EVALUATION OF CONTAMINATION OF WHEAT AND BREAD BY FUNGI AND MYCOTOXINS IN FEZ REGION OF MOROCCO

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ABSTRACT

The purpose of this study was first the determination of compliance of bread sale points in Fez city to hygienic principles and second analyze of bread sailed to mycotoxin and fungal contamination. The study was realized between January 2014 to April 2014.The results obtained show that the compliance for the five hygienic programs was 89.5% for personnel, 83.3% for commodity, 78.5% for method, 41.2% for environment and 23.52% for Materiel. For fungal analysis, the results indicated that the majority of samples were contaminated by *Aspergillus niger* and *Debaryomyces sp.* The level of aflatoxins (AF), deoxynivalenol (DON) and fumonisin toxins was determinate by using ELISA. The predominant contamination of bread was for aflatoxins with an average of 81.24µg/Kg in 88.8% of studied samples. For DON and fumonisin, about 38.8% of bread samples were contaminated by DON and 58.3% contaminated by fumonisin without exceeding the EU regulation. The wheat flours used to produce bread was analyzed and revealed same high contamination by Aflatoxins. In same conditions we have observed that local wheat flour from new harvest didn't contain any detectable Aflatoxins. In conclusion, these results, showed an important contamination of bread consumed by Moroccan by fungi and mycotoxins. This was related to origin of wheat used to produce bread and to incompliance to hygienic principles. The amelioration the good hygienic practices and the reduction of cereal storage period may probably reduce in future these contaminations observed.

Keywords: Hygienic programs – Mycotoxins – Cereal products- ELISA.

INTRODUCTION

Cereals represent the most important source of food in many countries (Pimentel & al., 2009); they can be contaminated by mycotoxins molecules, toxic secondary metabolites, as they play no clear role in the basic metabolic pathways used for growth and energy production. Some of these compounds have antibiotic properties such as penicillin, whereas others are potential toxins (Oueslati & al., 2012). Many species from the *Alternaria*, *Aspergillus*, and *Fusarium* genera, as well as some *Penicillium* species, and several others are known to produce mycotoxins (Zain & al., 2011) which are receiving a considerable attention worldwide as they show a wide range of pathological effects such as carcinogenicity, teratogenicity and mutagenicity (Oueslati & al., 2012). Fungus contamination is dependent on environmental factors like temperature, humidity, drought and inadequate storage conditions, but some surveys point to the possibility that it could be influenced by the method of farming, too (Kirincic & al., 2014).

Morocco is a North African country whose climate is characterized by high temperature and humidity level that seems to stimulate the toxigenic molds growth and their mycotoxins production, in which, cereals represent a staple food for population, therefore bearing high

social, economic and nutritional relevance. Moreover, cereals contribute to 12% approximately of the agricultural output and Moroccan households spend 25% of their food expenditure for this kind of products. On average, Morocco consumes 6 million tons of cereals each year. In addition, by 2020 the Moroccan population will require 8.5 million tons of cereals for the national consumption. Due to drought the country has endured during the last two decades, cereal yield production has been dramatically reduced in the range of 25–85% (National Institute of Agronomy Research of Morocco – INRA, 2002)leading to extensive importation from other countries. Thus, Morocco imports cereals for various countries particularly from France, USA, Canada, Brazil, Russia and Australia. It was reported that approximately 25% of cereals produced in the world are contaminated by mycotoxins (Zinedine & al., 2009). Preliminary surveys showed that Moroccan agricultural products including cereals appeared to be contaminated with spores of toxigenic strains of *Aspergillus.* Subsequently, a series of analysis supported by the Direction Frauds Repression (Ministry of Agriculture) between 1991 and 1992 showed that a corn sample was found contaminated with 18 μg/kg of AFB1 (Tantaoui & al.,1994) using Thin Layer Chromatography Technique. Recently, the analytical results of many studies in Morocco show that cereal and cereal products are contaminated by mycotoxins.

The aim of this study was to analyze fungal mycoflora and mycotoxin contamination of cereals produced in Fez, the Moroccan city.

METHODOLOGY

Realization of hygienic survey

The aim of our study was to analyze the level of compliance to hygienic principles at some bread sale points in Fez city (figure1). The study was conducted during a period of four months between January 2014 and April 2014. For this, a questionnaire was drawn up and used for evaluation of contamination by environment, by personal, and by equipment. The questionnaire was fulfilled basing on manager responses and self observation.

Samples

A total of 36 samples of cereal products commercialized in Fez city of Morocco were randomly purchased from popular markets and supermarkets. The samples included bread (N=23), wheat flour (N=6), biscuits (N=4), semolina (N=2) and corn product (N=1). Samples were directly taken to Laboratory for microbiological analysis and mycotoxin evaluation.

Fungal count, identification and toxigenic potential determination

Microbiological analyses were focused on the search of yeasts and molds, which represent the important fungal contamination of cereals (Larpent & *al.*, 1997). Twenty grams (20g) of sample were dispersed in 180 ml of 0.05% Tween 80 solution using a mixer. Samples were then homogenized for 10 min and aliquots were used for microbiological analysis (Tabuc & *al.*, 2007).

The isolation of yeasts and molds was realized by spreading a volume of 0.1 ml of the initial suspension on dishes containing the appropriate agar medium for each germ. PDA and YPG Mediums were used for mold and yeast isolation respectively and 25µg/ml of chloramphenicol was added in each medium for bacterial inhibition. Typical fungal colonies

were counted after 3.5 and 7 days of culture at 25°C and 31°C. Identification of the type of mold was performed according to the identification key published by Breton & *al.*,(1990).

Mycotoxins analysis

Samples were analyzed by the ELISA method. The RIDASCREEN (R-Biopharm AG', Germany) kits were used for determination of level of total aflatoxins (AFs) and deoxynivalenol (DON). The Agra-Qant (Romer Lab, Ferland) kit was used for determination of fumonisin (FM) levels. Mycotoxins extraction and tests was performed according to manufacturer's instructions. Each sample was extracted by appropriate solvent, methanol/water (70/30) for AF and FM, and distilled water for DON. The final extracts were diluted by distilled water and used for the specific determination by ELISA kits. The optical density was measured at 450 nm using ELISA 96 well plate reader. All standards and samples solutions were analyzed at least in duplicate. The calculations were made according to standard-curve realized in same conditions.

RESULTS

Hygienic evaluation of point of bread sales

In this study, samples were purchased from 19 points of sale of bread in Fez belonging to seven districts (figure 1). These areas were subject of this study investigating the hygienic status and the percentage of compliance to hygienic principles. This analysis was based on the responses of managers and on self observation. The investigation showed that the percentage of compliance for the five hygiene programs was 89.5% for Personal (e.g. the fact of blowing the nose, touching the dirt or smoking during the handling of the foodstuff), 83.3% for commodity (e.g. controlling the humidity and temperature in the storage areas), 78.5% for method (e.g. working and sales areas), 41.2% for environment (e.g. locals of storage and preparation of food) and 23.52% for Materiel (e.g. cleanliness of material used for preparation of foods and its presentation).

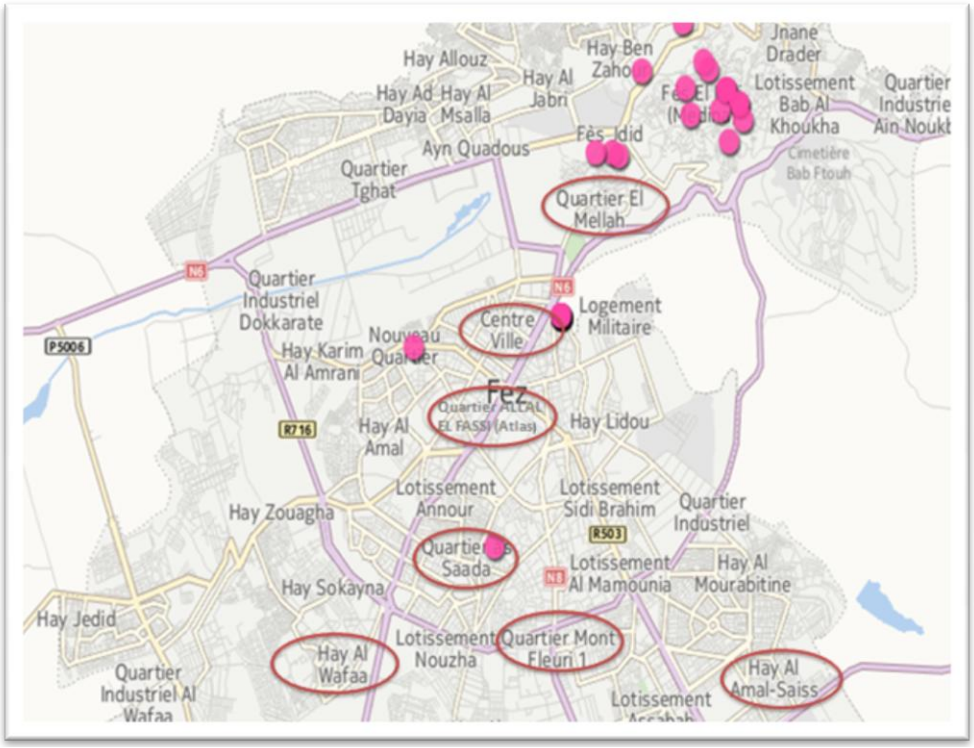

Figure 1: Areas visited during the investigation in the Fez city

Description of samples analyzed

The samples were collected from January 2014 to May 2014. The total samples was 36 including 64% bread samples, 17% wheat flours, 14% biscuits and 3% corn products.

Fungal population of cereal samples

We have analyzed 33 samples from 36 collected. The results show that from the 33 samples evaluated,13 samples (39,4%) were contaminated by fungal flora, of which 8 samples (24,2%) were contaminated by mold, 3 samples (9,1%) were contaminated by yeasts and 2 samples (6,1%) were contaminated by both molds and yeasts. The identification of Yeasts isolated from these samples showed that 60% were represented by *Debaryomyces sp* strains and an equal distribution (20%) was noticed for *Geotrichum candidum* and *Candida sp* strains.

The identification of molds showed that 57.1% of tested cereals were contaminated by *Aspergillus niger*, 28.6% of molds *Penicillium* species including *Penicillium notatum* and *Penicillium.sp, and* 14.3% of samples contaminated by *Fusarium.sp*. Because mycotoxins contamination depends on the fungal species developing on the substrate, a precise analysis of Aspergillus, Fusarium and Penicillium strains was done. Many fungal species isolated from cereal samples are known to be potentially toxigenic. Therefore, mycotoxins content of samples were analyzed.

Mycotoxin contamination of cereal products

Cereal samples were analyzed for aflatoxins (AF), deoxynivalenol (DON) and fumonisin (FM) contamination. Results reported in table 1 showed that AF contamination was mainly observed in 88.8% of samples analyzed (N=32) with a mean level of contamination of 42.0µg/kg. For bread samples, the AF level varied from 42µg/kg to 139µg/kg with an average of 81.24±0.85 (N=21). In other cereals samples, biscuits, wheat flours, semolinas and corn products, the levels were high than 100µg/kg.

For DON analysis, the levels measured varied from 7µg/kg to 92.5µg/kg. The average contamination in bread was 32.2µg/kg and in biscuits 52.7µg/kg and high level was obtained in wheat flour (sample F005). This later wheat flour was the most contaminated with FM with 484.8µg/kg followed by bread with an average of 74.7µg/kg varying from 5.6 to 133.8µg/kg. Three samples constituted of wheat flour and two bread samples were from cereals purchased from local and recent harvest of 2014. These samples didn't show any contamination by AF compared to other samples from imported cereals.

Table 1: Levels of cereal products contamination by aflatoxins (AF), deoxynivalenol (DON) and Fumonisin (FM). ND: Not Determinate.

	Cereal Products	AF (µg/kg)	DON (µg/kg)	FM (µg/kg)
Bread	B001	87,14 ±0,88	59,49±4,15	22,72± 0,74
	B002	102,47±0,76	70,11±10,13	ND
	B003	70,81±1,33	ND	60,23± 0,37
	B004	47,28±0,87	ND	50,94 ± 0,74
	B005	139,26±0,87	ND	88,45 ± 1,11
	B006	55,45 ±0,87	ND	ND
	B007	86,11 ±0,87	ND	ND
	B008	83,05 ±0,87	ND	ND
	B009	71,81 ±0,88	ND	ND
	B010	60,57 ±0,87	ND	ND
	B011	42,07 ±0,97	ND	ND
	B012	63,54 ±0,97	23,04 ± 5,16	50,57 ± 0,37
	B013	61,77 ±0,68	7,09 ±3,28	82,24 ± 0,75
	B014	103,73 ±0,64	ND	49,46 ± 0,74
	B015	47,64 ±0,51	ND	82,51 ± 0,37
	B016	60,71 ±0,73	ND	87,71 ± 0,37
	B017	86,11 ±0,87	25,62 ± 3,52	5,63 ± 0,74
	B018	72,83 ±0,87	ND	104,8 ± 0,37
	B019	110,64 ±0,88	ND	109,25 ± 0,37
	B020	116,78±0,88	ND	117,05 ±3,71
	B021	136,2±0,88	8,02± 2,34	133,77 ± 0,37
	B022	0	ND	ND
	B023	0	ND	ND
Wheat Flours	F001	152,65±0,05	ND	ND
	F002	149,29±5,16	ND	ND
	F003	103,56±0,81	ND	ND
	F004	138,9 ± 0,80	ND	ND
	F005	ND	91,52 ± 9,85	484,78 ± 4,23
	F006	0	ND	ND
Biscuits	B001	120,45 ±0,55	50,01 ± 2,11	25,43 ±1,67
	B002	116,5 ±0,6	47,66 ±0,23	188,71 ±4,23
	B003	113,13 ± 0,73	53,52 ± 0,87	112,58 ±4,23
	B004	125,55 ±0,57	55,41± 2,81	51,25 ±6,34
Semolinas	S001	118,45 ±0,65	45,78±2,11	42,79 ±2,11
	S002	105,86 ± 0,83	58,45±1,17	ND
Corn product	M001	120,75 ±1,26	7,79 ± 1,17	19,53 ± 4,23

DISCUSSION

This study reports in the first time the mycotoxin and the fungal contamination of bread and cereals products in Fez (Morocco). Many surveys done worldwide previously demonstrated that cereals could be contaminated with various fungal species, and that both fungal and

mycotoxins contaminations vary depending on the climate. Indeed, whereas the *Fusarium* species develop in the field on the living plants, *Aspergillus* and *Penicillium* mainly grows during storage (Miller & *al.*, 2002; Wilson & *al.*, 2002). In all cases, mould development and subsequent mycotoxin production is directly related to hydrothermal conditions (Tabuc & *al.*, 2007; Ramirez &*al.*, 2006). Because Morocco is located in North African country, which's Mediterranean climate may influence fungal species able to develop on cultures grown in this country, and both fungal and mycotoxins contamination of cereals may differ from those reported in other countries. Moreover, because fungal growth varies depending on the substrate, together bread, flour, biscuits, semolina and corn products were analyzed. We showed that *Aspergillus* fungi were very frequent contaminants in our samples.

This is in agreement with other study done in Italy and Spain (Giorni & *al.*, 2007*;* Medina & *al.*, 2007). This contamination in our case was related to long storage period since we haven't observed mycotoxin contamination of local cereals from recent harvest. All samples analyzed were from imported cereals with long storage periods.

Another study by Joshaghani shows that the species most commonly isolated fungi from samples of wheat were *Aspergillus Niger* (21.4%), followed by the species of *Fusarium spp* (17.8%) followed by *Penicillium spp* (8.9%) (Joshaghani & *al*,. 2013). In Iran, the fungal contamination of wheat samples collected from two different provinces still shows the dominance of three species of fungi including *Aspergillus spp*, *Fusarium spp* and *Penicillium spp* (Čonková & *al.*, 2006), also, an Algerian study in 2008 reveals the high frequency of contamination of wheat samples by species of *Fusarium*, *Penicillium*, *Aspergillus* (Riba & *al.*, 2008). The high frequency and abundance of *Aspergillus spp* species could be due to a failure in food production and/or preservation methods of the cereals.

Analysis of cereal products to their level of contamination by yeasts revealed the predominance of the species *Debaryomyce ssp* (60%) followed by *Candida sp* species and *Geotrichum candidum* species (20% for each). Indeed according to Joshaghani study in 2013, the main yeasts which can be present in the seeds of cereals and their by-products that are belonging to the species *Candida*, *Cryptococcus*, *Pichia*, *Sporobolomyces*, *Rhodotorula* and *Trichosporon* (Joshaghani & *al.*, 2013).

The presence of fungal species including species of the genus *Aspergillus*, *Fusarium* and *Penicelium* in the analyzed samples, which are recognized as major producer agents of mycotoxins in food mainly in grain products, suggests the likely presence of these molecules toxic in our samples. For this, three types of mycotoxins (aflatoxins, deoxynivalenol and fumonisin) were south in our sample using ELISA test. Our study shows that over 88% of samples contain a high concentration of aflatoxins ($>43\ \mu g/kg$) which widely grater to the European standard provided by the European Commission published in December 2006 (Regulation (EC) No. 1881/2006).This result is comparable to the study of Iqbal in 2014 where 41% of breakfast cereals samples were contaminated with aflatoxins (AFs) of which 8% of these samples present a higher rate of AFs to the standard set by the European Union in 2006 (Iqbal & *al.*, 2014) and other study published by Soleimany show that 50% of samples of cereals analyzed were contaminated by AFs (Soleimany & *al.*, 2011).

The results of deoxynivalenol assay showed that 38.8% of samples had a concentration lies between $3\mu g/kg$ and $101\mu g/kg$, however, 100% of positive samples presents a rate lower than the European standard (Regulation (EC) No. 1881/2006), these results are very close to those reported by Yanshen in China, in which 52.5% of the samples of commercial grain

analyzed have a concentration of deoxynivalenol between $7\mu g/kg$ and $534\mu g/kg$ remaining below the maximum limits set by European Union(Yanshen & *al.,* 2011, European Commission, 2006). Another study conducted in Morocco by Ennouari (Ennouari & *al.*, 2012) shows that from 81 of cereals tested, 9 samples (11.1%) were contaminated by DON which the concentration in samples positive ranged between $65\mu g/kg$ and $1310\mu g/kg$.

The results of fumonisin analysis show that 58.3% of samples were contaminated by fumonisin in a range between 4.9 and $489.01\mu g/kg$, however, all samples have a concentration below the European standard set by the European Commission (Regulation (EC) No 1881/2006). Ended, the previous reports from Bulgaria and other European countries describe the occurrence of *Fusarium moniliforme* in samples of infected grains in Bulgaria, suggesting potential for the presence of fumonisin in cereal grains (Radostina & *al.*, 2013).Taken together, these results prove that cereals produced in Morocco present a particular pattern of fungal mycoflora and mycotoxin contamination. Indeed, AFs appear have an important concentration in Moroccan cereals produced. This last point specially highlights that the carcinogenic mycotoxin AFs has still to be monitored in Morocco, since storage in temporary climatic conditions can lead to contamination of food mainly cereal products.

CONCLUSION

This study aims to assess initially the hygienic quality of some areas production of bread in Fez city. For this, we have prepared a questionnaire to bring out the hygienic conditions in visited stores. This first evaluation was completed by determination of fungal and mycotoxin analysis of cereal samples collected from these stores. These results show that high contamination may occur by observed unsanitary conditions especially cross-contamination, hygienic conditions of production, environment of the sale unity and equipment. The observation showed that all hygienic programs analyzed included major incompliance which must be ameliorated to enhance bread quality and decrease microbial contamination by these products. This may be realized by training cycles to introducing good hygienic practice in these small bread sale points.

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