Total aflatoxins and mould contamination in animal feed and bovine edible offal

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A B S T R A C T

Aflatoxins are toxic metabolites produced by moulds. These metabolites are known as dietary human carcinogens of fungal origin and have well-documented genotoxic effects in human and animals. The objectives of this study were firstly, to screen the incidence of mould contamination in the bovine edible offal and animal feed. Secondly, estimation of total aflatoxins in such samples was done. Thirdly, correlations between mould growth and aflatoxin production in the examined samples were analyzed. The achieved results declared high contamination percentages of the examined bovine edible offal and animal feed with different mould genera and aflatoxins. Intestine and liver samples had the highest mould counts and total aflatoxin residues as compared with other examined offal, respectively, while yellow maize either powdered or crushed had the highest contamination levels among examined animal feed. *Aspergillus spp.,* in particular, *Aspergillus niger*, and *Penicillium spp*. were the most predominant isolated moulds in all examined samples. Total aflatoxins exceeded the maximum permissible limits set by Food and Agriculture Organization in the examined liver and animal feed samples with variable percentages. Positive correlations between mould growth and aflatoxin residues were recorded in both edible offal and animal feed. Finally, possible health implications on human and animals were discussed.

Keywords: Aflatoxins, mould, offal, animal feed, health implications

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1. INTRODUCTION:

Aflatoxins (AFs) are secondary fungal metabolites, which are mainly produced by *Aspergillus (A.) flavus and A. parasiticus*. Total aflatoxins is a term that refers to the sum of four aflatoxins namely AFB1, AFB2, AFG1 and AFG2. Aflatoxins find their way to the human body through consumption of contaminated foods like agricultural crops, cereals, animal products (Kumar et al., 2016). Aflatoxins are a major source of disease outbreaks worldwide. In 1993, the International Agency for Research on Cancer (IARC) classified AFB1 and mixtures of AFs as Group 1 carcinogens (International Agency for Research on Cancer (IARC), 2002). In Egypt, AFs consumption raises the risk of liver cancer by more than ten-fold compared to either exposure alone in people with hepatitis B and C viral infection (Turner et al., 2008). Contamination of meat, meat products, agricultural crops and animal feed with mycotoxeginic moulds is a problem of magnitude worldwide, especially, in tropical and subtropical areas. The predisposing conditions for mould growth and mycotoxin production relate mainly to poor hygienic practices during handling, transportation and storage, high temperature and moisture content. Moulds cause various degrees of deterioration and decomposition of foods producing in many cases abnormal flavors and odors and in many cases mycotoxin production (FDA, 2001).

Edible offal is considered as a major economic source of animal derived protein, vitamins, essential trace elements and micronutrients. Offal is contributing to enjoyment of food with unique flavors, aromas and texture. Offal is very popular food in many Middle Eastern countries like Egypt. Moreover, offal like intestine is a major ingredient in the meat-products industry, sausage casings in particular (Darwish et al., 2014a). However, these animal byproducts might be exposed to a vast array of xenobiotics such as AFs and microorganisms like mycotoxeginic moulds. Edible offal might be contaminated with AFs due to either growth of aflatoxeginc moulds or exposure of animals during their lifetime to AFs through ingestion of contaminated animal feed (Darwish et al., 2016).

Animal feeds are the major source of animal exposure to mycotoxeginic moulds and aflatoxins. Moulds can invade and grow on virtually any type of animal feeds and human food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. Contamination of animal feeds and human foods by moulds can result in substantial economic losses to producer, processor, and consumer (FDA, 2001).

Thus, the objectives of this study were to screen the occurrence of mould contamination in bovine edible offal and animal feed marketed in Zagazig city, Sharkia, Egypt. Secondly, to estimate AFs residues in the positive samples. Thirdly, to analyze correlations between mould growth and aflatoxin production in the examined samples. Finally, to discuss the public health implications of the existed moulds and AFs.

2. MATERIAL AND METHODS:

2.1. Collection of samples:

One Hundred random samples; fifty each of bovine edible offal and animal feed. Bovine edible offal included 10 samples each of liver, rumen, intestine, kidney and muscle. Muscle samples were excised from the round. Offal samples were collected randomly from butchery at Zagazig city, Egypt. Animal feed samples included 10 samples each of whole, crushed, powdered yellow maize, bran and cotton seed oil cake were collected randomly from different animal feed shops at Zagazig city, Egypt. Samples were identified, packed and transferred to the laboratory, subjected to the mycological examination upon arrival and samples intended for measurements of aflatoxin residues were stored at -20 ºC.

2.2. Preparation of samples:

Twenty-five grams from each offal sample were aseptically excised. In case of animal feed samples, 25 g from each sample were weighed. Samples were prepared according to the method recommended by (American Public Health Association (APHA), 2001). In brief, all samples were homogenized in 225 ml of sterile buffered peptone water 0.1% at 2500 rpm for 2 min using a sterile homogenizer (type M-P3-302, mechanic, precyzina, Poland). Such homogenate represents the dilution of 10^{-1}, and then decimal dilutions were done.

2.3. Determination of the total mould count:

Malt extract agar media and Czapeck-Dox agar with 5% Nacl (Oxoid, Basingstoke, UK) were used as culture media for enumeration and isolation of different moulds. The total mould count (TMC) was determined by culturing duplicate plates on each media followed by incubation in dark at 25 ºC for 5-7 days. During the incubation time, the plates were examined daily for the star-shape mould growth, which is picked up under aseptic conditions with its surrounding cultivated medium and transferred into malt extract slope agar (Oxoid) then kept for further examination. Estimation of TMC was obtained by counting of the cultured agar plates of acidified malt extract agar and osmophilic moulds on Czapeck-Dox agar (American Public Health Association (APHA), 2001). Mould counts were converted into base 10 logarithms of colony forming units per g of samples (log 10 cfu/g).

2.4. Identification of isolated moulds:

The identification of isolated mould genera were carried out based on their micromorphological properties (Pitt and Hocking, 2009). In brief, the isolates were sub-cultured on malt extract agar and Czapeck-Dox agar, incubated at 25 ºC for 5-7 days. The identification of the colonies was carried out by careful observation and measurements of the macroscopical and microscopical characteristics of the mould colonies, which were recorded in data sheet.

2.4.1. Macroscopical examination:

The cultures were examined daily for the rate and pattern of growth during the incubation period. Observations were made for the consistency of the surface growth; the pattern of folding (rugae); the distinctness of the colony margin and for the presence of pigment either on the surface or the reverse of the colony or diffusing into the surrounding medium. Both the surface and backside of the colony were examined.

2.4.2. Microscopical examination:

From the periphery of 5-7 days old mould colony, a triangular piece was transferred to a clean glass slide. With two mycological needles, the piece of the colony was distributed with one or two drops of 70 % alcohol. One drop of lactophenol stain was added after evaporation of the alcohol. Then covered by a clean cover slide followed by gentle pressure to remove the excess of fluid and air bubbles as well as to depress the hyphae and other structures for facilitating microscopic examination. The prepared slides were examined under low power and oil immersion lens to characterize the measurements and morphological structures of the mould growth, concerning the

conidial stage, head, vesicle, sterigmata, conidiophore and conidia.

2.5. Calculation of mould relative density (RD %):

Calculation of the isolation relative density of different mould genera and species according was done according to the method of (Saleemi et al., 2010) using the following formula:
RD % = Number of isolates of a fungus or species/ total number of fungal isolates * 100

2.6. Quantitative estimation of total aflatoxins:

The quantitative estimation of total aflatoxins (B1+B2+G1+G2) by flourometer (VICAM. Series 4) was done following the method published before (Abd-Elghany and Sallam, 2015; El-Ghareeb et al., 2013) with slight modifications. In brief, 25 g of ground samples with 5 g 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.

2.7. Statistical analysis:

Statistical significance was evaluated using the Tukey–Kramer HSD test (JMP statistical package; SAS Institute Inc., Cary, NC). In all analyses, $P < 0.05$ was taken to indicate statistical significance (Gomez and Gomez, 1984).

3. RESULTS:

The recorded results in this study declared that mould contamination of bovine edible offal and animal feed was obvious. The incidence percentages of mould contamination of edible offal were 80%, 70%, 100%, 50% and 30% in the examined liver, rumen, intestine, kidney and muscle samples, respectively (Figure 1A). In case of examined animal feed samples, these percentages were 60%, 70%, 80%, 50% and 30% in the examined whole, crushed, powdered yellow maize, bran and cotton seed oil cake, respectively (Figure 1B). Total mould count (TMC) (log 10 cfu/g) was investigated in the examined samples as clear in figure 2. The mean ± SE values of TMC in the examined edible offal were 3.5 ± 0.15, 4.5 ± 0.55, 5.87 ± 0.93, 2.8 ± 0.33, 2.77 ± 0.54 log 10 cfu/g in the examined liver, rumen, intestine, kidney and muscle samples, respectively (Figure 2A). These values were 3.15 ± 0.33, 4.25 ± 0.88, 5.4 ± 0.45, 2.2 ± 0.44 and 2.5 ± 0.22 log 10 cfu/g in the examined whole, crushed, powdered yellow maize, bran and cotton seed oil cake samples, respectively (Figure 2B).

This study was extended to identify the prevalent mould genera in the examined samples. Relative density (RD %) of the isolated and identified moulds revealed that the prevalent mould genera in edible offal and animal feed, respectively, were *Aspergillus sp.* (35.29% & 28.41%); *Penicillium sp.* (29.41% & 22.73%); *Cladosporium sp.* (5.88% & 11.36%); *Fusarium sp.* (11.76% & 17.05%); and *Mucor sp.* (11.76% & 6.82) (Tables 1 &2). *Aspergillus sp.* was further identified into *A. niger, A. flavus, A. fumigatus, A. terreus* and *A. ochracous* in edible offal and animal feed. Total RD % of the isolated and identified Aspergilli in edible offal were 14.12%, 10.59% and 10.59% in *A. niger, A. flavus* and *A. fumigatus,* respectively. In animal feed samples, these percentages were 11.36%, 6.82%, 5.68%, 3.41% and 1.14% in *A. niger, A. flavus, A. fumigatus, A. terreus* and *A. ochracous,* respectively (Tables 1 & 2). Chemical analysis of offal samples revealed that AFs were detected only in liver, rumen and intestine samples with percentages of 40%, 30% and 30% (Figure 1A). Mean residual concentrations of total AFs (ppb) in these samples were 4.9 ± 0.52, 2.5 ± 0.14, 2.7 ± 0.23 ppb (Figure 3A). The incidence percentages of AFs contamination in animal feed samples were 30%, 40%, 50%, 30% and 30% in the examined whole, crushed, powdered yellow maize, bran and cotton seed oil cake samples, respectively (Figure 1B).

The recorded mean AFs residual concentrations in the aforementioned feed samples were 12.11 ± 2.25, 27.15 ± 2.5, 50.5 ± 3.7, 15.72 ± 1.8 and 4.45 ± 1.56 ppb, respectively (Figure 3B). Recorded results in figure 4 showed the percentages of samples exceeding recommended maximum permissible limits (MPL) of total aflatoxins by Food and Agriculture Organization (FAO, 2004), which were 4 ppb in edible offal and 10 ppb in animal feed. The results showed that 20% of liver samples exceeded MPL in the edible offal. However, in case of animal feed, 10%, 40%, 50% and 10% exceeded MPL in the examined whole, crushed, powdered yellow maize and bran samples (Figure 4).

Drawn scatter plots revealed positive correlation ($R^2 = 71$) between the incidence of mould contamination and aflatoxin production in

edible offal. Correlation coefficient in case of animal feed revealed positive value (R^2 = 66) between the incidence of mould contamination and aflatoxin production and total mould count and total aflatoxin residues (R^2 = 85) (Figure 5 A, B, C & D).

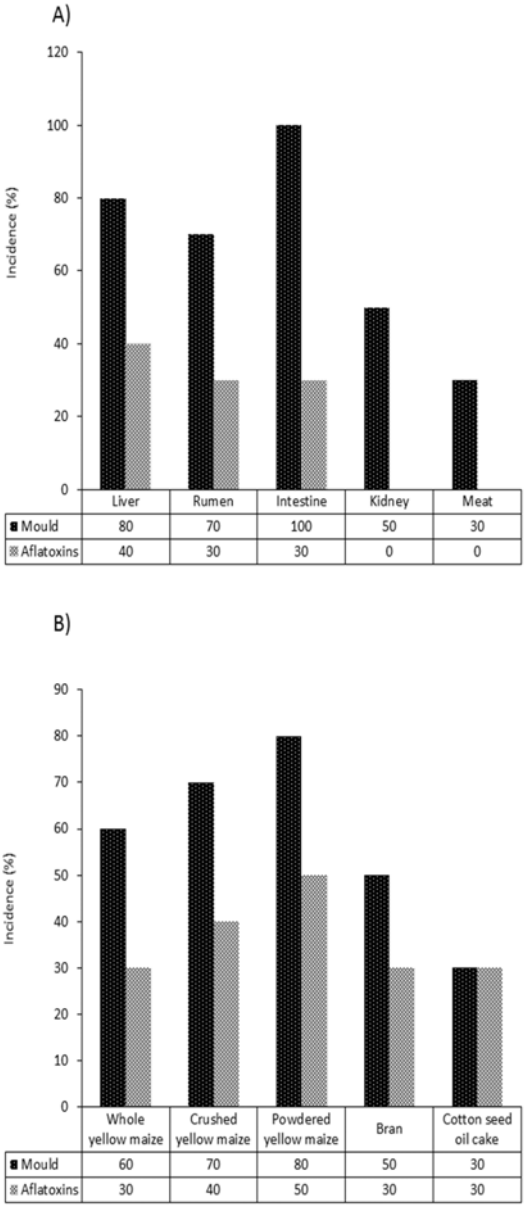

Figure 1: Incidence (%) of mould and aflatoxin contamination in bovine edible offal and animal feed.
A) Bovine edible offal; B) Animal feed

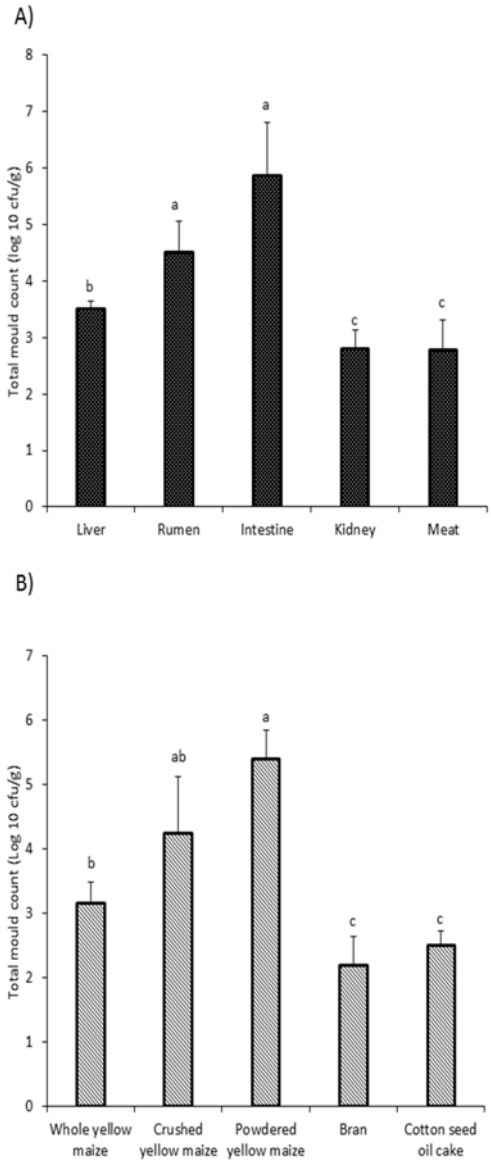

Figure 2: Total mould count in bovine edible offal and animal feed.
Total mould count in the examined A) Bovine edible offal; B) Animal feed. Values represent means ± SE (Log 10 cfu/g) of ten samples for each offal or animal feed. Each sample was measured in duplicates. Columns with different letters differ significantly at $P < 0.05$.

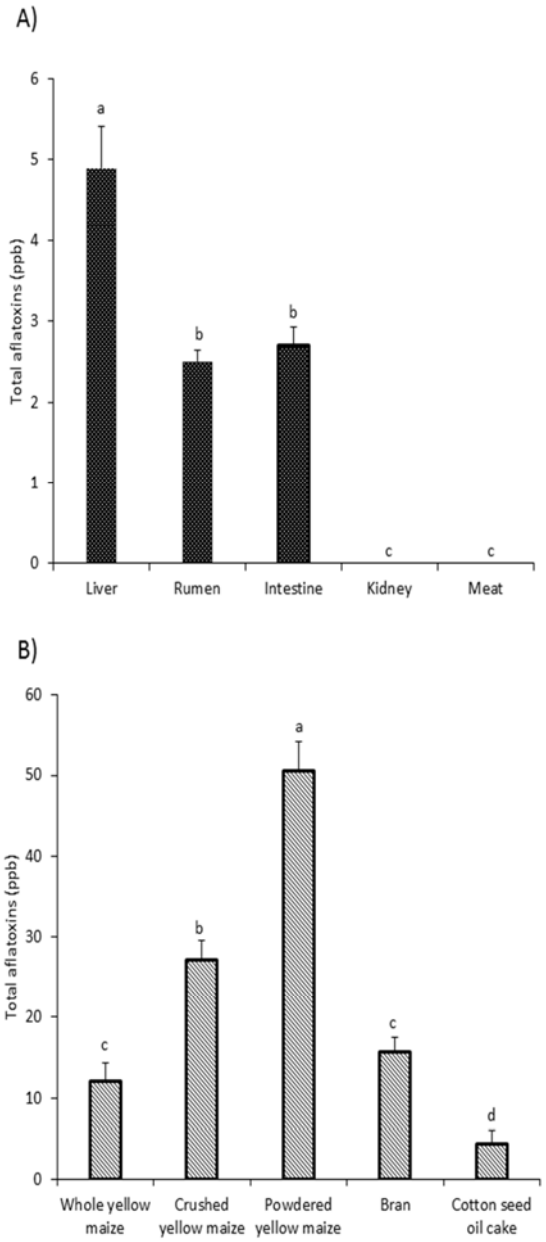

Figure 3: Total aflatoxin residues in bovine edible offal and animal feed.
Total aflatoxin residues (ppb) in the examined A) Bovine edible offal; B) Animal feed. Values represent means ± SE of ten samples for each offal or animal feed. Each sample was measured in duplicates. Columns with different letters differ significantly at $P < 0.05$.

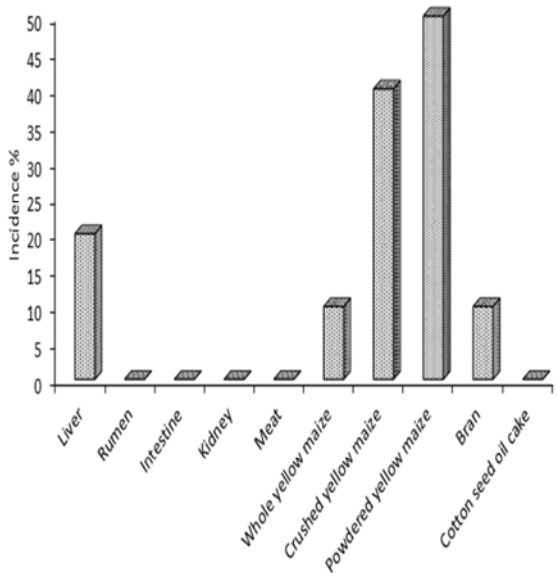

Figure 4: Incidence (%) of samples exceeding maximum permissible limits of total aflatoxins in bovine edible offal and animal feed.
Maximum permissible limits for total aflatoxins were retrieved from Food and Agriculture Organization (FAO) (2004) and were set as 4 ppb for offal and 10 ppb for animal feed.

Table 1. Number of different mould isolates and their relative density percentage (RD %) in the examined bovine offal

	Liver		Rumen		Intestine		Kidney		Muscle		Total	
	Number	RD %	Number	RD %	Number	RD %	Number	RD %	Number	RD %	Number	RD %
Aspergillus	6	7.06	10	11.76	12	14.12	1	1.18	1	1.18	30	35.29
A. niger	2	2.35	3	3.53	5	5.88	1	1.18	1	1.18	12	14.12
A. flavus	3	3.53	3	3.53	3	3.53	0	0	0	0	9	10.59
A. fumigatus	1	1.18	4	4.71	4	4.71	0	0	0	0	9	10.59
A. terreus	0	0	0	0	0	0	0	0	0	0	0	0
A. ochracous	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium	3	3.53	7	8.24	10	11.76	3	3.53	2	2.35	25	29.41
Cladosporium	0	0	2	2.35	3	3.53	0	0	0	0	5	5.88
Fusarium	2	2.35	2	2.35	5	5.88	1	1.18	0	0	10	11.76
Mucor	2	2.35	4	4.71	4	4.71	0	0	0	0	10	11.76
Others	1	1.18	2	2.35	2	2.35	0	0	0	0	5	5.88
Sum	14	16.47	27	31.76	36	42.35	5	5.88	3	3.53	85	100

RD %: Relative Density %
Values were neared to the second digit.

Table 2. Number of different mould isolates and their relative density percentage (RD %) in the examined bovine feed samples

	WYM		CYM		PYM		Bran		CSOC		Total	
	Number	RD%	Number	RD%	Number	RD%	Number	RD%	Number	RD%	Number	RD%
Aspergillus	4	4.55	5	5.68	12	13.64	2	2.27	2	2.27	25	28.41
A. niger	2	2.27	2	2.27	4	4.55	1	1.14	1	1.14	10	11.36
A. flavus	1	1.14	1	1.14	2	2.27	1	1.14	1	1.14	6	6.82
A. fumigatus	1	1.14	1	1.14	3	3.41	0	0	0	0	5	5.68
A. terreus	0	0	1	1.14	2	2.27	0	0	0	0	3	3.41
A. ochracous	0	0	0	0	1	1.14	0	0	0	0	1	1.14
Penicillium	4	4.55	5	5.68	7	7.95	2	2.27	2	2.27	20	22.73
Cladosporium	2	2.27	3	3.41	4	4.55	1	1.14	0	0	10	11.36
Fusarium	3	3.41	3	3.41	6	6.82	2	2.27	1	1.14	15	17.05
Mucor	1	1.14	1	1.14	2	2.27	1	1.14	1	1.14	6	6.82
Others	3	3.41	3	3.41	4	4.55	2	2.27	0	0	12	13.64
Sum	17	19.33	20	22.73	35	39.77	10	11.36	6	6.82	88	100

RD %: Relative Density %
WYM: Whole Yellow Maize
CYM: Crushed Yellow Maize
PYM: Powdered Yellow Maize
CSOC: Cotton Seed Oil Cake
Values were neared to the second digit.

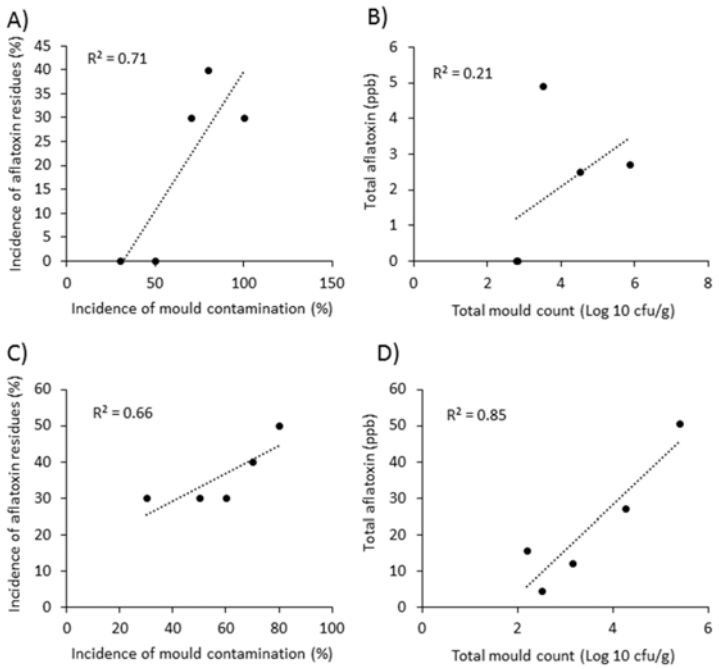

Figure 5: Correlations between mould contamination and aflatoxin residues in bovine edible offal and animal feed.

Scatter plots were drawn between: A) incidence of aflatoxin residues (%) and mould contamination (%) in bovine offal; B) Mean total aflatoxin residues (ppb) and Mean total mould counts (log 10 cfu/g) in bovine offal; C) incidence of aflatoxin residues (%) and mould contamination (%) in animal feed; D) Mean total aflatoxin residues (ppb) and Mean total mould counts (log 10 cfu/g) in animal feed. R^2 is the calculated correlation coefficient.

4. DISCUSSION

Mould contamination of edible offal may lead to their spoilage and production of mycotoxins with potential health hazards to human due to their carcinogenic effects, liver diseases and organ damage. AFs represent a major mycotoxin that threatens human and animal life, if ingested in high concentrations. Repetitive small concentrations of AFs are highly linked with liver failure and carcinogenesis (Darwish et al., 2014 b). Edible offal is contaminated with AFs as a result of mould contamination or as residues due to pre-slaughter exposure of the living animals to AF. Thus, we aimed to investigate the mould and AFs contamination status of the bovine edible offal and to link this with the contamination levels of animal feed by these toxic metabolites and microorganisms. The achieved results in this study revealed a clear mould contamination of the edible offal and animal feed. Intestine had significantly the highest contamination level and mould count among edible offal followed by rumen and liver samples. This may be attributed to the nature of these tissues as it is in a direct contact with animal ingesta and excreta, and even thorough cleaning with running water will not completely eliminate mould spores. Mould contamination of edible offal, meat and meat products is investigated and reported in different localities of the world such as Australia, Japan, Italy, Spain and Egypt (Darwish et al., 2014a; Hitokoto et al., 1978; Iacumin et al., 2009; King et al., 1986; Martín-Sánchez et al., 2011).

Mould contamination of edible offal in this study indicates inadequate sanitary measures performed during handling of these animal byproducts starting from the abattoir level. The conditions of the environment in the slaughtering halls, stores, refrigerators and butchery shops are very suitable for the development of moulds (Mizakova et al., 2002).

Animal feed samples were likely contaminated with mould spores. Maize, especially, powdered and crushed had significantly (p<0.05) the highest mould count and contamination level. Presence of microscopic moulds on the animal feed affects the nutritional and organoleptic quality of such feed (Cegielska-Radziejewska et al., 2013). High mould contamination level in the animal feed was previously reported in many localities worldwide such as Poland, Argentina and Uruguay

(Cegielska-Radziejewska et al., 2013) (Del Palacio et al., 2016; Greco et al., 2014).

Aspergillus and *Penicillium* species were the most predominant mould genera in this study either in edible offal or in animal feed. This may be attributed to their ability to grow over a wide range of temperatures besides they need a very low concentration of oxygen for growth as well as the spore germination (Plahar et al., 1991). The growth of *Cladosporium* species may be attributed to the ability of spores to survive at different adverse conditions such as minimal water activity (a_w; 0.85) and very low temperature up to -7 °C (Jay, 2000). *Fusarium* has worldwide distribution wherever crops such as maize and barley (Booth, 1971) and this might explain the presence of *Fusarium* in meat and edible offal especially in rumen and intestine. The contamination of offal and animal feed with other mould genera such as *Mucor* was clear because these moulds are considered as airborne mycoflora and they have the ability to grow over a wide range of temperature from -3 to 35 °C (Cappuccino and Sherman, 2005). *A. niger* and *A. flavus* were the dominant *Aspergilli*. It notes worthy that *A. flavus and A. parasiticus* are the major aflatoxeginc moulds. This might explain the high contamination level of examined samples with AF. *A. flavus* and *A. parasiticus* infect many crops in the field, during harvest, in storage, and during processing. *A. flavus* is dominant in corn, cottonseed, and tree nuts, whereas *A. parasiticus* is dominant in peanuts. *A. flavus* consists of mycelium, conidia, or sclerotia and can grow at temperatures ranging between 12 and 48 ºC (Hedayati et al., 2007). The isolated mould genera in edible offal samples in this study go in agreement with our previous report, as we could isolate four mould genera, namely, *Aspergillus, Penicillium, Cladosporium* and *Alternaria* from frozen chicken giblets and meat cuts (Darwish et al., 2016). In line with the achieved results of animal feed samples, Magnoli et al. (2002) isolated 15 genera of filamentous moulds from 120 samples of poultry feed. *Fusarium* and *Penicillium* were isolated in 67.5% of the samples and *Aspergillus* in 57.5% of them. Furthermore, Mngadi et al. (2008) recorded high mould contamination incidence (21 out of 23 samples) in the tested animal feed samples from South Africa, the most prevalent mould was *A. flavus* followed by *A. parasiticus*.

Mould contamination of edible offal and animal feed does not only reduce the nutritional and organoleptic quality of the final product, but also produce mycotoxins which have sever health implications on human and animal health beside the progressive economic losses. Estimation of AFs in this study revealed that liver had significantly the highest total AFs residues among examined offal samples with 20% of samples exceeding MPL set by Food and Agriculture Organization (FAO) (2004). This result seems reasonable, as liver is the organ of metabolism and detoxification of xenobiotics (Darwish et al., 2010). Estimation of total AFs residues in the animal feed samples revealed heavy contamination of these samples with AFs especially, in the powdered and crushed yellow maize samples, which exceeded MPL in 50% and 40% of examined samples respectively. Heavy contamination of animal feeds with AFs was corresponding to the recorded results in South Africa and Argentina (Greco et al., 2014; Mngadi et al., 2008).

Correlation coefficient values between mould contamination and AFs production reflected co-occurrence of mould growth and their metabolites. This finding agrees with the published reports in South Africa, Poland, Argentina and Uruguay (Cegielska-Radziejewska et al., 2013; Del Palacio et al., 2016; Greco et al., 2014; Mngadi et al., 2008).

Acute AFs toxicity results from consumption of large amounts of the toxin in a short period of time leading to death, while small doses over long time will result in chronic effects to the consumer. AFs bind to DNA and disrupt genetic coding, thus promoting carcinogenesis. In Africa, among other mycotoxins, AFs have been implicated in human diseases including liver cancer, Reye's syndrome, Indian childhood cirrhosis, chronic gastritis, kwashiorkor and certain occupational respiratory diseases (Darwish et al., 2014 b). In Egypt, aflatoxin-albumin (AF–alb) was detected in 24/24 samples from hepatocellular carcinoma (HCC)-negative individuals and 7/22 samples from HCC-positive cases (Turner et al., 2008).

In conclusion, the results achieved in this study confirmed co-occurrence of mould contamination and AFs production in edible tissues and animal feed samples suggesting that pre-slaughter exposure of living animals to AFs through ingestion of contaminated animal feed is the major cause of edible offal contamination with AFs. Concentrations of AFs in the animal feed exceeded MPL. Thus, strict legislations should be set to avoid importing contaminated grains. Furthermore, strict hygienic precautions should be taken during handling of offal, meat and animal feed during handling, processing and storage. Academics and governmental authorities to update the status of mould contamination and AFs in animal feed and tissues should perform continuous surveillance and monitoring studies.

5. ACKNOWLEDGMENTS

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6. **CONFLICT OF INTEREST:** The authors declare that there is no conflict of interest.

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