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AFLATOXINS ABSORPTION AND EXCRETION DYNAMICS IN DAIRY COWS: TECHNICAL STRATEGIES TO REDUCE METABOLITES CARRY OVER IN MILK

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RIASSUNTO

LE AFLATOSSINE SONO POTENTI SOSTANZE CANCEROGENE PRESENTI IN NATURA. L'AFLATOSSINA B1 VIENE POCO DEGRADATA NEL RUMINE ED È ESCRETA NEL LATTE COME AFLATOSSINA M1 CON UN CARRY OVER DEL 1-3%. NEL PRESENTE LAVORO È STATO STUDIATA L'APPARZIONE DELLE AFLATOSSINE NEL SANGUE CONSEGUENTE ALL'INGESIONE ORALE DI UN BOLO CONTAMINATO PER VERIFICARE COME QUESTE TOSSINE SONO ASSORBITE NEL TRATTO DIGESTIVO DELLE VACCHE DA LATTE. LA COMPARSA NEL PLASMA E NEL LATTE ATTRAVERSO UNA MUCOSA TIPICAMENTE NON DI ASSORBIMENTO PER DETERMINARE IL POSSIBILE MECCANISMO CHE REGOLA L'ASSORBIMENTO DELLE AFLATOSSINE È STATO UN ULTERIORE OGETTO DI STUDIO. UN' ALTRA PROVA È STATA EFFETTUATA CON VACCHE DA LATTE PER STUDIARE IL CARRY-OVER DELL'AFLATOSSINA B1 NEL LATTE IN RELAZIONE AL LIVELLO PRODUTTICO E ALLE CELLULE SOMATICHE, COME INDICATORE DI PROCESSI INFIAMMATORI NELLA MAMMELLA. LA CAPACITÀ SEQUESTRANTE DI DIVERSI TIPI DI ADSORBENTI È STATA COMPARATA IN PROVE IN VITRO CONDOTE IN DIFFERENTI CONDIZIONI SPERIMENTALI. ANCHE IL COMPORTAMENTO DEL COMPLESSO AFLATOSSINA-ADSORBENTE NEL TRATTO DIGESTIVO DI VACCHE IN LATTAZIONE È STATO STUDIATO IN VIVO PER MEZZO DELLA MISURAZIONE DELLA PRESENZA DI AFLATOSSINA M1 NEL LATTE. UNA PROVA IN VIVO È STATA EFFETTUATA PER VERIFICARE L'EFFETTO CHE LA PELLETTATURA O LA SEMPLICE MISCELAZIONE DI ADSORBENTI NEI MANGIMI PUÒ AVERE NEL MIGLIORARE L'EFFICIENZA DI SEQUESTRO.

ABSTRACT

AFLATOXINS ARE THE MOST POTENT NATURAL CARCINOGENIC COMPOUND PRESENT IN NATURE. AFLATOXIN B1 IS POORLY DEGRADED IN THE RUMEN AND IS EXCRETED IN MILK AS AFLATOXIN M1 WITH A CARRY-OVER RATE OF 1-3%. THE PRESENT WORK INVESTIGATED RATE AND SCHEDULE OF AFLATOXINS PLASMA APPEARANCE FOLLOWING AN ORAL CONTAMINATED BOLUS TO VERIFY HOW THESE TOXINS ARE ABSORBED IN THE GASTRO-INTESTINAL TRACT OF DAIRY COWS. AFLATOXINS PLASMA AND MILK APPEARANCES WERE ALSO INVESTIGATED USING A NON-ABSORBING MUCOSA TO UNDERSTAND THE POSSIBLE AFLATOXINS ABSORPTION MECHANISM THROUGH MUCOUS MEMBRANES. 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. SEQUESTERING CAPACITY OF DIFFERENT KINDS OF MYCOTOXINS SEQUESTERING AGENTS WERE COMPARED IN VITRO TRIAL CARRIED OUT AT DIFFERENT EXPERIMENTAL CONDITIONS. THE BEHAVIOUR OF THE AFLATOXINS-ADSORBENTS COMPLEXES THROUGH DIGESTIVE TRACT OF LACTATING DAIRY COWS WERE ALSO INVESTIGATED IN VIVO BY MEASURING APPEARANCE OF AFLATOXIN M1 INTO MILK. AN IN VIVO TRIAL WAS CONDUCTED TO VERIFY IF EFFECT OF PELLETIZING OR SIMPLY MIXING PROCESSES IS USEFUL TO IMPROVE MYCOTOXINS SEQUESTERING AGENTS EFFICACY IN DAIRY COW NUTRITION.

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

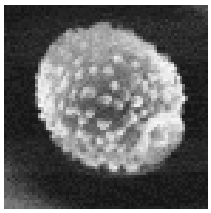
Manuscript for: *Journal of Dairy Science*

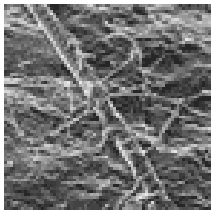
3 Aspergillus Growth and Aflatoxins Production

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).

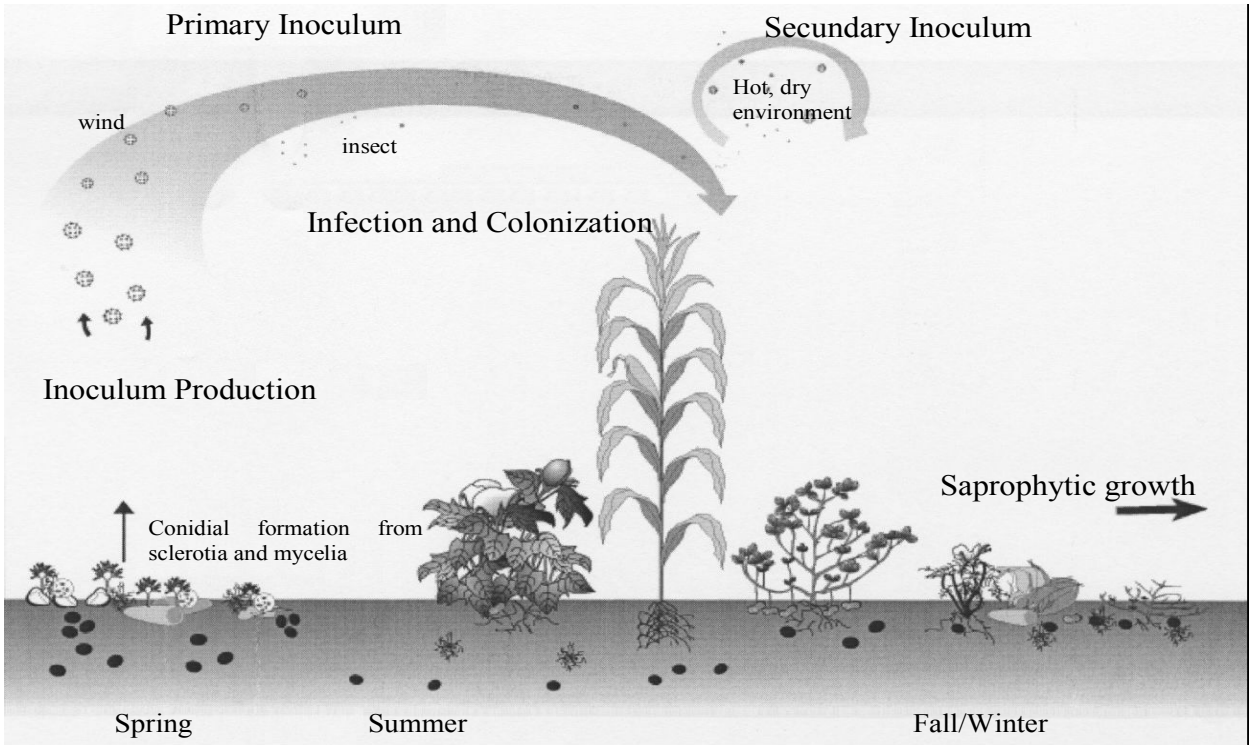
Aspergillus is well-known for its aggressiveness on stored crops, but also for its ability to produce aflatoxin in colonized seeds and grain and it is capable to grow on a large number of substrate (Scheidegger and Payne, 2003). However, it appears to colonize principally maize, cotton, peanut and their 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.

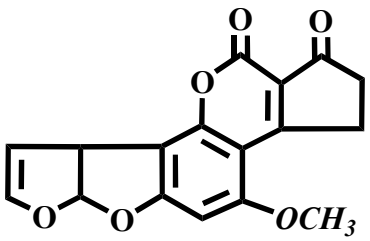
Mycotoxins associated with *Aspergillus* spp. include cyclopiazonic acid (CPA), ochratoxins, versicoloronins, sterigmatocystin, gliotoxin, kojic acid, nominine and citrin, with the principal group as aflatoxins.

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).

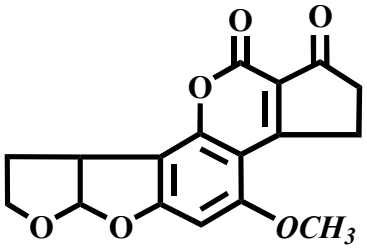
These fungi are capable to synthesize four types of aflatoxins: aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (figure 3-3). *A. flavus* is known to produce only AFB1 and

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).

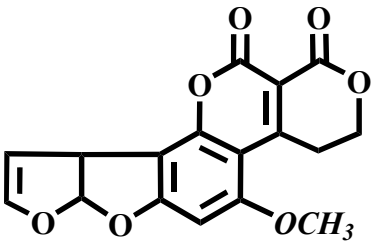
Figure 3-3. Chemical structures of the four parent aflatoxins: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2)



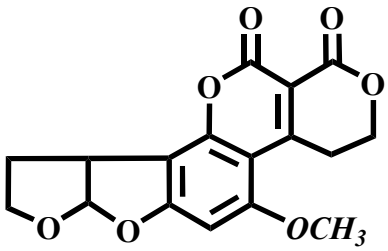
AFB_1



AFB_2



AFG_1



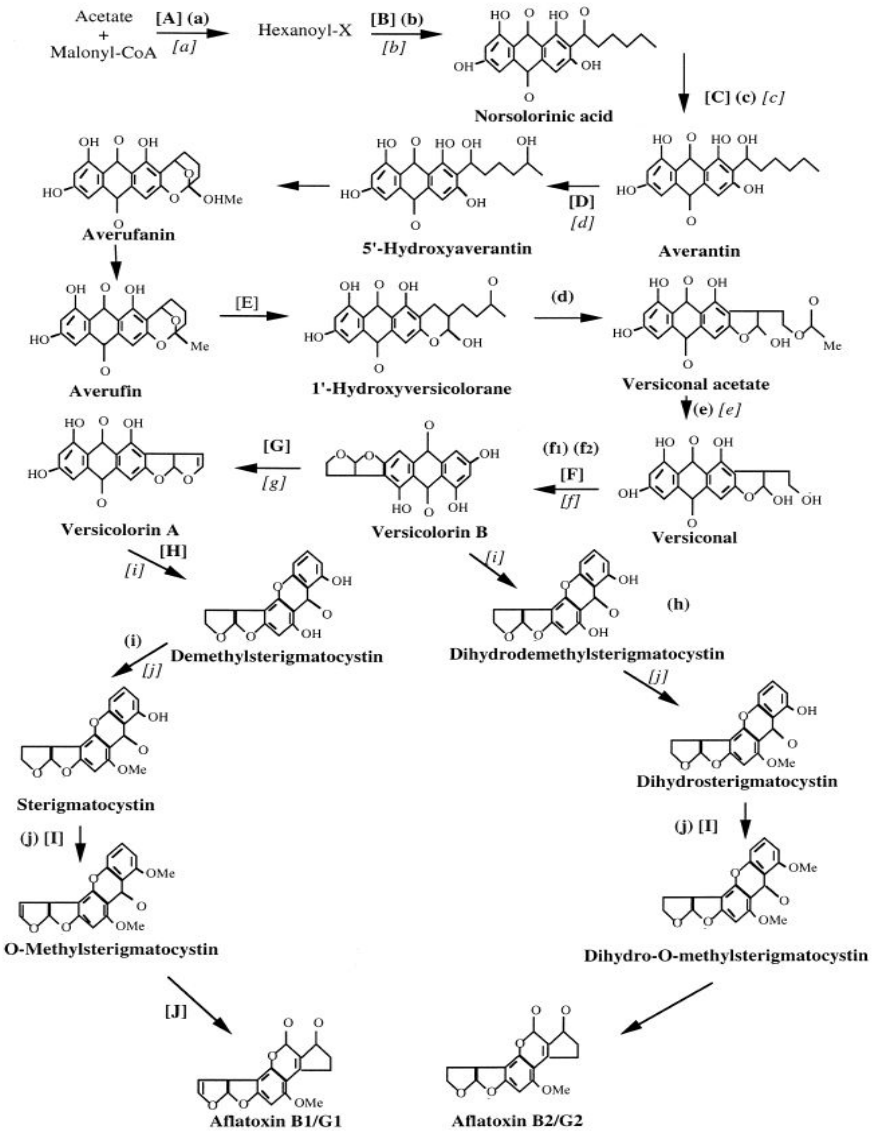
AFG_2

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.

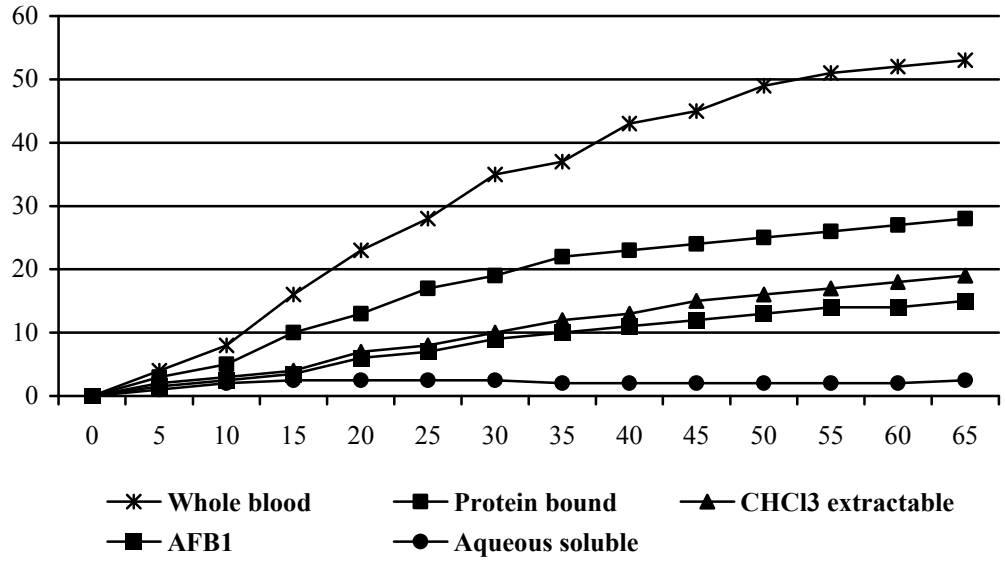
Results suggested that AFB1 was rapidly absorbed in the small intestine to the mesenteric venous blood. Similar considerations were reported in dairy cows after a single oral dose of 0.5 mg/kg BW of AFB1: the maximum AFB1 concentration in plasma was found at 12 and 60 hours

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.

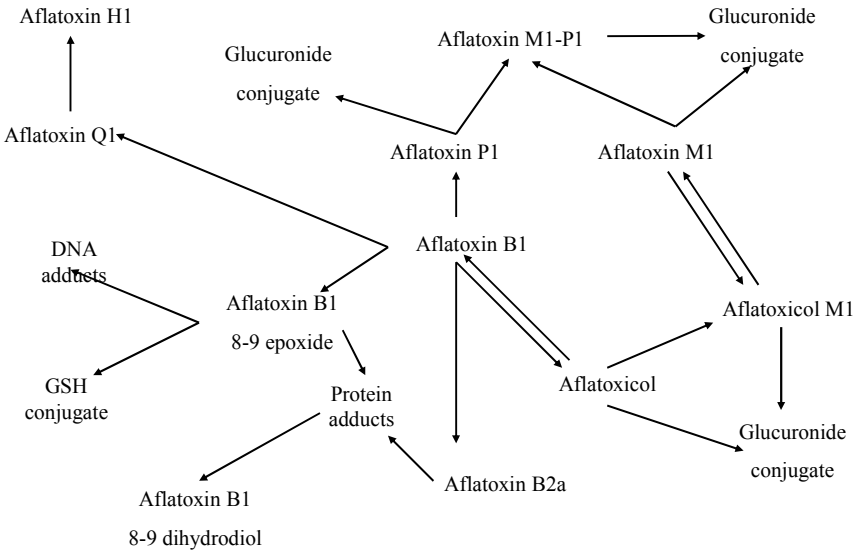
Wogan et al. (1967) studied the passage of aflatoxins from gastro-intestinal tract and absorption rate in the liver. These authors found that 30 minutes after an intra-peritoneal dose of 70 μg/kg of [^{14}C]AFB1, the liver contained approximately 17% of the total radioactivity, the kidneys 5% and the 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).

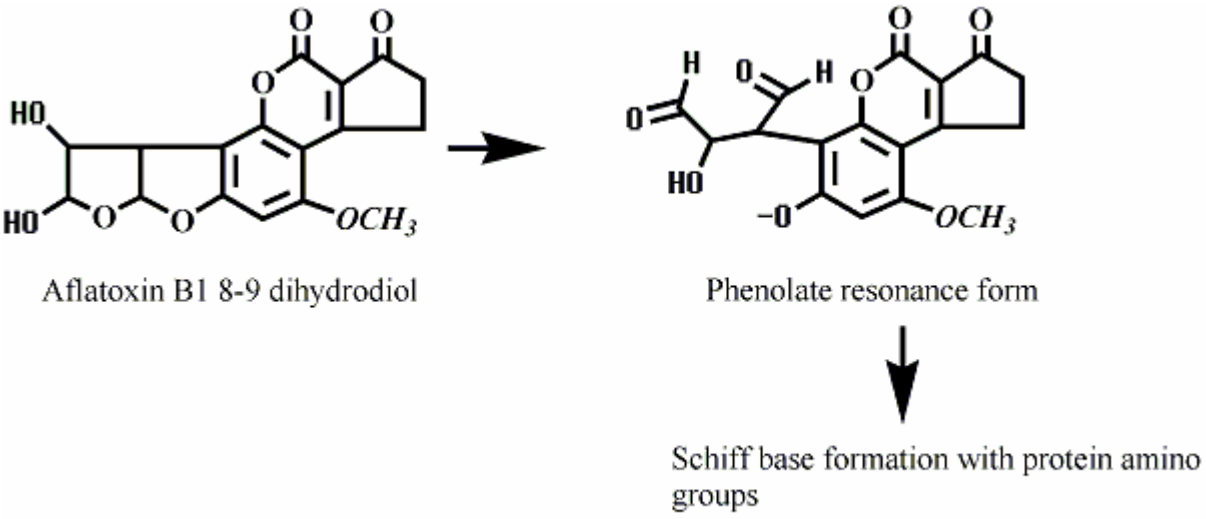
4.3.1 Epoxidation of Aflatoxins

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.

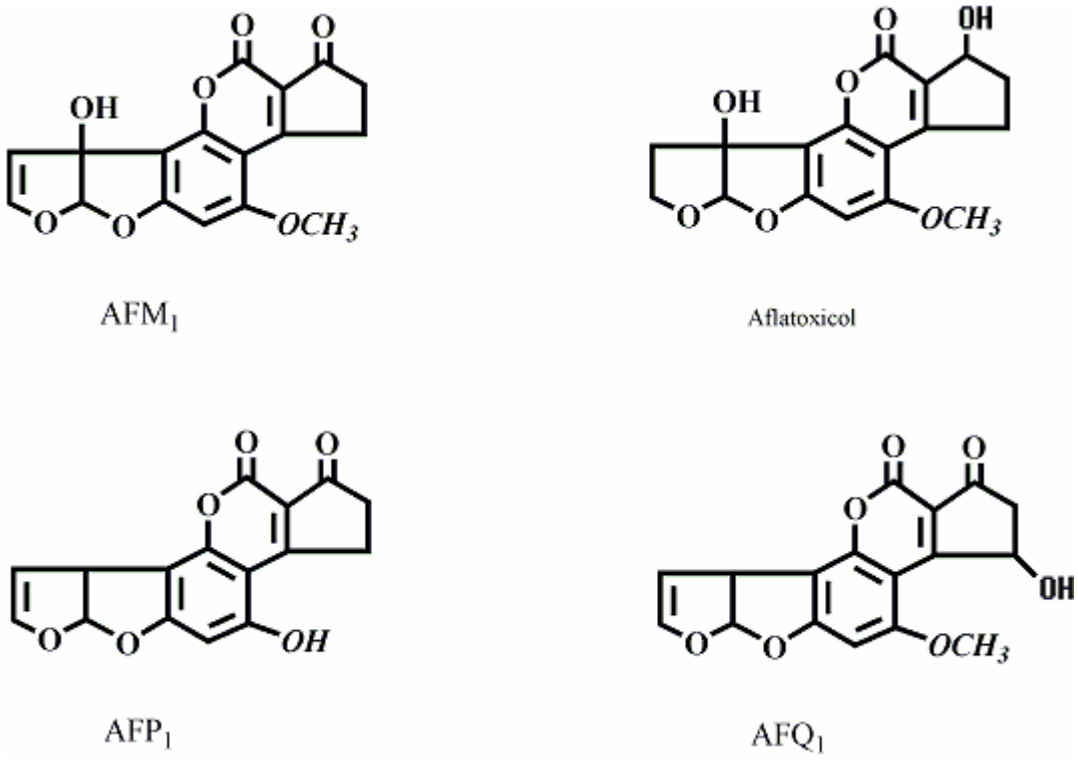
Other mechanisms of AFB1 activation that do not involve CYP have been demonstrated. Co-oxygenation of AFB1 can occur in the presence of arachidonic acid and prostaglandin H synthase, forming mutagenic metabolites. In particular, prostaglandin H synthase shown the same

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:

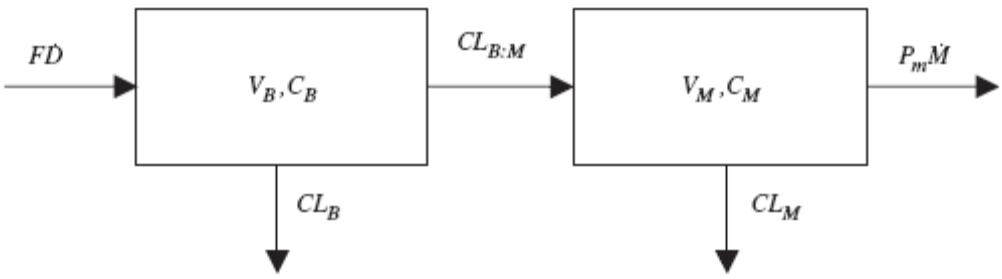
AFM1 (ng/Kg of milk) = 0.787 AFB1 (µg/cow/day) + 10.95
r = 0.92

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%.

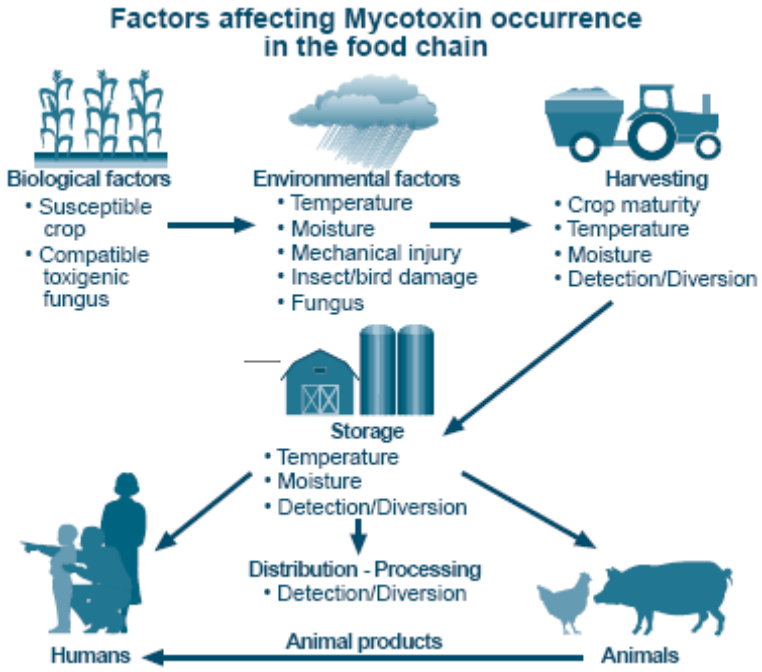
Ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are antioxidants capable to chemo-protection against aflatoxins (Kensler et al., 1994). Feedings studies with these compounds at levels used in anticarcinogenesis bioassays produced 60-90% reductions in the amount of AFB1 bound 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

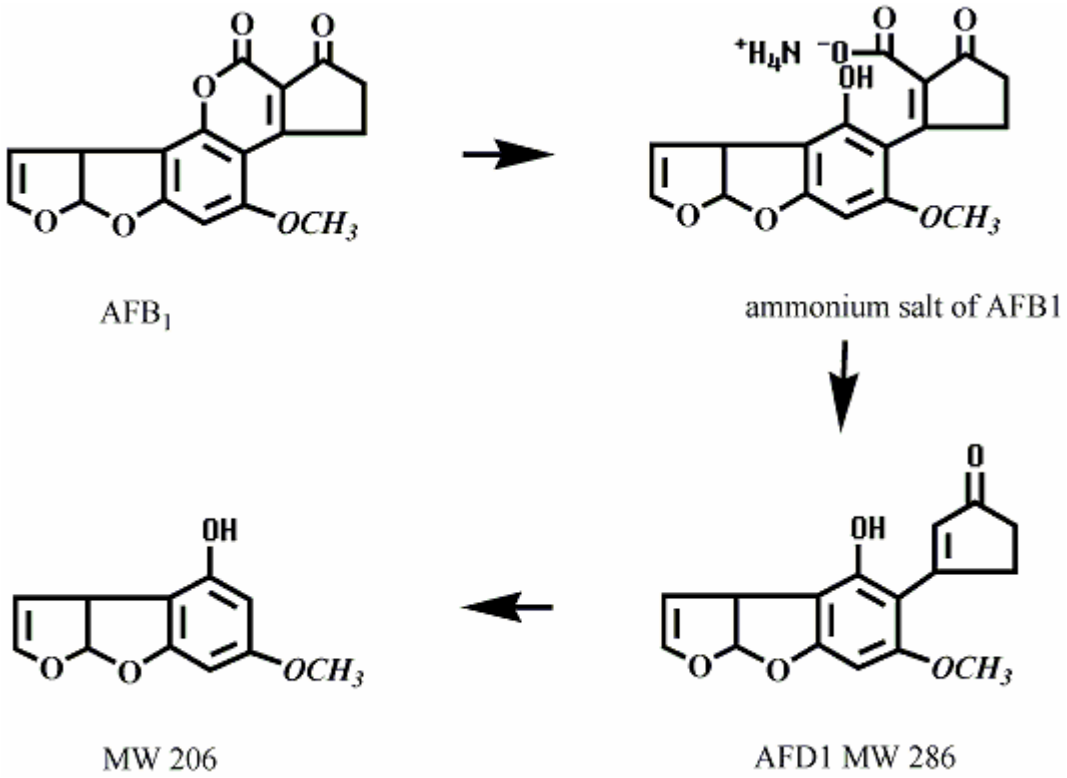
Numerous chemicals as acids, bases, aldehydes, bisulfite, oxidizing agents and various gases have been tested for their ability to degrade or detoxify aflatoxin. (Anderson, 1983; Goldblatt and Dollear, 1979; Hagler, 1991; Park et al., 1988; Phillips et al., 1994; Samarajeewa et al., 1991). Even if many 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.

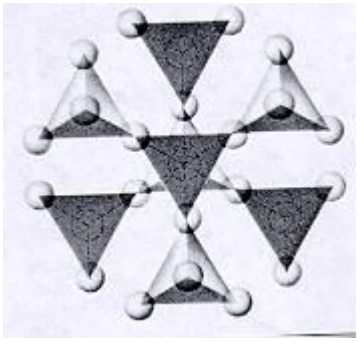
Adsorbents

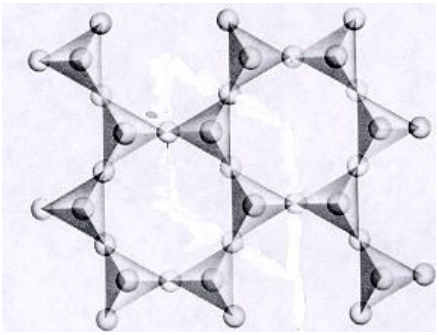
The largest and most complex class of SA includes clays. Several attempts of classifications for these sequestering compounds have been proposed (Phillips et al., 2004; CAST, 2003; Diaz and Smith, 2005). Generally clays are considered natural adsorbents chemically made of silicates and/or aluminosilicates (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

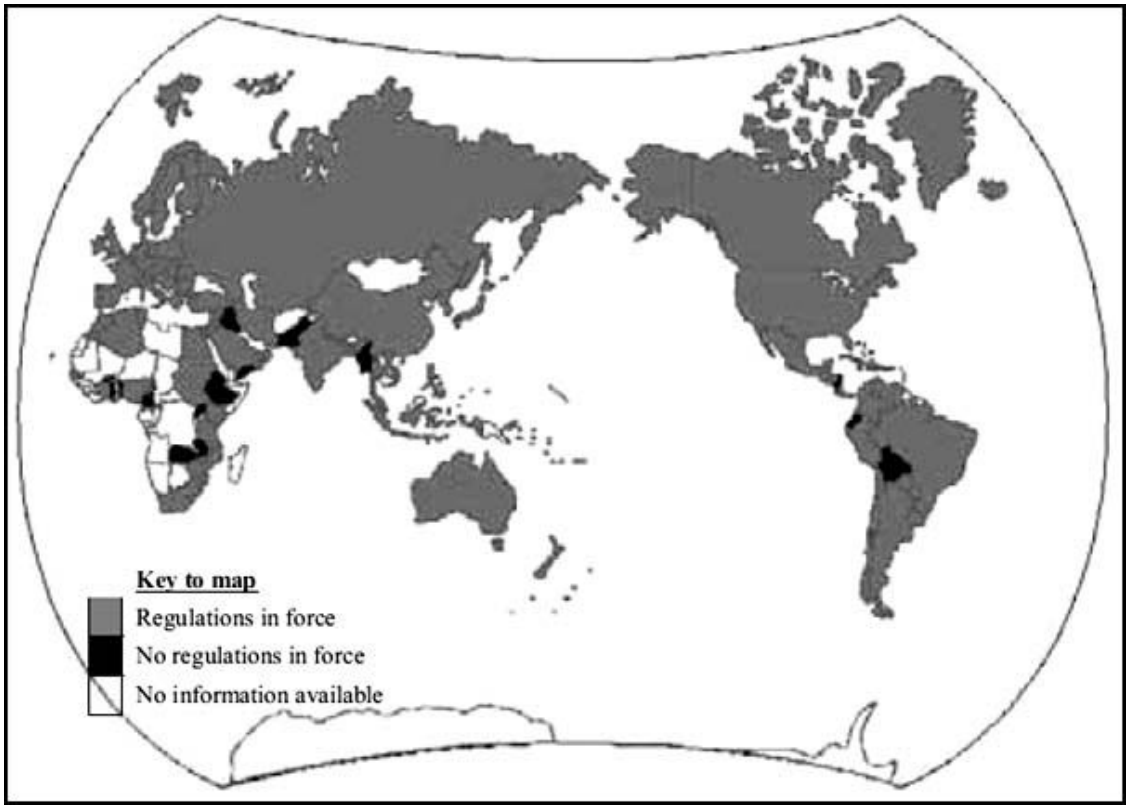
The first study of the *Saccharomyces cerevisiae* capacity to sequester aflatoxin was conducted by Stanley et al. (1993) on poultry. Also Devegowda et al. (1998) observed that the extracted yeast cell wall of *Saccharomyces cerevisiae* was able to bind *in vitro* a large range of mycotoxins. Then, many 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'

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10 Mucosal absorption of aflatoxin B1 in lactating dairy cows

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10.1 Abstract

The objective of this experiment was to monitor plasma levels of aflatoxin B1 (AFB1) aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1) in lactating dairy cows fed afltaoxin contaminated corn. Seven lactating Holstein cows were given a bolus of a naturally contaminated corn meal assuring an intake of 4.9mg AFB1, 1.01mg AFB2, 10.63mg AFG1 and 0.89mg AFG2. Vitamin A, at 1'000'000 IU, was also added as a biomarker of intestinal absorption. Blood samples were collected at 0, 15, 30, 60, 120, 180, 270 and 360 min after bolus. Plasma was analyzed by HPLC for AFB1, AFB2, AFG1, AFG2 and AFM1 concentrations. Within the considered time points, the peak plasma AFB1 concentration was obtained as soon as 15 minutes from drenching. The plasma AFM1 concentration was considerable as early as the first collection (15 minutes) and peaked at 270 minutes indicating both a rapid absorption of AFB1 through the rumen wall and metabolization into AFM1 in liver. The plasma palmitate level suggests the intestinal contribution to the aflatoxin plasma level after 120 min.

Key words: *Aflatoxins, absorption, blood, cow*

10.2 Introduction

The aflatoxins are secondary metabolites produced primarily by Aspergillus flavus and A. parasiticus. Aflatoxin M1 (AFM1) is the principal oxidized metabolite of AFB1 and it can be readily found in milk and urine of most mammalians after consumption of AFB1 (Wood, 1991). The aflatoxins, as a group (AFB1, AFB2, AFG1, AFG2 and AFM1), are classified as group 1 carcinogens (IARC, 2002). The European Union limits AFB1 allowed in animal feeds, concentrate

and the AFM1 in milk levels greater than 20 ppb, 5 ppb (EC, 2003a) and 0.05 ppb (EC, 2006), respectively. In the US the USDA permitted limit for AFM1 in milk is of 0.5 ppb (Berg, 2003). The AFB1 is promptly absorbed within the gastro intestinal tract of dairy cows (Polan et al., 1974), however, there are no work indicating the site of absorption in the gastro intestinal tract. The objective of this work was to monitor plasma levels of AFB1, AFB2, AFG1, AFG2 and AFM1 in lactating dairy cows following a single bolus of aflatoxins contaminated corn meal to determine sites of absorption.

10.3 Material and Methods

The experiment was carried out using seven lactating Italian Holstein Friesian cows housed at the CERZOO research and experimental center (San Bonico, PC, Italy). The research protocol and animal care were in accordance with the EC council directive guidelines for animals used for experimental and other scientific purpose (EEC, 1986). On the day of experiment animals were given via oral drench and before the morning meal a bolus of naturally contaminated corn meal (97.9±1.41 ppm AFB1, 20.2±0.5 ppm AFB2, 212.6±0.6 ppm AFG1, 17.7±0.3 ppm AFG2). The level of corn contamination assured a total intake of 4.9mg, 1.01mg, 10.63mg and 0.89mg/cow, for AFB1, AFB2, AFG1 and AFG2, respectively. The plasma retinol palmitate was used as a biomarker for the intestinal absorption (Bertoni et al., 2001) following the addition of vitamin A in reason of 1'000'000IU into the drench. Blood samples were taken before morning meal via jugular venipuncture at 0, 30, 60, 120, 180, 270 and 360 min after animal drenching. The blood was collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer tubes (Vacutainer systems, Belliver industrial estate, Plymounth, UK). Then, plasma was obtained by centrifugation (3'000 rpm for 15 minutes). The plasma fraction was isolated and stored at -20°C until analyzed for aflatoxins and relative metabolite by High Performance Liquid Chromatography (HPLC) analysis. The aflatoxins extraction was done by the immunoaffinity technique. Aflatoxins were extracted into chloroform (20mL plasma mixed with 90mL of chloroform). Then, the chloroform layer was evaporated to dryness and reconstituted in 1.5mL methanol and 35mL water. The elution were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with 5 mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

10.4 Results and Conclusions

The plasma AFM1 was detectable (45.1 ppt) as soon as 15 minutes from drenching (table 10-1). Since their low molecular weight (AFB1 = 312.27 and AFM1 = 328.27), the toxins are rapidly adsorbed through membranes by a passive mechanism and the transfer to blood and biological fluids of AFB1 is by passive diffusion of the polar component into the liquid phase and by diffusion or active transport of the non polar component into the lipid phase (Yiannikouris and Jouany, 2002). The AFB1 and AFM1 levels observed just after 15 minutes from drenching indicate both a rapid absorption of AFB1 through the rumen wall and metabolised in the liver into AFM1.

The measured plasma retinol palmitate suggests a probable early AFB1 absorption at rumen level and an AFB1 intestinal contribution to the AFM1 plasma level about 120 minutes after drenching (table 10-1 and figure 10-1). The AFB1:AFM1 ratio (1:3.4) was lowest at 270 from the beginning of treatment.

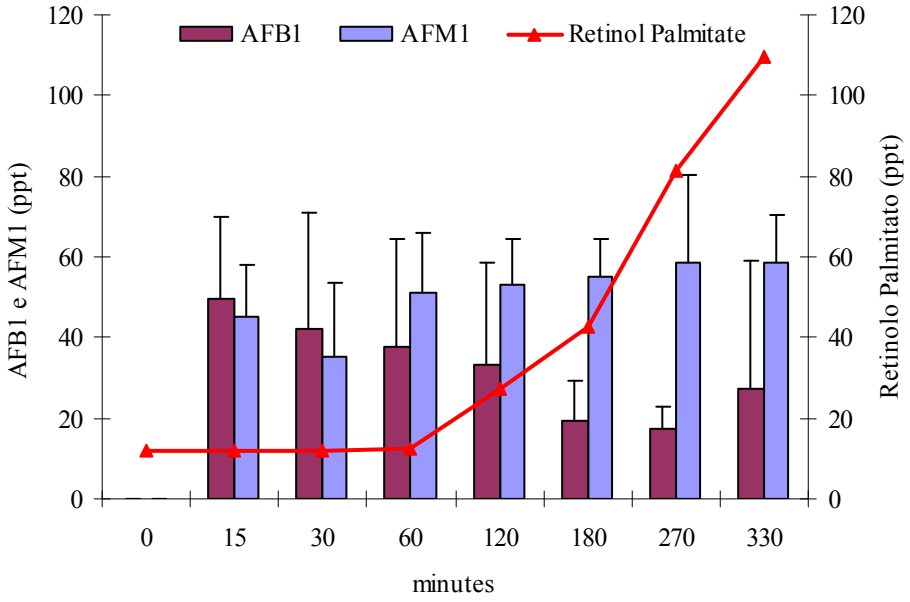
The data reveal important information for a better understanding of place and dynamics of aflatoxin absorption in dairy cattle that could be useful to minimize the AFM1 residues in products intended for human consumption.

Table 10-1. AFB1, AFB2, AFG1, AFG2, AFM1 and retinol palmitate plasma levels following treatment bolus drenching

Item	Time, minutes							
	0	15	30	60	120	180	270	360
AFB1, ppt	n.d.	49.5±21	42.2±29	37.8±27	33.0±26	19.2±10	17.4±5	27.2±32
AFB2, ppt	n.d.	9.0±7	8.0±8	6.8±5	9.2±8	5.5±3	4.4±3	7.2±8
AFG1, ppt	n.d.	24.2±17	20.2±14	24.1±21	48.0±82	14.6±10	12.5±7	43.8±84
AFG2, ppt	n.d.	3.6±3	3.2±3	3.6±2	8.5±10	2.8±2	2.7±2	7.8±10
AFM1, ppt	n.d.	45.1±13	35.3±19	51.0±15	53.2±11	55.1±9	58.7±22	58.7±12
Retinol palmitate, ppb	12.3±2	-	-	12.4±2	23.8±6	43.9±7	86.8±49	109.3±6

n.d. not detectable

Figure 10-1. AFB1 and AFM1 (ppt) and Retinol Palmitate (ppb) pattern after treatment bolus drenching



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

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11.1 Abstract

The objective of the experiment was to monitor plasma levels of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2) and M1 (AFM1) in lactating dairy cows fed a single oral bolus with aflatoxin natural contaminated corn meal (Trial 1). The possible aflatoxins (AFs) absorption through mucous membranes was also investigated using the vaginal mucosa (Trial 2). In trial 1, seven lactating Holstein dairy cows were given a single oral bolus of a naturally contaminated corn meal assuring an intake of 4.89 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2. Blood samples were collected at 0 and 5, 10, 15, 20, 25, 30 minutes after treatment. In trial 2 a similar aflatoxin dosage of trial 1 was provided through vaginal implant to eight lactating Holstein dairy cows.. Blood samples were collected at 0 and 15, 30, 60, 180, 360 minutes after treatment. Individual milk samples of six milkings, one before and five after treatment, were also collected. Plasma and milk samples were analyzed by HPLC for AFB1, AFB2, AFG1, AFG2 and AFM1 contents. In trial 1 AFB1 in plasma peaked (33.6 ng/L) as soon as 20 minutes after treatment. The plasma AFM1 was already detectable at 5 minutes (10.4 ng/L) and peaked at 25 minutes (136.3 ng/L). In trial 2 only AFB1 and AFM1 were detectable in plasma, starting from the first sampling time (15 minutes), with values of 10.7 and 0.5 ng/L, respectively. The AFB1 peaked at 30 minutes (23.9 ng/L). The AFB1 excreted in milk as AFM1 had the highest concentration (203.0 ng/L) in the first milking after treatment and decreased close to the starting values after 36 hours from treatment.

The prompt appearance of studied aflatoxins, and their metabolites, in plasma suggests absorption might also take place in mouth or oesophageal mucous membranes, before the rumen compartment.

Results support the hypothesis that the cytochrome P450 oxidative system, which is present in these tissues and in leukocytes, could be involved in the conversion of the AFB1 in AFM1. The

absorption of AFB1 through the vaginal mucosa confirms the passive diffusion as a probable mechanism for AFB1 absorption.

Key Words: *Aflatoxin B1, Aflatoxin M1, Dairy cows, Absorption, Blood, Milk*.

11.2 Introduction

Aflatoxins (AFs) are secondary metabolites produced principally by Aspergillus flavus and A. parasiticus either in pre or post-harvest conditions (Samapundo et al., 2007). These fungi can colonize and produce AFs on many food and feed products such as corn, cotton and peanut. The presence of AFs on feedstuffs can be severe and cause significant economic losses and potentially raise public health concerns (Norton, 1999). A. flavus and A. parasiticus are known to produce mainly four types of AFs: aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Scheidegger and Payne, 2003).

The AFB1 is considered one of the most potent known natural hepatic-carcinogen for mammals (Creppy, 2002). When adsorbed by lactating animals, the AFB1 is hydroxylated and its main metabolite, the aflatoxin M1 (AFM1), is excreted in the urine, feces and milk (Yiannikouris and Jouany, 2002).

The liver is the main site of AFB1 bio-transformation with the mitochondrial cytochrome P450 oxidative system (CYP) converting the AFB1 into AFM1 and other metabolites (Sudakin, 2003). The AFM1 preserves the same acute toxicity of AFB1 in rats (Pong and Wogan, 1971), with lower carcinogenic potential (Wogan and Paglialunga, 1974). Thus, the metabolic pathway leading to AFM1 formation can be considered a detoxification process (Yannikouris and Jouany, 2002).

The IARC (2002) classified AFB1, AFB2, AFG1, AFG2 and AFM1 as carcinogenic to humans (Group 1). The European Union allowed maximum limits for AFB1 concentration in feed materials, complete feedingstuffs and AFM1 in milk are set at 20 μg/kg, 5 μg/kg (2003/100/EC, 2003) and 0.05 μg/kg (2006/1881/EC, 2006), respectively.

The animal exposure to AFB1 occurs mainly with the ingestion of contaminated feeds (Sudakin, 2003), however skin (Rastogi et al., 2006) or inhalation (Jakab et al., 1994) exposures might also contribute. When absorbed, AFs presence in blood is prompt (Trucksses et al., 1983; Coulombe and Sharma, 1985) and can reach organs and peripheral tissues likely through a passive mechanism (Yannikouris and Jouany, 2002). The parent toxins (AFB1, AFB2, AFG1, AFG2) or the AFM1 have been detected in plasma of cows as soon as 15 minutes from oral administration of AFs naturally contaminated corn meal (Moschini et al., 2007).

The objective of this work was to monitor the early plasma levels of AFs in lactating dairy cows following a single oral bolus of AFs contaminated corn meal. The possible AFs absorption through mucous membranes was also investigated using the vaginal mucosa.

11.3 Materials and methods

11.3.1 Animals and samplings

Two experiments were carried out on lactating Italian Holstein Friesian dairy cows (seven cows in the first trial and eight cows in the second trial). Cows were housed at the CERZOO research and experimental center (San Bonico, Piacenza, Italy). The research protocol and animal care were in accordance with the European Community council directive guidelines for animals used for experimental and other scientific purpose (EEC, 1986).

Cows were housed in a free stall barn and had free access to water. The diet was formulated according to the nutrient requirements of dairy cattle (NRC, 2001) for an average cow weight of 600 kg, 140 days in milk (DIM) and a 35 kg milk yield (3.80% fat and 3.35% protein). The bulk of the diet on a dry matter basis was: corn silage (31.2%), dehydrated alfalfa hay (16.7%), grass hay (4.1%) and energy-protein supplement (48%). The diet was fed once a day (0900 h) and ad libitum (5% expected orts) as a total mixed ration (TMR).

Cows were milked twice a day (0230 h and 1330 h) and the individual milk yield was recorded at every milking (Afimilk system, Afimilk, Israel).

11.3.2 Trial 1: Aflatoxin as oral drench

On day of experiment seven cows averaging 185±43 DIM and 28.75±4.37 kg milk yield (mean ± sd) were given by oral drench and before the morning meal a bolus of AFs naturally contaminated corn meal for a total intake of 4.89 mg, 1.01 mg, 10.63 mg and 0.89 mg, respectively for AFB1, AFB2, AFG1 and AFG2. Cows were blood sampled by jugular venipuncture at 0, 5, 15, 20, 25, and 30 minutes from treatment. The blood was collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer tubes (Vacutainer systems, Belliver Industrial Estate, Plymounth, UK). Then, plasma was obtained by centrifugation (3’000 g for 15 minutes) and stored at -20°C until AFs analysis by High Performance Liquid Chromatography (HPLC)

11.3.3 Trial 2: AFs administration in vagina

Eight cows averaging 192±48 DIM and 27.78±4.13 kg milk yield (mean ± sd) were used in the experiment. AFs were extracted from 50 g of naturally contaminated corn meal with a methanol:water solution (80:20 v/v). Then, the methanol:water extract was dried under nitrogen, redissolved into 20 mL of water and adsorbed to a cotton wad. The cotton wad containing 4.89 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2 and was implanted directly in the vagina of the cows before the morning meal.

TMR samples were collected the day before treatment for AFs content determination. Individual milk samples were collected from four randomly selected cows at each milking and for six consecutive milking (the first sample before treatment). Then, samples were frozen at -4°C before HPLC analysis.

Blood samples were taken before morning meal by jugular venipuncture at 0, 15, 30, 60, 180 and 360 minutes after treatment and collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer tubes (Vacutainer systems, Belliver Industrial Estate, Plymounth, UK). Then, plasma was obtained and stored as in trial 1.

11.3.4 Sample Analysis

Extraction of the AFs from feeds was done by the immunoaffinity technique according to Arranz et al. (2006). Briefly, ten grams of dried feed were put in a methanol:water solution (80:20 v/v), shaken at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, five mL were eluted with 45 mL of bi-distilled water through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

Extraction of the AFs from milk was done by the immunoaffinity technique according to Mortimer et al. (1987). Briefly, 50 mL of defatted milk (centrifuged at 7'000 rpm for 10 minutes at 4°C) were filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water, and slowly eluted with 2.5 mL of methanol.

The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

Extraction of the AFs from plasma samples was done by the immunoaffinity technique. Obtained sample volume was recovered and plasma was put in a separatory funnel with 35 ml of chloroform. Then chloroform phase was separated and evaporated with Rotavapor (Büchi Labortechnik, Postfach, Switzerland). After the evaporation, AFs extracted were dissolved with one ml of methanol and 35 ml of water and the solution was passed through an immunoaffinity column (Aflatoxin Easy-extract) previously washed with 10 mL of a phosphate-buffered saline solution (PBS/2%, pH 7.4). The column was washed with five mL water, and slowly eluted with 2.5 mL of acetronitile. The column extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered before HPLC analysis.

11.3.5 Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFs in feed and in plasma was separated with a reverse-phase C18 Superspher column (4 μm particle size, 125 x 4mm i.d.; Merck, Darmstadt, Germany) at room temperature and isocratic conditions, with a mobile phase of water and acetonitrile:methanol solution (17:29 v/v) with a 64:36 (v/v) ratio. The flow rate was 1mL/min. Then, the AFB1 was detected by fluorescence, after postcolumn derivatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide perbromide (PBPB) at flow 0.1 mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFB1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, 5 μm particle size, 125 x 4 mm I.D.) at room temperature, with a water and acetonitrile (75:25 v/v) mobile phase made and the flow rate set at 1 mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFM1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

11.3.6 Statistical Analysis

Statistical evaluation of AFB1, AFB2, AFG1, AFG2 and AFM1 plasma and milk levels were carried out by paired t test. Differences between values at each sampling time after treatment versus the value at time zero were computed for considered parameters. By using the proc means of SAS (V9.1, Statistical Analysis Systems Institute Inc., Cary, NC) a mean and standard error of the obtained differences were computed for each parameters and the probability that the absolute value of the mean difference was greater than zero by chance alone was estimated.

11.4 Results and discussion

11.4.1 Trial 1: Aflatoxin as oral drench

This work follows a previous study on dairy cows (Moschini et al., 2007) in which the AFB1 and AFM1 were detectable in plasma as soon as 15 minutes after the ingestion of an AFs contaminated corn meal bolus.

In the current trial the TMR had a AFB1, AFB2, AFG1 and AFG1 contents of 1.7±0.4, 0.3±0.1, 0.5±0.2 and 0.1±0.1 μg/kg respectively, resulting in a non detectable level of AFs in plasma of lactating dairy cows (table 11-1).

The AFB1 ($P < 0.05$) was found in plasma as soon as five minutes after AFs ingestion (table 11-1). The result suggests a rapid absorption of AFB1 through the gastro-intestinal tract of cows and a quick oxidation of the toxin to AFM1, with a significant increase in plasma at 10 minutes compared to plasma samples collected before treatment ($P < 0.01$). As previously reported (Moschini et al., 2007), an early AFB1 absorption before the rumen wall could be involved before the intestinal contribution for AFB1 absorption, around 120 to 180 minutes after AFB1 ingestion.

For the studied sampling schedule of 0, 5, 15, 20, 25 and 30 the AFB1 plasma level was maximum (33.6 ng/L) at 20 minutes after exposure to AFB1, whereas the AFM1 peaked at 25 minutes from AFB1 exposure (136.3 ng/L). The AFB1 results agreed with our previous data in which the AFB1 plasma level peaked after 15 minutes from treatment (Moschini et al., 2007).

The AFM1 plasma levels reported by Moschini et al. (2007) were 45.3 ng/L at 15 minutes and 35.3 ng/L at 30 minutes from treatment. As in table 11-1, AFM1 plasma concentrations in the present work were 68.0 ng/L at 15 minutes and 135.3 ng/L at 30 minutes. Considering the standard deviations, the AFM1 values at 15 minutes could be considered similar, while a difference could be

addressed for the 30 minutes samples, with values four times higher in the present work and with, the same aflatoxin dosage being given to animals (4.89 mg/cows of AFB1).

However, differences in plasma AFs levels could result from high individual variability among animals, probably related either to differences in plasma volumes or to a different passage of the toxin through the membranes (Van Egmond, 1989; Veldman et al., 1992; Masoero et al., 2007) .

AFB2 and AFG1 were also detectable in plasma five minutes after the oral drench, with the maximum level at 30 minutes. Even in presence of higher AFG1 intake compared to AFB1 (10.63 vs. 4.89 mg/cow), in plasma the AFG1 content was lower than AFB1. The plasma level might be related to differences on absorption dynamics of the parent molecules. Very low concentrations and close to the detectable limit were observed for the AFG2 (table 11-1).

The AFM1/AFB1 plasma ratio moved from 0.99 at five minutes to 3.05 at 15 minutes and to 4.89 at 30 minutes from drenching. The pattern indicates an AFB1 conversion rate to AFM1 which overcomes the body capacity of getting rid of the AFM1, either through faeces, urine and milk (Yiannikouris and Jouany, 2002), and could lead to an accumulation of the metabolite within the blood pool.

The observed prompt absorption of the considered AFs and their appearance in blood, also as metabolite, are in agreement with previous works (Polan et al., 1974; Trucksess et al., 1987; Moschini et al., 2007). Authors suggested a rumen contribution to the AFs absorption before the intestinal tract.

Other ways of AFs passage to the blood compartment could be addressed too. In particular Coulombe and Sharma (1985), working with rats exposed to a single intra-tracheal or oral [H3]AFB1 doses, measured a peak AFB1 plasma concentration after one and three hours from treatment, respectively. Authors suggested that the absorption of non polar and lipid-soluble compounds, like AFs, is a rapid process either through the pulmonary tissues or in the gastro-intestinal tract.

Current results on plasma AFs and their metabolite presence as soon as five minutes after treatment suggest a rapid absorption even before the rumen compartment, through the mouth or oesophageal mucous membranes.

Once absorbed, the AFB1 is converted to AFM1 and other metabolites through processes mainly microsomal CYP mediated (Coulombe, 1993; Yannikouris and Jouany, 2002; Sudakin, 2003), and in particular in CYP3A4 and CYP2A6 forms (Gallhager et al., 1996; Pelkonen et al., 2000). These oxidative systems can be found in different tissues beyond the liver, like small intestine, pancreas, brain, lung, adrenal gland, kidney, bone marrow, mast cells, skin, ovary, testis and leukocytes (Krishna and Klotz, 1994; Chang and Kam, 1999; Lind et al., 2003). Thus, the

oxidation of the AFB1 seems related to either hepatic or extra-hepatic CYP (Coulombe, 1993). It has been reported a high oxidation activity of AFB1 within olfactory and respiratory tissues of cattle, sheep, swine and rat (Coulombe and Sharma, 1985; Tjälve et al., 1992; Larsson et al., 1994; Larsson and Tjälve, 1996). Thus, extra-hepatic conversion of the adsorbed AFB1 might justify the prompt appearance of AFM1 in plasma (table 11-1).

11.4.2 Trial 2: Aflatoxin administration in vagina

The TMR had an AFB1, AFB2, AFG1 and AFG2 contents of 2.3±0.5, 0.4±0.1, 0.9±0.3 and 0.2±0.1 µg/kg respectively, resulting in non detectable levels of AFs in plasma and in a a bulk milk AFM1 content of 2.3±2.0 ng/L of milk from exposed dairy cows.

The AFB1 and AFM1 plasma concentrations following the aflatoxin vaginal implant is reported in table 11-2. The AFB2, AFG1 and AFG2 were not detectable in collected plasma samples.

Both AFB1 and AFM1 were detected in plasma as soon as 15 minutes after treatment. For the considered sampling schedule, the maximum level for AFB1 (23.9 ng/L) was measured at 30 minutes from treatment, whereas no trend seemed detectable for AFM1, with similar concentrations in samples collected between 30 and 360 minutes from treatment (table 11-2).

The low molecular weight (312.27 formula weight) and the lipophilic characteristic of AFB1 could allow a mucosal passive diffusion through tissues as previously reported in different species (Kumagai, 1989; Coulombe, 1993; Hiesh and Wong, 1994; Yiannikouris and Jouany, 2002). Indeed there is a report of a faster pulmonary tissues AFB1 absorption in male rats compared to the gastro-intestinal tract toxin absorption (Coulombe and Sharma, 1985). These observations confirmed that the absorption and biotransformation rate of AFB1 could be affected by the administration route (Hsieh and Wong, 1994).

The prompt and continuous passage of the toxin through the vaginal mucosa observed in our trial might corroborate a passive diffusion process as a mechanism regulating the absorption of AFB1. This in agreement with Kumagai (1989), injecting [H3]AFB1 directly in stomach and in various sites of the small intestine, who found a rate of AFB1 uptake from gut to mesenteric venous blood nearly proportional to the AFB1 concentration.

The highest AFM1 concentration (203.0 ng/L) was measured in milk at eight hours after the treatment (figure 11-1). Then, the pattern of the AFM1 concentration in milk was downward through consecutive milk samples (80% lower in the second sample) up to a similar pre-treatment value at 32 hours from treatment. The AFM1 excretion pattern in milk were comparable to previous

reported data as consequence of a single AFB1 ingested dose (Battaccone et al., 2003) with maximum concentration in the first milking after dosing.

No AFM1 due to AFB1 administration was detected in the fifth milking after treatment.

The AFB1 in milk was found only in samples eight hours after treatment and at very low concentration (4.3 ng/L). The AFB1 was usually excreted in milk in buffalo cows fed an aflatoxin contaminated diet (Pietri et al., 2003), whereas in dairy cows the parent molecule was found in milk only following an AFB1 ingestion higher than 300 mg (Truckess et al., 1983). Our unpublished data confirm that lactating dairy cows ingesting 5, 15 and 50 mg of AFB1 excreted 107.2, 27.1 and 7.8 μg of AFB1 in milk, respectively.

11.5 Conclusions

AFs were quickly absorbed through the gastro-intestinal tract of cows. An early absorption might also take place in mouth or oesophageal mucous membranes, before the rumen compartment. Results support the hypothesis that the CYP oxidative system which is present in these tissues and in leukocytes could be involved in the conversion of the AFB1 in AFM1.

The absorption of AFB1 through the vaginal mucosa confirms a passive diffusion as a probable mechanism for AFB1 absorption.

Table 11-1. Plasma aflatoxins concentration (means ± standard deviation; ng/L) before and after an aflatoxin contaminated oral bolus[1] (n=7)

	Minutes from the oral bolus						
Aflatoxins	0	5	10	15	20	25	30
Aflatoxin B1 (AFB1)	n.d.[2]	10.5*±9.4	21.1*±20.2	22.3***±16.5	33.6***±22.8	25.7***±11.3	27.7***±14.7
Aflatoxin B2 (AFB2)	n.d.[2]	1.3*±1.6	3.2*±3.1	4.5*±4.4	3.7*±4.2	5.9±7.3	7.5*±8.9
Aflatoxin G1 (AFG1)	n.d.[2]	4.6*±5.1	12.0*±14.1	16.3±22.7	15.7±20.4	24.3±36.3	29.0±46.0
Aflatoxin G2 (AFG2)	n.d.[2]	0.5±0.7	0.7±0.8	0.3±0.6	0.7*±0.8	0.9*±1.0	0.6±0.9
Aflatoxin M1 (AFM1)	n.d.[2]	10.4±20.5	55.7**±49.8	68.0***±53.9	109.1***±54.0	136.3***±76.6	135.3***±81.7

[1]Aflatoxins presence into the oral bolus: 4.9 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2.

[2]n.d.= not detectable.

Differences with initial value (0 minute) being greater than zero. * $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$.

Table 11-2. Plasma aflatoxins concentration (means ± standard deviation; ng/L) before and after the aflatoxin contaminated vagina implant[1] (n=8)

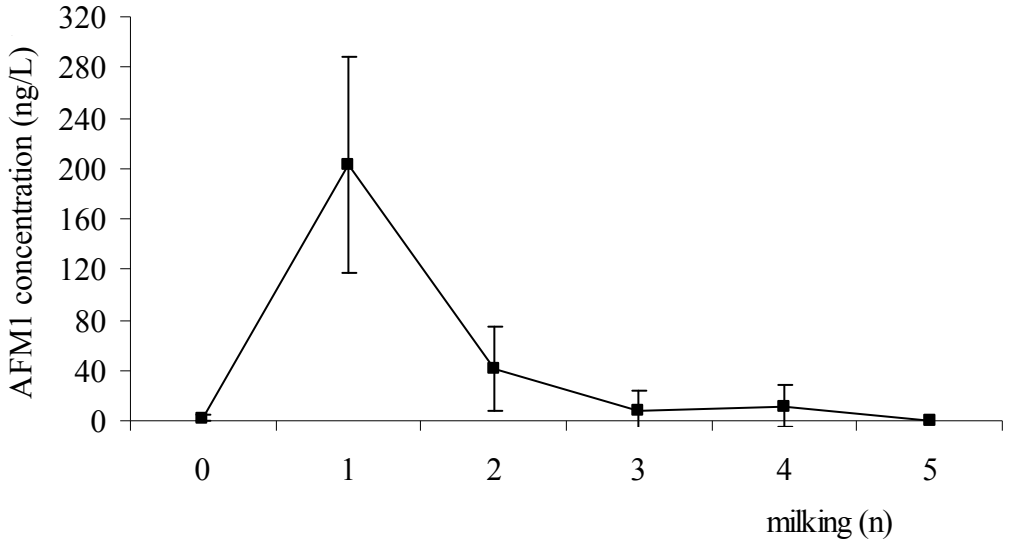
	Minutes from vaginal implant					
Aflatoxins	0	15	30	60	180	360
Aflatoxin B1 (AFB1)	n.d.[2]	10.7***±8.4	23.9*±27.2	11.8***±10.2	7.8*±7.0	4.4*±5.1
Aflatoxin M1 (AFM1)	n.d.[2]	0.5*±0.6	4.9±6.4	4.4*±5.2	2.8*±3.5	5.2*±6.2

[1]aflatoxins presence into the vaginal implant: 4.9 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2 .

[2]n.d.= not detectable.

Differences with initial value (0 minute) being greater than zero. * $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$.

Figure 11-1. Aflatoxin M1 (AFM1) milk concentration (means ± standard deviation; ng/L) at different milking from aflatoxin contaminated vaginal implant in lactating dairy cows (n=8)



Manuscript for: *Animal*

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

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12.1 Abstract

Aflatoxin M1 residues in milk are regulated in many parts of the world and can cost dairy farmers significantly due to lost milk sales. Additionally, due to the carcinogenicity of this compound contaminated milk can be a mayor public health concern. Thirty four lactating dairy cows were utilized to investigate the relationship between somatic cell counts (SCC), milk yield and conversion of dietary AFB1 into milk AFM1 (carry over). The AFM1 in milk increased as soon as the first milking after animal ingestion with a pattern of increment up to the observed plateau (between 7th and 12th days of AFB1 ingestion). There was a significant ($P < 0.01$) effect of the milk yield whereas no effect could be attributed to the somatic cell count level or to the milk yield*somatic cell count interaction. Similarly, the main effect of milk yield was also observed ($P < 0.01$) on the total amount of AFM1 excreted during the ingestion period. Although plasma concentration of Gamma-glutamyl transferase was significantly affected by aflatoxin administration levels of this liver enzyme were within the normal range for lactating dairy cows. The current data suggest that milk yield is the major factor affecting the total excretion of AFM1 and that somatic cell counts as an indicator of mammary gland permeability was not related to an increase in AFM1 carry over.

Keywords: *aflatoxins, dairy cows, milk, aflatoxin M1, somatic cell count*

12.2 Introduction

The aflatoxins are secondary metabolites produced primarily by Aspergillus flavus and A. parasiticus. Aflatoxins are common crop contaminants, with contamination occurring either in the field, during harvest or during storage. The most frequently affected crops are corn (Zea mais), cotton and peanuts and their by products. The major aflatoxins are aflatoxin B1 (AFB1), B2, G1 and G2. Because of their low molecular weight, once ingested these compounds are rapidly adsorbed in the gastro-intestinal tract through a non-described passive mechanism (Yiannikouris and Jouany, 2002) and quickly appears as a metabolite in blood after just 15 minutes (Moschini et al., 2006) and milk as soon as 12 hours post feeding (Diaz et al., 2004).

Aflatoxin M1 (AFM1) is the principal oxidized metabolite of AFB1 and it can be readily found in milk and urine of most mammalians after consumption of AFB1. The aflatoxins, as a group (AFB1, AFB2, AFG1, AFG2 and AFM1), are classified as group 1 carcinogens (IARC, 2002). The European Union allowable limits for AFB1 in animal feeds and concentrates are 20 µg/kg and 5 µg/kg respectively (EC, 2003a). Furthermore, the EC limits AFM1 in milk to levels not greater than 0.05 µg/L (EC, 2006). In the US AFM1 is regulated by the US Food and Drug Administration (FDA) at 0.5 µg/L.

In dairy cows the amount of AFM1 excreted into milk can be up to 3% of the AFB1 intake (Diaz et al., 2004) and is affected by milk yield (Pettersson et al., 1989; Veldman et al., 1992) and stage of lactation (Munksgaard et al., 1987; Pettersson et al., 1989; Veldman et al., 1992). Other factors that affect carry-over into milk include species differences (Battacone et al., 2003), animal variability (Egmond, 1989; Steiner et al., 1990; Veldman et al., 1992) and mammary alveolar cell membrane health (Lafont et al., 1983).

There is limited information about the effect of udder infection on AFM1 excretion into milk. It has been suggested that an increase in AFM1 carry over (CO) occurs due to Staphylococcus udder infection (Veldman et al., 1992) whereas a previous study showed a relationship between AFM1 milk carry over and milk somatic cell counts independent of the milk yield (Lafont et al., 1983). Mastitis increases the number of Somatic Cells in milk, alters milk composition (Walstra and Jenness, 1984) and may affect AFM1 carry over rate by increasing membrane permeability.

The objective of this work was to evaluate the effect of milk yield and somatic cells count, as indicator of udder inflammatory processes, on milk AFM1 carry over in lactating dairy cows.

12.3 Material and methods

12.3.1 Animals and Treatments

An experiment was carried out utilizing 34 Holstein multiparous cows housed at the CERZOO research and experimental center (San Bonico, PC, Italy). The research protocol and animal care were in accordance with the EC council directive guidelines for animals used for experimental and other scientific purpose (EEC, 1986).

Milk yield and SCC data for classification of animals as high or low was obtained as the average of three measurements during a 15 d pre-experimental period. Individual milk yield for the factorial arrangement was measured daily during the last week of the pre-experimental period. Milk yield (LY: < 30 kg/head per day, HY: > 30 kg/head per day) and milk somatic cells count (SCC) (LSCC: <350000, HSCC: > 350000) were used in a 2 x 2 factorial arrangement in a completely randomized design.

Cows were housed in a free stall barn and had free access to water. The diet was formulated according to the nutrient requirements of dairy cattle (National Research Council, 2001) for an average cow weighing 600 kg, 140 days in milk and a 35 kg milk yield (3.8% fat and 3.35% protein). The bulk of the diet (table 12-1) on a dry matter basis was: corn silage (31.2%), dehydrated alfalfa hay (16.7%), grass hay (4.1%) and energy-protein supplement (48%). The diet was fed ad libitum (5% expected orts) as a total mixed ration (TMR) daily (0900 h). Cows were milked twice a day (0230 h and 1330 h) and individual milk yield was recorded at every milking (Afimilk system, Afikim, Israel).

During the experimental period (10 days) cows were given, before the morning meal, a 300 g bolus containing 1.004±0.03 g/cow per day of a naturally contaminated corn meal. This allowed for an AFB1 intake from the contaminated bolus of 98.10±0.26 μg/cow per day.

TMR samples were collected on days 0 and 10 of the experimental period, dried at 55°C in a ventilated oven until constant weight, then ground with one mm sieve (Thomas-Wiley Laboratory Mill, Arthur H. Thomas Co., Philadelphia, PA) and frozen until aflatoxin analysis.

Individual milk samples were collected at each milking for 18 consecutive days (during the last two days of the pre-experimental period, the experimental period (10 d) and for six days at the end of the AFB1 ingestion period), then a representative sample for day of milking was obtained and stored at -18°C for subsequent analysis. Milk samples collected at days 3, 7 and 10 of the treatment period were analyzed for fat, protein and lactose contents (infrared analysis, Milkoscan Model FT120 Foss Electric, Hillerod, Denmark) and for SCC (Fossomatic 360 Foss Electric, Hillerod, Denmark).

12.3.2 Sample Analysis

AFB1 assay in Feeds

Ten grams of dried feed were put in a methanol:water solution (80:20 vol/vol), shaken at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, five mL were eluted with 45 mL of bi-distilled water through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

AFM1 assay in milk samples

Extraction was done by the immunoaffinity technique according to Mortimer et al. (1987). Briefly, 50 mL of defatted milk (centrifuged at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

12.3.3 Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFB1 was separated with a reverse-phase C18 Superspher column (four µm particle size, 125 x 4 mm i.d.; Merck, Darmstadt, Germany) at room temperature and isocratic conditions, with a mobile phase of water and acetonitrile:methanol solution (17:29, vol/vol) with a 64:36 (vol/vol) ratio. The flow rate was 1 mL/min. Then, the AFB1 was detected by fluorescence, after post-column dramatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide

perbromide (PBPB) at flow 0.1 mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFB1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, five μm particle size, 125 x 4 mm I.D.) at room temperature, with a water and acetonitrile (75:25 vol/vol) mobile phase, and the flow rate set at one mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFM1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

12.3.4 Blood samples

Blood samples were taken before morning meal via jugular venipuncture on day 0 and 10 of the experimental period. The blood was collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer (Vacutainer systems, Belliver industrial estate, Plymounth, UK). Then, plasma was obtained by centrifugation (3000 rpm for 15 minutes). The plasma fraction was isolated and stored at -20ºC until analyzed for albumin, globulin, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and bilirubin and Beta-hydroxybutyrate concentrations (Bertoni, 1999).

12.3.5 Carry over calculation

The carry over of AFM1 in milk was calculated as the percentage of the AFB1 consumed that was excreted as AFM1 in milk at the time when the toxin output in milk reached a steady-state.

12.3.6 Statistical Analyses

The AFB1 intake, AFM1 milk concentration and total excretion and the CO in milk were analyzed using the mixed procedure of SAS® (Statistical Analysis System Institute, 2001). A factorial arrangement was used and fixed effect in the model included the milk yield and the milk SCC. The animal, within milk yield and SSC interactions were included as random effects. The day of collection was a repeated measure (compound symmetry covariance structure).

The CO (%) was regressed on milk yield (Kg) over time of collection (day) and the linear equation was calculated.

Plasma parameters (pre-experimental and after AFB1 ingestion period) were compared using the paired t-test (Statistical Analysis System Institute, 2001).

12.4 Results

As expected, the low AFB1 concentration fed to animals did not cause negative health problems during the experimental period. The initial milk yield and the milk SCC for groups were (mean ± SD): 21.2 ± 3.8 and 127600 ± 161203, 21.7 ± 3.9 and 1171889 ± 676859, 41.8 ± 8.4 and 240000 ± 20075, 34.8 ± 4.6 and 2030667 ± 2451213; respectively for LY-LSCC, LY-HSCC, HY-LSCC, HY-HSCC.

The TMR had a base AFB1 content of 3.70 ± 0.21 μg/kg contributing to a bulk milk AFM1 content of 4.80±1.80 and 3.90±1.72 ng/L, respectively before and after the ingestion period.

The AFM1 in milk increased as soon as the first milking after animal ingestion with a pattern of increment up to the observed plateau (between 7th and 12th days of ingestion) as reported in figure 12-1. At day one of AFB1 ingestion the AFM1 contents in milk were 52.9 ng/L vs. 24.9 ng/L and 44.0 ng/L vs. 39.3 ng/L, respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups. Closer values among groups were observed from the third day until the end of the ingestion period with average AFM1 contents at the plateau condition of 65.8, 61.9, 66.7 and 59.2 ng/L respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups (table 12-2).

The AFB1 CO into milk calculated at the plateau was 2.32, 2.70, 1.48 and 1.29 % of the AFB1 consumed, respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups. There was a significant ($P < 0.01$) effect of the milk yield whereas no effect could be attributed to the somatic cell count level or to the milk yield x somatic cell count interaction. Similarly, the main effect of milk was also observed ($P < 0.01$) on the total amount of AFM1 excreted during the AFB1 ingestion period.

Plasma biochemistry for samples collected before and after the aflatoxin ingestion period are reported in table 12-3.

12.5 Discussion

The presence of AFM1 in milk was detectable from the first milking after the animal AFB1 ingestion (figure 12-1) which is in accordance with previous work (Allcroft et al., 1968; Diaz et al., 2004; Trucksess et al., 1983). In particular, in the early stage of increase of the AFM1 plateau (between 7th and 12th days of ingestion) there was an effect ($P < 0.01$) of the SCC. However, the

SCC effect was confined to the high yield groups ($P < 0.01$ and $P < 0.05$, respectively for the 1st and 2nd d of ingestion) due to the milk yield and SCC interaction being separated from the level of production (figure 12-1). Results suggest that high milk yield could intensify the effect of SCC on AFM1 CO. A previous report suggested a positive correlation between SCC and milk AFM1 content in dairy cows fed an AFB1 contaminated diet (Lafont et al., 1983). However, these authors did not report the effect of milk yield in their study.

The observed plateau developed later than in previous studies, in which the steady state for AFM1 was established at 24 (Frobish et al., 1986) and 76 hours (Polan et al., 1974, Diaz et al., 2004) from the initial AFB1 ingestion, but is in agreement with results reported by Battaccone et al. (2003) on sheep, in which the observed plateau condition was between day 9 and 13 from the first AFB1 ingestion. However, milk AFM1 levels in the present study were lower than previously reported maximum concentrations. Previous studies with dairy cows report a plateau at maximum AFM1 concentrations in milk (Frobish et al., 1986; Pettersson et al., 1989; Polan et al., 1974).

The AFB1 is promptly absorbed within the gastro intestinal tract of dairy cows and rapidly transferred as AFM1 into milk (Polan et al., 1974). Milking cows fed a 5 mg AFB1 bolus had detectable blood plasma AFM1 and AFB1 concentrations as soon as 15 minutes from treatment, indicating both a rapid absorption of AFB1 through the rumen wall and metabolism into AFM1 (Moschini et al., 2006). The authors in this study used the retinol palmitate plasma level as a marker for the intestinal adsorption (Bertoni et al., 2001) which indicated a probable AFB1 absorption at rumen level and an intestinal contribution to the AFM1 plasma level 120 minutes after drenching.

The adsorption and consequent transfer to blood and biological fluids is by passive diffusion of the polar component into the liquid phase and by diffusion or active transport of the non polar component into the lipid phase. Because of their low molecular weight (AFB1 = 312.27 and AFM1 = 328.27 formula weight), the toxins are rapidly adsorbed through membranes by a passive mechanism (Yiannikouris and Jouany, 2002). Upon adsorption, the body's ability for AFB1 detoxification is associated with the action of the liver microsomal cytochrome P-450 enzyme family and the enzyme S-glutation-transferase (Galtier, 1999). This system is effective within 7 and 351 µg/head/day (Munksgaard et al., 1987). The level of AFB1 being used in our trial was lower than 80 µg/kg body weight, a threshold value after which a decrease of feed intake was observed in calves (Lynch et al., 1971).

Milk yield was decreased when feeding 100 µg/kg AFB1 (Patterson and Anderson, 1982), and a considerable milk yield reduction was observed in cows fed 100 and 300 µg AFB1/kg body weight (B.W.) (Mertens and Wyatt, 1977). Similar results were obtained by (Applebaum et al., 1982). The level of AFB1 contamination used in our trial (0.16 µg/kg B.W.) was lower than the

indicated threshold value of 100 μg/kg B.W. for milk yield depression and no changes in milk yield pattern were observed during the AFB1 ingestion period, as would be expected (figure 12-2).

Several factors could affect aflatoxin CO. The variability observed among animals could be related to differences in rumen degradation activity (Westlake et al., 1989), difference in rumen biotransformation to aflatoxicol 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 differences in mammary gland permeability (Lafont et al., 1983).

Milk AFM1 content has been previously related to SCC (Lafont et al., 1983). However, more recent work suggests that milk yield is the main factor contributing to the total AFM1 excretion (Pettersson et al., 1989; Veldman et al., 1992). In our trial, the total AFM1 excretion and the CO in milk were affected by the milk yield and not by the SCC during the plateau period (table 12-2). This data suggest a higher AFM1 excretion in high milk yield cows compared to low milk yield cows.

Under the conditions of this experiment, in which all cows received AFB1, and the levels of AFB1 exposure, the relationship between CO (%) and milk yield (kg) can be described by the following (figure 12-3):

CO = - 0.326 + 0.077 x milk yield
RSD = 0.692
R2=0.58

The distribution of the residuals outlines the absence of any bias related to the milk yield on CO estimate. On average, the high milk yielding cows had a 1.81 fold increase in the CO to milk, which is in agreement with previous reported data in early and late lactating dairy cows (Lafont et al., 1983; Veldman et al., 1992). The inappropriate implementation of equations relating CO and milk yield could lead to erroneous conclusions in terms of maximum AFB1 daily intake to comply with the EU limit of AFM1 in milk. For instance, the plotting of the estimated CO obtained when applying the Veldman et al. (1992) equation to our milk yield against values calculated with our equation clearly indicate the presence of factors not accounted for (source of contamination, animal variability, etc..) in both equations which limits the equations to their respective trials for CO estimates (figure 12-4). A simple steady-state model has been proposed for the disposition of AFB1 and AFM1 in the lactating cow (Van Eijkeren et al., 2006). The model attempts to better define kinetics of carry over of AFB1 in feed to AFM1 in milk. However, the application of the model to our data did not fit: while daily intake of AFB1 was similar among different milk yielding groups

(LY, HY), the calculated AFM1 concentrations were 1.3 and 0.9-folds the corresponding observed mean levels, respectively for the LY and HY groups.

Mastitis as measured by high SCC could cause disruption of the tight junction of alveolar cell membranes in the mammary gland. Because of this reduction in the integrity of the blood-udder barrier, an influx of pro-inflammatory factors might further disrupt the tight junction and increased blood-udder permeability (Davis et al., 1999). Similar results were observed in sheep where distended udders were related to the pro-inflammatory factors have been found in the milk of sheep under similar circumstances (Colditz, 1988). Furthermore, anti-inflammatory factors from hyper-immunised cows reduced the cell membrane tight junction permeability (Stelwagen et al., 1997).

The animal arrangement for the SCC content in our trial obtained groups (LY-LSCC, HY-LSCC) with average SCC below or slightly over 100000/mL, which is considered a threshold value for a healthy udder (Steiner et al., 1990; Walstra and Jenness, 1984), and groups considerably higher in SCC (LY-HSCC, HY-HSCC) in which the integrity of tight junction were probably damaged allowing leaking of blood and milk components (Bruckmaier et al., 2004).

Thus, factors affecting the permeability of the blood-udder barrier, together with the low AFB1 molecular weight (312.27 formula weight), could regulate the excretion of AFM1 into milk, particularly in high producing dairy cows. From our data the increased mammary gland permeability as a consequence of inflammatory processes alone does not seem to explain the increase of the CO (table 12-2).

As previously reported (Diaz et al., 2004; Frobish et al., 1986), the AFM1 clearance at the end of the AFB1 ingestion period was fast, bringing the AFM1 below the legislative limit (50 ng/L) within 24 hours (all groups) and lower than 15ng/L (low somatic cells groups) within 48 hours from last day of ingestion.

The AFB1 ingestion period did induce changes in some of the evaluated plasma parameters (table 12-3). Even though the change in the Gamma-glutamyl transferase might suggest a damage of the liver at cellular level, the values for cows in this study were within the upper limit for cows at their stage of lactation (Bertoni et al., 2000).

12.6 Conclusions

The current data suggest that milk yield is the major factor affecting the total excretion of AFM1. In this study the CO calculated from a predictive equation was lower than previously reported for similar levels of AFB1 intake, although not outside the range. Previously reported differences in CO associated to membrane permeability due to inflammatory factors was only

detectable during the first days of AFB1 ingestion and only occurred in the high yielding cows on experiment. It is possible that the low molecular weight of aflatoxins could account for the absence of the SCC effect at plateau conditions.

Table 12-1. Ingredients and chemical composition of the basal diet

	Base diet
Ingredients (g/kg dry matter)	
Corn silage	312
Alfalfa hay, dehydrate	167
Grass hay	41
Cotton seed, whole with lint	85
Corn meal	183
Barley meal	66
Protein supplement[1]	103
Calcium soap[2]	9
Soybean meal	34
Chemical composition (g/kg dry matter)	
Crude Protein	162
Crude lipids	48.6
ADF[3]	204
NDF[4]	340
Calculated	
PeNDF[5]	266.8
NSC[6]	411.8
Forage (%)	52
Net energy lactation (MJ[7]/kg DM)	7.08

[1]Contains per kg of premix: Soybean meal 600 g, sunflower meal 300 g, mineral and vitamin supplement 100 g.; 120000 IU of Vitamin A; 9000 IU of Vitamin D3; 90 mg of Vitamin E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

[2]Megalac;

[3]expressed without residual ash.

[4]according to Van Soest et al. (1991) without sodium sulfite and with alpha-amylase; expressed without residual ash.

[5]PeNDF: Physical effective neutral detergent fiber (Mertens, 1997), calculated according to the contribution of the single feed present into the diet (concentrates were considered with PeNDF=0; whole cotton seeds PeNDF=70)

[6] Calculate as NSC= 100-(NDF+ASH+Crude protein+Crude Lipids)

[7]according to NRC, 2001

Table 12-2. AFB1 intake (µg), AFM1 milk concentration (ng/L) and total excretion (µg/cow/day) and carry-over (%) at plateau (7th – 12th day on AFB1 ingestion)

Item	Groups				s.e.	Main effects ($P <$)		
	HY-HSCC	HY-LSCC	LY-HSCC	LY-LSCC		Milk Yield	SCC	Milk Yield x SCC
AFB1 intake (µg)	98.0	98.3	98.0	98.1	0.064	0.455	0.007	0.141
AFM1 (ng/L)	65.8	61.9	66.7	59.2	4.908	0.915	0.500	0.832
Total AFM1 excreted (µg/cow/day)	2.27	2.66	1.45	1.27	0.177	0.001	0.756	0.360
Carry over (%)	2.32	2.70	1.48	1.29	0.180	0.001	0.767	0.366

Table 12-3. Blood parameters for cows with low somatic cell counts (LY-LSCC & HY-LSCC) before and after AFB1 ingestion

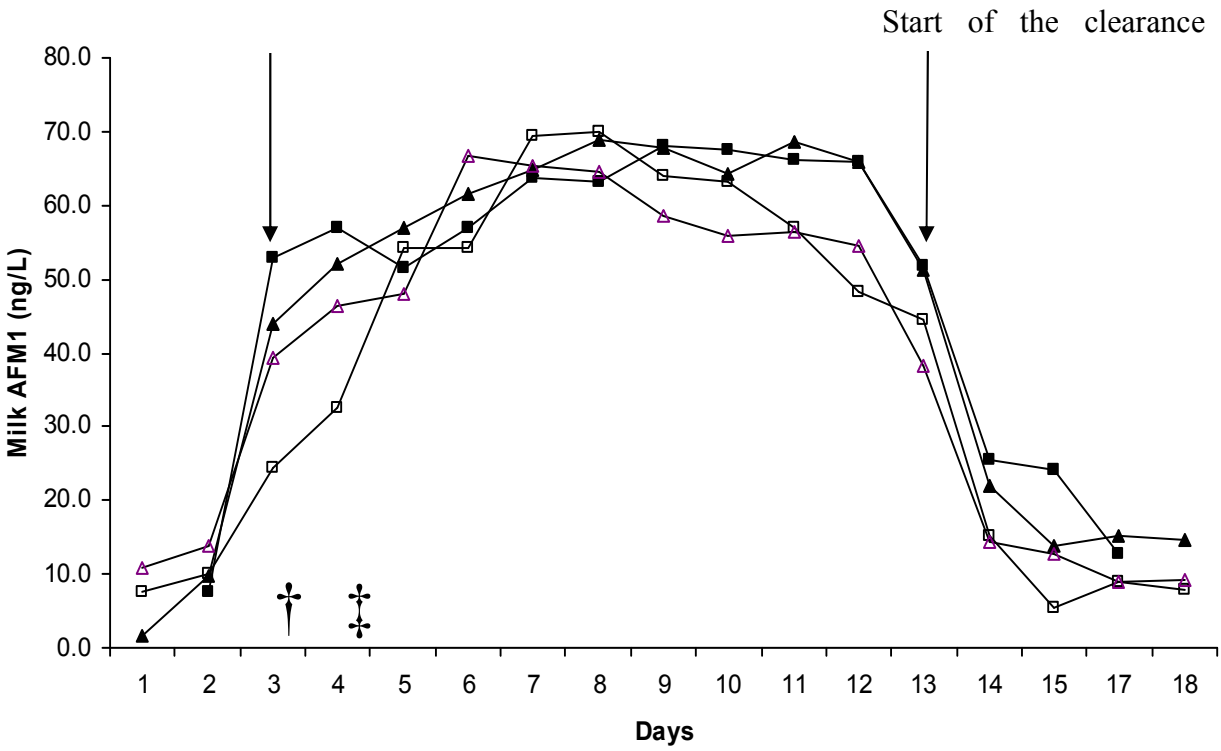
Parameter	Before ingestion	After ingestion	s.e.	P[1]	Range[2]
Albumin (g/L)	33.75	37.50	0.871	0.0012	32.3-35.9
Globulin (g/L)	33.83	38.50	1.469	0.0088	38.3-57.6
Aspartate aminotrasnferase (U/L)	63.17	68.25	3.903	0.2194	61.1-103
Gamma-glutamyl transferase (U/L)	26.58	29.58	0.739	0.0019	20.8-45.1
Bilirubin (µmol/L)	2.78	2.56	0.272	0.4261	1.63-4.58
Beta-hydroxybutyrate (mmol/L)	0.51	0.46	0.082	0.5357	0.16-0.75

[1]Cows consuming a 300g bolus containing 1.004±0.03 g/cow per day of a naturally contaminated corn meal. This allowed for an AFB1 intake from the contaminated bolus of 98.10±0.26 µg/cow per day

[2]For difference being different than zero

[3] Bertoni, G., 1999

Figure 12-1 AFM1 concentration in the milk of cows from different factorial arrangement: (■) HYHSCC, (□) HYLSCC, (▲) LYHSCC, (Δ) LYLSCC



†Somatic cells count effect ($P < 0.01$). Production and somatic cells count interaction effect sliced for the level of production significant ($P < 0.01$) in the HY group

‡Somatic cells count effect ($P < 0.05$). Production and somatic cells count interaction effect sliced for the level of production significant ($P < 0.05$) in the HY group

Figure 12-2. Milk yield of cows from different factorial arrangement: (■) HYHSCC, (□) HYLSCC, (▲) LYHSCC, (Δ) LYLSCC

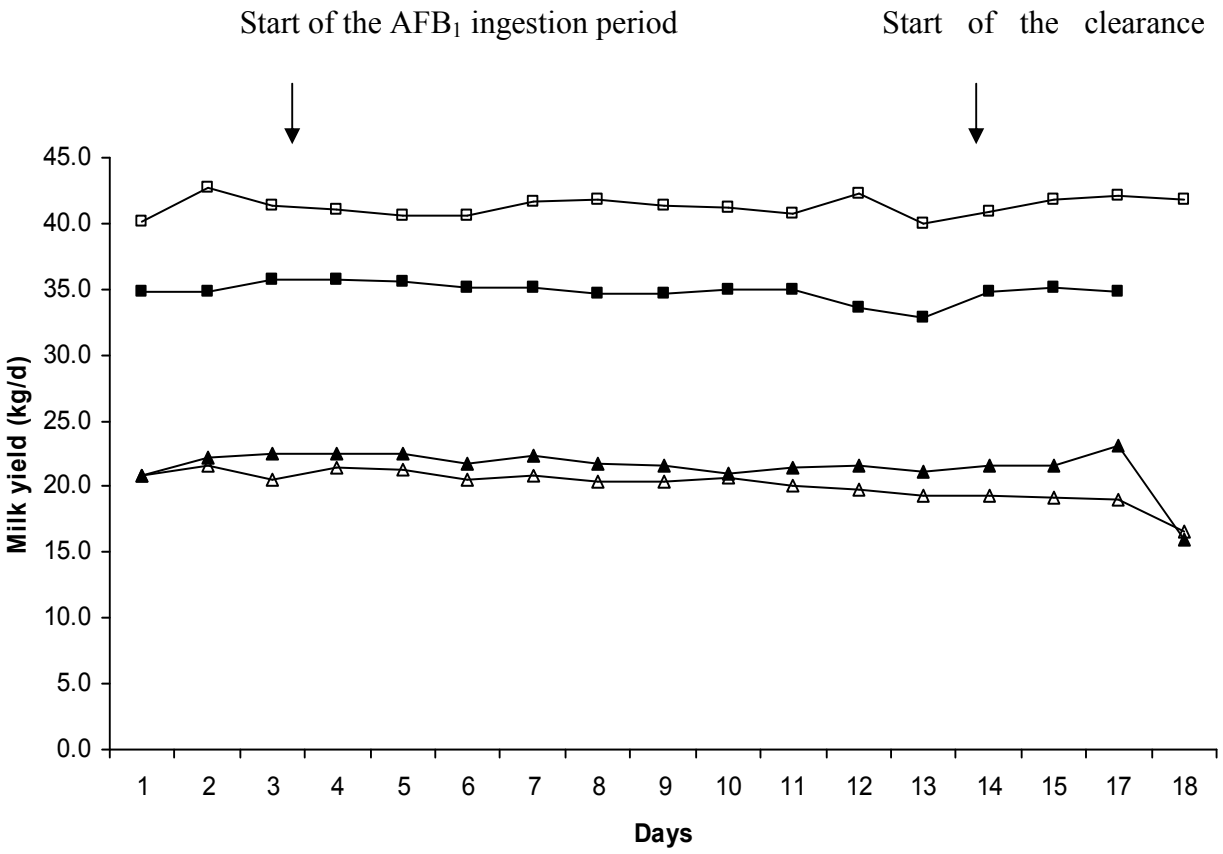


Figure 12-3. Plot of observed (■) and residuals (□) for carry-over versus milk yield (kg)

carry-over = - 0.3255 + 0.0769 * milk yield (RSD = 0.692, r2=0.58)

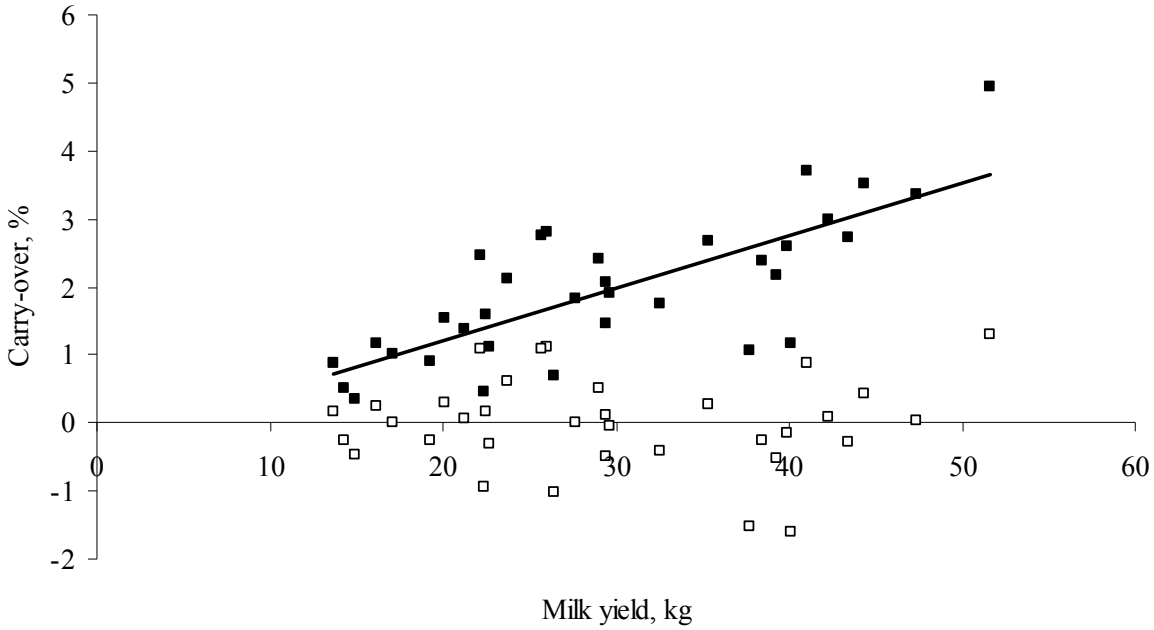
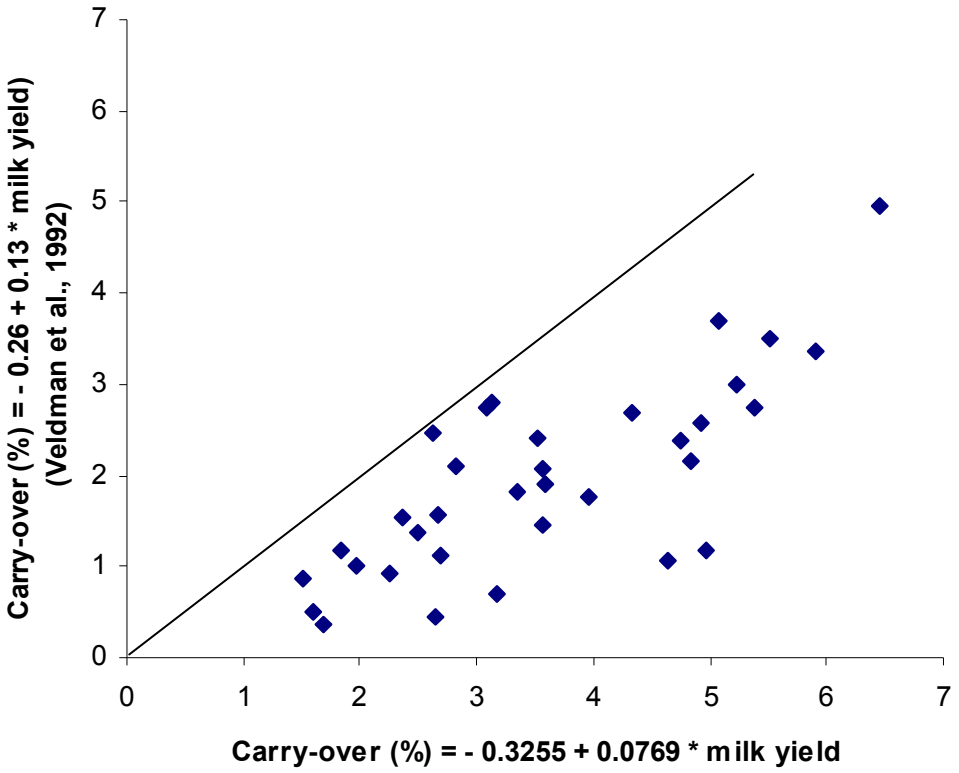


Figure 12-4. Plot of the predicted carry-over according to the obtained equation versus the predicted carry-over as proposed by Veldman et al. (1992)



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13 Effect of Rumen Fluid on in Vitro Aflatoxin Binding Capacity of Different Sequestering Agents and in Vivo Release of the Sequestered Toxin

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13.1 Abstract

Aluminosilicates (Atox® and NovasilTM Plus) and a yeast cell wall derivate (Mycosorb®) were used as sequestering agents (SAs) to verify their capacity on binding aflatoxin B1 (AFB1) *in vitro*. SAs were individually mixed at three different ratio with AFB1 (1:5000, 1:50000 and 1:500000, w/w) in water (CTR), rumen fluid of lactating cow with low rumen pH (LRS) or rumen fluid of dry cow with high rumen pH (HRS), then used in a 3x3x3 factorial arrangement of a completely randomized design. Complexes between AFB1 and SAs (AF:SA) were obtained by mixing one litre of AFB1 contaminate solutions (0.845, 0.790, 0.832 and 0.911 μg/mL) with 50 g Atox®, 150 g Mycosorb®, 50 g NovasilTM Plus and 4000 mL rumen fluid, respectively. The unbound AFB1 was eliminated from the AF:SA complexes after centrifugation and washing of precipitates. The strength of complexes were investigated *in vivo* by measuring the aflatoxin M1 (AFM1) in milk and the AFB1 recovery rate (RR) in milk as AFM1 in dairy cows fed-drench, before the morning meal, 300 mL/cow of the prepared AF:SA complex suspension for a total of 0.447, 0.360, 0.460 and 0.367 μg/mL AFB1, respectively for Atox®, Mycosorb®, NovasilTM Plus and contaminated rumen fluid (R-SA). In in vitro condition and at the 1:500000 AF:SA ratio Atox® and NovasilTM Plus sequestered over 0.87 and 0.98 of the AFB1 in CTR and rumen solutions (LRS and HRS), respectively. The efficacy decreased when the amount of clays was reduced, with higher values ($P<0.001$) for Atox® compared to NovasilTM Plus (0.50 vs. 0.28 in CTR; 0.58 vs. 0.16 in LRS and 0.44 vs. 0.27 in HRS). Mycosorb® had a lower sequestering efficacy ($P<0.001$) in all the tested experimental conditions, with 0.34 as the maximum value obtained in the CTR solution. When the prepared AF:SA complexes were given to cows, differences ($P<0.05$) were

observed for the total AFM1 excreted with amounts (ng) of 199, 870, 2394 and 1056, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Between used aluminosilicates, Atox® had the lowest RR value compared to NovasilTM Plus (0.002 vs. 0.017; $P<0.05$), however with no difference compared to Mycosorb® and R-SA. Higher amount ($P<0.05$) of AFB1 was released from the NovasilTM Plus AF:SA complex compared to the Atox® complex. R-SA showed a sequestering activity and a low release of the sequestered AFB1 from the AF:SA complex.

Keywords: *Aflatoxin B1; Aflatoxin M1; Dairy cows; Milk; Sequestering agents*

Abbreviations: ADFom, acid detergent fiber exclusive of residual ash; AFB1, aflatoxin B1; AFM1, aflatoxin M1; AF, Aflatoxin; CO, carry-over; CP, crude protein; CTR, water solution; DM, dry matter; F:C, forage to concentrate ratio; HRS, high rumen pH; HSCAS, hydrated sodium calcium aluminosilicates; LRS, low rumen pH; aNDFom, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash; NSC, nonstructural carbohydrates; PBS, phosphate-buffered saline; PeNDF, physical effective neutral detergent fibre; RR, recovery rate; R-SA, contaminated rumen; SA, sequestering agent; S.D., standard deviation; S.E.M., standard error of the mean; TMR, total mixed ratio; VFA, volatile fatty acid;

13.2 Introduction

Aflatoxins (AFs) are secondary metabolites produced primarily by Aspergillus flavus and A. parasiticus and are common contaminants of corn (Zea mais), cotton and peanuts and their by products, either in field or storage conditions. Aspergillus spp. have been described as ubiquitous (Scheidegger and Payne, 2003) and were isolated from all latitudes, however, higher frequencies were described in desert climates and at latitudes ranging from 16 to 35°, in tropical, subtropical and warm temperate climates (Klich et al., 1992).

The aflatoxins, as a group (aflatoxin B1, B2, G1, G2 and M1), are classified as group 1 carcinogens (IARC, 2002). Because of its lipophilic proprieties and low molecular weight, once ingested, aflatoxin B1 (AFB1) was rapidly adsorbed through the rumen wall and intestine (Moschini et al., 2007) by a non-described passive mechanism (Yiannikouris and Jouany, 2002) and quickly appeared, also as the metabolite aflatoxin M1 (AFM1), in blood just after 15 minutes (Moschini et al., 2007) and in milk at the first milking (Diaz et al., 2004) after intake of aflatoxin contaminated feeds. Efforts have been put in trying to understand major metabolites pathways (figure 13-1) of production and excretion following AFB1 intake.

The AFM1 excretion in milk occurred for several days after a single AFB1 dose (Battacone et al., 2003) with a carry-over (CO) rate even higher then 0.03 in dairy cows (Diaz et al., 2004). The excretion was affected mainly by milk yield and also by a different AFB1 liver metabolism in early and late lactation (Veldman et al., 1992; Van Eijkeren et al., 2006). The maximum allowed concentration of AFB1 in dairy animal feeds and concentrates is 5 μg/kg in the European Union (EC, 2003a). The concentration of AFM1 in milk is limited to 0.05 μg/kg in the European Union (EC, 2006) and 0.5 μg/kg in the US (Berg, 2003).

Within the food chain, the best way to avoid the risk of mycotoxicosis is the reduction of the mycotoxin production controlling harvesting conditions, grain maturity etc. (Huwig et al., 2001). Biological, chemical and physical strategies have been also developed to preserve feeds from post-harvest mycotoxins contamination.

The utilization of sequestering agents (SAs) capable of reducing the free toxin available for gastro-intestinal absorption is widely used for counteracting the biological negative effects of aflatoxin in diets. Huwig et al. (2001) summarized the binding capacity and affinity of several binders for AFs. Among most studied binders were smectite clay like hydrated sodium calcium aluminosilicates (HSCAS) (Phillips et al., 1990; Harvey et al., 1991), montmorillonites (Ramos and Hernandez, 1996), bentonite (Shell et al., 2000), zeolite (Piva, 1995); also activated carbons (Diaz et al., 2003) and yeast cell walls esterified glucomannans (Aravind et al., 2003; Karaman et al., 2005) had been used. The sequestering capacity of smectite clay are through electric elementary charges between the beta-carbonil portion of the aflatoxin molecule to sites containing aluminion ions (Phillips et al., 1990) or through hydrogen and van der Waals bonds between the single helix of β-D-glucans in yeast cell walls and the lactone groups of AFB1 (Yiannikouris et al., 2005; Jouany, 2007).

Several *in vitro* methods have been proposed to screen different SAs, however results were not always comparable to the *in vivo* responses having the pH, AFB1:sequestering agent (AF:SA) ratio, temperature (Ramos and Hermandez, 1996; Grant and Phillips, 1998; Lemke et al., 2001) and biological fluid (Ledoux and Rottinghaus, 1999) as important affecting factors. Differences of published *in vitro* works were: kind of AFs (AFB1 from standard or extracted with a methanol solution from natural aflatoxin contaminated feeds), dilution factor (AF:Volume ratio, μg/mL), AF:SA ratio, pH conditions. Also, authors did not consider the possible competition between AFs and other biological molecules for binding sites of SAs in the gastro-intestinal tract.

Even though works have been published about effects of SAs using the monogastrics and polygastrics models (Lemke et al., 2001; Spotti et al., 2005), there are few information about the adsorption mechanism and fate of the AF:SA complex in ruminants.

The objectives of the work were to compare the sequestering capacity, in water or in rumen fluid, of two aluminosilicates and a derived yeast cell walls esterified glucomannan, used at different AF:SA ratios. Also, the strength of the AF:SA complex was investigated *in vivo* by measuring the appearance of AFM1 into milk.

13.3 Materials and methods

13.3.1 AFB1 contaminated corn meal

The AFB1 contaminated corn meal was obtained after inoculation with Aspergillus flavus type strain MPVP 2092 (Istituto di Entomologia e Patologia Vegetale, Università Cattolica del Sacro Cuore, Piacenza, Italy). The AFB1 was produced at 25°C and 0.99 water activity (Giorni et al., 2007). The final AFB1 contamination of the corn meal was 82.21±0.01 mg/kg.

13.3.2 In vitro experimental design

Three commercial binders (Atox®, Grupo Tolsa, Madrid, Spain; NovasilTM Plus, Trouw Nutrition International, Verona, Italy; Mycosorb®, Alltech Italy, Bologna, Italy) were used to evaluate the *in vitro* AFB1 sequestering capacity. The Atox® and NovasilTM Plus products are sold (EC, 2003b) in the sub-classification "Binders, anti-caking agents and coagulants" whereas the Mycosorb® is sold as raw material for animal use. Binders, from now on identified as SA, were individually mixed at three different ratio with AFB1 (1:5000, 1:50000 and 1:500000, w/w) in water (CTR), rumen fluid of lactating cow with low rumen pH (LRS) or rumen fluid of dry cow with high rumen pH (HRS), then used in a 3x3x3 factorial arrangement of a completely randomized design. The rumen liquors were taken from two rumen fistulated cows three hours after the morning meal, filtered with a three layers cheese cloth, then stored at 39°C in anaerobic conditions.

Preparation of AFB1 solution

The AFB1 was extracted from a natural contaminated corn meal (82.21±0.01 mg/kg) using a water/methanol solution (20:80 v/v) in ratio of 1:100 (g:mL), at room temperature and light agitation (150 shake/min) for 120 minutes. The obtained concentration was 0.821 µg/mL of AFB1 (solution A).

Ten mL of the solution A were added to 2000 mL of water (CTR) or rumen fluids (LRS or HRS) and stirred for ten minutes to obtain the basal contaminated solutions (solutions B: calculated

AFB1 concentration of 4.1 µg/L). Solutions B were centrifuged (3500 g for 15 minutes), then supernatants were sampled for AFB1 (CTR, LRS and HRS) and aflatoxicol (LRS and HRS) analysis. AFB1 in precipitates were extracted into 10 mL chloroform and evaporated to dryness under nitrogen before being recovered into 1 mL acetonitrile:water solution (25:75 v/v) for HPLC analysis.

Then, twenty-seven 50 mL sub samples/solution were poured into a 150 mL glass beakers with a pre-weighted SA, three replicates for each SA and level (1:5000, 1:50000 and 1:500000, w/w). Samples were incubated at 39°C for one hour under gentle shake of five minutes every 15 minutes, then three 10 mL sub-samples were obtained for each beaker, cooled down at 4°C to stop any possible fermentation activity and centrifuged at 3500 g for 15 minutes.

The supernatant was recovered and the precipitate was suspended into 10 mL water before centrifugation at 3500 g for 15 minutes. The step was cycled three times and the recovered supernatant as whole was analyzed by HPLC for AFB1 content.

The AFB1 sequestered by SA in each sub-sample was obtained by difference to one hundred of the percentage of total AFB1 recovered in the supernatant over the AFB1 found in the solution B (water or rumen fluids).

13.3.3 In vivo trial

Preparation of the AF:SA complex

Solutions A were prepared for each AF:SA complex as previously described in the *in vitro* trial. The obtained AFB1 concentrations were 0.845, 0.790, 0.832 and 0.911 µg/mL respectively for Atox®, Mycosorb®, NovasilTM Plus and rumen fluid.

Atox® (50 g), NovasilTM Plus (50 g), and Mycosorb® (150 g) were mixed with 4000 mL water at room temperature, stirred for 60 min to complete hydration and suspension, then added 1000 mL of solution A. Amounts of SAs used for the AF:SA complexes preparation were based on the different sequestering efficacy observed in the *in vitro* experiment (table 13-4). This was to obtain a similar amount of bound AFB1 among the AF:SA complexes, then used in the *in vivo* trial.

The contaminated rumen (R-SA) was obtained by adding 1000 mL solution A to 4000 mL rumen fluid (LRS).

Solutions were gentle stirred for two hours, then each solution was divided into twelve fractions and centrifuged at 4000 g for 15 minutes. The supernatant was separated, measured and stored at 5°C until HPLC analysis for AFB1 content. The precipitate of each centrifugation tube was washed with 200 mL of distilled water, centrifuged and the supernatant recovered and analyzed

for AFB1 content. The washing was cycled until the supernatant AFB1 concentration was lower than 0.05 µg/L. For the twelve fractions, the number of washings needed were: five (12 L) for Atox®, seven (16.8 L) for NovasilTM Plus, 15 (36 L) for Mycosorb® and 16 (38.4 L) for the R-SA. Then, residues were pooled by solution and re-suspended into 1800 mL water (37°C) before animal drench (300 mL/cow).

13.3.4 Animals

The trial was carried out at the CERZOO research and experimental center (San Bonico, Piacenza, Italy). The research protocol and animal care was in accordance with the EC council directive guidelines for animals used for experimental and other scientific purposes (EEC, 1986).

The rumen liquor at low pH for the *in vitro* experiment was collected from a fistulated cow fed the same total mixed ratio (TMR) used in the *in vivo* experiment (LRS). A dry fistulated cow fed a TMR based on grass hay (700 g/kg), corn silage (200 g/kg) and concentrate (100 g/kg) on a dry matter (DM) basis was used as the high rumen pH liquor donor (HRS).

Twenty-four Holstein lactating cows were used in the *in vivo* experiment. Cows were housed in a free stall, had free access to water and were fed a TMR (table 13-1) formulated according to the nutrient requirements of dairy cattle (NRC, 2001) for an average cow weight of 600 kg, 140 days in milk and 33 kg milk yield (38.0 g/kg fat and 33.5 g/kg protein). The bulk of the diet on a DM basis was: corn silage (312 g/kg), alfalfa hay (167 g/kg), grass hay (41 g/kg) and energy-protein supplement (480 g/kg).

The diet was fed once a day (0800 hours) ad libitum (5% expected refusal). Cows were milked twice a day (0230 and 1330 hours) and the individual milk yield was recorded at each milking (Afimilk system, Afikim, Israel).

Animals (6 cows/treatment) were fed-drench 300 mL of the AF:SA complex suspension (0.447, 0.360, 0.460 and 0.367 µg AFB1/mL respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA) before the morning meal.

The recovery rate (RR) of AFB1 excreted in milk as AFM1 was calculated as the ratio total AFM1 in milk (sum of the AFM1 excreted in four consecutive milkings) over the total AFB1 ingested by the cow.The total AFM1 ingested was cleaned by the base diet contamination contribution. The total AFM1 excreted in milk was also cleaned of the contribution from the contamination of the base diet, estimated by the AFM1 content in milk measured in two consecutive milkings before the cow oral drench, and assumed as constant value for the four collected milkings after oral drench.

13.3.5 Milk and Feed samples

TMR samples were collected the day before treatment, dried at 55°C in a ventilated oven until constant weight, then ground with one mm sieve (Thomas-Wiley Laboratory Mill, Arthur H. Thomas Co., Philadelphia, PA) and stored for analysis.

Samples were assayed in duplicates according to AOAC (1990) for DM (procedure 930.15), crude protein (CP) (procedure 975.06), ash (procedure 942.05), crude lipids (procedure 954.02) and acid detergent fibre exclusive of residual ash (ADFom) (procedure 973.18). The neutral detergent fibre (aNDFom) assayed with a heat stable amylase and expressed exclusive of residual ash, according to Mertens (2002), without sodium sulfite and using the Ankom equipment (Ankom220, USA) for extraction and filtering.

Individual milk samples were collected at each milking and for six consecutive milkings (two before and four after treatment) for AFM1 content determination. Cumulative AFM1 excretion were individually calculated based on daily milk AFM1 concentration and milk yield for four milkings.

13.3.6 Aflatoxin Analysis

AFB1 and aflatoxicol assays

Ten grams of dried feed were extracted with 100 mL of a methanol:water solution (80:20 v/v), shacked at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher&Schuell 595 ½ filter paper (Dassel, Germany). Then, 5 mL were eluted with 45 mL of bi-distilled water and passed through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK).

One mL of the supernatant solution was added to 9 mL of phosphate-buffered saline (PBS) solution (pH 7.4) and passed throw an immunoaffinity column previously washed with 20 mL of a PBS solution.

The column was than washed with 5 mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75 v/v) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis for AFB1 (feed and supernatant) and aflatoxicol (supernatant) contents.

AFM1 assay in milk samples

The extraction was by immunoaffinity technique according to Mortimer et al. (1987). Fifty mL of defatted milk (centrifuged at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher&Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a PBS solution (pH 7.4). Then columns were washed with 5 mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75 v/v) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFB1 and aflatoxicol were separated with a reverse-phase C18 Superspher column (4 μm particle size, 125 x 4mm i.d.; Merck, Darmstadt, Germany) at room temperature (18°C) and isocratic conditions, with a mobile phase of water and acetonitrile:methanol (17:29 v/v) solution (64:36 v/v). The flow rate was 1mL/min. Then, the AFB1 and aflatoxicol were detected by fluorescence, after postcolumn derivatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide perbromide (PBPB) at 0.1 mL/min flow. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths for AFB1 and 360 nm excitation and 418 nm emission wavelengths for aflatoxicol. The standard stock solution was checked for AFB1 and aflatoxicol concentrations according to AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, 5 μm particle size, 125 x 4 mm I.D.) at room temperature (18°C), with a water and acetonitrile (75:25 v/v) mobile phase and flow rate set at 1 mL/min. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths. The standard stock solution was checked for AFM1 concentration according to the AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

13.3.7 Statistical Analyses

The *in vitro* data were analyzed as completely randomized design using the general linear model procedure of SAS® (Statistical Analysis System Institute, 2001). A factorial arrangement was used and fixed effects in the model included SA, solution and AFs:SA ratio.

The AFB1 intake, AFM1 in milk concentration and RR were analyzed as completely randomized design using the general linear model procedure of SAS®. Fixed effects were the different preparation of AF:SA complexes and the experimental unit was the single treated animal.

Both *in vitro* and *in vivo* means were considered different for $P<0.05$.

13.4 Results

13.4.1 In vitro experiment

Analytical parameters of rumen fluids showed the predictable effects due to the forage:concentrate ratio, energy and nonstructural carbohydrates on pH (6.1 and 6.7), N-NH3 (4.23 and 8.20 mg/100mL), total volatile fatty acids (138.57 and 124.56 mmol/L) and fatty acids molar concentration respectively for LRS and HRS (table 13-2).

Recoveries of AFB1

The average AFB1 recovered from the CTR was 0.93. The average AFB1 recovered in the supernatant after centrifugation of rumen fluids solutions were 0.53 and 0.53 and the average residual AFB1 extracted with chloroform from precipitate were 0.23 and 0.25, respectively for the LRS and HRS solutions. Thus, the total average AFB1 recovery (supernatant + precipitate) were 0.76 for LRS and 0.78 for HRS (table 13-3).

None of supernatants and precipitates analyzed for aflatoxicol content had concentration values over the detectable limit (0.03 μg/L).

Binding efficacy of SA

The *in vitro* AFB1 sequestering activities of used SA in different solutions and at different AF:SA ratios are reported in table 13-4. Data analyzed as a factorial arrangement showed the effect ($P<0.01$) of SA, solution, AF:SA, and of the first (SA x AF:SA; SA x solution, solution x AF:SA) and second (SA x solution x AF:SA) order interactions.

The higher sequestering capacity was observed for Atox® and NovasilTM Plus at the 1:500000 AF:SA ratio. Over 0.87 and 0.98 of AFB1 was sequestered by the SA, respectively in water and rumen solutions. The efficacy decreased when the amount of the SA was reduced with higher values for the Atox® compared to NovasilTM Plus, independently of the solution being used (0.50 vs. 0.28 in CTR; 0.58 vs. 0.16 in LRS and 0.44 vs. 0.27 in HRS). Comparing Atox® and NovasilTM Plus at the same level of inclusion no differences between the two SAs were observed at the higher dosage (1:500000), whereas the sequestering efficiency was higher ($P<0.001$) for Atox® compared to NovasilTM Plus for the lower and intermediate level of inclusion (1:5000 and 1:50000, respectively) independently of the used media solution (water or rumen fluids).

The Mycosorb® showed a lower ($P<0.001$) AFB1 sequestering capacity compared to the two aluminosilicate SAs in all experimental conditions (table 13-4), with the maximum efficacy (0.34) in CTR solution at the higher dosage (1:500000). When Mycosorb® was incubated at the same dose in the HRS media, its sequestering capacity decreased to the 0.02 at the 1:500000 dose.

13.4.2 In vivo experiment

Animals used in the trial had a daily milk yield of 27.5 ± 2.1 kg and a DM intake of 23.3 ± 0.8 Kg (mean ± S.D.).

The TMR had a base AFB1 content of 0.14 ± 0.01 µg/kg contributing to a bulk milk AFM1 content of 15.25 ± 9.10 ng/kg before treatment (mean ± S.D.).

During the AF:SA complex preparation for the *in vivo* trial the fraction of AFB1 not bound plus the AFB1 washed were 0.05, 0.18, 0.004 and 0.28, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Data indicated a non uniform strength of the AF:SA complex between tested SAs.

Mycosorb® sequestered 0.82 of the AFB1. The high AF:SA ratio used in this trial for the preparation of the AF:SA complex could be the key to get good sequestering performances if compared to results of the *in vitro* trial in which completely different dilution and AF to SA ratio were used.

The highest AFM1 milk concentration was detected for each SA as soon as the first milking after oral drenching (8.38 ± 7.65 µg/kg for Atox®, 26.78 ± 15.20 for Mycosorb®, 85.25 ± 82.56 for Novasil™ Plus and 35.65 ± 30.90 for R-SA) (figure 13-2). Differences ($P<0.05$) were also observed for total AFM1 excreted in four milkings after drenching and RR. The amount of AFM1 excreted (ng) were 199, 870, 2394 and 1056 and RR were 0.002, 0.008, 0.017 and 0.01, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA (table 13-5).

13.5 Discussion

Dietary supplementation with SA is the most practical and the most widely studied technique to mitigate the consequences of aflatoxin exposure (Diaz and Smith, 2005).

The HSCAS, a smectite clay (Philliphs et al., 1990), was reported to form AF:SA complex which reduced the absorption of the aflatoxin across the intestinal epithelium (Dawson et al., 2001; Abdel-Wahhab et al., 2002) and prevented negative effects due to mycotoxins intake in broilers (Pimpukdee et al., 2004), in pigs (Schell et al., 2000) and in goats exposed to 100 and 200 µg/kg of AFB1 (Smith et al., 1994).

When used in dairy cows (AFB1 content ranging from 55 to 200 µg/kg in TMR) the HSCAS and yeast derivate cell walls reduced from 24 to 65% the AFM1 in milk (Harvey et al., 1991; Nelson, 1993; Diaz et al., 2004). As far as we know there are no published works reporting on NovasilTM Plus and Atox® in dairy cows.

Mycosorb®, a yeast cell wall derived glucomannan, was reported to adsorb a large range of mycotoxins (Dawson et al., 2001) and it improved the performance in broilers (Aravind et al., 2003; Karaman et al., 2005) exposed to AFB1 ranging from 168 to 2000 µg/kg and in pigs (Casteel et al., 2003) fed 500 µg AFB1/kg of diet.

The AFB1 concentration used in our *in vitro* experiment was 4.1 µg/L, a level lower than what used in previous works (Galvano et al., 1996; Lemke et al., 2001; Diaz et al., 2003; Spotti et al., 2005; Ledoux and Rottinghaus, 1999) (table 13-6) and close to the concentration that could be found in the rumen of a cow with a daily AFB1 intake between 200 and 250 µg and considering a rumen liquid phase of 50-60 L. The level used was based on our preliminary observation of rumen AFB1 concentration on a dry cow at one (4.07 µg/kg) and three (5.91 µg/kg) hours after feeding a bolus containing 200 µg AFB1.

The amount of the SA used ranged between the on farm suggested dose as being effective (1:500000) and the suggested level for *in vitro* study (1:5000) as from Ledoux and Rottinghaus (1999). Compared to previous works (table 13-6), our *in vitro* experimental conditions were different particularly for the dilution factor (AF:Volume ratio, µg/mL), source of AFB1 (standard solutions or extracted from a natural contaminated feed), AF:SA ratios and type of the media solution (water, digestive enzyme solutions, intestinal or ruminal fluids).

The two aluminosilicates obtained in our trial a sequestering capacity higher than 0.80, a threshold value suggested by Ledoux and Rottinghaus (1999) to classify a SAs as efficient *in vitro*.

Similar results were reported by Phillips et al. (1988) using HSCAS and in which the chemisorptions of AFs to HSCAS involved the formation of a complex by the β-keto-lactone or bilactone system of aflatoxin with uncoordinated metal ions in HSCAS (Phillips et al., 1990).

Authors reported the maximum AFB1 binding capacity of one mg of HSCAS ranged from 200 to 332 nanomoles (63 to 105 µg), without effects of temperature and pH experimental conditions.

In vitro studies indicated that cell walls of several probiotic bacteria (i.e. lactobacilli) living in the intestine can bind AFB1 (Peltonen et al., 2000; Oatley et al. 2000; Gratz et al., 2005). This binding reduced the free AFB1 in the gastro-intestinal tract, delaying but not preventing the intestinal AFB1 absorption (Gratz et al., 2005).

The chlorophyllin, a water-soluble derivative of chlorophyll, was also effective in reducing the toxicity associated to AFs (Dashwood et al., 1998; Atroshi et al., 2002). Indeed, a chlorophyllin-AFs complex was actually the main mechanism responsible for chemoprotection (Breinholt et al., 1999).

Thus, rumen liquor intrinsic factors could justify the lower AFB1 recovery we observed in the supernatant after centrifugation of rumen fluids solutions and in the residual AFB1 extracted with chloroform from precipitate. Similar results on rumen solution were previously reported by Spotti et al. (2005). However, our results on aflatoxicol content after one hour incubation in rumen fluids should not exclude the possible presence of other AFB1 metabolites.

The yeast cell wall derivate (Mycosorb®) had a very low *in vitro* efficiency in all tested conditions (table 13-4). The pH and the kind of the solvent can reduce the ability of beta-D-glucans, a major component of the inner layer of the yeast cell wall, to complex mycotoxins, indicating the involvement of non-covalent binds (adsorption) rather than a real binding type (Yiannikouris et al., 2005). Indeed, a previous work by Dawson et al. (2001) suggested a pH of four as optimal for Mycosorb® activity along with 0.5 M phosphate, conditions that can be found in the gastro-intestinal tract of monogastric animals but not in the rumen. This specific SA itself had a slight acidifying property as observed during the AF:SA complex preparation (pH after binder mixing: 4.6). Thus, the higher pH values observed in the LRS and HRS solutions along with the considerable buffer capacity of the rumen liquor did probably counteract the acidifying property of Mycosorb® resulting in a negative environment for its specific sequestering activity, which lowered or zeroed (HRS solution) the binding efficiency. It has been reported that up to 90% adsorption of aflatoxins to yeast cells is dose-dependent in *in vitro* condition (Devegowda et al., 1994).

The efficacy of Mycosorb® was reported to be higher compared to the clay-based binders when used at low mycotoxin concentration, whereas the binding capacity was greater at high mycotoxin concentration (Dawson et al., 2001). Authors reported also an higher Mycosorb® efficiency with aflatoxin concentration ranging from 2 to 10 µg/mL at 37°C.

Concentrations used in our trial were lower than the above mentioned values and the binding efficiency of Mycosorb® was considerably low (table 13-4), not as high as the sequestering efficiency observed for Atox® and NovasilTM Plus, also when implemented at higher dosages.

Results of the *in vitro* experiment were partially contradicted by the *in vivo* data. In facts, NovasilTM Plus was less effective ($P<0.05$) in keeping bound the AFB1 in the gastro-intestinal tract compared to Atox®. Even though working in controlled environment, the *in vitro* operating conditions is still an attempt to resemble the real gastro-intestinal tract conditions.

The calculated RR value of bound AFB1 to AFM1 excreted in milk were 0.002 for Atox®, 0.008 for Mycosorb®, 0.017 for NovasilTM Plus and 0.01 for Rumen-SA. Animals were given 134.0, 108.0, 138.1 and 109.9 µg of bound AFB1, respectively for Atox®, Mycosorb®, NovasilTM Plus and Rumen-SA.

When assuming a CO rate of 0.03, an expected value in high yielding dairy cows (Veldman et al., 1992), based on the amount of AFM1 recovered in milk in our experiment we can estimate the fraction of AFB1 released from the complex drenched to animals. Calculated values were 0.05, 0.27, 0.58 and 0.30, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Data indicated differences in terms of bound strength within the AF:SA complexes used.

Therefore, data suggested an evidence of a labile AF:SA sequestering capacity which can lead to a release of some of the bound AFB1 in the gastro-intestinal tract, which was not supported by a previous work in different species (Phillips, 1990). However, Watts et al. (2003) did not find an effective prevention of HSCAS in chicks, suggesting a lack of efficacy of HSCAS against other mycotoxins.

Thus, more specific knowledge about the chemical and physical characteristics of Atox® and Novasil™Plus are needed to get additional answers about observed differences between *in vivo* and *in vitro* efficacies.

Up to now, no works have been published on this topic in cows and, as emphasize by Diaz and Smith (2005), a two step *in vitro* procedure must be used to correctly evaluate the efficacy of a SA: the initial sequestration (weak binding) and the desorption (strong binding). Chemical solvent extraction, like methanol or chloroform, have been used by several authors (Ramos-Girona and Gimenez, 1997; Kannewischer et al., 2006) to evaluate the strong binding.

The EU limit of AFB1 in dairy animal feeds refers to maximum allowed concentration (20 µg/kg for animal feeds and 5 µg/kg for concentrates) of the mycotoxin in marketed feeds, the same is true for the milk limit (0.05 µg/kg). However, there is no limit in terms of maximum intake of AFB1 by the animal if not the ultimate concentration of the produced milk. Thus, even feeding on regularly marketed feeds, in particular condition an high yielding dairy cow could easily intake over

40 µg AFB1/day, a value previously reported (Veldman et al., 1992) as the maximum amount for complying with the milk EU limit. Even though AFB1 CO modelling (Van Eijkeren et al., 2006) reports the effectiveness of the EU limits in preventing excessive levels of AFM1 in milk, high intake of AFB1 could justify the implement of effective SAs as a tool for complying with the AFM1 milk EU limit.

13.6 Conclusions

The *in vitro* efficiency of used SAs was related to the AF:SA ratio. There was a synergy between rumen fluid and SA on the reduction of AFB1 absorption in the gastro intestinal tract.

The discrepancy between the *in vitro* and the *in vivo* results outlines the need of further research on AF:SA complex behavior in the gastro intestinal tract.

Results suggest an higher level of inclusion for Novasil™ Plus or Mycosorb® to obtain a binding and a release efficiency similar to the Atox® product.

Table 13-1. Forage to concentrate ratio (F:C), composition (g/kg dry matter) and chemical composition (g/kg dry matter) of experimental diets fed to lactating (LRS) and dry cows (HRS)

Item	Lactating cows (LRS)	Dry cows (HRS)
F:C	40:60	80:20
Corn silage	312	200
Alfalfa hay, dehydrate	167	-
Grass hay	41	700
Cotton seed, whole with lint	85	-
Corn meal	183	30
Barley meal	66	30
Protein supplement[1]	103	20
Calcium soap[2]	9	-
Soybean meal	34	20
Chemical composition		
Crude protein	162	125
Crude lipids	48.6	25.2
ADFom[3]	204	320
aNDFom[4]	340	440
Calculated		
PeNDF[5]	266.8	405
NSC[6]	411.8	225

[1]Contains per kg of premix: Soybean meal 600 g, Sunflower meal 300 g, mineral and vitamin supplement 100 g.; 120000 IU of Vitamin A; 9000 IU of Vitamin D3; 90 mg of Vitamin E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

[2]Megalac;

[3]ADFom: acid detergent fibre expressed exclusive of residual ash.

[4]aNDFom: assayed with a heat stable amylase and expressed exclusive of residual ash, according to Mertens (2002), without sodium sulfite.

[5]PeNDF: Physical effective neutral detergent fibre (Mertens, 1997) calculated according to the contribution of the single feed present into the diet (concentrates were considered with PeNDF=0; whole cotton seeds PeNDF=70)

[6]NSC (nonstructural carbohydrates)= 100-(NDF+Ash+Crude protein+Crude lipids)

Table 13-2. Rumen pH, ammonia nitrogen ($N\text{-}NH_3$), volatile fatty acids (VFA), acetic/propionic and acetic+butyric/propionic molar ratios of rumen fluids from lactating (LRS) and dry (HRS) cows

Item	LRS	HRS
Rumen pH	6.10	6.70
$N\text{-}NH_3$ (mg/100 ml)	4.23	8.20
Acetic acid (mmol/L)	99.68	93.12
Propionic acid (mmol/L)	23.43	21.69
Butyric acid (mmol/L)	12.31	7.93
Total VFA (mmol/L)	138.57	124.56
Acetic/Propionic	4.25	4.29
(Acetic+Butyric)/Propionic	4.68	4.66

Table 13-3. Recoveries (mean ± S.D.) of aflatoxin B1 (AFB1) in supernatant, in rumen pellet and as total in water (CTR) and rumen fluids from lactating (LRS) and dry (HRS) cows

Solution	Recovery		
	Supernatant	Rumen pellet	Total
CTR	0.93 ± 0.05	-	0.93 ± 0.05
LRS	0.53 ± 0.03	0.23 ± 0.01	0.76 ± 0.02
HRS	0.53 ± 0.06	0.25 ± 0.02	0.78 ± 0.03

Table 13-4. *In vitro* sequestering efficiency of Atox®, Novasil™ plus and Mycosorb® used at several ratio with aflatoxin B1 (AF:SA) in water (CTR) and rumen fluids from lactating (LRS) or dry (HRS) cows

Solution	AF:SA	Sequestering Agent			S.E.M.[1]	Main effect (P)		First order interaction (P)				Second order interaction (P)
		Atox ®	Novasil™ plus	Mycosorb®		SA[2]	Sol[3]	AF:SA	SA x Sol	SA x AF:SA	Sol x AF:SA	SA x Sol x AF:SA
CTR	1:5000	0.50	0.28	0.09	0.02	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.006	< 0.001
	1:50000	0.66	0.53	0.34								
	1:500000	0.88	0.80	0.36								
LRS	1:5000	0.58	0.16	0.15								
	1:50000	0.86	0.70	0.17								
	1:500000	1.00	0.98	0.21								
HRS	1:5000	0.44	0.27	0.00								
	1:50000	0.90	0.78	0.00								
	1:500000	0.99	0.99	0.02								

[1]S.E.M.: standard error of the mean.

[2]SA: sequestering agent.

[3]Sol: solution.

Table 13-5. Milk yield, aflatoxin B1 (AFB1) ingested, cumulative aflatoxin M1 (AFM1) excreted in four milkings and recovery rate (RR) for Atox®, Mycosorb®, Novasil™ Plus and contaminated rumen fluid (R-SA) (n=6)

Item	Atox®	Mycosorb®	Novasil™ Plus	R-SA	S.E.M.[1]
Milk yield[2] (kg/day)	27.9	27.0	27.8	27.2	0.216
AFB1 ingested (µg/cow)	134.0	108.0	138.1	109.9	-
AFM1 excreted in 4 milkings (ng/cow)	199[a]	870[a]	2394[b]	1056[ab]	455
RR	0.002[a]	0.008[ab]	0.017[b]	0.010[ab]	0.348

[1]S.E.M.: standard error of the mean.

[2]P of the model not significant.

[a, b] Means within a row with different letters differ significantly (P<0.05).

Table 13-6. *In vitro* aflatoxin:sequestering agent (SA) ratio g/g (AF:SA), volume (mL) and dilution factor (µg/mL) reported by different authors and in our xperimental condition (present work)

Authors		AF:SA ratio (g/g)	Volume (ml)	Dilution (ug/mL)
Present work		1:5000 1:50000 1:500000	50	0.004
Spotti et al. (2005)		1: 250000	1	0.1
Ledoux and Rottinghaus (2005)		1: 5000	10	2
Lemke et al. (2001)	single-concentration absorption study	1:25	5	8
	Chemisorption index	1:1250	5	8
	GI model studies	1:312.5	40	10
	Modified Isotherm studies	1:2551	5	7.84
Diaz et al. (2002)		1:2000	1	5
Galvano et al. (1996)	test 1	1:10000	1	10
	test 2,3 and 4	from 1:40 to 1:500	5	from 0.1 to 1.25

Figure 13-1. Biotrasformation pathways in liver of absorbed aflatoxin B1 (AFB1): detoxification (continuous line) and toxic or carcinogenic (dashed line) processes (adapted from Yiannikouris and Jouany, 2002)

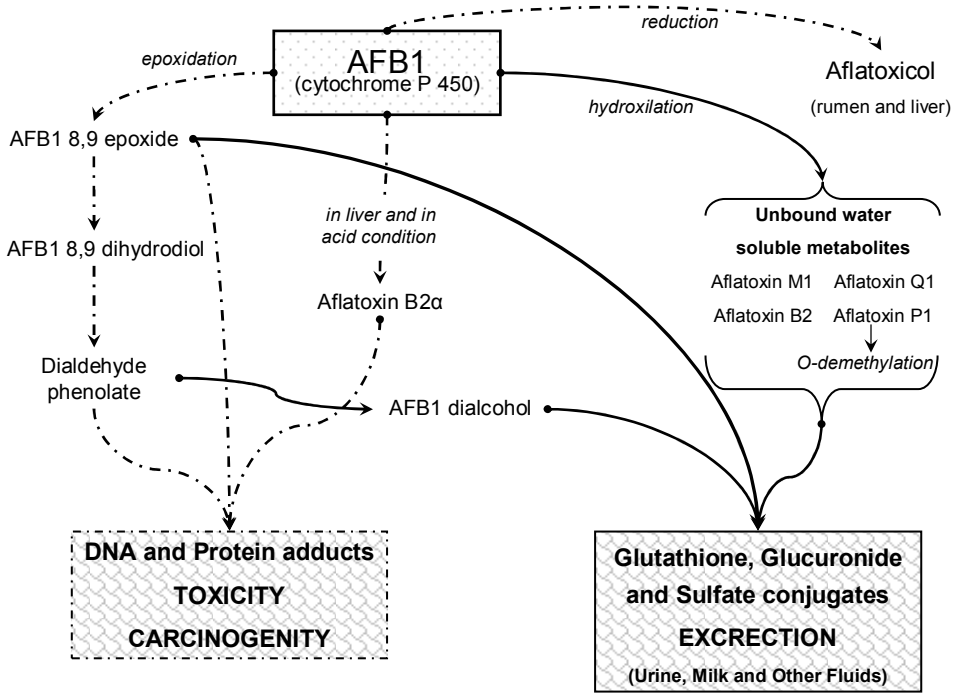
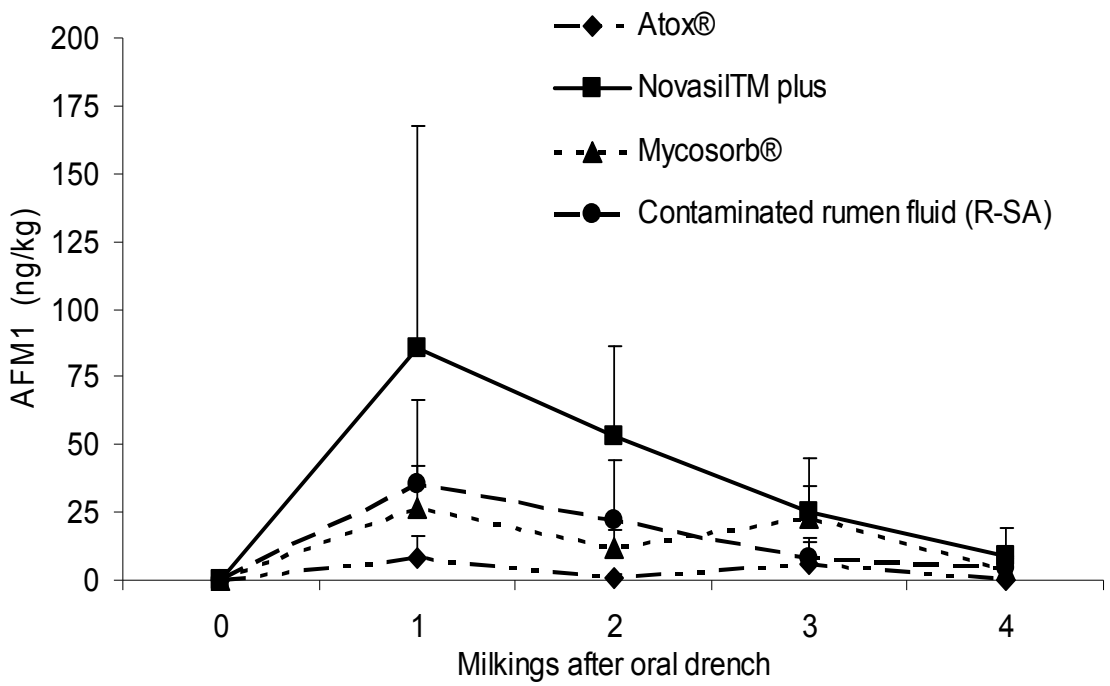


Figure 13-2. Milk AFM1 concentration (ng/kg) in four milkings after oral drench.



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14 Effects of Mixing and Pelletizing on the Efficacy of a Sequestering Agent in Reducing Aflatoxin M1 Excretion into Milk of Lactating Dairy Cows

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14.1 Abstract

Two experiments were carried out on lactating Holstein cows to determine the effect of different ways of inclusion of sequestering agent on the carry over of aflatoxin B1 (AFB1) from contaminated feeds to milk as aflatoxin M1 (AFM1). Aflatoxin B1 from natural contaminated corn meal (CC) and a pelleted mineral/vitamin protein supplement (PMX) made from contaminated corn meal were utilized. Diets fed to cows were based on corn silage (30%), alfalfa dehydrate hay (25%), grass hay (5%) and ingredients with CC (19%) and PMX (21%). In both experiments cows were fed AFB1 diets for nine days followed by five days of AFB1 free diets. In the first experiment cows were also assigned to four treatments in a replicated 4 x 4 Latin squares design. The sequestering agent was added directly to either the CC (CC-SQ) or the PMX (PMX-SQ) feeds (2.22% and 2.00% of SQ, respectively) or to a contaminated concentrate mix (46.88% CC and 52.07% PMX, 1.05% SQ). The four diets had a common forage source. The AFM1 concentration in milk reached a steady-state condition between the 7th and 9th days from AFB1 starting intake. Sequestering agent inclusion methods affected milk AFM1 concentration and the AFB1 carry over into milk. Higher AFM1 concentrations were observed in diet 3 and diet 4 (111.1 ng/kg and 120.4 ng/kg, respectively) compared to diet 1 (97.3 ng/kg), whereas diet 2 had the lowest AFM1 level (75.7 ng/kg). The lowest carry over (1.3%) was in diet 2 compared to 1.63% of diet 1, 1.95% of diet 3 and 2.03% of diet 4. No differences for carry over were observed between diets 3 and 4. In the second experiment two diets were used: a control diet (CC and PMX) without sequestering agent and diet 1 from experiment one. The presence of the

sequestering agent in diet 1 reduced both the milk AFM1 concentration (47%) and the carry over (44%) as compared to the control diet. Two methods for AFB1 extraction from feeds were tested based either on a methanol/water solution (80:20 vol/vol) or an acetone/water solution (85:15 vol/vol). When sequestering agent was added to feeds the acetone/water solution had a higher AFB1 extraction compared to the methanol/water solution.

Key words: *aflatoxin, carry over, dairy cow, sequestering agent*

14.2 Introduction

Crops such as corn (Zea mays L.), cotton or peanuts and their industrial by products are frequently contaminated by aflatoxins (AFs), hepatocarcinogens molecules (IARC, 2002) produced primarily by Aspergillus flavus and A. parasiticus (Scheidegger and Payne, 2003).

Aflatoxin B1 (AFB1), once ingested by mammals, is rapidly adsorbed in the gastro-intestinal tract and appears as aflatoxin M1 (AFM1), its principal oxidized metabolite, in plasma as soon as fifteen minutes after feeding (Moschini et al., 2007) and in milk as early as the first milking after AFB1 ingestion (Diaz et al., 2004). The AFB1 carry over (CO) into milk as AFM1 has been determined to be around 1-3% and is affected by milk yield and stage of lactation (Veldman et al., 1992; Diaz et al., 2004; Van Eijkeren et al., 2006).

The European Community (EC) allowable limits for AFB1 in animal feeds and concentrates are 20 μg/kg and 5 μg/kg respectively (European Commission, 2003). Furthermore, the EC limits AFM1 in milk to a level not greater than 0.05 μg/L (European Commission, 2006). In the US AFM1 is regulated by the US Food and Drug Administration (FDA) at 0.5 μg/L (Berg, 2003).

The use of sequestering agents (SQ) capable of binding mycotoxins molecules like AFB1 can reduce their absorption in the gastro-intestinal tract and therefore reduce their CO into milk. The addition of SQ is also a popular approach for reducing the negative effects associated with consumption of mycotoxin contaminated feeds (Diaz and Smith, 2005).

Hydrated sodium calcium aluminosilicates (Harvey et al. 1991; Phillips et al. 1988), montmorillonites (Ramos and Hernandez, 1996), bentonites (Schell et al., 2000), zeolites (Piva et al., 1995), activated carbons (Galvano et al., 1996; Diaz et al., 2004), and yeast cell wall extracted glucomannans (Aravind et al., 2003) have been reported to have a high affinity for AFs.

Compared to monogastric animals in which the SQ is normally included to the complete feed (meal or pellet), in lactating dairy cows the SQ is often added during total mixed ration

(TMR) preparation in the mixer wagon, with consequent dilution effect of the SQ respect at AFB1 which could lower the efficiency of the SQ.

One of the most frequently AFB1 contaminated feed in the farm is corn meal, which is normally added during the TMR preparation separately from the SQ. Only when buying a complete feed that includes a SQ would the product be added in advance by the feed mill.

Aflatoxins are highly heat stable (Hwang and Lee, 2006), however, feeds processing like roasting, autoclaving and extrusion-cooking can reduce AFB1 concentration in feeds (Hwang and Lee, 2006; Park and Kim, 2006; Castells et al., 2005). Recently, Oluwafemi (2004) observed a 20% decrease of AFB1 concentration in feeds treated at 100°C for 30 minutes.

No information is currently available on the effects of feed processing on the SQ and AFs interactions. High pressure the pelleting, expansion or extrusion are industrial processes that strongly impact the physical structure of feeds and could potentially have an effect on SQ efficiency.

The objective of this work was to compare the effect of pelleting or simply time of mixing of a commercial SQ either in from a contaminated corn meal or in the a diluted complete concentrate mix on the AFM1 CO into milk of lactating dairy cows.

14.3 Materials and Methods

14.3.1 Feed Preparation

A batch of 3000 kg of AFB1 naturally contaminated corn meal (CC) (32.13 ± 3.38 µg/kg) was obtain from a local producer. A 2000 kg batch of CC was added to 2.22% of the SQ (Atox, Grupo Tolsa, Madrid, Spain), then accurately mixed for three minutes (CC-SQ) in a industrial 3000 kg mixer (MO/30, Grespan, Treviso, Italy).

A mineral/vitamin protein premix (table 14-1), to be used as a separate component in the TMR formulation was prepared (PMX), 2.00% of the SQ was added to half of premix (PMX-SQ), then pelleted after steam conditioning (UMT 1200, Universal Milling Technology, Graz, Austria). The obtained AFB1 basal contamination of premixes was 4.13±0.71 µg/kg.

A 2500 kg batch of an AFB1 complete contaminated concentrate was obtained by mixing 52.07% PMX and 46.88% CC and adding 1.05% of the SQ. Then half of the batch (1250 kg) was pelleted (PC-SQ) after steam conditioning to obtain a 6mm pellet. The reminder of the material was utilized to prepare the MC-SQ that was to be mixed into the TMR formulation.

Feed processing, mixing and pelleting was carried out in a industrial feed mill plant in northern Italy (Ferrari mangimi S.r.l., Sarmato, Italy).

14.3.2 Animals and Diets

Eight multiparous Holstain Friesian cows (mean ± SD) of 604 ± 99 kg of BW, 32 ± 5 kg/d of milk production and 120 ± 22 DIM were utilized in two consequent experimental trials (trial 1 and 2). Trials were carried out at the CERZOO research and experimental center (San Bonico, Piacenza, Italy). The research protocol and animal care was in accordance with the EC council directive guidelines for animals used for experimental and other scientific purpose (European Communities, 1986).

Cows were fed utilizing an electronic gate feeder (American Calan Inc., Northwood, NH) and had free access to water. Animals were milked twice a day (0230 and 1330) and individual milk yield was recorded at every milking (Afimilk system, Afikim, Israel).

Trial 1

Cows were assigned to two balanced 4 x 4 Latin squares. The four diets (diet 1 to 4) fed in the Latin square were formulated according to the nutrient requirements of dairy cattle (NRC, 2001) and are reported in table 14-1. The single components were added in a mixer wagon (Data Ranger, American Calan Inc., Northwood, NH) and mixed for five minutes before distribution. The TMR was fed ad libitum (5% expected orts) once daily (0800). Orts were collected individually and weighted daily.

Each period of the Latin square had a nine days of AFB1 ingestion followed by a seven days of AFM1 clearance from milk periods in which animals were fed the base diet without AFB1 or SQ.

Trial 2

Two weeks after the end of trial 1 the same animals (mean ± SD: 603±102 kg of BW, 32.14 ± 4.91 kg/d of milk production, 185 ± 22 DIM) were randomly assigned to two treatment diets (four cows each) in a completely randomized design. Treatment diets were formulated according to the nutrient requirements of dairy cattle (NRC, 2001) and consisted of a base diet (forage and PMX) with AFB1 contaminated corn meal (CC) in the control diet (CTR) and a diet with contaminated corn meal and SQ (CC-SQ; diet 1) (table 14-1). The TMR preparation,

distribution and orts collection were as in trial 1. The aflatoxin ingestion period lasted nine days followed by a five days of clearance period.

14.3.3 Feed and Milk Sampling

Individual TMR samples and orts were collected at day 0, 3, 5, 7, 9, 11 and 14 for each experimental period. Single feeds entering the TMRs were sampled at day 0 and 7. Samples were dried in a ventilated oven (60°C) for 48 hours, ground with a 1 mm sieve (Thomas-Wiley Laboratory Mill, mod. 4, Arthur H. Thomas Co. Phyladelphia, PA), then analyzed for AFB1 content.

Individual milk samples were collected at each milking at day 0, 3, 5, 7, 9, 11 and 14, proportionally mixed by animal and day, analyzed for fat, protein and lactose content (Milkoscan Model FT120 Foss Electric, Denmark), then frozen at -20°C before AFM1 determination.

14.3.4 Sample Analysis

AFB1 Assay in Feeds

Ten grams of dried feed were mixed in 100 ml of a methanol/water solution (80:20 vol/vol) (Stroka et al., 1999), or in 100 ml of a acetone/water solution (85:15 vol/vol) (Arranz et al., 2006), shaked at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, 5 mL were eluted with 45 mL of bi-distilled water through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with 5 mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75 vol/vol) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

AFM1 Assay in Milk Samples

Extraction was done by the immunoaffinity technique according to Mortimer et al. (1987). Briefly, 50 mL of defatted milk (centrifugated at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4).

The column was washed with 5 mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile/water (25:75 vol/vol) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFB1 was separated with a reverse-phase C18 Superspher column (4 μm particle size, 125 x 4 mm i.d.; Merck, Darmstadt, Germany) at room temperature and isocratic conditions, with a mobile phase of water and acetonitrile/methanol solution (17:29 vol/vol) with a 64:36 (vol/vol) ratio. The flow rate was 1mL/min. Then, the AFB1 was detected by fluorescence, after postcolumn derivatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide perbromide (PBPB) at flow 0.1 mL/min. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, 5 μm particle size, 125 x 4 mm i.d.) at room temperature, with a water and acetonitrile (75:25 vol/vol) mobile phase and the flow rate set at 1.0 mL/min. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths.

The standard stock solutions was checked for AFB1 and AFM1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

14.3.5 Carry Over Calculation

The total excretion and CO of AFB1 into milk as AFM1 were obtained based on individual AFB1 daily intake, milk yield and AFM1 concentration. The days considered for calculation were at plateau condition, between 7th and 9th days on treatment.

14.3.6 Statistical Analyses

Data from trial 1 were analyzed by mixed procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC) as a 4 x 4 Latin square design, replicated two times, using the following model:

$$Yijkl = \mu + Di + Sj + Cl(j) + Pk + eijkl$$

where Yijkl = dependent variable (DMI, milk, fat, protein and lactose yield, AFB1 intake, AFM1 concentration and carry over), μ = overall mean, Di = mixed effect of the diet (i = 4), Sj = mixed effect of the square (j = 2), Ci(l) = random effect of cow (l = 4) nested within square i, Pk = mixed effect of the period (k = 4); and eijkl = residual error. Least squares means were separated into significant main effects by the PDIFF option of SAS. DMI, milk, fat, protein and lactose yield, AFB1 intake, AFM1 concentration and carry over data were analyzed as repeated measures in time using the MIXED procedure of SAS (Littell et al., 1996). The experimental unit was the cow.

Data from trial 2 were analyzed by the general linear model procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC) as a complete randomized design with the single cow as experimental unit. Pair wise comparisons among means were performed using T test. Differences were declared significant for $P < 0.05$.

14.4 Results

Analytical Assay of Feeds. The AFB1 extraction methods used for feeds analysis had considerable effects on final AFB1 amount when the SQ was added to the feed. Lower AFB1 extractions were observed when using the methanol/water compared to the acetone/water solvent solution (table 14-2). The discrepancy between the two methods was 72.1, 80.0 and 87.0%, respectively for MC-SQ, PC-SQ and CC-SQ feeds. Similar AFB1 values between methods were observed mixed concentrates.

14.4.1 In vivo Experiment

The average AFB1 contamination of the basal diet was 0.68 ± 0.04 μg/kg which contributed to a bulk milk AFM1 content of 13.7 ± 5.1 ng/kg.

Trial 1

Reported results on AFB1 contents of diets were obtained with the acetone/water solution extraction method (table 14-3). The calculated AFB1 intake for each cow was 171.3 μg/d.

The way of the SQ addition to feeds, pelleted or not, affected ($P < 0.05$; table 14-3) the milk AFM1 concentration (ng/kg) and the AFB1 carry over (%) to milk as AFM1. The AFM1 concentration decreased (22%; $P < 0.05$) in diet 2 whereas increased (14 and 24%; $P < 0.05$) respectively for the diets 3 and 4 compared to diet 1. No differences among groups were reported on AFB1 and dry matter intake or in milk yield. The lowest carry over (1.3%) was measured for diet 2, significantly lower ($P < 0.05$) than diet 1 (1.63%), 3 (1.95%) and 4 (2.03%); no differences were observed between diets 3 and 4.

Trial 2

Cows received 173.9 µg/d of AFB1 as average. The presence of the SQ mixed with the contaminated corn (2.22%) reduced ($P < 0.05$; table 14-4) the milk AFM1 concentration (47%) and the AFB1 carry over (44%) to milk as AFM1 compared to a control AFB1 contaminated diet (CTR) with no SQ addition. No differences were observed on AFB1 and dry matter intake and milk yield.

14.5 Discussion

14.5.1 Analytical Assay of Feeds

The different AFB1 values on SQ added feeds observed when using methanol or acetone extraction procedure can be misleading. In particular, the same feed could comply with the 5 µg/kg threshold, as the maximum AFB1 content allowed by the EU legislation in mixed feeds for animal use (European Commission, 2003), when analyzed for AFB1 content using the methanol/water extraction solution but at the same time could be over the limit when analyzed with the acetone/water extraction solution. Results suggest that the SQ when added to AFB1 contaminated feeds can reduce the AFB1 concentration to a level below the threshold value when the methanol/water extraction solution is being used. Also, the binding capacity of SQ seems not to have been changed by the physical processing of the feeds with the level of contamination utilized. Data outline that in feed analysis it is of paramount importance to know if the reported AFB1 contamination level was obtained in sequestering agent added feeds.

If the methanol/water solution is good for the AFB1 extraction in concentrate, the same analytical procedure seems inadequate to our experimental condition.

14.5.2 In vivo Experiment

The method in which the SQ was added to the diet affected the AFB1 carry over and the AFM1 concentration in milk (table 14-3; figure 14-1). The less effective way of counteracting the aflatoxin presence in feeds was observed when the SQ was added to the mineral/vitamin protein premix (PMX-SQ), and then to the base diet with the contaminated corn meal (diet 4). The measured AFB1 carry over was 2.03%. The low performance of the SQ could be caused by the physical separation between the sequestering agent, within the pellet of the premix, and AFB1 in the contaminated corn meal. However, diet 3 characterized by the concurrent presence of contaminated corn meal and the SQ and no pellet processing did not improve the AFB1 carry over and the total AFM1 in milk compared to the diet 4. Thus, in our conditions the physical contact between contaminated corn meal and SQ might not fully explain the SQ performance. Additionally, the pellet itself (diet 4) did not act as a time delay factor for SQ efficiency within the gastro-intestinal tract. Diet 3 represented the normal procedure for the SQ utilization on a commercial dairy farm.

The best performance was obtained in diet 2, which was similar to diet 3, except for a pelleting processing leading to a complete concentrate pellet containing the SQ and the contaminated corn meal. Under this processing condition the concentrate was steam conditioned to 80°C and 18% moisture (plus 6% the standard 12% moisture content of the corn meal), then pressure was increased during the pelleting processing up to a final specific weight of 0.713 (wt/vol). From our result it was clear that the physical processing was improving the strength of AFB1/SQ interactions.

The intermediate results obtained in diet 1 could have different implication. Even though the final content of the SQ in diets was similar, in terms of feeds preparation the dilution factors (aflatoxin and sequestering agent) were different. The diet 1 was obtained by adding to the same base diet the same amount of contaminated corn meal with double of the percentage content of the SQ. Thus, the result seemed to support the idea of a dilution factor that could improve the effect of the SQ.

If contact time between the AFB1 and the sequestering agent is the only factor affecting the binding performance, then we could think of that time as being spread between silo and rumen compartments. Giving a fixed rumen flow rate, the presence of a pellet compared to a meal could affect the time needed for rumen escaping of feed particles. This could in a way increase the time length of the SQ within the gastro-intestinal tract. However, results of diets 3 and 4 did not support this hypothesis. From our results it was impossible to prove the effect of time of a close contact between the SQ and AFB1 outside the rumen (silo compartment), however, the

dilution factor (diet 1) seemed more important than the time length within the rumen (diet 3) as by the AFM1 concentration and the AFB1 CO observed in both diets. The two diets had similar SQ content, however, there was a different SQ content mixed in the corn meal. The ratio between SQ and AFB1 in corn meal were 700 and 325 (wt/wt), respectively for diet 1 and 3.

Even though the dilution factor proved to be important for SQ performance compared to the normal sequestering agent usage in farm condition (diet 3), the effect was lower than the pelleting processing observed in diet 2 with half the dilution factor. These results underline the importance of processes occurring during the physical treatment leading to the conclusion that the pelleting step could be an important way of improving the SQ performance.

Results on calculated carry over could have been different according to the methods used for AFB1 determination of contaminated diets. Based on what observed on AFB1 feeds analysis when the two methods were used (table 14-2) it was clear a lack of the methanol/water solution to extract the AFB1 in presence of SQ. The result itself would not affect the amount of AFM1 getting into the milk, however, the ultimate result could be critical when complying with the maximum level of AFM1 into milk. The AFM1 content of milk from treated groups were all above the maximum EU limit of 0.05 µg/L (European Commission, 2006).

In trial 1 the estimated AFB1 intake (µg/cow/d) based on the methanol/water method was 39.49, 32.97, 45.03 and 149.3 respectively for diet 1, 2, 3 and 4. Considering a 1.5% carry over value of AFB1 into milk as AFM1 we could expect diets 1 to 3 to comply with the EC limit for AFM1 in milk. Then, diets would be considered legal and only diet 4, with the SQ not mixed directly with the contaminated corn meal, would be considered at risk and potentially blocked at the plant. However, the results from trial 1 proved this was not the case, with all diets leading to milk AFM1 concentrations over the allowed EC limit (table 14-3). Based on the estimated AFB1 intake (with the methanol/water method), the real total AFM1 in milk would justify carry over of 7.2, 6.9, 7.4 and 2.4%, respectively for diets 1, 2, 3 and 4; well above any reported except for diet 4. The calculated carry over from AFB1 intake estimated from value obtained with acetone/water extraction (table 14-3) was in agreement with previous work (Veldman et al., 1992; Diaz et al., 2004; Masoero et al., 2007).

It was clear from our data that only one of the analytical methods utilized for feed analysis in presence of SQ was as effective. Thus, when using the methanol/water extraction solution a competitive reaction between SQ and the organic solvent versus the AFB1 would benefit the SQ, though it would be later be released during the digestive processes.

The hypothesis of a weak extraction efficiency of organic solvents in presence of a clay type SQ can be considered observing the demonstrated efficiency smectite clay SQ in binding the

AFB1 in the gastro-intestinal tract of poultry (Pimpukdee et al., 2004) and swine (Phillips et al., 1988; Schell et al., 2000) and dairy cows (Harvey et al., 1991; Diaz et al., 2004). Also, previous data from our lab (M. Moschini, unpublished data) showed a different tendency of several adsorbents to release adsorbed AFB1 during the digestive processes.

Data from trial 2 showed that a SQ can be effectively utilized to reduce the CO of AFB1 into milk on dairy cows. The diet containing SQ was the same as diet 1 of trial 1 and it was chosen as alternative diet because of its performance in trial 1 and because it was not pelleted. The AFM1 concentration in milk of both groups consuming the same experimental diets was 97.3 ng/kg trial 1 (table 14-3) and 113.1 ng/kg in trial 2 (table 14-4) with an overlapping 0.95% confidence interval.

The AFM1 content in milk from cows fed the CTR was 215.1 ng/kg (table 14-4) very similar to 208.96 ng/kg calculated utilizing the Veldman et al. (1992) equation with an AFB1 intake of 174.00 µg/d.

14.6 Conclusion

The method utilized during SQ addition had a significant effect in the ability of the SQ agent to reduce the AFM1 carry over into milk. Additionally, it was clear that the physical processing method had an effect in improving the amount of AFB1 being sequestered by SQ. In addition, results supported the idea that a dilution factor can improve the effect of SQ in reducing the AFB1 available for absorption in the gastro-intestinal tract. The dilution factor proved to be important for SQ performance, however, the contribution seemed to be lower than that seen after pelletting. Results from our trials underline the need of an AFB1 extraction methodology working similarly in feeds either added or not different SQ to avoid negative drawback in farm condition.

Table 14-1. Ingredients (% of DM) of the five experimental diets (trial 1 and 2).

	Diets				
Ingredients (% DM)	CTR	diet 1	diet 2	diet 3	diet 4
Corn silage	30.0	30.0	30.0	30.0	30.0
Alfalfa hay, dehydrate	25.0	25.0	25.0	25.0	25.0
Grass hay	5.0	5.0	5.0	5.0	5.0
CC	19.0				19.0
CC-SQ[2]		19.0			
PMX[1]	21.0	21.0			
PMX-SQ[3]					21.0
MC-SQ[4]				40.0	
PC-SQ[4]			40.0		

[1]Contains per kg of premix: Soybean meal 600 g, Sunflower meal 300 g, mineral and vitamin supplement 100 g.; 120000 IU of Vitamin A; 9000 IU of Vitamin D3; 90 mg of Vitamin E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

[2]2.22% of the SQ.

[3]2.00% of the SQ.

[4]1.05% of the SQ.

[5]CC: naturally contaminated corn meal; CC-SQ: CC + 2% Atox®; PMX: mineral vitamin premix; PMX-SQ: PMX + 2% Atox®; MC-SQ: 55% PMX + 45% CC + 1% Atox®; PC-SQ: 55% PMX + 45% CC + 1% Atox® then pelleted.

Table 14-2. Aflatoxin B1 (AFB1) concentration (mean ± SD; µg/kg) in feeds used in trial 1 and 2 as obtained from two different extraction (methanol/water and acetone/water solutions).

Ingredients	n[1]	Methanol/water solution	Acetone/water solution
CC	8	30.60 ± 10.63	32.13 ± 3.38
CC-SQ	8	4.09 ± 2.31	31.47 ± 5.08
PMX	8	4.13 ± 0.71	5.64 ± 0.45
PMX-SQ	8	1.15 ± 1.43	3.82 ± 0.32
MC-SQ	8	4.74 ± 1.04	17.00 ± 0.45
PC-SQ	8	3.46 ± 0.70	17.33 ± 0.66
CC-PMX	16	17.16 ± 3.99	18.20 ± 1.62

[1]n = total number of analytic replicates.

[5]CC: naturally contaminated corn meal; CC-SQ: CC + 2% Atox®; PMX: mineral vitamin premix; PMX-SQ: PMX + 2% Atox®; MC-SQ: 55% PMX + 45% CC + 1% Atox®; PC-SQ: 55% PMX + 45% CC + 1% Atox® then pelleted.

Table 14-3. Trial 1: Mean of DIM (kg/d), milk production (kg/d), fat, protein and lactose (g/kg), aflatoxin B1 (AFB1) intake (µg/d), aflatoxin M1 (AFM1) milk concentration (ng/kg) and carry over in milk (%) at plateau condition (7th and 9th day on AFB1 ingestion period).

Item	Diets				SE
	diet 1	diet 2	diet 3	diet 4	
DMI, kg/d	23.06	22.73	23.09	23.42	0.2680
Milk, production kg/d	29.29	30.09	29.93	29.37	0.4173
Fat, g/kg	3.78	3.66	3.67	3.78	0.0884
Protein, g/kg	3.41	3.39	3.44	3.43	0.0291
Lactose, g/kg	5.10	5.12	5.10	4.99	0.0727
AFB1 intake, µg/d	172.42	170.42	170.39	171.85	0.7239
AFM1, ng/kg	97.3[b]	75.7[a]	111.1[c]	120.4[c]	3.6513
Carry over, %	1.63[b]	1.30[a]	1.95[c]	2.03[c]	0.0675

[a-c] Means within a row with different superscript differ ($P < 0.05$).

[5]CC: naturally contaminated corn meal; CC-SQ: CC + 2% Atox®; PMX: mineral vitamin premix; PMX-SQ: PMX + 2% Atox®; MC-SQ: 55% PMX + 45% CC + 1% Atox®; PC-SQ: 55% PMX + 45% CC + 1% Atox® then pelleted.

Table 14-4. Trial 2: Mean of DIM (kg/d), milk production (kg/d), aflatoxin B1 (AFB1) intake (µg/d), aflatoxin M1 (AFM1) milk concentration (ng/kg) and carry over in milk (%) at plateau condition (7th and 9th day on AFB1 ingestion period).

Item	Diet		SE	P-value[1]
	CTR	Diet 1		
DMI, kg/d	23.80	23.27	0.8581	0.6704
Milk, production kg/d	31.03	33.25	1.7470	0.3831
AFB1 intake, µg/day	174.00	173.79	0.3411	0.6699
AFM1, ng/kg	215.1[a]	113.1[b]	6.2708	<0.001
Carry over, %	3.81[a]	2.14[b]	0.1386	<0.001

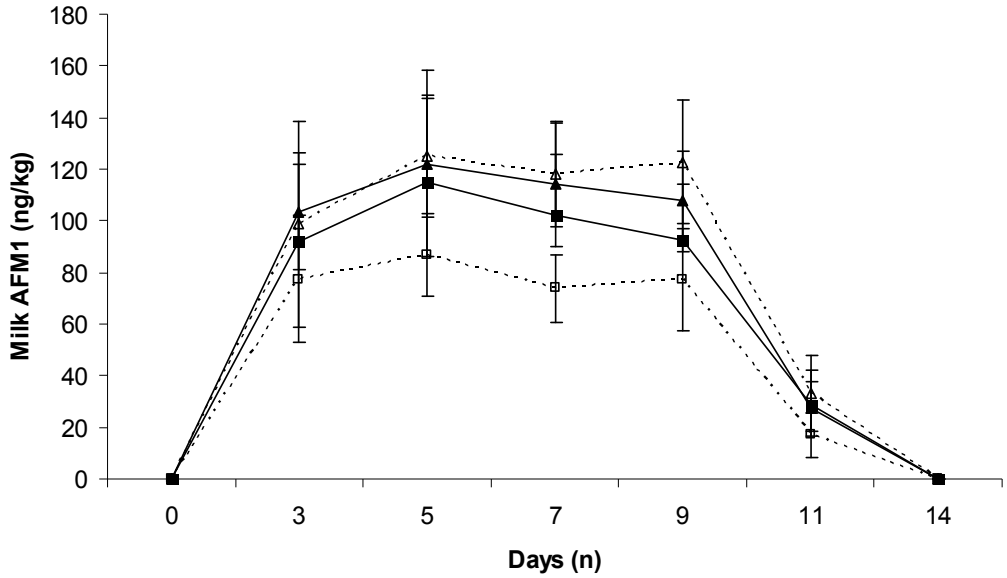
[a,b] Means within a row with different superscript differ ($P < 0.05$).

[1] *P*-value…

CC-PMX: naturally contaminated corn meal + mineral vitamin premix

CC-SQ: CC + 2.22% Atox®

Figure 14-1. AFM1 concentration (ng/kg) in the milk of cows from different diets: (■) diet 1, (□) diet 2, (▲) diet 3, and (Δ) diet 4. For each diets the point represents the mean of 8 data.



15 General Conclusion

This work focused on aspects related to aflatoxins absorption, biotransformation and excretion in milk of dairy cows. Also the use of mycotoxin adsorbents in dairy diets was studied *in vitro* and *in vivo* conditions to better understand their action mechanisms and improve their sequestering efficiency.

Data presented showed that aflatoxin B1 and other parent molecules (aflatoxin B2, aflatoxin G1 and aflatoxin G2) were quickly adsorbed through gastro-intestinal membranes of ruminants and then transferred to blood and other biological fluids. The speed with which aflatoxins appeared in blood is probably related to an early absorption that could take place in mouth or oesophageal mucous membranes and, successively, in the rumen compartment and in the intestinal tract. The appearance in blood of the principal aflatoxin B1 metabolite, the aflatoxin M1, just after five minutes from the consumption of an oral contaminated drench could represent another evidence of a quick absorption of the aflatoxins. However, this could reasonably establish that the oxidative system responsible of the aflatoxin B1 oxidation, which is present in these tissues and in the leukocytes, is active instantly after absorption.

The passive passage of aflatoxins through membranes was confirmed to be the most probable mechanism involved in absorption of these compounds, as proved by passage of the toxins through non absorbing mucosa like the vagina mucosa.

When absorbed by lactating dairy cows, part of aflatoxin B1 was transferred to the milk as aflatoxin M1. The aflatoxin M1 in milk increased as soon as in the first milking after initial aflatoxin B1 ingestion, up to a plateau observed after 6-7 days of continuous ingestion. The carry over of aflatoxin B1 in milk as aflatoxin M1 is known to be affected by several factors (species, animal variability, aflatoxin source, milk yield, stage of lactation and membrane permeability). The data proposed in this work support the idea that milk yield is the major factor affecting the total excretion of AFM1 and its carry over in milk of dairy cows, while changes of membrane permeability due to inflammatory process do not appear as a factor affecting significantly AFB1 carry over into milk.

The reported carry over values were lower than previously reported data from bibliography, which are in agreement with calculations used to define limits proposed by European Community for aflatoxin B1 in animal feedstuffs and aflatoxin M1 in milk.

Different commercial mycotoxins sequestering agents, usually used in dairy farms, were studied either *in vitro* or *in vivo* conditions. Data from the *in vitro* trial suggested that the circumstances in which these types of experiment were conducted (type of aflatoxins, solution media, dilution factor or aflatoxins:volume, aflatoxins:adsorbents ratio and pH experimental conditions) could strongly influence the sequestering efficacy of adsorbents *in vitro* and cause a different interpretation of results in diverse experimental conditions. Using the rumen fluid as solution media for *in vitro* evaluation, there was an increase in sequestering efficacy due to a probable synergism between rumen fluid, microorganism and adsorbents. This could cause the reduction of aflatoxins absorption when these binders are used on animals.

Testing the strength of the binding between adsorbents and aflatoxins directly in gastro intestinal tract of lactating dairy cows through the formation of an aflatoxin:adsorbent complex, the data clearly showed no correspondence in sequestering efficiency between *in vitro* and *in vivo* trial. This method was proposed to determinate the strength of bind and the fate of aflatoxin:adsorbents complex in gastro intestinal tract in the ruminants.

However, the addition of adsorbents to feedstuffs was shown to reduce the carry over of aflatoxin B1 in milk as aflatoxin M1. More importantly, it appears that the method and time of addition of the adsorbent into the feeds could further increase sequestering efficiency and improve the adsorbents performance. The physical processing methods (mixing or pelletizing) had an effect in improving the amount of aflatoxin B1 being sequestered more so than the time of inclusion of the adsorbents into mixer wagon at the moment of unifeed preparation. In addition, results supported the idea that a higher aflatoxin:adsorbent ratio can improve the effect of sequestering agents in reducing the aflatoxins B1 available for absorption in the gastro-intestinal tract.

Results from the trial have showed that the aflatoxins extraction methodologies usually used in laboratory practices (methanol or acetone water solutions) did not work similarly to determinate aflatoxins concentration in feeds in presence of sequestering agents. This could results in a mistake in analytic determination of aflatoxins level in feedstuff.

16 References

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