Wageningen Academic Publishers

Optimisation of aflatoxin B_1 reduction in pistachio nuts by kefir grains using statistical experimental methods

F. Ansari[1,2], K. Rezaei[3], F. Khodaiyan[1*] and A. Rahmani[2]

[1]Bioprocess and Biodetection Laboratory (BBL), Department of Food Science, Engineering and Technology, University of Tehran, 31587-77871 Karaj, Iran; [2]Department of Food Science and Technology, Standard Research Institute (SRI), 31745-139 Karaj, Iran; [3]Department of Food Science, Engineering and Technology, University of Tehran, 31587-77871 Karaj, Iran; khodaiyan@ut.ac.ir

Received: 3 March 2015 / Accepted: 12 July 2015

RESEARCH ARTICLE

Abstract

The presence of aflatoxin B_1 (AFB_1) in pistachio nuts has a serious impact on public health. Kefir grains (KGs) contain several probiotic microorganisms well known for their aflatoxin decontamination effects. In this study, the capacity of KG to remove AFB_1 from pistachio nuts was investigated and the detoxification method was optimised using certain statistical approaches. Five variables (toxin concentration, KG level, contact time, incubation temperature and pre-treatment heat level) were considered in the study. Pistachio samples were spiked with 5, 10, 15, 20 and 25 ng/g AFB_1 stock solutions and then treated with 5, 10, 15, 20 and 25% (w/w, based on the amount of pistachio paste) KG already heated at 70 and 110 °C. AFB_1 concentrations in the samples were determined using high-performance liquid chromatography. Results of the study indicated that: (1) KG caused significant decrease in AFB_1; (2) cell viability of KG was not necessary for its decontamination effect; (3) the amount of AFB_1 reduction depended on toxin concentration and contact time; and (4) the interactions between toxin concentration and KG level, toxin concentration and contact time as well as incubation temperature and contact time had significant effects on AFB_1 reduction. The optimum conditions for the highest AFB_1 reduction (96%, compared to the control sample) included a 20 ng/g toxin concentration, 20% KG level, 6 h contact time and 30 °C incubation temperature. To verify the findings of this study, the efficiency of KG needs to be confirmed *in vivo*.

Keywords: *Aspergillus flavus, Aspergillus parasiticus*, detoxification, mycotoxin

1. Introduction

Aflatoxins (AFs) consist of a large group of extremely toxic components that are produced by certain species of fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (Pereyra *et al.*, 2010) and are found in fungal-contaminated nuts (Wilson and Ryne, 1993). These microorganisms have been considered to be responsible for significant problems such as severe liver damage as well as mutagenic and carcinogenic effects (Li *et al.*, 2001; Wild and Turner, 2002). Among the four common AFs (B_1, B_2, G_1 and G_2), AFB_1 is the most widespread and the most poisonous AF that is categorised as group 1 human carcinogen (IARC, 1993; JECFA, 1998). Pistachio nuts are one of the most important commodities with the highest risk of AFB_1 contamination. Pistachio is cultivated in different regions of the world and provides economic benefits for many countries including Iran, the largest pistachio producer (FAO, 2012; Zheng, 2011). Natural occurrence of AFB_1 in pistachio nuts has been reported in different countries. In Qatar, 27.7% of 81 pistachio nut samples were found contaminated with AFs at >20 ng/g (Abdulkadar *et al.*, 2000). Set and Erkman (2010) reported that 50.5% (48 samples from the total of 95 samples) of unpacked pistachio nuts in Turkey during the years 2008-2009 were contaminated with AFB_1 at levels ranging from 0.007 to 7.72 ng/g. Similar levels of contamination were reported for total AF. Also, according to a report from Iran, during the years 2009-2011, 6.8% of pistachio nut sub-samples (556 from the total of 8,203 samples) were contaminated with AFB_1 levels higher

than the maximum level allowed for AFB_1 in Iran (5 ng/g) (Dini *et al.*, 2013).

According to EU legislation, contaminant levels shall be kept as low as can reasonably be achieved by following good practice at all the stages (EC, 1993). In this regard, several treatments including hydrogen peroxide, γ-radiation, ozone, sodium hydroxide, sodium hypochlorite, electrolysed NaCl anode and acidic electrolysed oxidising water have been applied developed to inactivate AFs (McKenzie *et al.*, 1997; Mukendi *et al.*, 1991; Patel *et al.*, 1989; Suzuki *et al.*, 2002; Zhang *et al.*, 2012). Most of these treatments involve certain difficulties such as a need for expensive equipment, high energy consumption and loss of product quality. In addition, the undesirable health impacts of these methods have not been fully evaluated (Phillips *et al.*, 1994; Samarajeewa *et al.*, 1990). Therefore, it is necessary to seek for some methods that are safe, effective and energy-saving to deactivate/remove AFs from foodstuffs. Use of certain bio-based approaches is preferred for such treatments using different bacteria mainly from the family of lactic acid bacteria (Bueno *et al.*, 2007; El-Nezami *et al.*, 1998; Gratz *et al.*, 2007; Haskard *et al.*, 1998, 2001; Hathout *et al.*, 2011; Hernandez-Mendoza *et al.*, 2009; Lee *et al.*, 2003; Peltonen *et al.*, 2001; Zuo *et al.*, 2013). Some strains of *Saccharomyces cerevisiae* were also reported as potential candidates to decrease AFB_1 (Bueno *et al.*, 2007; Devegowda *et al.*, 1996; Madrigal-Santillán *et al.*, 2006; Pizzolitto *et al.*, 2011, 2012; Rahaie *et al.*, 2010; Raju and Devegowda, 2000; Shetty *et al.*, 2007; Slizewska *et al.*, 2010). These microorganisms are capable of binding AFB_1 in liquid media involving the formation of a reversible complex between the toxin and microorganism surface (Bueno *et al.*, 2007; Lee *et al.*, 2003).

Kefir grains (KGs) are formed by the symbiotic association of lactic acid bacteria and/or yeasts containing probiotic microorganisms. A number of studies have utilised such microorganisms (*Lactobacillus plantarum, Lactobacillus casei* and *S. cerevisiae*) for decontamination of AFB_1 (Kakisu *et al.*, 2013; Leite *et al.*, 2012; Zhou *et al.*, 2009). Ismaiel *et al.* (2011) showed that KGs possess certain antimicrobial activities against *A. flavus* AH3 and consecutive repression of AF production. The reduction of AFB_1 in different food commodities is of the highest interest for human safety. To the best of authors' knowledge, no studies have so far been reported on the possibility of AFB_1 decontamination by KGs. Therefore, the objectives of this study were to evaluate the capacity of KGs to remove/deactivate AFB_1 from pistachio nuts. The effects of toxin concentration, KG level, contact time, incubation temperature and pre-treatment heat level on the reduction of AFB_1 were investigated using full factorial design followed by response surface methodology (RSM).

2. Materials and methods

Sample preparation

Dehulled, undamaged and mature dry pistachio nuts (Ahmad-aghaee cultivar from Kerman, Iran) were used to investigate the deactivation treatments in the current study. Preparation of subsamples for extraction, analysis and further analytical experiment were carried out at the Mycotoxin Laboratory located in the Department of Food Science and Technology at the Standard Research Institute (Karaj, Iran) and Faroogh Scientific Research Laboratory (Tehran, Iran).

To minimise the subsampling errors in the study, samples were mixed with water in a 1:1 (w:w) ratio and ground using a slurry machine to provide a uniform paste based on a standard method from ISIRI (2011). A 50-g aliquot of this paste was used for each experiment and analysis was performed by using a high-performance liquid chromatography (HPLC).

Activation and maintenance of kefir grains

KGs were obtained from Department of Food Science, Engineering and Technology (University of Tehran, Tehran, Iran). In the laboratory, they were propagated in pasteurised skimmed cow's milk at 23±2 °C for 24 h. To maintain grain viability, they were retrieved by sieving, re-inoculated into fresh milk and incubated under the same conditions on a daily basis (Tramšek and Goršek, 2007). Grains were then maintained at 4 °C for a short period of time (up to one week) before use in the treatments (Liu and Lin, 2000).

Preparation of kefir grains

For homogenisation of the KGs, 2.5, 5.0, 7.5, 10.0 and 12.5 g (respectively, equal to 5, 10, 15, 20 and 25% in pistachio paste samples) were ground and mixed with equal amounts of water using an appropriate high-speed blender (minimum 6,000 rpm). KGs were treated in three levels (N = non-heated at room temperature; H = heated at 70±2 °C using a controlled hot plate for 5-10 min; and U = heated at 110±2 °C using an autoclave for 10 min).

Sample treatment with kefir grains

According to the matrix design, to prepare contaminated samples, each pistachio paste portion (50 g) was thoroughly contaminated with working solution of AFB_1 to reach 5, 10, 15, 20 and 25 ng/g concentrations of AFB_1. The contaminated samples were then inoculated with 2.5, 5.0, 7.5, 10.0 and 12.5 g (w/w) of KGs, shaken and incubated at different temperature levels (20, 30, 40, 50 and 60 °C) for 0, 2, 4, 6 and 8 h while other parameters were maintained constant.

Chemicals and reagents

Standard solution of AFB_1 was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Methanol, *n*-hexane, acetonitrile, sodium chloride, potassium bromide, nitric acid were from Merck Chemical Company (Darmstadt, Germany). To produce phosphate-buffered saline, 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 and 8.0 g NaCl were dissolved in 900 ml water and the pH was adjusted to 7.4 using 0.1 M HCl or 0.1 M NaOH (as needed) and diluted (with water) to 1000 ml.

Extraction of residual aflatoxin B_1

For the extraction purposes, the test portions (25 g sample + 45 ml water) were combined with 180 ml of methanol and 50 ml n-hexane and shaken vigorously (at 8,000 rpm) for 3-5 min and then diluted by 130 ml water before being filtered through a glass microfiber filter. Aflatest immuno-affinity column (IAC; Faroogh Scientific and Research Laboratory, Theran, Iran) was used to clean up the samples. First, 10 ml of phosphate buffer saline was passed through the column to activate it for use in the subsequent stages and then 75 ml of the filtrate passed through at a rate of 1 drop/s. The column was then washed using 15 ml water and dried by applying vacuum for 10 s. Finally, elution of the column was performed using methanol in two steps. First, 500 µl methanol was applied on IAC and allowed to pass through by gravity and then 1000 µl additional methanol was poured on the column after one min and the eluate was collected in a vial. The eluate was finally diluted using 1,500 µl water before being analysed by HPLC (AOAC, 2000; ISIRI, 2011). Recovery of the method was determined for each representative commodity and those lying within 60-120% recovery were accepted (Fajgelj and Ambrus, 2000).

HPLC procedure

An HPLC system (Waters 2695; Waters Corporation, Milford, MA, USA) using post column derivatisation (PCD) involving bromination was applied to determine AFB_1 (AOAC, 2000; ISIRI, 2011). The HPLC system was equipped with a pump, a Waters 2475 multi-fluorescence detector and a Chromolit reversed-phase C18 analytical column (Merck Chemical Company; 20 cm × 4.6 mm × 4 µm). An electrochemical PCD system was applied using Farlib® EDC cell (Faroogh Scientific and Research Laboratory) for bromination purposes. 100 µl of each sample was injected into the HPLC system. Mobile phase was prepared by mixing water (600 ml), methanol (300 ml) and acetonitrile (200 ml) and adding 350 µl nitric acid (4 M) and 120 mg potassium bromide. All solvents were HPLC-grade. The flow rate was set at 2.5 ml/min. The detector was operated at 365 and 435 nm as excitation and emission wavelengths, respectively. For quantification purposes, a seven-point calibration curve was obtained on a daily basis using working standard solutions at different concentrations (0.4-7.2 ng/g). The limit of detection for AFB_1 determination was obtained at 0.1 ng/g.

Experimental design and statistical analysis

Screening experiments to select main factors were performed with combination of five variables (toxin concentration, KG level, contact time, incubation temperature and pre-treatment heat level) by use of a general full factorial design (using a first order model). Data analysis was carried out by the analysis of variance (ANOVA) combined with Tukey's mean comparison tests and step by step multiple regressions using the R statistical package (version R-3.0.3; https://cran.r-project.org/) at $P<0.05$.

A central composite design using RSM with four coded levels (main factors) was used to optimise significant factors. The results of the central composite design were fitted into a second-order polynomial equation by a multiple regression analysis. The fit of the polynomial model was expressed by the coefficients of determination R^2.

3. Results and discussion

Screening the significant factors for experimental design

According to the results of a preliminary study, pistachio nuts treated with KG showed a positive effect on the removal of AFB_1 ($P=3.05\times10^{-5}$) (Figure 1). Then, a full factorial design using four parametric variables (toxin concentration (X_1), KG level (X_2), contact time (X_3) and

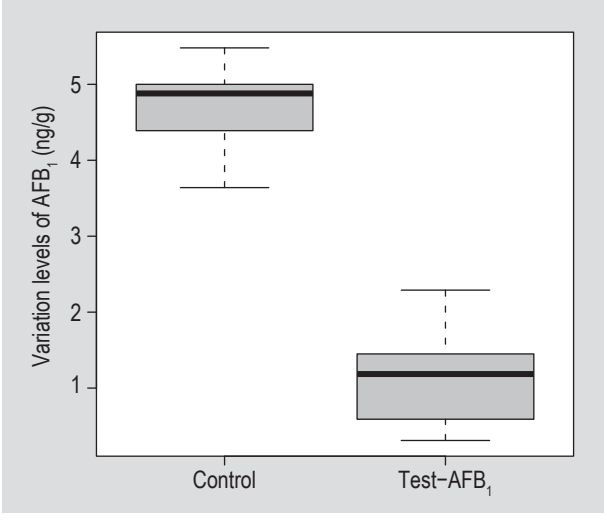

Figure 1. Range of variations in the preliminary study of the current work for the investigation of the effect of kefir grain (KG) on the reduction of aflatoxin B_1 (AFB_1). Control = samples with no KG treatment; Test = KG-treated samples ($P=3.05\times10^{-5}$).

incubation temperature (X_4)) and one non-parametric variable (pre-treatment heat level (X_5)) was applied to identify the main parameters (i.e. those having significant effects on the removal of AFB_1). Several stages were used to carry out the analysis of the data. Table 1 shows the results for the ANOVA based on a first order model. According to the results, KG (P<0.05), contact time (P<0.05), incubation temperature (P<0.01) and pre-treatment heat level (P<0.001) positively influence AFB_1 reduction. Then, means of the variables (X_1, X_2, X_3, X_4 in two levels and X_5 in three levels: N, H, U) affecting AFB_1 were compared against each other using Tukey's multiple comparisons of the means (Table 2). The statistical test factor, P-value, was used to evaluate the significance of the models at the 95% confidence level. The results indicated that there were significant differences between the two levels of KG, contact time and incubation temperature as well as the three levels of heating treatments. Further analysis using the coefficient estimates in the full factorial design (Table 3) indicated that the first-order model was significant and only 2.04% of the total variation was not explained by the model (R^2=97.96%). The value of adjusted coefficient of determination was also high enough (adjusted R^2=97.09%) to confirm the high significance of the model (Myers and Montgomery, 2002). Therefore, considering the above justifications, the first-order model for AFB_1 reduction was approved (Table 3). AFB_1 reduction determined for the first order model is shown in Equation 1:

$$Y = 50.46 + 0.62\,X_1 + 1.38\,X_2 - 0.75\,X_3 - 96.32\,X_{5N} + 6.39\,X_{5N}X_3 \quad (1)$$

where Y is the experimental response of AFB_1 reduction and X_1 is the toxin concentration, X_2 is the KG level, X_3 the contact time, and X_{5N} is the pre-treatment heat level at non-heated condition.

Table 3 and Equation 1 show that the main first-order factors (X_1, X_2, X_3) and X_{5N} and also the interactions of X_3 with X_{5N} have the most pronounced effects on AFB_1 reduction. Therefore, the main factors along with incubation temperature (X_4) obtained from ANOVA analysis in the screening test were used for further optimisation steps.

Comparing the means among the studied variables (Table 2) indicates that there were significant differences between the three levels of heating treatments on KGs (N, H and U) at 95% confidence level with the highest efficiency of AFB_1 reduction at H and U conditions and the lowest efficiency for the non-heated KG (N). However, using the data obtained for the coefficient estimates (Table 3) shows that there were significant differences (99.9% confidence level) between the AFB_1 levels in the non-heated grains (N) and grains heated at H level. These results are in good agreement with those of El-Nezami *et al.* (1998), Lee *et al.* (2003), Oatley *et al.* (2000), Rahaie *et al.* (2010), and

Table 1. Analysis of variance and coefficient estimates in the first-order model.

Variable	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value
Toxin concentration	1	59	59	1.39	0.244
Kefir grain level	1	276	276	6.53	0.014
Contact time	1	254	254	6.01	0.018
Incubation temperature	1	437	437	10.34	0.002
Pre-treatment heat[1]	2	44,714	22,357	529.92	<0.001

[1] Levels: H = 70±2 °C; U = 110±2 °C; and N = non-heated at room temperature.

Table 2. Multiple comparisons of the means for the variables in the first-order model.

Variable	Comparison	Differences	Lower	Upper	Adjusted-*P*
Toxin concentration	10-20 (ng/g)	2.21	-1.57	6.00	0.244
Kefir grain level	5-10 (g)	4.79	1.00	8.58	0.014*
Contact time	2-4 (h)	4.59	0.81	8.38	0.018*
Incubation temperature	30-40 (°C)	6.03	2.24	9.81	0.002*
Pre-treatment heat[1]	H-N	-61.38	-66.96	-55.79	<0.001*
	H-U	6.27	0.68	11.85	0.024*
	N-U	67.65	62.06	73.23	<0.001*

**P*<0.05.
[1] Levels: H = 70±2 °C; U = 110±2 °C; and N = non-heated at room temperature.

Table 3. Coefficient estimates in the first-order model.

Variable	Estimate	Standard error	t-value	*P*-value
Intercept	50.46	11.88	4.24	<0.001
Toxin concentration (X_1)	0.62	0.27	2.30	0.02
Kefir grain level (X_2)	1.38	0.54	2.56	0.01
Contact time (X_3)	-0.75	1.35	-0.55	0.58
Incubation temperature (X_4)	0.45	0.27	1.66	0.10
Pre-treatment heat-U (X_{5U})	25.52	16.80	1.51	0.13
Pre-treatment heat-N (X_{5N})[1]	-96.32	16.80	-5.73	<0.001
Interaction effects				
X_1X_{5N}	-0.65	0.38	-1.71	0.09
X_1X_{5U}	-0.54	0.38	-1.43	0.16
$X_{5N}X_2$	0.04	0.76	0.06	0.95
$X_{5U}X_2$	-1.33	0.76	-1.74	0.09
$X_{5N}X_3$	6.39	1.91	3.33	<0.01
$X_{5U}X_3$	2.77	1.91	1.44	0.15
$X_{5N}X_4$	0.72	0.38	1.88	0.06
$X_{5U}X_4$	-0.26	0.38	-0.69	0.49

[1] Levels: H = 70±2 °C; U = 110±2 °C; and N = non-heated at room temperature.
R^2=97.96%; Adj R^2=97.09%; $P<2.2\times10^{-16}$.

Shetty *et al.* (2007), who reported that heat-treatment of microorganisms enhance their AFB_1 binding abilities at 20-90%. In agreement with the above reports, Bueno *et al.* (2007), Haskard *et al.* (2001), Pizzolitto *et al.* (2011) and Topcu *et al.* (2010) reported no significant differences between the viable and non-viable microorganisms in eliminating AFB_1. Considering the above findings, KGs were heated under H conditions for further experiments in the current study.

The original concentration of AFB_1 was also considered as a factor for detoxification efficiency, which was not significant (Table 1 and 2) but the results of the coefficient estimates in Table 3 shows a positive effect on the AFB_1 reduction by KGs ($P<0.05$). Since the latter approach based on the coefficient estimates is more accurate, it was used for the evaluation of our data in the current study. Therefore, it can be concluded that the amount of toxin removed will increase with at higher AFB_1 contamination levels. Whether such decrease is sufficient to reduce the toxin level below the maximum tolerable level established for the pistachio nuts will be discussed in the later sections. El-Nezami *et al.* (1998), Haskard *et al.* (1998), Lee *et al.* (2003) and Pizzolitto *et al.* (2011) have also reported similar relationship between the toxin concentrations and the efficiencies of microorganisms.

Optimising the model for the best detoxification effect

A central composite design using four factors (X_1, X_2, X_3 and X_4) each at five levels and seven replicates at the centre point (to account for pure internal error) was applied for the optimisation of AFB_1 reduction in pistachio nuts. In these experiments, because of the results observed in the screening experiment and also the economic aspect which was related to the need for more energy consumption in the U condition (i.e. ultra-heated condition), KGs treated with 70±2 °C (H condition) were used for further optimisation. The design matrix for these factors in the optimisation runs was fitted to the data, as noted in the experimental section.

AFB_1 level is the main factor and other variables are set for its reduction. Maximum tolerable levels set for AFB_1 in pistachio nuts based on the Iranian standard is 5 ng AFB_1 in 1 g pistachio. That level for the European Union legislation is set at 8 ng/g (EC, 2010; ISIRI 2010). In this stage, the value of spiked AFB_1 was examined under the conditions of a maximum level (25 ng/g) and a minimum (5 ng/g). The highest value of AFB_1 reduction was observed at both higher and lower concentrations of KG in the first-order model (Table 3). The range of KG levels was also extended from 5-10 g (10–20%) to 2.5-12.5 g (5-25%). Such ranges for the contact time and incubation temperature were also extended from 2-4 h to 0-8 h and 30-40 °C to 20-60 °C, respectively, because of significant differences found between the two levels defined in the first-order model (Table 2) and in their interactions with other factors (Table 3).

Based on the ANOVA and coefficient estimates (Table 4) obtained from the second-order model the quadratic regression was significant (P=0.0001) and the lack-of-fit was insignificant (P=0.096). The value of the coefficient of determination (R^2) was 86.37% suggesting that the model (Equation 2) accurately represents the data in the experimental region:

$$Y = 86.44 + 3.25\ X_1 + 0.35\ X_2 + 1.58\ X_3 - 0.93\ X_4 + 1.04\ X^2_4 + 1.42\ X_1X_2 + 1.34\ X_1X_4 - 2.16\ X_3X_4 \quad (2)$$

Table 4 and Equation 2 show that toxin concentration and contact time have the most significant effects on the AFB_1 reduction (P<0.001 and P<0.01, respectively). The interactions between the applied factors in AFB_1 reduction (toxin concentration with KG: P<0.05; toxin concentration with incubation temperature: P<0.05; and contact time with incubation temperature: P<0.01) are shown in Figure 2. Reduction in AFB_1 increased with an increase in the toxin concentration and KG level (Figure 2A). But, it did not reach zero. Bueno *et al.* (2007) and Lee *et al.* (2003) suggested that a reversible process could be involved in AFB_1 reduction prohibiting a full removal of the toxin. At low concentrations of KGs (under 10%), increasing toxin concentration resulted in a maximum AFB_1 decontamination of 86%. This may be due to a saturation occurring in the binding equilibrium between the microflora of KG and AFB_1 (Pizzolitto *et al.*, 2011). On the other hand, at high toxin concentrations (20-25 ng/g), the reduction of AFB_1 increased with an increase in the KG level from 5 to 25%. With toxin concentration at 5 to 20 ng/g, an increase in AFB_1 reduction (from 72 to 88%) is observed at a contact time of up to ~6 h. At high toxin concentration levels (20-25 ng/g), a 90% AFB_1 decrease occurs in less than 1 h (Figure 2B).

When using low toxin concentrations (5-10 ng/g), AFB_1 reduction decreased with an increase in the temperature (up to ~50 °C). A 92-96% reduction in the AFB_1 level occurred at high toxin concentrations of 20-25 ng/g and at 50-60 °C incubation temperature (Figure 2C). Reduction of AFB_1 increased with an increase in the contact time at the lowest incubation temperature of ~20 °C. But, at higher temperatures (50-60 °C), the reduction was very fast (92% reduction in 0-1 h) (Figure 2D). According to the obtained results for the optimisation step (using the estimates in Equation 2(, KG level at 10 g (equal to 20% in pistachio paste samples), contact time at 6 h and an incubation temperature at 30 °C were the optimal conditions for the highest reduction in the AFB_1 level (96%) with an original toxin concentration at 20 ng/g. Such finding was examined using six replicates of contaminated pistachio paste. The obtained value (92%) was compared with that of the model (96%) using the t-test indicating a good agreement between these data (R^2=92.49).

Table 4. Analysis of variance and coefficient estimates in the second-order model.

Source of variation	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value
Linear	4	338.38	84.59	15.25	<0.001
Interaction	6	179.54	29.92	5.39	0.003
Square	4	44.45	11.11	2.00	0.142
Residuals	16	88.75	5.54		
Lack of fit	10	73.92	7.39	2.98	0.096
Pure error	6	14.84	2.47		
Term	**Estimate**	**Standard error**	**t-value**	***P*-value**	
Intercept	86.44	0.89	97.10	<0.001	
Toxin concentration (X_1)	3.25	0.48	6.76	<0.001	
Kefir grain level (X_2)	0.35	0.48	0.73	0.473	
Contact time (X_3)	1.58	0.48	3.30	0.004	
Incubation temperature (X_4)	-0.93	0.48	-1.95	0.068	
Interaction effects					
X_1X_2	1.42	0.58	2.41	0.028	
X_1X_4	1.34	0.58	2.27	0.036	
X_3X_4	-2.16	0.58	-3.67	0.002	
X_4^2	1.04	0.44	2.38	0.029	

R^2=86.37%; Adj R^2=74.44%; P=0.0001.

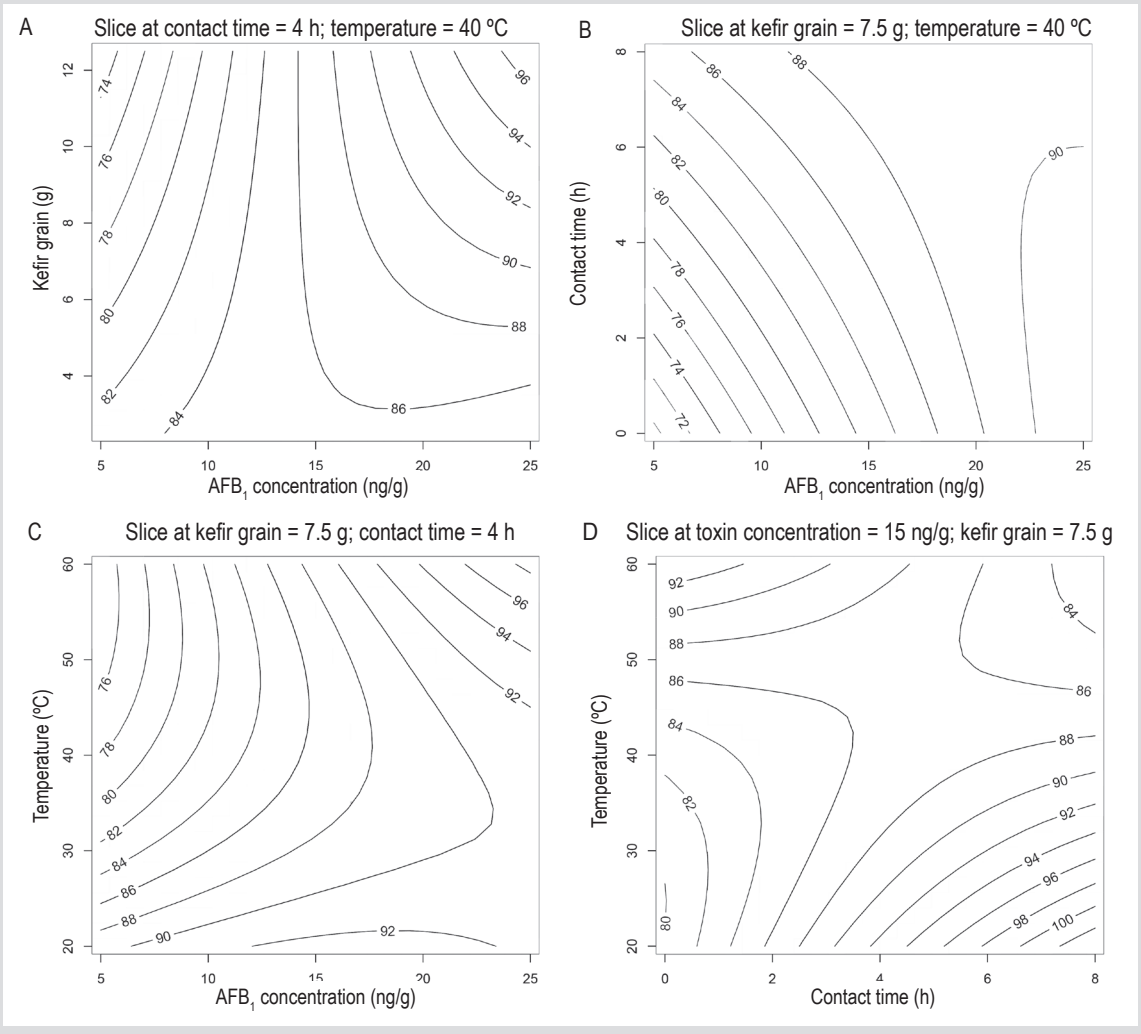

Figure 2. Contour plots of the model equation fitted to the data of the central composite design for the interaction between (A) kefir grains and aflatoxin B_1 (AFB_1), (B) AFB_1 and contact time, (C) AFB_1 and temperature, and (D) contact time and temperature.

4. Conclusions

The effects of five independent variables including toxin concentration, KG level, contact time, incubation temperature and pre-treatment heat level on AFB_1 reduction were investigated using a sequential optimisation strategy (a full factorial design followed by a central composite design). The results obtained for the pistachio paste (as used in the current study) indicated that KGs pre-treated at 70 °C caused significant reduction (P<0.05) in AFB_1 level in the contaminated pistachio samples. AFB_1 removal increased with increase in contact time (0 to 8 h) at 20 °C. But, at 50-60 °C the reduction was very fast (92% reduction in 0-1 h). The reduction level for AFB_1 was also dependent on toxin concentration (P<0.001). When applying such treatments for whole pistachio kernels, there might be some other parameters (such as available surface area) that need to be considered for more appropriate evaluation of the treatment efficiency for whole kernels.

The optimisation part of the study indicated that maximum AFB_1 reduction (96%) would be obtained at 20 ng/g toxin concentration, 20% KG (based on the amount of pistachio paste), 6 h contact time and 30 °C incubation temperature. These optimal conditions were then validated and a good accuracy was found among the results (R^2=92.49). Therefore, the optimal conditions obtained in this study were approved for the treatment of pistachio nuts with KGs pre-treated at 70 °C. As a final conclusion, the optimised detoxification conditions obtained in the current study can be suggested for routine AFB_1 detoxification in pistachio nuts. However, as a warning to the consumers, since the binding of AFB_1 with microorganisms is a reversible process (Bueno *et al.*, 2007; Lee *et al.*, 2003) the toxic effects observed from the treated pistachio with KG may be higher than what is expected in the current study. Therefore, future *in vivo* studies are required to verify the efficacy of KG on the consumed contaminated pistachios.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

We would like to express our appreciations to Prof. R. Sotudeh-Gharebagh and Prof. R. Nabizadeh at the University of Tehran for their kind support of this study. The authors would like to acknowledge the support provided by the Research Council of University of Tehran (Tehran, Iran) and also the Research Council of College of Agriculture and Natural Resources of University of Tehran (Karaj, Iran).

References

Abdulkadar, A.H.W., Al-Ali, A. and Al-Jedah, J., 2000. Aflatoxin contamination in edible nuts imported in Qatar. Food Control 11: 157-160.

Association of Official Analytical Chemists (AOAC), 2000. Aflatoxin B_1 and total aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder – immunoaffinity column LC with post-column derivatization. Official Methods of Analysis 999.07. (17th Ed.), Chapter 49. AOAC International, Gaithersburg, MD, USA.

Bueno, D.J., Casale, C.H., Pizzolitto, R.P., Salvano, M.A. and Oliver, G., 2007. Physical adsorption of aflatoxin B_1 by lactic acid bacteria and *Saccharomyces cerevisiae*: a theoretical model. Journal of Food Protection 70: 2148-2154.

Devegowda, G., Arvind, B.I.R. and Morton, M.G., 1996. *Saccharomyces cerevisiae* and mannanoligosaccharides to counteract aflatoxicosis in broilers. In: Proceedings of Australian Poultry, Science Symposium, Sydney, Australia, pp. 103-106.

Dini, A., Khazaeli, P., Roohbakhsh, A., Madadlou, A., Pourenamdari, M., Setoodeh, L., Askarian, A., Nader Doraki, Farrokhi, H., Moradi, H. and Khodadadi, E., 2013. Aflatoxin contamination level in Iran's pistachio nut during years 2009-2011. Food Control 30: 540-544.

El-Nezami, H., Kankaanpaa, P., Salminen, S. and Ahokas, J., 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B_1. Food and Chemical Toxicology 36: 321-326.

European Commission (EC), 1993. Commission regulation (EU) No. 315/1993, as regards laying down community procedures for contaminants in food. Official Journal of the European Union L037: 1-3.

European Commission (EC), 2010. Commission regulation (EU) No. 178/2010, amending regulation (EC) No. 401/ 2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. Official Journal of the European Union L52: 32-43.

Fajgelj, A. and Ambrus, A., 2000. Guidelines for single-laboratory validation of analytical methods for trace-level concentrations of organic chemicals. In: Fajgelj, A. and Ambrus, A. (eds.) Principles and practices of method validation. The Royal Society of Chemistry, London, UK, pp. 179-252.

Food and Agriculture Organization (FAO), 2012. Top production-pistachio, 2010. FAO, Rome, Italy.

Gratz, S., Wu, Q.K., El-Nezami, H., Juvonen, R.O., Mykkänen, H. and Turner, P.C., 2007. *Lactobacillus rhamnosus* strain GG reduces aflatoxin B_1 transport, metabolism, and toxicity in Caco-2 cells. Applied and Environmental Microbiology 73: 3958-3964.

Haskard, C.A., El-Nezami, H.S., Kankaanpää, P.E., Salminen, S. and Ahokas, J.T., 2001. Surface binding of aflatoxin B_1 by lactic acid bacteria. Applied and Environmental Microbiology 67: 3086-3091.

Haskard, C.A., El-Nezami, H.S., Peltonen, K.D., Salminen, S. and Ahokas, J.T., 1998. Sequestration of aflatoxin B_1 by probiotic strains: binding capacity and localisation. Revue de Medecine Veterinaire 149: 571.

Hathout, A.S., Mohamed, S.R., El-Nekeety, A.A., Hassan, N.S., Aly, S.E. and Abdel-Wahhab, M.A., 2011. Ability of *Lactobacillus casei* and *Lactobacillus reuteri* to protect against oxidative stress in rats fed aflatoxins-contaminated diet. Toxicon 58: 179-186.

Hernandez-Mendoza, A., Garcia, H.S. and Steele, J.L., 2009. Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B_1. Food and Chemical Toxicology 47: 1064-1068.

Institute of Standards and Industrial Research of Iran (ISIRI), 2010. Food and feed – mycotoxins-maximum tolerated level. National Standard 5925. ISIRI, Teheran, Iran.

Institute of Standards and Industrial Research of Iran (ISIRI), 2011. Food and feed – determination of aflatoxins B and G by HPLC method and clean up by imminoaffinity column-test method. National Standard 6872. ISIRI, Teheran, Iran.

International Agency for Research on Cancer (IARC), 1993. IARC Monographs on the evaluation of carcinogenic risks to humans, Volume 59. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines, and mycotoxins. IARC Press, Lyon, France, pp. 245-395.

Ismaiel, A.A., Ghaly, M.F. and El-Naggar, A.K., 2011. Milk kefir: ultrastructure, antimicrobial activity and efficacy on aflatoxin B_1 production by *Aspergillus flavus*. Current Microbiology 62: 1602-1609.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1998. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series 40: 897-913.

Kakisu, E., Bolla, P., Abraham, A.G., de Urraza, P. and De Antoni, G.L., 2013. *Lactobacillus plantarum* isolated from kefir: protection of cultured Hep-2 cells against *Shigella* invasion. International Dairy Journal 33: 22-26.

Lee, Y.K., El-Nezami, H., Haskard, C.A., Gratz, S., Puong, K.Y., Salminen, S. and Mykkänen, H., 2003. Kinetics of adsorption and desorption of aflatoxin B_1 by viable and nonviable bacteria. Journal of Food Protection 66: 426-430.

Leite, A.M.O., Mayo, B., Rachid, C.T.C.C., Peixoto, R.S., Silva, J.T., Paschoalin, V.M.F. and Delgado, S., 2012. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiology 312: 215-221.

Li, F.Q., Yoshizawa, T., Kawamura, O., Luo, X.Y. and Li, Y.W., 2001. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. Journal of Agricultural and Food Chemistry 49: 4122-4126.

Liu, J.R. and Lin, C.W., 2000. Production of kefir from soymilk with or without added glucose, lactose, or sucrose. Journal of Food Science 65: 716-719.

Madrigal-Santillán, E., Madrigal-Bujaidar, E., Márquez-Márquez, R. and Reyes, A., 2006. Antigenotoxic effect of *Saccharomyces cerevisiae* on the damage produced in mice fed with aflatoxin B1 contaminated corn. Food and Chemical Toxicology 44: 2058-2063.

McKenzie, K.S., Sarr, A.B., Mayura, K., Bailey, R.H., Miller, D.R., Rogers, T.D., Norred, W.P., Voss, K.A., Plattner, R.D., Kubena, L.F. and Phillips, T.D., 1997. Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. Food and Chemical Toxicology 35: 807-820.

Mukendi, N., Rollmann, B. and De Meester, C., 1991. Detoxification of aflatoxin B_1 by different chemical methods and evaluation of the effectiveness of the treatments applied. Journal de Pharmacie de Belgique 46: 182-188.

Myers, R.H. and Montgomery, R.C., 2002. Response surface methodology: process and product optimization using designed experiments. Wiley, New York, NY, USA.

Oatley, J.T., Rarick, M.D., Ji, G.E. and Linz, J.E., 2000. Binding of aflatoxin B_1 to bifidobacteria *in vitro*. Journal of Food Protection 63: 1133-1136.

Patel, U.D., Govindarajan, P.R.I.Y.A. and Dave, P.J., 1989. Inactivation of aflatoxin B_1 by using the synergistic effect of hydrogen peroxide and gamma radiation. Applied and Environmental Microbiology 55: 465-467.

Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J. and Salminen, S., 2001. Aflatoxin B_1 binding by dairy strains of lactic acid bacteria and bifidobacteria. Journal of Dairy Science 84: 2152-2156.

Pereyra, C.M., Cavaglieri, L.R., Chiacchiera, S.M. and Dalcero, A.M., 2010. Fungi and mycotoxins in feed intended for sows at different reproductive stages in Argentina. Veterinary Medicine International 1-7.

Phillips, T.D., Clement, B.V. and Park, D.L., 1994. Approaches to reduction of aflatoxins in foods and feeds. In: Eaton, D.L. and Groopman, J.D. (eds.) The toxicology of aflatoxins: human health, veterinary and agricultural significance. Academic Press, London, UK, pp. 383-406.

Pizzolitto, R.P., Bueno, D.J., Armando, M.R., Cavaglieri, L., Dalcero, A.M. and Salvano, M.A., 2011. Binding of aflatoxin B_1 to lactic acid bacteria and *Saccharomyces cerevisiae in vitro*: a useful model to determine the most efficient microorganism. In: Guevara-Gonzalez, R.G. (ed.) Aflatoxins – biochemistry and molecular biology. Intech, Rijeka, Croatia, pp. 323-346.

Pizzolitto, R.P., Salvano, M.A. and Dalcero, A.M., 2012. Analysis of fumonisin B_1 removal by microorganisms in co-occurrence with aflatoxin B_1 and the nature of the binding process. International Journal of Food Microbiology 156: 214-221.

Rahaie, S., Razavi, S.H. and Emam-djomeh, Z., 2010. The ability of *Saccharomyces cerevisiae* strain in aflatoxin reduction in pistachio nuts. Iranian Journal of Food Science and Technology 7: 81-88.

Raju, M.V.L.N. and Devegowda, G., 2000. Influence of esterified-glucomannan on performance and organ morphology, serum biochemistry and haematology in broilers exposed to individual and combined mycotoxicosis (aflatoxin, ochratoxin and T-2 toxin). British Poultry Science 41: 640-650.

Samarajeewa, U., Sen, A.C., Cohen, M.D. and Wei, C.I., 1990. Detoxification of aflatoxins in foods and feeds by physical and chemical methods. Journal of Food Protection 53: 489-501.

Set, E. and Erkmen, O., 2010. The aflatoxin contamination of ground red pepper and pistachio nuts sold in Turkey. Food and Chemical Toxicology 48: 2532-2537.

Shetty, P.H., Hald, B. and Jespersen, L., 2007. Surface binding of aflatoxin B_1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. International Journal of Food Microbiology 113: 41-46.

Slizewska, K., Nowak, A., Libudzisz, Z. and Blasiak, J., 2010. Probiotic preparation reduces the faecal water genotoxicity in chickens fed with aflatoxin B_1 contaminated fodder. Research in Veterinary Science 89: 391-395.

Suzuki, T., Noro, T., Kawamura, Y., Fukunaga, K., Watanabe, M., Ohta, M., Sugiue, H., Sato, Y., Kohno, M. and Hotta, K., 2002. Decontamination of aflatoxin-forming fungus and elimination of aflatoxin mutagenicity with electrolyzed NaCl anode solution. Journal of Agricultural and Food Chemistry 50: 633-641.

Topcu, A., Bulat, T., Wishah, R. and Boyacı, I.H., 2010. Detoxification of aflatoxin B_1 and patulin by *Enterococcus faecium* strains. International Journal of Food Microbiology 139: 202-205.

Tramšek, M. and Goršek, A., 2007. A novel approach for assuring and following inoculum activity during kefir grains growth studies – application of dynamic pH profiles. Acta Chimica Slovenica 54: 693-699.

Wild, C.P. and Turner, P.C., 2002. The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis 17: 471-481.

Wilson, D.M. and Ryne, G.A., 1993. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In: Eaton, D.L. and Groopman, J.D. (eds.) The toxicology of aflatoxins. Academic Press Inc., San Diego, CA, USA, pp. 309-325.

Zhang, Q., Xiong, K., Tatsumi, E. and Liu, H.J., 2012. Elimination of aflatoxin B1 in peanuts by acidic electrolyzed oxidizing water. Food Control 27: 16-20.

Zheng, Z., 2011. World production and trade of pistachios: the role of the US and factors affecting the export demand of US pistachios. University of Kentucky, Lexington, KY, USA, 123 pp.

Zhou, J., Liu, X., Jiang, H. and Dong, M., 2009. Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. Food Microbiology 26: 770-775.

Zuo, R.Y., Chang, J., Yin, Q.Q., Wang, P., Yang, Y.R., Wang, X., Wang, G.Q. and Zheng, Q.H., 2013. Effect of the combined probiotics with aflatoxin B_1-degrading enzyme on aflatoxin detoxification, broiler production performance and hepatic enzyme gene expression. Food and Chemical Toxicology 59: 470-475.