as affected by intraspecies**
2 **variability in *Aspergillus flavus***
3
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## Abstract

The probability of growth and aflatoxin $B_1$ ($AFB_1$) production of 20 isolates of *Aspergillus flavus* were studied using a full factorial design with eight water activity levels (0.84 to 0.98 $a_w$) 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, $AFB_1$ was extracted at different times from newly formed colonies (up to 20 mm in diameter). Although a total of 950 $AFB_1$ values over time for all conditions studied were recorded, they were not considered to be enough to build probability models over time, and therefore, only models at 30 days were built. The confidence intervals of the regression coefficients of the probability of growth models showed some differences among the 20 growth models. Further, to assess the growth/no growth and $AFB_1$/no- $AFB_1$ production boundaries, 0.05 and 0.5 probabilities were plotted at 30 days for all of the isolates. The boundaries for growth and $AFB_1$ showed that, in general, the conditions for growth were wider than those for $AFB_1$ production. The probability of growth and $AFB_1$ production seemed to be less variable among isolates than $AFB_1$ accumulation. Apart from the $AFB_1$ production probability models, using growth probability models for $AFB_1$ probability predictions could be, although conservative, a suitable alternative. Predictive mycology should include a number of isolates 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

53

## 54 1. Introduction

55 Mould spoilage and mycotoxin contamination of food products cause large economic
56 losses (Dantigny et al., 2005; Mitchell et al., 2016) and pose a serious risk to public health
57 (Marín et al., 2013). *Aspergillus* spp. is recognized as one of the most widely distributed fungal
58 genera in nature. The two most agriculturally important species are *Aspergillus flavus* and *A.*
59 *parasiticus*, which are found all over the world, being present in both the soil and the air
60 (Hedayati et al., 2007; Horn and Dorner, 1998; Wicklow et al., 1998). *A. flavus* is the major
61 causal agent of food and feed contamination with aflatoxins (AFs) (Klich, 2007). AFs ($B_1$, $B_2$, $G_1$
62 and $G_2$) are a group of toxic, mutagenic, carcinogenic and teratogenic secondary metabolites
63 which are health hazards to humans and animals (Bottalico, 1999). Aflatoxin $B_1$ ($AFB_1$) is
64 reported as the most toxic natural compound and is classified by the International Agency for
65 Research on Cancer (IARC) as a class 1 toxin (IARC, 2002) due to its demonstrated
66 carcinogenicity to humans.

67 Both fungal growth and mycotoxin production can be influenced by different factors such as
68 temperature, water activity ($a_w$), inoculum concentration, isolate, microbial interactions,
69 physiological state of the mould, genotype, etc., and these factors may affect in a different
70 way growth and mycotoxin production (Garcia et al., 2009). Aflatoxins are usually found in
71 foodstuffs such as cereals, nuts, spices, dried fruits and their by-products; the stability of such
72 commodities is maintained through moisture content reduction, while low temperature is not
73 usually applied for their preservation . For example, to prevent spoilage by storage fungi, the
74 moisture content of starchy cereal grains should be below 14.0% (about 0.70 $a_w$), although
75 temperature fluctuations and insect activity may enable grain rehydration during storage, if
76 safety management systems fail.

77 Populations of *A. flavus* in agricultural products and foods are complex communities that may
78 contain many different isolates (Mahmoud et al. 2016). Their growth and $AFB_1$ production
79 potential are known to vary (Abbas et al., 2004; Adhikari et al., 2016; Singh et al., 2015;
80 Yogendrarajah et al., 2016; Yousefi et al., 2009). There are many studies reporting the
81 variability among isolates in terms of growth and mycotoxin production (Abbas et al., 2005;
82 Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Pardo
83 et al., 2005, 2004; Parra and Magan, 2004; Romero et al., 2007; Singh et al., 2015;
84 Yogendrarajah et al., 2016). Garcia et al. (2011a) studied the growth and ochratoxin A (OTA)
85 production of thirty isolates of *Aspergillus carbonarius*. Their results showed a wide dispersion

86 in both growth rate and mycotoxin production, especially under marginal conditions. Foods
87 are generally stored under marginal conditions, of either $a_w$ or temperature, for fungal growth
88 and subsequent mycotoxin production. In these cases, growth is compromised but still occurs,
89 and thus knowing the growth and AFs production boundaries (growth/no growth and
90 toxin/no-toxin interface) of microorganisms becomes important for food safety.

91 Predictive models are helpful tools to estimate the safety and shelf-life of foods. Within these
92 predictive models, probabilistic models are used to predict the probability of growth or
93 mycotoxin production of a microorganism under different conditions (Tienungoon et al., 2000).
94 Logistic regression is a useful method for modelling boundaries between growth and no
95 growth of fungi, or mycotoxin production and no mycotoxin production (Aldars-García et al.,
96 2016a, 2015; Astoreca et al., 2012; Garcia et al., 2011c; García-Cela et al., 2014; Marín et al.,
97 2012, 2009; Tassou et al., 2009). An important aspect of predictive model development is
98 ensuring that the predictions made by the models are applicable to real situations. Thus,
99 predictive models should take into account suboptimal conditions (the usual storage food
100 environment) and strain variability because in natural ecosystems, different strains can occupy
101 the same niche. The objective of the present work was to determine if the intraspecies
102 variability detected for growth and toxin production in kinetic models can be overcome by the
103 use of probability models.

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

108

109 ## 2. Materials and methods

110 ### 2.1. Isolates

111 Twenty isolates of *A. flavus* isolated in previous years from maize grains, pistachio nuts, and
112 chilli were tested in this study. A preliminary study was conducted to characterize the isolates
113 in terms of their ability to produce $AFB_1$ on Potato Dextrose Agar (PDA) at 25 °C, after 7
114 incubation days. Isolates were categorized as “low AF producer” if $AFB_1$ levels were between
115 LOD and 100 ng/g agar and “high AF producer” for isolates that produced concentrations
116 higher than 100 ng/g agar. The results of this previous study and more details of the isolates
117 studied are shown in table 1.

118 Table 1. Description of the *A. flavus* isolates used in the present study.

119

| Category | Isolate code* | Origin | $AFB_1$ production after 7 days on PDA at 25 °C (ng/g agar)** |
|---|---|---|---|
| **High producers** | UdL-TA 3.268 | Pistachio nuts | 471.2 ± 62.9 |
| | UdL-TA 3.270 | Pistachio nuts | 114.8 ± 39.3 |
| | UdL-TA 3.318 | Maize grains | 1189.3 ± 55.6 |
| | UdL-TA 3.321 | Maize grains | 748.3 ± 297.9 |
| | UdL-TA 3.322 | Maize grains | 698.1 ± 68.1 |
| | UdL-TA 3.327 | Maize grains | 178.5 ± 44.7 |
| | UdL-TA 3.328 | Maize grains | 243.6 ± 57.6 |
| | UdL-TA 3.329 | Maize grains | 109.3 ± 8.5 |
| | UdL-TA 3.331 | Maize grains | 547.2 ± 59.9 |
| | UdL-TA 3.332 | Maize grains | 2114.6 ± 248.6 |
| **Low producers** | UdL-TA 3.244 | Chilli | 20.5 ± 3.1 |
| | UdL-TA 3.267 | Pistachio nuts | 25.9 ± 3.4 |
| | UdL-TA 3.269 | Pistachio nuts | 28.3 ± 6.8 |
| | UdL-TA 3.319 | Maize grains | 39.7 ± 2.7 |
| | UdL-TA 3.320 | Maize grains | 1.9 ± 0.9 |
| | UdL-TA 3.323 | Maize grains | 3.9 ± 2.8 |
| | UdL-TA 3.324 | Maize grains | 5.4 ± 3.0 |
| | UdL-TA 3.325 | Maize grains | 1.5 ± 0.5 |
| | UdL-TA 3.326 | Maize grains | 52.3 ± 1.1 |
| | UdL-TA 3.330 | Maize grains | 37.2 ± 10.1 |

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

122 ** Mean value ± standard deviation (n=3)

123

124 ## 2.2. Inoculum and media preparation

125 The twenty isolates were sub-cultured on PDA plates and incubated at 25 °C for 7 days to
126 obtain heavily sporulating cultures. After incubation, spores were collected by scraping the

127 surface of the plates, diluting them in sterile water adjusted to $a_w$ values of 0.84, 0.86, 0.88,
128 0.90, 0.92, 0.94, 0.96 and 0.98 with glycerol containing Tween 80 (0.05% v/v), and filtering
129 through sterile glass wool into a tube. Immediately, the total spore concentrations were
130 determined using a Thoma counting chamber and decimal dilutions (in sterile water adjusted
131 to the correspondent $a_w$ value with glycerol, containing Tween 80 (0.05% v/v)) and were
132 prepared to a final concentration of $10^2$ spores/mL for each $a_w$ and isolate.

133 The basic medium used in this study was Maize Extract Agar (MEA) adjusted to the 8 different
134 $a_w$. The medium was made by boiling 40 g of raw ground dry maize grains in 1 L distilled water
135 for 30 min. Next, the extract was filtered, and the amount of the evaporated water was added
136 to adjust the final concentration to 4% maize extract. The water activity of the media was
137 adjusted by the addition of certain amounts of glycerol-water to obtain the $a_w$ of each
138 treatment and 2% maize grain in the medium. Then, 12 g of agar were added per L of medium
139 (for each $a_w$), and the media was autoclaved and poured into 90 mm sterile Petri dishes, which
140 were prepared under aseptic conditions. The $a_w$ of each medium was checked with an AquaLab
141 Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003.

142 2.3. Experimental design

143 A 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
144 temperature (15, 20, 25, 30, 35 and 40 °C) levels was built to study the growth and $AFB_1$
145 production of several *A. flavus* isolates on MEA for 39 days. To carry out the experiment, not
146 all the 48 combinations within the $a_w$ and temperature range were studied. We aimed to study
147 those conditions where growth might occur (and then also mycotoxin production) but were
148 compromised. Thus only the 32 T-$a_w$ combinations were studied as shown in Figure 1.

149 2.4. Inoculation and incubation

150 The growth and $AFB_1$ production by *A. flavus* grown from single spores were assessed in the
151 present study. A 0.2 mL aliquot of $10^2$ spores/mL suspension was surface plated onto MEA
152 (2%) and spread with a sterile bent glass rod to obtain approximately 20 spores per Petri dish
153 (approximately 20 colonies originating from one spore each). Petri dishes with the same $a_w$
154 were enclosed in polyethylene boxes together with a glycerol–water solution at the same $a_w$ to
155 maintain the relative humidity inside the boxes. Plates were incubated at the required
156 temperature, and each Petri dish was checked daily (one Petri dish per isolate and T-$a_w$

157 combination, a total of 640 Petri plates per repetition, with three repetitions of the

158 experiment).

159 2.5. Growth assessment and $AFB_1$ determination

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

162 The presence of $AFB_1$ was determined at certain time intervals (maximum 39 days) depending
163 on how many colonies had arisen on each Petri dish. The size of the analysed colonies ranged
164 from 2 to 30 mm in diameter. Growth was assessed by measuring the perpendicular colony
165 diameter in millimetres. A 5-mm agar plug was taken for $AFB_1$ analysis from the centre of a
166 colony at appropriate time intervals. It has been shown for ochratoxin (not aflatoxin) that the
167 shorter the distance from the inoculum point, the higher the maximum toxin concentration
168 detected (each maximum takes place at a different time as the colony grows) (Valero et al.
169 2006). After sampling, the plates were taken back to incubation, for latter assessment of the
170 other colonies present on the Petri plates. Plugs were weighed and vortexed for approximately
171 5 seconds in 1 mL of methanol and left stationary. After 1 hour, extracts were vortexed again
172 and filtered (Millex$^R$ SLHV 013NK, Millipore, Bedford, MA, USA). Extracts were dried in a
173 nitrogen stream and stored at 4 °C until HPLC analysis. The analysis was carried out using a
174 previously described high performance liquid chromatography (HPLC) method (Aldars-García
175 et al., 2015). For the HPLC analysis, all extracts were resuspended with 0.5 mL of
176 methanol:water (50:50 v/v), and 100 µL was injected in the HPLC system (Waters, Milford, MA,
177 USA). The detection limit of the analysis was 0.1 ng/g of $AFB_1$, based on a signal-to-noise ratio
178 of 3:1.

179 2.6. Logistic models development

180 Logistic regression was used to calculate the probabilities of growth (Eq. 1) as a function of
181 time, temperature and $a_w$, and $AFB_1$ production (Eq. 2) as a function of temperature and $a_w$.
182 using Statgraphics Plus 5.1. Logistic regression describes the log odds of the event, which is the
183 natural logarithm of the probability of the event occurring (P) divided by the probability of the
184 event not occurring (1 − P).

185 The binary values (0=no visible growth/no $AFB_1$ detection; 1= growth/$AFB_1$ detection) were
186 adjusted by linear logistic regression, to obtain all the probability models, one for probability
187 of growth and one for $AFB_1$ production probability for each isolate (a total of forty probability
188 models).

189 $logit(P_G) = ln\ \frac{P_G}{1-P_G} = b_0 + b_1 t + b_2 T + b_3 aw + b_4 t^2 + b_5 tT + b_6 t\ aw + b_7 T^2 + b_8 t\ aw +$

190 $b_9 aw^2$ (1)

191

192 $logit(P_{AF}) = ln\ \frac{P_{AF}}{1-P_{AF}} = b_0 + b_1 T + b_2 aw + b_3 T^2 + b_4 aw^2 + b_5 T\ aw$ (2)

193

194 Where $P_G$ and $P_{AF}$ are the probability of growth or $AFB_1$ production (in the range of 0–1),
195 respectively, t is the time, T is the temperature in °C, aw is the water activity and $b_i$ are the
196 coefficients to be estimated.

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

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

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

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

205

206 Where n is the number of growing colonies and $n_T$ is the potential number of colonies which
207 could have arisen from the inoculated spores. A mean maximum value of 16 or 21 colonies was
208 calculated for the two different runs in which the experiments were performed. Those values
209 came from the experiments carried out at the best conditions for growth, where the observed
210 number of colonies was nearly constant. Graphics were performed using either Statgraphics
211 Plus 5.1 or JMP Pro 12.

212

213 ## 3. Results

214 The total number of conditions studied in the present work were 32 (Fig. 1) for each isolate.
215 The large number of isolates included in this study permitted a comprehensive investigation of
216 the relationships among growth, $AFB_1$ and isolate. All probability models developed described
217 satisfactorily the phenomena studied. The percentage of concordance (%C) of the models for
218 each isolate is shown in Table 2. The probability models included square and interaction terms.
219 Adding an interaction term to a model changes the interpretation of all of the coefficients. For
220 example, if there were no interaction terms, $b_2$ in eq. 2 would be interpreted as the unique
221 effect of temperature on the probability of growth. However, the interactions mean that the
222 effect of temperature on the probability of growth is different, for example, for different
223 values of $a_w$. So the unique effect of temperature on the probability of growth is not limited to
224 $b_2$ (in eq. (2)) but also depends on the values of other regression coefficients.

225

226 Table2. Percentage of concordance (%C) of the 40 logistic models developed with *A. flavus*
227 isolates.

| Isolate name | Probability growth models | Probability $AFB_1$ 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 |

228

229 **Effect of water activity and temperature on intraspecific differences in growth**

230 Firstly, $n/n_T$ was calculated for the different conditions and isolates, and it varied from 0 under
231 no growth conditions to 1 under the most suitable conditions. A polynomial model was fitted
232 to $n/n_T$ values for each isolate, including only 0 values surrounding the positive growth
233 conditions. Figure 2 shows a graphical example of one of these models. Regarding this fitting,
234 in several cases there was a certain disagreement between observed and predicted values at
235 40 °C, where observed data were 0 under certain $a_w$ levels (usually 0.92) and 1 when $a_w$
236 increased by 0.02 units (usually to 0.94). In such cases, the predicted values at 0.94-0.98 were
237 lower than the observed ones.

238 As an example, Table 3 shows the percentage of spores which led to growing colonies ($n/n_{T,}$) at
239 20 °C and 0.88 $a_w$. The maximum percentage of spores which developed to colonies informs us
240 about the ability of each isolate to initiate growth under a stressful environment. Almost all 20
241 isolates hardly grew under these marginal conditions; the maximum $n/n_T$ was up to
242 approximately 30%, except for isolates UdL-TA 3.270 and UdL-TA 3.318 and, to a lesser extent,
243 isolate UdL-TA 3.325. Notwithstanding this percentage, similar times to reach the maximum
244 number of colonies under these marginal conditions were obtained for the 20 isolates, with
245 most of them ranging between 15 and 16 days. This suggests a possible maximum period of
246 time of adaptation after which the spores are not able to initiate growth regardless of the total
247 % of germinated spores.

248 Table 3. The maximum proportion of spores (mean ± standard deviation) which initiated
249 growth ($n/n_T$) at 20 °C - 0.88 $a_w$ for the 20 studied isolates of *A. flavus* and the incubation time
250 (days) required.

| **Isolate name** | $n/n_T$* | **Time (d)** | **Isolate nameLOW16** | $n/n_T$* | **Time (d)** |
|---|---|---|---|---|---|
| UdL-TA 3.268 | 0.24±0.07 | 15.3±0.5 | UdL-TA 3.244 | 0.31±0.08 | 15.3±0.5 |
| UdL-TA 3.270 | 1.00±0.00 | 15.5±0.7 | UdL-TA 3.267 | 0.31±0.06 | 15.4±0.8 |
| UdL-TA 3.318 | 0.81±0.03 | 15.6±0.9 | UdL-TA 3.269 | 0.31±0.05 | 14.2±0.9 |
| UdL-TA 3.321 | 0.38±0.05 | 16.0±0.0 | UdL-TA 3.319 | 0.31±0.11 | 15.1±0.5 |
| UdL-TA 3.322 | 0.29±0.06 | 15.6±0.8 | UdL-TA 3.320 | 0.25±0.09 | 14.3±1.2 |
| UdL-TA 3.327 | 0.29±0.07 | 16.0±1.1 | UdL-TA 3.323 | 0.13±0.07 | 17.0±0.9 |
| UdL-TA 3.328 | 0.24±0.07 | 16.1±0.9 | UdL-TA 3.324 | 0.19±0.06 | 15.3±0.4 |
| UdL-TA 3.329 | 0.19±0.04 | 15.3±0.7 | UdL-TA 3.325 | 0.56±0.09 | 16.2±0.5 |
| UdL-TA 3.331 | 0.19±0.05 | 15.1±0.4 | UdL-TA 3.326 | 0.31±0.08 | 16.6±0.5 |
| UdL-TA 3.332 | 0.33±0.04 | 16.2±0.6 | UdL-TA 3.330 | 0.38±0.07 | 16.6±0.7 |

251 *Mean and standard deviations resulting from 2 to 3 replicates

252 Secondly, linear logistic regressions were carried out. Tables S1 and S2 (supplementary
253 material) show the regression coefficients of all models developed. Direct comparison of those

254 coefficients among isolates indicates the existing significant differences among the models
255 built for the different isolates. In particular, coefficients for the T x $a_w$ terms were clearly
256 different across isolates. This can be confirmed from Fig. 3, where a comparison among the 20
257 growth models is shown. 0.05 and 0.5 probabilities were plotted at 30 days for all of the
258 isolates as isopleths. 0.5 isopleth represents the points where 50% probability exists for
259 growth detection, while 0.05 isopleth describes the conditions under which growth is unlikely
260 to occur. These plots are based on total probability, once both the $n/n_T$ and logistic models
261 have been merged.

262 The growth/no growth boundaries varied among the tested *A. flavus* isolates. Fig. 3 shows
263 some differences in the curvatures of the contour lines for the 20 growth plots. In general, the
264 isolates grew under a wider range of $a_w$ when the temperature was between 25-35 °C. Greater
265 differences among the 20 isolates occurred when the temperature was <25 °C and >35 °C. Only
266 isolate UdL-TA 3.318 showed different behaviour, as its two isopleths showed a very different
267 curvature at 17-22 °C from the other isolates, indicating a lower optimal temperature for
268 growth.

269 **Effect of water activity and temperature on intraspecific differences in $AFB_1$ production**

270 The predicted $AFB_1$ production at probabilities of 5 and 50% for all isolates after 30 days is
271 shown in Figure 4. Some differences among isolates can be extracted from this figure.
272 Generally, temperatures below 30 °C encompass a wider range of $a_w$ for $AFB_1$ production. Only
273 2 isolates, UdL-TA 3.267 and UdL-TA 3.269, had a greater $a_w$ range for $AFB_1$ production at
274 higher temperatures. The different curvatures of the isopleths illustrate the variability in the $a_w$
275 and temperature tolerance of the different isolates for $AFB_1$ production. Both for the high and
276 low AF producing isolates (see Table 1), the shape of the isopleths was very similar for growth
277 and $AFB_1$ production. However, the conditions for $AFB_1$ production were narrower than those
278 for growth.

279 Table 4. Predicted probability of $AFB_1$ production by *A. flavus* isolates and the experimental
280 amount of $AFB_1$ (ng/g) detected in maize extract agar at two different temperature and $a_w$
281 combinations at day 30.

| Temp (°C) | $a_w$ | Isolate name | $AFB_1$ probability | $AFB_1$ (ng/g agar) | Temp (°C) | $a_w$ | Isolate name | $AFB_1$ probability | $AFB_1$ (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 |

282 nd: not detected
283

284 From Table 4, at 20 °C -0.92 $a_w$, 4 isolates out of 20 produced $AFB_1$, and the amount of $AFB_1$
285 produced ranged between 1.79 and 92.81 ppb (probability 0.22-0.35 in these cases). In the
286 same way, at 25 °C -0.90 $a_w$, 6 isolates out of 20 were able to produce $AFB_1$, ranging between
287 2.12-27.78 ppb (probability 0.24-0.49 in these cases). No correlation was found between
288 probability values and $AFB_1$ concentrations, confirming that the amount of toxin produced is
289 highly isolate dependent (Table 4).

290 These results show the high variability in the amount of $AFB_1$ produced, while one isolate
291 cannot produce the toxin at all, another isolate under the same condition is able to produce up
292 to 93 ppb. From the data, we can infer that predicting the kinetics of the amount of $AFB_1$
293 produced will be highly variable among isolates. On the other hand, probability models give a
294 more common trend, although differences still exist. Below a probability value of 0.22 no $AFB_1$
295 was detected; thus, we can use this value as a maximum limit below which $AFB_1$ production is
296 very unlikely to occur across the 20 isolates.

## 297 4. Discussion

298 Despite the known differences in growth and mycotoxin production by individual isolates, this
299 field has not yet been studied in detail for mycotoxigenic fungal species. Many sources can be
300 the cause of this intraspecies variability, e. g., molecular characteristics, geographical origin,
301 environmental conditions, etc. The present study compared the probabilities of growth and
302 $AFB_1$ production of 20 isolates of *A. flavus* using a wide range of T-$a_w$ combinations. In this

303 work, no effect was observed due to the isolation source of the isolates on the probabilities of
304 growth and AFB1 production.

305 It is important to highlight that the results of growth and $AFB_1$ production in this work
306 correspond to the behaviour of colonies arising from single spores. Aldars-García et al. (2016b)
307 modelled the probability of growth and $AFB_1$ production using single spores and a
308 concentrated inocula to assess the differences among them. The effect of inoculum
309 concentration greatly affected the outcome of the predictive models; growth/ $AFB_1$ production
310 occurred much earlier for the concentrated inoculum than for colonies arising from a single
311 spore (up to 9 days). That study demonstrated that the number of spores used to generate
312 data in predictive mycology experiments should be carefully controlled to predict as accurately
313 as possible the fungal behaviour in a foodstuff. Although the aim of the present work was to
314 assess the intraspecies variability, we tried to obtain predictions under conditions as similar as
315 possible to real food storage conditions.

316 Regarding the 20 probability growth models, the differences arise when the combination of
317 variables takes place, since the regression coefficients for the interaction factors are quite
318 variable among isolate models (Table S1, supplementary material). This means, for example,
319 that the use of a restrictive $a_w$ and temperature together have a greater effect on fungal
320 behaviour than restricting only one of these variables and that the adaptation and response is
321 isolate dependent. Other studies also suggest that the combination of factors can be restrictive
322 on the growth of *Aspergillus ochraceus* and OTA production, affecting different isolates in
323 different ways (Pardo et al., 2006). Furthermore, regression coefficients for the $AFB_1$
324 production probability models differed less among the 20 isolates (Table S2, supplementary
325 material) than the growth models. This was in general due to wider confidence intervals in the
326 $AFB_1$ models due to the lower number of observations and also because time was not included
327 as a variable in the $AFB_1$ models. When the twenty 0.05 probability lines were superposed (Fig.
328 5), it was clear that $AFB_1$ production was an event much more dependent on the isolate
329 involved than was growth. The lines in Figure 5a are closer than the lines in Figure 5b. A study
330 conducted with 8 isolates of *Penicillium expansum* in apples to study fungal growth and patulin
331 production showed this isolate-dependent mycotoxin production (Baert et al., 2007a). In that
332 study, the combination of stress factors, such as temperature and $O_2$ level, resulted in
333 differences in patulin production depending on the isolate.

334 Boundaries for growth/no-growth or $AFB_1$/no- $AFB_1$ production were variable among isolates.
335 Regions out of the temperature range of 20-35 °C showed the highest variability. These regions

336 correspond to marginal conditions. As has been reported several times, the differences among
337 isolates are more marked under marginal than under optimal conditions (Astoreca et al., 2007;
338 Baert et al., 2007b; Garcia et al., 2011b; Romero et al., 2010). As an example of these studies,
339 Garcia et al. (2011b) studied the impact of suboptimal environmental conditions on the
340 intraspecific variability of *A. carbonarius* growth and OTA production using 30 isolates and
341 found higher intraspecies variability under marginal conditions of growth.

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

346 In general, growth of *A. flavus* is unlikely for $a_w$ values under 0.85, regardless of the
347 temperature level. Certain combinations of T-$a_w$, especially those combinations which imposed
348 stress on the fungus, resulted in a significant decrease in the probability of growth and $AFB_1$
349 production. For example, 15 °C -0.90 $a_w$ or 35 °C -0.85 $a_w$ are almost not supportive of growth.
350 At temperatures beyond approximately 40 °C, a very small probability of $AFB_1$ production was
351 obtained. However, in this sense, high differences among isolates were found; the ability to
352 produce $AFB_1$ at high temperatures seemed to be very isolate-dependent.

353 The 20 predicted boundaries for 0.05 probability were plotted together with literature data for
354 both growth (Fig. 5a) and $AFB_1$ production (Fig. 5b). The two thicker lines in Figure 5a and 5b
355 represent the general behaviour of the isolates that grew/produced $AFB_1$ under the wider T-$a_w$
356 combinations and the narrower combinations. However, it proved difficult to find appropriate
357 literature data to which we could compare our logistic models, as no similar approach has
358 been employed so far for *A. flavus* colonies originating from single spores. Due to this
359 drawback, some literature data points in Fig. 5 showed a high probability of growth or toxin
360 production out and around the boundaries that we predicted. For growth, less agreement with
361 the literature data was found at higher temperatures (above 25 °C). The literature data were
362 obtained experimentally using inoculum sizes higher than 50 spores per inoculation point,
363 except for 4 data points extracted from Aldars-García et al. (2016b), which were at 25° C and
364 0.85 and 0.87 $a_w$, with approximately 100% probability of growth at 30 days for colonies
365 originating from single spores. The same conclusion was reached by Garcia et al. (2010) while
366 working with *A. carbonarius* and *P. expansum* at suboptimal conditions and different inoculum
367 levels. Their results showed that as conditions become limiting and the inoculum size
368 decreases, more variability in the growth probability is obtained. Therefore, we would expect

369 quite variable fungal behaviour under marginal conditions when one spore leads to growth.
370 Regarding $AFB_1$ production, more variability was found in the literature data, as is reflected in
371 Figure 5b (points) where, for example, some researchers detected $AFB_1$ at 30° C-0.82 $a_w$ but
372 others did not at 30° C -0.84 $a_w$. The 20 isopleths for $AFB_1$ production probability were more
373 widely distributed than those for growth. Moreover, Figure 5 clearly shows the narrower T-$a_w$
374 combinations that allow for $AFB_1$ production compared to those that allow for growth. In
375 general, comparison with literature data showed that, despite these differences, growth
376 boundaries are much more similar among isolates than those for $AFB_1$ production.

377 The amount of mycotoxins produced by fungi has been demonstrated to be highly variable
378 among isolates (Garcia et al., 2011b; Yogendrarajah et al., 2016). On the contrary, boundaries
379 for toxin/no-toxin seemed to be variable, but much less than the quantity of toxin produced.
380 Thus, taking into account the high variability in the amount of $AFB_1$ produced by different
381 isolates, modelling of the probability of toxin production seems a suitable alternative.
382 Boundaries for $AFB_1$ production were narrower than those for growth, thus the possibility of
383 using growth/no-growth models for predicting $AFB_1$ production may lead to unnecessary
384 rejection measures. However, the food industry needs to appropriately address mycotoxin
385 risk, and this will be a "fail-safe" scenario. Recent studies on aflatoxin biosynthesis gene
386 expression have shown that the two regulatory genes (*aflR* and *aflS*) were highly expressed at
387 maximum and minimum $AFB_1$ production. Conversely the two structural genes (*aflD* and *aflO*)
388 were highly expressed only at maximum $AFB_1$ production (Gallo et al. 2016). By contrast, a
389 strong correlation between the relative expression of the *afl*R and *afl*S genes and the
390 concentration of AFs (Peromingo et al. 2017). Both studies built kinetic models for aflatoxin
391 gene expression, which could also be investigated for earlier prediction of aflatoxin
392 production.

393 Results from Figures 3 and 4 show the possibility of encountering some isolates with a better
394 ability to grow and produce $AFB_1$ than others. Thus, when developing predictive models,
395 several isolates should be taken into account to overcome this issue. Working separately with
396 a large number of isolates may be tedious, and some authors have investigated the possibility
397 of using a cocktail of isolates as an inoculum (Aldars-García et al., 2015; Garcia et al., 2014).
398 Garcia et al. (2014) compared the growth among 25 isolates separately and an inoculum with
399 the 25 isolates together, and they concluded that the best adapted isolated led the behaviour
400 of the pooled inoculum, which would be equivalent to working with the fastest isolate in a
401 worst scenario situation. This approach would not be useful for quantitative risk assessment.

402 In conclusion, the results of this work showed that under marginal conditions, the combined
403 effect of temperature and $a_w$ had different effects on the 20 tested isolates of *A. flavus* in
404 terms of growth and $AFB_1$ production responses. Contour plots for each isolate satisfactorily
405 predicted the response studied. However, to build better models, more information
406 accounting for a wider range of isolates from different climatic conditions is required.

407

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412

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576 FIGURE CAPTIONS

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

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

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

582 Figure 4. The predicted $AFB_1$/no $AFB_1$ production boundaries after 30 days with respect to $a_w$
583 and temperature at probabilities of 0.05 and 0.5 for the 20 *A. flavus* isolates studied.

584 Figure 5a. The predicted growth/no-growth boundaries at 0.05 probability of the twenty *A.*
585 *flavu*s isolates at day 30 were plotted together with literature data (28-30 days). Points
586 represent the literature data;●, 0.9 < Probability < 1; ● 0.2 < Probability < 0.9; ●, 0.05 <
587 Probability < 0.2; ○, P=0. Data extracted from: Aldars-García et al. ,2016b; Marín et al., 2012,
588 2009.

589 Figure 5b. The predicted $AFB_1$ production/no $AFB_1$ production boundaries at 0.05 probability of
590 the twenty isolates at day 30 were plotted (grey lines) together with literature data (days
591 between 21 and 30). Points represent the literature data; ●, $AFB_1$ detected in all studies; ●,
592 AFB1 detected in some studies and not detected in other; ○, $AFB_1$ not detected. Data
593 extracted from: Astoreca et al., 2014; Lahouar et al., 2016; Mohale et al., 2013; Mousa et al.,
594 2013. Blue lines represent the general upper and lower limit for the boundaries at 0.05 of the
595 20 isolates used in the present study.

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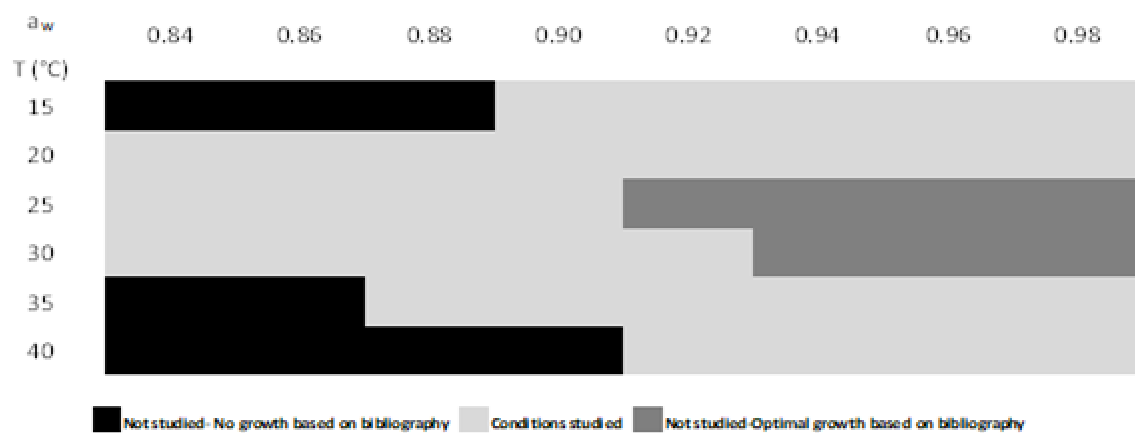


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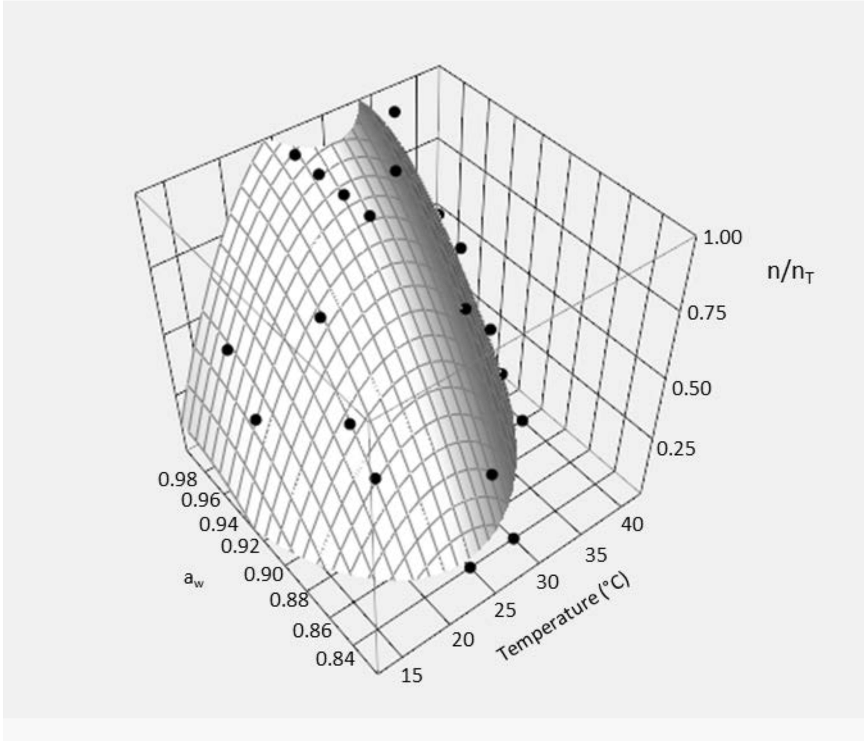


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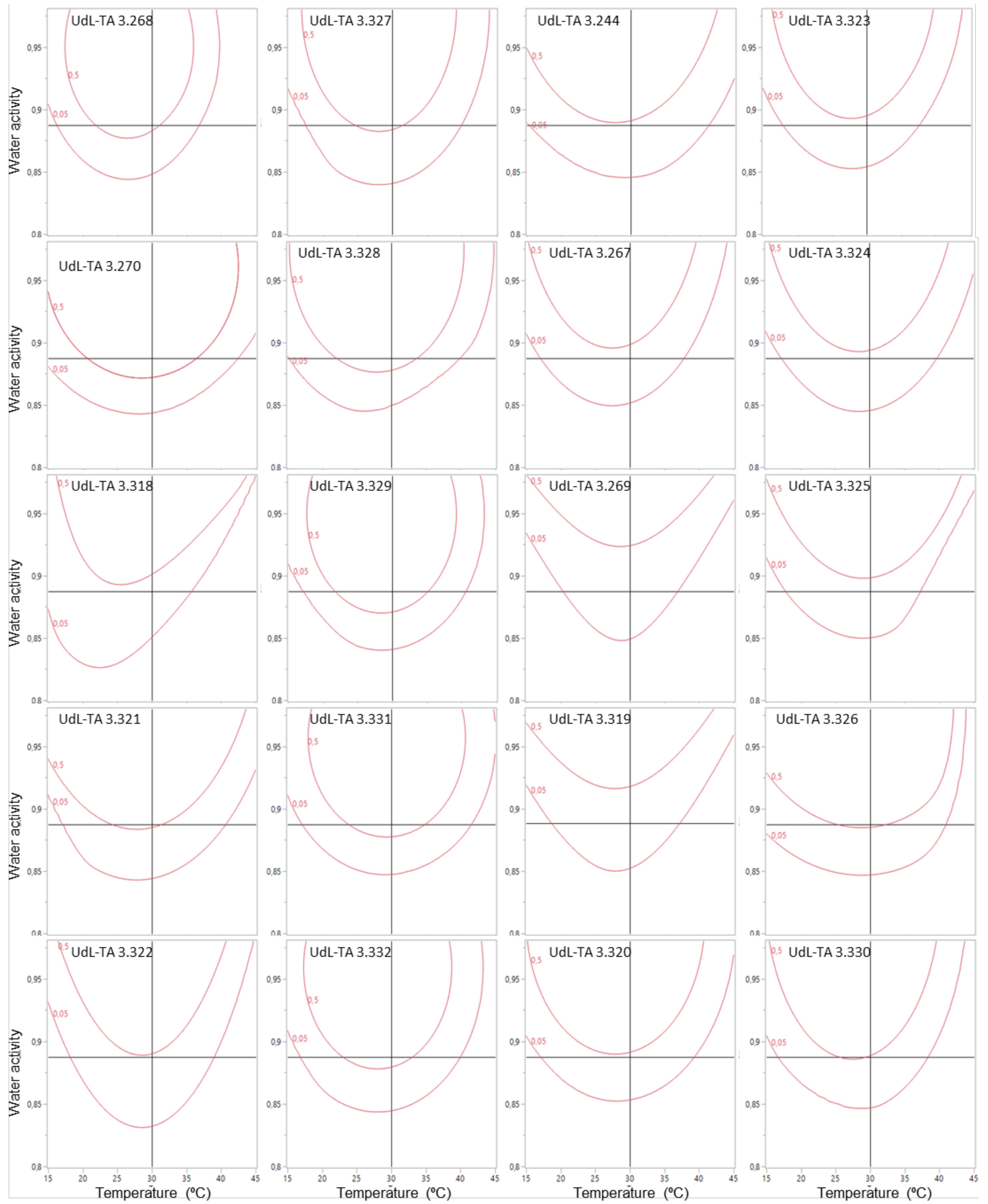


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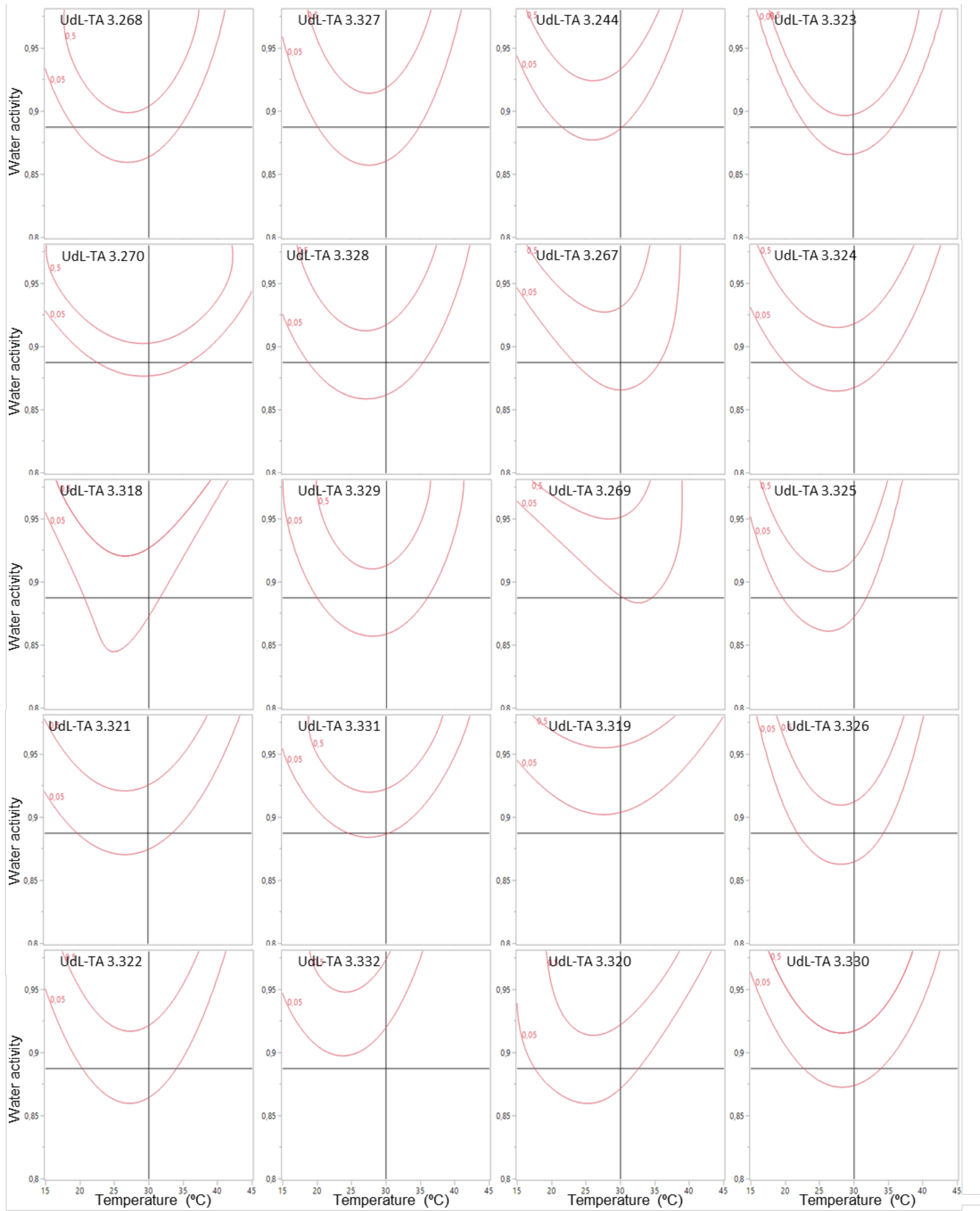


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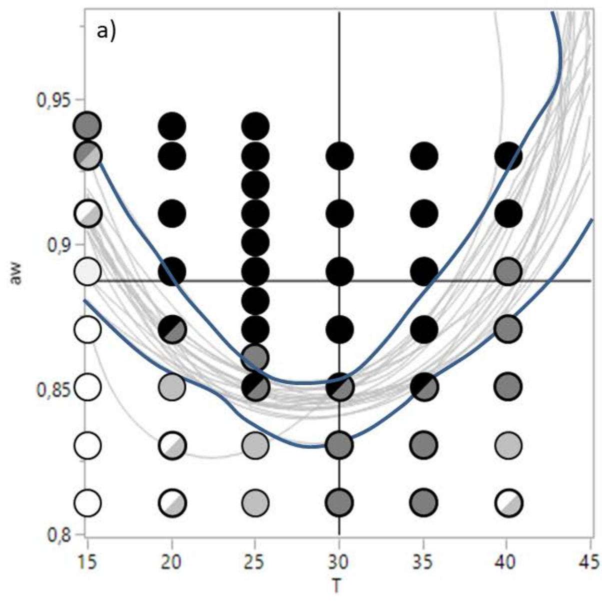


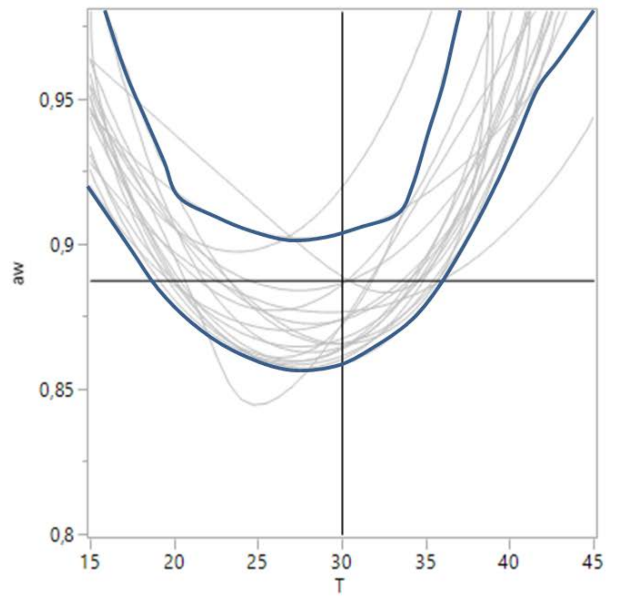


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