International Journal of Food Microbiology 333 (2020) 108777

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Modeling aflatoxin B_1 production by *Aspergillus flavus* during wheat malting for craft beer as a function of grains steeping degree, temperature and time of germination

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ARTICLE INFO

Keywords:
AFB_1
Model
Predictive mycology
Malted wheat
Artisanal beer

ABSTRACT

This study aimed to model the aflatoxin B_1 (AFB_1) production by *A. flavus* in wheat grains during malting for craft beer. A total of sixty-four different combinations of grains steeping degree (ST; 41, 43, 45 and 47%), temperature (13, 15, 17 and 19 °C) and time of germination (48, 72, 96 and 120 h), comprising the range of malting conditions that allow the production of quality malt, were assayed. AFB_1 was produced in a range of 15.78 ± 3.54 μg/kg (41% ST, 13 °C for 48 h) to 284.66 ± 44.34 μg/kg (47% ST, 19 °C for 120 h). The regression model showing an acceptable fit to the experimental data (adjusted R^2 0.84) for AFB_1 as a function of grains steeping degree, temperature and time of germination. Results showed that AFB_1 levels in wheat malt increase with increase of the temperature or time of germination. Within the range of tested malting conditions, no significant effects were observed for steeping degree on AFB_1 levels in wheat malt. The generated model is useful to estimate the AFB_1 levels in wheat malt. Findings highlight overall that if wheat grains are contaminated with *A. flavus*, AFB_1 might be produced in malt in levels above the limits set by regulatory agencies, regardless the steeping conditions used.

1. Introduction

Wheat craft beer is a non-filtered and unpasteurized alcoholic beverage characterized by the small-scale production using traditional beer brewing (Brewers Association, 2020a). Largely consumed worldwide, wheat beer is made with at least 50% malted wheat in addition to the water, barley malt, hops and yeast (Brewers Association, 2020b; Langos and Granvogl, 2016; Strong and England, 2015).

Wheat grains used to produce malt for craft beer can be infected by potentially toxigenic fungi in the field or during storage and mycotoxins can be produced if conditions are favorable (Neme and Mohammed, 2017). Studies of mycotoxins production during malting have primarily focused on barley and toxins produced by *Fusarium* species, which has been considered the main contaminant of this cereal (Jin et al., 2018a, 2018b; Mastanjević et al., 2018b). However, *Aspergillus flavus* producers of aflatoxins (AFs) have been isolated from several crops, including wheat grains (Al-Wadai et al., 2013; Riba et al., 2010; Schabo et al., 2020). AFs are harmful to humans and animals and the International Cancer Research Agency (IARC) recognizes particularly AFB_1 as a carcinogenic mycotoxin (Ostry et al., 2017).

In our previous study, we have demonstrated the production of AFs by an *A. flavus* strain isolated from wheat grains during standard conditions used for wheat malting (Schabo et al., 2020). The main concern regarding the production of AFs in the malt is their thermal stability (Raters and Matissek, 2008). Thus, once in the malt, AFs and particularly AFB_1 would not be destroyed during the brewing raising risks to human health by consumption of beer (Pascari et al., 2018).

Mathematical modeling has been used to predict the fungal growth in foodstuffs as a function of environmental conditions (Alonso et al., 2017; Nguyen Van Long et al., 2017; Sardella et al., 2018). Some

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https://doi.org/10.1016/j.ijfoodmicro.2020.108777
Received 21 March 2020; Received in revised form 5 June 2020; Accepted 29 June 2020
Available online 07 July 2020

studies reported *A. flavus* growth kinetics and presented the levels of aflatoxins produced in grains such as sorghum (Lahouar et al., 2016), paddy (Mousa et al., 2016), rice (Mousa et al., 2013) and peppercorns (Yogendrarajah et al., 2016). Mycotoxins production is rarely addressed directly because modeling has been applied to predict AFs production as a function of the *A. flavus* growth or biomass weight (Garcia et al., 2013; Mousa et al., 2016). However, a range of factors influences the fungal growth such as temperature, aeration, water activity, among others. Also, mycotoxins are produced by secondary metabolism pathways, thus growth models become a limitation because their production may occur under conditions that growth does not occur (Garcia et al., 2011; Marín et al., 2012; Pitt, 1993). Overall, from a food safety point of view, the main variable to be modeled is mycotoxin not fungal growth.

Steeping and germination are the key steps of malting. During the former, alternated controlled cycles of water immersion and aeration are applied to prepare the grains for germination (Pascari et al., 2019). Further, the germination induces the biochemical pathways for starch degradation, which is essential for the breakdown during mashing in the beer brewery (Kunze, 2004; Oliveira et al., 2012). As far as we know, no prior studies have focused on malt AFB_1 levels as a function of applied steeping and germination conditions for wheat malting used in brewing. Therefore, the present study was performed to model the produced AFB_1 levels by *A. flavus* in wheat grains during malting for craft beer as a function of grains steeping degree, temperature and time of germination, regardless of the fungal growth.

2. Material and methods

2.1. Raw material

Wheat (*Triticum aestivum* L.) grains (around 13 kg), purchased directly from a farmer in Terra Boa city, Paraná state, South Region of Brazil (23° 46′ 0.023″ S, 52° 26′ 52.12″ W) during the year 2018, were used for malting. Samples were tested for AFB_1, AFB_2, AFG_1 and AFG_2 by high performance liquid chromatography (HPLC), according to described in item 2.6, and certified as AFs-free. Before the experimental malting, grains were disinfected by 10-min immersion in 1% sodium hypochlorite and rinsed twice with sterile distilled water (Fiori et al., 2014).

2.2. Toxigenic fungal strain and growth condition

The toxigenic *A. flavus* CCDCA 11553 strain (AFB_1 and AFB_2 producer), belonging to the Microorganisms Culture Collection of the Food Science Department at Federal University of Lavras, CCDCA/WDCM1081, was used. This strain was originally isolated from wheat grains used in craft beer production, and has been shown previously to produce AFB_1 and AFB_2 during standard wheat malting conditions (Schabo et al., 2020).The strain is preserved in silica gel and stored at 4 °C (Perkins, 1962). *A. flavus* CCDCA 11553 was activated by place silica gel granules on the surface of malt extract agar (MEA) followed by incubation at 25 °C for 7 days.

2.3. Preparation of the fungal inoculum

To prepare the fungal inoculum, centrally inoculated MEA petri dishes with spores from the activated strain were incubated at 25 °C for 7 days to enable sporulation to take place. Spores were collected by scrapping using a mixture of sterile distilled water and 0.1% Tween 80 (Quimibras, Brazil). The suspension (~10 mL) was filtered through triple gauze to retain hyphae fragments (Wigmann et al., 2016) and the number of spores was verified using hemocytometer. A spore suspension of 10^6 spores/mL was prepared in a final volume of 200 mL for each combination of experimental conditions used for malting. This initial concentration was selected based on previous studies of fungal inoculation in cereal grains for malting (Habler et al., 2016; Hu et al., 2014; Pascari et al., 2019).

2.4. Experimental malting of wheat grains

A total of 64 different combinations of grains steeping degree (41, 43, 45 and 47%), temperature (13, 15, 17 and 19 °C) and time of germination (48, 72, 96 and 120 h), comprising the range of conditions ordinarily used during malting, were assayed. Sixty-four beakers were filled with 200 g of wheat grains previously disinfected. The inoculum (200 mL; 10^6 spores/mL) was added to the wheat grains. Wet and aeration periods were interspersed to obtain the expected steeping degree (ST, %) in a climate-controlled chamber (15 °C and 95–98% relative humidity). The 41% ST was achieved by immersion of the grains in the inoculum for 5 h, while the 43% ST was obtained by following a 19-h aeration period. To obtain the 45% ST, an additional 4-h wet steeping in sterile distilled water was needed. Finally, the 47% ST was reached after a subsequent 20-h aeration period.

For each ST sample, germination was subsequently performed for 48, 72, 96 or 120 h in 95–98% relative humidity climate-controlled chambers at 13, 15, 17 or 19 °C with twice daily turning to provide good aeration and to ensure a good germination yield. Sterile water (121 °C, 15 min) was sprayed each 12 h to avoid grain's surface dehydration. The green malt obtained for each combination of conditions was withered at 50 °C for 16 h, followed by kilning 1 h at 60 °C, 1 h at 70 °C, and finally 5 h at 80 °C according the malting standard procedures proposed by the Central European Commission of Brewing Analysis (MEBAK, 2017) for brewing. Rootlets were removed at the end of kilning. Samples (around 50 g each) of the obtained malt in each combination tested were collected following the sequence presented in Table 1. All procedures were performed in two distinct experiments.

2.5. Analysis of AFB_1 in wheat malt

Sample preparation, clean-up and determination of AFs by HPLC were performed according to Stroka et al. (2000). AFB_1 and AFB_2 analyses were performed using immunoaffinity clean-up columns considering the ability of the test strain to produce both AFs. Each sample (~50 g) was weighed and ground using a laboratory mill (IKA A11 basic, Sigma-Aldrich, St. Louis, USA). In each 12.5 g of sample was added 1.25 g of NaCl and 50 mL of the extraction solvent methanol: water solution (8:2, v/v) by blending for 30 min at high speed (10,000 rpm) using a horizontal shaker (New Brunswick Scientific Company, USA). Extracts were filtered through a quantitative filter paper (Nalgon, Germany), followed by another filtration through a glass microfiber filter (Vicam, Sweden) and 10 mL were diluted with 60 mL phosphate-buffered saline pH 7.0 (PBS). Diluted extracts were passed through immunoaffinity columns at a flow rate of 2–3 mL/min. Aflatoxin immunoaffinity columns (AflaTest-WB; Vicam, USA) were used for the clean-up of samples, strictly following the manufacturer's procedure. The column was then washed with 30 mL of distilled water and aflatoxins eluted with 1250 μL of methanol and 1750 μL of Milli-Q water. The eluate was filtered using a pre-cleaned PVDF syringe filter 13 mm × 0.22 μm (Pall Corporation, USA).

HPLC analyses were performed in an Agilent Model 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a fluorescence detector set at 362 nm (λ_{ex}) and 455 nm (λ_{em}) and a C18 column. Water: acetonitrile: methanol (6:2:3, v/v/v), with 119 mg of potassium bromide (KBr) and 350 μL of 4 M nitric acid per liter was used as mobile phase at a flow rate of 1 mL/min. The injection volume was 20 μL for both, standard solution (AFB_1, B_2, G_1 and G_2) and sample extracts, for 8-min total run time. An isocratic and reverse phase system associated with a Kobra Cell electrochemical reactor (R-Biopharm, Darmstadt, Germany) connected to a current of 100 μA for post-column derivatization of aflatoxins B_1 and G_1 was used. The limits of detection (LOD) and quantification (LOQ) for AFB_1 and AFB_2 were

Table 1
Aflatoxins (μg/kg) produced (mean ± standard deviation) by *A. flavus* during malting process at the different combinations of steeping degree, temperature and time for germination of grains.

Grain steeping degree (%)	Grain germination temperature (°C)	Grain germination time (h)			
		48	72	96	120
41	13	15.78 ± 3.54	42.58 ± 4.00	115.17 ± 32.09	145.42 ± 31.30
41	15	28.91 ± 0.22	86.10 ± 34.39	149.41 ± 24.79	133.08 ± 24.92
41	17	32.14 ± 1.75	95.39 ± 24.48	137.80 ± 55.57	178.95 ± 22.49
41	19	34.83 ± 0.97	100.52 ± 12.26	144.03 ± 19.24	257.81 ± 43.49
43	13	24.17 ± 1.08	36.72 ± 2.70	122.17 ± 31.05	137.14 ± 48.01
43	15	37.90 ± 0.06	25.80 ± 1.19	128.96 ± 18.73	143.54 ± 38.95
43	17	30.76 ± 0.31	99.45 ± 7.25	129.38 ± 38.80	164.33 ± 45.93
43	19	32.60 ± 3.30	129.65 ± 2.11	110.56 ± 30.75	145.89 ± 27.89
45	13	22.66 ± 7.91	34.85 ± 0.76	129.77 ± 37.87	133.17 ± 25.98
45	15	26.05 ± 0.10	87.45 ± 29.98	134.56 ± 24.16	132.06 ± 12.77
45	17	26.18 ± 4.11	90.58 ± 9.03	131.14 ± 45.98	139.82 ± 34.60
45	19	26.29 ± 2.06	99.52 ± 3.75	132.67 ± 26.29	202.21 ± 40.33
47	13	44.09 ± 0.69	26.01 ± 7.42	122.93 ± 10.82	131.97 ± 8.89
47	15	40.26 ± 0.69	44.44 ± 2.49	125.85 ± 43.44	140.02 ± 0.90
47	17	40.85 ± 7.21	126.50 ± 45.58	103.19 ± 11.97	155.55 ± 2.99
47	19	54.30 ± 7.89	111.52 ± 29.01	126.78 ± 1.98	284.66 ± 44.34

Table 2
Full and reduced models for the effects of steeping degree, temperature and time of germination on AFB_1 production[b] by *Aspergillus flavus* during wheat malting.

	Full model		Reduced model	
R^2	0.841428		0.841361	
$R^2_{adjusted}$	0.833499		0.836160	
F value	106.125473		161.760549	
RMSE	1.259		1.249	
No. of variables	64		64	
Variable[a]	Coefficient	*p*-Value	Coefficient	*p*-Value
Intercept	−5.290906	0.118062	−4.799002	0.000240
Steeping degree	0.011180	0.874366	–	–
Temperature of grain germination	0.371135	0.000002	0.371135	0.000002
Time of grain germination	0.100009	1.13E−24	0.100009	4.67E-25

[a] S, steeping degree (%); T, germination temperature (°C); t, germination time (h).
[b] Where sqrt(AFB_1) is the modeled variable.

determined following procedures of the Eurachem Guide (Magnusson and Örnemark, 2014). LOD and LOQ were 0.03 and 0.09 μg/kg for AFB_1 and 0.02 and 0.07 μg/kg for AFB_2. The concentrations of AFs in each sample collected during malting were presented in "dry basis", considering the moisture content of samples, which was determined by official gravimetric analysis (method 925.45b; AOAC, 2016).

2.6. Modeling of AFB_1 levels in wheat malt

Only AFB_1 levels were considered in modeling because of its importance to human and animal health. Determination of AFB_1 in wheat malt led to a data set, experimentally collected, of 64 entries. Each entry contains a combination of predictor variables (steeping degree, temperature and time of germination) and the corresponding value of the response variable (AFB_1 in μg/kg; Table 1). The square root transformation (sqrt) showed more uniform residuals relative to other transformations (data not shown). Transformed data were fitted to a multiple linear regression (MLR) model using the R programming language (R Core Team, 2019). The MLR equation was as follow:

$$\text{sqrt}(\gamma) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n \quad (1)$$

where γ is the level of AFB_1 produced, x_1 is the steeping degree (ST), x_2 is the temperature (T) and x_3 is the time (t) of grains germination) and β_n represents any of the corresponding coefficients. A *p*-value < 0.05 was needed for a parameter to be considered significant. The goodness of fit of the developed model was evaluated graphically, using Root Mean Square Error (RMSE) and the adjusted R^2. Parameters, their quadratic terms, or their interaction were retained in the model when statistically significant.

The equation for the MLR model considering only significant parameters was:

$$\text{sqrt}(\gamma) = \beta_0 + \beta_1 T + \beta_2 t \quad (2)$$

and was used to estimate the AFB_1 production by *A. flavus* during wheat malting as a function of grain steeping degree, temperature and time of germination.

3. Results and discussion

3.1. Modeling AFB_1 in wheat malt under tested malting conditions

AFB_1 concentration, produced by *A. flavus* in wheat malt samples was determined to assess the effects of currently proposed malting conditions and to create a data set of the response variable (AFB_1) for regression modeling (Table 1).

AFB_1 was detected in wheat malt obtained from all combinations of grains steeping degree, temperature and time of germination tested (Table 1). The levels of AFB_1 in wheat malt ranged from 15.78 ± 3.54 μg/kg (41% ST grains germinated at 13 °C for 48 h) to 284.66 ± 44.34 μg/kg (47% ST grains germinated at 19 °C for 120 h) (Table 1). These results show that all proposed conditions for wheat malting favored *A. flavus* production of AFB_1 above the maximum limits determined by the regulatory agencies. The European Commission recommends a maximum AFB_1 level of 2 μg/kg in all cereals and derived products (except maize, to be subjected to sorting or other physical treatment before human consumption) (European Commission, 2010). The Brazilian National Agency of Public Health Vigilance (ANVISA-Brazil) recommends 5 μg/kg as the limit for total AFs in cereals and cereal products such as malt (except maize and derivatives) but does not define the limit for AFB_1 levels yet (Brasil, 2011). It is worthy to mention that the recommended standard malting conditions (45% ST at 15 °C for 96 h) (MEBAK, 2017) are among the combinations tested.

Considering that the range tested of each parameter was defined based on realistic and feasible conditions to obtain a quality malt (Faltermaier et al., 2015; Mastanjević et al., 2018a), there was no ST condition considered safe to avoid AFB_1 production, or the possibility to

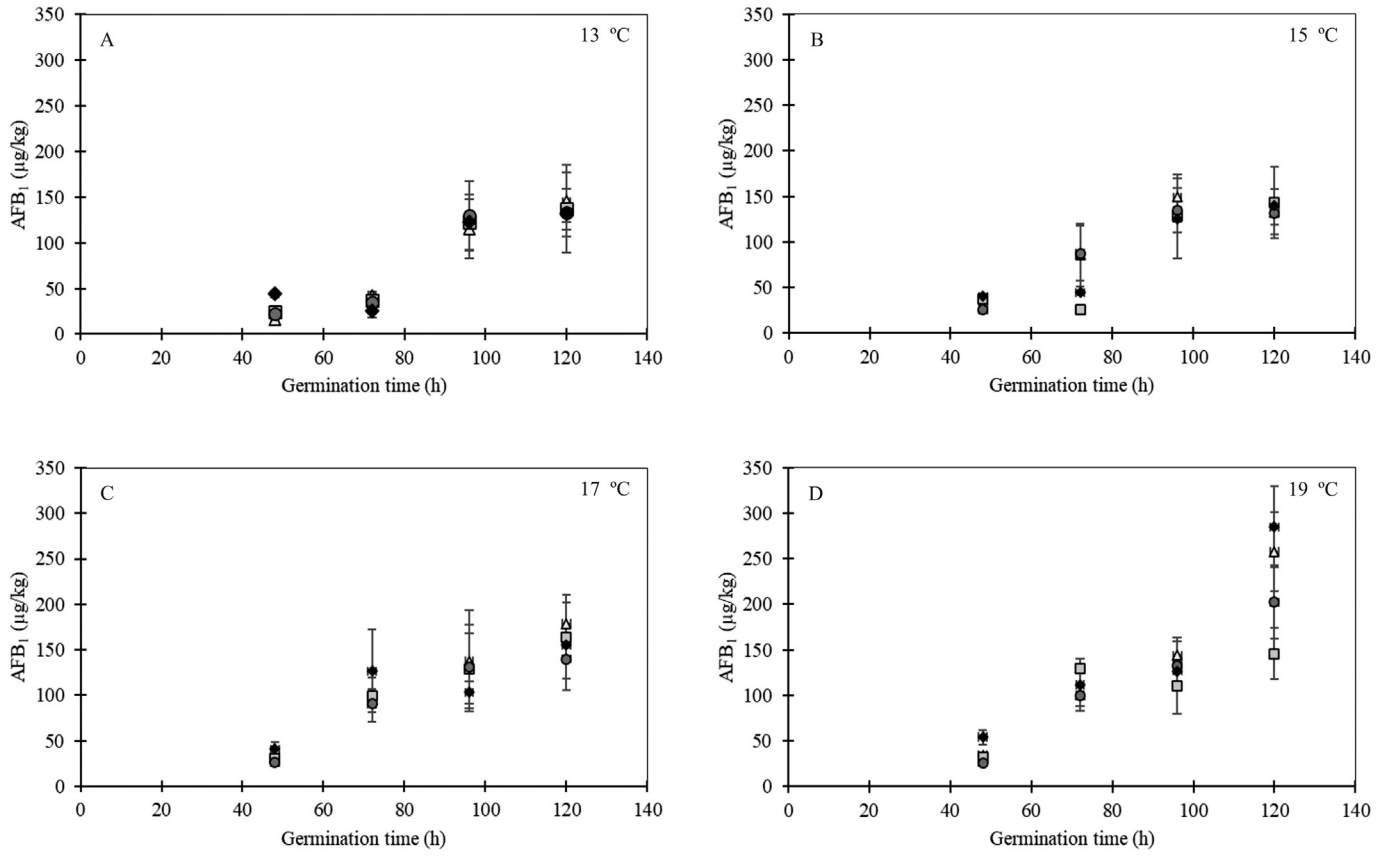

Fig. 1. Effects of distinct combinations of temperature and time of germination on AFB_1 production by *A. flavus* during wheat malting at distinct steeping degree conditions; (△) 41%, (□) 43%, (●) 45%, (◆) 47%.

establish a malting combination of parameters for production of free-AFB_1 malt for craft beer. Even the lowest ST tested (41%) provides a high moisture content in the grains, which is known to favor the mycotoxin production (Neme and Mohammed, 2017; Schabo et al., 2020). However, we opted to include the ST as a predictor because steeping is performed at 15 °C, a temperature below that considered in the range of AF production (~33 °C) for the > 34% moisture content (Milani, 2013). As observed, at an unfavorable temperature AFB_1 is produced in high moisture content grains.

The regression model showed relatively high-adjusted R^2 value (0.84) considering that AFB_1 is produced through secondary metabolism and maybe not related to growth metabolism (Table 2). The RMSE of 1.25 showed overall that the models fits well. The time of grains germination (t) had a highly significant ($p = 4.67E-25$) effect on the AFB_1 levels. The temperature of grains germination (T) also was significant ($p = 0.000002$), while ST did not have significant effects on AFB_1 levels in wheat malt (Table 2). The final model describing the AFB_1 production during wheat malting was:

$$\text{sqrt}\,(AFB_1) = -4.799002 + 0.371135T + 0.100009t\ (R^2_{adj}\,0.84) \quad (3)$$

The lack of significance observed for ST on the AFB_1 production within the range experimentally tested can be explained by the high significance of the temperature and time of grains germination (Table 2). In other words, since AFB_1 is produced in all ST conditions tested, the temperature and primarily the time of grains germination will define the levels in malt. Our model cannot predict if a sharp decrease in the ST (< 34%) of grains would result in lower AFB_1 levels in the malt, however in a practical point of view, it will not be relevant because the lowest level tested (41%) is the minimum moisture content needed for grains to germinate (Pascari et al., 2019).

During the wheat malting experiments, germination temperature was investigated over a range of 13 to 19 °C. No experiments at temperatures < 13 and > 19 °C were performed because it would extrapolate the conditions required for malting considering the quality parameters (Faltermaier et al., 2015; Jin et al., 2018a, 2018b; Mastanjević et al., 2018a). Fig. 1 shows AFB_1 concentrations within this range of temperature. An increase in temperature led to an increase in AFB_1. These are interesting results because all temperatures tested during germination were below the optimal temperature of AFs production (and fungal growth), despite a previous report of AF production at 15 °C on paddy (Mousa et al., 2011). However, the fact that germination occurred under controlled relative humidity (95–98%) probably favored the AFB_1 production in wheat grains.

In all tested experimental conditions, AFB_1 increased with the increase of germination time (Fig. 1). Based on the coefficient estimated and on their *p*-value (Eq. 3; Table 2), modeling the AFB_1 levels as a function of the germination time is more accurate than as a function of the germination temperature. In other words, the correlation between AFB_1 levels in malt and germination time is stronger than the correlation with the germination temperature of wheat grains (Table 2; Eq. 3). These results suggest that the time of germination of grains markedly affected AFB_1 levels in the wheat malt (Table 2). The longer the germination time, the greater the mycotoxin production (Fig. 1). A prior study suggested that a shorter germination period of barley grains during malting might reduce the formation of *Fusarium* toxins (Habler et al., 2016).

Therefore, within the range of conditions analyzed during the experimental wheat malting process, it can be predicted that the levels of AFB_1 in wheat malt increases with the increase of the temperature and time of germination. Considering that AFB_1 was produced in all tested ST conditions, the lowest germination temperature and the shortest time of grains germination would be applied, the lowest levels of AFB_1

would be in wheat malt. Further studies focusing on the transfer of AFB_1 from wheat malt to wheat beer during brewing would help to and evaluate the risk of beer contamination with AFB_1 through contaminated wheat grains.

4. Conclusions

AFB_1 was produced by *A. flavus* during malting under all combinations of grains steeping degree, temperature and time of germination tested. Levels of AFB_1 detected in all wheat malt samples were above the limits set by regulatory agencies for cereals and derived products. The regression model has an acceptable fit to the experimental data and showed that increased germination temperature and time generally result in increased AFB_1 levels in wheat malt. Steeping degree did not significantly affect AFB_1 levels in wheat malt within the range of conditions tested. The model can serve as a useful tool to estimate AFB_1 in wheat malt for use in craft beer. Our findings warn that if aflatoxigenic *A. flavus* species contaminate wheat grains for use in malting for craft beer, there are no realistic malting conditions which will prevent toxin formation.

Acknowledgments

Authors would like to thank the “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (Grants # CNPq, Grants #303437/2017-0 and #405644/2018-3) and “Coordenação de Aconselhamento de Pessoal de Nível Superior” (CAPES-Brazil; UFPB/CAPES/PrInt 88881.311776/2018-01 and PDSE Grant 88881.187242/2018-01).

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