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Review

Aflatoxins exposition in the agri-food industry workers

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Abstract
Aflatoxins are mycotoxins produced by some *Aspergillus* species. They are remarkably toxic and included among the most known carcinogenic substances. Article 139 of the Italian presidential decree of 30 June 1965, no. 1204 and subsequent amendments, includes hepatocellular carcinoma due to exposure to aflatoxin B1 in the list of diseases for which reporting is mandatory. Aflatoxins could contaminate food of plant and animal origin, therefore the areas of work most at risk are the agro-food and, in general, all the activities carried out in humid and poorly ventilated areas, subject to fungal contamination. Although the procedures for controlling aflatoxins in food are now fairly well established, there is still no full knowledge of the risk of workplace exposure. A review of the risks relating to exposure to aflatoxin of workers in the agri-food sector is presented here based on bibliographic data and surveys conducted in some Italian regions by local health authorities and by the National Institute for Insurance against Accidents at Work. To protect the health of workers in the sectors considered, we therefore consider it necessary to evaluate the presence of aflatoxins and inform workers about the application of good prevention practices and the use of adequate personal protective equipment.

Keywords
mycotoxins; *Aspergillus*; occupational exposition; hepatocellular carcinoma; Personal Protective Equipment

Introduction

Aflatoxins are mycotoxins, toxic molecules produced by the secondary metabolism of some microscopic filamentous fungi (moulds) belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genera. The moulds can proliferate, in appropriate temperature and humidity conditions, on foods of vegetable origin (cereals, legumes, fruits, nuts and dried fruit, spices, cocoa and green coffee) and those of animal origin (cheeses, sausages) following fungal contamination directly on the foodstuff, during the phases of production, processing, transport and storage. Aflatoxins can also be present in foods (milk, meat and eggs) deriving from animals breeded with contaminated feed or in some processed foods (beer and wine) due to contamination of the raw materials used (Pace et al., 2012).

In the early sixties of the last century, in turkey farms in England more than one hundred thousand cases of lethal poisoning of unknown etiology were occurred, which were labelled as 'Turkey "X" disease'. Soon it was attributed to a toxic groundnut meal imported from Brazil. From that point on,

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an extensive effort to find the cause eventually elucidated that a species of mould, called *Aspergillus flavus* Link, was involved and the hepatotoxic products of this mould, found also as components in the toxic groundnut meal, were called aflatoxins (Richard, 2008). After this event, the frequency of food poisoning caused by contaminating moulds has made known the nature and effects of aflatoxins and of various other toxic substances produced by the fungal secondary metabolism, to which it was given the name of 'mycotoxins'.

Aflatoxin production has incorrectly been ascribed to a long list of *Aspergillus* species and to species assigned to other fungal genera like *Fusarium* and *Penicillium*. However, authoritative reviews state that true aflatoxins are produced only by some *Aspergillus* species, while many reports of aflatoxins produced by several *Aspergillus* and non-*Aspergillus* species are intermediate compounds of aflatoxin biosynthesis (Varga et al., 2009; Adeyeye, 2019).

The genus Aspergillus

The genus *Aspergillus* (Ascomycota, Eurotiales) includes more than 340 species, divided into 20 or 21 sections. These species were traditionally associated with nine teleomorph genera, but phylogenetic data suggest that together with genera such as *Polypaecilum*, *Phialosimplex*, *Dichotomomyces* and *Cristaspora*, *Aspergillus* forms a monophyletic clade closely related to *Penicillium*. *Aspergillus* species are ubiquitous saprophytes, endophytes to opportunistic pathogens for plants and animals. Approximately 20 *Aspergillus* species are currently known as agents of opportunistic infections in humans (Samson et al., 2014). They develop on organic decomposing substances, and they are commonly isolated in the soil and into the silos on plant remains, animal tissues, domestic residues, compost, and hay bales. The species belonging to this genus are highly aerobic and grow in almost all oxygen-rich environments, usually on the substrate surface (Pfliegler et al., 2019). Many species develop from the decomposition of starchy foods (cereals, cotton seeds, peanuts, and nuts). They are among the organisms that contribute to the degradation of natural polysaccharides (De Vries et al., 2000; De Vries, 2003) and are important for the large-scale industrial production of both homologous and heterologous enzymes (Fawole and Odunfa, 2003).

Almost all *Aspergillus* are characterized by their peculiar anamorphic asexual reproduction *via* mitotic spores (conidia) with conidiophore ending with a swelling called vesicle; on the vesicle the phialides are formed, either directly or through a series of short sterile cells called metulae, and from each phialide a chain of spores are produced. The whole set of the vesicle, the phialides, and the spores, is called the 'aspergillary head'. Beside the anamorphic phase present in all *Aspergillus* species, several species can, under suitable conditions, reproduce sexually with cleistothecia containing asci with 8 meiospores (ascospores). In some species, there can also be a parasexual reproduction, the recombination of genes without sexual reproduction and meiosis, which was discovered precisely in *Aspergillus* (Pontecorvo et al., 1953a,b).

The conidia disperse in the air, favouring the dissemination as well as contamination of foodstuffs and animal feeds. In addition, they can pass through the respiratory tract to the pulmonary alveoli where, especially in immunosuppressed or compromised individuals, they can cause primary mycoses called 'aspergillosis'. Aflatoxins are produced by 13 species assigned to three sections of the genus *Aspergillus*: section *Flavi* [*A. flavus*, *A. pseudotamarii* Yoko Ito, S.W. Peterson, Wicklow & T. Goto, *A. parasiticus* Speare, *A. nomiae* Kurtzman, B.W. Horn & Hesselt, *A. bombycis* S.W. Peterson, Yoko Ito, B.W. Horn & T. Goto, *A. parvisclerotigenus* (Mich. Saito & Tsuruta) Frisvad & Samson, *A. minisclerotigenes* Vaamonde, Frisvad & Samson, *A. arachidicola* Pildain, Frisvad & Samson], section *Nidulantes* [*A. astellatus* (Fennell & Raper) Houbraken, Visagie & Samson, *A. venezuelensis* Frisvad & Samson, *A. oleicola* Frisvad, Zalar & Samson] and section *Ochraceorosei* [*A. ochraceoroseus* Bartoli & Maggi, *A. rambellii* Frisvad & Samson].

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Aspergillus flavus e *A. parasiticus* are xerophilic fungi and therefore more suitable for conditions of high temperatures and limited or absent rains (Payne, 1998). *Aspergillus parasiticus* seems to be more adapted to the soil environment, with a higher incidence in peanuts, while *A. flavus* seems to be more adapted to the aerial and leaf environment resulting prevalent in maize and cotton (Diener et al., 1987). The main factors influencing the population of these fungi are the temperature and the humidity of the soil. Under stressful conditions (thermal, water, biotic and abiotic), *A. flavus* and *A. parasiticus*, produce aflatoxins (*A. flavus* AFB1 and AFB2; *A. parasiticus* AFB1, AFB2, AFG1, and AFG2) which can contaminate agricultural products in the field, during the harvest or in post-harvest (storage and transformation processes) (Diener et al., 1987). *Aspergillus flavus* is also an opportunistic pathogen for humans and animals that can cause aspergillosis in immunocompromised individuals. It thrives at temperatures between 12 and 48 °C, with thermal optimum between 32-37 °C, and optimal humidity at 0.86-0.96 aw (Vujanovic et al., 2001; Causin, 2006; Pitt and Hocking, 2009). It is a metabolically very versatile organism capable of using different natural (plants, animals) or anthropogenic source substrates (Raper and Fennell, 1965; Hasan, 1999). Thanks to the production of a large amount of degrading enzymes, that allow it to readily exploit the available resources, *A. flavus* uses numerous organic compounds to grow (cellulose, pectins, lignin, tannins, cutin, starch, lipids, and proteins) (Olutiola, 1976; Betts and Dart, 1989; Guo et al., 1996; Mellon and Cotty, 2004; Batra and Saxena, 2005), on which it develops mainly the mycelium and sometimes the sclerotia (St. Leger et al., 1997).

Aspergillus flavus colonies commonly appear as powdery or fluffy masses of green-yellow or golden-red spores. In cereals and legumes, the infection affects small areas and a discoloration of these areas is often noted. The cycle of infection begins with the maturation of the fungus in the soil and the subsequent conidia dispersion, through wind or insect action. The spores penetrate inside the cereals grains or in the legumes thus infecting the seed. It is able to survive and overwinter in crop residues such as mycelium or sclerotia; these represent the source of new conidia that can start the infection cycle on new host plants. *Aspergillus flavus* strains can be divided into 2 groups based on the size of the sclerotia they produce: the S strains produce very small sclerotia (< 400 µm) and are capable of producing high quantities of aflatoxins, while the L strains produce fewer but larger sclerotia (> 400 µm) and their ability to produce aflatoxins is highly variable (Cotty, 1989). The diffusion phase of *A. flavus* in the surrounding environment starts at warm weather arrival, due to the huge quantities of conidia originated from the mycelium and the sclerotia. In the presence of high temperatures and low water activity (aw), typical conditions of agricultural crops in sub-tropical areas, *A. flavus* can be very competitive until becoming the soil dominant fungal species (Payne, 1998).

Moulds can proliferate, in appropriate temperature and humidity conditions (T = 25-32 °C, free water = aw 0.82 - 0.87), on foods of vegetable origin (cereals, legumes, nuts and dried fruits, some types of fruit, spices, cocoa and green coffee) and those of animal origin (cheeses, sausages), following fungal contamination directly on the foodstuff, during the phases of production, processing, transport and storage. Aflatoxins can also be present in foods (milk, meat and eggs) derived from animals fed with contaminated feed, or in some processed foods (beer and wine) due to contamination of the raw materials used and Commission Regulation (EC) No 401/2006 defines the methods of sampling and analysing for the official control of the levels of mycotoxins in foodstuffs (Trucksess et al., 1983; Williams et al., 2004; Knutsen et al., 2018). Their presence persists even if the life cycle of the mould has been interrupted, if this has been removed from the operations of processing the food or feed, or if the food has been cooked. A reduction of the contamination could happen with the milling of the cereals for impoverishment of aflatoxins in the innermost fractions of the grain and with the pushed roasting as that used on the coffee in Italy (Micco et al., 1992). It is possible, however, to carry out a decontamination and detoxification on food and feed using some treatments that could be physical (irradiation and extraction with solvents), biological (use of antagonist microorganisms) or chemical

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(ammoniation, prohibited in Europe, treatment with bisulfite, ozonation and interaction with chelating agents). *Aspergillus flavus* mostly grows on the aerial parts of plants; *A. parasiticus* is more adapted to the soil environment and has more limited distribution. The remaining 11 aflatoxigenic species are also AFs-producing species, but they are found less frequently. From a mycological perspective, there are qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species.

Aflatoxins

Chemically, aflatoxins are derivatives of difuranocoumarin and are defined as B1 (methoxy-difuro-coumarone), B2 (dihydro-derived methoxy-difuro-coumar-lactone), G1 and its dihydro-derived G2, while their hydroxylated metabolites of B1 e B2, which are found in milk from dairy animals fed with contaminated feed, are indicated respectively with M1 and M2 (EFSA, 2020). Aflatoxins have been chemically divided in two chemical groups, the difurocoumarocyclopentenone series and the difurocoumarolactone series (Bbosa et al., 2013a) (Table 1).

They are crystalline substances, soluble in moderately polar organic solvents (chloroform, methanol, dimethylsulfoxide), slightly soluble in water (10-30 μg ml^{-1}) and insoluble in non-polar organic solvents. Pure aflatoxins are stable in the absence of light and degraded by UV radiation. Aflatoxins have low molecular weight and high melting point. They are thermostable and present ubiquitously, aflatoxin B1 is produced between 24 °C and 25 °C, while the G1 between 29 °C and 30 °C. They are unstable at pH < 3 and > 10 and in the presence of oxidizing agents. The G series contains a lactonic ring, while the B series contains a cyclopentenoic ring, which is responsible for its greater toxicity. The order of acute and chronic toxicity is AFB1 > AFG1 > AFB2 > AFG2, reflecting the role played by epoxidation of the 8,9-double bond and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the lactone ring of the G series.

AFM1 and AFM2 are hydroxylated forms of AFB1 and AFB2 and are their main metabolites in humans and animals, may be present in the milk, serum, and urine of animals fed with feed contaminated with aflatoxin B1 (EFSA CONTAM, 2020). AFM1 is not directly produced by *Aspergillus*, but it derives from the metabolism of animals fed on aflatoxin B1 contaminated food. It appears in the milk about 4 hours after AFB1 ingestion by the cow and it is linked to the protein fraction of milk. AFM1 is excreted by the mammary glands and could be ingested by humans through milk and its derivatives because not any treatment can eliminate it, since it is a thermostable molecule. It disappears from milk, on average, within 3-4 days after stopping AFB1 administration. All the mammals that ingest AFB1 eliminate a quota as AFM1 in milk. In the dairy cow, the amount of AFM1 corresponds to 1-3% of the AFB1 ingested. The relationship between the amount of AFB1 taken with the diet and its concentration, or that of its metabolites present in the tissues is established by numerous studies. The amount of the AFM1 aflatoxins detectable in tissues is almost always negligible with the only exception of the milk.

Aflatoxins, in Italy, are a problem especially for imported products (peanuts, corn, wheat and cassava used to produce feed) from tropical and subtropical countries with a hot and humid climate, whereas local products are generally less contaminated due to different weather conditions to the best agronomic, harvesting and preservation techniques of the products. In addition, the insect presence often coincides with high levels of aflatoxins, especially in the case of the corn borer, *Ostrinia nubilalis* (Hübner), because insects are one of the main causes of contamination both for the conveyance of fungal spores and for the damage to the plant that increased its exposure to the fungal attack (Lillehoj et al., 1978). Even milk from cattle and sheep, contaminated with aflatoxins, can represent a source of exposure for humans through the direct ingestion of the food or its derivatives (cheese, yogurt, etc.) (Table 2).

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Table 1 - The aflatoxins produced by the *Aspergillus* species. The letters B and G derive from the type of fluorescence (blue or green) emitted when irradiated with UV rays (360 nm), while the letter M derives from milk. Blue aflatoxins B1 and B2 derived from *A. flavus* and *A. parasiticus*; green or blue-green aflatoxins G1 and G2 derived from *A. parasiticus*; blue-violet aflatoxin M1 (modified from Bbosa et al. 2013a)

Type of aflatoxin	*Aspergillus* species
Difurocoumarocyclopentenone series	
Aflatoxin B1 (AFB1)	*A. flavus*, *A. arachidicola*, *A. bombycis*, *A. minisclerotigenes*, *A. nomiae*, *A. ochraceoroseus*, *A. parasiticus*, *A. pseudotamarii*, *A. rambellii*, *A. venezuelensis*
Aflatoxin B2 (AFB2)	*A. arachidicola*, *A. flavus*, *A. minisclerotigenes*, *A. nomiae*, *A. parasiticus*
Aflatoxin B2a (AFB2a)	*A. flavus*
Aflatoxin M1 (AFM1)	*A. flavus, A. parasiticus* Metabolite of aflatoxin B1 in humans and animals and comes from the mother's milk
Aflatoxin M2 (AFM2)	Metabolite of aflatoxin B2 in milk of cattle fed on contaminated foods
Aflatoxin M2A (AFM2A)	Metabolite of AFM2
Aflatoxicol (AFL)	*A. flavus* Metabolite of AFB1
Aflatoxicol M1	Metabolite of AFM1
Difurocoumarolactone series	
Aflatoxin G1 (AFG1)	*A. arachidicola*, *A. flavus*, *A. minisclerotigenes*, *A. nomiae*, *A. parasiticus*
Aflatoxin G2 (AFG2)	*A. arachidicola*, *A. flavus*, *A. minisclerotigenes*, *A. nomiae*, *A. parasiticus*
Aflatoxin G2A (AFG2A)	Metabolite of AFG2
Aflatoxin GM1 (AFG1)	*A. flavus*
Aflatoxin GM2 (AFGM2)	Metabolite of AFG2
AFGM2A	Metabolite of AFGM2
Aflatoxin B3 (AFB3)	*Aspergillus* species not defined
Parasiticol (P)	*A. flavus*
Aflatrem	*A. flavus*, *A. minisclerotigenes*
Aspertoxin	*A. flavus*
Aflatoxin Q1 (AFQ1)	Major metabolite of AFB1 *in vitro* liver preparations of other higher vertebrates

To detect the aflatoxins presence in food and feed, chromatographic methods (High Performance Liquid Chromatography - HPLC and Thin Layer Chromatography - TLC) and enzyme immunoassays (Enzyme-Linked Immunosorbent Assay -ELISA) are used.

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Table 2 - Aflatoxins in foods. National Plan for the Official Control of Mycotoxins in Foods 2016-2018 (Italian Ministry of Health Directorate-General for Hygiene and Food Safety and Nutrition - Office 6 DGISAN). Modified from IARC, International Agency for Research on Cancer

Aflatoxins	Source	Toxic effects	Contaminated foods	IARC classification
AFB1, FB2	*Aspergillus flavus*	hepatitis	Peanuts and other legumes, corn and other cereals, oilseeds, nuts, dried fruits and derived products	Group 1 (evidence of human carcinogenicity)
AFG1, AFG2	*A. parasiticus,* others *Aspergillus* spp.	nephritis tumours		
AFM1, AFM2	Metabolites		Milk and its derivatives	

Toxicity of aflatoxins

In 1993 the International Agency for Research on Cancer, classified aflatoxin AFB1 in Group 1, that is carcinogen for humans and has a highly significant correlation with the incidence or mortality from hepatocellular cancer (HCC) (IARC, 2019). AFM1 has a similar structure and an acute toxicity comparable to that of AFB1, whereas his hepatic carcinogenicity, verified on trouts and rats, is approximately 2-8%. The AFM1 was classified by the IARC in Group 2B (possible carcinogenicity for humans) and its presence in milk raises some concerns because it is a food of large consumption (IARC, 2019).

The acute toxic effects of aflatoxins are known with the term 'aflatoxicosis' and their severity depends on the intensity of exposure, the age and nutritional status of the individual as well as the possible synergistic effect of other chemical agents to which the subject is exposed. The toxic and carcinogenic effects of aflatoxins have the liver as the main target organ (Peraica et al., 1999). In animals, young subjects are always much more affected than adults and polygastric mammals are generally more resistant than the monogastric ones, because of the detoxifying action of bacteria and protozoa (Upadhaya et al., 2009). Usually, acute intoxication symptoms are severe apathy, loss of appetite, a moderate to a high fever and death of the animal at variable times depending on the specific sensitivity. The liver appears pale, increased in volume, with necrosis of the parenchyma, the kidneys may have glomerulo-nephritic lesions, while in the lungs congestive phenomena are observed (Molina Alvarado et al., 2017). Chronic intoxication, instead, causes loss of appetite, slowing of growth and weight loss. The liver is significantly affected by the toxic activity, is congested and has haemorrhagic and necrotic areas (Zain, 2011). If the intoxication is prolonged, carcinogenic processes may occur. The kidneys are congested and occasionally haemorrhagic enteritis can be observed (Dhanasekaran et al., 2011). Depressive state and nervous disorders as motor incoordination, loss of balance and muscle spasms, also appear. The most dangerous and most commonly present aflatoxin is the AFB1 (World Health Organization, 2018). It shows high acute and chronic toxicity, carcinogenic activity in animals and potential harmful effects on humans, for which the LD_{50} is oscillating between 0.6-10 ppm (Dhanasekaran et al., 2011). The toxicity of the other aflatoxins is expressed as AFB1 equivalent. AFB1 is genotoxic and is considered the most potent hepatocarcinogen known (Bbosa et al., 2013a).

The toxic effects are attributable to:

- hepatotoxicity;
- bile duct hyperplasia;
- haemorrhage of the gastrointestinal tract and kidneys;
- hepatocellular carcinoma, especially in subjects with hepatitis;
- liver cirrhosis in children (Indian Childhood Cirrhosis, ICC), caused by M1 contained in breast milk;
- mutagenicity;
- immunosuppression (thymic hypoplasia, lymphopenia T) (Mohsenzadeh et al., 2016).

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The danger of the most well-known aflatoxins is mainly linked to the ingestion of contaminated food. Consumption of foods containing 0.2-10 ppm of aflatoxins appears to be from toxic to lethal. In lactating mammals, a small amount of B1 is excreted as M1 in milk (Coulter et al., 1984; El-Nezami et al., 1995). Ingested aflatoxins are absorbed along the gastrointestinal tract where they are metabolically activated or detoxified in the intestinal mucosa and in the liver (Kumagai, 1989). The presence of aflatoxins has been observed in human saliva (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2) (Verma and Chaudhari, 1998), which can be reabsorbed in the gastrointestinal tract and re-introduced into the bloodstream. This could explain some observed cases of aflatoxins recirculation in the organism, together with the enterohepatic circulation.

The aflatoxins absorption can also occur through the respiratory apparatus in the presence of inhalable dust contaminated or, at least experimentally, by contact with the skin. Inhalation absorption, often underestimated, could be decisive in occupational exposure if associated with particular environmental conditions during the processing and handling of contaminated products (high concentration of aflatoxins in the air during the processing of feed or spices). The possibility that AFB1, locally transformed into epoxide and once inhaled, acts directly on the lung tissue, has already been amply demonstrated (Kelly, 1997; He et al., 2006).

Since aflatoxins are non-volatile, inhalation exposure depends essentially on the inhalation of particulate matter both of fungal origin (spores) and of contaminated dusty substrates. Inhalation of this particulate may transport the aflatoxins to the pulmonary alveoli in which they can interfere with the immune responses or with the removal mechanism of foreign particles by the macrophages. These effects can potentially pave the way for infections (Viegas et al., 2013a).

Animal studies have shown that aflatoxins, after entering the blood flux, are mainly transported to the liver, through the portal circulation (Wilson et al., 1985). Probably due to the considerable permeability of the hepatocyte membrane, the liver has high efficiency in extracting AFB1 from the bloodstream, and it is the main organ in which the aflatoxins biotransformation and detoxification processes occur (Wilson et al., 1985). In addition, the kidneys can collect aflatoxins from the blood, although to a lesser extent than the liver (Wong and Hsieh, 1976; Hayes et al., 1977).

Several metabolic pathways in animals, including humans, involved in the biotransformation of AFB1 and other aflatoxins have been identified. These metabolic pathways can lead to activation, as 'first phase' reactions, producing from AFB1 and AFB2 the metabolites AFB2A, AFM1, AFM2, AFM2A and, from AFG1 and AFG2 to AFP1, AFQ1, and aflatoxicol or, as 'second phase' reaction, detoxification with the formation AFB1-8,9 dihyidrodiol, by glucurono-conjugation and sulfo-conjugation. The aflatoxins detoxification process (second phase) increases the water solubility of the compounds, favouring their excretion through bile and, to a lesser extent, through the urine and the milk. Both non-metabolized aflatoxins (AFB1, AFB2, AFG1, AFG2) and their metabolized forms (aflatoxicol, AFM1, AFM2) are mainly excreted through the faeces (approximately 80-90%) and, to a lesser extent, through the urine (10-20% approximately) (Eaton et al., 1994; Mykkänen et al., 2005).

The aflatoxins excreted with the faeces come mainly from the bile poured into the intestine through the biliary tract and, to a small extent, from the portion not absorbed in the lumen of the gastrointestinal tract, especially in the case of high doses. The soluble metabolites of AFB1, deriving directly from free circulating aflatoxins in the bloodstream, are excreted via the urine (Eaton et al., 1994).

Aflatoxins in the blood are partly linked to serum albumin and partly free, but only the free ones are able to pass through the cell membranes. The binding of AFB1 to albumin already at the site of absorption can therefore be considered as one of the major detoxification mechanisms, able to prevent the interaction of aflatoxin with the cell (Fig. 1) (Mykkänen et al., 2005; Yiannikouris and Jouany, 2002).

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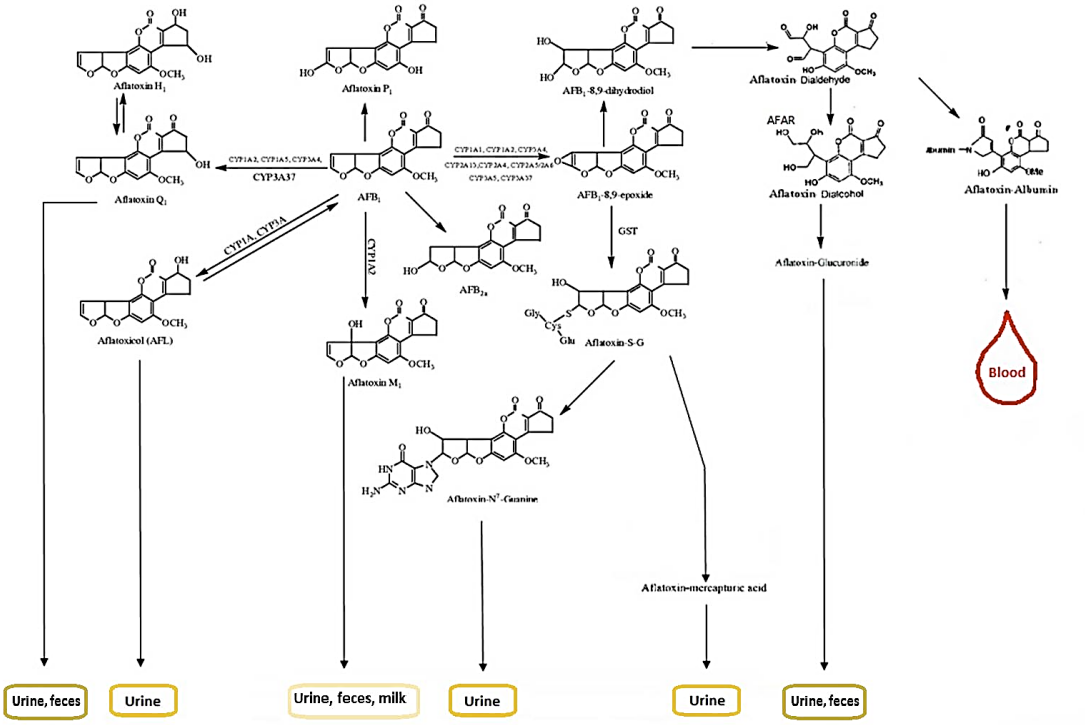

Fig. 1 - Metabolic AFB1 pathways modified from Bbosa et al. (2013b).

The most used method to measure human exposure to aflatoxins consists in quantifying their metabolites in body fluids (Makarananda et al., 1998; Wild and Pisani, 1998). The aflatoxin B1-albumin adduct can be detected in peripheral blood and has a half-life in the body of 30-60 days and, therefore, may represent a more reliable indicator of chronic exposure (Makarananda et al., 1998).

Several researches have investigated both molecular and cellular mechanisms involved in the aflatoxins toxic action (Yassein and Zghair, 2012). The effects are cellular migration, oxidative stress induction, apoptosis, carcinogenesis, aberrant expressions of tumour-suppressor genes and membrane receptors genes (Herath et al., 2006; Hou et al., 2013) (Fig. 2).

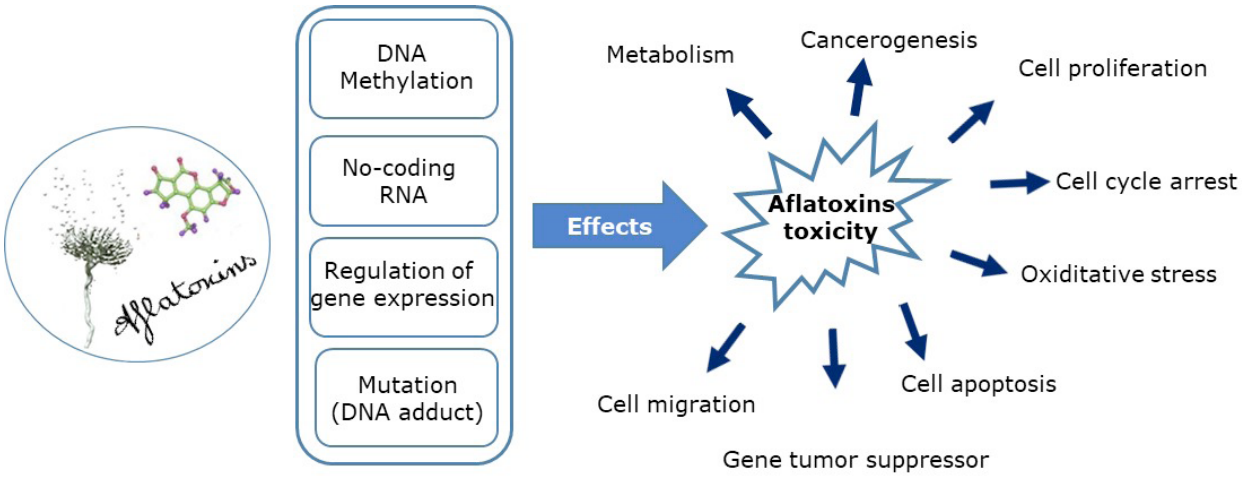

Fig. 2 - Aflatoxins toxic effects.

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AFB1 activation is important because the molecule itself is not carcinogenic and its toxic effects are attributable to the action of some reactive metabolites. The reactions take place mainly in the gastro-intestinal tract, in the liver, in the lungs and, to a lesser extent, in the kidneys. The main activation reactions (first phase) of AFB1 are mediated by the cytochrome P450 (CYP) enzyme system that catalyses oxidation reactions (Vermeulen, 1996). They occur mainly in the liver, but also in extra-hepatic tissues (respiratory and intestinal epithelium) (Larsson and Tjälve, 1996; He et al., 2006). After passing through the plasma membrane of hepatocytes, AFB1 is oxidized by CYP 450 microsomal to AFB1-8,9-epoxide, an electrophilic intermediate able to bind cellular DNA with great affinity (Swenson et al., 1977; Shimada et al., 1989; Ueng et al., 1995), causing nuclear damage (Hendrickse, 1991).

Another important discovered detoxification enzyme is aflatoxin B1-aldehyde reductase (AFAR) capable of reducing AFB1-dialdehyde (Knight et al. 1999; Primiano et al., 1996). In particular, a study on induced altered methylation by AFB1 on the thioredoxin reductase 1 (TXNRD1) and Ras-association domain family 1 isoform A (RASSF1A) genes, showed that the TXNRD1 gene is up-regulated, resulting in a reduction in the expression of AFAR and glutathione-S-transferase (GST) genes. This inhibition reduces the detoxification process, via glutathione, of metabolite AFB1-8,9-exo-epoxide. The result of this anomaly is an increase in the liver formation of DNA adducts (McLeod et al., 1997). The RASSF1A gene dysregulation can induce a condition of liver cirrhosis, the evolution of which in HCC is significantly associated with the presence of AFB1-induced DNA adducts (Zhang et al., 2012). Since the adducts formed by AFB1 with proteins are considered to be responsible for the acute toxicity of aflatoxin, AFAR could represent a fundamental detoxifying enzyme to attenuate toxic syndromes due to B1 exposure (Guengerich et al., 2002a,b).

The activated aflatoxins can interact with different molecules (DNA, RNA, proteins and carbohydrates), inducing genetic mutation phenomena, inhibition of enzymatic systems and alterations in the metabolism of the interferon involved in immune responses and anti-inflammatory reactions (Wong and Hsieh, 1976; Pier and McLoughlin, 1985; Smith and Moss, 1985). Aflatoxins have the ability to interfere with energy metabolism, inhibiting the activity of electron transport chains and altering the carbohydrates metabolism, with consequent modification of the hepatic glycogen metabolism (Doherty and Campbell, 1972, 1973; Kiessling, 1986).

Aflatoxin binding with steroid hormones receptor sites is also possible. Laboratory studies carried out on animals have shown that the formation of aflatoxin-DNA adducts and the consequent onset of liver cancer can be inhibited by the administration of agents capable of inducing glutathione S-transferases (GSTs) such as Oltipraz (a drug used in the 1980s against schistosomiasis), Phenobarbital or Ethoxyquin. The activated forms AFB1-dialdehyde and AFB2a, the hydrolytic product of AFB1, may covalently bind to proteins and determine a permanent inhibition of the enzymatic function. A loss of functionality of the proteins involved in biosynthetic pathways, in hormonal, neurotransmission, transport, and immune functions, compromising the life of the cell is also possible. Aflatoxins also inhibit protein synthesis and the proteins compromised in their functionality cannot be replaced. In the case of the proteins necessary for the lipids transport to the liver, a fatty liver degeneration follows (Terao and Ueno, 1978; Hsieh, 1987). Some proteins destined to the nucleus may bind to AFB1 in the cytoplasmic level and carry it to the microsomes, the activation site (Hsieh et al., 1977).

Activated aflatoxins are also transferred in other cellular sectors, such as in the endoplasmic reticulum ribosome, where they may covalently bind to other macromolecules. In human lung tissue, AFB1 would be activated mainly through the prostaglandin-H-synthase pathway and/or the lipoxygenase pathway, which can also catalyse the oxidation of AFB1 to B1-8,9-exo-epoxide (Battista and Marnett, 1985; Massey et al., 1995). AFG1 can induce oxidative stress in alveolar type II cells, increasing the production of reactive oxygen species (ROS) and thus inducing damage to mitochondria and DNA. It can also activate cell apoptosis through the c-Jun N-terminal kinase (JNK)

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and the p38+Mitogen-Activated+Protein+Kinases (p38 MAPK) signalling pathways.

The hypothesis that ROS-induced apoptosis is an important cytotoxic effect related to carcinogenesis associated with some mycotoxins has been confirmed (Wang and Yadav, 2006; Wild and Gong, 2010). The ROS, as known, induce DNA double-strand breaks, leading to genomic instability, mutations and neoplastic transformations that can play a critical role in carcinogenesis (Mills et al., 2003; Xie et al., 2005; Shen et al., 2012). The apoptosis induction is physiologically significant because alveolar type II cells repair the damaged alveolar epithelium, inducing the proliferation of type I alveolar cells.

An *in vivo* study, AFG1 intratracheal administration for some months, showed that aflatoxins could induce chronic lung inflammation, which may promote a microenvironment that fosters lung adenocarcinoma development (Liu et al., 2014). In fact, it has been observed that many factors could correlate with this hypothesis such as up-regulation of two regulators of inflammatory response, the signal transducer and activator of transcription factor 3 (Stat3) and nuclear factor-κB (NF-κB) expression. In particular, NF-κB induces the expression of several pro-inflammatory genes, including those encoding proinflammatory cytokines which may provide a microenvironment to contribute to lung adenocarcinoma: tumour necrosis factor alpha (TNF-a), interleukin 1b (IL-1b), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) mouse macrophage inflammatory protein 2 (MIP-2) / C-X-C motif chemokine ligand 2 (CXCL-2) and C-X-C motif chemokine ligand 1 (CXCL-1). In addition, the inflammatory response is confirmed by the increase in the number of macrophages, lymphocytes and alveolar type II cells located in the pulmonary epithelium. The aberrant activation of inflammatory response has been suggested in several human tumours (Morgillo et al., 2018). In addition, high expression levels of superoxide dismutase (SOD-2), hemoxygenase-1 (HO-1), two oxidative stress markers, and cyclooxygenase 2 (COX-2) have been detected, all of this sustains factors of the inflammatory process (Liu et al., 2014). The cytoplasmic monooxygenases enzymatic system is also responsible for the transformation of AFB1 into polar molecules. In fact, these enzymes can hydroxylate AFB1 to AFM1, which has remarkable carcinogenic activity *in vivo*.

The metabolite B1-8,9-exo-epoxide interacts with DNA to form AFB- DNA adducts causing DNA breakages or point-mutations and can alkylate proteins, this may result in a decline in the proteins function which can cause cell death or transformation (Hsieh et al., 1977). DNA synthesis is interrupted (DNA-dependent RNA-polymerase blocking), transcription, protein synthesis and cellular respiration are inhibited (cytochrome b and c blocking) and the mechanisms of cell growth, multiplication and metabolism are therefore altered.

The hypothesis that the aflatoxins' carcinogenic action is mainly related to phenomena of the regulation modification of recurrent cellular pathways is fairly well established. In particular, several epidemiological studies have shown some molecular mechanisms in the liver and the lung carcinogenesis, related to the exposure to AFB1 (Dai et al., 2017). The presence of mutation of arginine to serine at codon 249 in the tumour-suppressor p53 gene, induced by the metabolite B1-8,9-exo-epoxide, abrogating the function of the tumour suppressor gene is commonly observed in subjects with HCC (Kew, 2013).

An epigenetic mechanism of aflatoxins, related to toxicity, is aberrant methylation of the CpG islands located in the promoter regions of tumour suppressor genes. *S*tudies on mice have shown that p53 gene dysregulation AFB1-induced can also occur indirectly through promoter partial hypermethylation of the tumour-suppressor genes p16Ink4a and p19Arf. These two genes regulate pathways that cooperate on the induction of lung adenocarcinoma. In particular, the p16ink4a protein is a cyclin-dependent kinase inhibitor able to arrest the cell cycle, whereas the p19Arf protein regulates the p53 gene involved in the process of cell cycle arrest and apoptosis (Pomerantz et al., 1998; Massey et al., 2000; Tam et al., 2003).

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Aflatoxins play a role in carcinogenesis, also through the modulation of miRNAs expression (Livingstone et al., 2017). MicroRNAs (MiRNA) are small non-coding mRNAs that regulate the post-transcriptional gene expression in pivotal biological processes and, specifically, some of them have a role in aflatoxin-induced carcinogenesis. For example, the level of miR-34, and miR-138-1* might be up-regulated in HCC cells treated with AFB1. Remembering that miR-34 regulates the Wnt/b-catenin signalling pathways involved in cells proliferation, differentiation and migration, and miR-138-1* targets Pyruvate Dehydrogenase Kinase 1 (PDK1) which plays a role in cell proliferation, survival, metabolism and transformation, an aberrant modulation of these pathways is therefore conceivable that has an effect on carcinogenic development (Zhu et al., 2015; Wang et al., 2016). Additionally, AFB1 exposure was significantly correlated with up-regulation of miR-24 and miR-429. These miRNAs can modify the development and progression of HCC, in fact, the role of these miRNAs has been shown to regulate the carcinogenesis of a variety of cancers including HCC. The high expression of miR-24 and miR-429 related to HCC tumour tissues was significantly correlated with larger tumour size, tumour differentiation and with modified recurrence-free survival. The miR-24 and miR-429 overexpression progressed tumour cells proliferation, inhibited cell apoptosis, and developed the formation of AFB1-DNA adducts. This significant evidence indicates that these two miRNAs are potential diagnosis and prognosis biomarkers of AFB1-related HCC and may be a potential tumour therapeutic target (Huang et al., 2013a; Liu et al. 2014).

AFB1 also plays a central role in the expression of insulin-like growth factor-2 (IGF2) and IGF1 receptor (IGF-IR) in liver and lung cells (Ma et al., 2012; Cui et al., 2015). IGF2 is an important factor for the regulation of cell proliferation, growth, migration, differentiation and survival. Mounting evidences have demonstrated that the IGF/IGF-IR axis is involved in human cancer progression because the activation of signalling leads to increased DNA synthesis and cell migration (Scharf et al., 2003; Samani et al., 2007; Gallagher and LeRoith, 2011).

Occupational disease related to aflatoxin exposure

Mycotoxins in general and specifically aflatoxins are usually considered in relation to food and little considered at the level of occupational risk, even though the contaminated dust inhalation has proved to be an important source of occupational exposure (Baxter et al., 1981; Jakab et al., 1994; Kelly et al., 1997). At present, the human exposure to aflatoxins by inhalation, in the manufacturing and agriculture sectors, is probably considered to be jointly responsible for various pathological manifestations (Depico et al., 1977; Chan-Yeung et al., 1985, 1992; El Karim et al., 1986).

In particular, they are imputed of:

- some cancers in agricultural and food processing workers;
- the Organic Toxic Dust Syndrome (OTDS) very common in farmers and in people exposed to the inhalation of cereal dust, hay, fungi, bacteria and their metabolites, insects, mites, etc.;
- interstitial pneumonia in textile workers.

In one study it has been simulated, by intratracheal instillation, the inhalatory exposition to the AFB1, and its haematic concentration was analysed over time. It was found that absorption through the respiratory apparatus is faster than the oral administration, but that, after four hours, AFB1 concentration values in the blood tend to equalise (Coulombe and Sharma, 1985). If the tracheal dose was adsorbed in the dust before administration, the AFB1 permanence in the trachea was prolonged (Coulombe et al., 1991), increasing its retention time in the trachea and lungs.

AFB1, *in vitro*, is also able to penetrate the human epidermis (Riley et al., 1985, 1988). While *in vivo* studies by topical application in rats and rabbits have shown that a significant quantity of radioactive B1 was absorbed and transported by plasma proteins in various organs, including the liver

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(Joffe and Ungar, 1969; Wei et al., 1970). Dermal exposure to AFB1 can determine the formation of skin tumours and pre-neoplastic lesions in murine models (Rastogi et al., 2006).

Dvorackova and Pichova (1986), in a study, reported that AFB1 was present in the lungs of three workers, one from the agricultural sector and two from the textile industry, who died of pulmonary interstitial fibrosis, probably exposed for occupational reasons to AFB1 through the respiratory tract. Both Hepatitis B virus (HBV) infection and ethanol can potentiate the hepatotoxic and hepatocarcinogenic action of AFB1. Some authors suggest a multiplicative effect between aflatoxin and HBV infection in the risk of liver cancer (Fan et al., 2013), others an additive effect (Liu and Wu, 2010). In particular, chronic HBV infection would convert to hepatocellular carcinoma, as confirmed by epidemiological studies. In the presence of ethanol, on the other hand, AFB1 seems to trigger the cytochrome P450 increasing the production of reactive metabolites. In the absence of direct toxicological data, the risk for humans is currently assessed indirectly, starting from the animal toxicology data and from the statistical analysis of epidemiological data relating to populations at risk.

Risks for the workers

Attention and knowledge towards aflatoxins as an occupational risk factor are scarce "even in companies where inhalation exposure is relevant". The regulation concerning the possible occupational exposure to aflatoxins and the ways of preventing their harmful effects are also inadequate. Despite the fact that the procedures for the control of aflatoxins in food are now quite consolidated, there is not full knowledge of the risk of exposure in the workplace yet (Desai and Ghosh, 1989; Desai et al., 1990; Ferri, 2013).

In many companies, occupational exposure by inhalation is not negligible. The current standards do not establish limit values to aflatoxins occupational exposure *via* inhalation or cutaneous contact, but it is possible to estimate the healthiness of the workplace in relation to risk factors such as grain powders and flour, that are possible vehicles for aflatoxins, considering that a direct correlation between environmental dustiness and aflatoxins airborne concentration is not always demonstrable. In Table 3 some examples of occupational exposition limits values for cereal and flour powders internationally proposed or indicated from the competent bodies are reported.

The term "cereal dust" refers to the particulate matter originating from cereal processing (wheat, oats, barley, rye, sorghum, maize, rice, and various oilseeds). These dusts are generated by the seed movement, which may cause the release of quite fine or coarse particles, both from the surface of the seeds or from their inner part, after any breakage. The dust amount that cereals may emit during their handling and dimensional characteristics of the particles varies according to the seed and the processing considered (Boac et al., 2009; Reddy et al., 2009, 2011; Brochard, et al. 2010, Siruguri et al., 2012).

The possibility that aflatoxin B1, when inhaled, is locally transformed to epoxide that acts directly on the lung tissue, has already been amply demonstrated. Oluwafemi et al. (2012), in fact, report occupational aflatoxicosis due to the inhalation of fungal spores of *Aspergillus*. Such little attention paid to the aflatoxins occupational risk is demonstrated by:

- the limited number of epidemiological studies or experimental investigations on health effects and occupational exposure levels, even in the numerous companies that work, directly or indirectly, with contaminated food or feed;
- the absence of any specific prevention legislation to protect exposed workers and from the fact that, paradoxically, aflatoxins are not included in the EU list of occupational carcinogens.

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Risks are related to these processes:
• harvest (corn and other cereals);
• loading and unloading (ports, hauliers, etc.);
• Storage/silage;
• mechanical treatments;
• drying;
• feed production;
• animal breeding (avian species, swine, cattle, rabbits, dogs, fish and fur animals);
• analysis laboratories;
• biogas production;
• incineration.

Table 3 - Occupational exposition limits values for cereal and flour powders

Public authority	Inert powder (mg/m^3)	Cereals dust (mg/m^3)	Flours (mg/m^3)
ACGIH (USA) Oats, wheat, barley	10	4	0.5 ACGIH (2014): proposed limit for flours of cereal, corn etc.
NIOSH (USA) Oats, wheat, barley		4	
OSHA (USA) Oats, wheat, barley		10	
HSE (GB) Oats, wheat, barley, corn and rye, contaminants included		10	10
SCOEL (EU)			1 Recommendation SCOEL/SUM/123, December 2008: recommended value for cereals flour dust
SUVA (CH)		Sensitizing factor: impossible to establish a NOAEL for cereals dust (wheat, rye, etc.)	0.15 (excluding wheat and rye) (SUVA, 2015)
CTN CNAMTS		5 Cereals inhalable dust limits proposed by the National Technical Committee of the CNAMTS (French public insurance authority) 2004	

Studies have been carried out both internationally and in the European Union to assess the risks to which workers in agrifood and feed mills are subjected to Selim et al. (1998) found aflatoxins concentrations between 4 x 10^{-5} to 4.8 μg/m^3 in dust samples collected during different processes (harvesting and unloading, animal feeding, bin cleaning) in 28 United States farms. The higher

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aflatoxins concentrations have been detected during bins cleaning whereas during harvesting and unloading it has been the lowest. Aflatoxins may be mainly in *A. flavus* spores. Particularly in the southern of United States, truckers and farmers may be exposed to aflatoxins during harvesting. The amount of airborne aflatoxins may be related to the handling method. In India, in a study on environmental mycoflora of rice mills, fungi of the genus *Aspergillus*, including *A. flavus* (8%), were found, meaning that mill workers are subjected to occupational exposition by airborne aflatoxins (Desai and Ghosh, 2003).

Malik et al. (2014) determined the risk of occupational exposure to aflatoxins from the food-grain workers compared to not food-grain workers by detection of *Aspergillus* through microscopy and culture of bronchoalveolar lavage (BAL). Aflatoxins have been detected by serum samples analysis. About 47.8% of the food-grain workers and 11.4% of non-food-grain workers had chronic respiratory symptoms. In the two groups a significant difference in BAL culture for *Aspergillus* has been reported. Analysis have detected aflatoxins in 32.6% of food-grain workers and 9.1% of the not food-grain workers meaning that occupational exposure to aflatoxins in food-grain workers was found to be associated with the increased presence of respiratory symptoms.

In three feed mills in southwestern Nigeria, airborne fungi (*A. flavus*, *A. fumigatus* Fresen., *A. candidus* Link, *A. niger* Tiegh., *A. terreus* Thom and *Rhizopus* spp.) were analysed. Worker exposure to aflatoxins (B1, B2, G1 and G2) was studied by High-performance liquid chromatography (HPLC) analysis of blood samples compared to a control group. Higher levels of aflatoxin B1 have been observed in the blood of workers in sparsely ventilated establishments, showing that the risk of exposure to aflatoxins should be better considered among feed workers (Oluwafemi et al., 2012).

Mohgah et al. (2014) estimated the hepatotoxicity of aflatoxin B1 in workers exposed to wheat flour dust by detecting a significant increase in liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) compared to the controls, concluding that serum B1/albumin was significantly correlated with the duration of exposure and that its high serum levels determine hepatotoxic effects in exposed workers.

Several studies about aflatoxins exposure were carried out in Europe. A study on Danish workers exposed to aflatoxins present in imported crops in an animal feed production industry, showed an increase in hepatocellular carcinoma (HCC) among those who had a greater exposure to aflatoxin-contaminated row matter, in a period of 10 or more years preceding the cancer diagnosis (IARC, 1993).

Among the operators with greater seniority (> 10 years work), an excess of tumours to liver, biliary tract, salivary gland, and mediastinum was found compared to the general population, due to higher levels of aflatoxin B1 linked to blood proteins compared to not professionally exposed people (Olsen, 1988; Autrup et al., 1993). In the Netherlands, operator groups exposed to aflatoxins of peanut processing showed an increase in respiratory route cancer mortality compared to the control group of unexposed workers (Hayes et al., 1984; Ferri, 2013).

To characterize occupational exposure to multiple mycotoxins in swine production workers, in Portugal, Viegas et al. (2013a) examined biological samples (urine) of 25 workers and 38 environmental samples (23 air samples; 5 waste samples; 10 samples of feed). The results show that the occupational environment contributes to the exposure of workers to aflatoxins, as found through biomonitoring data and the high contamination present in food and waste samples. Another study was conducted in Portugal by Viegas et al. (2012) to verify the possible occupational exposure to aflatoxin B1 in poultry and swine production facilities. A group of 45 workers (34 of poultry farms; 11 of pig production plants) participated in this study, supplying blood samples. Furthermore, a control group (n = 30) composed of subjects without any kind of contact with agricultural activity was considered. The results suggest that exposure to B1 by inhalation occurs in both cases. However, poultry workers showed higher serum levels and a significant statistical difference was found between this group and the control group.

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For approximately thirty years, several studies have investigated the quantity of workers exposed to B1 in the workplace and in some studies, the number of cancer cases that developed seemed to correspond with that exposure. In general, it is expected that the primary route of exposure of these workers to B1 is respiratory although some authors note the possibility of dermal or oral exposure. In general, liver cancer was the primary cancer type although significant increases in respiratory and biliary cancer was also found (Hayes et al., 1984; Alavanja et al., 1987; Olsen et al., 1988; Ahmad and Khan, 1991) (Table 4).

Table 4 - Reports of occupational exposure to AFB1 (modified from Rushing and Selim, 2019)

Country	Occupation	Worker number	Positive for aflatoxin (n)	Positive for cancer (n)	Suspected route of exposure	Free AFB1 in serum (ng m^{-1})	Urinary AFM1 (ng m^{-1})	AFB1-albumin (pg mg^{-1})	Ref.
Netherlands	oil press	71	-	11	respiratory	-	-	-	Hayes et al. (1984)
Sweden	grain millers	2,649	-	310	-	-	-	-	Alavanja et al. (1987)
Denmark	animal feed	-	-	398	respiratory	-	-	44-100	Olsen et al. (1988)
Denmark	animal feed	45	7	-	respiratory	-	-	-	Autrup et al. (1991)
Portugal	poultry production	31	18	-	-	<1-4.23	-	-	Viegas et al. (2012)
Nigeria	feed mill workers	28	-	-	respiratory	73.4-189.2	-		Oluwafemi et al. (2012)
Egypt	textile workers	58	34	-	-	-	0-2.41		Saad-Hussein et al. (2013)
Portugal	swine production	28	21	-	respiratory, oral	<1-8.94	-	-	Viegas et al. (2013a)
Portugal	poultry and swine production	45	24	-		>1-8.94	-		Viegas et al. (2013b)
India	food-grain workers	46	15	-	respiratory	-	-	-	Malik et al. (2014)
Egypt	wheat handlers	190	-	-	-	-	-	0.06-0.11	Saad-Hussein et al. (2014)
Italy	feed production and sorting	29	23	-	respiratory	-	0-0.399		Ferri et al. (2017)

In the European Union, the CAREX (CARcinogen Exposure) was developed; it is a database that collects estimates of the number of workers potentially exposed to aflatoxins from 1990 to 1993.

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It represents an international information system on occupational exposures to known and suspected carcinogens that provides exposure data and estimates the number of workers exposed per country, carcinogen and industry (Kauppinen et al., 2000). Some studies have evaluated occupational exposures to aflatoxins in Italy about 123,000 employees and c.a. 20,000 interested companies (Tab. 5).

Table 5 – Flussi INAIL 2013. Companies and workers involved at risk of exposure to aflatoxins (modified from Ferri, 2013)

ATECO groups (A10d_Ateco 2007)	**Total workers**	**Companies**	**ATECO/NACE codes***
Cereals cultivation	3,343	1,619	A 0111; A 01111; A011110
Oily and mixed seeds cultivation (oily and not)	33,243	2,907	A 0112; A 01120; A 011200
Rice cultivation	246	191	A 0112; A 01120; A 011200
Spices, aromatic, and pharmaceutical plants cultivation	140	118	A 0128; A 01280; A 012800
Pigs, cattle and poultry breeding and their related feed production	3,404	1,311	A 014100; A 0142; A 01420; A 014200; A 0146; A 01460; A 014600; A 0147; A 014700; A 01499; A 014990; A 01500; A 015000
Support activities to vegetable production	10,868	6,079	A 01610; A 016100
Post-harvest activities	2,831	492	A 0163; A 01630; A 016300
Cleaning, sorting and other processing of seeds and grains	108	14	A 016401; A 016409
Refined or raw oil production from oily seeds or fruits (except corn oil)	1,303	193	C 10412; C 104120
Grain processing; starches and starchy products manufacturing	10,680	1,687	C 106; C 1061; C 10611; C 106110; C 10612; C 106120; C 10613; C 106130; C 10614; C 106140; C 1062; C 10620
Production of cocoa, chocolate, tea, coffee, confectionery, …	28,097	2,062	C 1082; C 10820; C 108200; C 1083; C 10830; C108301
Production of condiments and spices	1865	274	C 1084; C 10840; C 108400
Production of feed for farm animals	6,449	662	C 1091; C 10910; C 109100
Preparation and spinning of textile fibres	20,266	2,281	C 131; C 13100; C 131000
Repair and maintenance of other machines for agriculture, forestry and animal husbandry	5	2	C 331270
Total 2013 (in Italy)	122,848	19,892	

***ATECO** (ATtività ECOnomica) Statistical classification of economic activities in Italy adopted by from NACE (Nomenclature statistique des activités économiques dans la Communauté européenne) is the classification of economic activities in the European Union

In three food processing plants (cocoa, coffee, and spices) in Tuscany, Brera et al. (2002) collected and analysed a total of 44 samples (26 biological samples and 18 ambient air samples) to determine the concentrations of aflatoxins B1, B2, G1 and G2 in airborne dust. The samples showed

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aflatoxins contamination levels between under the detection limit to 0.08 ng. The wide range of toxin levels may depend on several causes, such as the distance between the worker and the stored raw materials, the job of the worker, the time of exposure and the amount of particulate sampled.

Ferri et al. (2017) conducted a study by analysing blood and urine samples, comparing the exposure to aflatoxins and aflatoxicol of 29 workers in a highly contaminated corn mill, with 30 workers from another mill not exposed to high concentrations of aflatoxins. The results of this study reveal the presence of a higher concentration of aflatoxins in exposed workers compared to unexposed controls.

Traverso et al. (2010) through the ARPAL laboratory (Agency for Environmental Protection of the Liguria Region) in Genoa (Italy), monitored airborne aflatoxins in a laboratory that analyses imported food products to verify mycotoxin contamination. Airborne aflatoxins during wet grinding phase of shelled peanuts from Vietnam was measured, to assess the exposition of operators. The analysis revealed very low concentration of aflatoxins (about 0.11 pg/m^3) but for their high toxicity, workers protection measures must be adopted, suggesting the introduction of threshold limits values for workplaces (Traverso et al., 2010). Airborne concentrations at the workplace are typically in the ng/m^3-range, but higher concentrations (up to ng/m^3) have been reported (Tab. 6).

Table 6 - Summary of airborne aflatoxin pollution levels in workplaces (in ng/m^3): survey by 22 different international studies Taken from "Aflatoxins: knowledge and prevention" (modified from AUSL Bologna, 2015)

Production sector	Aflatoxins	Airborne aflatoxins				N. studies-data
		min range (ng/m^3)	Min values median (ng/m^3)	max range (ng/m^3)	Max values median (ng/m^3)	
Cereal-growing	B1 - B2 total	nd-88 nd-384	nd 8	11.1-1,505 24-13,000	92 1,680	6-31
Feed mills	B1 total	nd-0.016 0.006-0.016	0.002 0.04	0.027-0.052 0.038-13.26	0.040 1.55	5-14
Peanut processing	B1	0.2-0.87	0.45	7.6-300	19	4-15
Coffee, cocoa, spices	B1 total	nd-nd nd-nd	nd nd	0.029-0.045 -	0.037 -	2-6
Processing/Grinding rice or corn	total	nd-8	0.05	7.39-28	19.11	2-8
Breedings (swine and poultry)	B1	5-124	64.5	0.08-4,849	421	2-5
Waste	B1	0.6-1.7	1.15	1.5-62.3	27.55	1-12

Current legislation

For the European Commission, aflatoxins are carcinogenic, genotoxic and teratogenic, the harmful effects on animal and human populations most exposed by food reasons 'are widely known since years', by having a direct causal role in 4.6 - 28.2% of all the cases of hepatocarcinoma. In fact, the European norms consider them as 'apply only to the food protection for consumers or for cattle'. There is no specific prevention regulation that protects exposed workers: aflatoxins are not included in the European list of occupational carcinogens.

There is a lack of attention/knowledge and poor regulation onto the possible occupational exposure to aflatoxins and on how to prevent their harmful effects. This "lack of attention" is demonstrated by a limited number of epidemiological studies or experimental investigations on

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health effects and occupational exposure levels, even though numerous companies treat, directly or indirectly, contaminated food and feed. It is also demonstrated by the absence of any specific prevention legislation to protect exposed workers and from the fact that, paradoxically, aflatoxins are not included in the EU list of occupational carcinogens.

The only (recent) specific regulatory link on the subject of occupational exposure is represented by the fact that since June 2014 INAIL, following the Decree of the Ministry of Labour of 10.06.14 which included hepatocellular carcinoma in the list of occupational diseases, with the obligation to complaint (pursuant to article 139 of Presidential Decree 1124/65), in the case of previous occupational exposure to Aflatoxin B1 (code I.6.45 - C22.0; Decree Min. Lav. 10.06.14), has classified it as an occupational disease among those eligible for compensation.

Furthermore, in the Ministry of Health Circular dated 16 January 2013 (Extraordinary operating procedures for the prevention and risk management of aflatoxin contamination ...) there is a reminder of the use of PPE among workers involved in the treatment of contaminated maize.

Workers prevention and protection

Aflatoxins are non-volatile substances that can contaminate workers by inhalation or transcutaneous absorption when carried by airborne dust created during handling or processing contaminated raw.

The diameter of airborne flour dust particles measured in mills and bakeries was found be between 0.05 to 21.3 μm that permits this particulate to reach all part of the respiratory routes (Sandiford et al., 1994). The aerodynamic sizes of flour dust particles measured from Stobnicka and Gorny (2015) were between ≤ 4 and 30 μm where the biggest particles were usually formed as agglomerates of smaller ones (Lillienberg and Brisman, 1994; Roberge et al., 2012). Kinetics of these particles in the lungs depends on their size, shape, density as well as on the respiratory volume.

According to Lillienberg and Brisman (1994) more than 50% of the mass of flour dust particles in the air has an aerodynamic diameter greater than 15 μm, but in dusty areas up to 20% of these particles has smaller aerodynamic dimensions (≤ 4 μm, Tiikkainen et al., 1996) characteristics of the respirable fraction.

The European Directives for workers' health protection (Directive 89/391/EEC; Directive 2004/37/EC) impose the risk assessment as the first step to prevent the onset of the professional diseases. While it is not usually possible to eliminate completely the risk, its reduction to the lowest level should be established as an ultimate target. If the risk assessment shows the possibility of exposure to a toxic substance, European Directives require risk mitigation as the first intervention. In this case, the reduction of the aflatoxin exposition could be carried out by reducing the use of contaminated products and reducing dust in the air by means of wet treatments or using automated processes. Subsequently the collective protection equipment such as air conditioning and dust extraction systems should be used. Several studies proved that poorly ventilated workplaces with high concentrations of airborne aflatoxin from strains of *A. flavus* resulted in an elevation in the blood levels of AFB1 in the exposed workers (Oluwafemi et al., 2012).

The samplings indicated that the use of an air conditioning system in the cabs of the combines was an effective control measure. During unloading, using a cab without an air conditioning system, the levels of airborne dust reached 231.1 mg/m^3. Aflatoxin levels varied greatly but levels as high as 195 parts per billion (ppb) were recorded. Much of the higher concentrations of aflatoxins may be in *A. flavus* spores, which can remain airborne for some period of time. Grain elevators located near towns may be a source of hazardous emissions for the population. During harvesting the grain handlers, truckers and farmers may be exposed to highly varying amounts of aflatoxin, particularly in the southern parts of the United States. Attempts were made to develop a mathematical relationship

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between the level of aflatoxin in airborne dust and the concentration of aflatoxin in bulk corn. The aflatoxin entering the air appeared to be related to the method of handling and the history of the corn being processed. Significant levels of aflatoxins were found in and around large commercial grain elevators (Malik et al., 2014). Only if no other system is possible to prevent workers exposure to aflatoxin, the PPEs should be used.

Aflatoxins are classified from cancerogenic to suspect cancerogenic so the ALARA (As Low As Reasonably Achievable) principle for the exposure risk must be respected. Exposure via all routes inhalation, dermal and ingestion, to hazardous substances at work should be eliminated or alternative substances, which are less hazardous should be used. Where elimination is not practicable, adequate protective measures should be put in place so that exposures are reduced to a minimum. The use of suitable protective measures at source should be the first choice to minimise the exposure. Such measures protect everyone in the workplace, whereas a respiratory protective device only protects the person who wears it. If adequate protective measures at source or any other administrative measures are not reasonably practicable or found to be inadequate for controlling inhalation exposures, then an adequate and suitable respiratory protective device should be used. PPEs to protect workers from the risks by professional exposure to aflatoxins are needed to avoid their exposition to the dust of contaminated products, particularly in some specific activities as (Ferri, 2013):

- cultivation and harvesting;
- processing (dryers, feed mills, mills);
- storage and sorting (ports, food consortia, animal husbandry);
- loading and unloading of goods;
- cleaning of facilities and equipment;
- waste disposal and composting (collection, transport, storage, processing, analysis of contaminated plants).

The first step for a correct protection from occupational exposure to aflatoxins is workers information about the connected health hazard. As request from the art. 28 of the legislative Decree 81/2008, the risks assessment permits to evaluate the processes that have exposition risks for workers and to choose the correct PPEs to protect them from these risks. In Europe, it is mandatory that all the PPEs have CE marking, that means that they must possess the essential health and safety requirements (Regulation (EU) 2016/425). These requirements are compulsory and they are provided in the Annex II of the Regulation 2016/425.

As known, aflatoxins are carcinogenic substances that could cause epathocarcinoma, recognized in Italy as a professional disease from the *National Institute for Insurance against Accidents at Work* (INAIL) (Decree of the Italian Ministry of Labor 2014.06.10, Tab. 1), and the workers, to avoid exposition risks, need to use the category III PPEs as provided in the Annex I of the same Regulation, i.e. equipment that protect against serious and deadly hazards. This means that PPEs must possess the CE declaration of conformity of the manufacturer, the technical construction documentation and the periodic verification of the manufacturer's quality system by the control institute. It is also mandatory for workers that use these PPEs to do a specific training. Furthermore, PPEs must be stored and maintained following the manufacturer's instructions for use and maintenance. In the case of processing of feeds contaminated with aflatoxins, PPEs must protect workers from dust inhalation and contact with the skin and mucous membranes (AUSL Bologna, 2015).

Respiratory protection

The PPEs for respiratory protection devices are distinguished in two types: breathing apparatus and filtering devices. Breathing apparatus are insulating from atmosphere air and supply breathable air to the wearer. Insulating PPE with an external air source supply are indispensable when the oxygen level is under 17% as recommended by European Committee for Standardization (CEN). Filtering devices

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purify the ambient air breathed using filters able to remove contaminants in the air.

The UNI EN 529:2005, guidelines for selection, use and maintenance of respiratory protective devices provides the criteria for the selection of the proper PPE based upon risk assessment. The best performances to protect the respiratory system could be achieved using isolating equipment as helmets or full masks with an external air source supply. Otherwise filtering devices could be used, as the powered air-purifying respirators (helmets with visors, visors or semi-masks with forced and pre-filtered air delivery) with P3 filters (high efficiency filters, catch 99.95% of the particles). Full masks equipped with P3 filters could also be used. All these PPEs simultaneously protect the eyes and the ocular mucosa from dust. They can be usefully utilized whenever there is a need for protection of the respiratory system against the inhalation of dust potentially contaminated with aflatoxins. The use of powered equipment is particularly indicated when the work has a particularly prolonged duration and/or has uncomfortable working environment (hot, wet) and/or the worker has individual intolerance (breathing difficulties) when uses masks with filters.

Other PPEs currently used to protect the respiratory apparatus from aflatoxins exposition are the Particulate Filtering Facepieces 3 (FFP3) (EN 149:2009; EN 529:2005). All masks and FFP3 must fit to the user's face and any particularity (glasses, beard, moustache, earrings, etc.) could reduce the protection offered to worker.

Eyes protection

If PPEs used to prevent respiratory exposition to aflatoxins are not eye-protecting, eye protection must be provided. The protection of the eyes and eyes mucosa from dust must take place through the use of protective glasses, preferably visors, with wraparound goggles or visors that must limit the visual field and the view of the user as little as possible; they must also be equipped with a degree of optical neutrality compatible with the nature of the activity and avoid the formation of condensation (EN 166:2004).

Skin protection

The skin should be protected wearing protective clothing from solid particles. These garments are full-body protective clothing (type 5), preferably disposable one-piece coveralls, with hood and front zipper, with elasticated wrist and ankle closure (EN ISO 13982-1:2011) and gloves (EN ISO 374-1-2-3:2016).

The circular of the Ministry of Health of 01.16.2013 "Extraordinary operating procedures for the prevention and risk management of aflatoxin contamination" provides for the use of PPE for workers involved in the treatment of contaminated maize (Ferri, 2013). All disposable PPE must be replaced and disposed of after work, while others must be properly cleaned and stored after each use. PPE must be replaced every time when worn out or broken. Workers should check the PPE before use according to the manufacturer's instructions verifying the correct donning and functionality.

Conclusions

Aflatoxins are a serious problem for human health, and it is not possible to evaluate this threat without paying great attention to the exposure to these compounds. The frequency and level of aflatoxins presence in the food chain have increased in the last decades, probably due to the changed global weather conditions, to the market globalisation, and to the worldwide deployment of mould. The social costs linked to an increase of health conditions like liver diseases, or the problems connected to crop destruction, can be more expensive than a preventive action to reduce aflatoxin presence. The studies evaluated allowed us to recognize that the occupational environment is contributing to the workers' total exposure to aflatoxins. This was confirmed by the high contamination found in food,

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feed and crops samples.

In many countries of the European Union, including Italy, there has also been an increase in mortality from respiratory tract cancer, liver and biliary tract cancers, salivary glands and mediastinum, in workers exposed to aflatoxins in the agri-food sector companies, in feed mills and in activities related to them, compared to the general population. The lack of an official recognition of aflatoxins as professional carcinogens, pursuant to art. 234 of Legislative Decree 81/2008, does not bind employers, if they find their presence in the production cycle, to treat them as such. The current legislation establishes the obligation to report in the case of hepatocellular carcinoma for subjects exposed to aflatoxins pursuant to art. 139 of Italian Presidential Decree 1124/65, based on the new table of occupational diseases. This possibility, although it may occur with a rare or exceptional event, reinforces the consideration that exposure to aflatoxins should be considered as an occupational risk and treated as effectively as possible.

To protect the health of workers, even if the aflatoxins are not on the list of occupational carcinogens, the risk assessment should include that of aflatoxin exposure and should inform workers about the application of good prevention practices to avoid/limit exposure, restore contaminated products, limit dust dispersion/pollution by aspiration and ventilation of work environments. Furthermore, it is essential to measure the dust levels and airborne aflatoxins in the workplace, since even if there are no occupational exposure limits neither nationally nor internationally, by knowing their concentration in the air it is possible to have a more precise idea of the risk magnitude in the studied workplace, and to evaluate and identify the priorities in the interventions of prevention. At the individual level it is essential to protect the exposed workers with the use of appropriate PPE, to evaluate the effectiveness of the adopted measures and of the residual risk and finally to establish an effective health surveillance protocol that foresees the possible biological monitoring, associated with evaluation of the eating habits of individuals, with reporting obligation in case of hepatocellular carcinoma and, of course, adequate integration, at international and national level, of the current legislation.

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