Individual and combined decontamination effect of fermentation and ultrasound on aflatoxin B_1 in wheat-based doughs: a preliminary study

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Abstract

In this work, a preliminary investigation was conducted to examine the effect of ultrasound (US), alone or in combination with fermentation, on the reduction of aflatoxin B_1 (AFB_1) at 50 ng/g in wheat flour-based doughs. The US treatment was performed by soaking flasks containing triplicate samples of non-fermented and fermented (18 h at 28°C; 85% relative humidity) doughs in US bath (40 kHz; 100 W/cm^2) at 35°C for 10, 30 and 60 min. Percentage reduction of AFB_1 levels as determined by high-performance liquid chromatography varied between 3.6% and 46.0% in non-fermented doughs, and from 6.7% to 61.7% in fermented doughs. The US treatments used in the experiment did not interfere with the titratable acidity of fermented doughs, hence confirming US as a promising technique to reduce aflatoxin levels in fermented wheat-based products. The future studies are required to determine other parameters, such as temperature and pH of the product, to optimize the decontamination process of AFB_1 as well as to evaluate possible effects of US on the sensory attributes of wheat-based products.

Keywords: aflatoxin B_1; wheat-based products; ultrasound; fermentation; decontamination

Introduction

Aflatoxins are toxic secondary metabolites produced by fungal species from the genus *Aspergillus*, especially *A. flavus*, *A. parasiticus* and *A. nomius* (de Oliveira *et al.*, 2014; Wochner *et al.*, 2018). Although more than 20 types of aflatoxins were identified, aflatoxin B_1 (AFB_1) is considered the major toxic metabolite produced by the fungi contaminating foods and feeds. The International Agency for Research on Cancer has classified AFB_1 as group 1 carcinogens (European Food Safety Authority [EFSA], 2020). Considering the toxic effects of AFB_1, proper prevention and control strategies in the feed and food chains are of fundamental importance to avoid health issues (Schrenk *et al.*, 2020). Prevention of aflatoxin contamination of foodstuffs can be achieved through the following two main approaches: inhibiting the fungal growth and subsequent aflatoxin biosynthesis, and/or detoxifying toxic compounds already produced by the fungi in food matrices (Ismail *et al.*, 2018). Strategies for inhibiting fungal growth include employing improved agricultural practices, antifungal agents, genetic engineering, and appropriate storage conditions (Corassin *et al.*, 2013). Decontamination of aflatoxins could be achieved by using physical, chemical and biological tools aimed at inactivating or degrading toxic substances in

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food matrices (Agriopoulou *et al.*, 2020). Physical techniques, such as heat-based inactivation, irradiation and ultrasound (US) application on food, have shown promising results for inactivating or degrading aflatoxins (Grenier *et al.*, 2014).

Wheat is a vital food ingredient consumed globally and utilized extensively in a wide range of food preparations. For example, wheat, because of its high nutritional values, is usually employed to produce daily-based breads, cookies, cakes and other essential edible products (Grenier *et al.*, 2014). Fermented wheat-based products are prepared by using two vital substances: starch, a carbohydrate polymer that serves as a significant energy source, and gluten, a protein predominantly found in wheat that imparts elasticity and softness to dough (Ma *et al.*, 2022; Xiong *et al.*, 2021). Consequently, developing effective technologies for decontamination of wheat containing aflatoxins has gained considerable interest in scientific and economic communities. Previous works investigating the effect of fermentation on the levels of aflatoxins in fermented wheat doughs indicated that this process could reduce levels of aflatoxin in final products (Noroozi *et al.*, 2020, 2022; Rashid Hudu *et al.*, 2022).

Among emerging technologies for processing of food, US has emerged as an innovative and promising method for different applications in the food industry (Abedi *et al.*, 2019; Dumuta *et al.*, 2022; Gavahian *et al.*, 2020). As the name suggests, ultrasound refers to inaudible sound waves with frequencies higher than those detectable by the human ear (20 kHz). Regarding its applications, high-frequency (>1 MHz) and low-intensity (<1 W cm^2) US, and low-frequency (20–100 kHz) with high-intensity (10–1,000 W cm^2) US have demonstrated notable uses in the food industry (Alarcon-Rojo *et al.*, 2015). The application of high-intensity US in food processing has been considered advantageous because of its operation at lower temperature with minimal impact on the physicochemical properties of foods (Nunes *et al.*, 2021). This technology has the potential to inactivate spoiling microbes and pathogenic enzymes in food products and beverages (Dumuta *et al.*, 2022). Moreover, US is an eco-friendly and non-polluting technique. Given the significant effect of US on aflatoxin degradation (Liu *et al.*, 2019b), possible interactions between fermentation and US detoxification have been hypothesized (Gavahian *et al.*, 2021). US can degrade AFB_1 by affecting the chemically stable furan moiety and by changing lactone ring in the main structure of AFB_1 (Gavahian *et al.*, 2021; Liu *et al.*, 2019b). Therefore, the aim of this study was to conduct a preliminary investigation to evaluate the effect of US, alone or in combination with fermentation, on the level of AFB_1 in wheat flour-based doughs.

Material and methods

Ingredients, reagents and equipment

Wheat flour samples were obtained in the summer of 2022 from local retailers at Pirassununga, São Paulo state, Brazil. Prior to the experiment, the collected wheat flour samples were submitted for aflatoxin analysis as described in subsequent sections, and were free of aflatoxins. The AFB_1 standard (Sigma-Aldrich, St. Louis, MO) was dissolved in acetonitrile and diluted to achieve a working solution containing 1.0-µg AFB_1/mL. This solution was used for spiking wheat flour samples with AFB_1 at 50 ng/g of AFB_1 for preparing contaminated doughs used in decontamination assays.

The spiking process was accomplished by transferring appropriate aliquots of AFB_1 working solution and wheat flour to a 250-mL beaker. The contents were mixed thoroughly and left in an oven at 50°C for evaporation of solvent. The US equipment (SolidSteel, Piracicaba, SP) used in the study had a frequency of 40 kHz with a power output of 100 W (Guo and Lei, 2020). AFB_1 analysis of the samples was conducted using a high-performance liquid chromatography (HPLC) system (Shimadzu®, Kyoto, Japan), equipped with a fluorescence detector (excitation: 360 nm, and emission: 440 nm) and a Kinetex C18 column (Phenomenex, Torrance, CA, USA) with a dimension of 4.6 × 150 mm (2.6 µm), preceded by a Shim-pack pre-column (5 µm, 4 × 10 mm). All reagents used in the experiment were of HPLC grade.

Preparation of wheat dough

Experimental wheat doughs were prepared following the procedures described by Mousavi-Khaneghah *et al.* (2023) and using the main ingredients as presented in Table 1.

All ingredients (AFB_1-contaminated wheat flour, sodium bicarbonate, vegetable fat, salt, and dry yeast) were mixed

Table 1. Main ingredients for preparing experimental wheat doughs.

Ingredients	Quantity
Wheat flour (spiked with aflatoxin B_1 [AFB_1])	100.0 g
Water	50.0 mL
Dry yeast (*Saccharomyces cerevisiae*)	1.0 g
Vegetable fat	14.0 g
Salt	0.8 g
Sodium bicarbonate	1.5 g
Total	**167.3**

in a glass container. The experimental doughs were prepared in duplicate lots; one lot was submitted directly for US treatments, and the other lot was reserved for fermentation prior to subjecting to US treatments. The fermentation of experimental doughs was conducted at 28°C for 18 h in a fermentation cabinet with 85% relative humidity (RH). In addition, fermented and non-fermented control doughs (without AFB_1) were prepared and subjected to the above-described US treatments.

Total titratable acidity (TTA) was evaluated as an indicator of fermentation process, using the method described by Li *et al.* (2013). TTA was measured in triplicate according to the method described by García-Mantrana *et al*. (2015) using 10-g dough fermented at 28°C for 2 h (relative humidity 85%) and mixed with 100 mL of acetone–water solution (5:95 ratio, v/v) for 5 min., and titrated subsequently. The results were expressed in volume (mL) of 0.1-N NaOH to titrate 10 g of fermented dough (García-Mantrana *et al.*, 2015).

Ultrasound treatment

Aflatoxin B_1 decontamination assays using the experimental doughs subjected to US treatments were performed as described by Liu *et al.* (2019b) with small modifications. Briefly, a 25-g aliquot of non-fermented and fermented doughs was weighed in Falcon tubes and introduced into a US bath set at 40 kHz, 100 W/cm^2 and 35°C for 10, 30 and 60 min. The tubes were removed from the US bath at the end of each treatment period and the samples were immediately submitted for AFB_1 determination.

Analysis of aflatoxin B_1

Aflatoxin B_1 in the samples was determined following the method recommended by manufacturer of the immunoaffinity columns (Aflatest®; Vicam, Milford, MA), followed by HPLC with fluorescence detector. The entire content of the sample in the Falcon tube (25 g) was mixed with 5 g of NaCl and 125 mL of methanol–water solution (70:30 ratio, v/v). After stirring the content in a shaker for 30 min, 15 mL of extract was centrifuged at 2,000 ×g and filtered through a filter paper into a beaker. Following this step, 30 mL of the ultra-purified water (Milli-Q; Millipore, Burlington, MA) was added to the extract, after which the mixture was homogenized and filtered again through a 1.5-µm filter membrane (Millipore, Burlington, MA). The collected 15-mL extract was passed through immunoaffinity column, connected to a vacuum system (flow set at 1–2 drops/s). After sample elution, the column was washed with 10-mL Milli-Q water. Then, 1 mL of HPLC-grade methanol was passed through eluted AFB_1, collecting the eluate in an amber vial. The eluate was evaporated to dryness under N_2 flow and derivatized with 100 µL of trifluoroacetic acid and 200 µL of *n*-hexane (Jager, 1990). The mixture was homogenized and placed in an oven at 35°C for 10 min., evaporated again, and finally re-suspended in 500 µL of ethanol–water solution (50:50 ratio, v/v). Final extracts were filtered through 0.45-µm polytetrafluoroethylene polymer (PTFE) filtering membranes (Millex; Millipore, Burlington, MA) and presented for chromatographic analysis.

The determination of AFB_1 was carried out by injecting a 20 µL sample extract into the HPLC system (Shimadzu 10 VP, Kyoto, Japan) as described above, using water–methanol–acetonitrile solution (60:20:20, v/v) as mobile phase with a flow rate of 1.0 mL/min. Under these conditions, the retention period of AFB_1 was approximately 3.8 min. Calibration curves were prepared with AFB_1 working solution and derivatized exactly as described previously to reach concentrations of 1.56, 3.125, 6.25, 12.5, and 25.0 ng/mL. The quantification of AFB_1 in samples was performed through the interpolation of the areas of chromatographic peaks and the regression equation of calibration curves. The limit of detection (LoD) and limit of quantification (LoQ) were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively.

Performance of analytical method

To evaluate the accuracy of the analytical method used for determining AFB_1 in wheat samples, recovery tests were carried out as described by Jager *et al.* (2016). Dough samples were prepared as described previously and spiked with AFB_1 working solution to achieve contamination levels of 5.0 and 50.0 µg/kg. The fortified samples were prepared in triplicate (n = 3) for each AFB_1 level, and subjected to the method described in previous sections to determine recovery proportions and coefficient variances of analytical results.

Statistical analysis

AFB_1 levels in the samples from different treatments were submitted to analysis of variance, according to the procedures established in the General Linear Model of SAS® (Vonesh, 2012). Tukey's test was used for comparison of mean values, when applicable, considering $\mu = 0.05$.

Results and Discussion

The TTA of fermented dough samples subjected to US treatments ranged between 3.0±0 mL and 4.0±0 mL (data not shown), and no differences ($P < 0.05$) were observed

between treatments. These results indicated that the fermentation process, as estimated by TTA (García-Mantrana *et al.*, 2015), was not affected by any US treatment employed in the present experiment. The data presented in this work agreed with the previous studies indicating that US at small frequencies associated with the fermentation resulted in improved enzymatic activity in various food products (Gavahian *et al.*, 2021).

Analytical performance for determining aflatoxin B_1 in wheat dough samples

The performance evaluation of the analytical method used in this study is crucial for ensuring reliability of the results. Table 2 presents concentration values, recoveries, and coefficients of variation obtained for the analytical method used for AFB_1 quantification in experimental dough samples. Data indicate that accuracy and precision attributes were satisfactory (Azevedo *et al.*, 2021), considering the average recoveries of 85.0% and 88.4% and coefficients of variation of 7.9% and 11.5% achieved for samples spiked with 5.0 ng/g and 50.0 ng/g, respectively.

The LoD and LOQ were 0.1 ng/g and 0.4 ng/g, respectively. The analytical method proved as effective and sensitive to determine the used concentrations of AFB_1 in experimental dough samples. Figure 1 shows typical chromatograms obtained for AFB_1 in wheat-based dough samples spiked with 50-ng/g toxin before and after 60 min of US treatment.

Ultrasound treatment of the fermented and non-fermented wheat doughs

The presence of AFB_1 in foods, including wheat-based products, is a major concern because of its harmful properties (EFSA, 2020; Schrenk *et al.*, 2020). Therefore, implementing suitable methods to reduce AFB_1 contamination in food products is an important issue to prevent health problems. Among these methods, US, as investigated in the present work, has emerged as a promising technique because of its easy availability, low cost, and effectiveness in reducing mycotoxins in foods (Dumuta *et al.*, 2022; Nunes *et al.*, 2021).

The concentration and percentage reduction of AFB_1 in fermented and non-fermented doughs treated with US at different periods are shown in Table 3. The non-fermented samples subjected to US treatment alone had significant reduction ($P < 0.05$) in AFB_1 levels, which decreased from 50.0 ng/g (nominal value) to 42.24±7.62 ng/g after 10 min, and further to 26.99±9.09 ng/g after 60 min. However, dough samples subjected to the combined methods of US and fermentation exhibited even lower ($P < 0.05$) levels of AFB_1, decreasing from 50.0 ng/g (nominal value) to 23.20±6.42 ng/g after 10 min, and to 19.33±3.78 ng/g after 60 min. Consequently, the percentage reduction of AFB_1 levels in non-fermented doughs varied between 3.6% and 46.0% and between 6.7% and 61.7% in fermented doughs (Table 3). Reduction in AFB_1 levels because of US treatment alone, as observed in this study, was consistent with findings of previous studies conducted on the application of US for decontamination of mycotoxins (Liu *et al.*, 2019a, 2022).

Ultrasound is a physical method that generates cavitation, resulting in localized high temperatures and pressures that can disrupt microorganisms (Khadhraoui *et al.*, 2021). These disruptions can also lead to changes in physicochemical properties, thereby enhancing the degradation of mycotoxins (Gavahian *et al.*, 2021; Liu *et al.*, 2019a). In addition, US treatment generates several free radicals, such as hydrogen atoms and hydroxyl, which can degrade AFB_1 molecules (Liu *et al.*, 2019a). Hence, reduction in AFB_1 levels observed in wheat dough samples treated with US is potentially attributed to a combination of these effects.

Formation of AFB_1-degraded products after US treatment was not assessed in the present study. However, Liu *et al.* (2019b) reported mass spectrometry data,

Table 2. Concentrations, recoveries and coefficients of variance of analytical method for determination of aflatoxin B_1 in wheat-based doughs.

Aflatoxin B_1 added (ng/g)	Aflatoxin B_1 obtained (ng/g)	Aflatoxin B_1 medium (ng/g)	Aflatoxin B_1 recovery (%)	Coefficient of variance (%)
0	0 0 0	0	-	-
5	3.84 4.12 4.79	4.25	85.0	11.5
50	47.40 40.45 44.75	44.20	88.4	7.9

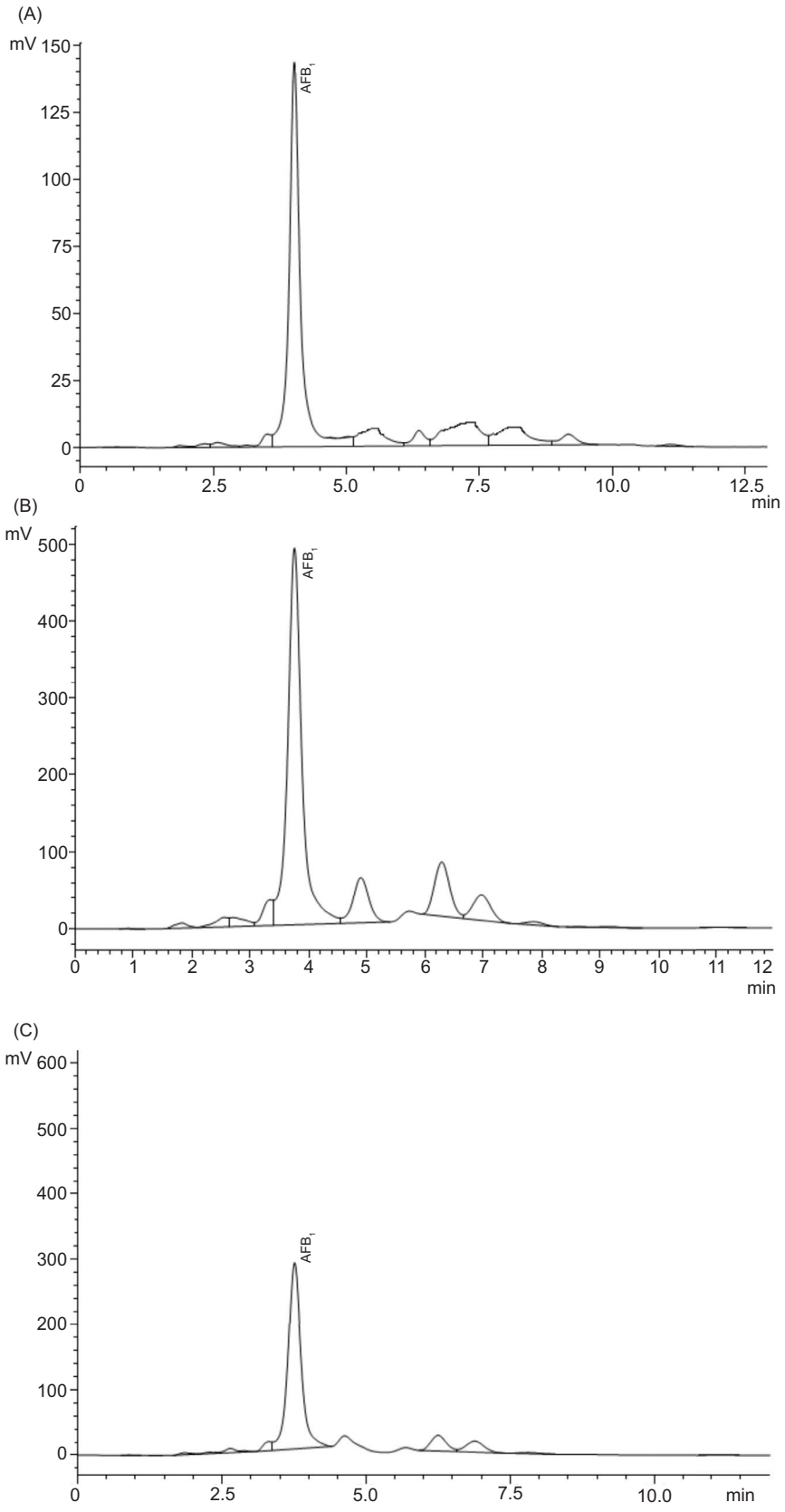

Figure 1. Chromatograms showing the retention time of aflatoxin B_1 (AFB_1) (nearly 3.8 min) in: (A) wheat-based dough spiked with 50 ng/g of AFB_1 before ultrasound treatment and fermentation; (B) non-fermented, spiked wheat-based dough after 60 min of US treatment (35.72 ng/g); and (C) fermented and spiked wheat-based dough after 60 min of US treatment (21.40 ng/g).

Table 3. Concentration and percentage reduction of aflatoxin B_1 in wheat-based doughs by ultrasound treatment submitted to different periods.

Type of dough	Applied ultrasound duration (min)							
	0		10		30		60	
	ng/g	%[1]	ng/g	%[1]	ng/g	%[1]	ng/g	%[1]
Non-fermented	48.21±3.42^{A,a}	3.6	42.24±7.62^{A,a}	15.5	37.89±1.83^{A,b}	24.2	26.99±9.09^{A,c}	46.0
Fermented	46.67±6.16^{A,a}	6.7	23.20±6.42^{B,b}	53.6	21.99±5.58^{B,b}	56.0	19.33±3.78^{B,b}	61.3

^{A,B,a,b,c}Different uppercase letters in the columns, or different lowercase letters in the rows differ by the Tukey's test at $P < 0.05$.
[1]Percentage reduction calculated in relation to the nominal concentration of AFB_1 before treatments (50.0 ng/g).

indicating that eight new degradation products were generated after 40 min of US treatment to degrade AFB_1. The structures of these eight reaction products resembled that of parent compound, with modifications in furofuran and lactone rings and the methoxy group of AFB_1 moiety (Liu *et al.*, 2019b).

Regarding the application of combined treatments of US and fermentation, an additional reduction effect was observed on the levels of AFB_1 in wheat dough samples (Table 3), with longer periods of US treatment resulting in greater reductions ($P < 0.05$). This suggests a possible synergistic interaction between the two methods that could enhance the decontamination efficiency of AFB_1 by US in the fermented product. However, the mechanism by which such potential interaction reduces the levels of AFB_1 in fermented wheat flour dough samples has to be determined.

The fermentation process lowers the pH of wheat dough (Casado *et al.*, 2017), which further inhibits the growth of microorganisms, such as AFB_1-producing fungi, rendering them more susceptible to the effects of US treatment (Khadhraoui *et al.*, 2021). However, the effect of US on microbial cells can vary according to different parameters, such as sonication time, temperature, and pH (Gavahian *et al.*, 2021). These variables could also impact the decontamination effect of US in fermented wheat doughs evaluated in the present study. The higher AFB_1 degradation activities observed in the US-treated fermented doughs could also be attributed to the stress exerted by the magnetic field of US and the application of combined fermentation, when compared to traditional approaches used in similar contexts (Gavahian *et al.*, 2021). However, the efficiency of sonication depends on factors such as the chemical structure of mycotoxins and possibly matrix-associated effects, which were not assessed in the present study.

Conclusion

Results of the present study indicate that US treatment, particularly when used in combination with fermentation, is an effective method for reducing AFB_1 levels in fermented wheat dough samples. Besides, the US treatment did not interfere with the TTA of fermented doughs. The best combination for decontamination of AFB_1 was fermentation associated with US application for 10 min, although the potential effects of temperature and pH on the decontamination process of AFB_1 were not assessed in this preliminary investigation. The future studies are required to optimize the process conditions of AFB_1 decontamination and determine possible effects of US on the sensory attributes of wheat-based products. Although US treatment reduces AFB_1 levels, it must not be considered as a substitute for good agricultural practices and proper storage conditions to prevent fungal growth and mycotoxin contamination.

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