Aflatoxin and Ochratoxin A residues in some meat additives

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Abstract: Contamination of meat additives with mould spores and mycotoxins like, aflatoxin and ochratoxin, is a major problem in many developing countries like Egypt as it leads to great public health hazards if consumed directly or added to the meat products. In this study, we investigated the contamination of some meat additives (Starch, soy flour, nutmeg, cumin, black pepper and red pepper) with different mould genera and subsequently screened their contamination potential with some mycotoxins like aflatoxin and ochratoxin A. Our results declared that, 100 % of the meat additive and spices were contaminated by mould. Aspergillus was the most predominant genus, (detected in 100% of samples), which identified into six species. *Aspergillus niger* and *Aspergillus flavus* were the most common Aspergillus species in this study. The means of aflatoxin residues in examined meat additive and spices arranged in descending manner as following: nutmeg > red pepper > soy-flour > starch > cummin > black pepper. However, ochratoxin A residues in examined meat additive and spices was ordered as following soy flour > nutmeg > starch > red pepper > cummin > black pepper. Great care should be taken during selection of the meat additives before introducing them to food commodities.
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1- Introduction

Meat and meat products are among the most important sources of high quality protein. They supply easily absorbed iron and are rich sources of vitamins like B group Meat processing has a wide variety of non-meat products (meat additive) referred to as binders or extenders and less frequently as fillers emulsifiers or stabilizers. These products are added to basic meat formulation for reducing the formulation cost and improving the meat product quality **(Bender, 1992)**.

Most popular meat products resulting from mixing of minced meat with filling materials which are added (salt - starch - skimmed milk powder - soy bean product - cumin- red, white and black pepper- ginger) where the end product obtained after cooking give the physical characteristics of required product.

Storage of meat additives in suitable condition such as (ambient temperature, relative humidity and air circulation) considered as favorable condition for mould development and mycotoxin production. Consumption of food contaminated with moulds and their toxic metabolites results in development of food-borne mycotoxicosis **(Miraglia *et al.*, 2009)**.

Mould toxicity has attracted attention, especially in the fields of agriculture and food industry. Microscopic filamentous fungi often contaminate vegetable and animal products, becoming a source of diseases in man and slaughter animals (**Laciakovei and Lack, 1994).** Mycotoxicosis is example of "poisoning by natural means" and thus is analogous to the pathologies caused by exposure to pesticides or heavy metals residues. The symptoms of mycotoxicosis depend on the type of mycotoxin, the amount and duration of the exposure, the age and health of the exposed individual, and many poorly understood synergistic effects involving genetics, dietary status, and interaction with other toxic insults (**Bennett and Klich, 2003).**

Aflatoxin, toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* fungi, are naturally occurring contaminants of food. Aflatoxins have been recognized as significant contaminants by the agricultural production community since the 1960s and control strategies have mostly eliminated harmful exposures in developed countries **(Guo, 2000).**

Ochratoxin A (OTA), the major compound, has been found in more than 10 countries in Europe and the USA. Ochratoxin formation by Aspergillus species appears to be limited to conditions of high humidity and temperature. Some Penicillium species may produce ochratoxin at temperatures as low as 5° C. OTA has been implicated in a human disease of kidney referred to as Balkan endemic nephropathy, characterized by tubular interstitial nephritis and associated with high incidence of kidney, pelvis, ureter and urinary bladder rumors in some eastern European countries (**Pfohl-Lesskowicz *et al.*, 2002).**

The regulation of mycotoxin in food and feed started in 1974, the number of countries with specific

regulations for mycotoxin has increased over the years. By the end of 2003, approximately 100 countries (covering approximately 85% of the world's inhabitants) had specific regulations or detailed guidelines for mycotoxin in food (**van Egmond *et al.*, 2007).** However, there is few available information about mycotoxin contamination in meat and meat products in Egypt. Thus, the aim of this work is to investigate the status of mould count and total aflatoxin (B1+B2+G1+G2) and ochratoxin A levels in most used meat additives in Egypt.

2. Materials and Methods

Sampling

One hundred and twenty samples of soy flour, starch, red pepper powder, nutmeg powder, cumin powder and black pepper powder (20 of each, sample weight is 100g for each) were collected from different markets from Sharkia and Cairo Governorates, Egypt. The samples were taken aseptically in sterile polyethylene bags and transferred to food control department; Faculty of Veterinary Medicine, Zagazig University, and then the following investigation were carried out

Estimation of total mould count and identification of the isolated mould genera

Preparation of serial dilution and counting of mould according to **APHA (1984).**The colonies of the moulds grown on malt agar were inoculated onto three different agars: czapek Dox agar, malt agar and malt agar containing 5% Nacl. The moulds were identified based on morphology and growth characteristics according to Samson and Pitt (2000) and Samson et al. (2004)

Quantitative estimation of total aflatoxins

The quantitative estimation of total aflatoxins (B1+B2+G1+G2) by flourometer (VICAM. Series 4) was done according to the manufacturer's instructions. In brief, 25 g of ground samples with 5 g salt (NaCl) were extracted in 100 mL methanol: water (80:20) three times. The extracts were diluted 4 times with DDW and filtrated using glass microfibre filter; 4 mL filtered diluted extract passed through AflaTest® -P affinity column at a rate of about 1-2 drops/second. Elution of affinity column by passing 1.0 mL HPLC grade methanol through column at a rate of 1-2 drops/second and collecting all of the sample elute (1 mL) in a glass cuvette. Then add 1.0 mL of AflaTest® Developer to elute in the cuvette. Mixing well and place cuvette in a calibrated fluorometer. Reading of aflatoxin concentration after 60 seconds. The detection limit from 0.1 ppb to 300 ppb.

Quantitative estimation of Ochratoxin

The quantitative estimation of ochratoxin by flourometer (VICAM. Series 4) was done according to the manufacturer's instructions. In brief, 50 g of ground samples were extracted in 100 mL methanol: water (80:20) three times. The extracts were diluted 4 times with phosphate buffer saline and filtrated using glass microfibre filter, 20 mL filtered diluted extract passed through OchraTest® -P affinity column at a rate of about 1-2 drops/second. Then 10 mL of 0.1% Tween 20/PBS passed through the column at a rate of 1-2 drops/second. Place glass cuvette (VICAM part # 34000) under OchraTestTM column and add 1.5 mL OchraTestTM elution solution into glass syringe barrel. and fill syringe barrel with 10 mL of PBS. Pass PBS through the column at a rate of 1-2 drops/second. Elute column at a rate of 1 drop/second, collect all of the sample eluate (1.5 mL) in a glass cuvette. Mix well, and place cuvette immediately into calibrated fluorometer. Read ochratoxin concentration after 60 seconds. The detection limit from 0.1 ppb to 100 ppb.

Statistical analysis

Statistical significances were evaluated using Tukey-Kramer HSD difference test(JMP) (SAS Institute, Cary, NC, USA), $P<0.05$ was considered to be significant.

3.Results and Discussion

Mycotoxin contamination of the meat additives is considered the major source of contamination of the manufactured meat products after that. Screening of this problem will help us to setup control strategies for this problem, which is very common in many developing countries like Egypt. In first, we investigated the mould growth in these meat additives. The results achieved in Fig.(1) revealed that the mean ± SD mould count in log10 CFU/gm of the examined meat additive and spices were 2.58 ± 0.26, 2.73 ± 0.32, 4.19 ± 0.20, 3.51 ± 0.35,1.97± 0.44 and 2.65 ± 0.163 log10 CFU/gm, respectively in examined soy-flour, starch, red pepper, nutmeg, cummin and black pepper. On the overall, 100 % of the meat additive and spices were contaminated by mould. Lower mould count detected in black pepper 0.80 ±0.37 log10 CFU/gm and in Ashanti pepper 2.47 ±0.36 log10 CFU/gm, while nearly similar mould count detected in nutmeg 3.45 ±0.20 log10 CFU/gm during survey on Mycobiota and aflatoxin conducted in the Nigerian spices **(Ezekiel *et al.*, 2013).**

The mould count in different spices coincides with those obtained by **Bokhari (2007)** in Saudi Arabia. Spices are largely produced in countries where tropical climates (high ranges of temperature, humidity and rainfall) are favorable to mycotoxin contamination. Furthermore, they are usually dried on the ground in the open air in poor hygienic conditions that even more promote growth of mould and production of mycotoxin (**Martins *et al.*, 2001**). There were significant ($p<0.05$) differences in the mould load on the meat additive and spices as red pepper had the highest count (4.19 $\log_{10}$CFU/gm) count while cumin was recorded as the least (1.97 Log

log_{10}CFU/gm). This may be attributed to the differences in the shape and structure of the spices which facilitate mould growth on it, the initial moisture in the product during drying process, the material of vehicle in which spices stored. Additionally, some spices have active principals against mould like piperine and pepper oil was found to which inhibit fungal growth in a dose-dependent manner **(Madhyastha and Bhat, 1984).**

Six mould genera isolated from the meat additive examined in this investigation. Aspergillus was the most predominant genus, (detected in 100% of samples), which identified into six species. *Aspergillus niger* and *Aspergillus flavus* were the most common Aspergillus species in this study as they emerged with percentages of 20% to 90% in cumin, nut meg and red pepper (Table 1). Our results goes in agreement with **Abdel-Hafez and El-Said (1997); El-Kady *et al.* (1992) and Abdulkadir *et al.* (2003)** who reported that *A. niger* and *A. flavus* were the most frequently encountered and widely distributed in spices. *A. ocharceus* and *A. fumigatus* were isolated in moderate frequency, while low frequency of occurrence reported in *A. parasiticus* and *A. ochraceous*. These species were commonly isolated from different kind of spices **(Moharram *et al.*, 1989; El-Kady *et al.*, 1992; Abdulkadir *et al.*, 2003)**.

In regards to other mould genera, Penicillium was detected in varying percentages, the highest level is reported in nutmeg samples 13(45%) and the lowest one in cummin and black pepper 1 (5%). The highest level of Cladosporium was detected by 4 (20%) in soy flour samples and the lowest one 1 (5%) in black pepper (Table 1). Alternaria, Mucor and Rhizopus were putative genera in this study as they recorded the lowest incidence in the examined samples. In correspondence with our results, **Omafuvbe and Kolawole (2004)** identified Aspergillus, Fusarium, Itersonilia, Botrydiplodia, Penicillium, Mucor, Candida, and Brettanomyces from spices In Nigeria. Similarly, Aspergillus, Penicillium and Rhizopus were common contaminants for all spices In Turkey **Erdogan (2004).** The variation of incidence among examined meat additive may be due to the hygienic condition during dryness of the product, storage condition and handling during distribution.

The prevalence of *Aspergillus flavus, Aspergillus parasiticus, Aspergillus ochraceus* and Penicillium considered mirror reflecting the possibility of aflatoxin and ochratoxin in the examined meat additive. The means of aflatoxin residues in examined meat additive and spices arranged in descending manner as following: nutmeg > red pepper > soy-flour > starch > cummin > black pepper (Table 2).

The level of aflatoxin in the spices were below the maximum permissible limit (10 ppb) of **European commission (EC) regulation (1998, 2002)** as guideline levels for spices, except only 3(15%) samples of nutmeg. Aflatoxin level was significantly higher in nutmeg compared with other spices. This may be due to the shape of nutmeg fruit, which facilitate the growth of mould in side it so when stored in favorable conditions aflatoxin production occur. Soy flour and starch samples were contaminated with measurable concentrations of aflatoxin with percentages of 45% and 60%, the concentration ranged from 2.9 to 8.1 and 1.2 to 3.9 ppb respectively. Only 2 (10%) samples from soy flour exceed the permissible level (4 ppb) established by **EC (1998, 2002)** as guideline levels for cereal. Our results goes in agreement with **Baydar *et al.* (2005)** who detected aflatoxin in cereal-flours and starches collected from markets and traditional bazaars in Ankara, Turkey in levels ranged from 0.03 to 3.16 ppb. However, lower concentrations were found in soybean paste in Korean market (**Ok *et al.*, 2007**).

The contamination percentages in examined spices were 70%, 60%, 30% and 20% with mean values of 2.01 ± 0.45, 4.14 ± 1.17, 0.64 ± 0.24 and 0.47 ± 0.22 ppb for red pepper, nutmeg, cummin and black pepper, respectively. These results was in line with the results reported In Spain as **Hernández Hierro *et al.* (2008)** found that the total aflatoxin residual in red paprika is 3.3 µg/kg. Additionally, **Santos *et al.* (2010)** found that aflatoxins contaminate 40% of 35 chilli samples, all at concentrations below the maximum allowable limits. In Korea, **Cho *et al.*** (2008) investigated 88 spice samples (including black pepper) and found aflatoxin contamination in only 13.6% (exclusive of black pepper) at concentrations below 5 µg/kg. Higher aflatoxin residues in powdered red pepper detected in turkey at range from 1.8 to 16.4 ppb (**Erdogan, 2004).** Higher aflatoxin levels were reported for cumin and black pepper in Egypt (**El-Kady *et al.*, 1995)** with a range of 8 to 35µg/kg. In Saudi Arabia, **Bokhari (2007)** detected aflatoxin in black pepper from 25 to 40µg/kg. Contrary to this finding, **Elshafie *et al.* (2002)** did not detect any aflatoxin in 15 selected samples out of 105 spices samples including cumin, black pepper collected from Sultanate of Oman.

The means of ochratoxin A residues in examined meat additive and spices arranged in descending manner as following soy flour > nutmeg > starch > red pepper > cummin > black pepper (Table 3). Ochratoxin A residues detected in soy flour and starch with percentages of 70% and 50% and mean ± SE values of 2.59 ± 0.57 and 1.93 ± 0.55ppb respectively. On comparing with the maximum residue limit of EC (2002) we found that 4 (20%) and

2 (10%) exceeded this limit. Nearly similar results obtained by **Baydar *et al.* (2005)** as they detected ochratoxin A in cereal-flours and starch collected from markets and traditional bazaars in Ankara, Turkey and levels ranged from 0.27 to 4.07 ppb. Lower ochratoxin A levels in cereals detected in Morocco **(Zinedine *et al.*, 2006).**

Ochratoxin A levels in other spices were 75%, 55%, 20% and 30% with mean values of 1.67 ± 0.47, 1.94 ± 0.51, 0.22 ± 0.10 and 0.21 ± 0.08 in red pepper, nutmeg, cumin and black pepper, respectively. Only 2(10%) and 3(15%) samples of red pepper and nutmeg exceeded the maximum residue limit of EC(2002). Higher ochratoxin A residues were recorded in Spain, as **Hernández Hierro *et al.* (2008)** found ochratoxin A in 67 % of red paprika samples, at concentration of 11.9 µg/kg. In North African countries, the foods most suspected to harbor ochratoxin A are domestic and imported cereals such as wheat and sorghum, olives, poultry products, and spices **(Grosso *et al.*, 2003).**

Aflatoxins are known as dietary human carcinogens of fungal origin and have a well-documented genotoxic effects **(Fapohunda *et al.*, 2008; Ezekiel *et al.*, 2011)**. There is a clear association between elevated exposure to ochratoxin A and cases of human nephropathies in Tunisia and Egypt (**Maaroufi *et al.*, 1995; Wafa *et al.*, 1998; Hassen *et al.*, 2004**). In conclusion, great care should be taken during selection of the meat additives before introducing them to food commodities.

Table (1): Incidence of isolated mould species and genera in examined meat additives (n=20)

	Soy flour	Starch	Red pepper	Nutmeg	Cumin	Black pepper
Aspergillus niger	13(65%)	11(55%)	18(90%)	17(85%)	4(20%)	6(30%)
Aspergillus flavus	7(35%)	8(40%)	12(60%)	16(80%)	4(20%)	5(25%)
Aspergillus parasiticus	0	2(10%)	3(15%)	4(20%)	1(5%)	0
Aspergillus fumigatus	3(15%)	2(10%)	4(20%)	7(35%)	2(10%)	1(5%)
Aspergillus ochraceus	11(55%)	6(30%)	7(35%)	9(45%)	2(10%)	2(10%)
Aspergillus terrus	3(15%)	2(10%)	9(45%)	2(10%)	0	0
Penicillium	5(25%)	6(30%)	8(40%)	13(45%)	1(5%)	1(5%)
Cladosporium	4(20%)	3(15%)	2(10%)	2(10%)	2(10%)	1(5%)
Alternaria	1(5%)	3(15%)	2(10%)	3(15%)	0	1(5%)
Mucor	3(15%)	0	3(15%)	0	2(10%)	0
Rhizopus	1(5%)	4(20%)	0	3(15%)	0	0

Table (2): Aflatoxin residues (ppb) in examined meat additives and spices (n = 20)

Samples	positive samples	Minimum	Maximum	Mean ± SE*	Number and percentages of samples exceed MRL**
Soy-flour	**9 (45%)**	**2.9**	**8.1**	**1.72 ± 0.50^{b}**	**2 (10%)**
Starch	**11 (60%)**	**1.2**	**3.9**	**1.33 ± 0.30^{b}**	**0**
Red pepper	**14 (70%)**	**1**	**6.8**	**2.01 ± 0.45^{b}**	**0**
Nutmeg	**12 (60%)**	**1**	**15**	**4.14 ± 1.17^{a}**	**3 (15%)**
Cummin	**6 (30%)**	**0.8**	**3**	**0.64 ± 0.24^{b}**	**0**
Black pepper	**4 (20%)**	**0.4**	**3.1**	**0.47 ± 0.22^{b}**	**0**

***Mean calculated based on total examined samples**

****European Commission Regulations (1998, 2002)** set a maximum residue limits for total aflatoxin 4 ppb for cereals and 10 ppb for spices.

Table (3): Ochratoxin A residues (ppb) in examined meat additives and spices (n = 20)

Samples	positive samples	Minimum	Maximum	Mean ± SE*	Number and percentages of samples exceed MRL**
Soy-flour	**14 (70%)**	**0.7**	**7.1**	**2.59 ± 0.57^{a}**	**4 (20%)**
Starch	**10 (50%)**	**0.8**	**7.8**	**1.93 ± 0.55^{ab}**	**2 (10%)**
Red pepper	**15 (75%)**	**0.9**	**8.3**	**1.67 ± 0.47^{ab}**	**2 (10%)**
Nutmeg	**11 (55%)**	**2.7**	**6**	**1.94 ± 0.51^{ab}**	**3 (15%)**
Cummin	**4 (20%)**	**0.6**	**1.4**	**0.22 ± 0.10^{b}**	**0**
Black pepper	**6 (30%)**	**0.3**	**1.3**	**0.21 ± 0.08^{b}**	**0**

***Mean calculated based on total examined samples**

****European Commission Regulations (2002)** set a maximum residue limits for Ochratoxin A 5 ppb.

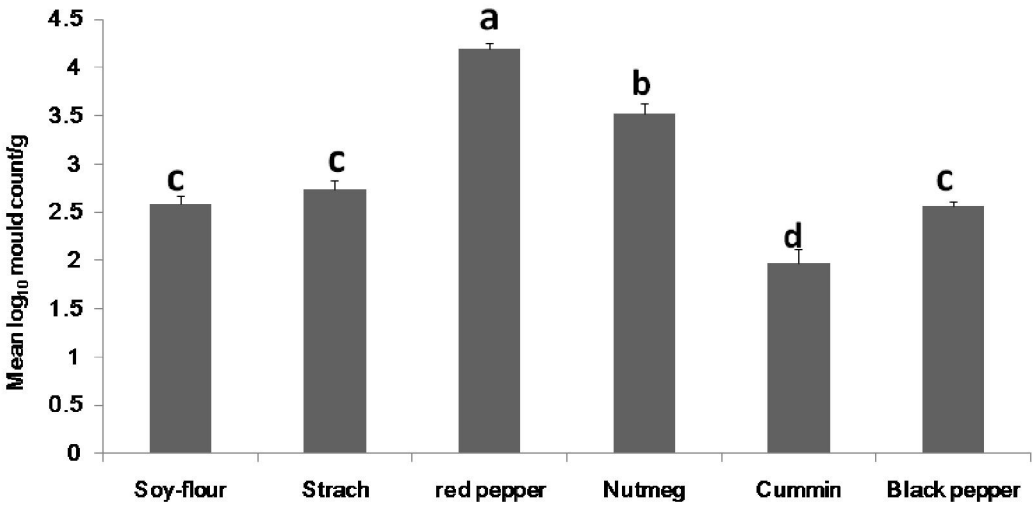

Fig. 1: Mould count in examined meat additives

Mean $\log_{10}$ mould count/g of examined meat additives samples. Data represent mean values ± SD. Columns carrying different letter are significantly different with each other ($p < 0.05$).

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