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Occurrence of aflatoxins in wheat flour specified for sangak bread and its reduction through fermentation and baking

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RESEARCH ARTICLE

Abstract

This study was conducted as a screening survey to determine the occurrence of total aflatoxins (TAF) levels in sangak flour samples provided from flour factories and bakeries and to assess the effect of commercial fermentation and baking practices on the most contaminated flour samples. In addition, the effect of fermentation by yeast (*Saccharomyces cerevisiae*) and a mixture of yeast and a lactobacillus strain (*Lactobacillus plantarum* LA7) in reducing aflatoxins in prepared sangak breads were compared. The competitive enzyme-linked immunosorbent assay method was employed to determine toxin levels in the samples. With contamination levels ranging from 6.68 to 34.47 ng/g, the majority of the tested samples were not considered safe. Mean aflatoxin contamination of examined samples was 18.06 ng/g. Considering the limits for TAF in wheat flour set by ISIRI and EC (15 ng/g), 56.7% of the samples were unacceptable. No significant correlation was found between ash content and TAF levels ($P>0.05$). A significant decrease in aflatoxin levels was seen during sangak bread manufacturing ($P<0.01$). Dough fermentation by yeast and the mixture of yeast and *L. plantarum* LA7 resulted in 33.7 and 40.7% decrease in aflatoxin levels of the contaminated dough, respectively. On average, the sangak bread made with the most contaminated flours contained 11.1 ng/g of TAF, which was above the established maximum residual level in Iran and European countries.

Keywords: aflatoxin, bread, dough, flour, *Lactobacillus plantarum*, mycotoxin

1. Introduction

Mycotoxins are low molecular weight (<700 Da) toxic chemical products formed as secondary metabolites by a few fungal species that readily colonise crops and contaminate them with their toxins in the field or after harvest (Andrade and Caldas, 2015). According to the Food and Agriculture Organization of the United Nations (FAO), 25% of the world grain supply is contaminated with mycotoxins (FAO, 1996). Some evidence on mycotoxin contamination of crops has been found in Iran (Behfar *et al.*, 2008; Kazemi *et al.*, 2007; Taheri, *et al.*, 2012).

Aflatoxins are difuranocoumarin derivatives produced through a polyketide pathway by many strains of *Aspergillus flavus, Aspergillus parasiticus* and the rare *Aspergillus nomius* and *Aspergillus tamari* (Goto *et al.*, 1997), *Aspergillus pseudotamarii* (Ito *et al.*, 2001) and *Aspergillus bombycis* (Peterson *et al.*, 2001). Although 20 types of aflatoxins have been identified, four main types, known as AFB_1, AFB_2, AFG_1 and AFG_2, occur naturally and are regarded as total aflatoxins (TAFs) due to their abundance in the environment. AFB_1 is classified by the International Agency of Research on Cancer as group 1 human carcinogen (IARC, 1993). Consumption of aflatoxin-contaminated food or feed results in acute or chronic health problems (Afsah-Hejri*et al.*, 2013) including acute aflatoxicosis or toxic hepatitis that is manifested by symptoms such as jaundice, diarrhoea, depression, low-grade fever, anorexia, liver damage and decreased essential

serum proteins synthesised by the liver. In severe cases, it leads to death (Zain, 2011). In addition, chronic dietary exposure to aflatoxins along with hepatitis B infection may increase the risks of hepatocellular carcinomas. The majority of human aflatoxicosis cases have been reported from developing countries in Asia and Africa (Afsah-Hejri *et al.*, 2013), due to improper production and storage of feed and food. In particular, lack of an integrated management system in the production of wheat and rice, which form the staple crops of the society, leads to the vulnerable food safety situation due to mycotoxin contamination (Khoshpey*et al.*, 2011). Wheat is one of the most important food crops in the world. Wheat and wheat-originated foods, namely bread, form the major part of diet for more than one third of the world's population (Riba *et al.*, 2010).

One of the most popular wheat breads in Iran is the sangak bread, which was invented about 500 years ago. This sourdough flat bread measures about 70-80 cm long, 40-50 cm wide and 3-5 mm thick; its surface is sprinkled with sesame or poppy seeds. During the manufacturing of sangak bread, whole-grain flour (91-95% extraction), salt, yeast (*Saccharomyces cerevisiae*) and water are mixed to a proper consistency and fermented for about 2 h. Contrary to other flat breads, sangak bread dough is more liquid and similar to a batter. Dough pieces weighing about 500 g are flattened on a special paddle docked with fingers and then baked for 3-5 min on the floor of either a traditional oven covered with small hot stones or an industrial rotary oven (Mosharraf *et al.*, 2009; Najafi *et al.*, 2012).

In Iran, few studies have been conducted to assess the mycotoxin contamination of wheat kernels or flour. These studies noted a wide range of contamination and some authors have reported that some samples were heavily contaminated by mycotoxins which exceeds the maximum permissible levels in crops (Hedayati *et al.*, 2005; Mahmoudi *et al.*, 2012).

A recent study, carried out in the North of Iran, showed that all sampled wheat grains contained aflatoxin residues, including 24% that had excessive concentrations (Mahmoudi *et al.*, 2012). However, in another study, wheat sampled in 14 towns from 5 provinces (Mazandaran, Gilan, Zanjan, Kermanshah, and Khuzestan) were contaminated with aflatoxin, at levels averaging 8.32 ng/g which was below the regulated level (Babaei-Razdari *et al.*, 2014).

However, no study has yet investigated the impact of domestic bread manufacturing in Iran on the residual level of mycotoxins. The aim of the present study was to determine the frequency of distribution of aflatoxins in wheat flour specified for sangak bread manufacturing in Iran. We also attempted to check the relationship between flour extraction rate and the observed concentration of aflatoxins. In addition, the effects of dough fermentation and baking on the most contaminated samples were also determined.

2. Materials and methods

Sample collection

Thirty wheat flour samples, specific for sangak bread manufacturing, were collected during two time periods (January 2013 and February 2014) from two factories and 24 bakeries in Isfahan and Shiraz (Iran). The selection of the wheat flour samples was done using a simple random method in accordance with national and international standards (ISIRI, 2004). Two kg samples were taken in accordance with the principles of random sampling, packed, transported, and stored at 4 °C.

Proximate composition of flour samples

Moisture content and ash levels were determined using standard methods (AACC, 2003). Flour extraction rate was roughly determined according to ash content, as recommended by document no. 103 of the Institute of Standards and Industrial Research of Iran (ISIRI, 2012). For pH and acidity determination, 15 g of homogenate flour samples, dough or bread were mixed with 100 ml of distilled water in a vial, which was then sealed and stirred for 10 min.

Preparation of sangak bread

Sangak bread was prepared with 500 g of each of the four most contaminated wheat flour. Two types of fermentation was applied: a common method in which 250 g wheat flour, 1.2% salt, 0.5% instant yeast and 230-250 ml water were thoroughly mixed manually. Each dough weighted about 500 g and used as a dough batch to produce a bread. The dough was allowed to rest at 30 °C for 2 h. Alternatively, the same procedure was followed except, a slurry of 250 µl of lactic acid bacteria was added to the dough in addition to yeast. Dough pieces of 500 g are flattened on a special paddle, docked with fingers, and then introduced in to a previously heated oven manually by an operator. Oven temperature of the revolving screen devices was set at 240 °C (Moghadam-Industry Co., Mashhad, Iran), and bread dough was baked until the internal temperature of the bread was 95 °C (about 3-3.5 min). The bread making procedure was repeated individually on the same batch of flour. Finally for each of the most contaminated flours, two numbers of bread, fermented in a similar way were prepared and used for aflatoxin determination.

Microorganism used for dough fermentation

S. cerevisiae was provided as instant yeast (Fariman Company, Mashhad, Iran). *Lactobacillus plantarum* LA7 was obtained from the microbial collection at the Isfahan University of Medical Sciences (Isfahan, Iran). The active culture of this strain was obtained by two successive 18-h incubations of the fresh culture in De Man-Rogosa-Sharpe broth at 35 °C under anaerobic conditions (NB203L; N-BIOTEK, Bucheon, Republic of Korea). The mixed culture broth containing 10^{10} cfu/ml was used for dough fermentation.

Aflatoxin extraction and determination

In this study, a competitive enzyme-linked immunosorbent assay (ELISA) commercial kit (5121 AFT; Europroxima, Arnhem, the Netherlands) was used for aflatoxin extraction and determination. Three grams of the sample (wheat flour, dough or bread) was accurately weighted and added to 9 ml of 80% methanol and was shaken at room temperature for 30 min. The mixture was then centrifuged (at 2,000×*g* for 10 min) and an aliquot of 50 µl of the supernatant obtained after centrifugation was diluted with 150 µl of dilution buffer, which was provided in the commercial kit. 50 µl of the prepared sample or standard solutions was added to the wells of a micro titre plate followed by addition of 25 µl of conjugated enzyme (aflatoxin-HRP) and 25 µl of antibody solutions. The micro titre plate was sealed and shaken manually for a few seconds. Then, it was incubated for 1 h in the dark at 37 °C, after which the wells of the plate were emptied and the plate was washed three times with rinsing buffer and then 100 µl of stop solution was pipetted into each well. After 30 min of incubation period, at 20-25 °C, 100 µl of stop solution was added to each well. The absorbance of the developed yellowish colour was immediately read at 450 nm using an ELISA reader (model ON-5657; Awareness Technology, Palm City, FL, USA). The ELISA test was performed in duplicate wells for each sample. Percentage maximal absorbance values, calculated for the standards, are plotted on the y-axis versus the TAF equivalent concentration (ng/ml) on a logarithmic x-axis R^2, and a standard curve was obtained at 0.9957 and then, the results converted to TAF as ng/g of sample. The detection limit was 0.5 ng/g. Using a flour sample, spiked with aflatoxin B_1 standard (1, 5 and 15 ng/g), recovery of the test kit was 99-137%, higher than precision data reported by the manufacturer.

Statistical analysis

Data were assessed by ANOVA and comparison of means was performed according to the paired t-test using SPSS (version 20.0; IBM Corp., Armonk, NY, USA). A significance value of α=0.05 or 0.01 was used to distinguish statistically significant differences. To evaluate the relationship between the extraction rate and ash content of tested flours and the concentration of TAF, correlation test using Pearson correlation coefficient, with an uncertainty value of α=0.05 was performed.

3. Results and discussion

Mean composition of the 30 tested flour samples is presented in Table 1. Based on ash content, flour extraction rate was highly variable amongst samples. Such results are surprising because flour samples were identified as whole-grain flours, which is a prerequisite for the production of sangak bread.

Occurrence of aflatoxins in sangak bread flour

Table 2 presents the frequency distribution of aflatoxins in flour. All flour samples were contaminated with aflatoxin, with an average value of 18.06 ng/g; minimum and maximum toxin levels were 6.68 and 34.47 ng/g, respectively (data not shown). Considering the established regulation on the maximum TAF residual limit in Iran and the EU (15 ng/g), 56.7% of the samples were out of the acceptable range (EC, 2006; ISIRI, 2002).

In previous studies conducted in different cities of Iran, diverse levels of aflatoxins in wheat and wheat flour samples were reported. In the Khuzestan province, in the south of Iran, high-performance liquid chromatography (HPLC) determination of different aflatoxins showed that all of the wheat flour samples, collected from flour factories in Ahvaz were contaminated with some aflatoxin residue but

Table 1. Composition of the 30 flour samples.[1]

	Min	Max	Mean (standard deviation)
Moisture (%)	11.57	15.40	14.03 (1.35)
pH	5.71	6.50	6.13 (0.42)
Ash (%)	0.73	1.63	1.21 (0.86)
Extraction rate (%)	67.5	96.5	84.70 (5.15)

[1] For each flour sample, each experiment was carried out in duplicate.

Table 2. Frequency distribution of aflatoxin contamination in flour samples.[1]

Sample	Distribution of sample n (%)			
	<10 ng/g	10-15 ng/g	15-30 ng/g	>30 ng/g
Flour (n=30)	2 (6.7)	11 (36.6)	13 (43.3)	4 (13.3)

[1] Aflatoxin concentration is presented on wet basis.

no case was found to exceed the maximum permissible level of TAFs for food (Behfar *et al.*, 2008). In the north of Iran, in Mazandaran (Mahmoudi *et al.*, 2012) and Golestan (Taheri *et al.*, 2012) provinces, two individual studies were carried out on wheat samples, which, respectively, reported that 100% and about 85% of the examined wheat samples contained aflatoxin. Using an ELISA commercial kit, one of the latter studies concluded that 24.3% of the examined wheat samples were unacceptable. However, in the other study, HPLC determination of aflatoxins in wheat grain revealed that contamination in all tested samples were below the regulated limit and mean TAF levels in the samples, collected in summer and winter, were 1.99 and 0.82 ng/g, respectively (Taheri *et al.*, 2012).

In the above studies, no details were given on the composition of wheat or flour samples. In contrast, the present study focuses on fibre-rich sangak bread flour, which is generally prepared with whole-grain flour and is more likely to be more heavily contaminated with mycotoxins present at the surface of the kernels (Cheli *et al.*, 2013).

Results of the present study show that occurrence of aflatoxins in the tested samples was higher than those found in Pakistan (Maliha *et al.*, 2010), South Africa (Mashinini and Dutton, 2006) and Qatar (Abdulkadar *et al.*, 2004). Several factors may be responsible for the higher concentration of TAF residue in the present study, including lack in manufacturing practices during handling and storage of wheat kernel and flour. It was mentioned that wheat kernel storage length in wintertime is an important factor to induce high level of aflatoxins in wheat. In Iran, a part of the cultivated wheat is harvested after summer time and it is usually scheduled to be kept in stocks for months. This might be another reason for the high aflatoxin levels observed in this study. Combination of imported and homemade wheat grain, which is normally carried out to achieve the desired protein quality, may partly be responsible for the occurrence of TAF in wheat (Sivaraman *et al.*, 2002).

Flour ash content and aflatoxin content

The frequency distribution of aflatoxins in relation to the ash content of the tested flour samples is depicted in Figure 1. No significant correlation was detected between the ash content and the amount of aflatoxin ($P>0.05$). In some previous studies, researches discussed such a relationship with controversial results. Cereal bran was

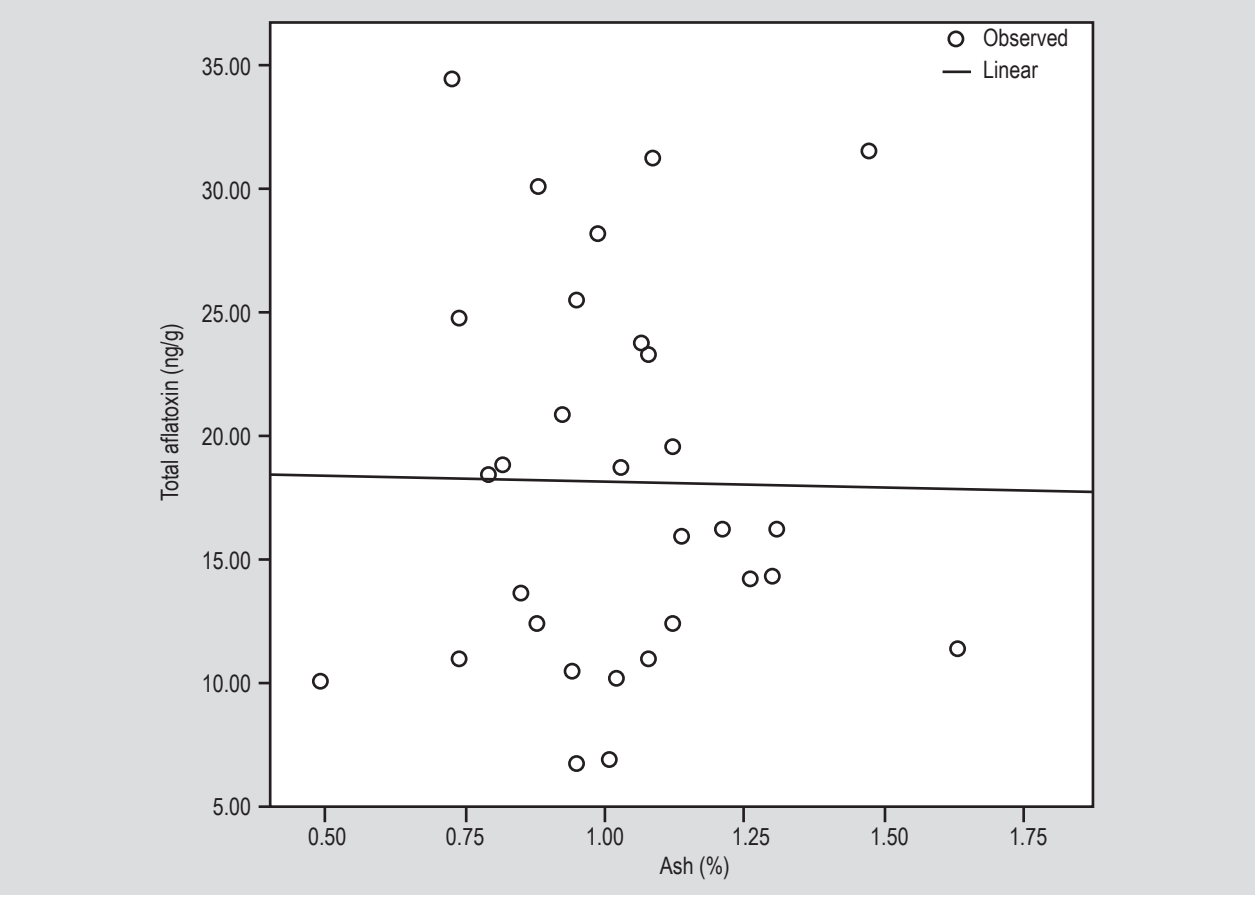

Figure 1. Total aflatoxin distribution in relation to the ash content of the tested flour samples.

stated to carry the highest concentration of mycotoxins in whole grain (Banu *et al.*, 2014; Castells et al., 2008; Thammawong et al., 2010; Vidal et al., 2013) and wheat processing techniques such as sorting, cleaning and debranning were stated to reduce the grain mycotoxin content before milling. However, some published data have denied any relationship in this regard (Cheli et al., 2013).

Effects of fermentation by yeast and yeast with *Lactobacillus plantarum* A7

The effect of dough fermentation with yeast (*S. cerevisiae*) alone or in combination with lactic acid bacteria *(L. plantarum* LA7) on the aflatoxin concentration in dough and bread are shown in Table 3. A significant decrease in aflatoxin levels was observed after fermentation ($P<0.01$).

Combination of yeast with lactic acid bacteria resulted in a 40.7% reduction in aflatoxin, which was significantly higher than 33% when yeast was used alone ($P<0.05$). However, whatever the microbial starter, the aflatoxin level was similar in bread.

The subject of mycotoxin binding by *S. cerevisiae* and lactic acid bacteria has been widely discussed in the literature (Gul *et al.*, 2005; Kabak and Ozbey, 2012; Lahtinen *et al*, 2004; Shetty and Jespersen, 2006). Although several species were investigated for such a potential in different types of food, *S. cerevisiae* was repeatedly reported to be the most efficient microorganism in quenching aflatoxin B_1 (Bento *et al.*, 2009) as well as other aflatoxins (Kabak and Ozbey, 2012). In general, lactobacilli strains have great impact on the physico-chemical, organoleptic and rheological characteristics of bread (Clarke and Arendt, 2005; Clarke *et al.*, 2004; Gul *et al.*, 2005). Some lactobacilli strains have been associated with aflatoxin removal capability and such ability has been associated with the cell-wall peptidoglycans (Lahtinen *et al.*, 2004). The peptides and glucans, which are released upon peptidoglycans activity, provide numerous easily accessible binding sites to quench toxins such as aflatoxins. Different binding mechanisms such as hydrogen bonding and ionic or hydrophobic interactions might be responsible for this adsorption (Huwig *et al.*, 2001). In a similar study, the capability of some probiotic bacteria to bind and remove aflatoxins from contaminated wheat during balady bread preparation, a traditional whole-grain bread in Egypt, was confirmed (Elsanhoty and Azeke, 2009).

Table 3. Aflatoxin levels according to sangak bread processing steps and using the four most contaminated flour samples.[1]

Processing step	Aflatoxins (ng/g)[2]	
	Yeast	Yeast + *Lactobacillus plantarum* LA7
Non-fermented dough	33.58^{a}	33.58^{a}
Fermented dough	22.27^{Ab}	19.92^{Bb}
Bread	17.85^{Ac}	16.48^{Ac}

[1] Mean aflatoxin content (dry basis) was obtained from sixteen data (4 samples × 2 repetitions × 2 measurements).
[2] Similar superscript capital letters in the same row are significantly different by paired t-test ($P<0.05$) and similar superscript lower letters in the same column are significantly different by ANOVA ($P<0.05$).

Considering the ISIRI (ISIRI, 2002) and EC limit (EC, 2006) for TAF in bread is set as 4 ng/g, all of the sangak bread samples that were prepared with the most heavily contaminated flour were not considered safe: they had 4 times the maximum acceptable level. Overall, it appears that sangak bread making might reduce by about 50% the aflatoxin level, although this was not sufficient to solve safety problems associated with the most contaminated flours.

Mycotoxins are relatively stable during cooking and processing and cannot be expected to decrease to safe levels in commercial cooking processes (Rao *et al.*, 1982; Sherif *et al.*, 2009). Chemical, biological and physical methods have been tried to reduce aflatoxin levels or reduce their content during cooking (Allam *et al.*, 2012; Rustom, 1997; Wu, 2004). Hwang and Lee (2006) showed that heating of wheat kernels, at 100 °C for 30 min reduced the aflatoxin level by about 38 and 17% depending on the wheat variety. They proposed higher temperature and longer treatment times to further reduce aflatoxins in kernels; in Korean steamed bread, aflatoxins could be eliminated up to 43% (Hwang and Lee, 2006). Thermal destruction of aflatoxin B_1 levels in maize was also reported (Ouluwafemi, 2004).

Result of the present study on aflatoxin reduction during bread preparation are in accordance with those obtained in balady bread by Elsanhoty and Azekeh (2009). However, with lactic acid bacteria, they had more success with more than 70% aflatoxin reduction.

In the present work, baking was not as efficient as the fermentation process to detoxify flour. Also, aflatoxin concentrations in the final products were not decreased to the safe levels during cooking and remained greater than the maximum permissible level in bread (4 ng/g). Differences in extraction according to the food matrix (flour, dough or bread) might also explain some of the data shown, which results in basis and error in the final results. Considering the lack of confirming data from more accurate analytical method, the likelihood of some under- or overestimation of the presented results could not be ignored.

4. Conclusions

This study showed that 56.7% of the sangak bread flour (bran-rich) in Iran carries aflatoxin levels at concentrations greater than the regulated maximum permissible level (15 ng/g). No significant correlation was detected between ash content and the concentration of aflatoxins in the tested flour samples (P>0.05). Comparing the common procedure of sangak bread preparation, applying or not lactic acid bacteria in combination with yeast, significantly decreased the aflatoxin levels in the fermented dough but no difference was seen in the sangak bread. Finally, despite the efficiency of both fermentation and baking techniques in aflatoxin reduction, none of these methods could improve the safety of heavily contaminated sangak bread flour.

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