1 General Introduction

In the early 1960s an acute hepatotoxic disease, then named turkey "X" disease, causing the death of more than 100'000 turkeys in Britain and focused the attention of many researchers on a new class of toxic compounds identified as mycotoxins. Mycotoxins are natural secondary metabolites of filamentous fungi that can cause intoxications in humans and animals, either after ingestion or inhalation or skin contact of mycotoxin contaminated feeds and food.

In a first step of investigation was found that toxin metabolites produced by some strains of Aspergillus flavus were the cause of the turkey "X" disease. Later on compounds were chemically characterized and designated as aflatoxins, from the acronym "Aspergillus FLAvus TOXINS".

These discoveries represent the begging of the modern mycotoxicology. Since then many other mycotoxins have been discovered, some were later found to be causes of intoxications in humans and animals while others remained just laboratory curiosities. Despite the fact that more than one thousand toxic metabolites from fungi are actually known, the mycotoxins causing health diseases and economic concerns can be grouped in few classes like aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone, cyclopiazonic acid and ergot alkaloids.

These mycotoxins are produced by a wide range of fungal species, mainly belonging to five genera: Aspergillus, Fusarium, Pennicillum, Claviceps and Stachybotrys. In many cases mycotoxins are produced in the field during the growing season; however fungi can also produce and increase mycotoxins content in post-harvested commodities during drying, storage and transport.

Direct exposure to mycotoxins in humans and animals are through consumption or contact with contaminated cereals, seeds, spices, fruits, beverages and other plant materials. Residues of mycotoxins, either as metabolites or not, can contaminate animal food by-products from animal exposed to contaminated crops, then indirectly entering the human food chain with consumption of milk, meat, eggs and their derivates.

Diseases caused by mycotoxins are referred to as "mycotoxicosis" and they can be acute, chronic and sub-chronic. Acute mycotoxicosis are reported rarely and mainly in developing countries. Recently a human mycotoxicosis outbreak caused by the consumption of maize highly contaminated with aflatoxins resulted in 317 confirmed cases and 125 deaths in Kenya. Chronic and sub-chronic mycotoxicosis are characterized by long-term exposure to low levels of contaminated food/feed and are most frequent both in developed or developing countries and can be considered as a worldwide problem.

Due to the fact that mycotoxins rarely cause acute intoxication disease and health emergencies, the risk perceived by the consumers is lower compared to other food-related threats such as pesticides, additives, heavy metals and microbial agents. However, mycotoxins in the human food chain have been responsible for many documented diseases, also in developed countries. Therefore, many countries have fixed legal limits for mycotoxins presence in food: more than 100 countries have specific regulations for mycotoxins, just few countries have no specific regulations and about 50 countries have no legislation on mycotoxins presence in feeds and in food.

Several approaches have been investigated to control mycotoxins contamination and to avoid or, at least, to reduce their presence in feeds and food. The most effective way to reduce the risk associated to mycotoxicosis is by the control of mould growth and consequently mycotoxins production in pre-harvest conditions. However, the post-harvest handling of grains gives additional opportunities to control mycotoxins presence in feeds and food. Even with current technologies and recent researches advances it is very difficult to prevent or predict mycotoxin contamination. The dietary supplementation with non nutritive mycotoxin adsorbent in one of the most used method to reduce negative effects of mycotoxins presence in animals feeds.

After the first confirmed mycotoxin outbreak, other mycotoxicosis have been documented particularly in correlation to animal diseases. Chronic and sub-chronic mycotoxicosis, more than acute types, are responsible for grave economic losses in animal farms through lowered productivity, decreased weight gain, decreased feed efficiency, decreased production (meat, egg and milk), immune system suppression, interference with reproductive performance and damage to target organs.

Overall, ruminants are more resistant to most mycotoxins than monogastric animals. This suggests the rumen, and the microbial population in it, plays an important role to counteract negative effects of mycotoxins. Zearalenone is degraded to two principal metabolites by rumen microorganism and the rate of carry over in milk, either as parent or derivate molecules, is very low and is considered not a real risk for the consumer, even if metabolites produced in the rumen are more estrogenic then parent compound. Trichothecenes and ochratoxins are metabolized in the rumen yielding less toxic compounds. Neither of these mycotoxins is efficiently transferred to milk. Fumonisins are apparently poorly metabolized in the rumen, however ruminants are considered to be very tolerant to these toxins and their carry-over into milk nearly negligible.

Aflatoxins are one of the most potent natural carcinogenic compounds present in nature. It has been indicated that they are rapidly adsorbed throughout the gastrointestinal tract of ruminants. Aflatoxin B1, the most toxic of the aflatoxins, is poorly degraded in the rumen and it is quickly excreted in milk as the metabolite aflatoxin M1 with a carry over rate ranging from 1 to 3 per cent. Due to the restrictive legislation in the UE, which limits aflatoxin M1 content in milk at 0.05 μg/L, several methods have been reported to reduce excretion of aflatoxin B1 as aflatoxin M1 in milk of dairy cows.

The present work is based on five article manuscripts. The aim was to study aspects related to aflatoxins absorption, biotransformation and excretion in dairy cows. Also technical strategies to reduce negative effects of aflatoxins and their metabolites carry over in milk of dairy cows were investigated.

Manuscripts 1 and 2: The present works investigated the rate over time of the aflatoxins plasma appearance following an oral contaminated bolus to verify where and when these toxins are absorbed in the gastro intestinal tract of dairy cows. The aflatoxins plasma and milk appearances were also investigated using a non absorbing mucosa to understand the possible aflatoxins absorption mechanism through mucous membranes.

Manuscript 3: A trial was carried out in lactating dairy cows to study the carry over of ingested aflatoxin B1 in milk as aflatoxin M1 in relation to milk yield and somatic cells count, the latter as indicator of udder inflammatory processes.

Manuscript 4: The sequestering capacity of different kinds of mycotoxins sequestering agents were compared in *in vitro* trials carried out at different experimental conditions. The behavior of the aflatoxins-adsorbents complexes through digestive tract of the lactating dairy cows were also investigated *in vivo* by measuring the appearance of aflatoxin M1 into milk.

Manuscript 5: An *in vivo* trial was conducted to verify if the effect of the pelletizing or simply the mixing processes is useful to improve mycotoxins sequestering agents efficacy in dairy cow nutrition.

2 Manuscripts proposed in this work

I. Mucosal absorption of aflatoxin B1 in lactating dairy cows

Manuscript for: *Italian Journal of Animal Science*

II. Aflatoxin B1 absorption in the gastro-intestinal tract and in the vaginal mucosa in the lactating dairy cows

Manuscript for: *Italian Journal of Animal Science*

III. Carry over of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts

Manuscript for: *Animal*

IV. Effect of rumen fluid on in vitro aflatoxin binding capacity of different sequestering agents and in vivo release of the sequestered toxin

Manuscript for: *Animal Feed Science and Technologies*

V. Effects of mixing and palletizing on the efficacy of a sequestering agent in reducing aflatoxin M1 excretion into milk of lactating dairy cows

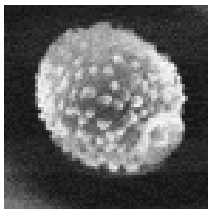
Manuscript for: *Journal of Dairy Science*

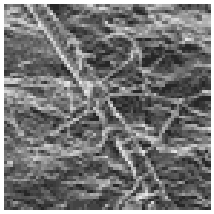
3 Aspergillus Growth and Aflatoxins Production

3.1 Introduction

Moulds are organisms without chlorophyll that are capable to grow in absence of light. The term mould includes all microscopic fungi species growing as multicellular filaments called hyphae (figure 3-1). A network of hyphea is called mycelium and is responsible for cementing kernels together, which results in a column. Fungi also produced spores or conidia capable of aerial diffusion and important for fungi survival and diffusion. Spores are usually dispersed passively by wind and rain, but also insect could serve as vectors transporting the spores with their bodies. An important characteristic of spores is that they can lay dormant for months or years until the proper condition for fungal development are available.

Figure 3-1. Individual spore (original magnification, o. m. x 3000), conidial structure (o. m. x 600) and several strands of hyphae (o. m. x 300) of *Aspergillus flavus*





Several moulds can produce mycotoxins. These fungi are defined as **mycotoxigenic fungi** (Santin, 2005) and are usually identified as non aggressive pathogens; however same species could invade and colonize plants either before or after harvesting, during transport or storage (Payne et al., 1988; Scheidegger and Payne, 2003) .

Under field condition stress reduces vigor of plants and could predispose to infestation and colonization by mycotoxigenic fungi (Bruns et al., 2003; CAST, 2003). In stored condition, two important factors can affect fungi colonization and mycotoxins production and they are temperature and moisture (Payne et al., 1988), even if substrate, oxygen (O_2) and carbon dioxide (CO_2) concentration and insect presence are still important (Scheidegger and Payne, 2003).

Main principally mycotoxigenic moulds are members of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, *Stachybotrys* and *Neotyphodium* genera (CAST, 2003; Santin, 2005). The genera *Aspergillus*, *Fusarium* and *Penicillium* are the largest number of mycotoxigenic fungi and they are usually classified as **storage fungi**, even if several species of *Aspergillus* spp.

and *Fusarium* spp. can damage crops directly in the field. The storage fungi are characterized to invade grains or seeds during storage and for their growth require low moisture conditions (13-18%) (Christensen, 1974; Santin, 2005).

In contrast *Claviceps* spp. and *Neotyphodium* spp. invade plant tissue mainly in field condition and require high moisture conditions (20-21%), so they are called **field fungi**. *Neotyphodium* spp. are also able to colonize plant as well as reproductive tissue (Santin, 2005).

3.2 Aspergillus Species Infection and Growth Condition

The genus *Aspergillus* represents a large family of fungi capable to live in very different ecological habitats. *Aspergillus* spp. appear to be abundant from 26° to 35° north and south equator (Klich et al., 1994), even if its members are distributed worldwide. These fungi are considered to be common in tropical, subtropical and warm temperate climates (Scheidegger and Payne, 2003).

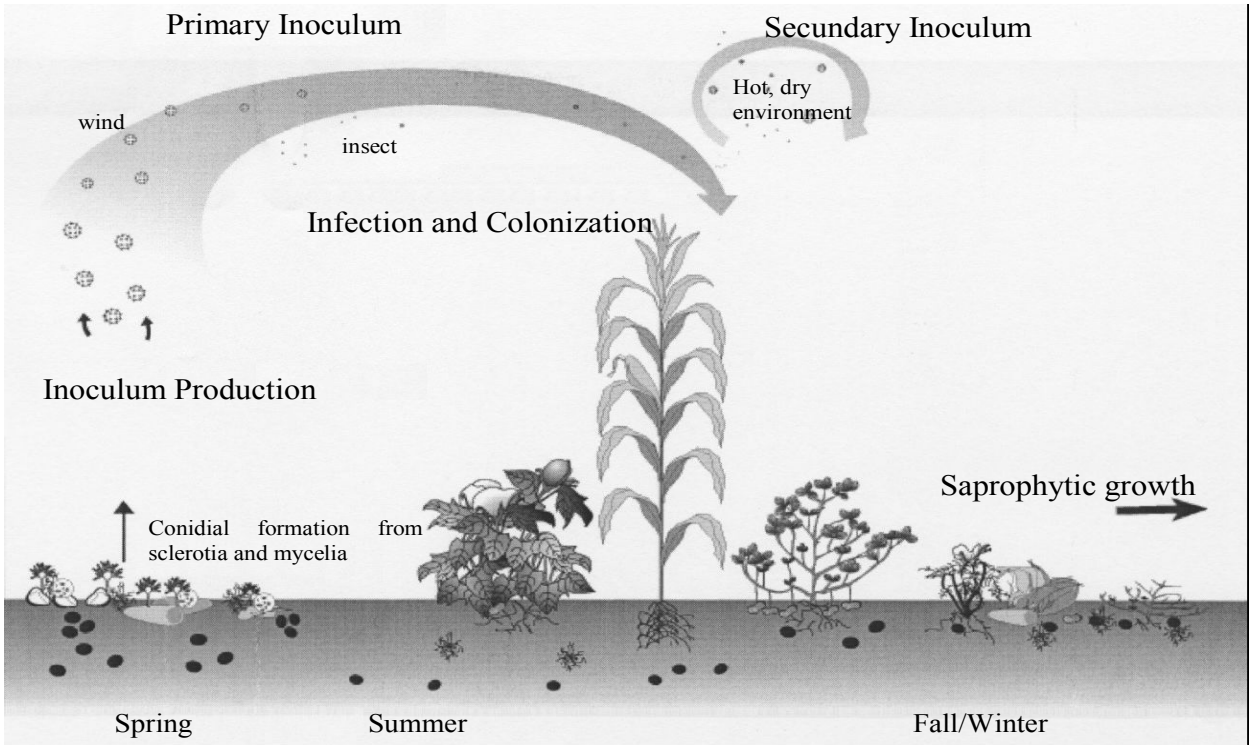
Aspergillus is well-known for its aggressiveness on stored crops, but also for its ability to produce aflatoxin in colonized seeds and grain and it is capable to grow on a large number of substrate (Scheidegger and Payne, 2003). However, it appears to colonize principally maize, cotton, peanut and their by-products (Wilson and Payne, 1994). *A. flavus* is the predominant species on all these feeds (Payne 1992; 1998), while *A. parasiticus* is common particularly on peanuts (Horn et al., 1994).

The infection process (figure 3-2) by *A. flavus* is well characterized in corn (Payne, 1998; Scheidegger and Payne, 2003): this mould is a soil-inhabiting fungus that reproduces by asexual conidia, therefore *A. flavus* appears to spend more of its life growing as a saprophyte in the soil (Scheidegger and Payne, 2003). Primary infection occurs through the dissemination and germination of conidia. Conidia are carried to the corn silks by wind, rain or insect. The fungus will colonize silk tissue and will grow down the silks to the kernels where it can infect developing kernels (Widstrom, 1996). With favorable environmental conditions the fungus may directly invade the seeds and cobs or enter through tissue wounds operated by insect. The infection usually does not occur until kernel moisture is below 32% (Payne, 1998), whereas aflatoxins may be produced until 15% moisture content (Payne, 1988).

When in presence of *A. flavus* plant contamination, it can grow and produce aflatoxin long after the harvesting time. While this appears to have severe consequences for the food and feed, saprophytic growth is also important to consider in the life cycle of this pathogen. Infected plant tissue such as corn kernels, cobs, and leaf tissue may remain in the soil and support the

fungus until the following season when newly exposed mycelium or sclerotia can give rise to conidial structures, thus producing the primary inoculum for the next infection cycle on the subsequent crop.

Figure 3-2. Diagram of the pre-harvest infection of cotton, corn, and peanuts by *A. flavus* (Scheidegger and Payne, 2003)



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The two principal factors affecting *Aspergillus* spp. contamination are temperature and moisture (Payne et al., 1998; Widstrom, 1996). Usually high temperatures and drought stress increase the airborne inoculum of the fungus (Jones et al., 1981; McGee et al., 1996). This is probably related to the optimum growth temperature and moisture conditions of the fungus.

A. flavus could grow over a wide range of temperatures (from 12 to 48°C); however its optimum growth condition is considered close to 37°C (Klinch et al., 2004). In an Italian research conducted on six northern Italian species the optimum growth conditions resulted ranging from 25 to 30°C (Giorni et al., 2007), lower than previously optimum temperature (Klinch et al., 2004).

Moisture is also important and can affect *Aspergillus* strains growth: these moulds can grow in a range of 13-18% RM and with water activity (a_w) higher to 0.73 value.

The relative concentration of O_2 and CO_2 are important factors acting on mould growth and possible mycotoxin production. A CO_2 value over 20% can depress fungi growth, whereas

the reduction of O_2 concentration below 10% lowers the *Aspergillus* colonization; levels lower than 1% can completely inhibit the mould growth (Ruiqian et al., 2004).

Another factor affecting crop contamination is plant nutrition, in particular sufficient level of nitrogen are known to be important in reducing the risk of aflatoxins presence (Jones, 1981). Plant stress resulting from unbalanced N-fertilization rates, associated with drought stress or leaching of mineralized N from the root zone due to excessive rain, were found to increase the incidence of aflatoxin contamination in maize (Santin, 2005).

To reduce contamination recommendations are to harvest maize grain at moisture content between 25.5 and 20.0% and then artificially dry it for safe storage to less than 15.5% within 48 hours for harvest (CAST, 2003; Santin, 2005). Fungal growth and mycotoxin production can flourish in few days if grains are not properly dried and cooled before being placed in storage. Even during transportation it is important to keep grain at a safe moisture and temperature conditions.

3.3 Aflatoxin Production

Mycotoxins are an important consequence of the fungal growth, even if the conditions regulating the toxins production by mycotoxigenic moulds are not still completely understood. Mycotoxins are produced as **secondary metabolite** of the fungi metabolism. The primary metabolites are considered compounds essential for fungi growth, while secondary metabolites are usually formed in the final stage of the exponential growth phase (CAST, 2003) and are related principally to the ecological relationship of the fungus with surrounding environment. Fungi produce mycotoxins under stressful conditions such as change in temperature, moisture, aeration or presence of aggressive agents.

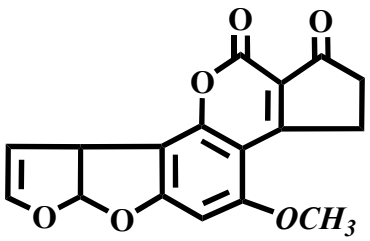
Mycotoxins associated with *Aspergillus* spp. include cyclopiazonic acid (CPA), ochratoxins, versicoloronins, sterigmatocystin, gliotoxin, kojic acid, nominine and citrin, with the principal group as aflatoxins.

The aflatoxins (AFs) can be produced by six species of *Aspergillus* section *Flavi*: *A. flavus*, *A. parasiticus, A. nomius, A. bombycis, A. pseudotamarii* and *A. tamari* (Goto et al., 1996; Ito et al., 2001; Varga et al., 2003). However, the two most important aflatoxin producer fungi are *A. flavus* and *A. parasiticus* which are also considered economically important (CAST, 2003).

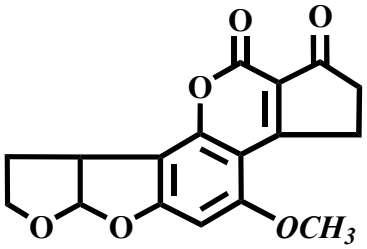
These fungi are capable to synthesize four types of aflatoxins: aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (figure 3-3). *A. flavus* is known to produce only AFB1 and

AFB2, while *A. parisiticus* could produce all aflatoxins (Pitt, 1989; Scheidegger and Payne, 2003). Over 4'000 aflatoxin-contaminated corn and peanut samples analyzed in Georgia (USA) showed that 79.6% of corn and 61.8% of peanut samples contained only AFB1 and AFB2 whereas 21.6% of corn and 35.7% of peanut samples contained all parent aflatoxins (Hill et al., 1985).

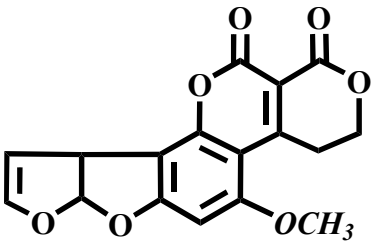
Figure 3-3. Chemical structures of the four parent aflatoxins: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2)



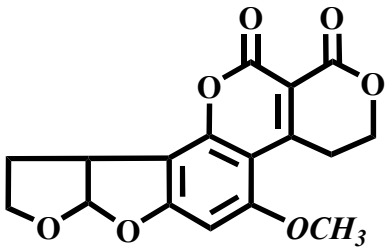
AFB_1



AFB_2



AFG_1



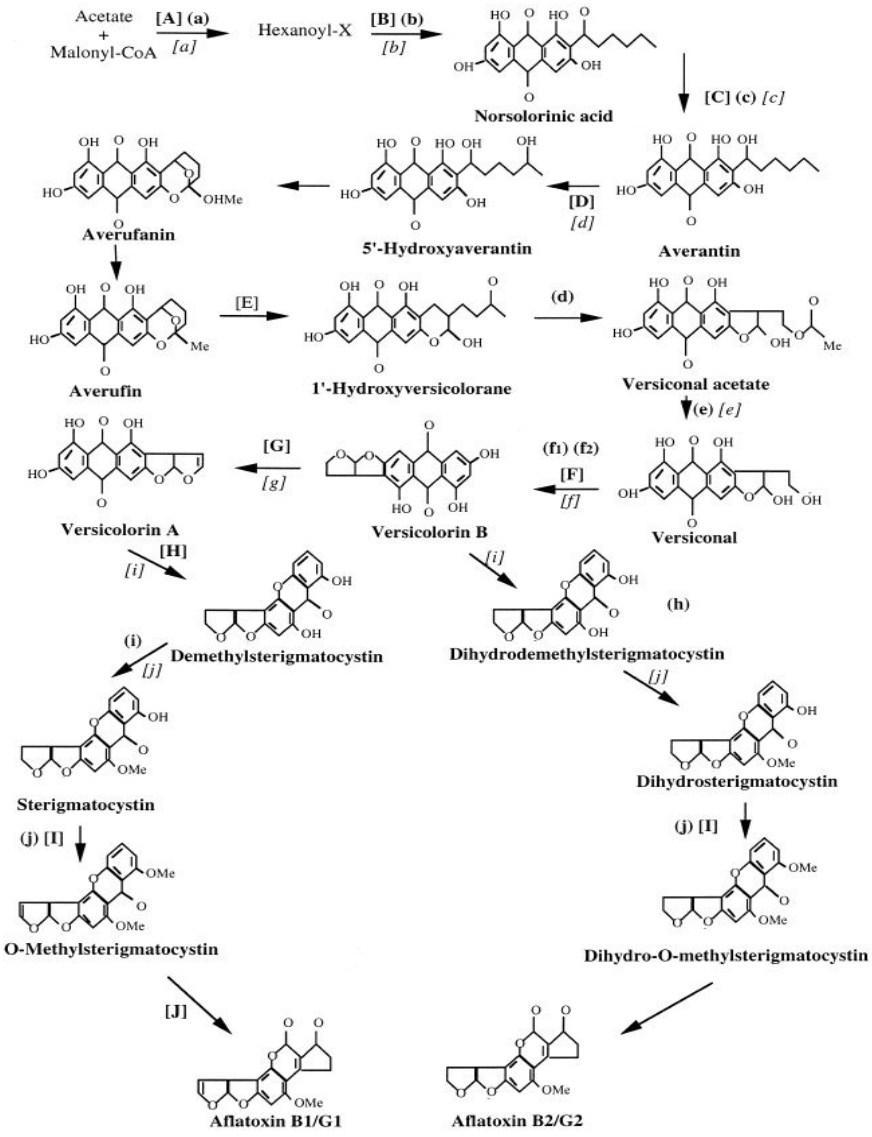
AFG_2

The factors affecting aflatoxin production can be grouped into three categories and precisely physical, nutritional and biological factors (Ruiqian et al., 2004). Physical factors include temperature, moisture and a_w, pH, light and aeration. Optimum temperature for mycotoxins production range between 25° and 30°C. However Giorni et al. (2007) reported on Italian strains of *A. flavus* optimum temperature of about 25°C. Aflatoxins production is favored by moist conditions. The a_w should be over a minimum value of 0.85, with an optimum value at 0.99 a_w (Somapundo et al., 2007)

The aflatoxins biosynthesis pathway is well understood and was recently reviewed (Trail et al., 1995; Minto and Townsend, 1997; Sweeney and Dobson, 1999). Briefly, acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase, which is then

extended by a polyketide synthase to norsolorinic acid, the first stable precursor in the pathway. The polyketide then undergoes approximately 12-17 enzymatic conversions, through a series of pathway intermediates, which are summarized in figure 3-4 (Sweeney and Dobson, 1999). Following the formation of versicolorin B, the pathway branches to form AFB1 and AFG1 which contain dihydrobisfuran rings and are produced from de-methyl-sterigmatocystin, and the other branch forms AFB2 and AFG2, which contain tetrabisfuran rings and are produced from di-hydro-de-methyl-sterigmatocystin.

Figure 3-4. Aflatoxins biosynthetic pathway. Enzymes involved: (a) fatty acid synthase, (b) polyketide synthase, (c) norsolorinic acid reductase, (d) versiconal hemiacetal acetate reductase, (e) esterase, (f1) versicolorin B synthase, (f2) versiconyl cyclase, (g) desaturase, (h) O-methyltransferase (MT-II), (i) O-methyltransferase, (j) O-methyltransferase (MT-I) (Sweeney and Dobson, 1999)



3.4 Occurrence of Aflatoxins in Raw Products

Aspergillus spp. grow on a large number of substrate, in particular *A. flavus* considered an ubiquitous mould (Payne, 1998; Scheidegger and Payne, 2003) and it could be found on cereals, seeds, spices and other plant material (Galvano et al., 2003). Aflatoxins contaminations are reported in feeds either before harvest or during time between harvesting and drying or during transport and in storage conditions (CAST, 2003; Santin, 2005).

The contamination is usually at low level (µg/kg), but sporadically it is possible to find higher level (mg/kg). The commodities considered at high contamination risk are corn, peanut, cotton, Brazil nuts, pistachios and copra, while low aflatoxin contamination risk plants are figs, almonds, pecans, walnuts and rainins.

Soybean, sorghum, millet, wheat, oat, barley and rice are less susceptible to *Aspergillus* spp. infection, but stored condition supporting mould growth, as high moisture and high temperature, may result in a contamination of this crops during storage (CAST, 2003). For example, 36% of stored sorghum samples were found to be positive to aflatoxin contamination in Uganda, with total level ranging from 1 to more than 1'000 µg/kg (D' Mello and Macdonald, 1997).

Cereals grain and related by-products are one of the most consumed food and million of people utilize these commodities as the primary source of carbohydrates for human and animals. Cereals are easily colonized by mycotoxigenic moulds because they represent a good substrate for fungi growth. Corn is the most frequently cereal contaminated by aflatoxin. The US Food and Drug Administration (FDA) reported that aflatoxins were found in the 26% of the corn samples in USA analyzed during 10 years, from 1987 to 1997, with the 22% of these samples at concentration higher than 20 µg/kg.

Also oilseeds are considered an excellent substrate for *A. flavus* growth and aflatoxin production. Peanuts and cottonseeds are the two principal aflatoxin contaminated crops. Al Adil et al. (1977) reported that *A. flavus* was isolated from 36% of samples of several legume seeds purchased on markets in Baghdad area. Peanut lots, analyzed from 1990 to 1996 to monitor aflatoxin concentration in USA, had a number of aflatoxin positive cases ranging from 22 to 41% (CAST, 2003).

Also spices were considered commodities with a high contamination risk. *A. flavus* was recorded in caraway (39.2%), karkade (38.3%) and peppermint (11.6%) in Egypt. However the aflatoxin contamination was low with the total aflatoxins amount ranged between 0.42-12.34 µg/kg (Soliman and Ismail, 1999).

3.5 Management of Grain to Avoid Mould Growth

3.5.1 Pre-Harvest Control Strategies

Agronomic and management practices assuring general health of the crops in filed can reduce pre-harvest aflatoxin contamination (Cole et al., 1995; Payne, 1998). Practices include timely planting, proper plant nutrition, mainly adequate amounts of nitrogen, avoiding drought stress, particularly during kernel filling, pests monitoring and proper harvesting (CAST, 2003; Bruns 2003; Santin, 2005).

Hybrids of maize more resistant than grain to mould should be planted where available in mycotoxins exposed areas. Inbred lines of corn with same resistance aflatoxins accumulations have been identified (Brown et al., 1998; Huang et al., 1997; Bruns, 2003). However, only few genotypes are available with a lower susceptibility to aflatoxins accumulation.

After seeding during crop development the stress of plants should be reduced. The principal stress condition associated to grain mould are insect damage, high moisture following a dry season, moisture stress early in the season and during grain fill (Santin, 2005).

Recent researches suggested bio-control agents as a tool to reduce aflatoxins contamination in pre-harvest condition (Dorner et al., 1997; Cleveland et al., 2003; Ruiqian et al., 2004). Plants in field are treated with non-aflatoxigenic strains of the *A. flavus*, since they live in the same ecological niches, these moulds are natural biocompetitor of the mycotoxigenic fungi. Bock and Cotty (1999) used a non-toxigenic strain (AF36) of *A. flavus* and found that an increase of this fungus caused a significant decrease of the toxigenic strains and aflatoxins production in the cottonseed. *Bacillus subtilis* can also inhibit the colonization of toxigenic *Aspergillus* spp. This inhibition may result from many factors including competition for space and nutrients (Ruiqian et al., 2004).

3.5.2 Post-Harvest Control Strategies

To minimize the mould growth and mycotoxins production in stored grain the moisture should be reduced to less than 15% rapidly after harvesting (Santin, 2003).

During harvest and post-harvest operations it should be avoid the mechanical damage of the grain: additional damage of the grain could mean entry sites for pathogenic moulds. Also insect damage, either in field or during storage, provides to increase presence of mycotoxigenic fungi, facilitates infection or promotes distribution throughout the grain mass. Moreover, same

insect can also act as vector for grain mould pathogens. Use of the fungi static agents could reduce this negative effect related to insect (Santin, 2003; CAST, 2003).

Organic acids (propionic, acetic, sorbic and benzoic acids) are usually used as mould inhibitor on stored crops because change pH of food preventing development of fungi (Dixon and Hamilton, 1981). The usages of these acids are related to the moisture content: with high moisture content more acid is needed. The form of the acid is also important, for instance the dissociated form of propionic acid does not fit for use since it is corrosive, whereas the undissociated form is the only form being effective in killing the fungi.

4 Metabolism and Mechanism of Action of the Aflatoxin

4.1 Absorption of Aflatoxins in the Gastro-Intestinal Tract and Passage in Blood Compartment

Humans and animals exposure to aflatoxins occurs through several routes like ingestion, that is considered the most prominent way of exposure (Sudakin, 2003), skin contact (Rastogi et al., 2006) or inhalation (Jakab et al., 1994).

Due to a relatively low molecular weight of the aflatoxins (AFB1 312.27 formula weight) and their lipophilic properties, the most probable mechanism of aflatoxins absorption is through a passive diffusion (Yiannikouris and Jouany, 2002). Several authors reported that AFB1 adsorption and excretion is a rapid process (Polan et al., 1974; Trucksess et al., 1983; Coulombe, 1993; Hiesh and Wong, 1994).

In one of the first study on aflatoxins absorption (Wogan and Newberne, 1967) authors reported that 20% and 60% of the AFB1 was excreted trough urinary and fecal routes within 24 hours of treatment in male rats. However, a small amount of the AFB1 was excreted in the first 8 hours suggesting a rapid passage through the gastro-intestinal mucosa as consequence of an oral AFB1 administration. Degan and Neumann (1978) reported that the female rats excreted about 10-30% and 60-65% of the total given [^{14}C]AFB1 into bile after 24 hours from oral and intra-peritoneal administration, respectively.

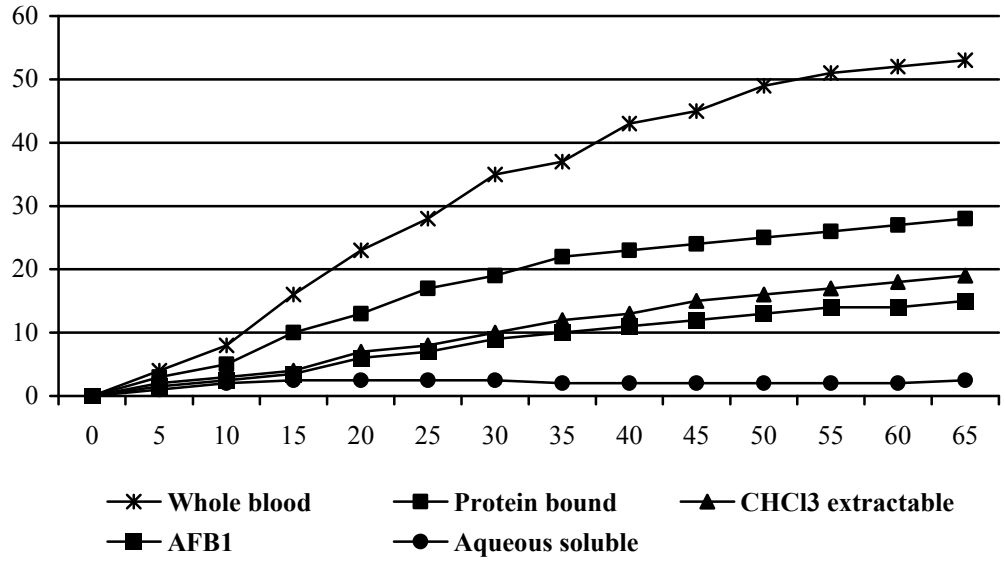
Results suggested that AFB1 was rapidly absorbed in the small intestine to the mesenteric venous blood. Similar considerations were reported in dairy cows after a single oral dose of 0.5 mg/kg BW of AFB1: the maximum AFB1 concentration in plasma was found at 12 and 60 hours

post dosing (Trucksess et al., 1983). Also in ruminants aflatoxins appeared to be absorbed rapidly in the gastro-intestinal tract after ingestion (Polan et al., 1974).

Kumagai (1989) reported the rate of AFB1 uptake by intestine tissue is nearly proportional to AFB1 concentration indicating that this toxin was absorbed by a probable passive diffusion. When the rate of AFB1 absorption was compared to AFG1, a less lipophilic molecula, the rate was lower, confirming that lipophilicity is a determinant factor affecting aflatoxin absorption.

A time concentration plot of the total radioactivity of three types of blood borne [^{14}C]AFB1 metabolites is shown in figure 4-1.

Figure 4-1. Absorption of [^{14}C]AFB1 from a ligated 10 to 15 cm duodenal section of small intestines in male rats. The absorbed radioactivity was shown as the percentage of that in the administered dose of 0.05 mg/kg (Hsieh and Wong, 1994)



The curves of comparison of the different [^{14}C]AFB1 metabolites in blood suggested that absorption of AFB1 from duodenal section of the intestine of rats in the mesenteric venous blood and conversion of AFB1 in the different metabolites, took place as a first order processes. The residual radioactivity found in the small intestines at 65 minutes after aflatoxin administration was 45% of the administered dose, in which 39% was present as free AFB1 and the remaining amount (61%) as water soluble and protein bound metabolites. No other chloroform extractable metabolites were found after this time. The AFB1 residue in the small intestine most likely is derivate from the lumen content. The low aqueous soluble and protein bound content indicated a rapid transport of biotransformation products from intestinal lumen to the mesenteric venous blood.

4.2 Aflatoxins Distribution in the Organism and Concentration in Liver and Kidneys

After absorption in the gastrointestinal tract, the AFB1 apparently enters in the liver through the hepatic portal blood supply (Wilson et al., 1985). Given the high efficiency of this organ to extract free absorbed AFB1 from blood, probably due to the high permeability of the hepatocyte membrane for AFB1 (Eaton et al., 1994), liver appears to be the principal organ involved in the aflatoxins biotransformation and detoxification (Hsieh and Wong, 1994).

Muller and Petzinger (1988) showed that AFB1 permeation occurs by non-ionic diffusion in rat hepatocytes. Authors suggest that the rate of the permeation is determined by the composition of the lipid domains in the cell membranes. Species differences in lipid composition of the membrane would be a factor determining the hepatic uptake of AFB1. Also kidneys are able to concentrate the free AFB1 from blood compartment (Hsieh and Wong, 1994). Thus, two organs (liver and kidneys) are considered the principal target organs related to metabolism of the AFB1.

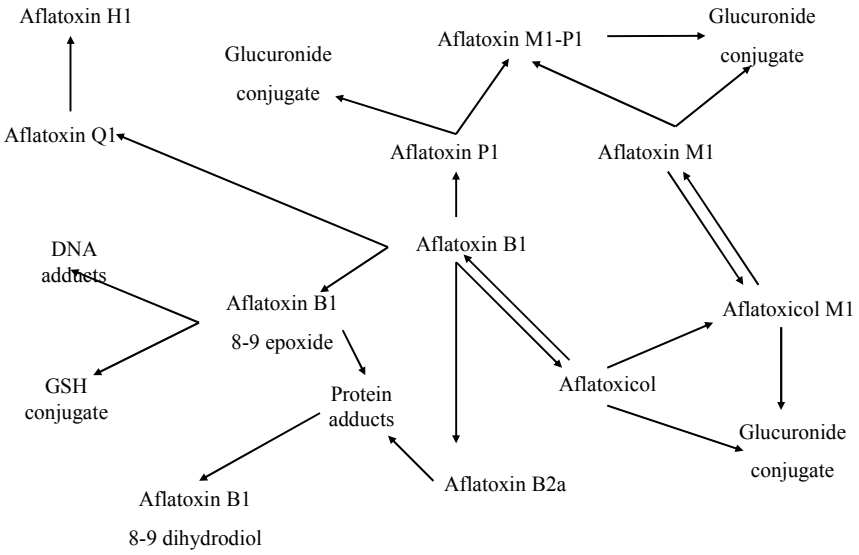
Wogan et al. (1967) studied the passage of aflatoxins from gastro-intestinal tract and absorption rate in the liver. These authors found that 30 minutes after an intra-peritoneal dose of 70 μg/kg of [^{14}C]AFB1, the liver contained approximately 17% of the total radioactivity, the kidneys 5% and the carcass 27% in rats. Other organs as adrenal glands, brain, heart, pancreas, spleen, thymus and testis contained levels lower than 0.5%. During the next 90 minutes, the radioactivity in the kidneys and liver decreased rapidly to less than 1% and 10% after 2 hours from the treatment, respectively.

The activation of the mechanisms of aflatoxin conversion in the liver appear to be very fast: after 2 hours from AFB1 ingestion the amount retained in the liver was converted in polar molecules for 12%, in no polar metabolites for 3% and in covalently bound adducts for more than 70% of liver retained aflatoxins (Holeski et al., 1987).

4.3 Biotransformation of Aflatoxins

Several pathways have been identified involving bio-transformation of the AFB1 and other aflatoxins when these compounds are absorbed by human or animal organisms. These pathways could conduct to activation or modification of the AFB1 molecule and to a conjugation of the toxin to obtain less toxic compounds respect to the parent compound. The toxicity and carcinogenicity of the AFB1 are determinate by how the toxin is bio-transformed in the organism. The figure 4-2 summarizes the principal studied pathways.

Figure 4-2. Bio-transformation pathways for aflatoxin B1 (adapted by Eaton et al., 1994)



Many researches have been focused particularly on the metabolic alteration of AFB1, due to toxic and carcinogenic proprieties of these aflatoxins and to the highest concentration often found in food and feed (CAST, 2003). Similar pathways are similar for other aflatoxins like AFB2, AFG1 ad AFG2.

Activation of AFB1 and other aflatoxins to different metabolites appears to be mediate principally by cytochrome P450 (CYP) oxidative enzymatic system. The CYP is present in the organism in several forms and tissues. Those involved in AFB1 bio-transformation appear to be CYP 1A2, CYP 2A6, CYP 2C, CYP 3A1, CYP 3A2 and 3A4 (Shimada and Guengerich, 1989; Coulombe, 1993; Yannikouris and Jouany, 2002; Sudakin, 2003). More active cytochromes are CYP 3A4 and CYP 1A2 forms (Gallhager et al., 1996; Pelkonen et al., 2000), however the relative importance of the various CYP forms in aflatoxins bio-activation to different metabolites is still uncertain (Eaton et al., 1994).

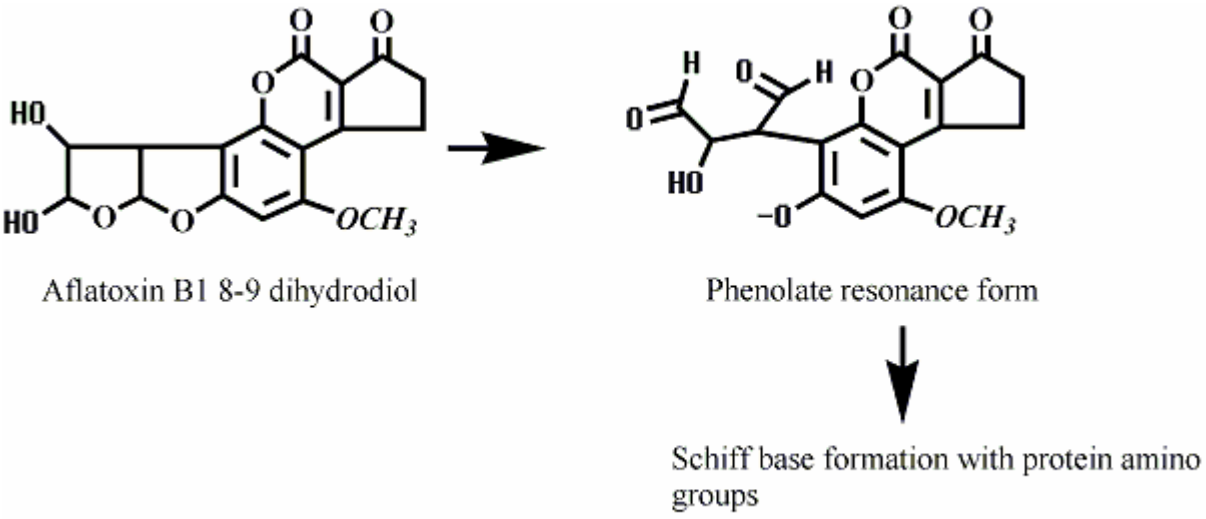
4.3.1 Epoxidation of Aflatoxins

CYP dependent epoxidation of the terminal furan ring double bond of AFB1 and AFG1 generates very potent electrophilic species that are capable to bound nucleic acids of DNA, particularly guanine, forming adducts (Essigmann et al., 1977; Baertschi et al., 1989). The first step of epoxidation pathways is the formation of AFB1-8,9-epoxide metabolite. Due to its instability in

aqueous system, the epoxide form disappears completely in less than 5 seconds (Eaton et al., 1994) and it has not been still isolated from biological fluid, but its formation is inferred from the isolation of products of its reaction with biological nucleophiles, as DNA and glutathione (GSH) (Essigmann et al., 1977). The reaction between DNA and AFB1-8,9-epoxide is probable related to carcinogenic activity of aflatoxins (CAST, 2003).

The hydrolysis of the epoxide is theorized to form the AFB1-8,9-dihydradiol, that is capable to form Schiff base adducts with protein amino groups, particularly lysine (figure 4-3). The AFB1-lysine adduct is the principal protein adduct found in the plasma albumin after AFB1 exposure *in vivo* (Sabbioni et al., 1987). This reaction can be involved in the mechanism of toxicity of the AFB1.

Figure 4-3. Schiff base formation between protein amino groups and AFB1-8,9-dihydradiol (by Eaton et al., 1994)



Covalent binding to DNA is generally a property of all aflatoxin derivates, included hydroxylated metabolites of the AFB1 as aflatoxin M1 (AFM1) and aflatoxin P1 (AFP1), presumably due to an epoxidation of the molecule. The AFM1 and AFP1 epoxides were identified in liver extracts following AFB1 administration in rats (Essigmann et al., 1983). Aflatoxin Q1 (AFQ1) indeed is a relatively poor substrate for epoxidation (Eaton et al., 1994).

The adducts formed from AFB2 are identical *in vivo* to those identified following AFB1 administration in rat (Groopman et al., 1981), consistent with hypothesis that AFB2 can be reduce to AFB1 and then epoxidized.

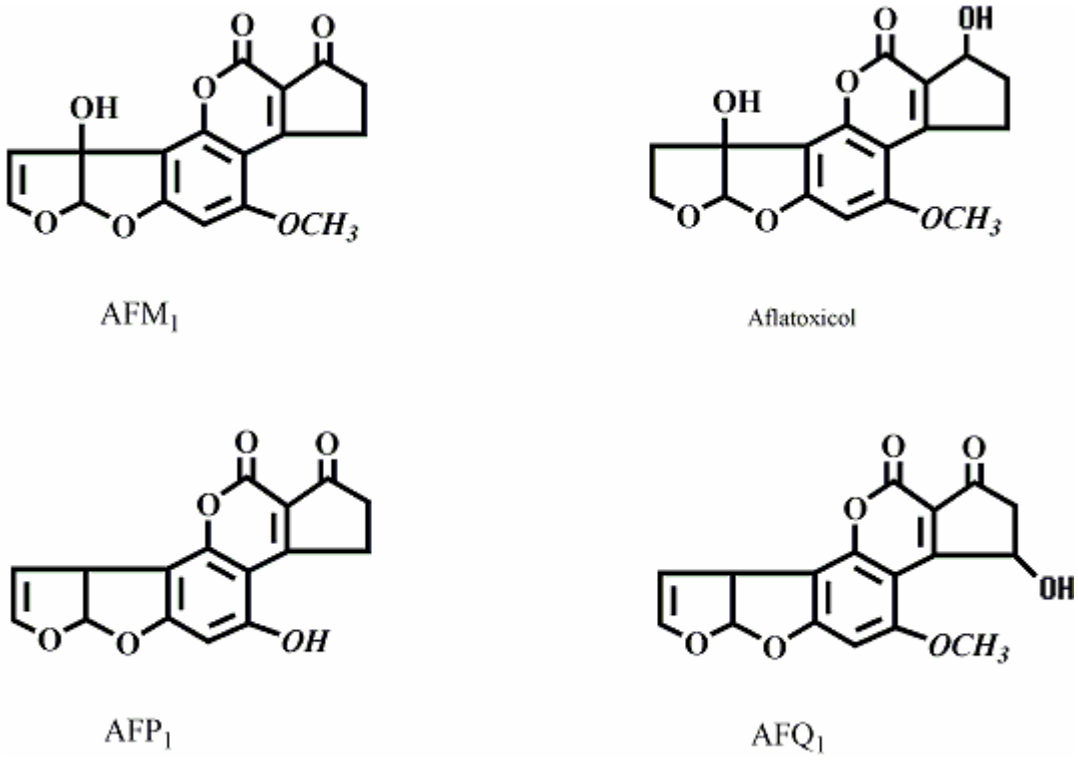
Other mechanisms of AFB1 activation that do not involve CYP have been demonstrated. Co-oxygenation of AFB1 can occur in the presence of arachidonic acid and prostaglandin H synthase, forming mutagenic metabolites. In particular, prostaglandin H synthase shown the same

amount of activity of CYP in the kidney microsomes (Liu and Massey, 1992), appearing to significantly contribute to the activation of AFB1 in these organs. Also lipoxygenases and UV light can activate AFB1 to DNA binding metabolites (Liu and Massey, 1992).

4.3.2 Hydroxylation, O-Demethylation and Reduction of Aflatoxins

Oxidation of AFB1 by microsomal CYP forms, principally CYP1A2 and CYP3A4, produce similar hydroxylated metabolites that have generally lower biological activity than the parent toxin and therefore considered as detoxification products (Eaton et al., 1994; Yannikouris and Jouany, 2002; Riley and Pestka, 2005). The principally hydroxylated metabolites of the AFB1 are AFM1, AFQ1, AFP1 and aflatoxicol (AFL) as shown in figure 4-2 and 4-4.

Figure 4-4. Chemical structures of the aflatoxin M1 (AFM1), aflatoxin Q1 (AFQ1), aflatoxin P1 (AFP1) and aflatoxicol (AFL).



The AFM1 was identified as a metabolite of AFB1 in milk and urine in several species. This toxin has less biologically activity than the parent molecula, but nevertheless is a relatively potent carcinogen. A level of exposure of 50 µg/kg of AFM1 induced a 33% incidence of liver tumours while 50 µg/kg of AFB1 induced 95% in the rats (Hsieh et al., 1974). Coulombe et al. (1982) reported that AFM1 had approximately a 2% of the mutagenic potency of AFB1, while the acute

toxicity is similar to AFB1 (Pong and Wogan, 1982). Also hydroxylation of the AFB2 conducts to formation of the AFM2, a metabolite found in milk of dairy cow after AFB2 ingestion.

AFQ1 is a detoxification product of AFB1 having lower acute toxicity, mutagenicity and carcinogenicity than the parent molecule (Hsieh et al., 1974; Coulombe et al., 1982). The reaction that conducts to AFP1 is, instead, an O-demethylation of the AFB1 possible in same species (Wong and Hsieh, 1980). This product is much less toxic than AFB1 and shown little mutagenic activity (Coulombe et al., 1982).

Hydration of the double bound of the furan ring of AFB1 results in the formation of AFB2α. The reaction can occur without enzymatic system under acid condition and may occur in the stomach after oral ingestion of aflatoxin. Microsomal oxidation has been also suggested as a source of these compounds.

Reduction of the AFB1 forms AFL (Detroy and Hesseltine, 1970). This metabolite has been found in the rumen of dairy cows after AFB1 ingestion (Trucksess et al, 1983), however it could be formed also in the liver (Yiannikouris and Jouany, 2002). AFL formation does not appear as a significant detoxification pathway since the two molecules have the same carcinogenicity and similar mutagenicity (Yiannikouris and Jouany, 2002). The metabolite aflatoxicol-M1 (AFL-M1) results by either oxidation of the AFL or reduction of the AFM1 (Eaton et al., 1994).

4.3.3 Conjugation of Aflatoxins with Glutathione, Glucuronides and Sulphate

GSH conjugation is one important detoxification mechanism (CAST, 2003) that can influence the susceptibility of different species to the toxin effect of AFB1. The conjugation reaction is mediated by citosolic glutathione-*S*-transferase (Eaton et al., 1994). The glutathione S-transferase can bind the GSH with AFB1-8, 9-epoxide causing a reduction in the carcinogenic and toxic properties of this compound and favouring the excretion of the toxin via bile (Yiannikouris and Jouany, 2002).

Also the glucuronides have been found conjugate in the bile with different AFB1 metabolite, as AFL or AFL-M1. Glucuronide conjugates with AFL and AFL-M1 are reported to be the principal biliary metabolites of AFB1 in trout (Loveland et al., 1984). Thus, enterohepatic circulation of AFL could prolong the effective half-life of AFB1. The AFP1 has been found as conjugate with glucuronides in the rat too (Eaton et al., 1993). The sulphonides represent another detoxification pathway when they are conjugated with aflatoxins. The reaction takes place in the hepatocytes (Ch'ih et al., 1983; Yiannikouris and Jouany, 2002).

4.3.4 Aflatoxins Metabolism in Rumen

The rumen was found to put in action a degradation of the mycotoxins; in particular Engel and Hagemeister (1978) reported that the cow rumen was able to degrade more than 42% of used AFB1 *in vitro* condition. The result was not confirmed by other researches (Kiessling et al., 1984; Westakle et al., 1989).

Recent *in vitro* studies indicated that cell walls of several intestinal lactobacilli bacteria can bind AFB1 (El-Nezami et al. 1998; Peltonen et al., 2000; Oatley et al. 2000; Gratz et al., 2005). Authors suggested the activity of intestinal bacteria may reduce the free AFB1 in the gastro-intestinal tract, just delaying but not preventing the AFB1 absorption (Gratz et al., 2005). The chlorophyllin, a water-soluble derivative of chlorophyll, is also capable to reduce the toxicity associated to AFs, forming a chlorophyllin-AFs complex (Dashwood et al., 1998; Atroshi et al., 2002). These indications could justify the results found by Engel and Hagemeister (1978), even if it is not possible to consider a degradation of the toxin.

Some metabolites of AFB1, as AFM1 and AFL, were usually found in rumen fluid after an oral administration of the parent toxin to cows (Trucksess et al., 1983) and buffalo species (Fedele Vincenzo, personal communication). However, Auerbach et al. (1998) in an *in vitro* study concluded that rumen microbes are capable to produce only AFL. A rumino-hepatic pathway was theorized by authors to justify the presence of AFM1 in the rumen.

Since the rumen degradation of the AFB1 is considered a less important pathway (Jouany and Diaz, 2005) and the produced metabolite has the same toxicity of the parent toxin, it may be concluded that ruminants are not so efficient against aflatoxin.

5 Excretion of AFB1 and its Metabolites

Excretion of AFB1 and its metabolites occurs primarily through bile, followed by the urinary pathway (Eaton et al., 1994). In lactating animals, a little amount of the AFB1 is also excreted as AFM1 and other hydroxylate metabolite in milk.

Wong and Hsieh (1980) reported that the total excretion of [^{14}C]AFB1 100 hours after intravenous dosing in male mouse, rat and monkey were 80%, 72% and 73%, respectively. The excretion of total radioactivity was most extensive during the first 24 hours after dosing confirming that absorption, distribution in the organism, bioconversion and final excretion of aflatoxin are very fast processes.

The elimination pathways of aflatoxins, either parent molecules or derivate metabolites, play an important role in carcinogenic properties: the pattern of urinary metabolites was correlated with the relative species susceptibility for effects of these toxins. The mouse, less susceptible specie, produced the most water-soluble urinary metabolites, whereas less was produced by monkey and rat, more susceptible than mouse (Eaton et al., 1994).

5.1 Bile and Feces Excretion

Coulombe and Sharma (1985) reported that after an oral [H^3]AFB1 dose the 55% of the radioactivity was excreted cumulatively in the feces and 15% in the urine 23 days after dosing. Similar results were reported by Wong and Hsieh (1980) that found a cumulative excretion of 53% of the administered dose in feces and 19% in urine for the 4 days following the intravenous dosing. These results confirmed the idea that biliary excretion pathways represent the primary route of aflatoxins elimination. The rate of biliary excretion peaked at 30 minutes and decrease by 2 hours from the treatment (Wong and Hsieh, 1980).

The major biliary AFB1 metabolite was the AFB1-glutathione (CAST, 2003), which accounted for 49-57% of the total biliary radioactivity after an intra-peritoneal administration of [H^3]AFB1 (Holeski et al., 1987). AFP1-glucuronide was also identified in the bile, accounting for 4-15% of total biliary radioactivity in rats.

5.2 Urinary Excretion

The urinary pathways appeared to be another important excretion way for several species. Approximately 10-20% of intravenous AFB1 administered to rats at high level, ranging from 0.4 to 1 mg/kg, is excrete in urine 24 hours after treatment (Groopman et al., 1988). The three principal metabolites found in urine were AFM1, AFP1 and the principal DNA adducts, the AFB1-N^7-guanine (Eaton et al., 2004; Yiannikouris and Jouany, 2002). These soluble AFB1 metabolites in urine derived directly by blood circulating AFM1 and AFP1 (Jouany and Diaz, 2005).

Groopman et al. (1988) reported that on the total aflatoxins excreted in the rat urine the AFM1 resulted the principal recovered metabolite representing about 40-50% of total amount. The AFP1 represent less than 10%, whereas AFB1-N^7-guanine represented 16%.

A dose-dependent correlation between AFB1 and AFB1-N^7-guanine excreted in urine was observed in male rats (Bennett et al., 1981). AFB1-N^7-guanine excretion was rapid with 80% of total excretion of this adducts found in the urine during the first 48 hours from aflatoxin

administration, in agreement with rapid excretion of hydroxylate metabolites (Essigmann et al., 1983). Also AFQ1 is found in urine after AFB1 ingestion, but this metabolite was found only in treated mouse (Eaton et al., 1994).

In humans, AFM1 is apparently the major AFB1 metabolite found in urine of AFB1 exposed individuals. In the early 70s, Campbell et al. (1970) identified AFM1 in urine of individuals exposed to highly contaminated peanut butter in Philippines. These results are in agreement with Groopman et al. (1985) and Zhu et al. (1987) that found AFM1 in urine of Chinese people after AFB1 ingestion. The results obtained by these authors indicated that 1.23-2.18% of total AFB1 is excreted in males as AFM1, whereas lower level was found in female. As in rat, the three principal metabolites found in humans urine was the two detoxification metabolite (AFM1 and AFP1) and the DNA adduct (AFB1-N^7-guanine).

5.3 Carry Over of AFB1 as AFM1 in Milk

Since the early 1960s, when modern mycotoxicology was born (CAST, 2003), several studies have been carried out to establish the carry over (CO) of AFB1 in milk as AFM1 in lactating cattle. In the 1960s and 1970s most of these studies have been conducted at high (mg range) daily intake levels, on relatively low milk yields and with analytic methods not still well developed. Only later, from the 1980s to the 2000s, the studies were carried out at low (sub mg range) levels, with higher milk yields and with improved analytic methods.

One of the first study effectuated to determinate the CO of AFB1 in milk was conducted by Van de Linde et al. (1964) using high and low milk yielding cows. Cows were given a contaminated AFB1groundnut meal for a period of 18 days. These authors found that the AFM1 was detected in milk after 12-24 hours from the first ingestion of AFB1 and the total amount of AFM1 excreted into milk was less than 1% of ingested AFB1.

After this study other researches were conducted to evaluate the CO value and AFM1 excretion trend of AFB1 in milk (Masri et al., 1969; McKinney et al., 1973; Polan et al., 1974). Kiemeier et al. (1977) reported that the CO value varied from animal to animal, from day to day, from one milking to the next and, in the same milking, with the stage of lactation. The CO of AFB1 resulted ranging from 0.2 to 4.0% with an average value around 1% (McKinney et al., 1973; Polan et al., 1974). The studies of these years suggested that AFM1 concentration in milk increased for 1-3 days after a constant daily AFB1 intake before reaching a steady-state condition (Frobish et al., 1986; Polan et al., 1974). When AFB1 was removed by the diet, the decrease of the AFM1 concentration in the milk was rapid and toxin became undetectable just after 2-4 days (4-8 milking).

In the 80s and 90s more information on AFM1 excretion in milk became available. Veldman et al. (1992) found that the carry over value can range from 0.3% to 6.2%. These authors suggested that the primary factor affecting AFM1 excretion in the milk was the milk yield level. A very significant relationship between milk yield and CO rate was found on 12 cows for an AFB1 intake ranging from 5 to 80 µg/cow/day (Veldman et al., 1992).

CO (%) = (0,013 x Kg of milk) – 0,026
r = 0.99

The equation reported by Veldman et al. (1992) shown as CO of AFB1 in milk as AFM1 is unaffected by AFB1 intake levels, but indeed related to milk yield. The results are in agreement with other authors operating in different experimental conditions (Diaz et al., 2004; Battacone et al., 2003).

Another equation proposed by these authors (Veldman et al., 1992) put in relation AFB1 ingested per day by cows and the AFM1 content in milk. The equation is:

AFM1 (ng/Kg of milk) = 1.2 AFB1 (µg/cow/day) + 1.9
r = 0.93

Veldman et al. (1992) assumed that the higher excretion efficiency of the high yield cows was the result of the greater permeability of the cell membranes of the alveoli of the mammary gland. Also Jouany and Diaz (2005) reported a relationship found by Petterson (1997) based on 10 determinations collected from five trials carried out in controlled condition. The equation is:

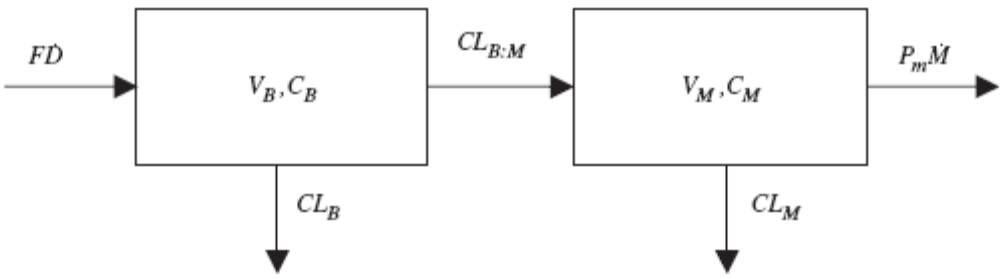
AFM1 (ng/Kg of milk) = 0.787 AFB1 (µg/cow/day) + 10.95
r = 0.92

The EU limit of AFB1 in dairy animal feeds refers to maximum AFB1 allowed concentration of 20 µg/kg for animal feeds and 5 µg/kg for concentrates (EC, 2003). In milk the maximum concentration allowed is 0.05 µg/kg (EC, 2006); while in the USA the allowed AFM1 concentration in milk is regulated by the US Food and Drug Administration (FDA) at 0.5mg/l. To respect the European AFM1 limit into milk, Veldman et al. (1992) recommended a maximum intake of 40 µg of AFB1/cow/day, while Petterson (1997) a higher value near to 50 µg of AFB1/cow/day.

These recommendations have to be putted in relation to farm condition and particularly productive performances of the dairy cows. Several factors have been found to affect aflatoxin CO in ruminant as the differences between species (Battacone et al., 2003); individual variability of lactating animals (Munksgaard et al., 1987; Pettersson et al., 1989; Van Egmond, 1989; Steiner et al., 1990; Veldman et al., 1992), rumen degradation activity (Westlake et al., 1989), hepatic and rumen biotransformation to AFL and other metabolites other than AFM1 (Auerbach et al., 1998), differences in term of induction of the enzymatic AFB1 oxidation system (Steiner et al., 1990) and in the mammary gland permeability (Lafont et al., 1983; Veldman et al, 1992).

A new approach was recently proposed to calculate the carry over of AFB1 into milk (Van Eijkeren et al., 2006). The figure 5-1 summarized the kinetic model of the carry over of AFB1 to AFM1. The conclusions of the authors were: 1) CO is positively correlated with milk production, 2) given a fixed AFB1 intake the corresponding AFM1 level in milk is negatively correlated to milk production and 3) CO is independent of AFB1 level in feed.

Figure 5-1. Kinetic model of the carry-over of AFB1 to AFM1. A fraction F of the daily dose D enters the system across the gut wall. AFB1 is cleared by excretion and biotransformation to toxicologically non-interesting metabolites (clearance CL_B) and to AFM1 (clearance $CL_{B:M}$). AFM1 is cleared by biotransformation to non-interesting metabolites or by excretion through toxicologically non-interesting pathways (clearance CL_M) or by excretion through milk (P_mM), that has a milk/plasma partition coefficient P_m and a daily production of M. Plasma concentrations of AFB1 (C_B) and AFM1 (C_M) are the amounts of AFB1 and AFM1 divided by their distribution volume (V_B and V_M, respectively) (Van Eijkeren et al., 2006)



6 Fate of Aflatoxin M1 during Milk Processing

The treatments that are common in the dairy industry can be separated into two distinct processes: 1) those that do not involve separation of milk components, such as heat treatment, low temperature storage and yoghurt preparation; and 2) those that separate milk components, such as concentration, drying and cheese or butter production.

When contaminated milk is processed to dairy products the toxin is transferred to the resulting manufactured products (Barbieri et al., 1994; Yaroglu et al., 2005). Several studies examined the stability during heat process as pasteurization (Allcroft and Carnaghan, 1962; Patel et al., 1981; Brackett and Marth, 1982). Supporting the idea that heat treatments do not cause a change in amount of AFM1 in heat treated milk and other dairy products. Also the low temperatures, either cool or frozen storage, do not seem to affect AFM1 concentration in milk (Yousef and Marth, 1989; Galvano et al., 2005).

The effect of the manufacture of cultured dairy products, such as the preparation of kefir or yoghurt, on AFM1 concentration was studied by Wiseman and Marth (1983). Even if the results varied, the general trend was that these processes also do not lead to a significant decrease in AFM1 content.

Several investigations have been published in which the effects of the removal of water on AFM1 were studied, including those that involved heat (spray drying or roller drying) as well as freeze-drying. These studies were reviewed by Galvano et al. (2005). Severe losses of AFM1 were reported, whereas other studies revealed that milk concentration did not affect AFM1 content substantially.

In the cream manufacturing process, a part of the aqueous phase is separated. Since AFM1 is considered a semi-polar component, it occurs predominantly in the no fat fraction, suggesting a lower concentration in the cream than in the milk from which it is made. A small proportion of AFM1 is carried over to cream and yet a smaller proportion to butter. No losses of AFM1 occur, since the remainder of AFM1 remains in skim milk and buttermilk.

The cheese manufacturing involves several processes. In the first phase, the conversion of milk into pressed curd, AFM1 seems not to be degraded since the total amount of AFM1 in whey and curd is approximately the same as in the original milk (Yousef and Marth, 1989). AFM1 seems to occur predominantly with casein, however, causing the cheese curd to contain a higher concentration than the whey. The association of AFM1 with casein is also manifested in a higher concentration of AFM1 in cheese than in the milk from which the cheese is made. Yousef and

Marth (1989) expressed the ratio as concentration of AFM1 in milk on concentration of AFM1 in cheese and called this index the enrichment factor (EF).

The EF varies within 2.5-3.3 in soft cheese and 3.9-5.8 in hard cheese. These values can be consequence to the condensation of the raw material occurring during cheese manufacturing (Galvano et al. 2005, JEFCA, 2001)

During the second phase of cheese manufacturing, the ripening process, some discrepancies in AFM1 stability were noticed but, in general, AFM1 did not seem to be degraded during ripening of most cheeses.

7 Aflatoxicosis in Humans and Animals

The name aflatoxicosis indicates the disease resulting from exposure of humans or animals to aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1 and other metabolites). The negative mechanism of aflatoxins involves their metabolism to reactive intermediates which bind to nucleic acids and proteins (DNA- and protein-adducts) with consequent disruption of transcriptional and translation processes (CAST, 2003). The response of humans and animals to aflatoxins exposure is related to the rate of the metabolism and type of metabolites being produced (Riley and Pestka, 2005).

Aflatoxicosis can results in an acute, chronic or sub-chronic forms. While the acute aflatoxicosis is related to a high ingestion of the toxins over a short time, chronic or sub-chronic aflatoxicosis are associated to a low exposure over a prolonged time (CAST, 2003).

7.1 Acute Aflatoxicosis in Humans

Acute aflatoxicosis in humans resulting from aflatoxins ingestion has been manifested as an acute hepatitis (Krishnamachari et al., 1975; Ngindu et al., 1982; Shank, 1977) usually associated with highly contaminated food. In some cases exposure was sufficient to find aflatoxins in target tissues as liver and kidneys (CAST, 2003). Typical but non specific changes in patients with acute aflatoxicosis include several diseases like jaundice, low-grade fever, depression, anorexia, diarrhoea, fatty degenerative change in the liver, resulting by histopathology examination.

Tenderness near the liver was evident in patients with acute aflatoxicosis, aflatoxin-caused hepatitis in Kenya; ascites may develop (Ngindu et al., 1982). Mortality reached 25% in outbreaks in India (Krishnamachari et al., 1975). Samples of liver obtained from dead patients contained detectable levels of AFB1. Recently between January and June in the year 2004, the Kenya

Ministry of Health (MOH) and partners identified 317 cases of acute hepatic failure in Eastern Kenya, with 125 death (Azziz-Baumgartner et al., 2004). Also, authors identified serum aflatoxin B1–lysine adduct and positive hepatitis B in death people.

Two humans diseases of no well defined aetiology have been related to the consumption of aflatoxin contaminated food: kwashiorkor and Reye's syndrome. Kwashiorkor is characterized by hypo-albuminemia, fatty liver and immunosuppression and it has been geographically associated with the seasonal occurrence and distribution of aflatoxins in food (Hendrickse et al., 1983; 1985). The aetiology of Reye's syndrome appears to be more problematic. The disease, which includes an acute encephalopathy with fatty acid degeneration of the viscera, has been associated with aflatoxins because these mycotoxins have been found in Reye's syndrome patients in South Asia, East Europe and USA (Becroft and Webster, 1972; Chaves-Carballo et al., 1976; Dvorackova et al., 1977; Ryan et al., 1979; Shank et al., 1977).

7.2 Acute Aflatoxicosis in Animals

Fungal toxins produced a wide range of disease in animals that could result in an economic loss in farms and an increase of hazard for humans consuming contaminated food. The diagnosis of mycotoxicosis, either in humans or animals, is based on knowledge gained in experimental studies conducted with specific toxins and on different animal species. The symptom of a particular mycotoxicosis may be changed by environmental and other factors (sex, nutrition and breed) resulting in a more difficult detection of the disease with no definite consequences of specific toxin intake (CAST, 2003).

In particular, acute aflatoxicosis in cattle could consist in decrease of feed intake, a strong reduction in milk production in lactating animal, weight loss, lower rumen activity and liver damage (CAST, 2003). These consequences could be all or only in part co-present in animal subject to acute aflatoxicosis.

However, same indication on mycotoxins potencies can be obtained by studies of LD_{50} which is the single dose of a substance needed to cause 50% mortality in a definitely animal population. In table 7-1 is reported LD_{50} values for AFB1 in some species. The most resistant species against AFB1 appeared to be mouse (Roebuck and Maxuitenko, 1994). Other interesting data are reported by authors compared the acute toxicity, expressed as LD_{50}, of the four parent molecules (AFB1, AFB2, AFG1 and AFG2) in ducks and in rats as shown in table 7-2. The AFG2 appeared to be the less toxic compound, while AFB1 was confirmed to be the most toxic and the most carcinogenic of the four aflatoxins.

Table 7-1. Acute toxicity of AFB1 (Roebuck and Maxuitenko, 1994)

Species	Strain	LD_{50} (mg/kg)	Sex	Age (days)	Route[a]
Duck	Pekin	0.34	M, F	1	p.o.
Rabbit	Dutch Belted	0.3	M, F	90	p.o./i.p.
Trout	Rainbow	0.81	M, F	270	i.p.
Guinea pig		1.4	M, F	250	i.p.
Rat	Fisher	from 0.75 to 8	M, F	from 2 to 70	i.p/s.c.
	Sprague-Dawley	1.36	M, F	1	p.o
Mouse	CFW Swiss	From 1.36 to >150	M, F	from 1 to 100	p.o./i.p.
Hamster	Syrian	10.2	M	30	p.o.
Chicken	Australia	15-18	M	21	p.o.

[a] p.o., *per os*; i.p., intraperitoneal; s.c., subcutaneous

Table 7-2. Acute toxicity of naturally occurring aflatoxins in duck (50 g weight) and in rat (200 g weight) (Roebuck and Maxuitenko, 1994)

Species	Strain	Aflatoxins	LD_{50} (mg/kg)	Sex	Route[a]
Duck	Pekin	AFB1	0.73	M	i.p.
		AFB2	1.76	M	i.p.
		AFG1	1.18	M	i.p.
		AFG2	2.83	M	i.p.
Rat	Fischer	AFB1	1.16	M	p.o.
		AFB2	>200	M	p.o
		AFG1	1.5-2.0	M	p.o.
		AFG2	>200	M	p.o.

[a] p.o., *per os*; i.p., intraperitoneal

7.3 Chronic Aflatoxicosis in Humans

Chronic exposure to low levels of aflatoxin ingestion is the most frequent and realistic situation occurring either in humans or animals (CAST, 2003).

Chronic aflatoxicosis in humans usually implies the association of the mycotoxin with hepatocellular carcinoma. The IARC (International Agency for Research on Cancer) considered aflatoxins as carcinogenic substance for humans and they are classified as group (AFB1, B2, G1, G2 and M1) in group 1 carcinogens (IARC, 2002). Several studies have correlated aflatoxin exposure with this carcinoma in different geographical areas (Shank et al., 1972; Peers and Linsell, 1973; van Rensburg et al., 1974; Peers et al., 1976, van Rensburg et al., 1985).

Essigmann et al. (1977) determined that aflatoxin B1 can form a DNA adduct whose excision product, aflatoxin B1-N^7-guanine, occurs in urine of individuals coming from high liver cancer risk area who were presumably exposed to aflatoxin. Sabbioni et al. (1987) discovered that aflatoxin B1 binds to proteins and forms lysine adduct that can be found in serum of individuals for a considerable period of time, with a half-life of 20 days. Monoclonal antibodies have been developed to quantify aflatoxin B1 adducts in humans (Groopman et al., 1988) and are used in attempts to measure exposure of populations to aflatoxin.

Most researches examined the hepatitis B surface antigen (HBsAg) as well as aflatoxin exposure in relation to incidence of hepatocellular carcinoma. Most found an aflatoxin effect independent of HBsAg prevalence (Peers et al., 1987; Sun and Chu, 1984; Yeh et al., 1989).

More recently, biomarkers have been used to understand the aetiology of tumours, such as mutations of the tumour suppressor gene (p53) which is commonly mutated in humans cancers. This development is reviewed by Scholl and Groopman (1995). The outcome is that aflatoxin has been linked to specific p53 mutations where is a G → T transversion in the third position of codon 249. These specific mutations in tumours can provide important evidence as to their cause. A specific biomarker for aflatoxin is related to human liver cancer and that HBV and aflatoxin B1 interact as risk factors for liver cancer (Scholl and Groopman, 1995).

7.4 Chronic Aflatoxicosis in Animals

Chronic exposure of dairy and beef cattle to aflatoxin contaminated feed may result in decreased feed efficiency, immunosuppression and lower reproduction (CAST, 2003; Riley and Pestka, 2005). Aflatoxins affect rumen function either *in vitro* or *in vivo* condition decreasing cellulose digestion, volatile fatty acid and proteolysis (Dvorak et al., 1977; Fehr and Delage, 1979).

Other diseases observed in ruminants as consequence of the aflatoxins ingestion are diarrhoea, mastitis, respiratory disorder, hair loss, prolapsed rectum and decreased feed consumption (CAST, 2003).

The immune system is a target of several important mycotoxins (Bondy and Pestka, 2000). Aflatoxins can have negative effects on functionality of immune system predisposing cattle to infectious disease. The general effects of aflatoxins on immune system are related either to cellular response (reduction in phagocytosis of macrophages, in lymphoblast genesis, in delayed of coetaneous hypersensivity) or humoral factors (reduction of IgA and IgG plasma concentration, in complement activity and bactericidal activity of plasma) (CAST, 2003).

The ingestion of aflatoxin contaminated feeds by poultry can cause hemorrhagic lesions in principal organs and tissues (Forgacs and Carll, 1962). Also haemoglobin, packed cell volume and circulating erythrocytes significantly decrease after AFB1 ingestion (Tung et al., 1975).

In experimental condition aflatoxins resulted teratogenic (CAST, 2003). High prenatal mortality, appearance of malformations and aborts are some of the consequences of an intra-peritoneal dose of 4 mg of AFB1/kg during pregnancy period in rat.

7.5 Chemo-Protection against Aflatoxins

Protection against aflatoxins effects are principally related to glutathione conjugation of the toxin (Roebuck and Maxuitenko, 1994). Glutathione status was demonstrated to modulate the toxicity of AFB1.

Oltipraz is a drug competitive and irreversible inhibitor of CYP 1A2 and 3A4 and its use in humans can blocks the oxidative system of AFB1 to epoxide and hydroxylated derivates (JECFA, 2001; Kensler et al., 1999). A reduction of 77% of AFM1 excretion in urine during dietary intervention with oltipraz was found in rats (Scholl et al., 1996). Liu et al. (1988) reported a reduction of the toxicity of AFB1 with oltipraz: in an experiment carried out on rats, the use of this drug reduced mortality from 83% to 36%.

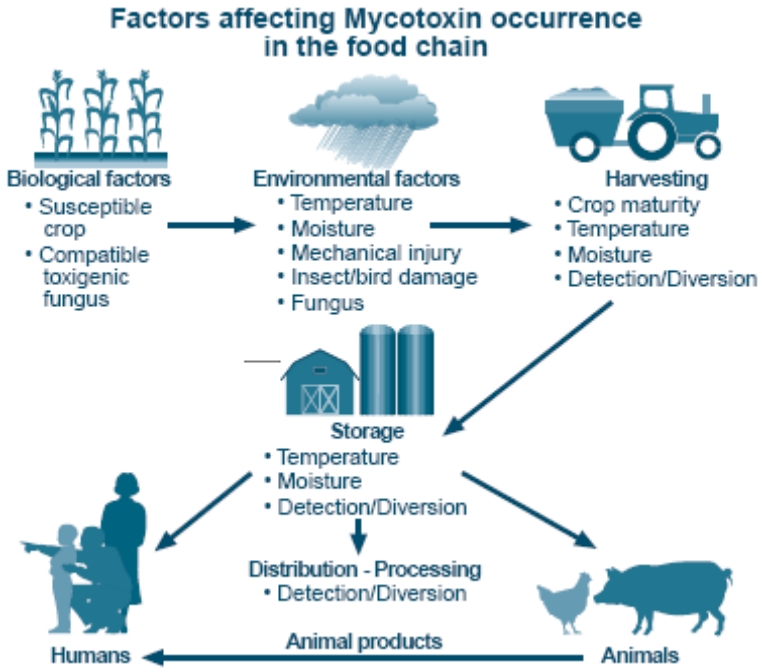
Ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are antioxidants capable to chemo-protection against aflatoxins (Kensler et al., 1994). Feedings studies with these compounds at levels used in anticarcinogenesis bioassays produced 60-90% reductions in the amount of AFB1 bound to hepatic DNA. The ethoxyquin has been shown to increase the activity of glutathione-*S*-transferase 3 to 5-fold in the cytosol of rat liver cells (Kensler et al., 1986).

However, many other compounds are reported to be effective in the reduction of the aflatoxin toxicity. Their use conditions in animals and different response is reviewed by Kensler et al. (1994).

8 Decontamination and Detoxification Strategies for Neutralization of Aflatoxins in Food and Feeds

The mycotoxin presence in the food chain is due to many factors capable to promote fungi growth and mycotoxins production either in pre or post-harvest conditions as shown in figure 8-1.

Figure 8-1. Factors affecting mycotoxin occurrence in the food and feed chain (Pestka and Casale, 1989)



Several strategies have been investigated to avoid mycotoxins occurrence in each ring of food chain, from field to food. The simplest strategy is based on the prevention of mycotoxins formation in feeds (Huwig et al., 2001). Even with current technologies in developed countries, it is very difficult to prevent mycotoxins contamination either in pre-harvest condition or during transport and storage of feeds (CAST, 2003). Therefore, several tools for the neutralization of mycotoxins have been developed to preserve feeds from post-harvest mycotoxin contamination.

These practices could represent a mycotoxin decontamination processes, refers to methods by which the mycotoxins are removed and/or neutralised from contaminated feeds, or mycotoxin

detoxification processes, refers to methods by which the toxic properties of the mycotoxins are removed (Diaz and Smith, 2005). The decontamination and detoxification procedures have to respect same guidelines (CAST, 2003):

- ✓ to be effective in the inactivation, destroy or removal of the mycotoxins
- ✓ not results in the deposition of toxic or carcinogenic/mutagenic substances, metabolites or by-products in the feeds and food
- ✓ retain nutrient value and feed acceptability of the products or commodities
- ✓ not result in significant alterations of the product's technologies properties
- ✓ to be economical and technologically convenient and not alter the cost of final product
- ✓ destroy fungal spores to avoid a late contamination

Decontamination or detoxification procedures are usually divided in physical, biological and chemical methods.

8.1 Physical methods

8.1.1 Mechanical Separation and Density Segregation

A physical separation of a contaminated products (broken kernels, infected plant part, etc.) from a lot could reduce toxin level. The method is considered not very practical, due to incomplete removal of mycotoxin contaminated grains (Natarajan et al., 1975; Phillips et al., 1994). However, significant decreases in aflatoxin levels from electronic and hand sorted peanuts have been reported (Natarajan et al., 1975). Even though complete removal of all residual contamination can not be expected with a variety of mechanical methods of separation, aflatoxin concentrations may be markedly decreased following rigorous treatment strategies.

Density segregation of contaminated grain and oilseeds involves sorting and delineating good versus contaminated kernels by flotation. This method decreased aflatoxin concentrations either in experimental or practice conditions as reviewed by Cole (1989) and Phillips et al. (1994). This procedure may be compatible with current wet milling practices and alkaline processing of corn (Hagler, 1991). It should be noted, however, that the appearance and weight of a particular kernel do not always indicate the presence or absence of mycotoxin.

Combinations of methods involving hand picking and density segregation of contaminated grains can result in a decrease of the 70 to over the 90% of aflatoxin and fumonisins (Vasanthi and Bhat, 1998).

8.1.2 Thermal Inactivation

Aflatoxins are resistant to thermal inactivation (Hwang and Lee, 2006), so they are not completely destroyed by heat treatments as boiling water, autoclaving and extrusion-cooking (Hwang and Lee, 2006; Park and Kim, 2006; Castells et al., 2005). Partial destruction of aflatoxin may be accomplished by oil roasting or dry roasting peanuts and oilseed meals (Marth and Doyle, 1979) or roasting corn (Conway et al. 1978). In a study by Lee (1989), roasting conditions and initial aflatoxin concentration in raw peanuts determined the degree of mycotoxin reduction, with a decrease ranging from 45% to 83%. Another study using roasting demonstrated that aflatoxin concentrations could be decreased in nuts and oilseed meals and in corn (Conway et al., 1978).

However, aflatoxin drop is not complete and uniform and it is affected by temperature, heating interval, and moisture content (Mann et al., 1967). Recently, Oluwafemi (2004) observed a 20% decrease of AFB1 concentration in feeds treated at 100°C for 30 minutes.

8.1.3 Irradiation

Exposing peanut oil to UV light has been reported to decrease aflatoxin levels (Shantha and Sreenivasa, 1977), but UV light has been reported to activate these chemicals to mutagen compounds (Stark et al., 1990). Applying UV light for 20 minutes at 25°C the AFM1 concentration in contaminated milk was decreased of the 89.1% in the presence of 0.05% peroxide, compared to 60.7% without peroxide (Yousef and Marth, 1989). Also in this case concern was raised because treatment could cause peroxidation leading to more toxic products.

Also sunlight after 14 hours of exposition destroyed between 77 and 90% of the AFB1 added to groundnut flakes, although only 50% of the toxin was destroyed in the naturally contaminated product (Shantha, 1987). The gamma irradiation indeed is not able to degrade aflatoxin in contaminated peanut meal (Feuell, 1977).

Microwave irradiation also has been suggested as a method for the detoxification of certain mycotoxins in model systems and in foodstuffs. Farag et al. (1996) report that AFB1, B2, G1, and G2 respond to microwave treatment in both model and food systems. The rate of aflatoxins destruction was positively correlated with the power setting and exposure time.

8.1.4 Solvent Extraction

Aflatoxins can be extracted efficiently from contaminated grains using selected solvent mixtures. This method has a minimal effect on nutritional value of the contaminated feeds (Goldblatt and Dollear, 1979; Rayner et al., 1977). Among tested solvents, ethanol, aqueous acetone, isopropanol, hexaneethanol, hexane-methanol, hexane-acetone-water and hexane-ethanol-water combinations resulted effective in the reduction of the aflatoxin level, but these treatments are often considered cost prohibitive and not enforceable for most practical applications (Shantha, 1987).

8.2 Biological Methods: Microorganism and Chemoprotection

The uses of no toxigenic strains of *A. flavus* and *A. parasiticus*, that are considered a biological method of aflatoxins decontamination, are reported in paragraph 2.4.1.

Also other microorganisms (yeasts, muolds and bacteria), screened for their ability to modify or inactivate aflatoxins, were tested in post-harvest condition. *Flavobacterium aurantiacum* (NRRL B184) was shown to significantly remove aflatoxin from a liquid medium without producing toxic by-products or metabolites (Ciegler et al. 1966). These same investigators also determined that certain acid producing moulds could catalyze hydration of aflatoxin B1 to B2a, which is a less toxic product than parent toxin.

Substances donor to methyl group to aflatoxins as choline and methionine, often used conjugate with folate, could modify toxin molecules and reduce their negative effects (Phillips et al., 2004). Also nutrient as saturated fat, vitamins (vitamin A, folic acid, carotene, etc.) and selenium could chemoprotect humans and animals organism modulating the hepatocarcinogenicity of AFB1 (CAST, 2003).

8.3 Chemical Methods

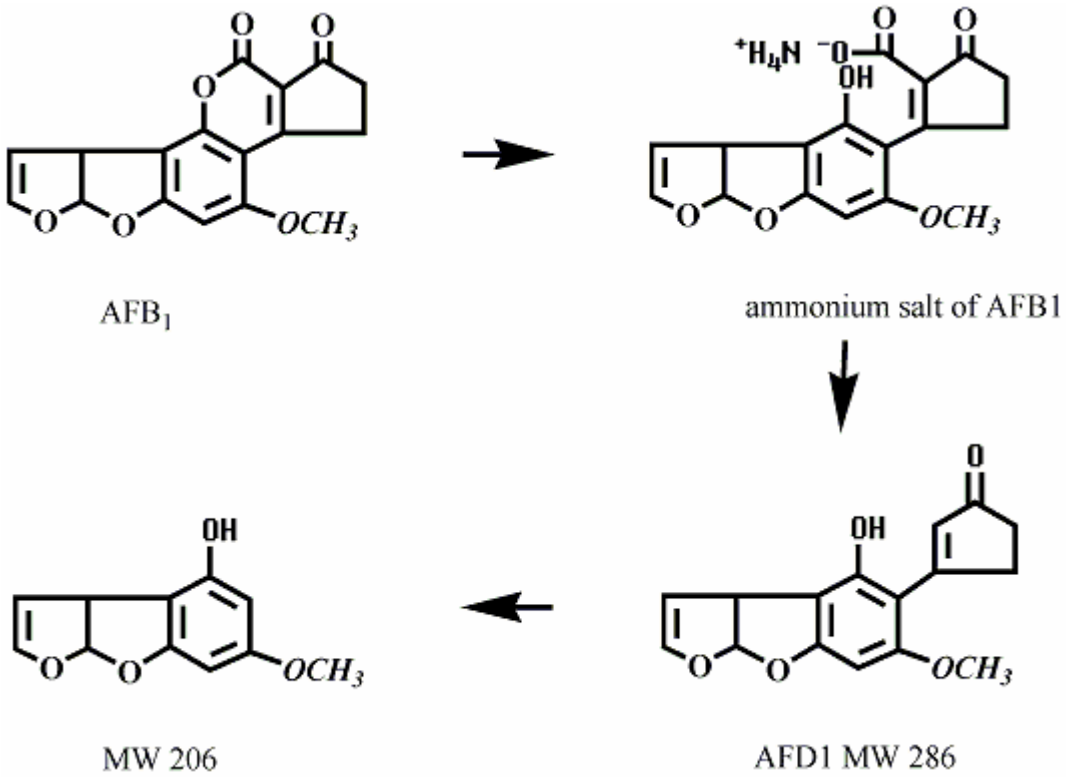
Numerous chemicals as acids, bases, aldehydes, bisulfite, oxidizing agents and various gases have been tested for their ability to degrade or detoxify aflatoxin. (Anderson, 1983; Goldblatt and Dollear, 1979; Hagler, 1991; Park et al., 1988; Phillips et al., 1994; Samarajeewa et al., 1991). Even if many of these compounds are able to destroy aflatoxins, they may result not applicable or potentially harmful. The most investigated and used chemical strategies for reduction of the

aflatoxins include ammoniation, ozonization, and reaction with food grade additives such as sodium bisulfite.

8.3.1 Ammoniation, Ozonizazion and Treatment with Bisulfite

Ammoniation is a feasible method to detoxify aflatoxins contaminated products and involves use of gaseous ammonia (NH_3) or ammonium hydroxide (NH_4^+). This method has been shown to decrease aflatoxin level also more than 99% in feeds (Masri et al., 1969; Park et al., 1984; Phillips et al., 1994). Ammoniation resulted in the conversion of aflatoxin B1 to less toxic products including aflatoxin D1 and a derivative with molecular weight 206 as shown in figure 8-2 (Phillips et al., 1994). Two different ammoniation procedures are currently utilized: a high-pressure and high-temperature process (HP/HT) and an atmospheric pressure and ambient temperature procedure (AP/AT).

Figure 8-2. Proposed major reaction from the ammoniation of AFB1 (Phillips et al., 1994)



Promising results have been achieved in aflatoxin detoxification using accepted food additives as sodium bisulfite. These compounds are able to react with AFB1, AFG1 and AFL to form water-soluble products (Doyle and Marth 1978a,b; Hagler et al. 1982). The reaction between bisulfite and AFB1 form a no toxic adduct for organism (Phillips et al., 1994).

Another recent studied method to reduce mycotoxin is based on reaction with ozone (O_3) gas, a powerful oxidant with a preference for double bonds. Studies by Maeba et al. (1988) reported that O_3 chemically degraded and detoxified AFB1, B2, G1 and G2 as pure standards *in vitro* model. O_3 is a fairly stable gas but in an aqueous environment its half-life drops to approximately 20 minutes (CAST, 2003). O_3 oxidizes aflatoxins liberate oxygen and therefore can be classified as a no persistent chemical residual compound. However, it must be generated at the location of its intended use. Ozonization may help remediate bulk quantities of corn at a minimal cost with minimal destruction of important nutrients. These findings indicate a potentially practical approach to the remediation of unprocessed corn contaminated with aflatoxin (McKenzie et al., 1998).

8.3.2 Sequestering agents

Dietary supplementation with sequestering agents is one of the most studied and utilized method to avoid negative effects due to the mycotoxin ingestion in animals (CAST, 2003; Diaz and Smith, 2005). The sequestering agents (SA) are considered compounds capable to bind toxin molecules forming a stable complex in the gastro-intestinal tract between aflatoxins and SA through electronic elementary charges, hydrogen bond and Van der Waals bonds (Phillips et al., 1990; Yiannikouris et al., 2005; Jouany, 2007) in the gastro-intestinal tract.

However, a SA should be effective against several mycotoxins reducing or limiting bio-disponibility of these toxins for humans and animals. It represents an economic method to counteract negative effects due to mycotoxins ingestion and it is practical because SA are just a small amount in the animal diets, free of impurities, off-flavour and off-odours (CAST, 2003; Phillips et al., 1994).

These compounds are before tested *in vitro* to verify their ability to sequester mycotoxins in controlled conditions and after, if this first step of investigation results positive, they are used in *in vivo* conditions. In many cases a correlation between *in vitro* and *in vivo* results has not been found (Rotter et al., 1989; Dwyer et al., 1997; Diaz et al., 2004). This may be related to a specific characteristic of the tested SA or to the inadequacy of the *in vitro* models in predicting response in animals (Diaz and Smith, 2005).

Several *in vitro* methods have been used and proposed to screen different SAs, but the experimental conditions (kind of aflatoxins - standard or natural toxin -, dilution factor - aflatoxin and experimental volume ratio -, aflatoxin and SA ratio, pH conditions, temperature and biological fluids where SAs were tested) rarely are comparable each other (Ramos and Hermandez, 1996; Grant and Phillips, 1998; Lemke et al., 2001; Ledoux and Rottinghaus, 1999).

Also, authors did not consider the possible competition between AFs and other biological molecules for binding sites of SA in the gastro-intestinal tract and this appears to be an extremely simplification of the *in vitro* experiments respect *in vivo* conditions (Diaz and Smith, 2005). Even if few works have been published about effects of SA using the monogastrics and polygastrics models (Lemke et al., 2001; Spotti et al., 2005), there are not information about the adsorption mechanism and fate of the AF:SA complex in ruminants and monogastrics.

To evaluate sequestering efficiency of a SA, two-step process should be utilized: the first step to evaluate the mycotoxins sequestered by SA forming the complex (calculated as difference between found and initially administered toxin) while the second step to control if sequestered mycotoxin are released by complex after exposure to a second solvent system (Diaz and Smith, 2005). Total efficiency of SA is evaluate by comparing the initial sequestration (weak binding) and successive desorption (strong binding). In any case, a first screening of SAs in *in vitro* experiment is necessary to avoid a most expensive test conducted in *in vivo* conditions.

The principal classes of SA are silicates, activate charcoals, polymers, chlorophyll products and yeast wall derived products.

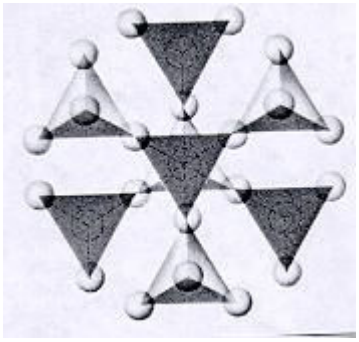
Adsorbents

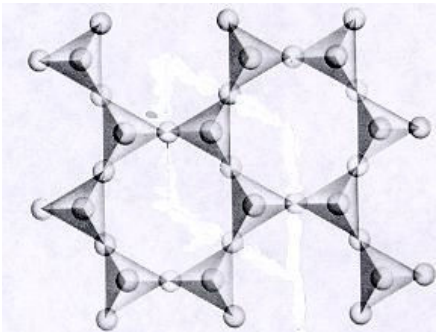
The largest and most complex class of SA includes clays. Several attempts of classifications for these sequestering compounds have been proposed (Phillips et al., 2004; CAST, 2003; Diaz and Smith, 2005). Generally clays are considered natural adsorbents chemically made of silicates and/or aluminosilicates (Jouany, 2007). Minerals are generally divided according to structure of their silicate anions into several groups (Nesosilicates, Sorosilicates, Cyclosilicates, Inosilicates, Phyllosilicates and Tectosilicates). The most representative classes of minerals used as SA are: tectosilicates and phyllosilicates.

The tectosilicates are characterized by a three-dimensional structure (figure 8-3). In the tectosilicates group there is zeolite, that is an important and highly studied SA (Jouany, 2007). The phyllosilicates clay minerals are characterised by two-dimensional sheets of corner sharing SiO_4 and AlO_4 tetrahedra. Each tetrahedron shares three of its vertex oxygen atoms with other tetrahedra. In clays the tetrahedral sheets are always bounded to octahedral sheets formed from small cations, such as aluminium or magnesium, coordinated by six oxygen atoms (figure 8-3). Phyllosilicates can be categorised in different sections depending on the way that tetrahedral and octahedral sheets are packaged into layers. If there is only one tetrahedral and one octahedral sheets in each layer the clay is known as a 1:1 phyllosilicates. Kaolinite and serpentine are a 1:1 phyllosilicates. The alternative, known as a 2:1 clay, has two tetrahedral sheets with the unshared vertex of each sheet pointing

towards each other and forming each side of the octahedral sheet. Illite, montmorillonite, sepiolite are categorized as 2:1 clays.

Figure 8-3. Basic units for tectosilicates and phylosilicates (from Missouri State University web site, dr. Bill Hames)





Zeolites were sometimes found efficient to counteract aflatoxin exposure *in vivo* conditions on several animal models (Dvorak, 1989; Scheideler, 1989; Sova et al., 1991). In a recent experiment of Harvey et al. (1993) only two zeolites on five tested resulted effective to alleviate the decrease in body weight due to aflatoxins ingestion. Zaghini et al. (1998) fed broiler chickens with a high dose (2.5 ppm) of clinoptilolite, a type of zeolite clays. This clay caused a significant reduction in the liver concentration of AFB1. Mayura et al. (1998) tested the clinoptilolite on pregnant rats because of the sensibility of rat embryos versus aflatoxins. The authors suggested that clinoptilolite may interact with dietary components that modulate aflatoxins. Nada (1998) found a reduction in AFM1 excretion in rat urine after the administration of an aflatoxin contaminated diet, probably because less aflatoxin is available in the gastro-intestinal tract of these animals.

Hydrate sodium calcium aluminosilicate (HSCAS), considered a montmorillonite by CAST (2003) and a natural zeolite by Diaz and Smith (2005), is the most studied mycotoxins sequestering agents. It appears to bind positively charge or cationic compounds (Jouany, 2007). Many *in vitro* tests are conducted by Phillips et al. (1987, 1988, 1990, 1991, 1994) to verify the high capacity of the NovaSil® to sequester AFB1 (Trouw Nutrition International), a typical HSCAS recently modifies and sells as NovaSil® Plus. The high affinity of these compounds are associated to stable electric elementary charges between the beta-carbonil portion of the aflatoxin molecule and the sites containing aluminion ions in the HSCAS. Phillips et al., (1988) determined that the maximum quantity of AFB1 that could be bound was 200 to 332 nmol (63 to 105 µg) per mg of HSCAS and that the reaction reached an equilibrium after 30 minutes. Smith et al. (1994) found a decrease in AFM1 level into milk of dairy cows after an administration of HSCAS. Ramos and Hernandez (1997) reviewed the *in vivo* capacity of the HSCAS to sequester aflatoxins in poultry, swine and ruminats.

Montmorillonite is the main constituent of bentonite (Diaz and Smith, 2005). Dvorak (1989) testing a bentonite in several fluid media (water, saline solution, serum, stomach fluid and rumen fluid) demonstrated the high capacity of this clays to sequester AFB1. These considerations were tested by several authors *in vivo* experiments (Lindemann et al., 1993; Schell et al., 1993; Abdel-Wahhab et al., 2002). The bentonites resulted effective also *in vivo* and caused positive effects on daily feed intake, blood urea, total protein, activities of aminotransferase (AST) and alkaline phosphatase (ALP) in swine exposed to mycotoxins.

Activate Charcoals

Activated carbon is a form of carbon heated without air and then treated with oxygen to open millions of pores. The activated carbon has been studied for its ability to bind aflatoxins (Piva CAST, 2003). The sequestering properties of activated carbons depended by pore size, surface area and mycotoxins structure (Galvano et al., 2001). Galvano et al. (1996b) investigated numerous activated carbons to determine their affinities for AFB1 and other mycotoxins in aqueous solution. The results for AFB1 varied according to the type of activated carbon used, suggesting that those with a high methylene blue index and a low surface acidity could adsorb greater than 99% of the AFB1 present *in vitro* condition. In an experiment on lactating dairy cows, the activated carbons decreased the carry over of AFB1 as AFM1 in milk from 40.6 to 73.6% when included in the diet at concentrations of 2.0% (Galvano et al., 1996a). These results were not confirmed by Diaz et al. (2004) that did not find a reduction in AFM1 in milk of dairy cows at 0.25% of the feed. However, the industries that produce activate carbons do not find any economic suitability advantage in their application for this use (Pietri Amedeo, personal communication), so these compounds are not used in practical farm condition.

Cholestyramine

Cholestyramine, an insoluble quaternary ammonium anion exchange resin (CAST, 2003), is usually used in humans medicine for reducing cholesterol and for absorbing bile acids in the gastrointestinal tract. *In vitro* cholestyramine are able to bind ochratoxin A (OTA) and zearalenone (Ramos et al., 1996; Diaz and Smith, 2005). No studies are reported on aflatoxins.

Chlorophyll Products

Chlorophyllin is a water soluble derivative of the green plant pigment chlorophyll. These substances has been shown effective in reducing the toxicity associated with aflatoxins (Atroshi et

al., 2002; Dashwood et al., 1998). Breinholt et al. (1995) suggested that the formation of a chlorophyllin – aflatoxin complex is the main mechanism responsible for chemoprotection.

Yeast Wall Derived Products

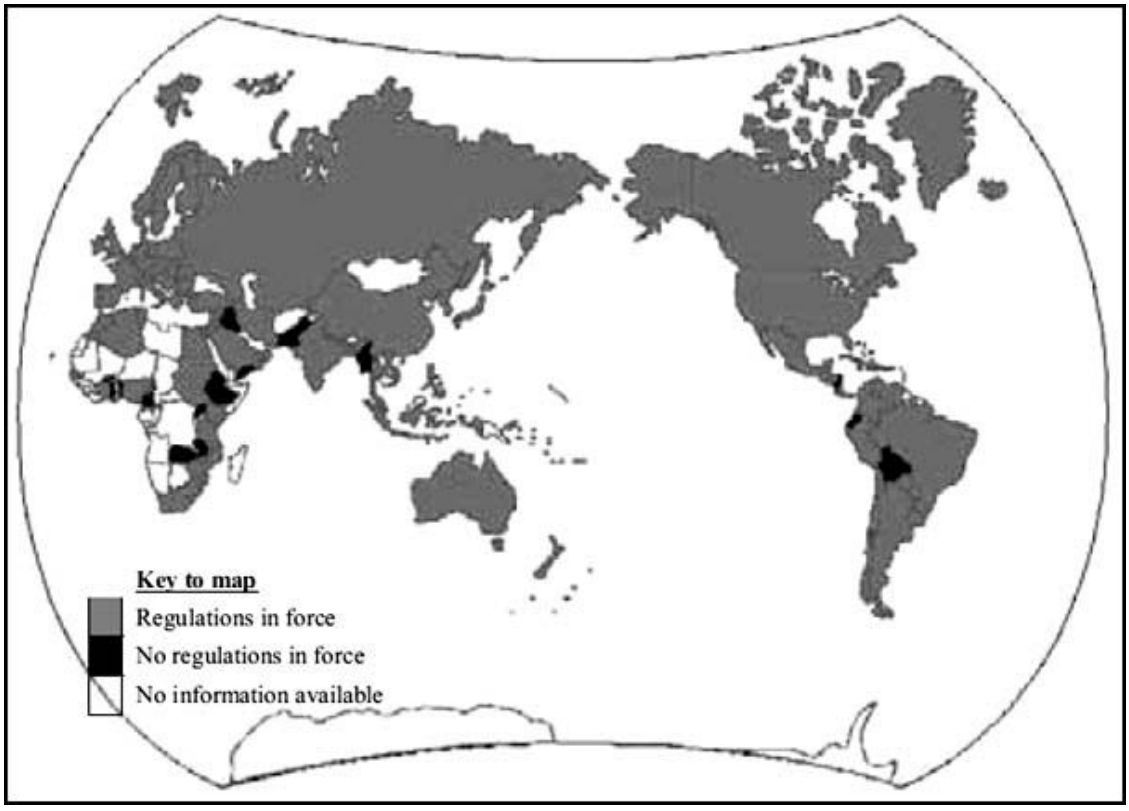
The first study of the *Saccharomyces cerevisiae* capacity to sequester aflatoxin was conducted by Stanley et al. (1993) on poultry. Also Devegowda et al. (1998) observed that the extracted yeast cell wall of *Saccharomyces cerevisiae* was able to bind *in vitro* a large range of mycotoxins. Then, many studies have been carried out in several species with commercial products containing an active binder based on yeast cell wall (Aravind et al., 2003; Casteel et al., 2003; Karaman et al., 2005). Diaz et al. (2004) discussed the efficacy of an esterified glucomannan to reduce the 59% of the AFM1 in milk of lactating dairy cows. Recently, Yiannikouris et al. (2004) demonstrated that the β-D-glucan fraction of yeast cell wall is directly involved in the binding process of mycotoxins, and that the structural organization of β-D-glucans modulates the binding strength (Yiannikouris et al., 2004a). Hydrogen and van der Waals bonds have been evidenced in the glucans-mycotoxin complexes (Yiannikouris et al., 2004b, c), and are stable at the pH condition of the digesta all along the digestive tract, even if the maximum absorption occurs at a pH of 4 (Diaz and Smih, 2005). Probably, a similar chemical mechanism is involved in the binding process of mycotoxins by lactobacilli described by El-Nezami et al. (1998, 2000). The recommended dose for the extracted active yeast compounds is in the range of 1–2 kg/tonne of feed, which corresponds to a daily dose of 20 g/day for a dairy cow.

Organic binders are efficient against a larger range of mycotoxins than inorganic binders, which make them more useful to the most frequent cases of multi-contaminated feeds (Jouany et al., 2007). Also they are biodegradable and do not accumulate in the environment after being excreted by animals. On the contrary, clays which are incorporated at a higher rate than organic binders, accumulate in manure and then in field during spreading and can harm soils and pastures.

9 Regulation of Aflatoxins in the European Community

Thirteen nine Europe countries, accounting for approximately 99 percent of the European's population, were known by FAO to have specific mycotoxin regulations. Figure 9-1 shows the occurrences of regulatory limits for various mycotoxins in Europe and other continents in food and feed respectively.

Figure 9-1. Countries with and without regulations for mycotoxins (FAO, 2003)



Compared to other regions of the world, Europe has the most extensive and detailed regulations for mycotoxins in food and feeds. The current legislation in the European Union refers to the allowed limit of aflatoxins in foodstuff (EC, 2006) and AFB1 and others parent aflatoxins (AFB2, AFG1 and AFG2) in the feeds (EC, 2003a) reported in table 9-1 and table 9-2.

Table 9-1. Maximum levels for aflatoxin contaminants in foodstuffs (reported by EC, 2006)

Foodstuffs [1]		Maximum levels (μg/kg)		
2.1	**Aflatoxins**	B_1	Sum of B_1, B_2, G_1 and G_2	M_1
2.1.1	Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8,0 [5]	15,0 [5]	—
2.1.2	Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0 [5]	10,0 [5]	—
2.1.3	Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0 [5]	4,0 [5]	—
2.1.4	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
2.1.5	Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0	4,0	—
2.1.6	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.7, 2.1.10 and 2.1.12	2,0	4,0	—
2.1.7	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
2.1.8	Raw milk [6], heat-treated milk and milk for the manufacture of milk-based products	—	—	0,050

Table 9-2. Maximum levels for aflatoxin contaminants in feedstuffs (reported by EC, 2003a)

Undesirable substances	Products intended for animal feed	Maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %
(1)	(2)	(3)
'7. Aflatoxin B1	All feed materials	0,02
	Complete feedingstuffs for cattle, sheep and goats with the exception of:	0,02
	— complete feedingstuffs for dairy animals	0,005
	— complete feedingstuffs for calves and lambs	0,01
	Complete feedingstuffs for pigs and poultry (except young animals)	0,02
	Other complete feedingstuffs	0,01
	Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs)	0,02
	Complementary feedingstuffs for pigs and poultry (except young animals)	0,02
	Other complementary feedingstuffs	0,005'