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Isolation and identification of some fungal species and assessment of Aflatoxins in dairy animal's ration and milk

Eman, F. Mohamed and Mohamed, H. Gaffer

Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center of Egypt , Shebin El koom Branch, Egypt.

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ABSTRACT

The current investigation was conducted to investigate the relationship between ingestion of aflatoxin already formed in ration and level of aflatoxin M1 secreted in milk. A total of 300 samples were collected from animal ration, cow, and buffalo milk samples (100 samples for each from the same farm) from different dairy farms in El Menofia Governorate. The Mean mould count in the examined ration samples, cow milk samples and buffalo milk samples were 4.03 ± 0.40, 3.34 ± 0.31 and 2.85 ± 0.26log cfu/ml, respectively. Identification of mould isolates in ration samples, cow milk samples and buffalo milk samples were ***Aspergillus niger*** isolated in a percentage of 15, 30 and 28, respectively, while, ***Aspergillus flavus*** isolated in a percentage of 20, 12 and 14, respectively. On the other hand, the number of ***Aspergillus fumigatus*** isolated in a percentage of 8, 8 and 12 of examined samples, respectively. The number of ***Penicillium species*** of the same samples were 4, 25 and 18, respectively. ***Fusarium species*** were isolated in a percentage of 25, 20 and 15, respectively. The number of ***Mucor species*** isolated in a percentage of 28, 5 and 13, respectively .The mean level of aflatoxin in 57 ration samples was 29.578 ± 0.80 (PPb)/kg. while the mean level of aflatoxin M1 was 0.079 ± 0.003ppb/kg and 0.068 ± 0.002ppb/ kg. In cow and buffalo milk samples respectively. In conclusion, The levels of contaminated animal's feed and milk obtained in this study with aflatoxin (AF) and AFM1 should serve as a cautionary call for strict control of raw materials and feed samples to prevent exposure of livestock to aflatoxin contaminated feed which could result in the release of AFM1 into milk and ultimately cause human endangerment through consumption of contaminated milk.

INTRODUCTION

Milk is regarded as a very healthy and well -balanced diet, particularly for kids (**Alahlah et al. 2020).** Due to its high nutritional content and favorable health benefits, animal milk is an important food-grade liquid in human nutrition. **(Akinyemi *et al.* 2021).**The most significant class of mycotoxins is known as aflatoxin (AFB), and it is generated by the fungus *Aspergillusflavus*, *Aspergillus parasiticus*, and

Corresponding author: Eman, F. Mohamed; Food Hygiene Lab., Shebin El koom Lab., Animal Health Research Institute, Agriculture Research Center of Egypt , Egypt.
E-mail address: fathyeman662@yahoo.com
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Aspergillus nominus **(Asi et al., 2012).**When *Aspergillus flavus* and *A. parasiticus* colonise food and crops such maize, peanuts, cottonseed, sunflower seeds, and tree nuts, they generate aflatoxin B1, as well as aflatoxin B2, G1, and G2 **(Alshannaqand Yu, 2017).**When ruminant animals eat contaminated feed with mycotoxin, the forestomach partially degrades the aflatoxin B1 eaten by the animals before it reaches the circulatory system. The remaining portion is converted by the liver into monohydroxy derivative forms, not only AflatoxinM1, but also aflatoxinM2, aflatoxinM4, and other metabolites such as Aflatoxicol in lower amounts, followed by release into the milk by the mammary glands **(Frazzoli et al. 2017).**

When AFB1 is consumed by dairy cows, excretion occurs within 12 to 24 hours later, and the depuration period lasts for around two to three days (**Creppy, E. 2002).** Depending on the type of dairy cows used and the volume of milk produced, between 1 and 6% of the ingested AFB1 is excreted through milk. Highly producing breeds have greater carry-over rates (**Tsakiris et al. 2013).** Aflatoxin M1 (AFM1) is the most dangerous toxin found in milk and dairy products, which is difficult to eliminate during processing due to its thermal stability (**Milićević et al. 2019**).The presence of AFM1 in milk and milk products, even in trace amounts, is a cause for worry, owing to the fact that these products are frequently ingested by children, who are more vulnerable to the harmful effects of aflatoxins due to their immature metabolic and immunological systems (**Fakhri et al. 2019a).** Due to its hepatotoxicity and probable carcinogenicity, regulatory organizations set maximum allowable amounts of AFM1 in milk must not exceed 50ng kg^{-1}. (**FAO/WHO Codex Alimentarius, 1995; European Community, 2006).**

Farmers and food processors in local production chains are encouraged to utilize fluormeter to assess the quality of raw materials before processing foods meant for human consumption, This suggests that the fluorometer may be more successful in determining aflatoxins prior to food preparation **(Mariamu 2019).**

This study aimed to evaluate the mycological aspect of ration samples collected from some dairy farms and evaluate also the mycological status of milk produced from these farms, also measuring the aflatoxin content of examined ration samples as well asin milk produced from these farms to detect the correlation between levels of aflatoxin present in ration ingested by animals and Aflatoxin M1 produced in milk. Also comparison between the amount of aflatoxinM1 secreted in milk of cow and buffalo fed the same ration.

MATERIALS AND METHODS

Collection of samples

Three hundred samples were collected from animal's ration and cows and buffalo's milk samples (100 samples from each and from the same farm) from different dairy farms in El Menofia Governorate. The animal's ration samples were separately collected in sterile polyethylene bags, while milk samples in sterilized Falcon tubes. All samples of ration and milk were delivered in ice box to Animal Health Research Institute laboratory without delay for the following examination:

Determinations of the total mould count according to total viable counts were tested according to the method of **International Dairy Federation (IDF), (1991).**

Isolation and identification of isolated mould species according to **Cruickshank et al. (1975**).

Determination of aflatoxin in animal feed samples and aflatoxin M1 in both types of milk samples using immuno-affinity method **(Scot and Trucksess 1997)**. Series-4 Fluorometer (VICAM^{TM}) was used in this procedure. This method depends upon sample extraction by using methanol (80%) and column chromatography by passing the filtered extract through Afla Test affinity column in case of feed samples and afla MI FL+ affinity column in case of milk samples.

Statistical analysis

The obtained study's data were statistically analyzed according to **Feldman et al. (2003).**

RESULTS

As shown in table 1, mould count in the examined ration samples was ranged from 1.20 to 6.86 with mean value of 4.03 ± 0.40 log cfu/ gm.,while the mould count in cow's milk samples was ranged from 1.14 to 5.54 with mean value of 3.34 ± 0.31log cfu/ml. in buffalo milk samples was ranged from 0.95 to 4.76with mean value of 2.85 ± 0.26log cfu/ml.

Table 2 demonstrated the classification of mould species isolated from examined ration and milk samples. The number of *Aspergillus niger* isolated from ration, cow and buffalo milk samples were 15, 30 and 28 at percentages of 15%, 30% and 28%, respectively. While the number of *Aspergillus flavus* isolated from ration, cow and buffalo milk samples were 20, 12 and 14 at percentage of 20%, 12% and 14%, respectively. On the other hand the number of *Aspergillus fumigatus* species isolated from ration, cow and buffalo milk were 8, 8 and 12 at percentages of 8%, 8% and 12%, respectively.

The number of *Penicillium species* isolated from ration, cow and buffalo milk samples were 4, 25 and 18 at percentages of 4%, 25% and 18%, respectively. The number of *Fusarium species* isolated from ration, cow and buffalo milk were 25, 20 and 15 at percentages of 25%, 20% and 15%, respectively. The number of *Mucor species* isolated from ration, cow and buffalo milk samples were 28, 5 and 13 at percentages of 28%, 5%and 13%, respectively.

Table 3 illustrated the level of total aflatoxins in 57 ration samples (exceed permissible limits) that was ranged from 23 to 48 with mean of 29.578 ± 0.80 PPb/kg. In case of cow milk samples (34) (exceed permissible limits) aflatoxin M1 ranged from 0.053 to 0.120 with mean value of 0.079 ± 0.003ppb/kg. While in case of 23 buffalo milk samples (exceed the permissible limits) aflatoxin M1 ranged from 0.052 to 0.092 with mean of 0.068± 0.002 ppb/ kg.

Table 4 illustrated that the number of positive samples of cow and buffalo samples (exceed the permissible limits) were 34 and 23 at percentage of 59.65% and 40.35%, respectively. While negative samples in cow and buffalo milk samples were 23 and 34at percentage of 40. 35 %and 59.65%, respectively.

DISCUSSION

Aflatoxin is a naturally occurring toxin produced by certain types of mould that can be found in food **(Hell et al. 2000).** There is a strong relationship between the level of Aflatoxin in the animal feed and the level of Aflatoxin in the milk produced by those animals. This is because animals that consume contaminated feed can pass the toxins on to humans through their milk (**Rahimi et al. 2010).**

The current study presented that the result of mean value of mould count in ration samples was 4.03 ± 0.40 log cfu/ gm , this result was higher than that obtained by **(Mohammad et al. 2015)** who found that mean value of mould count was 3.63 log cfu/gm in dairy feed samples. Feed samples were contaminated with fungi and some toxigenic isolates were responsible about Mycotoxin production that cause serious health problems for animals **(Hassan et al. 2014).** Chronic exposure to aflatoxins in dairy cows can affect performance, damage liver function, weaken immunological function, and increase vulnerability to illnesses (**Fink 2008).** In addition, Aflatoxin B1 (AFB1) can potentially affect reproductive function by lowering bull sperm viability and DNA integrity and causing damage to the bovine preimplantation embryo (**Jiang et al. 2019).**

The mean value of mould count in cow milk samples was 3.34 ± 0.31 log cfu/ ml that agreed with results obtained by (Amentie *et al.*, 2016), but higher results were recorded by (Gurmessa, 2015) who found that the mean mould count in cow milk samples was 4.363 ± 0.038 log cfu/ml

The mean value of mould count in buffalo milk was 2.85 ± 0.26 log cfu/ ml, this agree with the results obtained by (Soomro *et al.,* 2016).Contamination of foods and feeds with toxigenic fungi is a great complex problem, it affects economy by reduction of livestock production, increase animal health care and increase veterinary costs **(Hussein and Brasel 2001).**

The isolated mould species were classified into *Aspergillus niger* which isolated from ration, cow and buffalo milk in percentages of15, 30 and 28, respectively. *Aspergillus niger* is a type of mould that can pose a danger to humans in certain situations. There are some potential risks associated with *Aspergillus niger* as it is described as a producer of a wide variety of toxic metabolites, including ochratoxins, a group of secondary metabolites that are classified as possible human carcinogens **(Blumenthal, 2004; Tian et al. 2015).** In addition to generating toxins, *Aspergillus niger* is a significant contributor to allergies because it is a significant source of the allergen proteins necessary for the emergence of allergic symptoms, especially in atopic people **(Gabriel et al. 2016b).**

Aspergillus flavus species isolated from ration, cow and buffalo's milk in percentages of 20, 12 and 14 respectively .*A. flavus* is a human pathogen, allergen, and mycotoxin producer. It is the second leading cause of invasive aspergillosis and the most common cause of superficial infection.*A. flavus* produces aflatoxins, the most toxic and potent hepatocarcinogenic natural compounds ever characterized .Aspergillosis is an infection caused by Aspergillus, including *A. flavus*, and it can affect the lungs, sinuses, and other organs in the body **(Hedayati et al. 2007).**

On the other hand, the number of *Aspergillus fumigatus* species isolated from ration, cow and buffalo's milk in percentages of 8, 8 and 12, respectively, one of the most common types of airborne saprophytic fungus is *Aspergillus fumigatus*. Numerous conidia of this fungus are regularly inhaled by people and animals. The only infection seen in immune competent hosts are aspergilloma and allergic broncho pulmonary aspergillosis, which are unusual clinical disorders. In immunocompetent hosts, the conidia are often removed by innate immune processes. Thus, For many years, *A. fumigatus* was thought to be a fragile pathogen. However, with a surge in immunosuppressed individuals, there has been a sharp rise in severe and typically deadly invasive Aspergillosis, making it the most widespread mould infection in the world **(Jean-Paul, 1999).**

Penicillium species isolated from ration, cow and buffalo milk at percentages of 4, 25 and 18, respectively.

Some *Penicillium species* produce mycotoxins that are known carcinogens, while others cause measurable organ damage when inhaled, when ingested. Immunosuppressive and cancerous effects are among the harmful consequences of ingesting penicillium toxins, in addition to cytotoxic, nephrotoxic, and tremorgenic effects (**Giancarlo and Antonia 2017**).

Fusarium species isolated from ration, cow and buffalo milk at percentage of 25, 20 and 15, respectively.

The principal toxins generated by these *Fusarium species* are fumonisins and trichothecenes, which can harm human and animal health if they get into food chains and impact cereal crops. *Fusarium species* have serious health effects on both people and animals, including a wide spectrum of infections and toxic byproducts **(Nelson et al. 1994).**

Fusarium species produce a wide range of diseases in humans, from superficial infections like keratitis and onychomycosis to locally invasive and disseminated infections (**Marcio and Elias 2007).**

Mucor species isolated from ration, cow and buffalo milk at percentages of 28, 5 and 13, respectively.

Mucor is a mould genus that may be found in soil, digestive systems, plant surfaces, decaying vegetable waste, and iron oxide residue in the biosorption process. However, only a few thermo tolerant species are medically significant, and human infections are rare. Although their inability to develop at temperatures beyond 32°C casts doubt on their viability as human pathogens, their pathogenic role may be restricted to cutaneous illnesses **(Orlowski, 1991).**

Zahra et al . (2019) recorded that Aspergillus, Fusarium, Mucor and Penicillium species isolated from milk samples at percentage of 22, 42, 29 and 3, respectively.

Fungi can come from dairy farms via polluted water and equipment used for milk collection, storage, and transportation .Raw milk and dairy products can get contaminated by various microorganisms which come from milk handlers or the soil, water, skin, and hair of animals **(Lendenbach and Marshal, 2009).**

To minimize contamination, strong sanitary precautions should be implemented during the production, processing, and distribution of milk and its products, to reduce milk contamination with various yeasts and moulds, dairy farms must undergo routine examination by experts **.(Saadia et al. 2018).**

The mean level of aflatoxin in ration obtained from ration samples was 29.578 ± 0.80 ppb, nearly similar results recorded by (**Fernanda et al. 2022)** who found that mean level of aflatoxin in ration was 26.0 ± 0.4 PPb. While higher results achieved by (**Seham et al. 2016**) who denoted that the mean level of aflatoxin in ration samples was 32.06 ± 1.47 ppb and results obtained by (**Anjelina et al. 2022**) who found that mean aflatoxin in ration samples was 24.00 to 76.23 ppb.

We found that 57% of the examined ration samples were exceeding the permissible limits recommended by Food And Drug Administration (**FDA), (2019**) that must not exceed 20 PPb/kg.

Aflatoxins were formed by the fungus *Aspergillus flavus* and *Aspergillus parasiticus* when the temperature was between 24 and 35° C and the moisture content was over 7% **(Williams et al. 2004).** Aflatoxin concentrations exceeding 2 mg/kg diet can have serious consequences for animal development, productivity, and health. Several studies have found changed plasma metabolite concentrations in ruminant animals fed aflatoxin-contaminated diets, which can imply alterations in immune response or damaged animal health (**Sulzberger et al. 2017).**

The presence of total aflatoxin and aflatoxinM1 in contaminated animal feeds and milk, respectively should serve as a wake-up call for thorough monitoring of raw materials and feed samples to prevent cattle exposure to aflatoxins contaminated feeds, which would lead to AFM1 excretion in milk and eventually human exposure through consumption of contaminated milk **(Kangethe and Langa 2009).**

The mean aflatoxin M1 obtained from cow milk samples was 0.079 ± 0.003 ppb/ kg , nearly similar results obtained by (Iqbal *et al.,* 2022) who found that the mean aflatoxin M1 in cow milk samples was 82.4 ± 7.8 ppt /L. but lower results obtained by **(Fernanda et al. 2022)** who found that the mean aflatoxin M1 in cow's milk samples was 32.0 ± 1.0 ppt/ L, (**Raghda, et al. 2022**) who found the mean of aflatoxin M1 in examined cow's milk samples was 40.27 ± 3.996 ppt/L and (Asmaa *et al.,* 2017) who found that the mean aflatoxin M1 in cow milk samples was 35.68 ± 10.90 ppt/L. But higher results were recorded by (**Kirino et al. 2016)** who found that the mean aflatoxin M1 in cow's milk was 128.7 ppt/L.

When Mycotoxins are present in the feed provided to animals, they are metabolized, transferred to the animal tissues, and contaminate animal-derived foods like dairy products, especially raw milk **(Negash, 2018).**

The mean value of aflatoxin M1 in examined buffalo samples was 68.52± 2.495 ppt/L, lower results obtained by (**Ghareeb et al. 2013**) who summarized that the mean aflatoxin M1 in buffalo samples was 62.9 ± 32.1 ppt/l, **(Esam et al. 2022)** who found that the mean aflatoxin M1 in buffalo samples was 40.27±3.996 ppt/L and **(Shaker and Elsharkawy 2014**) who denoted that the mean concentration of aflatoxin M1 in raw buffalo milk from Sohag was 64.49 ± 16.8 ppt/L.

The degree of feed-to-milk transfer (carry-over) is determined by a variety of nutritional and physiological parameters, including animal health, hepatic biotransformation competence, and for individual animals, day to day and milking to milking. Consumption of much larger volumes of concentrated feeds in high-yielding cows may result in carry-over percentages as high as 6.2% (**Veldman et al. 1992**).

The most important observation that 34 cows (59.65%) fed ration from the positive ration (57%) that exceed the permissible limits recommended by Food And Drug Administration **(FDA) (2019**) secrete Aflatoxin M1 in milk, on the contrast there are 23 cow (40.35%) fed from the same ration but they don't secrete Aflatoxin M1 in milk. On the other hand there are 23 buffalo (40.35%) fed ration from the positive ration (57%) that exceed the permissible limits recommended by Food And Drug Administration (**FDA) (2019**) secrete aflatoxin M1 in milk, on the contrast there are 34 buffalo (59.65 %) fed from the same ration but they don't secrete aflatoxin M1 in milk. This suggests that buffaloes excrete aflatoxin M1 in milk less than cows do.

CONCLUSIONS:

From this study we concluded that poor storage conditions of animal ration with suitable conditions enhance multiplication of mould species that will produce several types of mycotoxins, when the animal eat from this ration containing already formed aflatoxin B1, they transferred in liver into aflatoxin M1 then excreted in milk so there is a strong relationship between the level of aflatoxin in the animal feed and the level of aflatoxin in the milk produced by those animals. This is because animals that consume contaminated feed can pass the toxins on to humans through their milk

RECOMMENDATIONS :

Therefore, strict measures must performed to reduce aflatoxin contamination in feed and prevent or reduce the transfer of toxins to milk to protect animal and human health and improve dairy safety and dairy industry profitability as periodical training of farmers about the proper storage conditions of ration and proper training for good hygienic conditions during milking, proper cleaning and sanitizing milking utensils. Culling diseased animals from milking until complete recovery.

Table 1. Statistical analysis results of mould count in examined ration log cfu/gm and milk samples log cfu/ml. n=100

Samples		Min.	Max.	Mean ± SE
Ration		1.20	6.86	4.03 ± 0.40
Milk	Cow	1.14	5.54	3.34± 0.31
	Buffalo	0.95	4.76	2.85 ± 0.26

Mean ± SE: Mean ± Standard Error

Table 2. Identification of mould species isolated from rations and milk samples. n=100

Mould species		Ration samples		Milk samples			
				Cow		Buffalo	
		No.	%	No.	%	No.	%
Aspergillus	*niger*	15	15	30	30	28	28
	flavus	20	20	12	١٢	14	14
	fumigatus	8	8	8	8	12	12
Penicillium species		4	4	25	25	18	18
Fusarium species		25	25	20	٢٠	15	15
Mucor species		28	28	5	5	13	13

Table 3. Statistical analysis of total aflatoxins &aflatoxin m1 in both ration and both types of milk samples, respectively .n=100

Type of sample		Number of positive samples	Max.	Min.	Mean ± SD
Ration samples		57	48ppb	23ppb	29.57± 0.80
Milk samples	Cow	34	0.120ppb	0.053ppb	0.079± 0.003
	Buffalo	23	0.092ppb	0.052ppb	0.068± 0.002

ppb : Part Per Billion

Table 4. Correlation between level of aflatoxin in ration samples (57 %) exceed limits recommended by Food and Drug Administration (FDA), (2019) and levels of aflatoxinM1 in milk samples of cow and buffalo eat from this ration.

		No.	%
Cow milk	Positive % *	34	59.65
	Negative % **	23	40.35
Buffalo milk	Positive % *	23	40.35
	Negative % **	34	59.65

Positive % *: Afla M1 in milk exceed permissible levels according to The European Commission (EC) 2006.
Negative % **: Afla M1 in milk within permissible levels according to The European Commission (EC) 2006.

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