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Aflatoxins in foods

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Abstract

Aflatoxins are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*. The aflatoxin group is comprised of aflatoxin B_1 (AFB_1), B_2, G_1 and G_2. In addition, aflatoxin M_1 (AFM_1), a hydroxylated metabolite of AFB_1, is excreted in the milk of dairy cows consuming an AFB_1-contaminated ration. AFB_1 has shown extreme acute and chronic toxicity and carcinogenic activity in animals; the acute toxicity of AFM_1 is nearly equal to that of AFB_1, but its potential carcinogenic hazard is about one order of magnitude less than that of AFB_1. The International Agency for Research on Cancer classified AFB_1 as a human carcinogen (group 1) and AFM_1 as a possible carcinogen (group 2A). Recently, the possibility of a synergistic carcinogenic interaction between HBV chronic infection and dietary exposure to AFB_1 arose from the observation of their co-existence in countries with high incidences of HCC and was confirmed by further experimental and epidemiological studies. However, the carcinogenic potency of AFB_1 is considered much lower in populations where chronic hepatitis infections are rare.

For the first time in 2003, significant problems arose in Italy, due to the aflatoxin contamination of maize. The summer was extremely hot and dry and A. flavus is very competitive under these conditions as the plants are stressed. Maize grain is normally utilized in the food supply for dairy cows and as such led to the severe and widespread contamination of milk with AFM_1. In the following years (2004-2006), different climatic conditions as well as better compliance with guidelines by farmers, led to a dramatic reduction of the problem.

Key words: aflatoxins, production, toxicity, diffusion, legislation

Introduction

Many food crops such as cereals, nuts, dried fruits and legumes and their products are susceptible to fungal attack either in the field or during storage. Some of these fungal species can produce as secondary metabolites a diverse group of chemical substances known as mycotoxins.

The three important genera associated with mycotoxin production are *Aspergillus*, *Penicillium* and *Fusarium* (Table 1) [1].

Mycotoxin-producing fungi can contaminate commodities destined for use in animal feed and human food products (Table 2). Although there are geographic and climatic differences in the production and occurrence of mycotoxins, exposure to these substances is worldwide, with

Table 1. Commonly encountered toxigenic fungi and toxins they produce.

Fungi	Produced mycotoxins
Genus *Aspergillus*	
A. flavus	Aflatoxins B_1, B_2, cyclopiazonic acid
A. parasiticus	Aflatoxins B_1, B_2, G_1, G_2
A. ochraceus (A. alutaceus)	Ochratoxin A, citrinin, penicillic acid
A. clavatus	Patulin, other neurotoxins
Genus *Penicillium*	
P. verrucosum	Ochratoxin A, citrinin
P. expansum	Patulin
Genus *Fusarium*	
F. graminearum, F. culmorum, F. poae, F. sporotrichioides	Trichothecenes (DON, nivalenol, diacetoxyscirpenol, T-2 toxin), zearalenone
F. verticillioides (moniliforme), F. proliferatum	Fumonisine
Genus *Claviceps*	
C. purpurea	Alcaloids (ergotoxins)

Table 2. Commonly encountered mycotoxins and frequently contaminated foods.

Mycotoxin	Foods
Aflatoxins	Maize and derived products, groundnuts, almonds, dried figs, spices, ...
Aflatoxin M_1	Milk and milk products
Ochratoxin A	Cereals, pork, coffee, cocoa, beer, grape, wine, spices, liquorice
Fumonisine	Maize and derived products
Trichothecenes	Cereals and derived products
Zearalenone	Cereals, maize
Patulin	Apples
Ergotoxins (*Claviceps* alkaloids)	Rye, cereals

Table 3. Some toxic effects caused by mycotoxins.

Mycotoxin	Effect
Aflatoxin B_1	Genotoxic, carcinogenic, hepatotoxic, teratogenic, immunosuppressive
Aflatoxin M_1	Genotoxic, carcinogenic, hepatotoxic
Ochratoxin A	Nephrotoxic, teratogenic, immunosuppressive, carcinogenic
Fumonisin B_1	Neurotoxic, carcinogenic, cytotoxic
Trichothecenes	Immunosuppressive, dermatotoxic, haemorrhagic
Zearalenone	Oestrogen-like effect
Patulin	Cytotoxic, immunosuppressive
Ergotoxins	Neurotoxic

much of the world food supply contaminated to some extent [1,2].

If mycotoxins only affected animal productivity, it is doubtful if they would have aroused much interest, however it was their carcinogenicity, with consequent implications for human health, which evoked global concern. The results of a huge number of research studies show that mycotoxins elicit profound and very diverse effects in all classes of livestock as well as in humans (Table 3) [3].

In terms of exposure and severity of chronic disease, especially cancer, mycotoxins appear at present to pose a higher risk than anthropogenic contaminants, pesticides, and food additives (Table 4). This was based on a comparison of tumour potency and exposure [4].

Table 4. Rating health risks from foods (Kuiper-Goodman, 1998).

Acute	Chronic
High	
Microbiological	Mycotoxins
Phycotoxins	Anthropogenic contaminants
Some phytotoxins	Some phytotoxins
Mycotoxins	Unbalanced diet
Anthropogenic contaminants	Phycotoxins
Pesticide residues	Food additives
Food additives	Pesticide residues
	Microbiological
Low	

Although evidence of mycotoxicosis can be traced to ancient times, the impetus for mycotoxin research was not provided until 1960, when 100,000 turkeys died in the United Kingdom from acute necrosis of the liver and hyperplasia of the bile duct after consuming a groundnut meal infected with *Aspergillus flavus*. The research which followed this event led to the identification and isolation of the aflatoxins (AFs) [2].

Aflatoxin production, toxicity and legislation

The aflatoxin group is comprised of aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2, respectively). In addition aflatoxin M_1 (AFM_1) has been identified in the milk of dairy cows consuming an AFB_1-contaminated ration.

The main members of *Aspergillus* section *Flavi* able to produce AFs are *A. flavus* and *A. parasiticus* [5]. These are closely related fungi and difficult to distinguish from each other. It is now generally accepted that *A. flavus* produces only AFB_1 and AFB_2, while *A. parasiticus* produces all the four principal AFs (AFB_1, AFB_2, AFG_1 and AFG_2) [6,7]. However, Gabal et at. [8] reported a high percentage of *A. flavus* strains producing AFG_1 and a minor group also producing AFG_2. In most isolates, AFB_1 is produced in the greatest quantities; however, in both species of *Aspergillus*, there are strains which are non-aflatoxigenic.

Recent ecological studies carried out on isolates obtained from maize produced in northern Italy [9]

showed that the growth of the *Aspergillus* section *Flavi* was optimal at between 25°C and 30°C, while AFB_1 production was optimal at 25°C. Regarding water activity (a_w), 0.99 a_w was optimal for both growth and AFs production, while the only aflatoxin produced in the driest conditions tested (0.83 a_w) was AFB_1. The risk of aflatoxin contamination is, consequently, much greater in commodities produced in the tropical and sub-tropical regions.

Nearly all of the interest in AFs has focused on AFB_1, primarily due to its extreme acute and chronic toxicity and its carcinogenic activity in animals, in addition to its potential effects in humans. Initiation of tumours by AFB_1 has been described in ducklings, rats, ferrets, trout, guinea pigs, mice, and sheep. The International Agency for Research on Cancer (IARC, 1993) has classified AFB_1 as a human carcinogen (group 1) [4].

As AFs can never be completely removed from the food supply, many countries have defined maximum residue levels in foods and feeds. Within the EU, harmonized regulations exist for AFs in various foodstuffs (Table 5), as well as for AFM_1 in milk (Table 5) and AFB_1 in various feedstuffs: in order to limit AFM_1 contamination of milk, a maximum limit of 5 µg/kg has been fixed for AFB_1 in complete feedstuffs for dairy animals [10,11].

Aflatoxin B_1 and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most widespread malignancies that has the fourth highest mortality rate worldwide and is estimated to cause approximately half a million deaths annually. Geographical areas of interest include Asia, southern China and sub-Saharan Africa, but warnings of increasing levels of HCC incidence has been recently observed also in the USA [12]. Chronic hepatitis B or C infections, exposure to dietary AFB_1 and alcoholic cirrhosis have been demonstrated to be, in order of importance, the major risk factors in the multi-factorial aetiology of HCC.

Early epidemiological studies conducted in areas with high HCC incidence, basically investigated the possible correlation between AFB_1 dietary exposure and the occurrence of HCC. The involvement of other factors, such as hepatitis B virus (HBV) infection, was verified only in successive studies. Recently, the possibility of a synergistic carcinogenic interaction between HBV chronic infection and dietary exposure to AFB_1 arose from the observation of their co-existence in countries with high incidences of HCC and was confirmed by further experimental and epidemiological studies [13].

Recent studies have highlighted that AFB_1 induces epigenetic changes involved in the molecular pathogenesis of HCC, including somatic mutations in the p53 tumour suppressor gene (TP53). Liver cytochrome P-450 enzyme system metabolizes AFB_1 leading to the formation of the electrophilic intermediate AFB(1)-8,9-epoxide, which can easily bind to guanine in DNA, resulting in typical mutations such as G:C to T:A transversions at the third base in codon 249 of TP53.

The detection of this mutation in plasma and tissues was used as a reliable biomarker to assist researchers in understanding the role of AFB_1 [14]. It was found that this mutation is common in HCC from areas of high exposure to AFB_1, whereas it is absent from HCC in regions with negligible levels of AFB_1 exposure, thus providing evidence of a carcinogenic role for the toxin [15]. Sufficient data led researchers to conclude that chronic hepatitis B or C infections, exposure to dietary AFB_1 and alcoholic cirrhosis are, in order of importance, the major risk factors in the multi-factorial aetiology of HCC. However, the carcinogenic potency of AFB_1 is considered much lower in populations where chronic hepatitis infections are rare.

Aflatoxin M_1 in cow milk and milk products

Mammals who ingest AFB_1-contaminated diets eliminate into milk amounts of the main hepatic 4-hydroxylated metabolite known as "milk toxin" or AFM_1. AFM_1 residues in milk are a variable

Table 5. Maximum levels of aflatoxins. Commission Regulation EC no 466/01, 2174/03 and 683/04.

Product	Maximum level aflatoxin (µg/kg)		
	B_1	$B_1+B_2+G_1+G_2$	M_1
Groundnuts, nuts and dries fruit and processed products thereof, intended for direct human consuption or use as an ingredient in foodstuffs	2.0	4.0	-
Cereals (including buckwheat) and processed products thereof, intended for direct human consuption or use as an ingredient in foodstuffs	2.0	4.0	-
Dietary foods for special medical purposes intended specifically for infants	0.10		0.025
Milk (raw milk, milk for the manufacture of milk based products and heat-treated milk .....)	-	-	0.05
Infant formulae and follow-on formulae, including infant milk and follow-on milk			0.025

percentage (0.3-6%) of AFB_1 ingested. AFM_1 is usually considered to be a detoxification product of AFB_1, however its acute toxicity is nearly equal to that of AFB_1; as regards the potential carcinogenic hazard, it is about one order of magnitude less than that of AFB_1 [16]; the International Agency for Research on Cancer [4] classified AFM_1 as a possible human carcinogen (group 2B).

The joint Expert Committee on Food Additives (JECFA), a scientific advisory body of the World Health Organization (WHO) and the FAO, was requested to examine exposure to AFM_1 and to conduct a quantitative risk assessment to compare the application of two standards for contamination of milk (0.05 µg/kg and 0.5 µg/kg, limits currently applied in the EU and in the USA respectively). The calculations showed that, with worst-case assumptions, the projected risks for liver cancer attributable to use of the proposed maximum levels of AFM_1 of 0.05 µg/kg and 0.5 µg/kg are very small, and that there is no significant health benefit from reducing a 0.5 µg/kg limit to 0.05 µg/kg [17].

According to Stoloff [18], milk has the greatest demonstrated potential for introducing AFs residues from edible animal tissues into the human diet. Literature data on AFM_1 contamination of milk and milk products are copious and surely much larger than any other mycotoxin/food related data (for extensive reviews see Fremy and Dragacci [19]; Galvano et al. [20]; Van Egmond [16]). In the last decade, the incidence of AFM_1 contamination seems to have been reduced both by increasing the accuracy of detection procedures and by the setting of stricter regulatory limits (mainly in the EU) for AFB_1 in feeds and AFM_1 in milk. The incidence of AFM_1 contamination is often higher in commercial milk than in raw farm milk, because of the dilution of uncontaminated bulked milk by a few contaminated samples. For the same reason high AFM_1 contamination levels in commercial milk seldom occurs. Nevertheless, the occurrence of AFM_1 in cow milk and milk products is widespread, even if contamination levels do not seem to be a serious health hazard according to the current scientific fund of information. The above considerations have been confirmed by recent surveys conducted in many countries [21-27]. However, as AFM_1 may or may not be present in dairy products in a particular year depending on the weather for that period, widespread and frequent monitoring programs performed by accurate and reliable analytical techniques still remain the primary means of protecting milk consumers. When dairy products are manufactured from milk contaminated with AFM_1, the toxin is transmitted to the resulting products. AFM_1 is stable in raw milk and processed milk products and is generally unaffected by pasteurization or processing into cheese, yogurt, cream and butter. Association of AFM_1 with casein causes the cheese to contain a higher concentration of the toxin than the whey; this concentration is 2.5- to 3.3 times higher in many soft cheeses and 3.9- to 5.8 times higher in hard cheeses than that in milk from which these cheeses were made [28].

Milk powder for infant formula is another route of exposure to AFM_1. Galvano et al. [20] reviewed literature data from 1980 to 1995. Although only limited literature data were available, the authors concluded that the incidence and contamination level of AFM_1 in dried milk and infant formulae was not likely to be a health hazard, however, since infants are more vulnerable and sensitive than adults, monitoring of infant foods should be repeated more frequently and extensively. After 1995 other surveys have been conducted, two in Italy [25,26] and one in Brazil, [29], Korea [30], Turkey [31] and India [32]. Consistent with data on liquid milk, only low levels of contamination were found, apart from a recent report from India noting that almost all samples tested exceeded the EC limit. However, Aksit et al. [31] correctly concluded that, although AFM_1 concentrations in infant formula were found to be within acceptable limits for most countries, its presence must be carefully evaluated, because future influences of very small amounts of AFM_1 on the growing organism have not been fully elucidated.

Aflatoxins in fruit and vegetables: the situation in Italy before 2003

The available data from the literature has shown that, before 2003, aflatoxin occurrence in fruit and vegetables for human consumption had been highlighted, especially in imported products (dried fruit, spices and herbs). Based on data collected by Moretti et al. [33], 20% of fruit and vegetable samples were found to be positive (Table 6); for cereals and derivates, the prevalence was 29%. In 1998, Miraglia et al. [34] examined aflatoxin

Table 6. Occurrence of aflatoxins in food and feed in Italy (1989-1996).

Product	No. Positive/ total samples	Range µg/kg
Dried fruit and derivates	187/1373	<0.1-1870
Spices and derivates	117/158	0.1-504
Cereals and derivates	56/193	0.1-24.6
Herbs	7/20	0.2-1.5
Miscellaneous	12/12	0.5-6.4
Olive oil	3/35	0.1-11.2

contamination data for a number of foodstuffs (whole cereals, whole wheat, wheat meals, bran, breakfast cereals, peanuts, peanut-based products, pistachio nuts and other nuts, dried fruit, dried figs, spices, confectionery, soft drinks, beer, olive oil and dairy products) and reached the conclusion that the levels of contamination in Italy were similar to those found in other European countries.

Aflatoxins in animal feed and dairy products: the situation in Italy before 2003

The contamination of animal feed, milk and dairy products with AFs must be considered as a whole, because of their strict interdependence.

Out of 533 maize samples for animal consumption collected in northern Italy during the time period of 1995-1999, Pietri et al. [35] reported high positive rates (42.9%), although the levels were generally very low. The only exceptions were 2 samples contaminated with AFB_1 over 100 μg/kg and 5 samples over 20 μg/kg. Low maize contamination was confirmed by Minervini et al. [36]. These authors analysed 197 samples of dairy cow feed and observed that they were all below 5 μg/kg (i.e., the EU limit for feedstuffs for animals during lactation). The same authors analysed 124 maize samples of which only 9 showed values of between 5 and 20 μg/kg, with no values found above 20 μg/kg. The Technical Services of the Lombardy Region (1999-2000) analysed 830 samples of dairy cow feed [37], on average, all feedstuffs contained AFB_1 levels below 5 μg/kg. If AFB_1 alert values are set between 2 μg/kg (limit for foodstuffs) and 5 μg/kg, the feedsftuffs generally falling within this range are cotton seeds and maize gluten meal. With regards to maize, 9 samples out of 124 were found to show values of AFB_1 over 5 μg/kg, but all were below 20 μg/kg.

Milk contamination provides fundamental information on aflatoxin contamination in animal feed. Data collected by different authors and with different methods show different patterns (Table 7). Palermo et al. [38] observed a 29% prevalence of AFM_1 between 1981 and 2001, with 8 samples exceeding the EC limit. Moretti et al. [33] observed that all AFM_1 values between 1994 and 1999 were below 50 ng/kg, except for two cases, both with a prevalence of 45%. Albertini et al. [39] reported that 65% of the samples of raw milk for human consumption were below 20 ng/kg. Pinelli et al. [40] summarised the results of approximately 5000 inspections a year carried out by a large dairy factory (Parmalat) from 1998 to August 2003. Continuous reduction of contamination was observed: in 1998 samples with AFM1 >20 ng/kg were slightly over 25%, but by the first few months of 2003 the rate had dropped to values below 5%. Pietri et al. [27] reported inspections of milk, carried out in the period 1993-1999, to be used for the production of Parmigiano-Reggiano, a production limited to a small area in northern Italy. Some data variability was shown over the various years, with a reduction of AFM_1 contamination from 1993 onwards. Such a reduction is also the result of measures undertaken by the Parmigiano-Reggiano cheese Consortium for the benefit of safeguarding the production of this cheese, establishing a maximum threshold of 3 μg/kg for AFB_1 in animal feed. Values of AFM_1 in milk over 50 ng/kg should not be considered surprising, since EU regulations only came into force on 1 January 1999. Finally, organic milk showed higher levels of contamination than conventional milk [41].

Aflatoxins in maize and milk: an emerging problem in 2003

Maize

In Italy, maize is widely grown in the northern regions, where the main concern is contamination with fumonisins, produced by *F. verticillioides*,

Table 7. Milk contamination with AFM_1 in Italy from 1981 to 2003.

Source	Palermo et al.,	Moretti et al.,	Albertini et al.,	Pinelli et al.,	Pietri et al.,	Serraino et al.,
	2001 [38]	2004 [33]	2002 [39]	2005 [40]	2003 [27]	2003
Survey period	1981-2001	1994-1999	2001	1998-2002	1993-1999	1999-2001
No. Samples	2434	1381	1285	5000/yr	332	1850
	cow	Buffalo	cow	cow	cow	cow
No. Positive	718	627	1134		317	
% Positive	29	45	88		95.5	
Max. ng/kg	930	77			406	130
Mean ng/kg					18	27- 30
>20 ng/kg % [1]			35	28		
>50 ng/kg % [2]				2	8	

[1] *AFM_1, 20 ng/kg: alert value for many dairy factories*

[2] *AFM_1, 50 ng/kg: maximum admissible level of AFM_1 (EU limit)*

with the incidence being high in most years. Deoxynivalenol is detected only sporadically, especially in rainy years with temperature levels lower than usual for these regions, when *F. graminearum* becomes dominant [35]. For the first time in 2003, significant problems arose due to aflatoxin contamination of maize.

The summer was extremely hot and dry, maize plants were stressed by drought and there was a widespread reduction in the crop cycle length, with rapid drying and early ripening. As a consequence farmers harvested grain with a lower humidity than usual and frequently stored it without any drying or cleaning. Based on the available knowledge *A. flavus* should have been very competitive in this situation with plants stressed due to dry and hot weather. This was confirmed by analysis in September of samples from different maize-growing areas in northern Italy. A survey of 110 samples, initially planned to monitor the occurrence of fumonisins, showed that 75% of the samples tested positive for AFB_1 with a mean and a maximum value of 4.4 and 154.5 μg/kg, respectively.

Milk

Maize grain is normally utilized in the feed rations for dairy cows at the rate of 5-6 kg per cow per day. The feeding of dairy cows with contaminated maize led to the severe widespread contamination of milk with AFM_1. The problem was immediately identified by manufacturers of milk for human consumption and by health inspectors. For example, in Lombardy (northern Italy) 4,321 inspections were carried out over a period of 20 days in early October 2003 on samples of milk from different farms (2,061) and dairy factories (808). Over 33% of the samples were above the threshold of 0.05 μg/kg of AFM_1. Several thousand tonnes of milk above the legal limit were discarded. Farmers were not allowed to sell their milk immediately after a positive test, and had to wait until their milk was found, at a later inspection, to be back to an acceptable level. Systematic analysis of maize and raw materials used in dairy cow feeds and a well-run information campaign rapidly reduced the problem. In fact, based on evidence collected by Lombardy Region inspectors, the percentage of samples over the value of 0.05 μg/kg (or 50 ng/kg) of AFM_1 dropped to 2.5% in dairy factories and to 18.5% in farms in the period November 2003 - January 2004.These data match our laboratory data (over 800 samples), which found 14% of milk samples above the threshold over the period October 2003 – February 2004, with a peak of 27% in October.

After the first alert

In the following years (2004-2006) different climatic conditions as well as better compliance with guidelines by farmers, led to a dramatic reduction of the problem. Nevertheless, some cases of kernel contamination above the EU limit did occur during these years. This was not expected, because the weather conditions were not particularly dry or hot. A point to be considered is undoubtedly the increase of over-wintering inoculums of *A. flavus*, with particularly high rates in 2003. Contaminated samples were mainly associated with early-maturing maize hybrids, grown without irrigation and harvested around mid August or before. It is Probable that periods of drought, even if short, affected these crops and created favourable conditions for *A. flavus*.

Conclusions

AFs are considered unavoidable food and feed contaminants. Controlling the infection by *Aspergilli* is, of course, the most desirable method of reducing the incidence and level of aflatoxin contamination. Control measures are often based on what is known about factors that are conducive for the growth of the mould.The most important factors that promote the production of AFs in foods and feeds are moisture and high temperature. Rapid drying of the commodity immediately after harvesting and storage under appropriate conditions are both part of proper management strategies to minimize contamination. However, weather conditions can not be controlled; therefore, even with the best agricultural practices, pre-harvest contamination can occur.

Before 2003, in Italy the aflatoxin problem was predominantly considered a problem of imported products; however, climatic changes are altering the situation and the aflatoxin risk for a summer crop like maize is increasing, in connection with drought and high temperatures. As a consequence, the contamination risk for maize-derived products and for milk will be higher than in the past.

Risk management sets priorities for risk reduction in line with a variety of available options. These range from prevention of mould growth through modifying of agricultural practices, setting of regulatory limits (already in force in many countries) in grains destined for food and feed use, to diversion into alternate uses. There can be high economic costs associated with all of these, but prevention through adopting a HACCP approach is probably the most effective.

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