CHAPTER 1

Introduction

Fungi, developing from spores and found ubiquitously in the environment, can grow on almost any organic matter. These fungi produce structurally diverse metabolites, mycotoxins, that occur as both food and animal feed contaminants worldwide (Ramos & Hernández, 1996). The *Fusarium*, *Penicillium* and *Claviceps* species of fungi generally contaminate feeds prior to harvest with mycotoxins such as those belonging to the trichothecene family (T-2 toxin, HT-2 toxin and vomitoxin) and the ergot alkaloids, respectively. *Aspergillus* and *Penicillium* species are commonly found in stored grain or food and produce aflatoxins and ochratoxins, and citrinin, respectively (Marquardt, 1996).

Mycotoxins can cause serious health problems and production losses in livestock (Ramos & Hernández, 1996). Aflatoxin is the most prevalent and economically significant mycotoxin. It is found in maize, peanuts, cottonseed, millet, sorghum and other feed grains (Phillips, 1999). Like many microbial secondary metabolites, the aflatoxins are a family of closely related compounds (Moss, 1996) that include aflatoxin B_1, B_2, G_1 and G_2, but aflatoxin B_1 (AFB_1) is usually in the highest concentration and is the most toxic. Aflatoxin is stable once formed in grain, and is not degraded during normal milling and storage (Brown, 1996). AFB_1 is known as a potent hepatotoxin and hepatocarcinogen and the liver is considered to be the primary target for aflatoxins (Towner *et al*., 2000), but it also affects other organ systems (Coulombe, 1994). The immune system is a highly sensitive indicator of aflatoxicosis in poultry (Giambrone *et al*., 1985b) affecting both cellular and humoral immune reactions (Giambrone *et al*., 1978).

Practical methods for detoxifying mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. A variety of physical, chemical, and biological techniques have been employed but with limited success (Edrington *et al*., 1997). The most recent approach is the use of non-nutritive adsorptive materials (enterosorbents), which bind the aflatoxin molecule and reduce aflatoxin absorption. Ideally, the adsorbent should have a high affinity for the specific mycotoxins, resulting in the formation of a strong complex with a low possibility of dissociation and should also have a high capacity for binding to prevent saturation (Ramos & Hernández, 1996; Edrington *et al*., 1997). Ledoux & Rottinghaus (1999) are of opinion that the addition of adsorbents to contaminated feed to selectively bind the mycotoxin during the digestive

process, allowing the mycotoxin to pass harmlessly through the animal is, at present, the most promising and practical approach. The major advantages of adsorbents include low cost, safety and the ease with which they can be added to animal feeds, but according to Ramos & Hernández (1997) this strategy would only be effective if these materials had the ability to adsorb a large number of chemically distinct mycotoxins.

Research indicates that a number of adsorbents are capable of binding aflatoxin and reducing or preventing its toxic effects. However, not all adsorbents are equally effective in protecting livestock against the toxic effects of aflatoxin and several adsorbents have been shown to impair nutrient utilisation (Chung *et al*., 1990; Kubena *et al*., 1993; Scheideler, 1993). Dale (1998) noted that many of the adsorbents on the market today have not been adequately tested for *in vivo* efficacy, but are used based on *in vitro* adsorption data only. *In vitro* tests may not always be a reliable indicator of ability to bind a mycotoxin (Scheideler, 1993; Dwyer *et. al.,* 1997; Ledoux & Rottinghaus, 1999). Therefore, it is important that adsorbents be subjected to extensive *in vivo* evaluation to determine both efficacy and impaired nutrient utilisation from the diet (Ledoux & Rottinghaus, 1999).

Humic acids are substances widely distributed in nature and are present in soils, natural waters, river, lake and sea sediments, peat, brown and brown-black coals and other natural materials as a product of chemical and biological transformations of animal and plant residues (Novák *et al*., 2001). The humic acids in peat have been known since ancient times for their therapeutic properties such as anti-inflammatory, antiviral, oestrogenic and profibrinolytic activity (Schepetkin *et al*., 2002). Humic acids in peat caused stimulation of lymphatic system cells (Obminska-Domoradzka *et al*., 1993a & b), thymus activity (Madej *et al*., 1993a & b), neutrophil function (Riede *et al*., 1991) and phagocytic activity of granulocytes (Jankowski *et al*., 1993). The oxidative polymerization of o- and p-diphenols produced synthetic humic acids, which showed similar therapeutic activities (Klöcking, 1994). It was also demonstrated that humic acids have the ability to adsorb heavy metals (Madronovà *et al*., 2001), herbicides (Leone *et al*., 2001), mutagens (Sato *et al*., 1987a & b), monoaromatic compounds (Nanny & Maza, 2001), polycyclic aromatic compounds (Kollist-Siigur *et al*, 2001), minerals (Fein *et al*., 1999) and bacterial DNA (Crecchio & Stotzky, 1998). In recent years, it has been observed that dietary intake of humates promote growth in poultry (Bailey *et al*., 1996; Shermer *et al*., 1998). Kocabağli *et al*. (2002) found that dietary humates significantly improved body weight and feed conversion of broilers.

A South African company, Enerkom, developed an effective large-scale regeneration process for humic acids from coal. This technology can economically regenerate large quantities of pure, high quality humic acids by reversing the process whereby coal was formed. Humic acids produced in this way are called oxihumic acids. Chemically oxihumic acids differ only marginally from humic acids obtained from other sources (Cloete *et al*., 1990; Dekker *et al*., 1990; Cronjé *et al*., 1991; Bergh *et al*., 1997).

The hypothesis of this study was that, because of the adsorbing abilities of humic acids to a wide range of compounds, oxihumate would bind mycotoxins in the digestive system to ameliorate the toxic effect thereof on the animal. The effectiveness of oxihumate to adsorb mycotoxins was evaluated to determine the possibility of developing it as a commercial mycotoxin binder to be used in the preventative management of contaminated poultry feedstuffs. This was done by (1) evaluating the *in vitro* affinity and adsorption capacity of oxihumate to aflatoxin (AF) B_1 and G_2 by studying their Langmuir and Freundlich adsorption isotherms; (2) determining the efficacy of oxihumate as an aflatoxin binder in broiler feeds *in vivo* and (3) investigating the ability of oxihumate to prevent the inhibiting effect of aflatoxin on lymphocyte proliferation *in vitro*.

CHAPTER 2

Literature Review

1. Mycotoxins

Fungi develop from spores and are ubiquitous in the environment, being present in the soil, on the walls of storage bins, on grain handling equipment, and within the home environment. Fungi can grow on almost any organic matter and often produce highly toxic compounds referred to as mycotoxins. Fungi are able to invade the plant during growth and during storage and as a result a wide range of pre- and post-harvest fungi may contaminate feeds. The major genera of mycotoxin-producing fungi are *Aspergillus*, *Claviceps*, *Penicillium* and *Fusarium*. The *Fusarium*, *Penicillium* and *Claviceps* species of fungi generally contaminate feeds prior to harvest and are often referred to as "field fungi" while *Aspergillus* and also the *Penicillium* species are commonly found in stored grain or food and are referred to as "storage fungi". These fungi are able to produce a wide array of different and related mycotoxins. Important toxins produced by field fungi are for example those belonging to the trichothecene family (T-2 toxin, HT-2 toxin and vomitoxin) and the ergot alkaloids. The toxins produced by storage fungi of greatest concern are the ochratoxins (mainly ochratoxin A), citrinin and the aflatoxins, particularly aflatoxin B_1 (AFB_1) (Marquardt, 1996).

1.1 Environmental factors affecting mycotoxin formation

Fungal growth and mycotoxin production are consequences of an interaction between the fungus, the host and the environment. The appropriate combination of these factors determines the amount of colonization of the substrate and the type and amount of mycotoxin produced (Pitt *et al*., 2000). The synthesis of any particular mycotoxin depends not only on the species but also on the strain (Sweeney & Dobson, 1998). Under field conditions, stress and subsequently reduced vigour often predispose crop plants to infestation and colonization by toxigenic fungi. Water stress, high temperature stress and insect damage of the host plant are major determining factors in mould infestation and toxin production under field conditions. In stored grain, factors which are likely to affect mycotoxin formation include moisture content of the substrate, environmental temperature, exposure time, damage to seed, oxygen availability, carbon dioxide concentrations, composition of the substrate, fungal abundance, prevalence of toxigenic strains, spore loads, microbial interaction and invertebrate vectors particularly insects. Spoilage, fungal growth and mycotoxin formation result from the complex interaction of these factors (Marquardt, 1996; Pitt *et al*., 2000).

Temperature and moisture are of particular importance in fungal growth and mycotoxin production. Although three important genera of fungi (*Fusarium*, *Penicillium* and *Aspergillus*) have worldwide distribution, it is possible to generalize that *Penicillium* is of particular importance in temperate parts of the world. *Fusarium* is often associated with cold climates while *Aspergillus* is most common in the tropics. The growth of storage fungi is greatly influenced by the water content of the substrate. At low moisture content (usually less than 14 to 16%) most storage fungi do not grow or grow very slowly. At high moisture content, usually above 25 to 30% moisture, other competing microorganisms such as bacteria tend to displace fungi. Maximum mycotoxin formation may be reached at moisture levels of 20-25% (Marquardt, 1996).

1.2 Mycotoxins and health

Mycotoxins have become increasingly important in our understanding of food safety and food poisoning. The major problem with mycotoxicosis for the clinician is that few produce overt signs of poisoning, and therefore mycotoxicosis is very difficult to diagnose (Pitt *et al*., 2000). The impact of fungal toxins upon animals extends beyond their obvious effect in producing death in a wide variety of animals that are likely to consume mycotoxin-contaminated grains or feeds. The economic impact of lowered productivity, reduced weight gain, reduced feed efficiency, subtle damage to vital body organs and interference with reproduction is many times greater than that of immediate morbidity and mortality. Potential threats of cancer induced by mycotoxins in feeds and human foods, along with the unknown subtle effects of these mycotoxins, are coupled to the universal concerns about health risks (Marquardt, 1996). Consumption of some mycotoxins, at levels that do not cause overt clinical mycotoxicosis, may suppress immune functions and decrease resistance to infectious disease. The sensitivity of the immune system to mycotoxin-induced immunosuppression arises from the vulnerability of the continually proliferating and differentiating cells that participate in immuno-mediated activities and regulate the complex communication network between cellular and humoral components (Corrier, 1991).

1.3 Ochratoxins

Ochratoxin is a general term describing a group of compounds (ochratoxin A, B and C). Ochratoxins are derivatives of isocumarin linked to L-b-phenylalanine and are classified as pentaketides. Ochratoxin A has been reported in many plant products, especially in Europe, where the highest frequencies and levels of ochratoxin A contamination are found in cereals (Pitt *et al*.,

2000). Ochratoxin A has also been reported in wine and grape juices (Ueno, 1998). It is possible that ochratoxin A can be transferred to meat from animals eating contaminated feeds (Pitt *et al*., 2000). The high incidence of ochratoxin in human blood serum (Jiménez *et al*., 1998) and milk (Jonsyn *et al*., 1995) reflects a widespread exposure of humans to ochratoxin A. According to Pitt *et al*. (2000) the World Health Organization has proposed a maximum limit for ochratoxin A of 5 µg/kg in cereals.

Ochratoxin A was originally isolated as a metabolite from *Aspergillus ochraceus* (Van der Merwe *et al*., 1965), but Hesseltine *et al*. (1972) also isolated it from other related *Aspergilli* species. Pitt *et al*. (2000) reported that *Penicillium verrucosum* is the only *Penicillium* species producing ochratoxin A. *Penicillium verrucosum* is the main producer of ochratoxin A in temperate climates. This is a cold climate fungus, growing between 0 and 31 °C, with an optimum at 20 °C. It grows in the pH range 2-10, with an optimum between 6 and 7. The ochratoxin A producing *Aspergilli* species grow in the range 8-37 °C (Pitt *et al*., 2000).

The ochratoxins, particular ochratoxin A, are acutely toxic to several species of animals including poultry (LD_{50} of 3.3 mg/kg body weight) and pigs (1.0 to 6.0 mg/kg LD_{50} value). Ruminants are much more tolerant to ochratoxin A as the microorganisms in the rumen are able to readily convert it into alpha ochratoxin, a non-toxic metabolite. The toxicity of the different forms of ochratoxin greatly differs from each other (Marquardt, 1996).

Ochratoxins have been demonstrated to have nephrotoxic effects on all mammalian species (Pitt *et al*., 2000). Hasinoff *et al*. (1990) suggested that an ochratoxin A-iron chelate catalyzed the formation of a reactive oxygen species, such as hydroxyl radicals, and that this complex in the presence of NADPH-cytochrome P-450 reductase system stimulated lipid peroxidation. The main clinical pattern associated with acute ochratoxicosis include, initial anorexia, weight loss followed by passage of clots of bloodstained mucus and death. Birds dying of acute toxicosis show symptoms of listlessness, huddling, occasional diarrhoea, ataxis and prostration. Chronic effects in pigs have been observed at ochratoxin A dietary concentrations of greater than 2.3 mg/kg feed. This was associated with reduced feed consumption, growth reduction, increased water consumption and polyuria (Marquardt, 1996; Pitt *et al*., 2000). Ochratoxin has been shown to cause renal disturbances, which are associated with damage to the proximal tubules and thickening of the glomerular basement membrane, and are manifested by a reduced ability to produce concentrated

urine. Ochratoxin is also a potent teratogen, producing gross malformation of the foetus, can affect carbohydrate metabolism particularly in chickens, is able to suppress the immune system and is one of the most potent mycotoxin carcinogens that has been identified. The toxic effects of ochratoxin are also greatly influenced by the nature of the diet and by age and species of animals (Marquardt, 1996; Santin *et al*., 2002).

1.4 Fusarium toxins

Fusarium species are capable of producing several mycotoxins, including the fumonisins, zearalenone, the fusarins, moniliformin and the trichothecenes. The trichothecenes included T-2 toxin, fusaric acid, deoxynivalenol, nivalenol, 3-acetyl deoxynivalenol, diacetoxyscirpenol and HT-2 toxin (D'Mello *et al*., 1999; Li *et al*., 2000).

Fusarium graminearum is a plant pathogen known to produce deoxynivalenol (also referred to as vomitoxin), nivalenol and zearalonone. It grows on gramineous plants, especially wheat, and is the most widely distributed *Fusarium* species (Marasas *et al*., 1984). *F. graminearum* grows optimally at 24-26 °C and optimum pH values are temperature dependent. The pathogen, *Fusarium monoliforme*, which produces fumonisin B_1, grows optimally between 22.5-27.5 °C (Pitt *et al*., 2000).

Zearalenone and its metabolites possess oestrogenic activity in pigs, cattle and sheep (D'Mello *et al*., 1999) and therefore it is possible that this toxin may play a role in hormonal balance and mammary cancer in regions with high zearalenone ingestion (Pitt *et al*., 2000). According to Etienne & Dourmad (1994) zearalenone reduces embryonic survival, and sometimes foetal weight, when administered above a threshold level. It may also affect the uterus by decreasing LH and progesterone secretion and by altering the morphology of uterine tissues.

Exposure to fumonisin B_1 in maize causes leukoencephalomalacia in horses (Marasas *et al*., 1988). It also causes pulmonary oedema in pigs (Harrison *et al*., 1990) and damage to the central nervous system, liver, pancreas, kidneys and lungs in a number of animal species (Pitt *et al*., 2000). Decreased performance and immune responses have been shown in calves consuming fumonisin B_1 (Osweiler *et al*., 1993). Sydenham *et al*. (1990) found a significant positive correlation between fumonisin intake and rates of human oesophageal cancer in Transkei and southern Africa. According to Pitt *et al*. (2000) fumonisins cause neurological diseases in humans by altering

sphingolipid metabolism and disrupting the barrier function of endothelial cells. Fumonisin B_1 caused poor performance, elevated free sphinganine and sphinganine: sphingosine ratios, increased organ weights, decreased immune responses and organ lesions in chicks (Ledoux *et al*., 1992; Qureshi and Hagler, 1992; Javed *et al*., 1993; Weibking *et* al., 1993a, Li *et al*., 1997; Li *et al*., 2000), turkey poults (Weibking *et al*., 1993b and c) and ducklings (Bermudez *et al*., 1995). Zacharias *et al*. (1996) observed dysfunction of sphingoid metabolism in chick embryos exposed to fumonisin B_1.

Moniliformin caused poor performance, increased serum pyruvate levels, enlarged hearts and cardiac lesions in broilers (Ledoux *et al*., 1995; Reams *et al*, 1997), turkey poults (*Ledoux et al*., 1993; Li *et al*., 2000) and ducklings (Morris *et al*., 1997).

General signs of trichothecene toxicity in animals include weight loss, decreased feed conversion, feed refusal, vomiting, bloody diarrhoea, severe dermatitis, haemorrhage, decreased egg production, immunosuppression and death. Commercial diets containing 4.8 to 16 mg T-2 toxin/kg resulted in oral lesions, increased spleen and pancreas weight, decreased bursa weight and reduced growth rates when fed to day-old broiler chicks for three weeks. The toxicity of different forms of the trichothecenes differs greatly and is influenced by species of animal (Rotter *et al*., 1995; Marquardt, 1996; Pitt *et al*., 2000). Various trichothecenes inhibit hepatic protein synthesis to different degrees and cause an increase of tryptophan in both blood and brain. Tryptophan is a precursor of the neurotransmitter serotonin and the serotonergic neurons are important mediators in such behaviours as appetite, muscular coordination and sleep (Pitt *et al*., 2000).

According to Berek *et al*. (2001) T-2 toxin, produced mostly by *Fusarium sporotrichioides*, almost completely suppressed IL-2 and IL-5 production at 5 ng/mL or more. Marin *et al*. (1996) reported that T-2 toxin inhibited cytokine production and proliferation. Deoxynivalenol modulated cellular and humoral immune function by altering mitogen-induced proliferative response of murine lymphocytes, viability of splenic lymphocytes and host resistance (Bondi & Pestka, 1991; Meky *et al*., 2001). Berek *et al*. (2001) found that nivalenol and deoxynivalenol had an inhibitory effect on the mitogen-induced blast transformation and natural killer reaction. Natural killer reactions play a significant role in immunological defense reactions against malignancy and tumor cells. Thuvander *et al*. (1999) studied the effect of all four trichothecenes (T-2 toxin, diacetoxyscirpenol, nivalenol and deoxynivalenol) on human lymphocyte cultures. All four of the tested trichothecenes

effectively inhibited mitogen-induced lymphocyte proliferation and immunoglobulin production in human lymphocytes in a dose-dependent manner with limited variation in sensitivity between individuals. Enhanced immunoglobulin production was observed in cell cultures exposed to low doses of the trichothecenes. Combined exposure to two of the toxins resulted in additive or antagonistic effects, which indicates that the total intake of toxins should be taken into account in risk assessment.

1.5 Citrinin

Citrinin, produced by the filamentous fungus, *Monascus ruber* (Hajjaj *et al*., 2000), causes kidney damage in laboratory animals similar to pig nephropathy, and may interact synergistically with ochratoxin A as it often occurs with this toxin (Marquardt, 1996; Vrabcheva *et al*., 2000).

1.6 Ergot alkaloids

Ergotism is a classic mycotoxicosis caused by the plant parasitic fungi of the *Claviceps* species. The fungi produce a sclerotium in small grains such as rye, barley and wheat (Marquardt, 1996). The sclerotia (ergots) contain alkaloids that can be divided structurally into three groups, e.g. simple lysergic acid amines, peptide derivatives of lysergic acid amides and the clavines. The most biologically active are those with a peptide moiety, especially ergotamine and ergotoxine. The actions of ergot alkaloids are varied and complex. In general, the effects result from actions as partial agonists or antagonists at adrenergic, dopaminergic and serotonergic receptors. The spectrum of alkaloid effects and the differences in activity among individual compounds are a function of chemical configuration resulting in differences in potencies at various receptors and differences in the manner in which each interacts with different receptors (Bakau & Bryden, 1998).

Ergot alkaloids have many effects on animals and birds including neurohormonal, peripheral and central nervous effects. The peptide derivatives of lysergic acid are stimulatory of smooth muscles (both vascular and non-vascular). This direct action is often expressed in the form of vasoconstriction, local ischemia and gangrene (Bakau & Bryden, 1998).

Bragg *et al*. (1970) and Rotter *et al*. (1985) found that young chicks are very susceptible to ergot toxicity, tolerating dietary levels up to 0.3 – 0.8% before growth, feed intake and feed conversion efficiency are affected. Ingestion of higher dietary levels can result in poor feathering, nervousness, incoordination and gangrene of the foot and beak (Bragg *et al*., 1970). Ergot toxicity affected egg

production, egg shape and egg shell thickness, at levels greater than 1.2% in the diet of layers (Johnson & Sell, 1976). According to Bakau & Bryden (1998) the clinical expression of ergotism is affected by ambient temperature. Birds fed ergotamine and exposed to a high temperature were most affected with respect to growth rate, feed intake and feed conversion efficiency. However, birds ingesting ergotamine at a high temperature had less severe gangrenous lesions and they did not develop foot lesions. It is possible that the high temperatures counteracted the vasoconstrictive effects of ergot alkaloids on peripheral blood vessels, preventing ischemia, necrosis and gangrene.

1.7 Aflatoxins

The aflatoxins are the toxins of greatest concern worldwide. The aflatoxins are potent liver toxins and their effects vary with dose, length of exposure, species, breed and diet or nutritional status. These toxins may be lethal when consumed in large doses. Substantial doses produce an acute toxicity and chronic exposure to low levels can result in cancer (Marquardt, 1996).

Aflatoxin will be discussed in more detail in a separate section.

1.8 Limits and regulations for mycotoxins

The contamination of agricultural commodities with mycotoxins may present a serious health hazard to both humans and animals. Although worldwide regulations exist to control aflatoxins in animal feed, similar legislation is not available for any of the *Fusarium* mycotoxins, including the carcinogenic fumonisins (Placinta *et al*., 1999). However, many countries have established some measures to control the contamination of these toxins in food and animal feedstuff. These limits are imposed to protect animal health as well as the health of potential consumers of contaminated animal products and to limit financial losses due to the adverse effects of mycotoxins on animal productivity (Marquardt, 1996). In the USA, advisory directives exist for concentrations of deoxynivalenol in grains and by-products. A value of 10 mg/kg has been set for such feeds offered to cattle and chickens and a level of 5 mg/kg for pigs (Trucksess *et al*., 1995).

In 1961/62 the FAO and WHO established Codex Alimentarius to elaborate international food legislation, including provisions for mycotoxins in foods and feeds. Chemical contaminants and toxins are handled by the Codex Committee for Food Additives and Contaminants. The Codex Alimentarius system for developing legislation concerning contaminants including mycotoxins in food is based upon the Codex General Standard for Contaminants and Toxins in Food, which also

covers feeds and raw commodities. The principles for laying down Maximum Limits for contaminants and toxins in foods and feeds within Codex Alimantarius are agreed, and work is in progress to establish the Maximum Limits for some mycotoxins, in particular in cereals. The body responsible for the risk assessment component of the Codex Alimentarius risk analysis process is the FAO/WHO Joint Expert Committee on Food Additives. In the European Union, a similar process is in progress. The EU regulations and proposals are roughly similar to the worldwide Codex legislation, but contain more detail (Berg, 2002).

No allowance has been made in the past for additive or synergistic effects arising from co-occurring mycotoxins (D'Mello *et al*., 1999; Thuvander *et al*., 1999). A synergistic toxicity existed between aflatoxin and ochratoxin A (Huff *et al*., 1983; Raju & Devegowda, 2000), between aflatoxin and T-2 toxin (Raju & Devegowda, 2000) and between AFB_1 and fumonisin B_1 (Pozzi *et al*., 2000; Carlson *et al*., 2001), to increase their general toxicity. Huff *et al*. (1986) also found that the effects of a combination of aflatoxin and deoxynivalenol on broiler performance and health were more severe than the individual effects of these mycotoxins. Sandhu *et al*. (1995) discovered that body hepatitis was more severe with concurrent inclusion of aflatoxin and ochratoxin, and it is therefore necessary to take animal health into consideration. Also of cardinal importance is the choice of performance and biochemical criteria when selecting tolerance criteria (D'Mello *et al*., 1999).

The most difficult and controversial aspect of the risk assessment for humans, especially for carcinogenicity data, is the extrapolation of toxicology data from animals to humans, using safety factors or other methods, to arrive at an estimate of safe intake. In the extrapolation of animal toxicity data to humans, it is important to consider species differences, such as differences in absorption and binding to plasma and tissue constituents. These latter species differences may affect the mobility of the mycotoxin between different body compartments, and the ultimate target tissue. Species differences in biotransformation and in plasma and tissue half-life are also important (Kuiper-Goodman, 1991).

Overall risk extrapolation from animal data to humans involves (Kuiper-Goodman, 1990):

1. Extrapolation from high doses within the experimental range of animal experiments to low doses usually outside the experimental range to which humans might be exposed;
2. Extrapolation from test species to humans; and
3. Extrapolation to the most sensitive subgroup of humans.

1.9 Prevention of mycotoxin production

Prevention of mycotoxin production includes the use of proper cultural practices such as minimizing the amount of crop residues in the field, so as to reduce the source of inoculum when susceptible crops are grown. Crop rotation can also reduce the impact of crop diseases while the development of crops that are genetically resistant to fungal infection can be a long-term solution (Marquardt, 1996). Substantial effort has been made to identify maize genotypes that resist infection by *Aspergillus flavus* (Brown *et al*., 1995). Tubajika & Damann (2001) found that drought-tolerant maize genotypes might be used as potential sources of resistance to *Aspergillus* ear rot and aflatoxin production.

Avoiding mycotoxin accumulation in stored grains and oil seeds depends primarily on the moisture content, as fungal growth will not occur on dry seeds. Antifungal agents, if of low toxicity, can also be used to reduce fungal growth and mycotoxin contamination. Blasticidin A showed strong inhibitory activity toward aflatoxin production by *Aspergillus parasiticus* (Sakuda *et al*., 2000). High moisture grains have been treated with volatile organic acids such as propionic and acetic acids (Marquardt, 1996). Van den Berghe *et al*. (1990) tested the effect of a mould-inhibitor, based on propionic acid, lactic acid, acetic acid and ascorbic acid, which was added to a feed at the rate of 500 g/ton. The mould inhibitor affected mould count significantly when compared with the control for the starter feed at two, four and six weeks of storage and for the finisher feed at two weeks of storage.

Phenolics are secondary plant metabolites synthesized via the phenylpropanoid biosynthetic pathway. These compounds are building blocks for cell wall structures and serve as defence against pathogens (Hahlbrock & Scheel, 1989). Eugenol (4-allyl-2-methoxy phenol), the active principle of cloves (*Syzygium ar*omaticum) and of medicinal and aromatic plants, such as *Ocimum sanctum* and *Pimenta racemosa*, is generally used as a food-flavouring agent (Jayashree & Subramanyam, 1999). Eugenol exerts its non-mutagenic and non-carcinogenic properties through its antioxidant effects, including the inhibition of Cu^{2+}-H_2O_2 catalysed lipid peroxidation (Nagashima, 1989). Jayashree & Subramanyam (1999) found that eugenol inhibited aflatoxin production by *Aspergillus parasiticus* (strain NRRL 2999) in a dose-dependent manner up to a concentration of 0.75 mmol/L without inhibiting growth. When the mould was grown for three days in the presence of 0.45 mmol/L eugenol, *in vivo* activities of components of polysubstrate mono-oxygenase were decreased at idiophase, concominant with decreased activities of enzymes involved in free radical scavenging,

lipid peroxidation and maintenance of redox potential. These results indicated that anti-aflatoxigenic actions of eugenol might be related to inhibition of the ternary steps of aflatoxin biosynthesis involving lipid peroxidation and oxygenation.

Hua *et al.* (1999) evaluated the effects of three phenolics acetosyringone, sinapinic acid and syringaldehyde on aflatoxin biosynthesis in *Aspergillus flavus*. All three phenolics inhibited AFB_1 biosynthesis with acetosyringone being the most effective (4 mmol/L reduced aflatoxin biosynthesis by 96%).

1.10 Detoxification and modified feeding strategies

Various detoxification procedures have been employed with limited success. This includes mechanical separation of infested grain, thermal inactivation, irradiation, solvent extraction and microbial inactivation. Many chemicals have been tested for their ability to structurally degrade and/or inactivate aflatoxins including numerous acids, bases, aldehydes, bisulfates and various gases. Ammoniation, under certain conditions, has resulted in a significant reduction in the level of aflatoxins in contaminated peanut and cottonseed meals (Marquardt, 1996). No adverse effects were observed in production rate, egg quality, reproduction, feed efficiency, mortality rates (Hughes *et al*., 1979) or antibody titres against Newcastle disease (Boulton *et al*., 1982) after chickens were fed aflatoxin-contaminated maize that had been ammoniated. Neal *et al*. (2001) subjected a sample of peanut meal, highly contaminated with aflatoxins, to decontamination by two commercial ammonia-based processes. It was concluded that the aflatoxin levels in the peanut meal had been reduced by both the ammonia-based processes to acceptable levels, but different effects *in vivo* were noticeable when incorporated into animal diets. Other than ammoniation, many of the techniques proposed to remove mycotoxins are currently perceived to be impractical, ineffective and/or potentially unsafe for large-scale utilisation (Marquardt, 1996).

Various dietary treatments have also been employed to reduce the toxicity of mycotoxins. This includes the use of chemisorbents with the capacity to tightly bind and immobilize mycotoxins in the intestinal tracts of animals. These treatments may not be cost effective and may bind other nutrients (Marquardt, 1996).

Nutrients can also affect the toxicity of mycotoxins. For example, free-radical scavenging compounds such as vitamin E and C and β-carotene have been shown to reduce the toxicity of

ochratoxin A, while compounds that enhance lipid peroxidation such as dietary iron, copper and unsaturated fats tend to have an opposite effect. Vitamin E additions to the diet at high concentrations have been shown to dramatically reduce the production of lipid peroxides and the corresponding toxicity of ochratoxin A (Marquardt & Frohlich, 1992). Haazele *et al*. (1993) demonstrated that ascorbic acid supplementation (300 mg/kg) of laying hen diets, which contained 3.0 mg ochratoxin A/kg, partially ameliorated the toxicity effects, including the negative effects on egg mass production and rigidity of eggshells. Malaveille *et al*. (1994) reported that a water-soluble form of vitamin E completely quenched the genotoxic effects of ochratoxin A in *Escherichia coli.* Dietary use of selenium, in combination with ascorbic acid and α-tocopherol, provided protection against acute toxicosis caused by the trichothecenes, deoxynivalenol and T-2 toxin (Rizzo *et al*., 1994). Grosse *et al*. (1997) observed that vitamin A, C and E reduced DNA adducts in kidney and liver by 70 to 90% in mice exposed to ochratoxin and zearalenone. Hoehler & Marquardt (1996) showed that T-2 toxin stimulates lipid peroxidation in yeast cells due to an increased free radical generation that might be partially counteracted by an antioxidant such as vitamin E, but not vitamin C. Based on a study on rats, Atroshi *et al*. (1995) concluded that selenium, vitamin E and vitamin C act as antioxidants and free radical scavenger systems that protect the spleen and brain against membrane damage caused by T-2 toxin and deoxynivalenol.

Another management strategy is to feed the toxin-contaminated feed to animals that can best tolerate them. Non-pregnant and older animals, particularly ruminants, tend to be more resistant to certain toxins or are better able to metabolize toxins compared to other classes of livestock. Animals to be marketed, however, should be free of mycotoxins (Marquardt, 1996).

2. Aflatoxins

The aflatoxin problem is long-standing and inextricable (Phillips, 1999). Aflatoxin is the most prevalent and economically significant mycotoxin apt to be consumed by poultry. It is found in maize, peanuts, cottonseed, millet, sorghum and other feed grains. *Apergillus flavus* produces the majority of the toxin, which explains the origin of its name, but aflatoxin is also produced by *A. parasiticus*. Moss (1996) mentioned a more recently recognized third species, *A. nomius*. The fungi are found widely in the environment, contain toxigenic and non-toxigenic strains, and produce aflatoxin in warm (30-35 °C) and high humidity (water activity of 0.90-0.99) conditions. Aflatoxin contamination is thus more likely in grains grown or handled in the tropics or subtropics. Stressing

host plants by insect damage, drought, poor nutrition or delayed harvest increases aflatoxin production (Brown, 1996). According to Moss (1996) the aflatoxin levels of foods for human consumption often exceed the upper limit of 30 µg/kg initially established by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO). Meanwhile, many developed countries had set legislative or guideline levels that were more stringent than the limits set initially by the FAO/WHO. Grains and oil seeds, adulterated with aflatoxins at concentrations higher than 20 µg/kg are condemned in the United States. However, in lesser-developed countries with limited supplies of food, it is not always feasible, nor practical, to condemn and destroy mouldy grains. Thus, these practices may result in enhanced aflatoxin exposure and a higher incidence of aflatoxin-induced disease in humans and animals.

Like many microbial secondary metabolites, the aflatoxins are a family of closely related compounds (Moss, 1996). Naturally occurring aflatoxin consists of aflatoxins B_1, B_2, G_1 and G_2, but AFB_1 is usually in the highest concentration and is the most toxic. According to Moss (1996) AFB_1 is an acute toxin as well as being carcinogenic and immunosuppresive but in contrast to T-2 toxin, the precise nature of the response to the consumption of aflatoxin is dependent on species, sex and age. Aflatoxin is stable once formed in grain, and is not degraded during normal milling and storage (Brown, 1996).

According to Lanza *et al*. (1980) chickens of different ages showed differential susceptibility to different aflatoxin doses. Quezada *et al*. (2000) found that one-week-old broilers were more susceptible to aflatoxin than 4-week-olds. These findings are in agreement with the results reported by Edrington *et al*. (1997).

There are also large species differences, with ducks being 10 times more sensitive than chickens, and turkeys intermediate between the two (Brown, 1996). Giambrone *et al*. (1985b) found that aflatoxin is highly toxic to 2-week-old turkeys and mildly toxic to 2-week-old broilers when fed daily for 5 weeks at 0.4 mg/kg and 0.8 mg/kg feed. The day-old duckling and the adult dog are remarkably sensitive to the acute toxicity of AFB_1, with LD_{50} values of 0.35 and 0.5 mg/kg body weight respectively, while others, such as the adult rat and the mouse, are more resistant (LD_{50} ca. 9 mg/kg). Not all animals respond to the carcinogenic activity of aflatoxin, but for the rat and rainbow trout, AFB_1 is one of the most carcinogenic compounds known. Indeed, as little as 4 µg/kg in the diet of rainbow trout gave rise to 25% incidence of hepatoma within 12 months. There have

been a number of isolated cases of deaths in humans attributed to aflatoxin in food. A particularly tragic demonstration of the acute toxicity of aflatoxin in humans was reported in India in 1974, when a large outbreak of poisoning occurred, involving nearly 1000 people, of whom nearly 100 died (Moss, 1996). Moss (1996) estimated the LD_{50} for AFB_1 in humans as approximately 5 mg/kg body weight, thus lying somewhere between that for the dog and the rat.

Many animal studies have shown that the female is more resistant to all aspects of the toxicity of aflatoxin than the male, and these observations may also be true of men and women. Reports in 1987 of studies from the former Czeckoslovakia of patients with primary liver cancer had shown, by using a radioimmunoassay, that AFB_1 was detected in the livers of 27 of 34 patients studied. Although dietary exposure to aflatoxin also occurs in India, the incidence of liver cancer is less than in Africa and it may be possible that genetic differences may play a role here. There is still a lot to learn about the role of aflatoxin in liver diseases in different parts of the world.

Practical methods to detoxify mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. A variety of physical, chemical, and biological techniques have been employed but with only limited success (Edrington *et al*., 1997).

2.1 Absorption

Aflatoxins are very liposoluble compounds and are therefore readily absorbed from the site of exposure into the bloodstream. Absorption of ^{14}C-labelled aflatoxin administered orally to laying hens occurred very rapidly from the gastrointestinal tract (Sawhney *et al*., 1973). Absorption of aflatoxin from the respiratory system has been reported in workers at feed mills, although there have been no studies to determine the quantitative importance of this route of absorption of aflatoxins in poultry (Leeson *et al*., 1995).

2.2 Metabolism

After absorption from the small intestine, AFB_1 readily binds to plasma albumin, which serves as the major transporter of AFB_1 in blood (Yatin & Sachan, 2001). When one sees a diverse range of responses to the toxic effects of a compound it is a reasonable assumption that the compound is metabolized in the animal body and that the resulting toxicity is the final result of this metabolic activity. This is certainly the case with AFB_1, from which a very wide range of metabolites is formed in the livers of different animal species (Moss, 1996). AFB_1 is oxidized by microsomal

mixed-function oxidase (cytochrome P450) to several water-soluble metabolites. It is generally believed that the formation of AFB_1-8,9-epoxide, an active metabolite, and its subsequent covalent binding to DNA, RNA and proteins plays a critical role in both acute and chronic toxicity (Choy, 1993; Eaton & Gallagher, 1994). Animals, which fail to produce the epoxide are relatively resistant to both, but those animals which produce the epoxide, but do not effectively metabolize it further, may be at the highest risk to the carcinogenic activity of AFB_1, but relatively resistant to the acute toxicity. Those animals that not only produce the epoxide but also effectively remove it with a hydrolase enzyme, thus producing a very reactive hydroxyacetal, are most sensitive to the acute toxicity (Moss, 1996). In most animal models examined, glutathione S-transferases are probably the most critical AFB_1-detoxicity enzymes. The extreme sensitivity of animal species such as turkeys, to AFB_1 may be due to a combination of efficient activation by cytochrome P450 and deficient detoxification as a result of the absence of AFB_1-specific glutathione S-transferase activity in the liver (Klein *et al*., 2000). The glutathione S-transferase isozyme M1a-1a appears to be the most active in conjugating 8,9-epoxide in humans (Johnson *et al*, 1997). This isozyme is expressed as null phenotype in approximately 50% of the Caucasian population, and individuals so expressing it may be at an increased risk of developing chemically induced cancers (Ryberg *et al*., 1997).

The epoxide of AFB_1 is known to react with guanine residues in DNA, and can cause subsequent depurination, while the hydroxyacetale derivate reacts with proteins through such residues as lysine. The parent molecule may thus be seen as a very effective delivery system, having the right properties for absorption from the gut and transmission to the liver and other organs of the body. It is, however, the manner in which it is subsequently metabolized *in vivo* which determines the precise nature of the animal's response (Moss, 1996).

An alternative biotransformation of AFB_1 to aflatoxicol is mediated by a reductase, and the product can be reconverted to AFB_1 by a dehydrogenase. AFB_1 reductase and aflatoxicol dehydrogenase are not well studied, but this mechanism is considered to be very important because aflatoxicol may play a role as a reservoir of AFB_1 in some organisms (Lee *et al*., 2001).

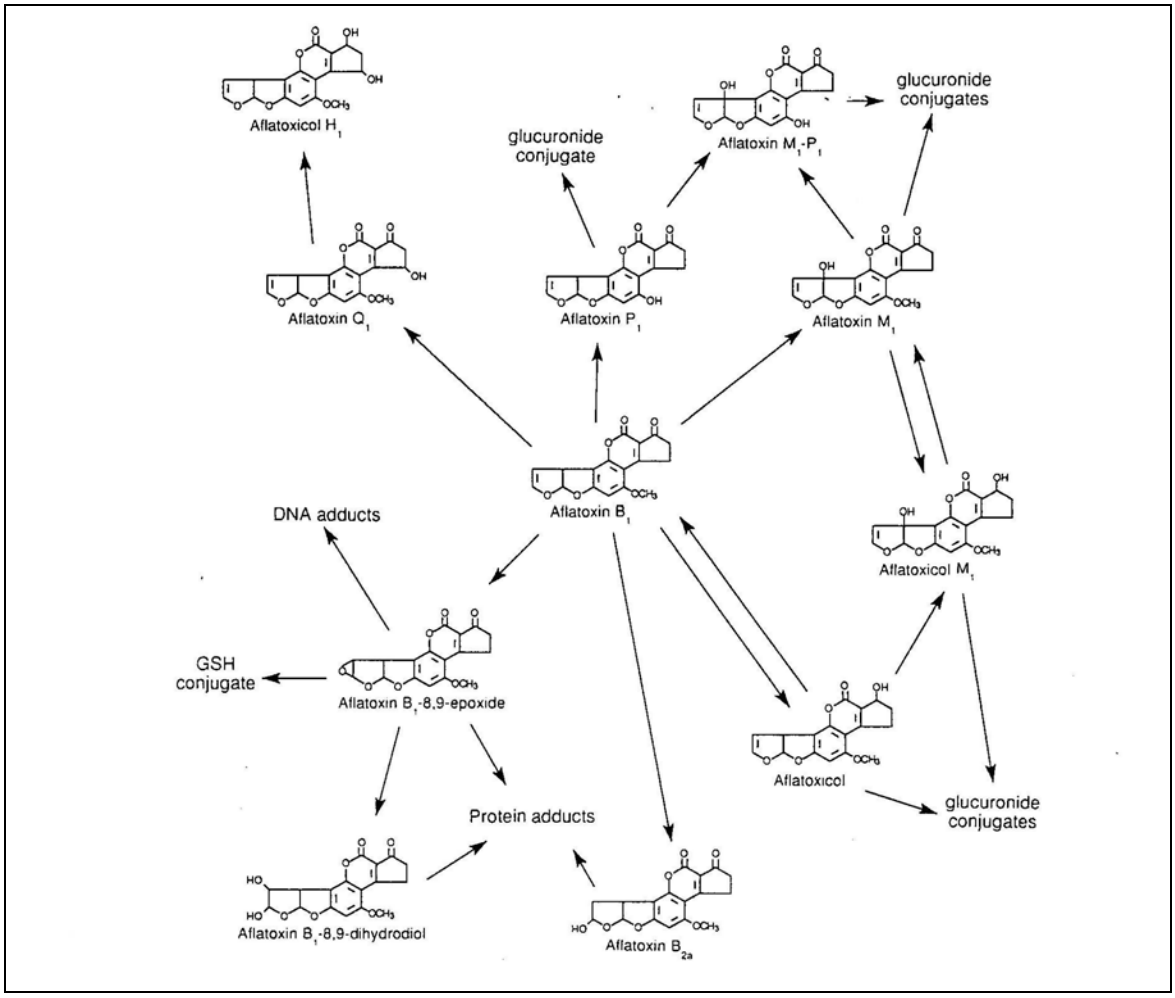

Figure 1.1. Biotransformation pathways for aflatoxin B_1 (Eaton *et al.*, 1994)

2.3 Elimination

Aflatoxin is rapidly excreted in the bile and urine and does not accumulate or persist in body tissues. This perhaps explains the rapid recovery of egg production and hatchability after cessation of toxin ingestion (Brown, 1996). Truckess *et al.* (1983) fed 18 laying hens an aflatoxin-contaminated diet (8 mg AFB_1/kg feed) for seven days after which half of the group was sacrificed, while the remainder was sacrificed after feeding an aflatoxin-free diet for seven more days. AFB_1 and its metabolite aflatoxicol were detected in the eggs and edible tissues of all hens sacrificed seven days after exposure. Liver and ova contained the highest levels of AFB_1 and aflatoxicol. Seven days after withdrawal, only trace amounts of aflatoxicol were detected in eggs and no aflatoxin residues were found in edible tissues.

2.4 The effects of aflatoxins in animals

2.4.1 Liver

AFB_1 is known as a potent hepatotoxin and hepatocarcinogen and the liver is considered to be the primary target for aflatoxins (Towner *et al*., 2000). The toxic metabolites of aflatoxin bind to nucleic acids and nucleoproteins, essential to cellular viability, and result in an excessive build-up of hepatic lipids, with enlargement of the liver, proliferation of bile duct epithelium (Adav & Godinwar, 1997), necrosis (Kichou & Walser, 1994) and hepatocellular carcinoma (Hamilton, 1978; Van Rensburg *et al*., 1985; Peers *et al*., 1987; Bammler *et al*., 2000).

AFB_1 must be transformed by cytochrome P450 enzymes to the reactive intermediate AFB-8,9-epoxide to exert its hepatocarcinogenic effect. Significant species differences exist in susceptibility to AFB-induced carcinogenesis. For example, rats are highly sensitive, whereas mice are quite resistant. The resistance of mice to AFB hepatocarcinogenicity appears to be due solely to the constitutive expression of a specific form of glutathione S-transferase, mGSTA3-3, with unusually high catalytic activity toward AFB-8,9-epoxide (Buetler *et al*., 1992).

With high-dose acute exposure, fat accumulates as clear vacuoles in the cytoplasm of hepatocytes in a dose- and time-dependent fashion, and results in grossly evident large, friable, yellow livers. With continued exposure intra-hepatic biliary epithelial hyperplasia occurs as an attempt to regenerate the hepatic parenchyma when the parenchymal cells themselves have lost their capacity. Such hepatobiliary hyperplasia resulted in a significant increase of alanine aminotransferase, gamma-glutamyltransferase and total bilirubin (Zaky *et al*., 1998). Extramedullary haematopoiesis may also occur in response to a toxin-induced anaemia. Petechial haemorrhages or bruises after trauma are increased due to decreased clotting factor synthesis and increased capillary fragility (Brown, 1996).

Adav & Godinwar (1997) found that the administration of AFB_1 to chickens caused a significant increase in liver microsomal protein, electron transport components and drug metabolizing enzymes. No alteration, however, was found in the activity of alanine and aspartate aminotransferases.

In a study on young rabbits, Verma & Raval (1996) observed a reduced glycogen content of the liver and skeletal muscle after consumption of feed contaminated with 15 mg aflatoxin/kg feed, for 60 days. This could be due to enhanced glycogenolysis (breakdown of glycogen in liver). Aflatoxin consumption also caused hyperglycaemia in the rabbits, possibly the result of accelerated glycogenolysis, gluconeogenesis (production of glucose in the liver) or release of glucose from the liver into the bloodstream. In addition, the activities of certain enzymes such as aspartate amino transferase and alanine aminotransferase, which are responsible for the transamination of amino acids, increased in the liver. This indicated enhanced utilisation of proteins or amino acids in the process of gluconeogenesis during aflatoxicosis. The significant increase of lactate dehydrogenase activity, a cytoplasmic enzyme present in the liver, indicated the increased conversion of lactate to glucose.

Free amino acid content of plasma and urine increased markedly and progressively in aflatoxin-fed rabbits. Hyperaminoacidaemia could be due to reduced utilisation of amino acids in the biosynthesis of proteins and/or increased degradation of proteins. Aflatoxin is known to reduce protein biosynthesis by forming adducts with DNA, RNA and protein; and to inhibit RNA synthesis and DNA dependent RNA polymerase activity, as well as causing degranulation of endoplasmic reticulum. Extensive cellular necrosis in liver and kidney could also cause a reduction in total number of cells thus impairing protein synthesis and secretion (Verma *et al*., 1997).

In a study from Anh Tuan *et al*. (2002) Nile tilapia received diets containing up to 100 mg AFB/kg feed for eight weeks. Lesions were observed in the liver but not in the spleen, heart, kidney, stomach or intestine. This is consistent with previous reports that the liver is the organ in fish most susceptible to injury by AFB (Hendricks, 1994). Fish fed a diet containing 10 mg AFB/kg had pleomorphic nuclei and lipofuscin in the liver, and these changes were more severe in fish fed a 100 mg AFB/kg diet. Livers of fish fed 100 mg AFB/kg had basophilic, spindle-shaped cells that were mitotically active. However, these basophilic cells surrounded ballooning necrotic cells and did not form well-defined nodules. El-Banna *et al*. (1992) observed vacuolization and necrosis of hepatocytes in Nile tilapia fed 0.05-0.2 mg AFB/kg in the diet. Hepatic necrosis and fibrosis in Nile tilapia fed AFB were also reported by Chávez-Sánchez *et al*. (1994).

Although the vast majority of the work on AFB_1 toxicology has focused appropriately on hepatic toxicity, AFB_1 also elicits significant effects in other organ systems. In most cases, such effects in

organs other than the liver are secondary. Factors such as route of administration, dose, and frequency of dose and species, strain, age, and sex of animal appear to affect the degree of extrahepatic involvement (Coulombe, 1994).

2.4.2 Respiratory system

According to Baxter *et al*. (1981) numerous surveys have shown that AFB_1 is virtually always present in grain dusts at levels often exceeding those found in contaminated food. Evidence linking inhaled AFB_1 to human lung cancer is tenuous. An epidemiological study found that workers in a Dutch peanut oil processing plant exposed to AFB_1-contaminated dusts experienced a significantly greater incidence of upper respiratory (trachea and bronchus) as well as liver tumors than unexposed subjects (Hayes *et al*., 1984). Isolated case studies also have described the development of tumors in people working with AFB_1-contaminated dusts (Dvorakova, 1976) and a significantly higher amount of aflatoxins in the sera of patients with lung cancer (Cusumano, 1991).

AFB_1 appeared in the blood more rapidly when it was dosed intratracheally instead of orally, although the overall plasma concentration-time plots of AFB_1 did not differ significantly between the two routes at later time periods. Following oral exposure, the amount of AFB_1 distributed into lungs and associated tissues was about half that distributed into the liver (Coulombe & Sharma, 1985a). Dust-absorbed AFB_1 administered intratracheally, distributed into the liver, kidney, spleen, thymus and brain as well as into the lung and major airways. At three hours post-administration, the level of AFB_1-DNA adducts in the trachea was higher than in the liver (Coulombe *et al*., 1991).

Ultrastructural analysis of upper airway tissues cultured with AFB_1 indicates that this mycotoxin is selectively toxic to only one population of airway cells. In cultures of hamster and rabbit tracheal epithelium, AFB_1 elicited significant ultrastuctural changes in the nonciliated cells but other cell types, such as the ciliated and basal cells were relatively unaffected. This selective toxicity is probably largely the result of the distribution of cytochrome P450 isozymes in the lung and airways, where nonciliated cells contain the majority of the components of the cytochrome P450 system. Nonciliated cells in rabbit upper airways contain two forms of rabbit lung pulmonary cytochrome P450 isozymes that are active in converting AFB_1 to mutagenic species (Coulombe *et al*, 1986).

As in the liver, considerable species differences exist with respect to AFB_1 activation, detoxification, and action in the lung. Cultured epithelial cells or microsomes from rabbit trachea

were more active in converting AFB_1 to DNA-binding species than those from hamster, in which AFB_1-detoxifying enzymes and reactions predominate. Tissues from rat trachea were relatively inactive in AFB_1 activation and detoxification (Ball & Coulombe, 1991).

According to Coulombe (1994) these *in vivo* and *in vitro* studies indicate that inhaled AFB_1 may be a significant health hazard, but further studies are necessary to establish the risk associated with inhaled AFB_1.

2.4.3 Renal System

Various portions of the renal nephron are exposed to AFB_1 or metabolites because a significant portion of ingested mycotoxin is excreted via the urine (Coulombe, 1994). In cattle given radiolabeled AFB_1, renal tissue contained the highest concentration of AFB_1 and metabolites (Hayes *et al*., 1977). Early experiments of Epstein *et al*. (1969) with laboratory rats demonstrated that oral AFB_1 is a renal carcinogen. The ultrastructural characteristics of renal neoplasms were characterized in Wistar rats receiving 1-3 mg AFB_1/kg feed (Merkow *et al*., 1973). The tumors, which were present in 50% of the animals receiving 3 mg AFB_1/kg feed, were most likely of tubular origin and were characterized by loss of apical orientation of brush borders.

Although the mouse is the most resistant animal known to the acute and chronic effects of AFB_1, the kidney appears to be the major target organ of AFB_1 in this species. Shortly after administration, this toxin causes massive hemorrhagic lesions of the kidneys. A single dose of AFB_1 suppressed [^{14}C]orotic acid incorporation into RNA by 50% and inhibited RNA synthesis in mouse kidney, but showed none of these effects in the liver (Akoa *et al*., 1971). In agreement with this observation, the level of AFB_1-DNA adducts in the kidney was about six times higher than that found in the liver after a single injection in the mouse. Controversially, in the rat, in which the main target organ is the liver, the level of adducts in the kidney was a small fraction of that seen in the liver (Croy & Wogan, 1981).

A single dose of AFB_1 (100 µg/kg) significantly decreased glomerular filtration, glucose reabsorption, and tubular transport of electrolytes and organic anions in Wistar rats 24 hours after administration, indicating that AFB_1 might exert its effects on glomerular basement membrane and renal tubules (Grosman *et al*., 1983). Ikegwuonu *et al*. (1980) focused on AFB_1-induced biochemical changes in the kidney and demonstrated that ip AFB_1 depressed activities of renal

glutamate-oxaloacetate and pyruvate transaminases, as well as alkaline phosphatase, in rats. In cultured kidney cell lines, AFB_1 induced aggregation and loss of chromatin, mitochondrial degeneration, and loss of microvilli (Yoneyama *et al*., 1987).

The susceptibility of poultry to AFB_1 is likely to be caused by the effect of this mycotoxin on the renal system. A fatty and hemorrhagic kidney syndrome that commonly occurs in chickens in Africa has been linked to AFB_1 (Dafalla *et al*., 1987). Thickening of glomerular basement membrane, abnormal development of glomular epithelial cells, and degenerative changes in renal tubular cells were observed in male broiler chicks given 2.5 – 5 µg/kg AFB_1 (Mollenhauer *et al*., 1989).

According to Verma & Kolhe (1998) the raised levels of creatinine in the serum and urine of aflatoxin-fed rabbits indicated the increased transformation of phosphocreatine to creatinine in muscle, which might be due to lower utilisation of phosphocreatine in muscular contraction. The kidneys normally rapidly excrete creatinine, and the increased concentration in the serum indicated severe kidney damage.

Verma & Raval (1997) observed increased activities of acid phosphatase in the kidney of aflatoxin-fed rabbits. This enzyme is possibly responsible for the reabsorption of nitrogenous materials other than urea and uric acid from the lumen. Increased acid phosphatase might thus enhance the reabsorption of amino acids and proteins present in ultrafiltrate.

2.4.4 Gastrointestinal System

The gastrointestinal tract is exposed to AFB_1 initially via the diet and subsequently to AFB_1 metabolites from the bile, which is the major route of excretion of this toxin (Coulombe, 1994). Experimentally, AFB_1 is a modest colon carcinogen. A small incidence of colon carcinomas occurred when AFB_1 was administered to Fisher rats either orally or via the diet (Wogan & Newberne, 1967), but this incidence was increased by concurrent low dietary vitamin A (Newberne & Rogers, 1973; Newberne & Suphakarn, 1977).

Autrup *et al*. (1979) used cultured colon tissues to study AFB_1 metabolism and activation in this tissue. Human colon tissue was capable of activating AFB_1 to form DNA adducts. As in hepatic and pulmonary systems, the principal AFB_1 adduct formed in cultured colon was the AFB_1-N^7-

guanine adduct (Autrup *et al*., 1979). Colon tissues formed fewer adducts than liver *in vivo*, although the qualitative patterns of adducts seen in the colon were similar. Small intestinal microsomes from the rat activated AFB_1 to mutagens and, based on cytochrome P450 content, were as efficient as hepatic microsomes. However, unlike hepatic microsomes, the AFB_1-activating ability of intestinal microsomes was not affected by the *in vivo* administration of phenobarbital or β-naphthoflavone (Walters & Combes, 1985).

In domestic animals, dietary AFB_1 alters many aspects of rumen function. For example, acute AFB_1 exposure decreased rumen motility in cows (Cook *et al*., 1986). AFB_1 reduced cellulose breakdown and production of volatile fatty acids and ammonia in both *in vivo* and *in vitro* rumen model systems (Mertens, 1977). Such effects on the gastrointestinal tract of domestic animals may contribute to the decreases in weight gain, feed conversion, and milk production observed when animals are fed AFB_1-contaminated feed (Coulombe, 1994).

2.4.5 Nervous System

AFB_1 may decrease the free tryptophan pool or alter biosynthesis of serotonin (Coulombe, 1994). In chickens, however, a single dose of 3 mg AFB_1/kg feed increased brain serotonin concentrations (Ahmed & Singh, 1984). Concentrations of brain catecholamines are altered by AFB_1. In rats, repeated intragastric AFB_1 resulted in decreases in dopamine in several brain regions (Coulombe & Sharma, 1985b). In chickens, a single dose of AFB_1 caused a depression in the concentration of norepinephrine (Ahmed & Singh, 1984). According to Coulombe (1994), the biochemical alterations in the central and peripheral nervous systems have been postulated to contribute to some of the many signs of overt toxicity in domestic animals that are observed after consumption of AFB_1-contaminated feeds, for example, changes in feeding patterns and appetite.

In addition to the biochemical effects, exposure to AFB_1 also causes tumors of the nervous system (Coulombe, 1994). Ip administration of AFB_1 elicited tumors in the central and peripheral nervous systems of pregnant Sprague-Dawley rats (Goerttler *et al*., 1980).

2.4.6 Reproductive system

Aflatoxicosis alters the reproductive efficiency of both male and female domestic animals, particularly poultry. Sexual maturity of young male Japanese quail, as assessed by plasma testosterone concentrations and testicular weight, was delayed when they were fed a diet containing

10 mg AFB_1/kg feed soon after hatching. Age differences with respect to AFB_1 sensitivity were observed. Quail given the AFB_1 diet between 7 and 21 days of age recovered earlier than those treated between 14 and 28 days after hatching (Ottinger & Doerr, 1980). AFB_1 also affects the reproductive potential of mature poultry. Reductions in semen volume, testes weight, spermatocrit, and plasma testosterone resulted when mature male leghorn chickens were given a diet containing 20 mg AFB_1/kg feed (Sharlin *et al*., 1981) and when *Babcock* breeder cocks received a ration with 5, 10 and 20 mg total aflatoxin/kg (Ortatatli *et al*., 2002). Pathologic changes in ovaries of laying hens given 8.1 mg AFB_1/kg feed were observed, which caused cessation of egg production from the first day until the end of the 21 day-feeding period. Histopathological studies of the testicles revealed no abnormalities (Hafez *et al*., 1982a). Qureshi *et al*. (1998) found that aflatoxin dietary exposure of broiler breeder hens resulted in embryonic mortality and reduction in hatchability compared to controls.

A single dose of 150 µg AFB_1 to rats significantly depressed Leydig cell function and responsiveness to human chorionic gonadotropin, although neither sperm production nor testosterone secreting ability was affected (Egbunike, 1982). More severe effects were seen in male and female rats chronically treated with AFB_1, for example, severe testicular degeneration, impaired spermatocytogenesis, smaller litter sizes and higher embryo mortality (Egbunike *et al*., 1980). Ikegwuonu *et al*. (1980) and Egbunike (1981) also reported altered *in vivo* biochemical end points such as sperm or testicular enzyme activities in rats due to AFB_1 treatment. A daily dose of 50 µg AFB_1/kg body weight, administered intraperitoneally daily to Swiss albino mice, reduced male fertility drastically. Sperm concentration in the epididymis and sperm motility decreased whereas sperm abnormalities increased. Epididymal function towards the post-testicular sperm maturation process was also altered by AFB_1 (Agnes & Akbarsha, 2003).

Hafez *et al*. (1982b) determined the effect of aflatoxin on semen quality of buffalo bulls. The addition of aflatoxin in the diet resulted in a marked decrease of percentage of live spermatozoa and a very high increase in sperm abnormalities.

2.4.7 Embryotoxicity

The carry-over of aflatoxin from layer feed to eggs has been demonstrated, where 500 and 600 µg/kg AFB_1 in the diet of layers resulted in a concentration of 100 ng/kg egg (Oliveira *et al*., 2000) and 300 ng/egg AFB_1 (Sudhakar, 1992), respectively. Aflatoxins have been reported to cause a

variety of disturbances in chick embryos, from early embryonic death to organ malformations (Kemper & Luepke, 1986), resulting in serious economic losses and decreased productivity in the hatchery (Çelik *et al*., 2000a). Trace amounts of aflatoxin found in eggs may cause functional defects in the immune system of the developing embryo with subsequent immunodeficiency (Neldon-Ortiz & Qureshi, 1992b).

The chicken embryo has been proposed to be one of the ideal materials for testing the embryotoxicity, mutagenity, teratogenity and carcinogenity of a variety of harmful agents (Ubeda *et al*., 1994).

Çelik *et al*. (2000a) evaluated the embryotoxicity of mixed aflatoxins and AFB_1 by a modified chick embryotoxicity screening test. Mixed aflatoxins and AFB_1 were injected into the eggs just prior to incubation at doses of 10, 100 and 1000 ng/egg and resulted in embryonic mortality and increased early deaths. In the groups that received 100 ng/egg mixed aflatoxins and AFB_1 an abnormal development was seen, with a protruded central region, corresponding to the *area pellucida* of the blastoderm. Roll *et al*. (1990) investigated AFB_1 and AFG_1 in NMRI mice for embryotoxic and teratogenic activity. AFB_1 produced moderate retardation in foetal development and a dose-related increase of cleft palates, wavy ribs and diaphragm changes. AFG_1 caused reduction of foetal weights, increase of diaphragm changes and malformations of kidneys. Veselý *et al*. (1983) reported that the sensitivity of embryos to aflatoxin administration decrease with age and established the rank order for embryotoxicity as $B_1 > G_1 > M_1 = B_2 > G_2$.

2.4.8 Cytotoxicity

Calcium plays an important role as an intracellular regulator of many physiological and pathological processes. Normally intracellular calcium homeostasis is achieved by the concerted operation of plasma membrane calcium translocases and intracellular compartmentalization systems. Disturbances of the mechanisms can result in enhanced calcium influx, release of calcium from intracellular stores and inhibition of calcium extrusion at the plasma membrane, which can lead to an uncontrolled rise in the intracellular calcium concentration. Such sustained increases in intracellular calcium will obliterate the transient calcium responses, compromise mitochondrial function, cytoskeletal organization and ultimately activate degradative processes and thus cell injury. Intracellular calcium accumulation may thus be a common step in the development of cytotoxicity (Nicotera *et al*., 1992). Verma *et al*. (1998) reported a rise in intracellular calcium

concentration in the organs of aflatoxin-fed rabbits. The rise in cytosolic calcium may affect the cytoskeleton and thereby damage cellular integrity either directly or through calcium activated proteases.

Verma & Raval (1991) revealed a concentration dependent increase in the rate of haemolysis, indicating AFB_1–induced cytotoxicity, which could be due to lipid peroxidation of plasma membrane, permeability alterations and cell lysis.

2.4.9 Immune System

Persistent observations that consumption of AFB_1-contaminated feed lowers disease resistance in domestic animals encouraged investigations into the possible effects of aflatoxin on the immune system (Coulombe, 1994). Several reports showed that the immune system is a highly sensitive indicator of aflatoxicosis in poultry (Giambrone *et al*., 1985b). Aflatoxin-induced immunosuppression may be manifested as depressed cellular (Giambrone *et al*., 1978) and humoral immune reactions. Although a definite explanation for the immune suppressive effect of aflatoxin has not yet been found, Corrier (1991) proposed the following possible mechanisms. Aflatoxin depresses protein synthesis via inhibition of RNA polymerase, which results in suppression of specific immunoglobulin synthesis (Giambrone *et al*., 1985a,b). Nonspecific factors such as complement, interferon and nonspecific serum protein concentrations also decrease as a result of liver damage (Tung *et al*., 1975; Stewart *et al*., 1985).

The supplementation of 0.1 mg or more per kg feed, of a mixture of AFB_1 and AFB_2 to young broiler chicks (Giambrone *et al*., 1985a, b) and rats (Raisuddin *et al*., 1993) led to reductions in cell-mediated immunity, as measured by a delayed hypersensitive skin test. A depression of phagocytic efficiency was observed in chickens fed 0.3 (Kadian *et al*., 1988) and 2.5 mg AFB_1/kg feed (Ibrahim *et al*., 2000). Steers fed a maize ration naturally contaminated with AFB_1 showed decreases in cutaneous hypersensitivity (Richard *et al*., 1983) and Kadian *et al*. (1988) observed a depression of delayed hypersensitivity in aflatoxin-fed chickens. Long-term AFB_1 treatment of weanling rats caused, in addition to a depletion of cell populations of the thymus, reduced bone marrow and red and white cell counts, a depression of macrophage numbers, with reduced phagocytic capacity (Raisuddin *et al*., 1990).

Ubosi *et al*. (1985) observed a reduction in thymus weight in Leghorn chickens receiving AFB_1 at a concentration of 1.83 mg or more per kg feed and a reduction in bursa weight at a concentration of 0.966 mg or more per kg feed. Giambrone *et al*. (1985b) couldn't find an effect of aflatoxin (up to 0.8 mg/kg feed) on antibody synthesis or on thymic or bursal development. Lymphoid organs of chicks given 2.5 mg aflatoxin/kg feed were markedly reduced in size (Çelik *et al*., 2000b). In those animals, lymphoid cell depletion in cortical and medullary areas of both thymuses and bursal follicles were prominent.

Boulton *et al*. (1982) found that layer-breeders fed a diet containing 0.5 mg aflatoxin/kg showed a significant decrease in haemaglutination-inhibition antibody titres over a 12-week period, after a single Newcastle disease vaccination. Similar suppression of the immune response was found in male broiler chickens fed 2.5 mg aflatoxin/kg feed (Ibrahim *et al*., 2000). Azzam & Gabal (1997) also observed a highly significant reduction in antibody titres against infectious bursal disease after vaccination.

AFB_1 inhibits lymphoblastogenesis in tests that rely on mitogen-stimulated uptake of [^{3}H]thymidine into various lymphocyte populations (Coulombe, 1994). A significant depression was found in T cell-dependent functions of splenic lymphocytes isolated from CD-1 mice, treated *in vivo* with up to 0.7 mg AFB_1/kg feed for 2 weeks (Reddy & Sharma, 1987). These workers also found a similar T cell-specific toxicity in a later study (Reddy & Sharma, 1989). In addition, they discovered that the natural killer cell function of the peripheral blood lymphocytes was affected by AFB_1 treatment. Total lymphocyte and T lymphocyte counts were significantly lower in chicks fed 0.3 and 1 mg AFB_1/kg feed (Ghosh *et al*., 1990; Ghosh *et al*., 1991) and 2.5 mg total aflatoxin/kg feed (Çelik *et al*., 2000b).

Aflatoxin dietary exposure of broiler breeder hens at a level of 1 and 5 mg aflatoxin/kg feed resulted in a depressed secondary antibody response in the progeny chicks against sheep red blood cells. Less anti-*Brucella abortus* antibodies occurred in the progeny chicks from the hens fed 5 mg aflatoxin/kg feed. Furthermore, phagocytosis of sheep red blood cells and reactive oxygen intermediate production by macrophages, important for the killing of microorganisms, from aflatoxin progeny chicks were reduced as compared with the control chicks. This study implied that the maternal transfer of aflatoxin residues to eggs reduced the posthatch immunological potential of chicks and their resistance to disease challenges (Qureshi *et al*., 1998). According to Nicolas-

Bolnet *et al*. (1995) the exposure of embryos to aflatoxin metabolites via maternal transfer may affect the differentiation and maturation processes of immature progenitor cells, which is crucial for the establishment of various haematopoietic lineage cells such as lymphocytes and macrophages.

Neldon-Ortiz & Qureshi (1992a) conducted a study to determine the *in vitro* effects of direct and activated AFB_1 exposure on various chicken peritoneal macrophage parameters. Direct exposure of macrophages to AFB_1 did not reduce macrophage phagocytic potential suggesting that macrophages were unable to activate AFB_1 presumably due to the absence and/or very low levels of endogenous mixed function oxidase (MFO). MFO's are necessary for the activation of AFB_1 into toxic metabolites thereby causing functional alterations. When chicken macrophage cultures were exposed to AFB_1 in the presence of a MFO system in vitro, even low concentrations of AFB_1 (1.0 µg) caused significant reduction in macrophage adherence potential and 5 µg AFB_1 caused almost complete sloughing, indicating cell death. Macrophages that remained adhered exhibited significant morphological alterations. These data suggested that AFB_1 was metabolized by the MFO system and that these metabolites proved to be more toxic to macrophage functional potentials. Ghosh *et al*. (1991) also found that the macrophages from chickens given 0.3 and 1 mg AFB_1/kg feed had a reduced phagocytic activity.

Sahoo & Mukherjee (2001) intraperitoneally injected 1.25 and 5.00 mg purified AFB_1/kg body weight into Indian major carp (*Labeo rohita*) fingerlings. After the trial period of 90 days the aflatoxin-treated fish revealed a reduction of total protein, globulin levels and bacterial agglutination titres.

2.4.10 Meat quality

Integumentary pigmentation of broiler chickens is a major determinant of acceptance by consumers and therefore a concern of the poultry industry. Aflatoxin decreased the plasma oxycarotenoid concentration and caused pale skins under field conditions (Osborne *et al*., 1982). This deleterious effect of aflatoxin on pigmentation in broilers can be associated with an impaired absorption of lutein. Aflatoxin probably decreases the ability to form micelles in the amount and character required for optimal absorption of lutein from intestinal contents (Schaeffer *et al*., 1988).

Huff *et al*. (1983) evaluated the effect of aflatoxin on bruising and bloody thigh syndrome in broiler chickens by simulating poultry industry live-haul catching and crating procedures. The broilers

were carried by the right leg, so that the left leg and thigh area were considered to be nonstressed. The severity of the bruises on the medial surface of the thigh and also on the breast was scored in a blind manner on a scale from 0 to 3. Aflatoxin-fed broilers showed an increased incidence and severity of thigh and breast bruises. This susceptibility to bruising did not recover after aflatoxin-treatment was discontinued and the broilers were placed on a toxin-free diet for 3 weeks.

2.5 Clinical Signs of aflatoxicosis in chickens

Aflatoxicosis does not usually induce mortality directly although high concentration levels (more than 10.0 mg aflatoxin/kg feed in chickens) may be lethal. The most economically significant effects of aflatoxicosis in growing birds are decreased growth and poor feed conversion (>1.0 mg aflatoxin/kg feed). There is also a marked decrease in resistance to infections such as salmonellosis, coccidiosis, infectious bursal disease and candidiasis, with resultant increased condemnations at processing (> 0.5 mg aflatoxin/kg feed). Poultry manifesting aflatoxicosis may also have failure of normal pigmentation and increased bruising (0.5 mg aflatoxin/kg feed). Intoxicated adult hens have decreased egg production and the hatchability of those eggs that are produced, is reduced (>2.0 mg aflatoxin/kg feed). In adult breeder males, testicular weights and sperm counts are reduced. Insemination of hens with semen from affected males has shown decreased fertility in some studies, and no significant reduction in others (Brown, 1996).

Table 1.1. Clinical signs of aflatoxicosis in broilers

Parameter	Significant Effect	Aflatoxin concentration	Reference
Body Weight Gain	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	2.5 mg AFB_1/kg feed	Scheideler (1993)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	2.5 mg AFB_1/kg feed	Miazzo *et al*. (2000)
	Decrease	3.5 mg total aflatoxin/kg feed	Harvey *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Decrease	0.1 and 0.2 mg total aflatoxin/kg feed	Azzam & Gabal (1997)

	Decrease	2.5 mg total aflatoxin/kg feed	Huff *et al*. (1983)
	Decrease	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
	Decrease	2.5; 5 and 10 mg AFB_1/kg feed	Dalvi & McGowan (1984)
Feed Intake	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
	Decrease	2.5; 5 and 10 mg AFB_1/kg feed	Dalvi & McGowan (1984)
Feed Conversion Ratio	Increase	2.5 mg AFB_1/kg feed	Scheideler (1993)
(feed/ body weight gain)	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
	Decrease	0.8 mg AFB_1/kg feed	Giambrone *et al*. (1985b)
Serum Alkaline Phosphatase	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
Serum Creatine Kinase	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
Serum δ-glutamyltransferase	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Serum Lactate Dehydrogenase	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
Serum Aspartate Aminotransferase	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Serum Alanine Aminotransferase	Increase	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
Serum Lactic Dehydrogenase	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
Total Serum Protein	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Decrease	3.5 mg total aflatoxin/kg feed	Harvey *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Decrease	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)

	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci et al. (1998)
Serum Albumin	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	0.3 and 1 mg $_{AFB1}$/kg feed	Ghosh *et al*. (1990)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci et al. (1998)
Serum Globulin	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Decrease	0.3 and 1 mg AFB_1/kg feed	Ghosh *et al*. (1990)
Serum Creatinine	Increase	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
Serum Calcium	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
Serum Phosphorus	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Serum Sodium	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
Serum Chloride	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
Serum Potassium	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
Serum Cholesterol	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Decrease	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci et al. (1998)
Serum Uric Acid	Decrease	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)

	Increase	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Serum Urea Nitrogen	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
Serum Glucose	Decrease	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
Serum Triglycerides	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Decrease	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
Haematocrit	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Haemoglobin	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Thrombocyte count	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Lymphocyte %	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Monocyte count	Decrease	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
White blood cell count	Increase	2.5 mg total aflatoxin/kg feed	Keçeci *et al*. (1998)
Liver Lipid	Increase	2.5 mg AFB_1/kg feed	Scheideler (1993)
Relative Liver Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Increase	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
	Increase	2.5 mg AFB_1/kg feed	Miazzo *et al*. (2000)
	Increase	5 mg total aflatoxin/kg feed	Okotie-Eboh *et al*. (1997)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Relative Kidney Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Relative Heart Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)

	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
Relative Gizzard Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Relative Spleen Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	5 mg AFB_1/kg feed	Rosa *et al*. (2001)
Relative Pancreas Weight	Increase	5 mg total aflatoxin/kg feed	Bailey *et al*. (1998)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1993)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
Relative Proventriculus Weight	Increase	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
	Increase	4 mg AFB_1/kg feed	Ledoux *et al*. (1999)
	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
Relative Diverticulum Weight	Decrease	3 mg total aflatoxin/kg feed	Santurio *et al*. (1999)
Relative Bursa of Fabricius Weight	Decrease	3.5 mg total aflatoxin/kg feed	Kubena *et al*. (1990a)
Adrenal size	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)
Mortality	Increase	5 mg total aflatoxin/kg feed	Kubena *et al*. (1998)
	Increase	0.3 mg AFB_1/kg feed	Raju & Devegowda (2000)

2.6 Factors affecting AFB_1 toxicity

Numerous factors can influence the magnitude of response in vertebrates to exposure to AFB_1. One of the more striking modulating effects on AFB_1 toxicity is associated with dietary components that the animals consume prior to or following exposure to AFB_1 (Cullen & Newberne, 1994).

2.6.1 Environmental Temperature

Manning & Wyatt (1990) studied the effect of acclimation to environmental temperatures (10-12; 14-16 and 28-30 °C) on the resistance of broiler chicks to acute aflatoxicosis. Chicks acclimated to

10-12°C were more resistant to a single oral dose of aflatoxin (8 mg aflatoxin/kg of body weight) after 10 and 20 days than chicks acclimated to 28-30 °C. Chicks acclimated to 14-16 °C required 20 days of acclimation before an increase in resistance was observed. Acclimation to 10-12 °C for 14 days followed by acclimation to 28-30 °C for seven days resulted in no increase in the resistance of the chicks to aflatoxin, indicating that the resistance conveyed by cold acclimation was temporary. The cytochrome P-450 content was significantly increased after acclimating the broilers to 10-12 °C, suggesting that stimulation of the microsomal mono-oxygenase system after cold acclimation may be responsible for the increased resistance of the broilers to acute aflatoxicosis.

2.6.2 Dietary and Nutrient Effects

(i) Amino Acids

Protein and vitamin content of the diet, hormonal status, and treatment with pharmacologically active compounds, among other changes, modify the response of experimental animals to acute AFB_1 toxicity. These responses – whether measured as animal mortality, histopathological alterations in the liver, or clinical evidence of liver damage – all register differences compared with untreated animals. Many of these factors also have been studied with respect to their effects on AFB_1-induced hepatocarcinogenesis, the metabolism of AFB_1, the biochemical response to AFB_1 toxicity (especially in terms of inhibition of nucleic acid and protein synthesis), and the metabolism and pharmacokinetics of AFB_1. In an early study of Newberne *et al*. (1966) with ducklings, the addition of 1% arginine and 0.8% lysine to the diet with, but not without, aflatoxin sharply decreased weight gain and increased mortality. A profound increase was indicated in sensitivity of ducklings to aflatoxin when dietary concentrations of arginine and lysine were increased only slightly above normal. The addition of glutathione or cysteine to the diet as sources of sulfhydryl groups had no effect on toxicity. Autoclaving aflatoxin-contaminated peanut meal that was used as the source of the toxin decreased toxicity and markedly increased weight gains of ducklings over a 9-day period.

N-acetylcysteine, the acetylated variant of the amino acid, L-cysteine, has been used safely in humans and in other mammals as an antidote against several toxic and carcinogenic agents (Valdivia *et al*., 2001). Valdivia *et al*. (2001) showed that broilers treated with AFB_1 plus N-acetylcysteine were partially protected against the deleterious effects on performance, liver and kidney damage and biochemical alterations induced by AFB_1 in broiler chickens.

(ii) Protein

Rats fed a low protein diet (4% casein) and given 50 µg of an undefined aflatoxin preparation exhibited a significant degree of toxic liver lesions within 3 weeks, compared with rats fed a high-protein diet (20% casein). These results were confirmed with the observation that a high protein diet (16% casein) protected monkeys from aflatoxin liver injury in comparison with animals on a 1% casein diet (Cullen & Newberne, 1994).

Protein A of *Staphylococcus aureus* is a potent immunostimulant and anticancer agent and has no toxic effects, even after multiple inoculations (Ray *et al*., 1984). Protein A has demonstrated its ability to decrease toxicity of drugs like cyclophosphamide (Zaidi *et al*., 1990) and environmental toxicants like carbon tetrachloride (Singh *et al*., 1990). Raisuddin *et al*. (1994) observed that protein A provided protection to rats from AFB_1-induced immunotoxicity. Various parameters showing suppression of cell-mediated immunity following AFB_1 exposure were reverted back towards normality in protein A-treated animals. Zaky *et al*. (1998) found that protein A counteracted the effect of AFB_1 on haematocrit, globulin and bilirubin in broilers. According to Raisuddin *et al*. (1994) the protective effect of protein A on AFB_1 toxicity is caused by inhibition of the activity of liver cytochrome P450 mixed function oxidase system that activates AFB_1 to form the active metabolite that binds to cell DNA and RNA.

(iii) Choline and Methionine

The effect of a choline-deficient/low-methionine diet on hepatic aflatoxin-DNA adduct burden in male Fisher rats dosed with a carcinogenic regimen of AFB_1, was examined in a model by Schrager *et al.* (1990). After three weeks of ingestion of a choline-deficient/low-methionine diet or a control semi-purified diet (sufficient time for enzyme activity to be altered), the rats were administered a carcinogenic regimen of 25 µg [^{3}H] AFB_1 for five days/week over two weeks. Six choline-deficient and four control diet rats were killed two hours after each dose so liver DNA could be isolated. In addition, hepatic DNA was isolated from animals 1, 2, 3, and 11 days after the last [^{3}H] AFB_1 administration. High-performance liquid chromatography (HPLC) analysis of aflatoxin-DNA adducts was performed to confirm radiometric determinations of DNA binding levels. No significant quantitative differences in AFB_1-DNA adduct formation were observed among the dietary groups after the first exposure to [^{3}H]AFB_1; however, total aflatoxin-DNA adduct levels in the choline-deficient animals were increased significantly during the multiple-dose schedule. When total aflatoxin-DNA adduct levels were integrated over the ten day dose period, a 41% increase in

adduct burden was determined for the choline-deficient animals. This increase in DNA damage is consistent with the hypothesis that DNA damage is related to tumor outcome, but the biochemical basis for this effect is not known.

(iv) Carnitine

L-carnitine is a carrier of acyl groups, particularly the long-chain fatty acids, across the cellular compartments. It was reported that carnitine in combination with coenzyme Q_{10} offered significant protection against oxygen radicals induced by mycotoxins including AFB_1 in bacteria (Atroshi *et al*., 1998). When a carnitine-supplemented diet (4 g/kg) was fed to rats for 6 weeks followed by a single oral dose of AFB_1 (1 mg/kg feed), there was a significant reduction in the concentrations of AFB_1 adducts of hepatic DNA and RNA (Sachan & Yatim, 1992).

AFB_1 binds to serum albumin and hepatic DNA in a dose-dependent manner (Sabbioni *et al*., 1990). AFB_1 has been shown to have the highest affinity for liver microsomes followed by the cytosol, mitochondria and nuclei (Ewaskiewicks *et al*., 1991). Yatim & Sachan (2001) found that carnitine increased the retention of AFB_1 by plasma proteins and they concluded in their study that the lowered binding of AFB_1 to DNA in isolated hepatocytes can be attributed to less free AFB_1 available for uptake and metabolism by liver cells.

(v) Antioxidants

According to Souza *et al*. (1999), the principal manifestations of AFB_1-induced toxicity are lipid peroxidation and oxidative DNA damage, which could be mitigated by antioxidants. Verma & Nair (1999) found that aflatoxin treatment caused a significant dose-dependent increase in lipid peroxidation, which could be due to reductions in the activities of superoxide dismutase, glutathione peroxidase and catalase. The levels of glutathione, total ascorbic acid and reduced ascorbic acid also declined significantly. Shen *et al*. (1994) measured malonaldehyde concentrations in liver homogenate as well as in subcellular fractions. Malonaldehyde is one of the end products from the chain reactions of lipid peroxidation. They showed a significant, persistent and dose-dependent increase of malonaldehyde after AFB_1 administration in mice, suggesting that AFB_1 caused lipid peroxidation in the liver. In this study, liver cell damage caused by AFB_1 was measured by the increase of serum alanine aminotransferase and serum aspartate aminotransferase activities.

In an *in vivo* study in rats Shi *et al*. (1994) demonstrated that selenium inhibits AFB_1-DNA binding and adduct formation. Shen *et al*. (1994) showed that the pretreatment of rats with selenium and vitamin E significantly inhibited the increase of malonaldehyde in liver homogenate induced by AFB_1. A concomitant decrease of serum alanine aminotransferase and serum aspartate aminotransferase activities was also observed. Shen *et al*. (1994) suggested that the vitamin E inhibits lipid peroxidation by breaking the chain reaction initiated by •OH. In a similar study Verma & Nair (1999) supplemented adult Swiss strain male albino mice with vitamin E prior to aflatoxin treatment and found that the change in glutathione, total ascorbic acid and reduced ascorbic acid were significantly less after exposure to aflatoxin. The effect was more pronounced in animals treated with a low dosage of aflatoxin (25 µg aflatoxin/animal/day) than in those receiving a high dose (50 µg aflatoxin/animal/day). These findings (and also those of Verma & Nair, 2001) suggest that vitamin E pre-treatment significantly inhibited aflatoxin-induced lipid peroxidation. The protective effect of vitamin E against lipid peroxidation is mainly due to increased non-enzymatic and enzymatic antioxidants.

Carotenoids (carotene and xanthophylls) are excellent antioxidants with antimutagenic and anticarcinogenic properties. They occur naturally in some foods such as carrots, red tomatoes, butter, cheese, paprika, palm oil, corn kernels and red salmon (Piva & Galvano, 1999). Okotie-Eboh *et al*. (1997) fed broiler chicks diets containing 0, 0.01 and 0.02% β-carotene or canthaxanthin with or without 5 mg aflatoxin/kg feed. Neither of the two carotenoids was effective at overcoming the growth-depressing effects of aflatoxin. Canthaxanthin significantly increased the concentrations of cholesterol, total protein, uric acid and triglyceride, all of which were significantly depressed by aflatoxin. It also reduced the relative liver weights and creatine kinase activity of the aflatoxin-treated chicks. This data suggested that β-carotene is not effective at ameliorating aflatoxicosis in broiler chickens but that canthaxanthin may be effective with respect to certain blood clinical chemistry indicators. It might be possible that the cartenoids, canthaxanthin and astaxanthin, exert their protective effects altering AFB_1 metabolism toward pathways that lead to the formation of aflatoxin M_1, a less toxic metabolite (Piva & Galvano, 1999).

Crocetin is a cartenoid isolated from the seeds of Cape jasmine (*Gardenia jasminoides*). Lin & Wang (1986) have observed a protective effect of crocin (the digentibiose of crocetin) on the hepatic damage induced by AFB_1 and dimethylnitrosamine. The cytotoxicity and DNA-adduct formation of rat microsome-activated AFB_1 in fibroblast cells were significantly inhibited by

pretreatment with crocetin (Wang *et al*., 1991). Consistent elevation in the cytosolic glutathione (GSH) levels and the activities of GSH S-transferase and GSH-peroxidase were also observed. A possible mechanism for crocetin inhibition could thus be mediated through its antioxidant properties.

Verma *et al*. (2001) evaluated the ameliorative role of vitamin A on aflatoxin-induced cytotoxicity *in vitro*. The addition of vitamin A in incubation medium significantly reduced aflatoxin-induced hemolysis.

An *in vitro* study by Verma *et al*. (1999) revealed that pretreatment of an erythrocyte suspension with ascorbic acid significantly decreased aflatoxin-induced hemolysis. The solution chemistry of the interaction of aflatoxin with ascorbic acid in aqueous solutions showed enhanced conversion of AFB_1 and aflatoxin G_1 to the less toxic aflatoxin B_2 and aflatoxin G_2, respectively.

The effects of nutrient antioxidants on acute toxicity of AFB_1 are sometimes variable. For example, oral treatment of rats with 300 mg/kg of ubiquinone, vitamin K_1, menadione or α-tocopherol immediately after an oral dose of 7 mg AFB_1/kg did not alter the 50-75% mortality within the various treatment groups (Rogers & Newberne, 1971). Okotie-Eboh *et al*. (1997) couldn't find any ameliorating effects of β-carotene on aflatoxicosis in broilers. The same study concluded that canthaxanthin might be somewhat effective with respect to certain clinical blood chemistry indicators. In addition, vitamin A deficiency or supplementation was not a factor in the severity of aflatoxin-produced liver damage in female rats (Reddy *et al*., 1973). Vitamin deficiency in males, however, increased the susceptibility of the liver to aflatoxin damage; this increased susceptibility was overcome largely by vitamin A supplementation. Pretreatment of rats fed a control diet with carotene 30 min before an LD_{50} dose of 7 mg AFB_1/kg feed, reduced the 7-day mortality from 80% to less than 10% (Newberne *et al*., 1974). McLeod *et al*. (1997) reported that Fischer 344 rats fed on a diet that was deficient in selenium were more resistant to the hepatocarcinogen AFB_1 than those that were fed on a selenium-sufficient diet. According to the authors the protection conferred by selenium deficiency was associated with the hepatic expression of an aldehyde reductase and gluthathione S-transferase that efficiently metabolize the mycotoxin.

(vi) Food components and additives

Numerous food components, ingredients or additives with or without overall antioxidant properties have been investigated to verify their chemoprotective properties (Piva & Galvano, 1999). One of the mechanisms by which these compounds may exert putative anticancer effects is through interaction with cytochrome P450 enzymes in the liver to reduce activation of procarcinogen substrates to carcinogens (Tsyrlov *et al*., 1994). Another mechanism may be by promoting the transformation of AFB_1 into nontoxic products (Lee *et al*, 2001).

Ellagic acid is a phenolic compound that occurs naturally in some foods such as strawberries, raspberries and grapes. A wide range of *in vivo* and *in vitro* assays demonstrated that it has both antimutagenic and anticarcinogenic activity (Piva & Galvano, 1999). Ellagic acid inhibited AFB_1-induced mutagenicity in *in vitro* tests on salmonella cells by formation of an AFB_1-ellagic acid chemical complex (Loarca Piña *et al*., 1996; Loarca Piña *et al*., 1998). Dietary addition of ellagic acid to rats (Soni *et al*., 1997) significantly reduced the number of gammaglutamyl transpeptidase-positive foci induced by AFB_1, which is considered as the precursor of hepatocellular neoplasm. Soni *et al*. (1997) also observed the same effects when certain food additives such as turmeric and garlic were used. Curcumin, the diferuloylmethane present in turmeric and other *Curcuma* species, strongly inhibited the formation of the AFB_1 reductase product, aflatoxicol, by chicken liver cytosols. It was established that β-diketone groups linked with two benzyl moieties were essential for inhibition of aflatoxicol formation (Lee *et al*., 2001).

In tests performed by Aboobaker *et al*. (1994) on rats given a synthetic diet containing various food-associated phenolic compounds, a marked decrease was observed in the ability of liver microsomes to catalyze reactions of AFB_1, leading to its activation and DNA adduct formation. Flavonoids, an example of such a phenolic compound, (Cholbi *et al*., 1991; Galvez *et al*., 1995; Keli *et al*., 1996) and lignans and neolignans (Haraguchi *et al*., 1996) have antioxidant properties and can inhibit lipid peroxidation (Pulla Reddy & Lokesh, 1992). These substances often serve as hepatoprotective agents in the prevention of toxicity caused by certain drugs and environmental chemicals wherein pathogenic oxidative and lipid peroxidative reactions are believed to play an essential role (Hara *et al*., 1991). Souza *et al*. (1999) found that 72 hours after a single 1 mg/kg intraperitoneal dose of AFB_1, the concentration of malondialdehyde, the product of lipid peroxidation in liver homogenates, and serum levels of alanine aminotransferase and aspertate aminotransferase were significantly elevated. The bioflavonoid ternatin (4'5-dihydroxy-3,3',7,8

tetramethoxyflavone) has been isolated from the dried flower heads of *Egletes viscosa* L. (Asteraceae). Subcutaneous ternatin (25 mg/kg) pretreatment greatly reduced AFB_1-induced increases in the levels of serum enzymes and elevated malondialdehyde levels in a manner similar to oral vitamin E, a standard antioxidant. Further, histological changes induced by AFB_1 such as hepatocellular necrosis and bile duct proliferation were markedly inhibited in animals pretreated with ternatin or vitamin E. Other naturally occurring flavones, quercetin, fisetin, nobiletin and tangeritin protect cultured rat liver epithelial-like cells against AFB_1-induced cytotoxicity and inhibited the binding of [3H] AFB_1 to cellular DNA (Schwartz *et al*., 1979).

Lee e*t al.* (2001) tested the effects of 34 flavonoids on AFB_1-8,9-epoxide formation in mouse liver and found that galangin and rhamnetin were the most potent, followed by the parent compound flavone. Naringenin also showed strong inhibition of epoxide formation, whereas the other flavanones tested did not show activity. The authors found that, for polyhydroxylated flavonoids, an increasing number of hydroxyl groups decreased the effectiveness.

A range of natural dietary constituents showed *in vitro* chemoprotective actions toward AFB_1, including garlic oil, ethoxyquine, indole-3-carbinol, phenethyl isothiocynate (Manson *et al*., 1997) and S-methyl methanethiosulfonate, a compound present in the juice of cabbage and onion (Ito *et al*., 1997). Cavin *et al*. (1998) identified two diterpenes (cafestol and kahweol), present in green and roasted coffee beans, as potentially chemoprotective agents against AFB_1-induced carcinogenisis by modulation of multiple enzymes involved in carcinogen detoxification. According to Reen *et al*. (1997), piperine (1-piperoylpiperidine), the major alkaloid constituent of pepper (*Piper nigrum*), has considerable potential as protective agent against the carcinogenic effects of AFB_1. AFB_1 toxicity is bioactivated by the cytochrome P450 monooxygenases (CYP450). In cultured rat cells piperine dramatically reduced $CYP4502B_1$ activity and counteracted toxicity.

Synthetic phenolic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been widely used for many years as antioxidants to preserve and stabilize the freshness, nutritive value, flavour and colour of foods and animal feed products (Williams *et al*., 1999). BHA and BHT are known to inhibit AFB_1-DNA binding, thereby protecting the liver against the chronic effects of the carcinogen (Allahmeh *et al*., 1989). Pretreatment of animals with BHA increased the specific activities of the detoxification enzymes, glutathione S-transferase and epoxide hydratase in hepatic tissues in mice (Rahimtula *et al*., 1982).

These antioxidants probably acquire in part their anticarcinogenic property by inducing cellular detoxification pathways. However, quantitative and qualitative alterations in cytochrome P450 by these antioxidants cannot be ruled out (Rahimtula *et al*., 1982; Allahmeh *et al*., 1989).

Chlorophyllin, a water-soluble form of chlorophyll, which is used extensively as a food colourant and has numerous medicinal applications, is an effective anticarcinogen in experimental models including aflatoxin-induced hepatocarcinogenesis. Chlorophyllin is thought to form molecular complexes with carcinogens, thereby blocking their bioavailability. Administration of chlorophyllin three times a day led to a 50% reduction in the median level of urinary excretion of aflatoxin-N^7-guanine compared to placebo. This excreted DNA adduct biomarker is derived from the carcinogen aflatoxin-8,9,-epoxide, and is associated with increased risk of developing liver cancer in prospective epidemiological studies (Egner *et al*., 2003).

2.6.3 Microsomal enzyme inducers

Microsomal enzyme induction is an important toxicological phenomenon, because it leads to an accelerated biotransformation of certain toxicants *in vivo*, thus altering the duration and intensity of toxic effects. Participation of hepatic microsomal enzymes such as P450 in the metabolism of AFB_1, has been demonstrated by several researchers (Mgbodile *et al*., 1975; Reen *et al*., 1997). Data obtained from a study by Dalvi & McGowan (1984) indicated that dietary AFB_1 caused a dose-dependent loss of cytochrome P450. The administration of phenobarbital intermittently in the drinking water of aflatoxin-fed chickens caused a substantial increase in cytochrome P450 content, enhancing the metabolisms and elimination of absorbed AFB_1. As a result, an improvement in feed consumption and weight gain was noted in the affected chickens.

Hao & Brackett (1988) have reported the degradation of AFB_1 in food products by *Flavobacterium aurantiacum*. *F. aurantiacum* removed 82% AFB_1 from partially defatted peanut milk, and 40% of AFB_1 from phosphate buffer after 24 hours. Line *et al*. (1994), using ^{14}C-labeled AFB_1, provided the first evidence that AFB_1 removal was through degradation and not through binding by the bacterium. Smiley & Draughon (2000) investigated the mechanism of degradation and found that it is probably linked to a protein with typical enzyme characteristics. The results from this study also indicated that the component within the crude protein extract of *F. aurantiacum*, responsible for AB_1 degradation, might be stable in a purified form when isolated and used in the food industry as a means of removing aflatoxins from contaminated foods. Although the maximum amount of AB_1

degradation occurred at pH 7, 22 to 45% degradation was still observed at the other pH values tested, making it applicable to a wide range of foods.

2.6.4 Non-nutritive absorptive materials

Practical methods to detoxify mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. A variety of physical, chemical, and biological techniques have been employed but with limited success (Edrington *et al*., 1997). These include soil and crop management techniques; genetic and molecular control of mould growth and aflatoxin formation; various food and feed processing techniques; biological controls; methods of direct chemical degradation of the molecule; nutritional modulation of toxicity and hepatic cancer; and various other chemopreventive interventions (Phillips, 1999). Chemoprevention, chemoprotection or chemoprophylaxis are terms that describe novel chemical or dietary interventions that can alter the susceptibility of humans and animals to the actions of carcinogens and block, retard, or reverse carcinogenesis or disease (Kensler *et al*., 1994). Very promising chemical interventions that can modify the hepatotoxicity and carcinogenicity of aflatoxin, include modified nutrient and endocrine status, as well as non-nutrient dietary additives and various pharmacological interventions, as viable approaches to the reduction of human cancers (Kensler *et al.*, 1994). A recent approach to detoxify mycotoxin-contaminated grain has been the addition of non-nutritive absorptive materials (enterosorbents) to the diet in order to reduce the absorption of mycotoxins from the gastrointestinal tract (Edrington *et al*., 1997). Ledoux & Rottinghaus (1999) are of opinion that the addition of adsorbents to contaminated feed to selectively bind the mycotoxin during the digestive process, allowing the mycotoxin to pass harmlessly through the animal is, at present, the most promising and practical approach. The major advantages of adsorbents include low cost, safety and the ease with which they can be added to animal feeds.

Research indicates that a number of adsorbents are capable of binding aflatoxin and reducing or preventing its toxic effects. However, not all adsorbents are equally effective in protecting livestock against the toxic effects of aflatoxin and several adsorbents have been shown to impair nutrient utilisation (Chung *et al*., 1990; Kubena *et al*., 1993; Scheideler, 1993). Dale (1998) noted that many of the adsorbents on the market today have not been adequately tested for *in vivo* efficacy, but are used based on *in vitro* data only. *In vitro* tests may not always be a reliable indicator of ability to bind a mycotoxin (Scheideler, 1993; Dwyer *et. al.,* 1997; Ledoux & Rottinghaus, 1999). Therefore, it is important that adsorbents are subjected to *in vivo* evaluation to

determine both efficacy and impaired nutrient utilisation from the diet (Ledoux & Rottinghaus, 1999).

According to Ramos & Hernández (1997) the management of mycotoxin problems associated with animal production, using adsorbent materials will only be an effective strategy if these materials have the ability to adsorb a large number of chemically distinct mycotoxins.

(i) Activated Charcoal

Activated charcoal is a nonsoluble powder formed by pyrolysis of different kinds of organic materials. The use of activated charcoal as an oral antidote for the treatment of poisonings is well established. Charcoal acts as an insoluble carrier that nonspecifically adsorbs a wide variety of molecules, thereby preventing their absorption. This property has been used to prevent the gastrointestinal absorption of various xenobiotics and to increase their direct elimination. It has been used as an antidote against poisoning from the early XIX century, but its used has been limited (Ramos *et al*., 1996).

Adsorption of chemicals onto charcoal depends on several factors, of which pore size, surface area, chemical nature of the xenobiotic, charcoal dose, pH and gastrointestinal contents being the most important (Ramos *et al*., 1996). Specific surface area measurements for commercial activated charcoals may vary from 500 m^2/g (with lignin as starting material) to over 2,000 m^2/g (with oil as starting material) (Diamadopoulus *et al*., 1992). Superactivated charcoal differs from activated charcoal in that the particle size is reduced, thereby increasing surface area up to 3,500 m^2/g (Ramos *et al*., 1996), and the carrier base for the charcoal is chemically modified during the manufacturing process (Edrington *et al*., 1997).

Several studies were done on different kinds of animals over the years to test the efficacy of activated charcoal as an antidote against different mycotoxins (Ademoyero & Dalvi, 1983; Dalvi & Ademoyero, 1984; Dalvi & McGowan, 1984; Abdelhamid *et al*., 1990; Edrington *et al*., 1996; Galvano *et al*., 1996; Galvano *et al*., 1997; Galvano *et al*., 1998). Decker & Corby (1980) made the first observations about the potential applications of adsorptive materials in the field of aflatoxicosis. They demonstrated that activated charcoal was able to adsorb AFB_1 *in vitro* in an efficient manner at neutral pH. Hatch *et al*. (1982) used activated charcoal as an antidote against a lethal dose of AFB_1 in goats (3 mg of AFB_1/kg of body weight). Treatment with activated charcoal

resulted in a 100% survival rate with a dose of aflatoxin corresponding to an LD_{90-100}. Furthermore, bile duct proliferation and serum aspartate aminotransferase responses to AFB_1 were decreased and the average percentage of hepatic destruction was only 3% against the 25% of nontreated goats. This study probably proved the therapeutic effect of activated charcoal in an acute poisoning situation, and postulated the adsorbent prevents gastrointestinal absorption of aflatoxins. The fact that the faeces of charcoal-treated animals contained large concentrations of AFB_1 and its metabolite aflatoxin M_1, while in the control animals they only found small amounts of these toxins further supported this hypothesis.

Edrington *et al*. (1997) suggested that the addition of dietary superactivated charcoal is marginally effective in alleviating some of the toxic affects associated with aflatoxin, but was of little benefit when T-2 toxin was fed to growing broilers. Jindal *et al*. (1994) described 200 mg activated charcoal/kg feed to be moderately effective in improving body weights, feed consumption and feed conversion ratios where broiler chicks were fed a diet containing 0.5 mg AFB_1/kg from day 1 to day 42.

Some work, however, (Kubena *et al*, 1990a; Edrington *et al*., 1996) reported no significant differences in performance of broilers or turkey poults following the addition of charcoal to rations including aflatoxin. Huwig *et al*. (2001) stated that the failure of charcoal to diminish the effects of mycotoxins might be due to the fact that activated charcoal is a relatively nonspecific adsorbent and hence, essential nutrients are also adsorbed.

(ii) Bentonite

Bentonite originates from the weathering of volcanic ash *in situ* and consists primarily of montmorillonite. The composition of bentonite may vary from one deposit to another, mainly because of the interchangeable ions, e.g., Na^+, K^+, Ca^{++} and Mg^{++}. It has a layered crystalline microstructure, which allows adsorption of other molecules and accounts for its swelling abilities when added to water. This adsorbent compound has a variety of industrial, engineering, agricultural and miscellaneous uses, including the clarification of beverages and water and the decoloration of oils (Ramos *et al*., 1996).

An *in vitro* study from Rosa *et al*. (2001) indicated that a sodium bentonite from southern Argentina had a high ability to adsorb AFB_1 from aqueous solution. The authors proceeded to evaluate this

compound (at a concentration of 0.3%) for its ability to reduce the effects of 5 mg AFB_1/kg of feed in the diet of growing broiler chickens from 30 to 52 days of age. The bentonite ameliorated the effects of AFB_1 treatment as measured by body weight gain and feed conversation. However, the biochemical indicators and histopathological findings showed that the amelioration of the effects of aflatoxin was not as great as might have been predicted. Similar results were shown by Santurio *et al*. (1999), where the addition of 5% sodium bentonite to an aflatoxin-contaminated diet (3 mg total aflatoxins/kg of feed) led to an improvement of body weight, feed intake and feed efficiency but not of biochemical variables or relative organ weights. Bentonite supplementation in an aflatoxin-contaminated diet was, however, significantly effective in ameliorating the negative effects of aflatoxin on certain immune response parameters (Ibrahim *et al*., 2000).

Lindemann *et al*. (1993) found that the addition of different amounts of sodium bentonite to growing pig diets containing 0.8 mg AFB_1/kg resulted in an improvement of average daily gain and average daily feed intake. Sodium bentonite at a concentration of 2% in the diet greatly reduced the uptake of aflatoxin in trout (Ellis *et al*., 2000). Schell *et al*. (1993a) demonstrated that an addition of 1% of a sodium bentonite in control and contaminated diets of growing pigs did not affect the absorption and retention of K, Cu, Mn, Ca, P, Zn and Fe and lowered Mg and Na absorption. Feeding clay with AFB_1 resulted in a partial restoration of performance and liver function without greatly influencing mineral metabolism. The inability of the clay to restore all functions indicated different degrees of sensitivity of the functions to aflatoxins.

Carson & Smith (1983) demonstrated that a 2.5% addition of bentonite in a diet contaminated with 3 g of T-2 toxin/kg feed greatly increased feed consumption and final body weights of rats in a 2-week study, while 10% completely overcame the toxicosis. On the other hand, Williams *et al*. (1994) found that the addition of 20 or 50 g of bentonite/kg of feed contaminated with zearalenone (3.0 mg/kg) and nivalenol (11.5 mg/kg) was not effective in overcoming either the oestrogenic or depressed performance effects in pigs. They postulated that the dietary concentration of mycotoxin used in this study was excessively high and therefore a strong adverse reaction developed.

(iii) Zeolite

Zeolites are crystalline, hydrated aluminosilicates of alkali and alkali-earth cations, having infinite three-dimensional structures. They are further characterized by a high ability to lose and gain water

reversibly and to exchange constituent cations without major changes in structure (Ramos *et al*., 1996).

Many studies have been made to determine if the use of zeolites in the diet has a beneficial effect on aflatoxin-fed chickens or not. Scheideler (1989) showed that *in vitro* commercial zeolites were able to absorb from 58 to 74% of the aflatoxins present in a gastric content solution, depending on the type of zeolite tested. The same author also demonstrated in an *in vivo* study with chickens that zeolites alleviated depression of growth in chickens fed with a diet containing 2.5 mg AFB_1/kg. Some differences were, however, found in liver lipids, bone ash, and serum P and Cl, depending on the zeolite used (Scheideler, 1989; Scheideler, 1993). Harvey *et al*. (1993) used five commercially available zeolitic ore compounds at 0.5% concentration, to evaluate their ability to reduce the deleterious effect of 3.5 mg of aflatoxins/kg of feed on growing broiler chickens in a 3-week experiment. Three of them were not able to diminish the toxicity of high concentrations of aflatoxins. Zeomite™ mordenite ore reduced the toxicity by 41% as measured by weight gains, liver weight and serum biochemical measurements, which compares favorably with its *in vitro* binding capacity to aflatoxin. SC Zeolite™ reduced the aflatoxin-mediated body weight losses by 29%. Miazzo *et al*. (2000) evaluated four different synthetic zeolites *in vitro* for their ability to adsorb AFB_1 from an aqueous solution. Zeolite NaN was selected for a subsequent *in vivo* study because of its high affinity and its stable association with AFB_1. This zeolite proved to offer almost total protection against the effects caused by aflatoxin by virtue of body weight measurements.

(iv) Hydrated sodium calcium aluminosilicate

Among all aluminosilicates tested with regard to mycotoxin adsorption, a hydrated sodium calcium aluminosilicate (HSCAS) from natural zeolite has been the most extensively studied because of its promising aflatoxin-binding capacity. This is a phyllosilicate whose positive-charge deficiencies create the potential for adsorbing positively charged or cationic compounds (Ramos *et al*., 1996). HSCAS clay acts as an aflatoxin enterosorbent that tightly and selectively binds these poisons in the gastrointestinal tract of animals, decreasing their bioavailability and associated toxicity. The dicarbonyl system of aflatoxin is essential for tight binding by HSCAS (Phillips, 1999).

The *in vitro* experiments carried out with radiolabelled AFB_1 by Phillips *et al*. (1988) demonstrated that of 38 different adsorbents tested (a variety of aluminas, zeolites, silicas, phyllosilicates and chemically modified phyllosilicates), HSCAS was able to form the most stable complex with this

mycotoxin. The HSCAS adsorbed more than 80% of AFB_1 present in the medium. The complex was stable in water at pH 2,7 and 10 and at temperatures of 25 and 37°C. Scheideler (1993), however, found that only 55% of radiolabelled AFB_1 being adsorbed when dissolved in methanol. *In vitro* study results of Ledoux *et al*. (1999) indicated that hydrated sodium aluminosilicates (Improved Milbond-TX®) was able to bind 100% of AFB_1 at pH 3 to 9.

A large number of experiments were done on chickens (mainly broiler chicks) where HSCAS was added at a concentration of 0.25% to 1% in diets with or without aflatoxins (Davidson *et al*., 1987; Kubena *et al*., 1987; Kubena *et al*., 1988; Phillips *et al*., 1988; Doerr, 1989; Scheideler, 1989; Kubena *et al*., 1990a and b; Araba & Wyatt, 1991; Huff *et al*., 1992; Kubena *et al*., 1993; Lindemann *et al*., 1993; Scheideler, 1993; Abo-Norag *et al*., 1995; Kubena *et al*., 1998; Ledoux *et al*., 1999). HSCAS has also been assayed in a great variety of other animals, including turkey poults (Kubena *et al*., 1991), pigs (Colvin *et al*., 1989; Harvey *et al*., 1989; Lindemann *et al*., 1989; Beaver *et al*., 1990; Lindemann *et al*., 1993; Schell *et al*., 1993b), dairy cows (Harvey *et al*., 1991b), lambs (Harvey *et al*, 1991a) and rats (Mayura *et al*, 1998; Abdel-Wahhab *et al*., 2002). In every case, HSCAS eliminated or ameliorated the toxic effects of the aflatoxins on animal performance, and on several biochemical and histopathological parameters.

Although HSCAS was demonstrated to be very effective with regard to preventing aflatoxicosis, its efficacy against zearalenone (Bursian *et al*., 1992), ochratoxin A (Huff *et al*., 1992; Santin *et al*., 2002), T-2 toxin (Kubena *et al*., 1990a; Kubena *et al*., 1998), diacetoxyscirphenol (Kubena *et al*., 1993) and deoxynivalenol (Patterson & Young, 1993) was not significant.

(v) Biological binding agents

Devegowda *et al.* (1996) demonstrated up to 77% *in vitro* binding of *Saccharomyces cerevisiae* to aflatoxin in a dose-dependent manner. Devegowda *et al*. (1998) reported that modified mannanoligosaccharide (Mycorb, Alltech Inc.) derived from the cell wall of *S. cerevisiae* had even higher binding capacity (95% aflatoxin, 80% zearalenone, fumonisin up to 59% and vomitoxin up to 12%). Newman (2000) found that Mycosorb™ demonstrated a high binding affinity for aflatoxin and other toxins when compared to either activated charcoal or HSCAS. According to Newman (2000) interference with mineral metabolism, in contrast to many of the clay-based adsorbents, has not been noted due to more specific binding characteristics of Mycosorb.

Savage *et al*. (1995) and Smith *et al*. (2000) fed turkeys mycotoxin-contaminated diets and concluded that Mycosorb™ showed promise in promoting growth and feed intake of affected poults. Diaz *et al*. (1999) showed that Mycosorb™ reduced milk aflatoxin concentrations by 58% in dairy cows consuming aflatoxin-contaminated diets when included at 0.05% of the diet dry matter. Esterified-glucomannan (derived from the cell walls of *Saccharomyces cerevisiae*) improved the body weight and food intake of broilers after ingestion of three different mycotoxins (aflatoxin B_1, ochratoxin and T-2 toxin). It also decreased the liver and adrenal weight and serum γ-glutamyl transferase activity, and increased serum protein, cholesterol and blood haemoglobin (Raju & Devegowda, 2000). Stanley *et al*. (1993) found a significantly improved performance for chicks fed an aflatoxin-contaminated diet together with 0.1% *Saccharomyces cerevisiae*. These beneficial effects of mannanoligosaccharide can be attributed to its ability to bind the mycotoxins irreversibly (Devegowda *et al*., 1996) and to stimulate the immune response (Savage *et al*., 1996)

Some dairy strains of lactic acid bacteria were found capable of removing AFB_1 from contaminated liquid media via a rapid process involving the removal of approximately 80% of AFB_1 immediately upon contact without further incubation (El-Nezami *et al*., 1998). The authors found that heat-treated bacteria had the same ability to remove AFB_1 as viable bacteria and, therefore, metabolic degradation by viable bacteria has been ruled out as a possible mode of action under the experimental conditions tested. All the Gram-positive strains of bacteria tested were more efficient than *Escherichia coli* suggesting that the ability of bacteria to remove AFB_1 be dependent on cell wall structure. Haskard *et al*. (2001) also suggested that surface components of these bacteria are involved in binding because of the accessibility of bound AFB_1 to an antibody in an indirect competitive inhibition enzyme-linked immunosorbent assay. Temperature and bacterial concentration affected the binding response, but not pH across the range of pH 4 to 6 (El-Nezami *et al*., 1998). El-Nezami *et al*. (2000) tested the ability of *Lactobacillus rhamnosus* strains GG and LC-705 to remove AFB_1 from the intestinal luminal liquid medium using a chicken intestinal loop technique. In this study, the GG strain of *L. rhamnosus* decreased AFB_1 concentration by 54% in the soluble fraction of the luminal liquid within one minute. The LC-705 strain removed only 44% of aflatoxin under similar conditions. The complexes formed *in vitro* between the *L. rhamnosus* strains and AFB_1 were stable under luminous conditions for a period of one hour. Peltonen *et al*. (2001) found removal of more than 50% AFB_1 by two *Lactobacillus amylovorus* strains and a *Lactobacillus rhamnosus* strain after a 24-hour incubation period. The binding was reversible and AFB_1 was released by repeated aqueous washes.

Hirano *et al.* (1994) demonstrated that bovine serum albumin provided protection against AFB_1 toxic effects by binding to the toxin in the intestinal tract. In studies on day-old chicks a marked reduction in histological and biochemical symptoms of exposure to AFB_1 and toxin level in the plasma and liver, was observed.

Chlorophyllin, a water-soluble derivative of chlorophyll, has been shown to be potently antimutagenic against various mutagens including heterocyclic amines (Arimoto *et al*., 1993) and AFB_1 (Dashwood *et al*., 1991). Arimoto *et al*. (1993) provided evidence that the suppressing effects arise by a complex formation between the porphyrin-like structure of chlorophyllin and the planar molecular surfaces of these compounds. Arimoto-Kobayashi *et al*. (1997) reported that the water-insoluble form of chlorophyllin, chlorophyllin-chitosan, is a stable material in organic solvents and on drying, and thus is superior to chlorophyllin as an adsorbent for planar compounds.

The addition of an enzyme-linked polyvinylpolypyrrolidone (*Mycofix® Plus*) to an aflatoxin-containing diet significantly increased T-lymphocyte and splenic plasma cell counts. Polyvinylpolypyrrolidone forms a hydration hull around its particles that attracts polar molecules, such as aflatoxin. Mycofix® Plus has been combined with enzymes capable of degrading mycotoxins by breaking particular functional groups (Çelik *et al*., 2000b).

3. Humic Acids

Humic substances are ubiquitous, and are found wherever matter is being decomposed, or has been transposed as in the case of sediments. Four main fractions of humic substances can be identified according to solubility (Hayes *et al*., 1989):

(i) *Fulvic acids* are the fraction of humic substances that is soluble under all pH conditions (mM: < 500 Da)

(ii) *Hymatomelanic acids* are the fraction of humic substances that is soluble in short chain alcohol (i.e. ethanol) (mM: 5 000 – 11 000 Da)

(iii) *Humic acids* are the fraction of humic substances that is not soluble in water under acidic conditions (pH < 2) but is soluble under higher pH values (mM: >12 000 Da)

(iv) *Humin* is the fraction of humic substances that is not soluble in water at any pH value (mM: > 70 000)

Humic acids are widely spread in nature and are present in soils, natural waters, river, lake and sea sediments, peat, brown and brown-black coals and other natural materials as a product of chemical and biological transformations of animal and plant residues (Novák *et al*., 2001). Peat is organic soil formed as a result of incomplete disintegration and humification of died marsh plants in conditions of high humidity (Schepetkin *et al*., 2002). The humic acids in peat, which have been well known for their therapeutic properties in ancient times, showed several characteristics such as anti-inflammatory, antiviral, oestrogenic, profibrinolytic and heavy metal-binding activities. The oxidative polymerization of o- and p-diphenols produced synthetic humic acids, with similar therapeutic activities (Klöcking, 1994).

3.1 The structure of humic acids

The humic acids dissolved or dispersed in aqueous solution can show a size distribution and spontaneous changes in their conformational and aggregation states in relation to pH, ionic strength and presence of di- or multivalent ions. The molecular features of humic acids also depend on their age and origin (Niemeyer *et al*., 1992). In general, an increase in the soil age leads to changes in the structure of humic acids, becoming progressively more aromatic and less rich in polysaccharide and lignin derivatives. Francioso *et al*. (2001) found a specific pattern of functional groups for humic acid samples from different origins. Humic acids from peat contain a high amount of oxygenated groups (-COOH, O-CH_3, -C=O and OH in phenols). Humic acids extracted from leonardite and lignite showed a lower content of sugar-like components and polyethers. On the other hand, the aromatic structures were ubiquitous in all samples. It is this physicochemical property of low-molecular humic substances that is responsible for some reactions occurring in tissues, such as the antiviral effects, antibacterial effects, desmutagenic effects and anti-inflammatory effects.

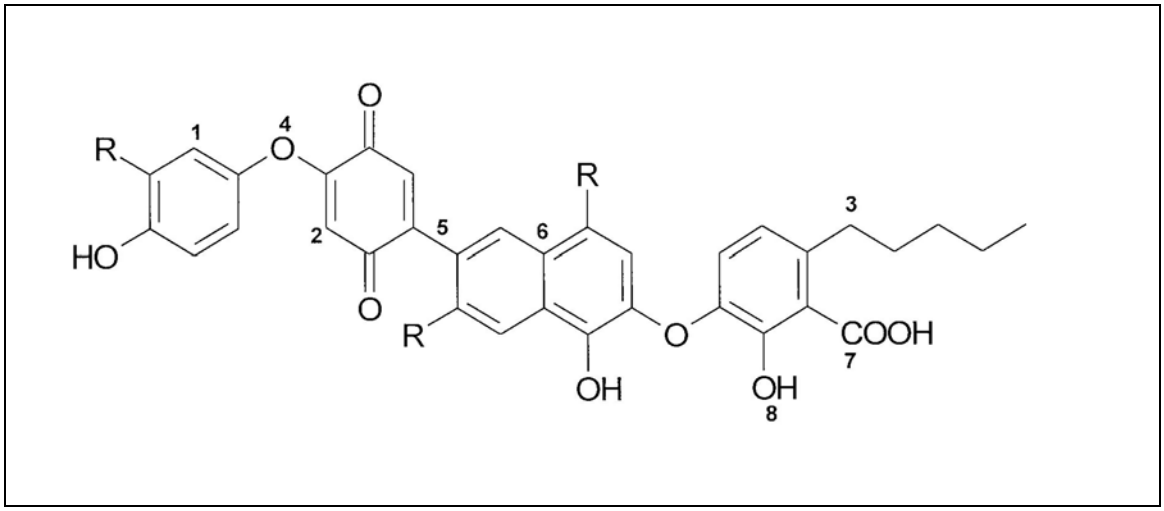

Figure 1.2. Structural characteristics of a model humic substance. 1. aromatic ring; 2. chinoid ring; 3. aliphatic side-chain produced by breaking up of ring structure; 4. O-bonds; 5. C-bonds; 6. C-C-bonds; 7. carboxyl groups; 8. phenolic-OH groups (Riede *et al*., 1991)

3.2 The therapeutically value of humic acids

3.2.1 Antiviral activity

Different studies showed that humic acids are effective against both naked and enveloped DNA viruses. *In vitro* studies by Thiel *et al*. (1977) and Schiller *et al*. (1979) revealed that ammonium humate, isolated from peat water, had strong antiviral activity against herpes simplex virus type 1 and type 2. According to Klöcking (1994) humic acid-like polymers were effective against cytomegalovirus and vaccinia virus. However, the polymers proved to be inactive against polio virus type 1, Semliki forest virus, parainfluenza virus type 3, reovirus type 1 and Sindbis virus. The effective antivirally concentrations of humic acid-like polymers were below the cytotoxic concentration, indicating that the polymers have a selective antiviral effect. As for the cytomegalovirus it appears to be likely that the polyanionic humic acids occupy positively charged domains of the viral envelope-glycoproteins, which are necessary for virus attachment to the cell surface.

Schneider *et al*. (1996) found that synthetic humic acids selectively inhibited the AIDS-causing infectious agent, human immunodeficiency virus (HIV) type 1. The humic acid analogue inhibited the infectivity of HIV particles by interference with a V3 loop-mediated step of virus entry.

3.2.2 Antiinflammatory effect

Human and veterinary medicine exploited the anti-inflammatory effect of humic acids for a long time by various external and internal peat applications. The main indications in human medicine were rheumatoid arthritis as well as chronic and subchronic inflammations of genitals. There are also reports on successful treatment of dermatitis, eczema, burns and infected wounds (Klöcking, 1994).

A possible biochemical explanation of the anti-inflammatory effect of humic acids may be the inhibition of the lipoxygenase path of the arachidonic acid cascade. Arachidonic acid is released because of plasma membrane lesions and is then degraded to leukotrienes. These leukotrienes act as inflammation mediators, which cause increased vascular permeability, formation of oedema, leukocyte infiltration, enzyme release and superoxide formation (Klöcking, 1994).

Iubitskaia & Ivanov (1999) reported that sodium humate balneotheraphy for osteoarthrosis patients had produced analgetic, anti-inflammatory and lipid modulating effects and it improved metabolic processes.

3.2.3 Effect of humic acids on the immune system

Obminska-Domoradzka *et al*. (1993a; b) found that the administration of Tolpa Peat Preparation TPP, a mixture rich in humic substances, to immunologically mature mice causes functional stimulation of the lymphatic system cells. Daily administration of 1 mg/kg TPP for 12 weeks enhanced the response to sheep erythrocytes significantly. TPP caused morphological changes of the thymus in mice (the central lymphatic organ where maturation and differentiation of T-lymphocytes occur), which indicated thymus activity stimulation (Madej *et al*., 1993a and b). Jankowski *et al.* (1993) performed a randomized, double blind study to assess the therapeutic efficacy of TPP in 39 patients with recurrent respiratory tract infections. The phagocytic activity of granulocytes was significantly stimulated after six months of treatment.

Riede *et al*. (1991) tested three different low-molecular humic substances (two naturally occurring humates and one synthetically prepared humate) on neutrophil function. They were all capable of stimulating certain functions of human neutrophils, such as the respiratory reaction with hydrogen peroxide as main product. It was suggested that the humic substances acted as signals to change dormant human neutrophils into activated cells.

3.2.4 Humate as a growth promoter

Humate forms chelates with trace minerals to enable uptake of nutrients by plant cells and is therefore considered as a fertilizer for growing plants (Kocabağli *et al*., 2002). The beneficial effects of humic acids on plant growth and crop yields may also be attributed to improvements in soil properties and structure (Kahsnitz, 1992) and to increased microbial populations and biologically active metabolites such as plant growth regulators (Tomati & Galli, 1995; Doube *et al*., 1997). Atiyeh *et al*. (2002) used humic acids derived from earthworm-processed organic wastes on tomato and cucumber seedlings. Plant growth increased significantly in terms of plant heights, leaf areas, shoot and root dry weights with increasing concentrations of humic acids incorporated into the medium, but often decreased when the concentrations exceeded 500-1000 mg/kg. According to Atiyeh *et al*. (2002) these growth responses were probably due to hormone-like activity of humic acids or plant growth hormones adsorbed onto the humates.

Using humates in animal nutrition has a very short history. In recent years, it has been observed that dietary intake of humates promote growth in poultry (Bailey *et al*., 1996; Shermer *et al*., 1998). Kocabağli *et al*. (2002) investigated the effects of dietary humates on live performance, carcass weights and abdominal fat pads of broilers. The humate was added to the diets at 2.5 g/kg feed. At 42 days of age, body weights and feed conversions of broilers were significantly affected by the dietary humate treatment. There was no difference in carcass yield or abdominal fat pad percentage due to feeding humate.

3.3 Adsorption of humic acids to various compounds

3.3.1 Heavy metal-binding activity

The interactions of humic stubstances with metals play an important role in metal mobility and bioavailability in the environment. Adsorption is the most promising technique for the removal of harmful heavy metals from aqueous environments. The ability of humic acids to adsorb heavy metals has been demonstrated in a number of investigations. According to Madronová *et al*. (2001) the capacity of a humic acid depends on its origin and pre-treatment. After oral application, binding to humic acids reduced the toxicity of metal ions. Thus, it is possible that humic acids can reduce the risks of heavy metal intoxication. It was observed that small humic acid concentrations (0.1%) in food were sufficient to significantly reduce the incorporation of lead and cadmium in rats (Klöcking, 1994).

Madronová *et al*. (2001) conducted a study to confirm the possibility of using humic acids for the separation of heavy metals from wastewater. The adsoption efficiency was dependent on the composition of the solution and the pH. The adsorption efficiency deteriorated with decreasing pH, especially for Pb and Cd, and slightly for Ni and Zn, but not for Cu.

In a study by Liu & Gonzalez (2000), a system consisting of lead (II), copper (II) and cadmium (II) as typical heavy metal pollutants, and purified Aldrich humic acid were selected as prototypes to model the environmental system. The results showed that pH and ionic strength are the most important variables in controlling metal adsorption on humic acid, but the concentration of humic acid may also be of importance in some cases (Tanaka & Senoo, 1995). A high complexation capacity of humic acid for the metals was found, in the sequence of Pb>Cu>Cd. According to Ibarra *et al*. (1979) a higher pH, and also higher atomic weight and valency favoured metal retention by humic acid. Several other studies confirm the potential of humic acid to adsorb cadmium (Riffaldi & Levi-Minzi, 1975; Sohn & Rajski, 1990; Fu *et al*., 1992) and lead (Seki *et al*., 1990). Riffaldi & Levi-Minzi (1975) proved that 50% of Cd that is adsorbed on humic acid is in an exchangeable form and 50% in coordination complexes. Higher adsorption constants of Cd were associated with the sedimentary humic acids which contained spectroscopically detectable amino acid nitrogen, suggesting that these additional adsorption sites provided by amino acid nitrogen are responsible for the greater adsorption constant values of sedimentary vs. soil humic acids (Sohn & Rajski, 1990). According to Fu *et al*. (1992) humic acids could explain all of the binding of cadmium by sediments. Ran *et al*. (2001) also found an adsorption effect of humic acids on gold.

3.3.2 Adsorption of herbicides and pesticides

Humic substances play an important role in influencing the fate and behaviour of herbicides in soils. Various mechanisms or combination of mechanisms have been shown to operate simultaneously in the adsorption process, depending on the chemical, structural and functional properties of the interacting humic macromolecule and herbicide (D'Orazio *et al*., 1999). Nègre *et al*. (2001) performed adsorption isotherms of the imidazolinone herbicides, imazapyr, imazethapyr and imazaquin on a soil humic acid at pH 2.8 and 4.0. At both pH, adsorption increased according to the lipophilic character of the molecules (imazapyr < imazethaper << imazaquin). Leone *et al*. (2001) studied the adsorption of the same herbicides on two ferrihydrite-humic acid binary systems (containing 4% and 8% humic acid) and on ferrihydrite alone. Imidazolinone adsorption on pure

ferrihydrite and on the 4% ferrihydrite-humic acid system decreased with increasing herbicide hydrophobicity (imazaquin < imazethaper < imazapyr). However, on the 8% ferrihydrite-humic acid system the order of adsorption was nearly the same, indicating that higher amounts of humic acid on ferrihydrite increase the hydrophobicity of the surface and thus increases its affinity for the herbicides, especially for imazaquin. Leone *et al.* (2002) observed that even though pure smectite cannot adsorb herbicides, it modifies the adsorption capacity of ferrihydrite. The mutual interaction of active phases such as humic acid, ferrihydrite and smectite alters the characteristics of the resulting surface and hence the adsorption process. Both Nègre *et al*. (2001) and Leone *et al*. (2001) found that the extent of adsorption was higher at lower pH values because of the partial ionization of the carboxylic groups of both the herbicides and the humic acids at increasing pH (Nègre *et al*., 2001). No differences between adsorption isotherms at 10 ºC and 25 ºC were observed at pH 4.0, indicating that adsorption involved very weak bonds. However, at pH 2.8, adsorption was higher at 10 ºC than 25 ºC indicating an exothermic process. The extent of adsorption was correlated with the amount of carboxylic and aromatic groups of the humic acid, suggesting that hydrogen bonding and/or charge-transfer complex formation could take place (Senesi *et al*., 1997; Nègre *et al*., 2001).

Triallate is a carbamothioate herbicide widely used to control annual and perennial grasses. The adsorption of triallate was between two and four orders higher for a humic acid isolated from pig slurry than that for humic acid isolated from two different soil types. The adsorption could be described in all cases by linear isotherms. The types of binding mechanisms appeared to be related to the compositional, structural and functional properties of the humic acid. Hydrophobic bonds possibly prevailed in the interaction of triallate with pig slurry humic acid, whereas adsorption of triallate onto soil humic acids possibly involved charge-transfer and ionic bonds, with the participation of fluorophore groups of the humic acid macromolecules (D'Orazio *et al*., 1999).

It should be noted that the characteristics of soil humic acids change with the depth of the soil. These changes may affect the adsorptive behaviour of the humic acid and may therefore have variable reactivity with a specific compound at different depths. Such reactivity variation with depth should be considered in predictive models for fate and transport of compounds and assessment of availability and risk of a contaminated site (Xing, 2001).

3.3.3 Adsorption of mutagens

Humic acids inhibited the mutagenicity of various mutagens and may play an important role in natural purification. According to Sato *et al*. (1987a) the inhibitory effect was desmutagenic, heat-resistant and increased with an increase of the humic acid molecular weight. The desmutagenic effect of humic acid was caused by adsorption of the mutagen, such as benzo[*a*]pyrene and 3-aminoanthracene, and not by decomposition of the mutagen. Humic acid exerted a desmutagenic effect on mutagens directly before they act on cells (Sato *et al*., 1986). The adsorption activity was greatest at its critical micelle concentration and the adsorbed mutagen was released by ultrasonication (Sato *et al*., 1987a & b). Cozzi *et al*. (1993) also observed a desmutagenic rather than antimutagenic activity of humic acids when tested in combination with the well-known mutagens, mitomycin C and maleic hydrazide. Humic acids in the form of potassium humate, exerted a strong inhibitory effect on the formation of the mutagen N-methyl-N-nitrosourea when present during the nitrosation of N-methylurea but didn't reduce the mutagenicity of this specific mutagen (Badaev *et al*., 1989).

3.3.4 Interactions between monoaromatic and polycyclic aromatic compounds and humic acids

Nanny & Maza (2001) demonstrated that solution-phase, noncovalent interactions between deuterated monoaromatic compounds (phenol-d_5, pyridine-d_5, benzene-d_6) and humic acids are a function of solution pH, percent aromaticity, and the monoaromatic functional group. Strong interactions were found at lower pH values.

The adsorpion of 45 polycyclic aromatic compounds (PAC) to humic acid increased with the size of the PAC and the number of lipophilic substituents, but decreased when polar substituents were present. Other factors impacting the PAC binding may be specific interactions with humic acid and the ionic strength of the aqueous phase (Kollist-Siigur *et al*., 2001).

3.3.5 Adsorption of humic acid to mineral particles

The adsorption of humic acid to mineral particles, such as aluminium (Fein *et al*, 1999; Elfarissi & Pefferkorn, 2000), can be characterized by specific and electrostatic interactions and by polydispersity effects. Humic acid adsorption onto mineral surfaces was the strongest when the humic acid was negatively charged and the mineral surface was positively charged, with the adsorption decreasing as the concentration of positively charged mineral surface sites decreased

with increasing pH (Fein *et al*., 1999). Vermeer *et al*. (1998) found that the adsorption was low at a high pH and low salt concentration and the humic acid molecules were adsorbed relatively flat on the surface. At low pH and high salt concentration a substantial fraction of the adsorbed humic acid was not in direct contact with the surface which resulted in a relatively high adsorption and a large layer thickness.

3.3.6 Humic acid adsorption onto bacteria surfaces

Fein *et al*. (1999) measured adsorption of humic acid onto aerobic gram-positive *Bacillus subtilis* bacterial species. The adsorption was strongly pH-dependent, increasing with decreasing pH over the pH range 2.5 – 11.5. The interaction was dominantly hydrophobic. Crecchio & Stotzky (1998) reported on the equilibrium adsorption and binding of DNA from *Bacillus subtilis* on humic acids extracted from a forest soil. Adsorption of DNA on humic acid was maximal after 2 and 4 h at pH 3.0 and 4.0, respectively. The adsorption of 50 μg of DNA on increasing concentrations of humic acid reached a plateau with 2 and 3 mg of humic acid at pH 3.0 and 4.0, respectively.

3.4 Toxicology studies with humic acids

Treatment of rats with 1000 and 2000 mg/kg feed of a low-molecular synthetic humic acid during different stages of pregnancy caused no signs of toxicity to either mothers or foetuses. A humic acid dose of 5 g/kg feed over a period of 100 days also didn't affect the reproduction parameters of rat males (Lange *et al*. 1996).

According to Kühnert *et al*. (1989) the use of humic acid products is exceptionally safe with no danger of toxicity or any side effects such as allergy or resistance.

3.5 The derivation of humic acids from coal

Coal arises from the accumulation of vast quantities of plant remains and their subsequent decomposition and consolidation. The first phase of the decomposition involves microbial activity and the formation of peat in waterlogged environments. In the second phase the peat becomes overlain by sediments and subjected to temperatures up to 200 °C and very high pressure. Over geological periods of time this results in the successive formation of lignite and brown coal, bituminous coals of increasing maturity, and ultimately anthracite. The age of mature coals ranges from 2 x 10^6 to 250 x 10^6 years, with anthracites the oldest and most mature and brown coals the youngest. The maturation of coal is accompanied by changes in composition and in many of its

properties. The most important changes in composition involve a progressive increase in the carbon content, from about 70% in low-rank coals to 95% in anthracites, and a corresponding decrease in the oxygen content (Lawson & Stewart, 1989).

There are basically two primary methods for the extraction of humic acids from coal (Lawson & Stewart, 1989):

(i) Direct Extraction of humic acids

Less mature coals, including brown coals and lignites, may contain significant amounts of acidic groups, substantial proportions of which may be extracted into aqueous alkaline media as alkali metal humates. Dilute aqueous alkali appears to be the most favoured extractant, since it is selective in dissolving only acidic material, leaving both mature coal substances and inorganic material undissolved (Lawson & Stewart, 1989). Dryden (1951) studied organic liquids as coal solvents. Mature coals are reasonably soluble at moderate temperatures only in solvents such as pyridine and 1,2-diaminoethane. Brown coals and lignites contain materials such as waxes, bitumens and humic acids, which are soluble in benzene or benzene-ethanol mixures. When organic solvents are used to extract humic acids from coal, extraction is usually incomplete, and subsequent alkali treatment is necessary to separate the humic acids from non-acidic soluble substances (Verheyen & Johns, 1982).

Humic acids are normally extracted from coal at moderate temperatures with dilute sodium or potassium hydroxide solutions. Air may be excluded to avoid oxidation. The alkaline extract, which is usually separated from insoluble residue by filtration or centrifugation, contains a complex mixture of molecules, which after acidification, are separated into fulvic acids (soluble in water), hymatomelanic acids (soluble in ethanol) and humic acids (insoluble in water and ethanol). The yield of humic acids extracted from coal decreases as the coal rank increases. (Lawson & Stewart, 1989). According to Verheyen & John (1982) the rank also influences the character of the humic acid; humic acids from higher rank coals have fewer functional groups and greater aromaticity.

Bernacchi *et al*. (1996) obtained humic-like substances by heating mineral coal at relatively low temperatures (180 °C), followed by alkalization with a process resembling an inversion of the natural diagenetic transformation. The compounds obtained reveal physical and chemical properties quite similar to those of natural humic and fulvic acids. Novák *et al*. (2001) used common techniques and procedures readily applicable in industry (alkaline extraction, acidic

precipitation, membrane separation) to prepare cost-effective samples of humic acids from low-rank young brown coals, not usable as fuel, of the North-Bohemia coal fields in the Czech Republic.

(ii) Oxidation of coals

Humic acids may be obtained from mature, alkali-insoluble coals by oxidation, which leads to elimination of small molecules, including CO_2, and introduces sufficient acid groups to confer solubility. Different oxidizing agents may be used, including oxygen (air) (Bergh *et al*., 1997), nitric acid (Moliner & Galivan, 1981) and performic acid (Raj, 1980).

Estevez *et al*. (1990) studied dry oxidation of coals by air to increase the humic acid content of four different Spanish coals. The best results for humic acid production were obtained at the highest temperatures and after longer times. Lower-rank coals yielded the highest humic acid concentrations, but due to the severe loss of weight of these coals, the net increase was lower than that obtained from subbituminous coals. With alkaline oxidation of coals, the best humic acid production was also obtained with the highest temperature, longest time and lowest alkali concentrations (Juan *et al*., 1990). Bergh *et al*. (1997) oxidized South African bituminous coal with oxygen at 180 °C under constant pressure (4MPa) for one hour.

3.6 Oxihumate-K S35

A South African company, Enerkom, developed an effective large-scale regeneration process for humic acids from coal. This technology can economically regenerate large quantities of pure, high quality humic acids by reversing the process whereby coal was formed. Humic acids produced in this way are called oxihumic acids. Chemically, oxihumic acids differ only marginally from humic acids obtained from other sources (Cloete *et al*., 1990; Dekker *et al*., 1990; Cronjé *et al*., 1991; Bergh *et al*., 1997).

Van Rensburg *et al*. (2002) studied the effects of oxihumate on HIV-1 replication as well as the development of viral resistance in cell culture when exposed to oxihumate for an extended time period. Oxihumate inhibited HIV-1 infection of MT-2 cells with an IC_{50} of 12.5µg/mL. Treatment of free and cell-attached HIV with oxihumate irreversibly reduced infectivity, while the susceptibility of target cells to the virus was not impaired by treatment prior to infection. The infectivity of the HIV particles was inhibited by interference with CD4-binding and the V3 loop

mediated step of virus entry. No viral resistance to oxihumate developed over a 12-week period *in vitro*.

Jooné *et al*. (2003) found that oxihumate increased the *in vitro* proliferative response of phytohaemagglutinin-stimulated human lymphocytes, from a concentration of 20 µg oxihumate/ml and upwards. This response was even more significant in the lymphocytes from HIV-infected patients. Similar effects were observed *ex vivo* following administration of a dosage of 4 g oxihumate per day to HIV-positive individuals for two weeks. Stimulation of the proliferative response of lymphocytes by oxihumate is associated with an increased production of IL-2, as well as expression of the IL-2 receptor in the setting of decreased production of IL-10.

Bergh *et al*. (1997) carried out primary acute toxicity studies on humic substances derived from coal by oxidation. Apart from local irritation caused by their acidic nature, neither of these fractions exhibited significant acute toxicity in the tested animals. Botes *et al*. (2002) gave dosages of up to 8 gram of oxihumate per day to HIV-1 infected individuals for a period of two weeks. No adverse effect was observed in any of the patients and it was concluded that oxihumate is safe and non-toxic to human subjects.

The production of oxihumate-K S35

Reactive bituminous coal is pulverized to < 250 µm particle size in a ball mill. The powered coal is then mixed with water to produce a slurry which is continuously fed into a series of three reactors where the oxidative conversion takes place at elevated temperature and pressure conditions. The heat generated during the exothermal oxidation reaction is removed through heat exchangers in the reactor. After the coal slurry reached the desired level of oxidation it is cooled by passing it through a heat exchanger into a drum. Dissolved CO_2 gas is allowed to escape. The oxiproduct is pumped into a filter press to separate the solids from the aqueous filtrate. The filtrate contains 2.5% oxifulvic acid. The solid product contains oxihumic acid, is reslurried in water to a density of 30% and reacted with 40% potassium hydroxide solution until a pH of approximately 9 is reached. The slurry becomes a gel and is spray-dried to form potassium oxihumate-K S35 (Adapted from SA patent 88/4770, described by Bergh *et al*, 1997).

CHAPTER 3

In Vitro Binding Of Oxihumate To Different Mycotoxins

Abstract

Mycotoxins have become an important issue for the grain industry and animal producers with a growing interest in the decontamination and remediation of highly contaminated feedstuffs. A recent approach to detoxifying mycotoxin-contaminated grain is the use of non-nutritive adsorbents, which bind the mycotoxin and thereby reduce their absorption from the gastrointestinal tract. Research showed that humic substances have a strong affinity to bind with several compounds. A South African company developed an effective large-scale regeneration process for very pure and high quality humic acids, called oxihumic acids, from coal. The main objective of this study was to evaluate the *in vitro* affinity and adsorption capacity of oxihumate to aflatoxin (AF) B_1 and G_2 by studying their Langmuir and Freundlich adsorption isotherms. The Freundlich isotherm fitted the data better than the Langmuir isotherm. The data showed adsorptions by oxihumate of approximately 10.3, 7.4 and 11.9 mg AFB_1/g oxihumate at pH 3, 5 and 7, respectively, and 1.2, 2.6 and 8.5 mg AFG_2/g at pH 3, 5 and 7, respectively. The results of this study indicated that oxihumate could be considered as a potential mycotoxin binder and additional studies are warranted to assess its efficacy under *in vivo* circumstances.

Introduction

Mycotoxins are a group of structurally diverse fungal metabolites that occur as both food and animal feed contaminants worldwide. Fungi such as *Aspergillus*, *Fusarium*, *Penicillium*, and *Claviceps* species often infect economically important crops and forages in the field, during storage, transportation and processing. The mycotoxins produced by these fungi can cause serious health problems and production losses in livestock. Mycotoxins have therefore become an important issue for the grain industry and animal producers with a growing interest in the screening of agricultural commodities for mycotoxins and the decontamination and remediation of highly contaminated feedstuffs. Grains contaminated with low levels of mycotoxins can be used safely in livestock and poultry feed, but feed containing higher levels of mycotoxins may reduce efficiency of production and increase susceptibility to infectious diseases. Very high levels may cause an acute mycotoxicosis outbreak with resultant high mortality rates and serious losses.

Practical methods to detoxify mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. A variety of physical, chemical, nutritional and biological techniques have been employed but with only limited success (Edrington *et al*., 1997). The most recent approach is the use of non-nutritive adsorbents, which bind the aflatoxin molecule and thereby reduce their absorption from the gastrointestinal tract, avoiding the toxic effects for livestock and the carryover of these fungal metabolites into animal products (Ramos *et al*., 1996; Raju & Devegowda, 2000). A few of these, for example hydrated sodium calcium aluminosilicate (Ledoux *et al*., 1999), activated charcoal (Dalvi & Ademoyero, 1984), bentonite (Santurio *et al*., 1999), zeolite (Miazzo *et al*., 2000) and mannan oligosaccharide (Raju & Devegowda, 2000) was extensively studied with promising, but varying results. When employing adsorbent compounds to hinder the gastrointestinal absorption of these mycotoxins, two points have to be kept in mind. Firstly, the chosen compound should have a high affinity for the specific mycotoxins, resulting in the formation of a strong complex with a low possibility of any dissociation. Secondly, the chosen compound should have a high capacity to prevent saturation of the compound (Ramos & Hernández, 1996). The search is still on for an effective tool to counteract the problem of aflatoxicosis in farm animals.

Humic substances are ubiquitous, and are found wherever matter is being decomposed, or has been transposed as in the case of sediments (Hayes *et al*., 1989). The humic acids in peat, which have been well known for their therapeutic properties since ancient times, have shown anti-inflammatory, antiviral, oestrogenic and profibrinolytic activity (Klöcking, 1994). Research showed that humic acids have a strong affinity to bind with several compounds. The ability of humic acids to adsorb heavy metals has been demonstrated in a number of investigations (Madronová *et al*., 2001). Humic substances are also known for their ability to bind herbicides (Nègre *et al*., 2001), mutagens (Sato *et al*., 1987a; Cozzi *et al*., 1993), monoaromatic (Nanny and Maza, 2001) and polycyclic aromatic compounds (Kollist-Siigur *et al*., 2001), minerals (Elfarissi & Pefferkorn, 2000) and certain bacterial species (Fein *et al*., 1999).

A South African company, Enerkom (Pty) Ltd, developed an effective large-scale regeneration process for humic acids from coal. This technology can economically regenerate large quantities of pure, high quality humic acids by reversing the process whereby bituminous coal was formed. Humic acids produced in this way are called oxihumic acids. Chemically oxihumic acids differ

only slightly from humic acids obtained from other sources (Cloete *et al*., 1990; Dekker *et al*., 1990; Cronjé *et al*., 1991; Bergh *et al*., 1997).

The main objective of this study was to evaluate the affinity and capacity of oxihumate to adsorb different mycotoxins *in vitro*, for the purpose of developing it as a commercial mycotoxin binder. To describe the different kinds of adsorption processes several mathematical approaches have been developed. One of the most efficient ways of investigating surface adsorption is through the use of isotherms, in which the amount of compound adsorbed per unit of weight of adsorbent is plotted against the concentration of the compound in the external phase, at a fixed temperature and under equilibrium conditions (Ramos & Hernández, 1996). Multiple isotherm equations have been proposed for the modelling of the adsorption of compounds in aqueous solutions to the surfaces of solids (Kinniburgh, 1986). Grant & Phillips (1998) was able to use multiple isotherm equations to determine not only capacity and affinity, but also average capacity, enthalpy of binding, heterogeneity coefficient, multiple site distribution coefficients and multiple site capacity of HSCAS for AFB_1. Langmuir and Freundlich isotherms are the most extensively used (Ramos & Hernández, 1996). The Langmuir equation is most applicable to a single ligand adsorbing to a single type of site on a particular adsorbent (Langmuir, 1916). In this study the Langmuir and Freundlich adsorption isotherms of oxihumate for AFB_1 and aflatoxin G_2 (AFG_2) were determined to measure the affinity and capacity of oxihumate for each of these aflatoxins.

Materials and Methods

Reagents – Oxihumate was provided by Enerkom (Pty) Ltd, Pretoria, South Africa and MycosorbTM purchased from Alltech, Inc., Pretoria, South Africa. Aflatoxins, zearalenone, ochratoxin A, vomitoxin, and the ergopeptine alkaloids were purchased from Sigma (St. Louis, MO). All solvents and reagents were purchased from Fisher Scientic (St. Louis, MO). Primary stock solutions of each mycotoxin (1,000 ppm) were prepared in methanol. Mycotoxin test solutions for adsorption tests were prepared by adding methanol stock solutions to 0.1 M phosphate buffer adjusted to the desired pH. Mycotoxin concentrations were based on the relative ease of analysis by HPLC and cost of mycotoxin rather than levels known to cause problems in livestock.

Equipment - High pressure liquid chromatography (HPLC) analyses were performed on a Perkin Elmer 250 pump with a Perkin Elmer ISS200 autosampler, fluorescence detection with a Hitachi

F1200 fluorescence spectrophotometer and UV detection with a Perkin Elmer LC90 detector. Separations were achieved on a Hypersil®BDS 3µ C_{18} column (100 x 4.6mm) or a Perkin-Elmer 3 cm C_{18} column (3 um particle size) with a flow rate of 1mL/min.

Experiment 1

Experiment 1 was done as a pilot study to determine possible binding capabilities of oxihumate to different mycotoxins.

Duplicate aliquots of 0.1 M phosphate buffer (adjusted to pH 3) containing 2 mg/L zearalenone, 2 mg/L AFB_1, 2 mg/L vomitoxin, 2 mg/L ochratoxin A or 250 µg/L ergopeptine alkaloid mix in solution (10 mL) were added to 15 mL screw cap test tubes to which had been added 0.5, 1, 10 and 50 mg of oxihumate. 10 mL aliquots of 0.1 M phosphate buffer (pH 3) containing 2 mg/L AFB_1 were also added to 15 mL screw cap test tubes with 0.5, 1, 10 and 50 mg of Mycosorb™, a commercially available mycotoxin binder (Alltech, Inc.). Mycosorb™ was used as a control to provide a standard for comparison. In order to eliminate exogenous peaks, controls were prepared by adding 10 mL of 0.1 M phosphate buffer plus 100 mg of adsorbent (oxihumate and Mycosorb™) to test tubes. The tubes were placed on a rotator shaker for 30 minutes at room temperature. Each mycotoxin test solution and controls were then centrifuged at 3000 rpm for 10 minutes and 2 mL of the aqueous supernatant was added to autosampler vials and analysed for mycotoxin levels by HPLC. The mobile phases and detection wavelengths for each mycotoxin are listed in Table 3.1.

The percentage mycotoxin bound for each sample was calculated from the difference between the initial and final concentration of mycotoxin in the aqueous supernatant, by the following equation:

$$\% \text{ bound mycotoxin} = 1 - (\text{Sample peak height} / \text{Mean standard peak height}) \times 100$$

Table 3.1. HPLC conditions used for the analysis of different mycotoxins

Mycotoxin	Mobile Phase	Detection
Aflatoxin B_1	water:MeOH:isopropanol (40:17:2)	Fluorescence (F): excitation wavelength (ex) - 365 nm emission wavelength (em) - 430 nm
Zearalenone	water:MeOH (55:45) with 1% acetic acid	F: ex - 274 nm em - 465 nm
Ochratoxin A	MeOH:water:acetic acid (60:40:1)	F: ex - 365 nm em - 450
Vomitoxin	water:MeOH (9:1) with 3 g KCl/L	UV: 224 nm
Ergot	ACN:water (40:60) with 200 mg NH_4CO_3	F: ex - 250nm em - 415 nm

Experiment 2

In Experiment 2, the binding of oxihumate to AFB_1 and AFG_2 was determined under different pH conditions and a study of the Langmuir and Freundlich adsorption isotherms was carried out. Mycosorb™ was also used in this experiment as a control.

Adsorption experiments

Stock methanolic solutions were prepared at a concentration of 1 mg/mL for AFB_1 and 0.5 mg/mL for AFG_2.

The adsorbents (oxihumate and Mycosorb™) were weighed in duplicate into clean 15 mL screw cap test tubes and 10 mL of 0.1M phosphate buffer (pH 3, 5 or 7) containing either 2 mg/L AFB_1 or 0.5 mg/L AFG_2 was added. The tubes were vortexed and placed on a rotator shaker for 30 minutes at room temperature. Control treatments were carried out for each experiment. One control was run without adsorbent to investigate any possible non-specific binding of aflatoxins and another control was run with an oxihumate sample in buffer without mycotoxin in order to eliminate exogenous peaks.

The suspensions were centrifuged at 1700 rpm for 5 minutes. The aqueous supernatants were added to autosampler vials and analysed for aflatoxin levels by HPLC. The mobile phase was run at 1

mL/min. For AFB_1 analysis a mobile phase of water:MeOH:isopropanol at 40:17:2 and for AFG_2 at 45:15:5 were used. An aliquot of the original buffered aflatoxin test solution was used as the HPLC standard for each mycotoxin.

At pH 7, the oxihumate was soluble to a greater degree than at the lower pH's, noted by the darker colour of the supernatants and could not be analysed directly. The 10 mL supernatant of these samples was extracted with 5 mL chloroform. The chloroform was filtered through anhydrous sodium sulphate and dried. The aflatoxin was dissolved with 1 mL methanol, after which the volume was made up to 10 mL with buffer (pH 7). This solution was then added to the auto-sampler vials for analyses.

Fitting of models to data

Aflatoxin adsorption data for oxihumate was examined using the Langmuir (Giles et al., 1974) and Freundlich isotherms (Ramos & Hernández, 1996). The classical Langmuir isotherm is expressed by the equation (Giles *et al*., 1974):

$$C_s/C_a = (C_s/k_1) + (1/k_1k_2)$$

where C_a is the amount of aflatoxin adsorbed per unit of weight of adsorbent (mg/g); C_s is the concentration of unadsorbed aflatoxin at equilibrium (mg/L); k_1 is the capacity constant for a complete monolayer and k_2 is the affinity constant of the adsorbent for each aflatoxin studied.

Rearranging:

$$C_s/C_a = (1/k_1)Cs + (1/k_1k_2)$$

A plot of C_s/C_a verse C_s gives a straight line of slope $1/k_1$ and an intercept of $1/k_1k_2$ where k_1 is the maximum aflatoxin adsorbed per unit weight of oxihumate.

The empirical Freundlich isotherm is given by the equation (Hermosín *et al*., 1991):

$$C_a = k_1C_s^{k2}$$

where C_s is the equilibrium concentration of aflatoxin in solution (mg/L), C_a is the amount of aflatoxin adsorbed per unit weight of oxihumate (mg/g), k_1 is a constant related to the capacity of the adsorbent for each aflatoxin, and k_2 is a constant related to the affinity of the adsorbent for each aflatoxin. Converting to logs and rearranging:

$$log\ C_a = log\ k_1 + k_2\ log\ C_s$$

Plotting $log\ C_a$ verse $log\ C_s$ gives a line with a slope of k_2 and a intercept of $log\ k_1$.

Experiment 3

In Experiment 3, the binding of AFB_1 by oxihumate was determined when mixed with a commercial poultry feed to simulate practical conditions. If a high proportion of oxihumate would be bound by the feed, it could refrain the oxihumate from binding with the mycotoxin. The adsorption experiments were carried out at pH 3 and 7. 10 mg of oxihumate and 2.86 g of feed were weighed into clean 15 mL screw cap test tubes to give a concentration of 3.5 g oxihumate/kg feed. 10 mL of 0.1M phosphate buffer (pH 3 or 7) was added to the tubes. The tubes were vortexed and shaken for 30 minutes at room temperature to allow binding of oxihumate to the feed. AFB_1 was then added to the tubes at a final concentration of 2 mg/L. The tubes were vortexed and shaken for 30 minutes at room temperature. The suspensions were centrifuged at 1700 rpm for 5 minutes. The aqueous supernatant was added to autosampler vials and analysed for aflatoxin levels by HPLC. A control treatment without feed and a standard treatment with feed and AFB_1 only, were also included. All tests were run in triplicate.

Experiment 4

Experiment 4 was carried out to determine the stability of the aflatoxin-oxihumate adsorption complex in the presence of a series of solvents. 10 mg oxihumate was weighed into clean 15 mL screw cap test tubes and 10 mL of 0.1M phosphate buffer (pH 3) containing 2 mg/L AFB_1, was added to each tube. The tubes were vortexed and shaken for 30 minutes at room temperature. The suspensions were centrifuged at 1700 rpm for 5 minutes. The aqueous supernatants were removed and 10 mL of chloroform, acetonitrile or acetone was added. After centrifugation 5 mL of solvent was removed and evaporated. 1 mL of methanol was added to each tube and vortexed, after which 4 mL of buffer (pH 3) was added to restore the original volume. Samples were added to autosampler vials and analysed for aflatoxin levels by HPLC. All tests were run in triplicate.

Results

Experiment 1

Oxihumate showed a high affinity for AFB_1, zearalenone, ochratoxin A, ergosine, ergotamine, ergocornine, ergocryptine and ergocristine (Tables 3.2 and 3.3), but did not bind to vomitoxin (results not shown). The binding capacity of Mycosorb™ to AFB_1 at pH 3 proved to be considerably less than that of oxihumate (Table 3.2).

Table 3.2. *In vitro* binding of aflatoxin B_1 by oxihumate and Mycosorb™

Aflatoxin B_1 (2 mg/L) at pH 3 in 10 mL buffer			
mg oxihumate	**% bound**	**mg Mycosorb™**	**% bound**
50	100%	50	38%
10	100%	10	11%
1	47%	1	2%
0.5	17%	0.5	2%

Table 3.3. *In vitro* binding (%) of mycotoxins[1] by oxihumate at pH 3 in 10 mL buffer

Mycotoxin	**mg oxihumate**			
	0.5	**1**	**10**	**50**
Zearalenone	23	39	94	100
Ochratoxin A	26	42	95	99
Ergosine	26	69	100	100
Ergotamine	48	80	100	100
Ergocornine	25	68	100	100
Ergocryptine	15	68	100	100
Ergocristine	34	82	100	100

[1] 2mg zearalenone and ochratoxin A/L buffer and 250µg ergot alkaloid/L buffer

Experiment 2

The adsorption and equilibrium concentrations in solution of AFB_1 and AFG_2 at room temperature and at different pH levels for different levels of oxihumate are presented in Tables 3.4 and 3.5 and of Mycosorb™ in Table 3.6. Each value is the average of two replicates compared to a control without added oxihumate.

Table 3.4. ***In vitro*** **binding of AFB_1 (2 mg/L) by oxihumate at pH 3, 5 and 7**

mg oxihumate in 10 mL buffer	% Bound		
	pH 3	pH 5	pH 7
50	99.8	99.8	89.7
10	95.4	91.9	85.5
5	83.7	85.5	82.1
4	75.8	82.6	81.3
3	71.2	81.4	76.4
2	58.2	59.9	74.0
1	46.7	36.4	58.1
0.8	43.5	28.7	55.0
0.6	39.9	24.5	42.9
0.4	27.5	18.1	33.5
0.2	18.3	11.0	21.1
0.1	11.1	6.2	12.4
0.08	8.3	5.2	9.8
0.06	5.6	3.5	8.0
0.04	4.3	2.6	4.9

Table 3.5. ***In vitro*** **binding of AFG_2 (0.5 mg/L) by oxihumate at pH 3, 5 and 7**

mg oxihumate in 10 mL buffer	% Bound		
	pH 3	pH 5	pH 7
50	97.2	98.2	94.3
10	83.3	89.7	92.4
5	68.9	75.5	82.2
4	65.8	75.8	80.0
3	55.6	66.9	69.8
2	41.8	55.4	63.6
1	29.1	40.4	38.9
0.6	16.7	25.4	29.8
0.4	12.4	8.6	13.5
0.08	2.5	3.4	5.5
0.04	1.1	1.7	1.5

Table 3.6. *In vitro* binding of AFB_1 (2 mg/L) by Mycosorb™ at pH 3, 5 and 7

mg Mycosorb™ in 10 mL buffer	% Bound		
	pH 3	pH 5	pH 7
50	26.5	27.6	23.0
10	4.6	7.2	2.9
5	0.7	2.0	1.5

The data (Tables 3.7 – 3.12) were used to construct isotherm plots of oxihumate for each aflatoxin (Figures 3.1 – 3.24) in order to determine the binding capacity and binding affinity constants, which are presented in Table 3.13. The binding capacity and affinity constants of Mycosorb™ for AFB_1 are presented in Table 3.14.

Table 3.7. Aflatoxin B_1 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFB_1 to oxihumate at pH 3

Oxihumate concentration (mg/mL)	mg AFB_1/L	mg AFB_1/g oxihumate	Log mg/L	Log mg/g	(mg/L)/(mg/g)
5.0	0.00	0.40	-2.49	-0.40	0.01
1.0	0.09	1.91	-1.04	0.28	0.05
0.5	0.33	3.35	-0.49	0.52	0.10
0.4	0.48	3.79	-0.32	0.58	0.13
0.3	0.58	4.75	-0.24	0.68	0.12
0.2	0.84	5.82	-0.08	0.76	0.14
0.1	1.07	9.35	0.03	0.97	0.11
0.08	1.13	10.87	0.05	1.04	0.10
0.06	1.20	13.29	0.08	1.12	0.09
0.04	1.45	13.73	0.16	1.14	0.11
0.02	1.63	18.30	0.21	1.26	0.09
0.01	1.78	22.22	0.25	1.35	0.08
0.008	1.83	20.70	0.26	1.32	0.09
0.006	1.89	18.76	0.28	1.27	0.10
0.004	1.91	21.52	0.28	1.33	0.09

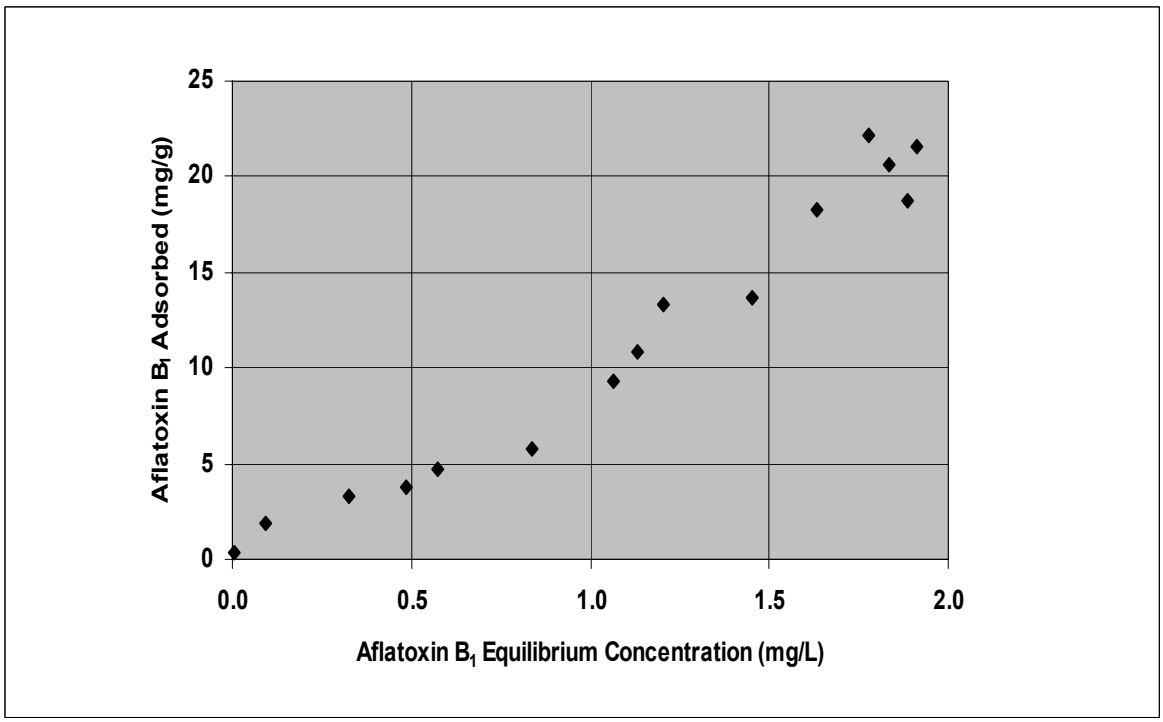

Figure 3.1. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 3

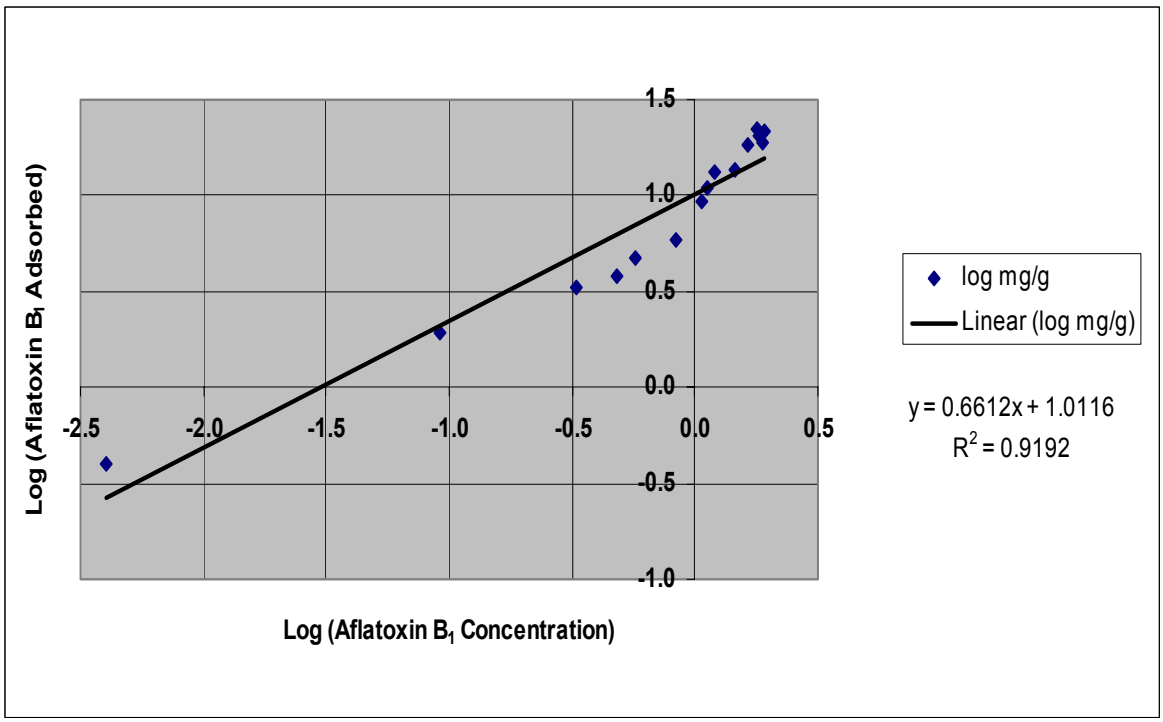

Figure 3.2. Log-log plot of the isothermal adsorption of aflatoxin B_1 to oxihumate at pH 3

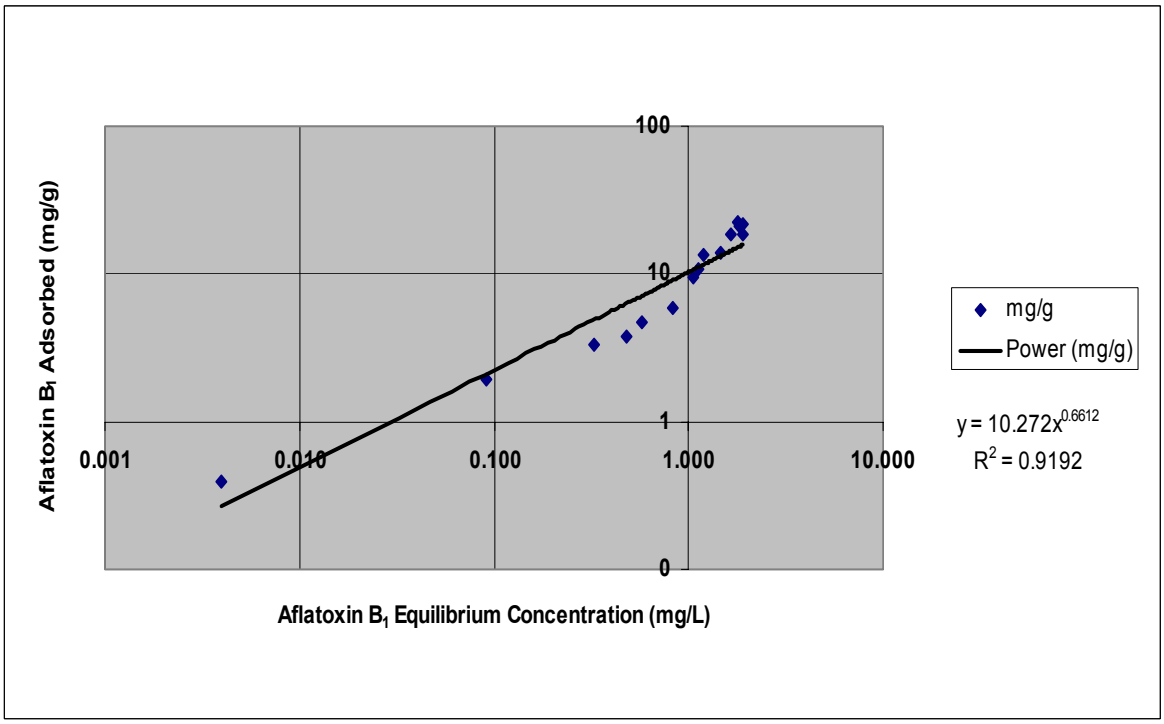

Figure 3.3. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 3

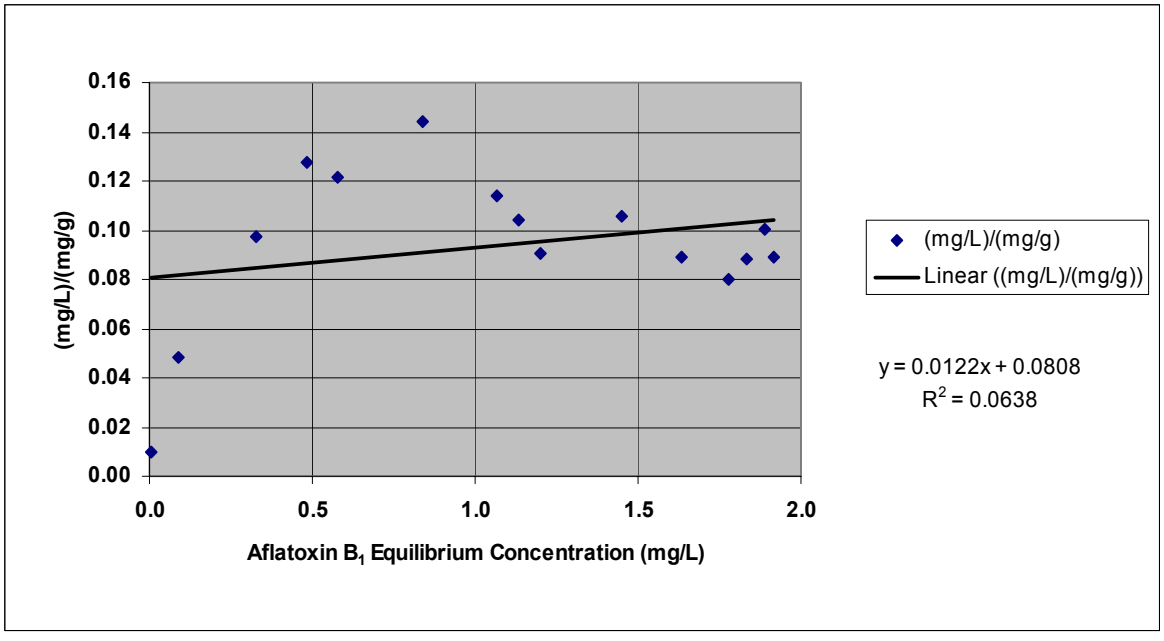

Figure 3.4. Isotherm plot of (mg/L)/(mg/g) against aflatoxin B_1 equilibrium concentration (mg/L) for aflatoxin B_1 adsorption to oxihumate at pH 3

Table 3.8. Aflatoxin B_1 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFB_1 to oxihumate at pH 5

Oxihumate concentration (mg/mL)	mg AFB_1/L	mg AFB_1/g oxihumate	Log mg/L	Log mg/g	(mg/L)/(mg/g)
5.0	0.00	0.40	-2.49	-0.40	0.01
1.0	0.16	1.84	-0.79	0.26	0.09
0.5	0.29	3.42	-0.54	0.53	0.08
0.4	0.35	4.13	-0.46	0.62	0.08
0.3	0.37	5.43	-0.43	0.73	0.07
0.2	0.80	5.99	-0.10	0.78	0.13
0.1	1.27	7.28	0.10	0.86	0.17
0.08	1.43	7.18	0.15	0.86	0.20
0.06	1.51	8.18	0.18	0.91	0.18
0.04	1.64	9.06	0.21	0.96	0.18
0.02	1.78	11.05	0.25	1.04	0.16
0.01	1.88	12.46	0.27	1.10	0.15
0.008	1.90	13.08	0.28	1.12	0.14
0.006	1.93	11.63	0.29	1.07	0.17
0.004	1.95	13.08	0.29	1.12	0.15

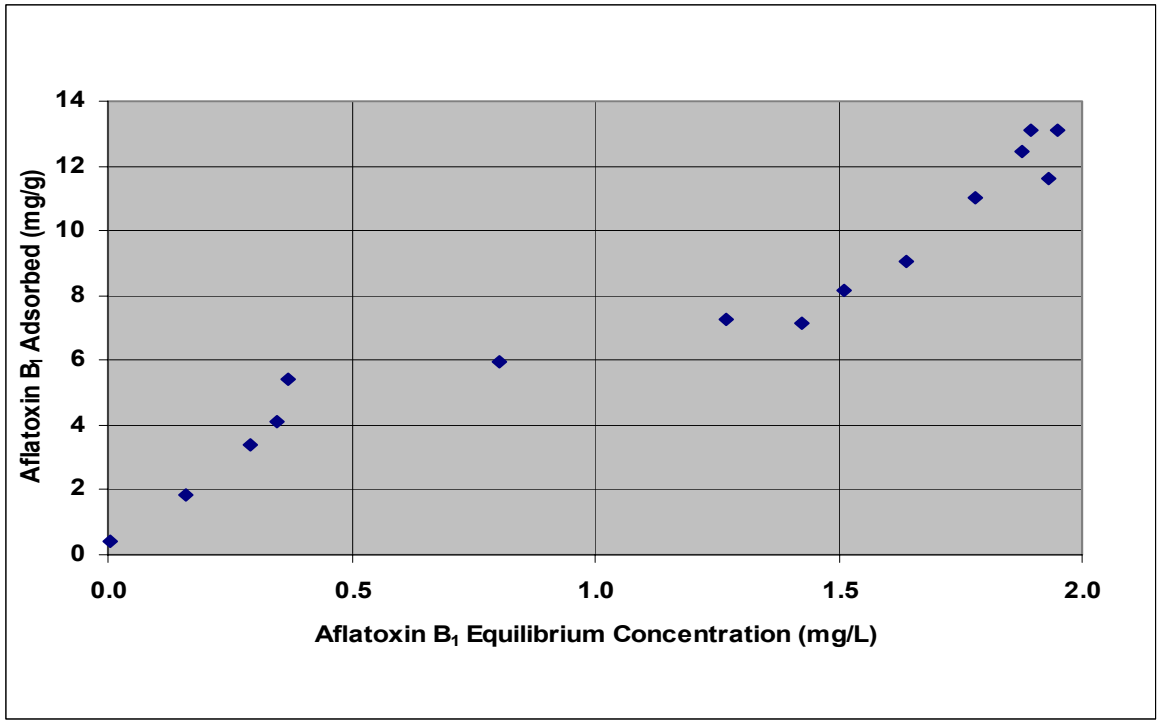

Figure 3.5. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 5

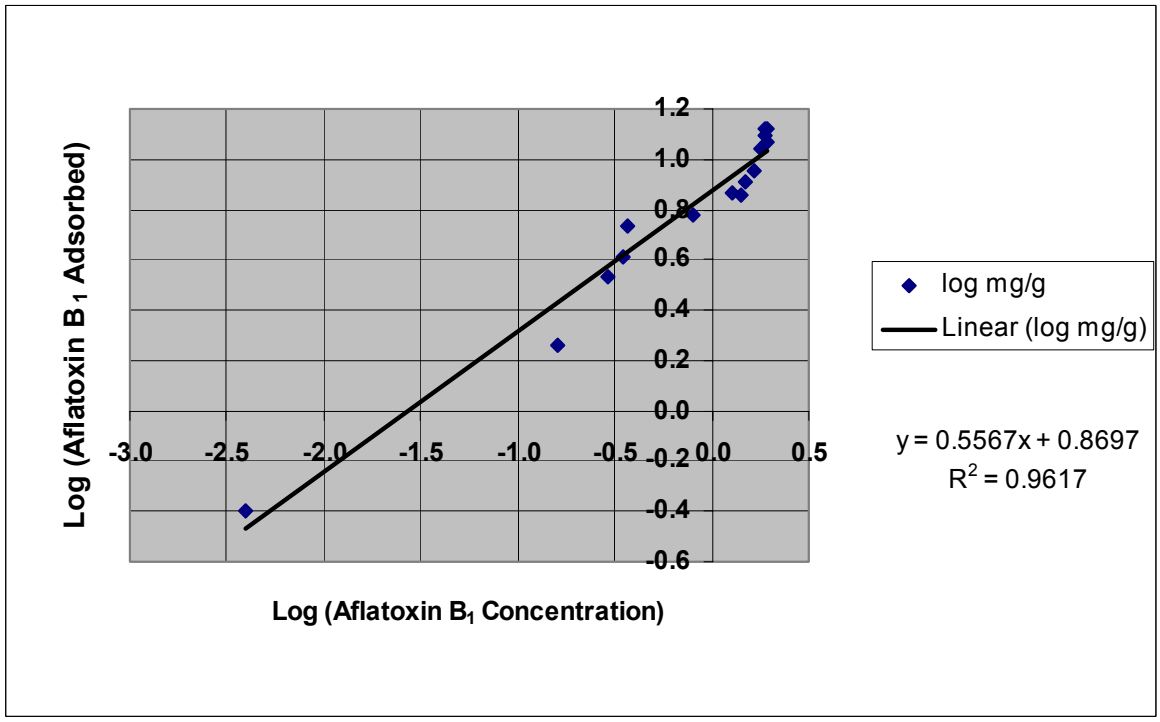

Figure 3.6. Log-log plot of the isothermal adsorption of aflatoxin B_1 to oxihumate at pH 5

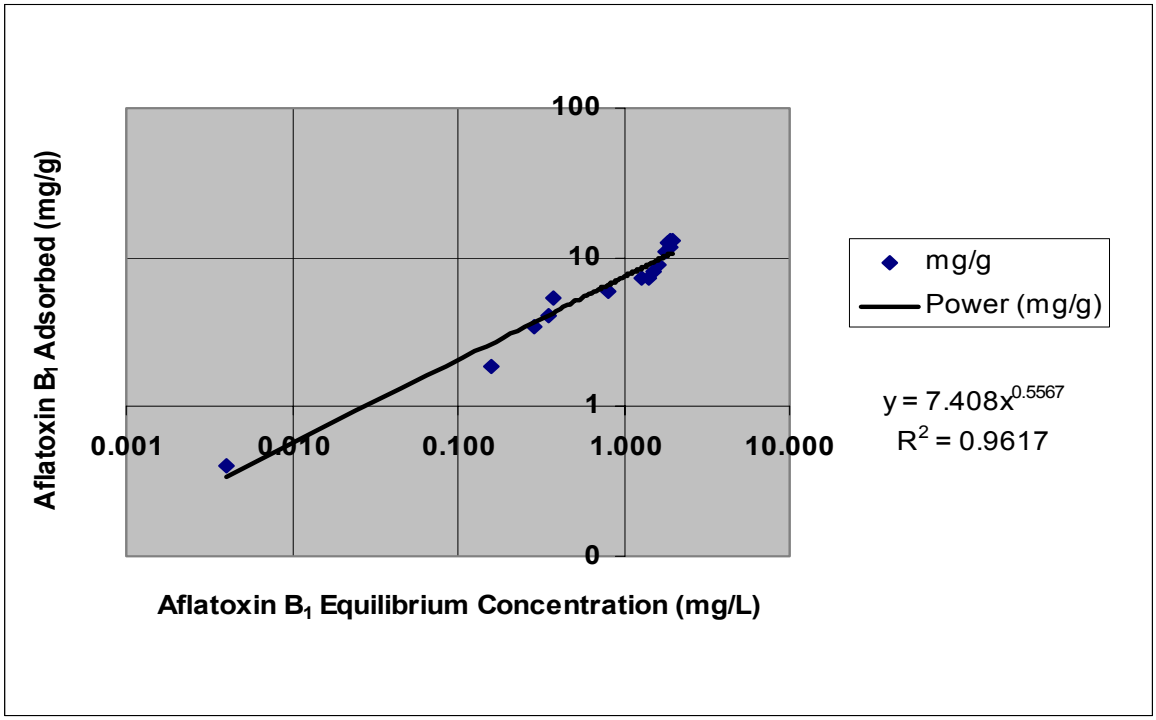

Figure 3.7. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 5

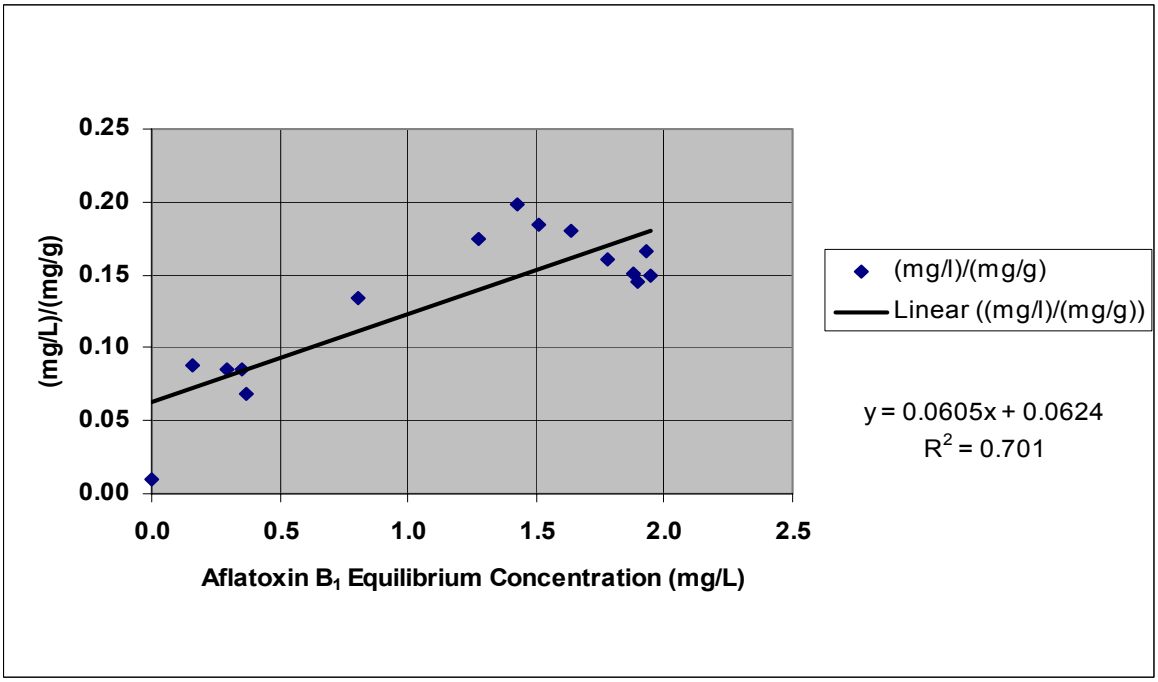

Figure 3.8. Isotherm plot of (mg/L)/(mg/g) against aflatoxin B_1 equilibrium concentration (mg/L) for aflatoxin B_1 adsorption to oxihumate at pH 5

Table 3.9. Aflatoxin B_1 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFB_1 to oxihumate at pH 7

Oxihumate concentration (mg/mL)	mg AFB_1/L	mg AFB_1/g oxihumate	Log mg/L	Log mg/g	(mg/L)/(mg/g)
5.0	0.21	0.36	-0.69	-0.44	0.57
1.0	0.29	1.71	-0.54	0.23	0.17
0.5	0.36	3.28	-0.44	0.52	0.11
0.4	0.37	4.07	-0.43	0.61	0.09
0.3	0.47	5.10	-0.33	0.71	0.09
0.2	0.52	7.40	-0.28	0.87	0.07
0.1	0.84	11.63	-0.08	1.07	0.07
0.08	0.90	13.75	-0.05	1.14	0.07
0.06	1.14	14.30	0.06	1.16	0.08
0.04	1.33	16.73	0.12	1.22	0.08
0.02	1.58	21.09	0.20	1.32	0.07
0.01	1.75	24.73	0.24	1.39	0.07
0.008	1.80	24.44	0.26	1.39	0.07
0.006	1.84	26.67	0.26	1.43	0.07
0.004	1.90	24.44	0.28	1.39	0.08

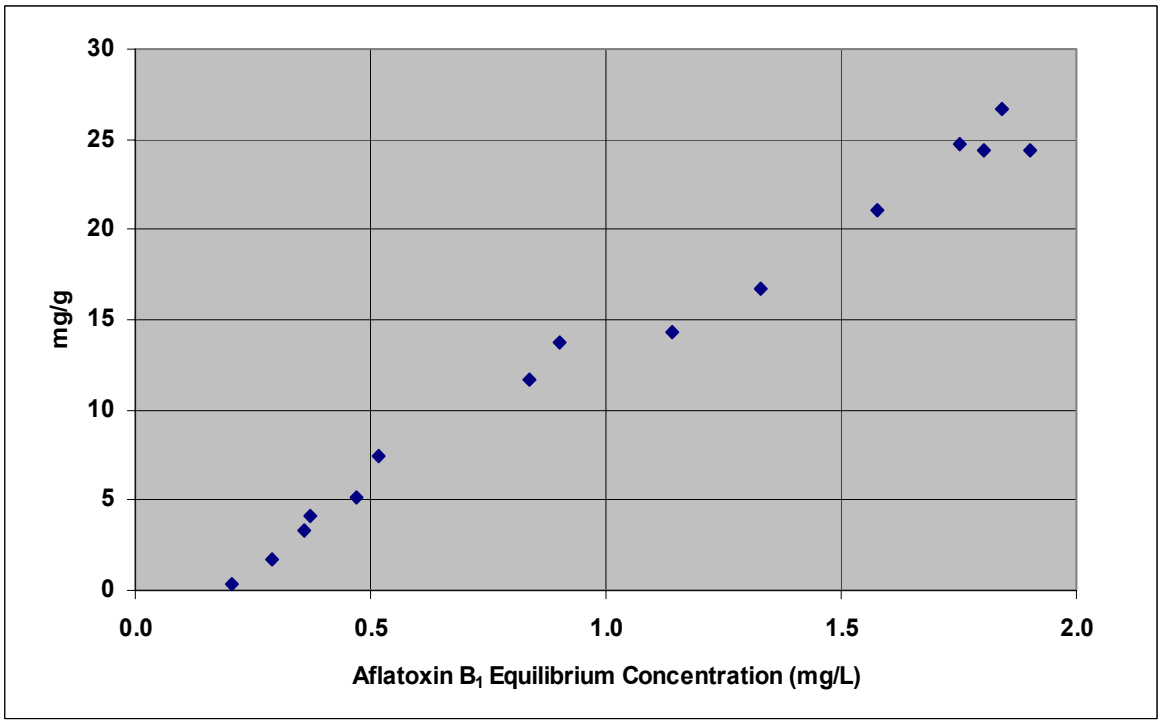

Figure 3.9. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 7

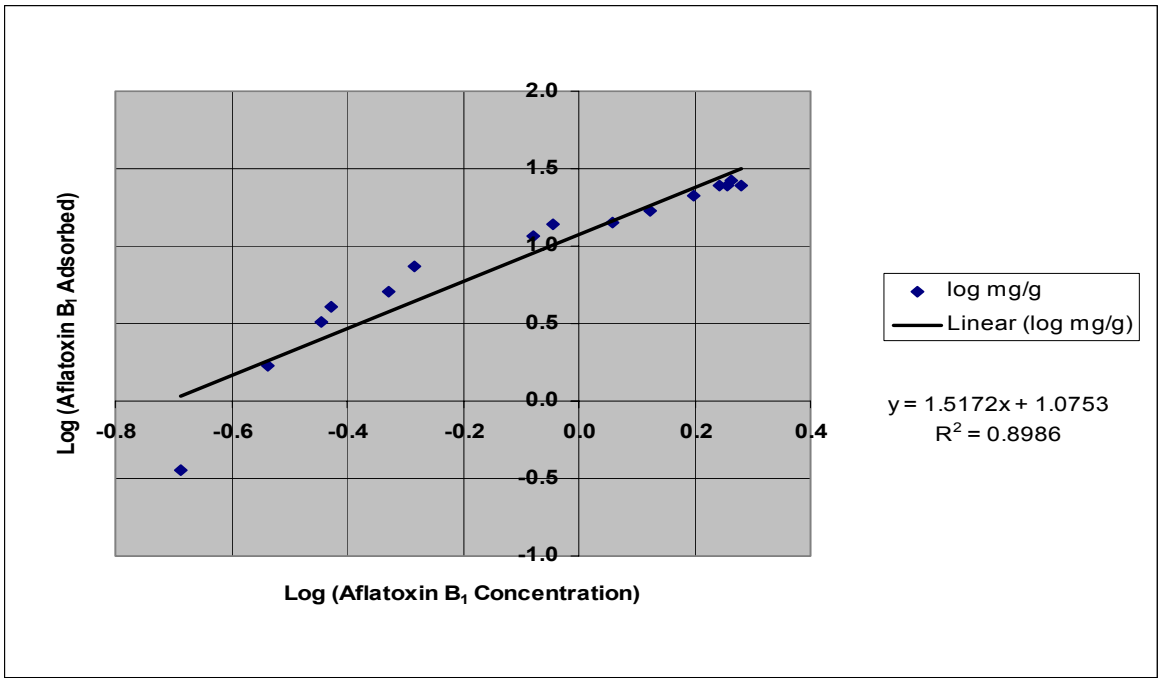

Figure 3.10. Log-log plot of the isothermal adsorption of aflatoxin B_1 to oxihumate at pH 7

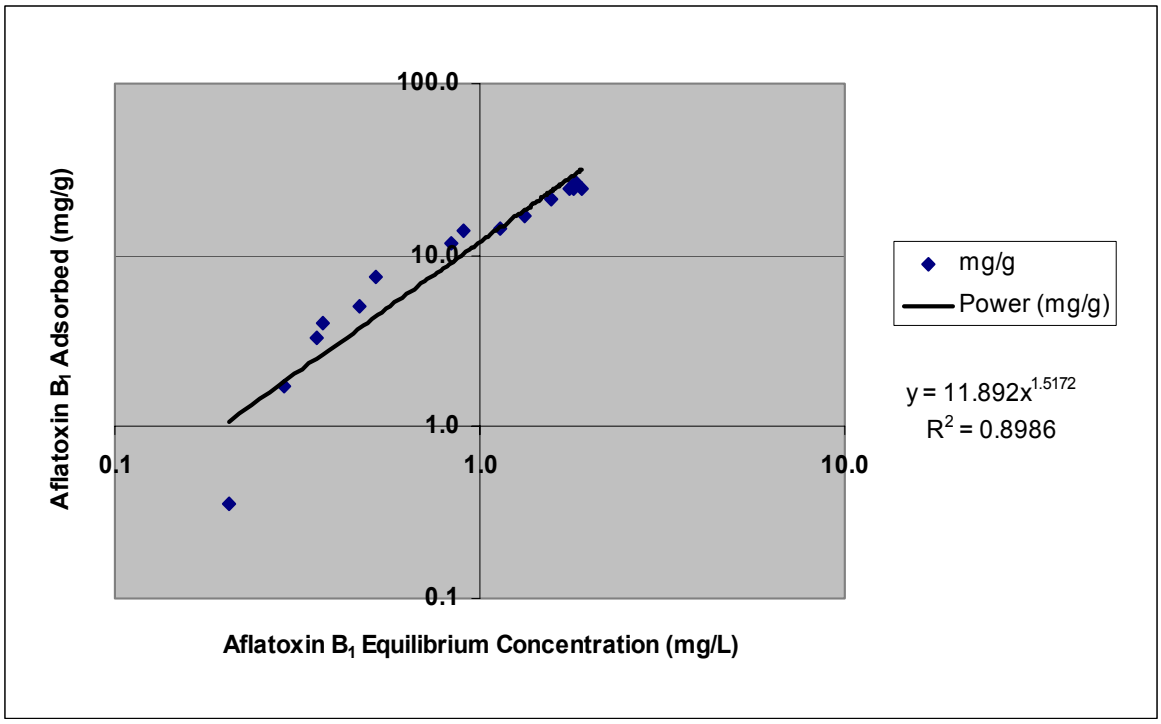

Figure 3.11. Isotherm plot for aflatoxin B_1 adsorption to oxihumate at pH 7

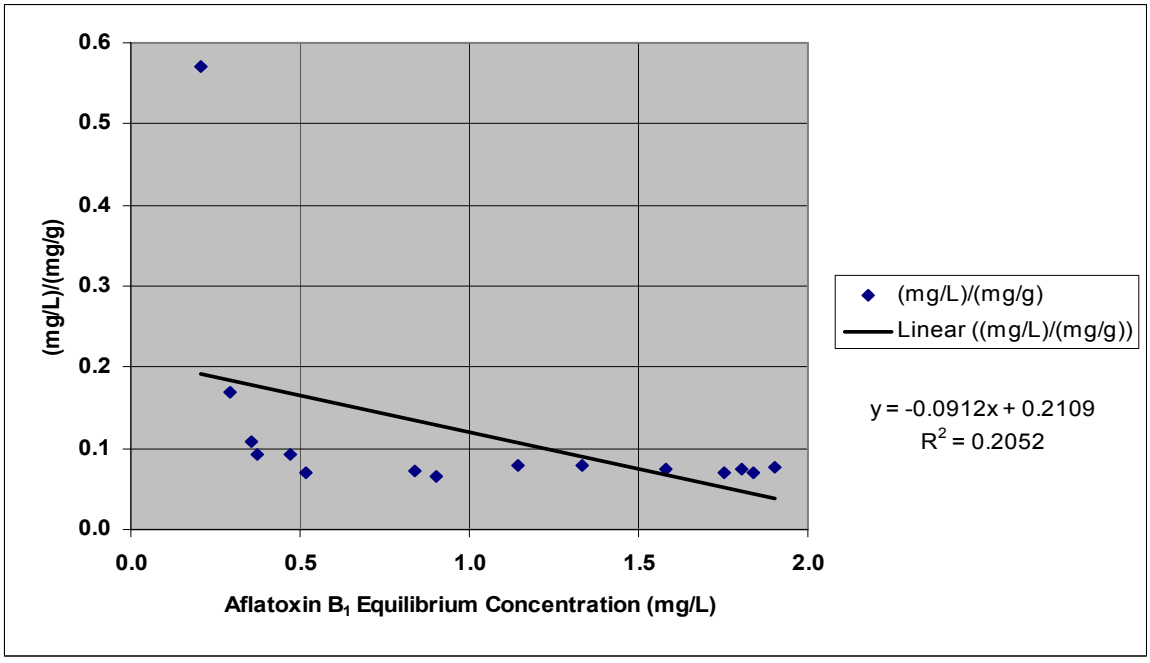

Figure 3.12. Isotherm plot of (mg/L)/(mg/g) against aflatoxin B_1 equilibrium concentration (mg/L) for aflatoxin B_1 adsorption to oxihumate at pH 7

Table 3.10. Aflatoxin G_2 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFG_2 to oxihumate at pH 3

Oxihumate concentration (mg/mL)	mg AFG_2/L	mg AFG_2/g oxihumate	log mg/L	log mg/g	(mg/L)/(mg/g)
5.0	0.06	0.01	-1.25	-2.01	5.81
1.0	0.33	0.04	-0.48	-1.38	8.00
0.5	0.62	0.07	-0.21	-1.16	9.02
0.4	0.68	0.08	-0.17	-1.08	8.31
0.3	0.89	0.09	-0.05	-1.03	9.56
0.2	1.16	0.10	0.07	-0.98	11.14
0.1	1.42	0.15	0.15	-0.84	9.75
0.06	1.67	0.14	0.22	-0.86	12.00
0.04	1.75	0.16	0.24	-0.81	11.27
0.008	1.95	0.16	0.29	-0.80	12.27
0.004	1.98	0.14	0.30	-0.85	14.00

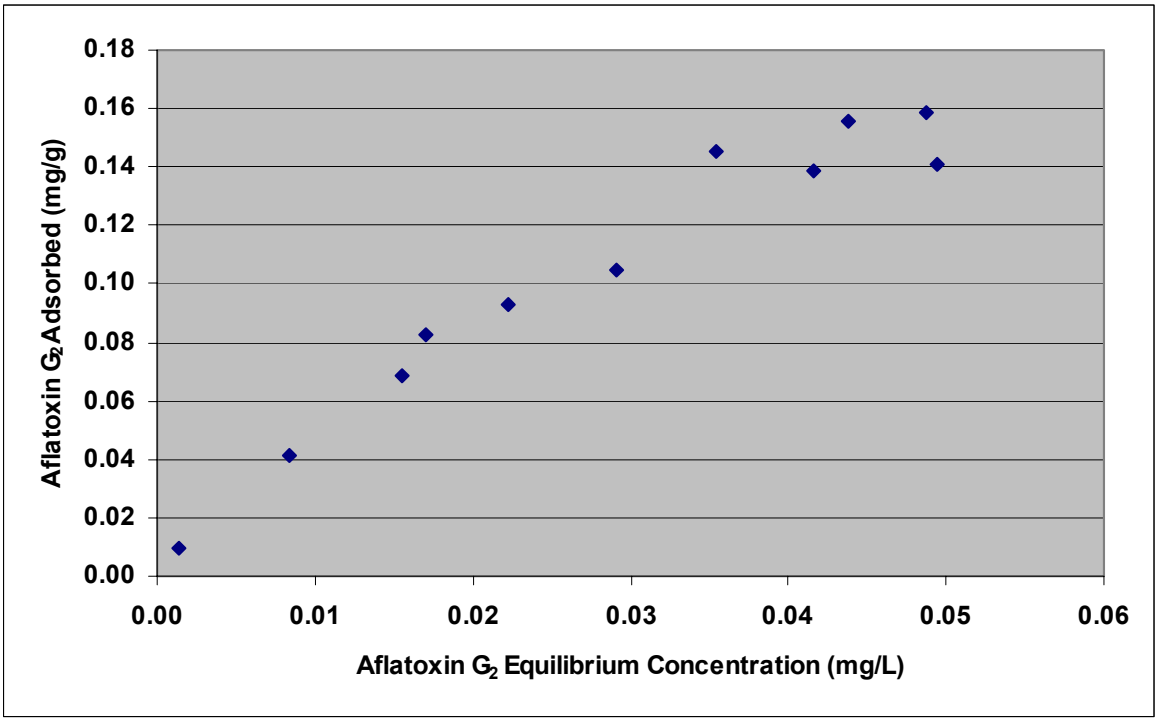

Figure 3.13. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 3

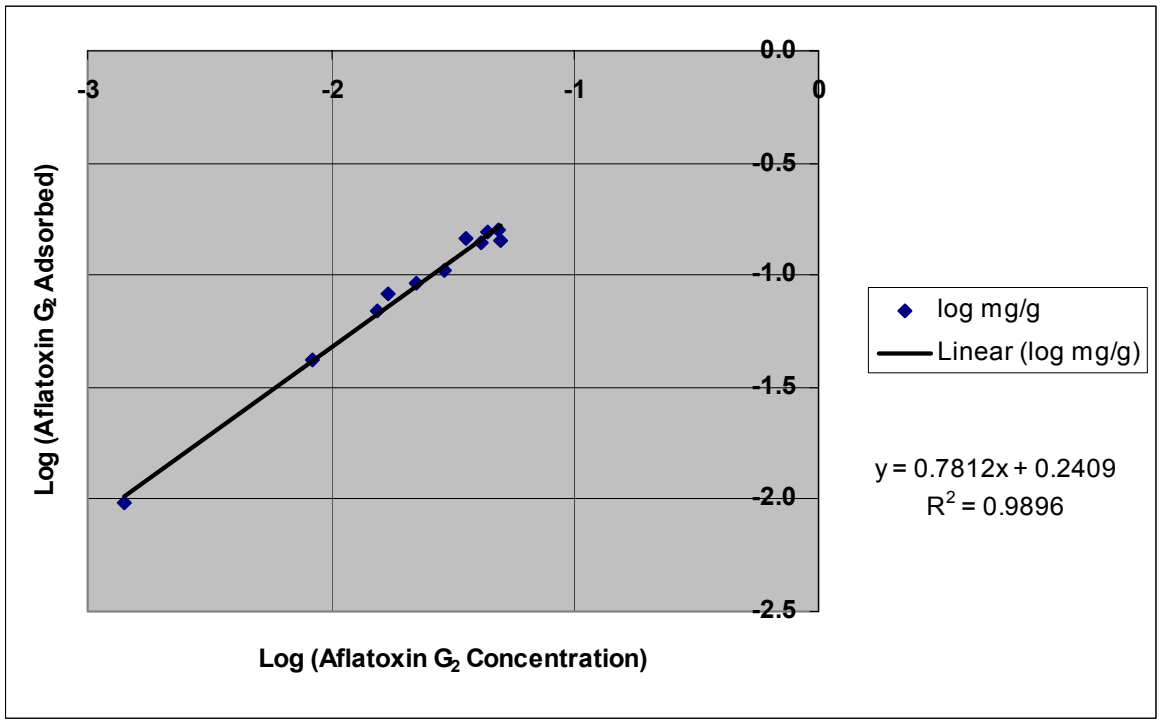

Figure 3.14. Log-log plot of the isothermal adsorption of aflatoxin G_2 to oxihumate at pH 3

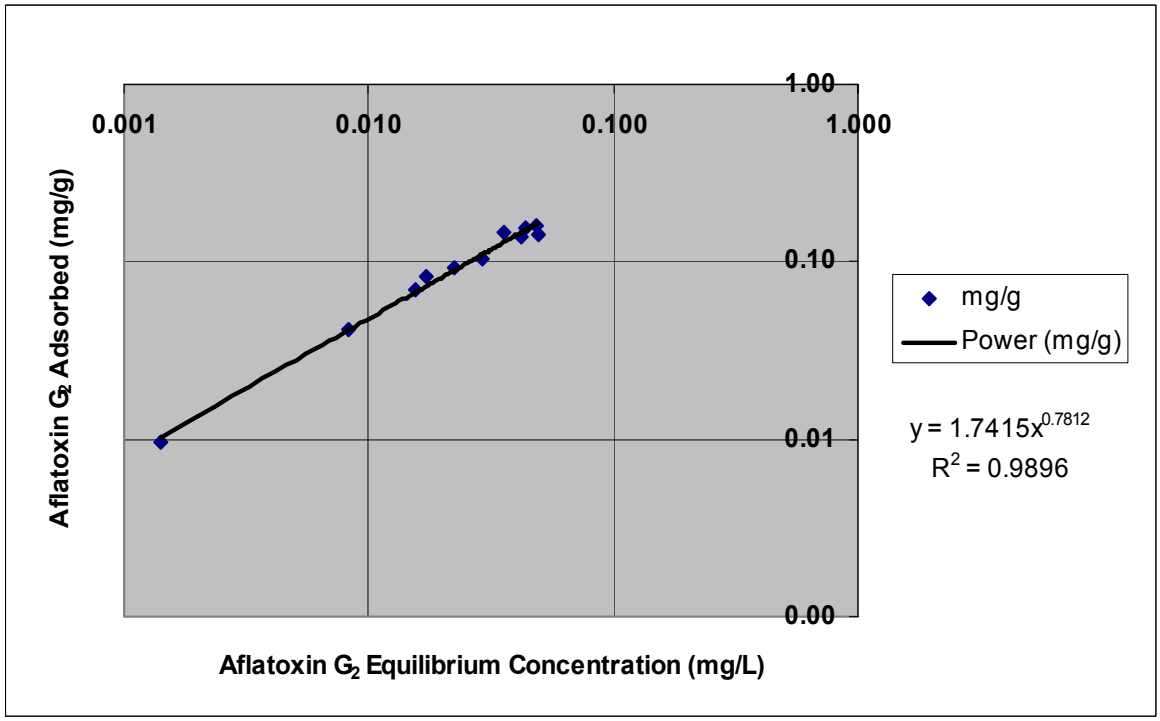

Figure 3.15. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 3

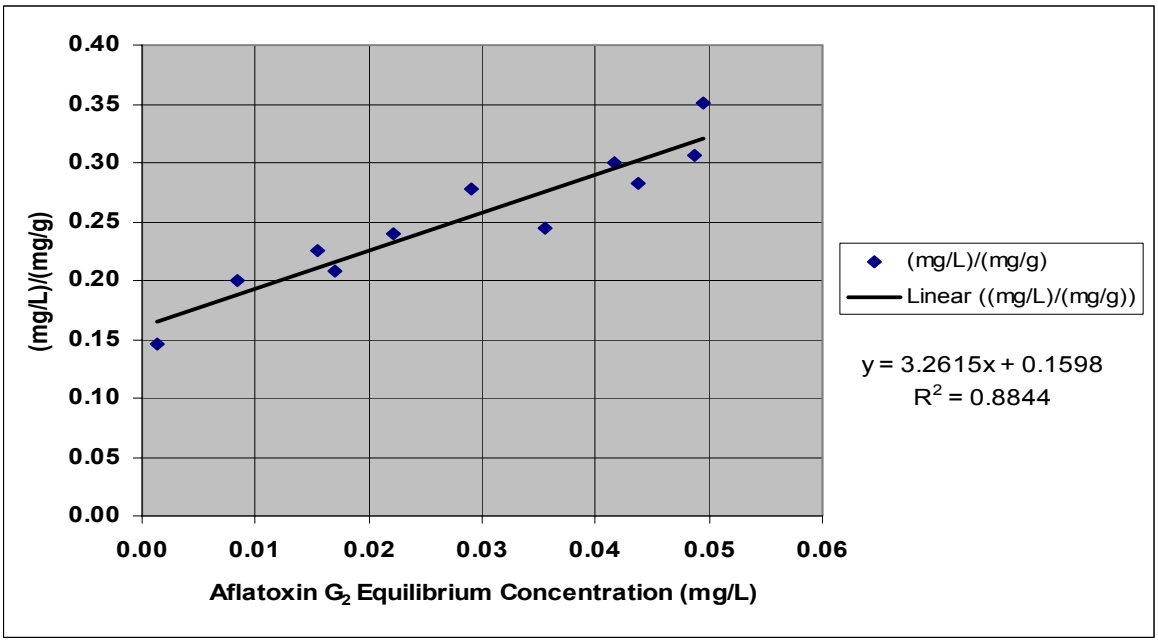

Figure 3.16. Isotherm plot of (mg/L)/(mg/g) against aflatoxin G_2 equilibrium concentration (mg/L) for aflatoxin G_2 adsorption to oxihumate at pH 3

Table 3.11. Aflatoxin G_2 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFG_2 to oxihumate at pH 5

Oxihumate concentration (mg/mL)	mg AFG_2/L	mg AFG_2/g oxihumate	log mg/L	log mg/g	(mg/L)/(mg/g)
5.0	0.04	0.01	-1.44	-2.01	3.73
1.0	0.21	0.04	-0.69	-1.35	4.59
0.5	0.49	0.08	-0.31	-1.12	6.48
0.4	0.48	0.09	-0.32	-1.02	5.10
0.3	0.66	0.11	-0.18	-0.95	5.94
0.2	0.89	0.14	-0.05	-0.86	6.44
0.1	1.19	0.20	0.08	-0.69	5.91
0.06	1.49	0.21	0.17	-0.67	7.04
0.04	1.83	0.22	0.26	-0.67	8.47
0.008	1.93	0.21	0.29	-0.68	9.21
0.004	1.97	0.21	0.29	-0.68	9.37

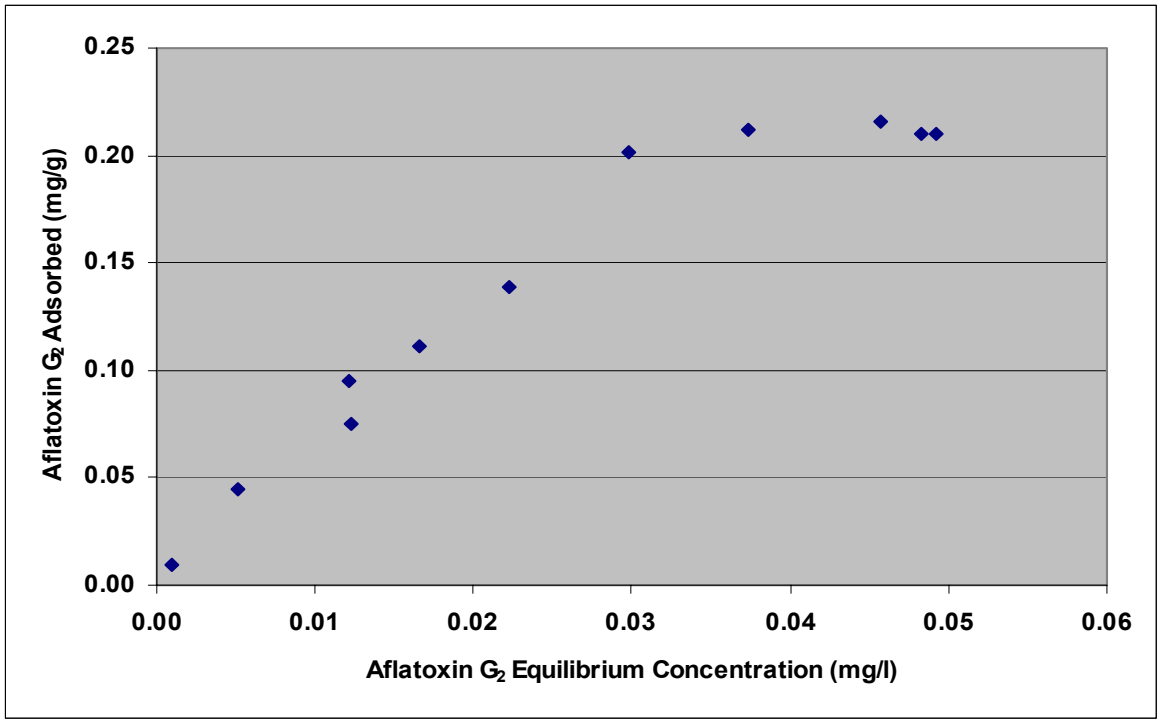

Figure 3.17. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 5

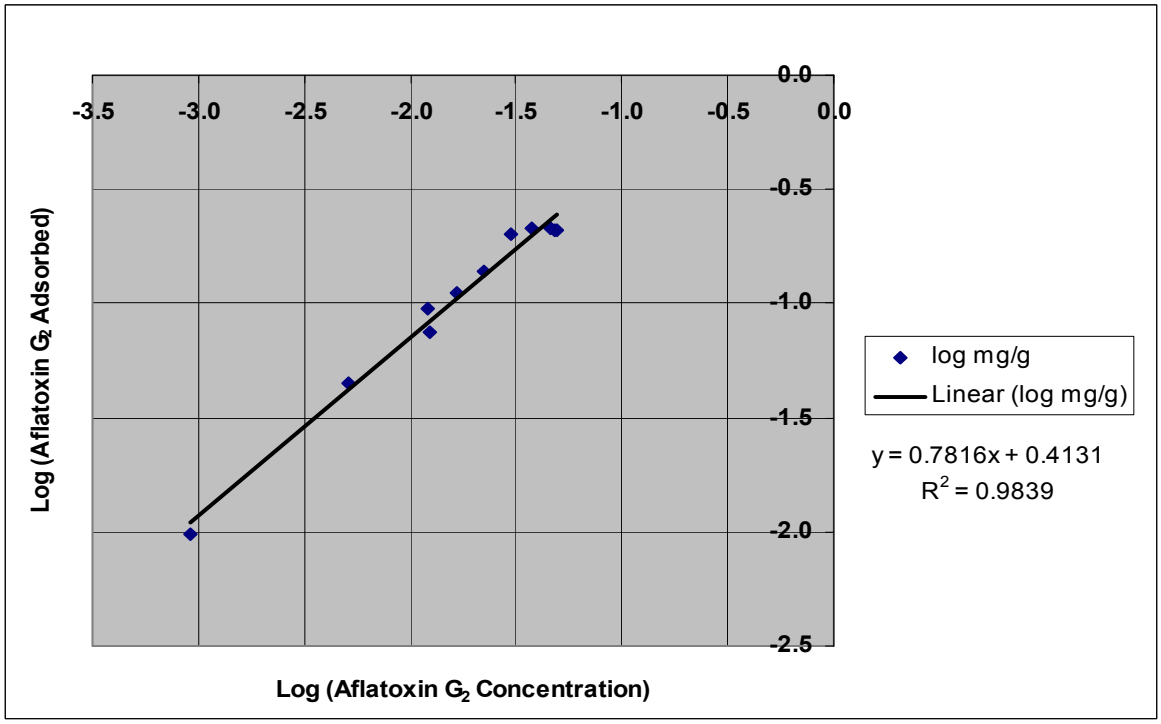

Figure 3.18. Log-log plot of the isothermal adsorption of aflatoxin G_2 to oxihumate at pH 5

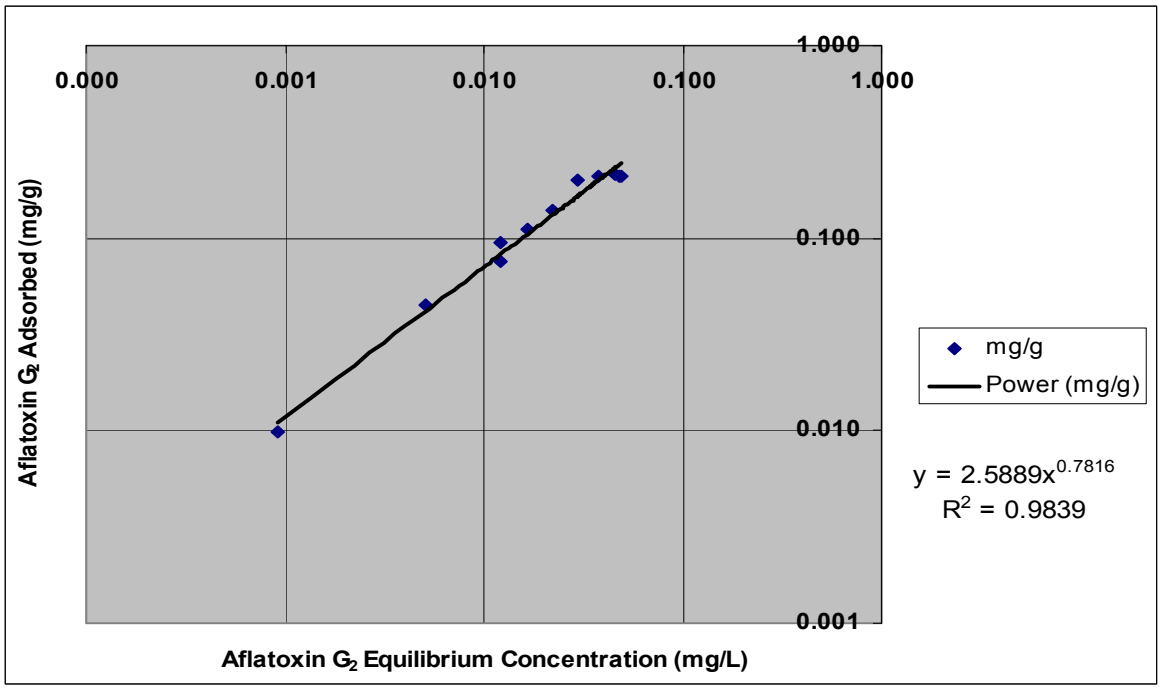

Figure 3.19. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 5

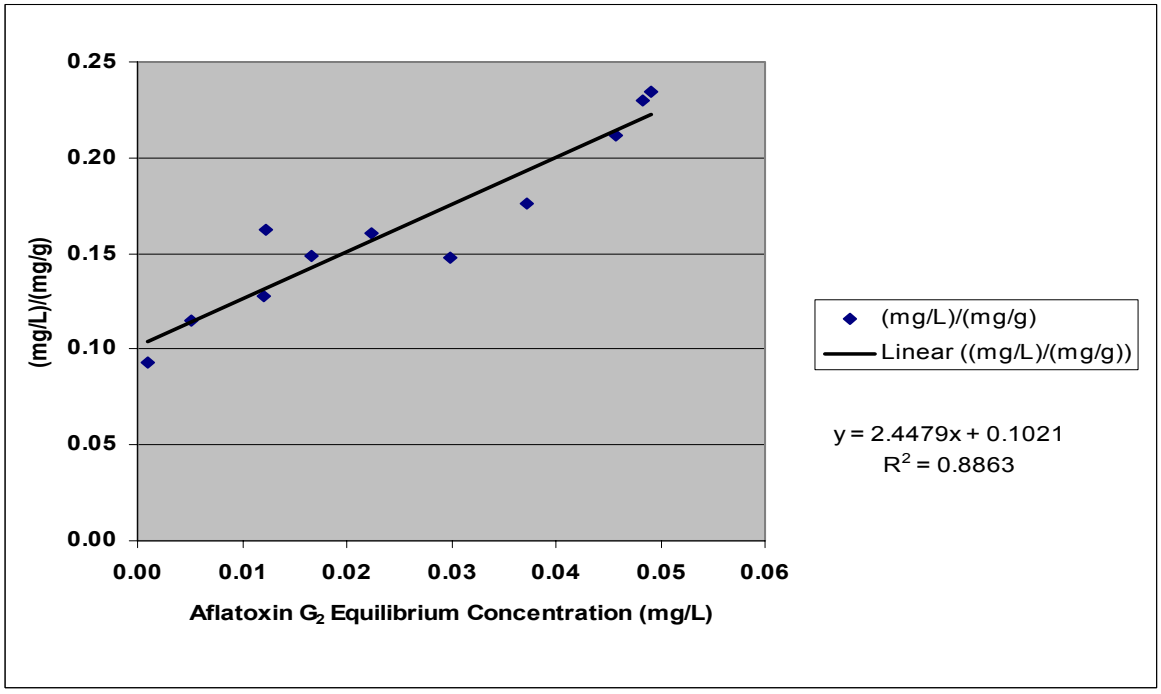

Figure 3.20. Isotherm plot of (mg/L)/(mg/g) against aflatoxin G_2 equilibrium concentration (mg/L) for aflatoxin G2 adsorption to oxihumate at pH 5

Table 3.12. Aflatoxin G_2 equilibrium concentrations (mg/L), mg/g adsorbed from solution for oxihumate and data for isotherm plots of AFG_2 to oxihumate at pH 7

Oxihumate concentration (mg/mL)	mg AFG_2/L	mg AFG_2/g oxihumate	log mg/L	log mg/g	(mg/L)/(mg/g)
5.0	0.11	0.01	-0.94	-2.03	12.05
1.0	0.15	0.05	-0.82	-1.34	3.28
0.5	0.36	0.08	-0.45	-1.09	4.33
0.4	0.40	0.10	-0.40	-1.00	4.00
0.3	0.60	0.12	-0.22	-0.93	5.19
0.2	0.73	0.16	-0.14	-0.80	4.57
0.1	1.22	0.19	0.09	-0.71	6.28
0.06	1.40	0.25	0.15	-0.60	5.65
0.04	1.73	0.34	0.24	-0.47	5.15
0.008	1.89	0.34	0.28	-0.47	5.55
0.004	1.97	0.36	0.29	-0.44	5.42

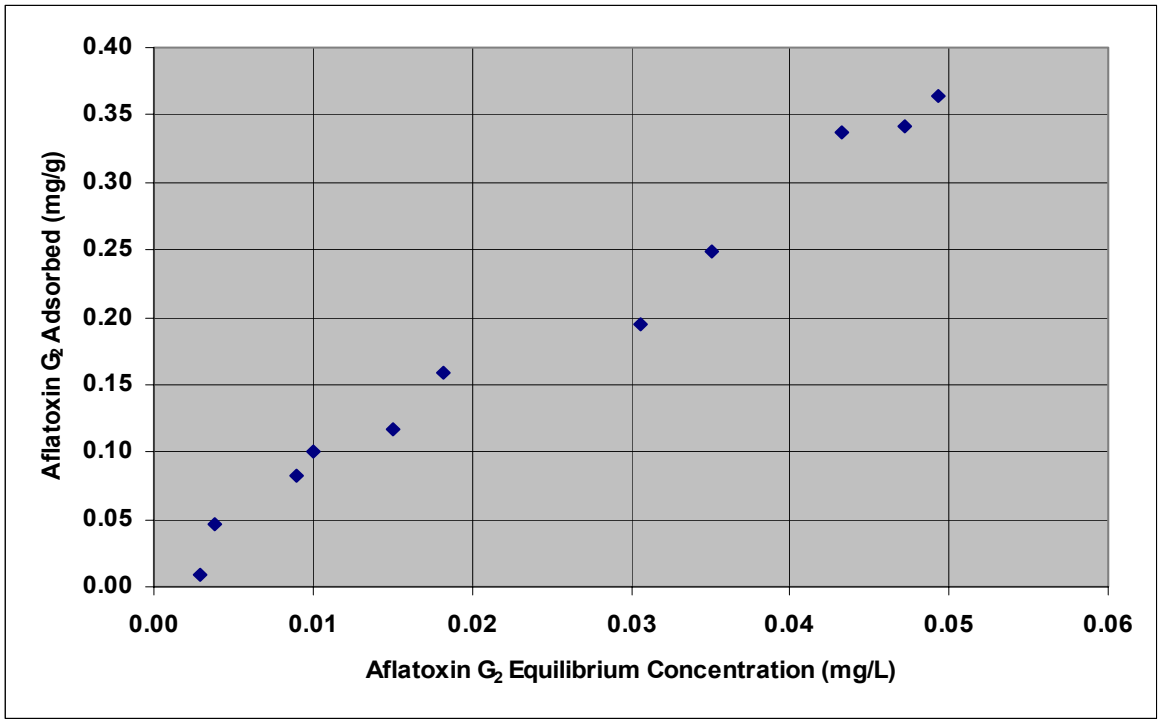

Figure 3.21. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 7

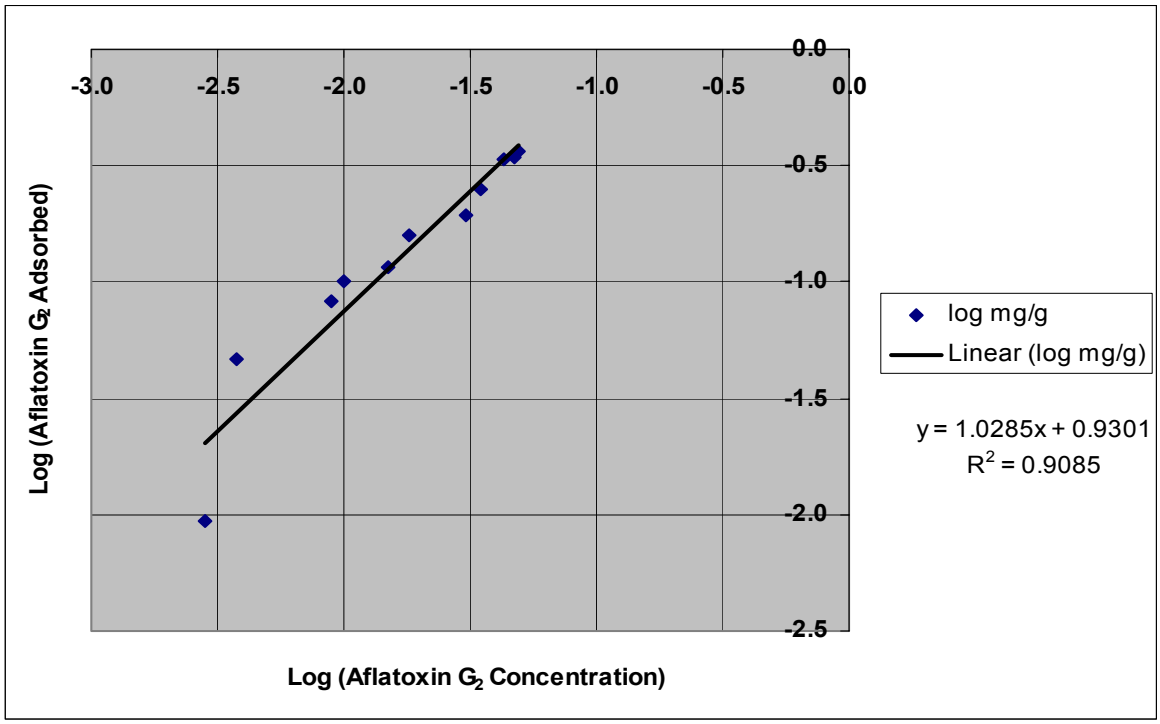

Figure 3.22. Log-log plot of the isothermal adsorption of aflatoxin G_2 to oxihumate at pH 7

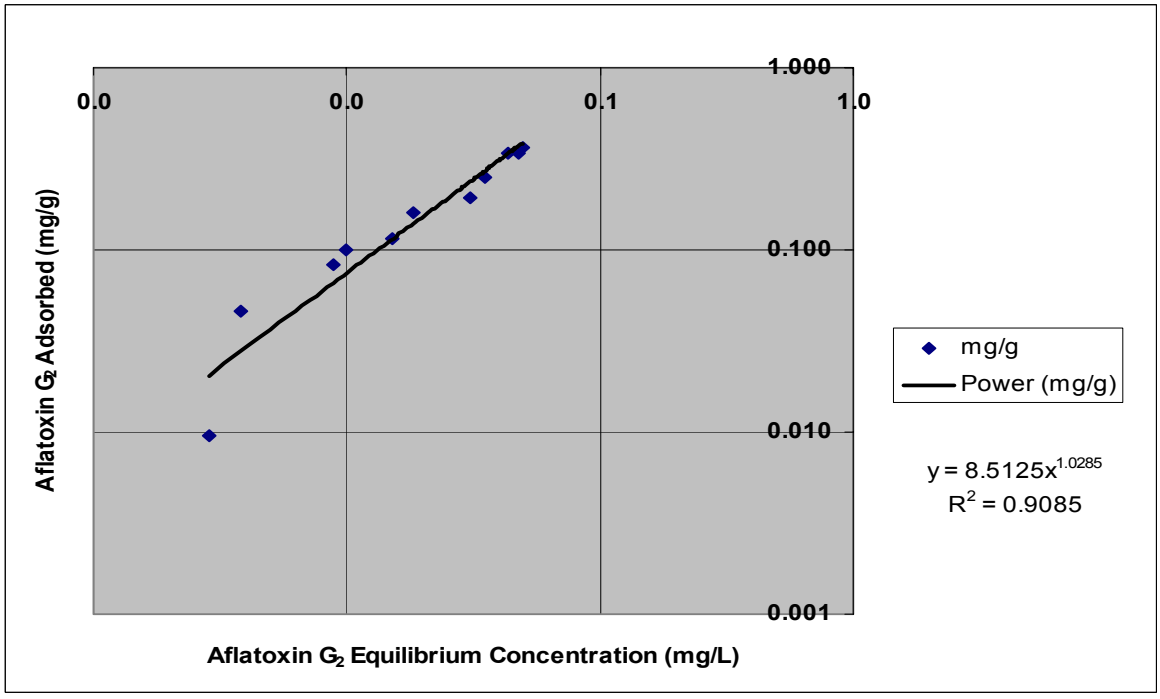

Figure 3.23. Isotherm plot for aflatoxin G_2 adsorption to oxihumate at pH 7

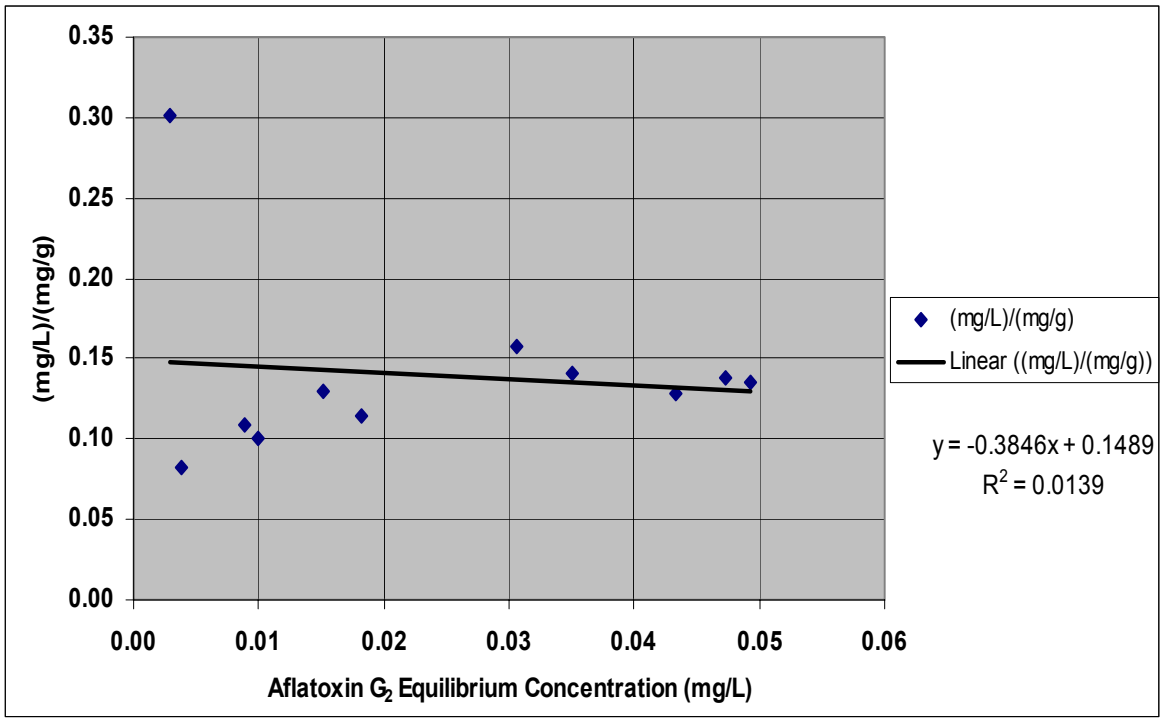

Figure 3.24. Isotherm plot of (mg/L)/(mg/g) against aflatoxin G_2 equilibrium concentration (mg/L) for aflatoxin G_2 adsorption to oxihumate at pH 7

Table 3.13. The Langmuir and Freundlich oxihumate adsorption isotherm parameters obtained for AFB_1 and AFG_2 at pH 3, 5 and 7 at room temperature

	Aflatoxin B_1			Aflatoxin G_2		
	pH 3	**pH 5**	**pH 7**	**pH 3**	**pH 5**	**pH 7**
Freundlich isotherm $C_a = k_1.C_s^{k2}$						
k_1 (mg/g)	10.268	7.408	11.892	1.174	2.589	8.513
k_2	0.614	0.557	1.517	0.781	0.782	1.029
R^2	0.913	0.9627	0.899	0.990	0.984	0.909
Langmuir isotherm $C_s/C_a = (C_s/k_1) + (1/k_1k_2)$						
k_1 (mg/g)	81.967	16.529	-10.965	0.307	0.0494	2.600
k_2	0.151	0.970	-0.432	0.510	5.384	-0.065
R^2	0.064	0.701	0.205	0.884	0.702	0.014

C_a: amount of aflatoxin adsorbed per unit weight of adsorbent (mg/g); C_s: concentration of unadsorbed aflatoxin at equilibrium (mg/g); k_1: capacity constant; k_2: affinity constant; R^2: coefficient of determination

The Langmuir and Freundlich oxihumate adsorption isotherm parameters obtained for AFB_1 and AFG_2 at pH 3, 5 and 7 at 25°C are given in Table 3.13. In Table 3.14, the Langmuir and Freundlich Mycosorb™ adsorption isotherm parameters for AFB_1 at pH 3, 5 and 7 are shown. The coefficients of determination (R^2) are also shown.

The Freundlich oxihumate adsorption isotherm fitted the data better than the Langmuir oxihumate isotherm, as demonstrated by the higher R^2 values. The data show adsorptions of 10.3, 7.4 and 11.9 mg AFB_1/g oxihumate at pH 3, 5 and 7, respectively. Oxihumate adsorbed 1.2, 2.6 and 8.5 mg AFG_2/g at pH 3, 5 and 7, respectively. Oxihumate is thus more effective at adsorbing AFB_1 than AFG_2 (Table 3.13).

For Mycosorb™, the Freundlich isotherm fitted the data better than the Langmuir at pH 3 and 7, while at pH 5 the Langmuir fitted better (Table 3.14). However, results were poor, with the best adsorption shown as 204 µg/g Mycosorb™ at pH 7.

Table 3.14. The Langmuir and Freundlich Mycosorb™ adsorption isotherm parameters obtained for AFB_1 at pH 3, 5 and 7 at room temperature

	Aflatoxin B_1		
	pH 3	**pH 5**	**pH 7**
Freundlich isotherm $C_a = k_1.C_s^{k2}$			
k_1 (mg/g)	0.145	0.136	0.204
k_2	-0.803	-0.407	-1.847
R^2	0.822	0.050	0.986
Langmuir isotherm $C_s/C_a = (C_s/k_1) + (1/k_1k_2)$			
k_1 (mg/g)	0.012	0.067	0.025
k_2	-0.722	-1.477	-0.917
R^2	0.410	0.289	0.991

C_a: amount of aflatoxin adsorbed per unit weight of adsorbent (mg/g); C_s: concentration of unadsorbed aflatoxin at equilibrium (mg/g); k_1: capacity constant; k_2: affinity constant; R^2: coefficient of determination

Experiment 3

Experiment 3 showed that oxihumate mixed with poultry feed, adsorbed the AFB_1 with the same efficacy as in buffer alone at both pH 3 (Table 3.15) and pH 7 (Table 3.16).

Table 3.15. Binding of AFB_1 (2 mg/L) by oxihumate (3.5 g/kg feed) when mixed with poultry feed (pH 3)

% Bound		
Oxihumate + AFB_1	**Feed + AFB_1**	**Feed + oxihumate + AFB_1**
92.00	44.88	92.91

Table 3.16. Binding of AFB_1 (2 mg/L) by oxihumate (3.5 g/kg feed) when mixed with poultry feed (pH 7)

% Bound		
Oxihumate + AFB_1	**Feed + AFB_1**	**Feed + oxihumate + AFB_1**
82.24	41.34	87.54

Experiment 4

In Experiment 4, the stability of the aflatoxin-oxihumate adsorption complex was tested in the presence of a series of solvents. The results (Table 3.17) showed that the complex was stable in the presence of chloroform but a high degree of rupturing of the complex took place in the presence of acetonitrile (61%) and acetone (75%).

Table 3.17. The rupture (%) of the oxihumate-AFB_1 complex after mixing with different solvents

% Rupture			
Buffer	**Acetonitrile**	**Acetone**	**Chloroform**
0.00	60.94	74.68	3.00

Discussion

Aflatoxin is a relatively low molecular weight, lipohilic molecule that appears to be absorbed rapidly (Kumagai, 1989) and completely (Wogan *et al*., 1967) from the gastrointestinal tract. Adsorbents used to hinder the gastrointestinal absorption of mycotoxins should, therefore, have a high affinity for the specific mycotoxins, resulting in the formation of a strong complex to minimize the risk of any rupture of the complex. The chosen compound should also have a high capacity to prevent saturation (Ramos & Hernández, 1996).

In the first experiment, oxihumate showed a high affinity for AFB_1, zearalenone, ochratoxin A, ergosine, ergotamine, ergocornine, ergocryptine and ergocristine, but not vomitoxin. This is an important finding as some commercially available binders, such as hydrated sodium calcium aluminosilicate (HSCAS) decrease the toxicity of aflatoxin (Phillips *et al*., 1988; Kubena *et al*., 1990a,b), but are ineffective against other mycotoxins (Huff *et al*., 1992). During the management of a mycotoxin problem the lack of a specific binder to reduce the toxicity of various mycotoxins and mycotoxin combinations could become important. The concept that binder materials can be used to manage mycotoxin problems associated with poultry production is a valuable hypothesis, but to be truly effective these materials must have the ability to adsorb a large number of chemically distinct mycotoxins, such as oxihumate proved to have in this *in vitro* experiment.

The study of the isotherm, in which the amount of compound adsorbed per unit weight of the adsorbent is plotted against the concentration of the compound in the external phase under equilibrium conditions, is the most useful mathematical approach to describe the different kinds of adsorption processes. Langmuir and Freundlich isotherms are the most extensively used, giving a measure of the affinity and capacity of the adsorbent for each compound adsorbed (Ramos & Hernández, 1996).

The Langmuir and Freundlich oxihumate adsorption isotherm parameters were obtained for AFB_1 and AFG_2 at pH 3, 5 and 7. The Freundlich oxihumate adsorption isotherm fits the data better than the Langmuir oxihumate isotherm, as demonstrated by the higher coefficients of determination values. According to Ramos & Hernández (1996) this might indicate the presence of adsorption centres within the oxihumate with different affinities for aflatoxin, resulting in a heterogeneous adsorbent surface or the co-existence of different adsorption mechanisms. In the concentration

range assayed, a better fit of data to the Freundlich isotherm shows that most of the adsorbed molecules are filling the more active centres of the adsorbent, and do not saturate the adsorbent material (Ramos *et al*., 1996).

The data show adsorptions of 10.3, 7.4 and 11.9 mg AFB_1/g oxihumate at pH 3, 5 and 7, respectively. Oxihumate adsorbed 1.2, 2.6 and 8.5 mg AFG_2/g at pH 3, 5 and 7, respectively. Oxihumate is thus more effective at adsorbing AFB_1 than AFG_2. According to Decker & Corby (1980) activated charcoal adsorbed 10 mg AFB_1/g, while a gram of hydrated sodium calcium aluminosilicate adsorbed 62-103 mg AFB_1 (Phillips *et al*., 1990). Ramos & Hernández (1996) demonstrated that 1 g of montmorillonite silicate was able to adsorb about 1 mg AFB_1. The maximum AFB_1 adsorption capacity of sodium bentonite from southern Argentina was estimated as 45 mg/g at pH 2 (Rosa *et al*., 2001). In this study, Mycosorb™ demonstrated poor adsorption of AFB_1, with the highest adsorption shown as 204 µg/g Mycosorb™ at pH 7. Various authors, however, reported that Mycosorb™ had a high binding affinity for mycotoxins *in vitro* (Devegowda *et al*, 1998; Newman, 2000).

The third experiment showed that oxihumate mixed with poultry feed, adsorbed AFB_1 with the same efficacy as in buffer alone at pH3 and pH 7, suggesting that oxihumate does not bind feed molecules and, thus, all oxihumate particles mixed into mycotoxin-contaminated feed, should be available for the formation of an oxihumate-mycotoxin complex. The complex may, however, not be very stable as it was ruptured to a high degree in the presence of acetonitrile (61%) and acetone (75%), but not chloroform (3%). The failure of chloroform to rupture the complex may be attributed to the high capacity of oxihumate to hold water in and around its structure with a consequential gel formation, thus preventing the hydrophobic chloroform from penetrating the water layer and as the aflatoxin in association with oxihumate is water-insoluble, the complexes cannot be reached by the chloroform.

Addition of oxihumate to the initial composition of feedstuffs should ameliorate aflatoxicosis efficiently by inducing the formation of an oxihumate-aflatoxin complex that prevents aflatoxin absorption from the gastrointestinal tract. *In vitro* binding tests may, however, not always be a reliable indicator of ability to bind a mycotoxin. Therefore, it is important that adsorbents be subjected to *in vivo* evaluation both with respect to efficacy and to determine if impaired nutrient utilisation occurs (Ledoux & Rottinghaus, 1999).

CHAPTER 4

Efficacy Of Oxihumate As An Aflatoxin Binder *In Vivo*

Abstract

Industrial efforts to protect their animals from the effects of mycotoxicosis have created an entrance point into the market for many different products. There is great variability in the efficacy of adsorbents *in vivo*, even though the compounds may show great potential for binding to the toxins *in vitro*. The *in vivo* efficacy of oxihumate as an aflatoxin binder was evaluated in the present study after results obtained during *in vitro* binding studies showed that it has a high mycotoxin adsorption capacity and therefore definite potential for lessening the effects of aflatoxicosis in livestock.

Humic acids are substances widely found in nature and are present in soils, natural waters, river, lake and sea sediments, peat, brown and brown-black coals and other natural materials as a product of chemical and biological transformations of animal and plant residues. Research has shown that humic acids have a strong affinity to bind with several compounds. A South African company developed an effective large-scale regeneration process for very pure and high quality humic acids, called oxihumic acids, from coal. In this study, oxihumate was effective in diminishing the adverse effects caused by AFB_1 on body weight. Oxihumate also showed some protective effects with respect to liver damage, as indicated by liver enlargement, colour and liver lesions. Enlargement of the stomachs and hearts of intoxicated broilers was reduced by the addition of oxihumate in the contaminated feed. Oxihumate was also effective in diminishing other effects of aflatoxin, as there was apparent protection noted for some of the haematological and serum biochemical changes associated with aflatoxin toxicity. The protective effect of oxihumate appears to involve sequestration of aflatoxin in the gastrointestinal tract and a reduction in bioavailability of aflatoxin. These results suggest that oxihumate can alleviate some of the toxic effects of aflatoxin in growing broilers, and when used with other sound mycotoxin management practices, may prove beneficial in the preventative management of aflatoxin-contaminated feedstuffs for poultry.

Introduction

Aflatoxins, produced mainly by *Aspergillus flavus* and *A. Parasiticus,* are often encountered in food and animal feedstuffs at alarming concentrations in different parts of the world (Jelinek *et al*.,

1989). The aflatoxin problem is long-standing and inextricable (Phillips, 1999). Aflatoxin is the most prevalent and economically significant mycotoxin apt to be consumed by poultry. In lesser-developed countries with limited supplies of food, it is not always feasible, nor practical, to condemn and destroy mouldy grains. Thus, these practices may result in enhanced aflatoxin exposure and a high incidence of aflatoxin-induced diseases in humans and animals.

Naturally occurring aflatoxin contains aflatoxins B_1, B_2, G_1 and G_2, with aflatoxin B_1 (AFB_1) usually the highest and most toxic. AFB_1 is an acute toxin as well as carcinogenic and immunosuppressive, but the precise nature of the response to the consumption of aflatoxin is dependent on species, sex and age (Moss, 1996). Aflatoxin is stable once formed in grain, and is not degraded significantly during normal milling and storage (Brown, 1996).

Practical methods to detoxify mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. A variety of physical, chemical, nutritional and biological techniques have been employed but with only limited success (Edrington *et al*., 1997). The most recent approach is the use of nonnutritive adsorbents, which bind the aflatoxin molecule and thereby reduces their absorption from the gastrointestinal tract, avoiding the toxic effects for livestock and the carryover of these fungal metabolites into animal products (Ramos *et al*., 1996; Raju & Devegowda, 2000). A few of these, for example hydrated sodium calcium aluminosilicate (Ledoux *et al*., 1999), activated charcoal (Dalvi and Ademoyero, 1984), bentonite (Santurio *et al*., 1999), zeolite (Miazzo *et al*., 2000) and mannan oligosaccharide (Raju & Devegowda, 2000) was extensively studied with promising, but varying results. The search is still continuing for an effective tool to counteract the problem of aflatoxicosis in farm animals.

Humic substances are ubiquitous, and are found wherever matter is being decomposed, or has been transposed as in the case of sediments (Hayes *et al*., 1989). The humic acids in peat have been known since ancient times for their therapeutic properties such as anti-inflammatory, antiviral, oestrogenic and profibrinolytic activity (Schepetkin *et al*., 2002). Research showed that humic acids have a strong affinity to bind with several compounds. The ability of humic acids to adsorb heavy metals has been demonstrated in a number of investigations (Madronová *et al*., 2001). Humic substances are also known for their ability to bind herbicides (Nègre *et al*., 2001), mutagens (Sato *et al*., 1987a; Cozzi *et al*., 1993), monoaromatic (Nanny and Maza, 2001) and polycyclic

aromatic compounds (Kollist-Siigur *et al*., 2001), minerals (Elfarissi & Pefferkorn, 2000) and certain bacterial species (Fein *et al*., 1999).

A South African company, Enerkom (Pty) Ltd, developed an effective large-scale regeneration process for humic acids from coal. This technology can economically regenerate large quantities of pure, high quality humic acids by reversing the process whereby coal was formed. Humic acids produced in this way are called oxihumic acids. Chemically oxihumic acids differ only marginally from humic acids obtained from other sources (Cloete *et al*., 1990; Dekker *et al*., 1990; Cronjé *et al*., 1991; Bergh *et al*., 1997).

In vitro studies proved oxihumate to be an effective adsorbent of AFB_1, zearalenone, ochratoxin A and the ergopeptine alkaloids. It is, however, important to adequately test a potential adsorbent also for its *in vivo* binding abilities, as results in the past have indicated that the *in vitro* and *in vivo* efficacies are not necessarily correlated (Ledoux & Rottinghaus, 1999). The objective of this study was to evaluate the effects of oxihumate on growth performance and various liver morphological, serum biochemical and haematological variables in broiler chickens exposed to AFB_1.

Four different experiments were run in this study. The objective of the first experiment was to determine the most effective oxihumate inclusion level to ameliorate the effects of aflatoxin. The second experiment was done to ensure the safety of oxihumate in broilers at the specific dietary level decided on after the first experiment. The purpose of the third experiment was to extensively test oxihumate's efficacy against aflatoxicosis, not only at the aflatoxin level used during experiment 1, but also at a lower level closer to expected levels in practice. During this experiment oxihumate's performance was also compared against a well-known commercially available mycotoxin binder (Mycosorb^{TM}). High levels of antioxidants, alone and in combination with oxihumate were included in the aflatoxin-contaminated diets during the last experiment to test for further enhancement of protection against aflatoxicosis.

Materials and Methods

Aflatoxin Production

For the production of aflatoxin, *Aspergillus parasiticus* strain NRRL 2999 (kindly donated by Prof. W.M. Hagler, College of Agriculture and Life Sciences, North Carolina State University) was

grown on rice as described by Shotwell *et al*. (1966). This strain is very stable and consistently yields high levels of aflatoxin, especially AFB_1, even after many transfers. Potato dextrose agar was mixed with distilled water (39g of agar/L water) and autoclaved. Petri dishes were plated with potato-dextrose-agar and inoculated with spores of NRRL 2999 and incubated for seven days at 28 °C. By seven days, the cultures had a heavy crop of green conidia.

Fermentations were carried out in 500 mL cotton-stoppered Erlenmeyer flasks containing 38 g of polished rice. After 15 mL of distilled water was added to the rice, the cotton stoppers were covered with aluminium foil and the mixture was allowed to set for 2 hr at room temperature to allow water to imbide into the rice grains. The flasks were then autoclaved at 15 psi for 15 min and cooled. Each flask was then inoculated by adding a small piece of agar with culture material directly to the rice. The flasks were shaken by hand to mix the inoculum with the rice grains and then allowed to set at room temperature for 24 hours. The flasks were again shaken by hand to break up the rice and then placed on a shaker at 28 °C at 300 rpm for seven days. At 48 hours after inoculation, the rice kernels showed small white areas at the sites where the fungi had begun to grow. Shortly afterwards, the rice assumed a bright yellowish colour, which darkened to a dull light brown. These colour changes occurred in all successful fermentations. Sporulation did not occur except on the wall of the flask above the fermenting rice.

Fermented rice was autoclaved to stop all growth and dried by placing the contents of the flasks into stainless steel pans in a forced air oven at 40°C for 24 hours. The dried rice was then ground to a fine powder and stored. After several batches of aflatoxin-contaminated rice were produced, it was thoroughly mixed and tested for aflatoxin levels by HPLC.

One gram of culture material was weighed into a polypropylene wide mouth screw cap bottle and 100 mL acetonitrile:water (9:1) was added. The sample was placed on a rotator shaker for 30 min. Approximately 10 mL of the extract was filtered through Whatman #4 filter paper and 2-3 mL of filtrate was applied to an M224 cleanup column. Column eluant was mixed with an equal volume of water and examined by high pressure liquid chromatography (HPLC). HPLC analyses were performed on a Perkin-Elmer Model 250 liquid chromatograph equipped with an autosampler, a Hypersil®BDS 3µ C_{18} column (100 x 4.6mm) and fluorescence detection (em – 365 nm; ex – 430 nm) with a Hitachi fluorescence spectrophotometer. The mobile phase used was water:methanol:isopropanol (40:20:2) and was run at 1 mL/min.

The ground rice was added to the ration in the required proportion. The concentration of ground rice never exceeded 1% of the total diet.

Experiment 1: Different dietary inclusion levels of oxihumate

The objective of the first experiment was to determine the most effective oxihumate inclusion level to ameliorate the effects of aflatoxin, included in the diet at 2 mg AFB_1/kg, a level known to cause detrimental effects in poultry.

Birds and Diets

Day-old male Ross broiler chicks were purchased from a commercial hatchery for use in all *in vivo* experiments. Birds were kept on the same commercial broiler starter diet from day one to seven days of age. On day seven 240 chicks of similar weight were wing-banded and randomly assigned to 24 pens in an environmentally controlled broiler house. Birds were maintained on a 23-hour light and 1-hour dark schedule and allowed to consume feed and water *ad libitum*. The birds were divided into 8 treatment groups, with 3 replicates per treatment and 10 birds per replicate. A commercial broiler diet (3 phase) was used as basal diet for all treatments and the oxihumate and aflatoxin-contaminated rice powder were mixed thoroughly into the treatment diets (Table 4.1)

Table 4.1. Dietary treatments used in Experiment 1 to determine the most effective oxihumate inclusion level to ameliorate the effects of aflatoxin

Group	Treatment
Group 1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4	2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5	2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6	2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7	2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8	2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

Mortalities were recorded as they occurred. The experiment was terminated when the broilers reached the age of 42 days.

Body weight

The birds were individually weighed on day 7, 21, 35 and 42, using a Precisa XT2200C balance.

Haematocrit

On day 38, all chicks were bled by cardiac puncture. Whole blood was collected in EDTA blood tubes for haematocrit determinations. Haematocrit was determined with a Jouan Microhaematocrit Centrifuge (The Scientific Group, SA Scientific Products (Pty) Ltd. Trading, South Africa).

Serum albumin

Serum samples were collected for albumin analyses. Albumin concentration was measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA Systems Manual (Method No. SM4-0131E94, May 1994). This albumin method is based on the work of Doumas *et al*. (1971) who automated the original manual method of Rodkey (1965).

Relative liver, heart and stomach weight

At the termination of the study, all chicks were killed by cervical dislocation and the liver, heart, proventriculus and gizzard were removed and weighed. The contents of the proventriculus and gizzard were removed before weighing them together to determine the stomach weight. The organ weights were expressed as a percentage of body weight.

Liver lesions

A sample of the right lobe of the liver was taken from all the birds for histopathological examination. The samples were fixed in 10% neutral buffered formalin and subjectively judged for liver lesions by the Pathology Laboratory, Faculty of Veterinary Sciences, Onderstepoort, South Africa. The evaluation was done as a double-blinded study. Two opposite sections of all formalin fixed samples were examined. The samples were macroscopically judged for colour after formalin fixation. The microscopic appearance of the samples was evaluated for fatty degeneration, hepatocyte necrosis, bile duct proliferation, fibrosis, architectural disturbances, anisonucleosis, chromatin margination, prominent nucleoli, mitosis, nodular hyperplasia and heterophil and

lymphocyte aggregation. The presence of nodular hyperplasia was confirmed with special staining (GRI) on test sections. The degree of severity of each of the different lesions was expressed as 0 (no lesions); 1 (mild); 2 (moderate) or 3 (severe). The numeric values of the lesions for each sample were added and used as a basis for comparison between birds.

Experiment 2: Toxicity of oxihumate in broilers

The objective of the second *in vivo* experiment was to determine the toxicity of oxihumate at a concentration of 3.5 g/kg feed in broilers.

Birds and Diets

Day-old male Ross broiler chicks were kept on the same commercial broiler starter diet from day one to seven days of age. On day seven 80 chicks of similar weight were wing-banded and randomly assigned to 8 pens in an environmentally controlled broiler house. Birds were maintained on a 23-hour light and 1-hour dark schedule and allowed to consume feed and water *ad libitum*. The birds were divided into 2 treatment groups, with 4 replicates per treatment and 10 birds per replicate. A commercial broiler diet (3 phase) in mash form was used as basal diet for both treatments. The first treatment group received a control diet and the second treatment group received the same diet, but with 3.5 g of oxihumate/kg of feed, mixed thoroughly into the feed.

The experiment was terminated when the birds reached the age of 35 days. Mortalities were recorded as they occurred.

Body weight

The birds were weighed individually on days 7, 14, 21, 28 and 35. A Precisa XT2200C balance was used.

Haematocrit

Haematocrit was determined by the same method as used in Experiment 1.

Serum profile

Albumin – Standard procedures, as described in Experiment 1, were used for measuring of albumin levels in the serum.

Total Serum Protein (TSP) - Serum samples were collected for TSP analyses. TSP concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA Systems Manual (Method No. SM4-0147E94, May 1994). This total method is based on the work of Skeggs & Hochstrasser (1964) who automated the manual method of Weichselbaum (1946).

Globulin – Serum globulin values were calculated as the difference between TSP and albumin.

γ-Glutamyltransferase (GGT) (E.C. 2.3.2.2) - Serum samples were collected for GGT analyses. GGT concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA® Systems Manual (Method No. SM4-0142E94, May 1994). GGT was first demonstrated in human serum by Goldbard *et al*. (1960) and Szewczuk & Orlowski (1960). The Technicon RA® system method follows the modified procedure of Szasz (1976).

Creatine Kinase (CK) (EC 2.7.3.2) - Serum samples were collected for CK analyses. CK concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA® Systems Manual (Method No. SM4-0140E94, May 1994). The CK method is an adaptation of the IFCC reference method (Szazs *et al*, 1976).

Anti-New Castle Disease Virus (NDV) Antibody Titres

On 22 days of age the chicks were vaccinated against NDV using Nobilis® ND Clone 30, a live freeze-dried vaccine against NCD in fowls, obtained from Intervet S.A. (Pty) Ltd (Edenvale, South Africa). The vaccine was dissolved in Intervet Oculo-Nasal Diluent and administered intra-ocular by means of the standardized dropper provided with the vaccine. Vaccination was only done after the chicks reached 3 weeks of age to eliminate the possibility of carried-over paternal immunity against NDV. Blood samples were obtained 2 weeks after vaccination and the serum was stored at –20 °C until analysis.

An enzyme-linked immunosorbent assay (ELISA) kit (BioChek, Gouda, Holland, Catalogue Code CK116) was used to measure primary antibody production against NDV. Microtitre plates have been pre-coated with inactivated NDV antigen. Chicken serum samples were diluted and added to

the microtitre wells where any anti-NDV antibodies present bound and formed an antigen-antibody complex. Non-specific antibodies and other serum proteins were washed away. Anti-chicken IgG labeled with the enzyme alkaline phosphatase was then added to the wells to bind any chicken anti-NDV antibodies originally bound to antigen. After another wash to remove unreacted conjugate, substrate was added in the form of pNPP chromogen. A yellow colour developed if anti-NDV antibody was present and the intensity is directly related to the amount of anti-NDV present in the sample. The colour intensity was measured by a Ceres 900 Bio-Tek EIA Microtitre Plate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) with a 405 nm filter.

Experiment 3: The effect of oxihumate on aflatoxicosis in broilers

The purpose of the third experiment was to extensively test oxihumate's efficacy against aflatoxicosis, not only at 2 mg but also at 1 AFB_1/kg feed, a lower level closer to expected levels in practice. During this experiment oxihumate's performance was also compared against a well-known commercially available mycotoxin binder (Mycosorb^{TM}), both included at 3.5 g/kg feed.

Birds and Diets

Day-old male Ross broiler chicks were kept on the same commercial broiler starter diet from day one to seven days of age. On day seven 420 chicks of similar weight were wing-banded and randomly assigned to 28 pens in an environmentally controlled broiler house. Birds were maintained on a 23-hour light and 1-hour dark schedule and allowed to consume feed and water *ad libitum*. The birds were divided into 7 treatment groups, with 4 replicates per treatment and 15 birds per replicate. A commercial broiler diet (3 phase) was used as basal diet for all treatments and the oxihumate, Mycosorb^{TM} and aflatoxin-contaminated rice powder was mixed thoroughly into the different treatment diets (Table 4.2)

Mortalities were recorded as they occurred. The experiment was terminated when the broilers were 42 days of age.

Body weight

The birds were weighed individually on day 7, 14, 21, 28, 35 and 42. A Precisa XT2200C balance was used.

Table 4.2. Dietary treatments used in Experiment 3 to determine the effect of oxihumate on aflatoxicosis in broilers

Group	Treatment
Group 1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb^{TM}/kg feed
Group 2	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb^{TM}/kg feed
Group 3	1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb^{TM}/kg feed
Group 4	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb^{TM}/kg feed
Group 5	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb^{TM}/kg feed
Group 6	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb^{TM}/kg feed
Group 7	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb^{TM}/kg feed

Haematocrit

Haematocrit was determined by the same method as used in Experiment 1.

Serum profile

Albumin, TSP and GGT – Standard procedures, as previously described in Experiments 1 and 2, were used for the measuring of albumin, TSP and GGT activities in the serum.

Aspartate Aminotransferase (AST) - Serum samples were collected for AST analyses. AST concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA® Systems Manual (Method No. SM4-0137E94, May 1994). The Technicon RA® system AST method is based on work by Karmen (1955) who originated a procedure that coupled malate dehydrogenase and NADH to the aminotransferase reaction. Bergmeyer *et al*. (1978) modified this procedure to eliminate side reactions and to optimize substrate conditions.

Liver lesions

Liver lesions were quantified as described in Experiment 1.

Relative liver, heart and stomach weight

The liver, heart and stomach weights, as percentage of body weight, were determined as described in Experiment 1.

Experiment 4: The effect of oxihumate, alone and in combination with elevated dietary levels of antioxidants on aflatoxicosis

High levels of antioxidants (four times higher than standard levels of antioxidants normally added to broiler rations with 120 mg Vit C/kg feed) (Table 4.4), alone and in combination with oxihumate, were included in aflatoxin-contaminated diets during the fourth experiment to test for further enhancement of protection against aflatoxicosis in broilers.

Birds and Diets

Day-old male Ross broiler chicks were purchased from a commercial hatchery. The birds were kept on a commercial broiler starter diet from day one to seven days of age. On day seven 378 chicks of similar weight were wing-banded and randomly assigned to 27 pens in an environmentally controlled broiler house. Birds were maintained on a 23-hour light and 1-hour dark schedule and allowed to consume feed and water *ad libitum*. The birds were divided into 9 treatment groups, with 3 replicates per treatment and 14 birds per replicate. A commercial broiler diet (3 phase) was used as basal diet for all treatments and the oxihumate, Mycosorb^{TM}, antioxidants and aflatoxin-contaminated rice powder were mixed thoroughly into the different treatment diets (Table 4.3).

Table 4.3. Dietary treatments used in Experiment 4 to determine the effect of oxihumate, alone and in combination with elevated dietary levels of antioxidants on aflatoxicosis

Group	Treatment
Group 1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1]; 0 g Mycosorb^{TM}/kg feed
Group 2	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2]; 0 g Mycosorb^{TM}/kg feed
Group 3	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1]; 0 g Mycosorb^{TM}/kg feed
Group 4	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2]; 0 g Mycosorb^{TM}/kg feed
Group 5	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1]; 0 g Mycosorb^{TM}/kg feed
Group 6	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2]; 0 g Mycosorb^{TM}/kg feed
Group 7	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1]; 0 g Mycosorb^{TM}/kg feed
Group 8	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2]; 0 g Mycosorb^{TM}/kg feed
Group 9	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1]; 3.5 g Mycosorb^{TM}/kg feed

[1] standard antioxidant levels
[2] 4 times standard antioxidant levels + 120 mg Vit C/kg feed

Table 4.4. Antioxidant levels in the diets of Experiment 4 to determine the effect of oxihumate, alone and in combination with elevated dietary levels of antioxidants on aflatoxicosis (values given per kg feed)

Antioxidant	Starter Standard	Starter High	Grower Standard	Grower High	Finisher Standard	Finisher High
Vitamin A (IU)	12 000	48 000	10 000	40 000	9 000	36 000
Vitamin E (IU)	40	160	40	160	30	120
Vitamin C (mg)	-	120	-	120	-	120
Manganese (mg)	70	280	70	280	70	280
Zinc (mg)	50	200	50	200	50	200
Copper (mg)	10	40	10	40	8	32
Iron (mg)	25	100	25	100	25	100
Selenium (mg)	0.2	0.8	0.2	0.8	0.2	0.8

Mortalities were recorded as they occurred. The experiment was terminated when the broilers were 42 days of age.

Body weight

The birds were weighed individually on days 7, 14, 21, 28, 35 and 42. A Precisa XT2200C balance was used.

Haematocrit

Haematocrit was determined by the same method as used in Experiment 1.

Serum profile

Albumin, CK and GGT – Standard procedures, as previously described in Experiments 1 and 2, were used for the measuring of albumin levels and CK and GGT activity in the serum.

Liver lesions

Liver lesions were quantified as described in Experiment 1.

Relative liver, heart and stomach weight

The liver, heart and stomach weights, as percentage of body weight, were determined as described in Experiment 1.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of the Faculty of Natural and Agricultural Sciences, University of Pretoria (EC010607-006).

Statistical Analysis

Body weight

A repeated measure analysis of variance with the GLM model (Statistical Analysis Systems, 1994) was used to determine the significance (5%) between treatment- and pens within treatment effects for the repeated weekly body weights. Least square means and standard errors (±SE) were calculated.

Other analyses

An analysis of variance with the GLM model (Statistical Analysis Systems, 1994) was used to determine the significance between treatment- and pens within treatment effects for the unbalanced data. Least square means and standard errors (±SE) were calculated for treatments. Significance of difference (5%) between least square means was determined by the Bonferroni test (Sameuls, 1989).

Results

Aflatoxin Production

Aflatoxins were produced by fermentation of rice by the NRLL2999 strain of *Aspergillus parasiticus* under constant stirring and controlled temperature. With the fermentation method a total of 1,116 mg aflatoxin/kg of rice was obtained containing 82.58% AFB_1, 3.17% AFB_2, 13.55% AFG_1 and 0.70% AFG_2.

Experiment 1: Different dietary inclusion levels of oxihumate

Body weight

Table 4.5. Effect of different levels of oxihumate on the body weight (g) of birds fed 2 mg aflatoxin B_1 (AFB_1)/kg feed (± standard error of the mean)

Group	Treatment	Day 7	Day 21	Day 35	Day 42
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed	172.8[a] (2.3)	772.6[a] (12.3)	1658.1[a] (28.4)	2197.7[a] (40.3)
2	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed	167.4[a] (2.5)	629.0[b] (13.2)	1057.1[b] (30.5)	1374.3[b] (43.1)
3	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed	165.6[a] (2.9)	652.1[b] (15.3)	1089.0[bc] (35.2)	1481[bc] (49.9)
4	2 mg AFB_1/kg feed; 6 g oxihumate/kg feed	167.8[a] (2.3)	639.8[b] (12.3)	1091.7[bd] (28.4)	1473.1[bc] (40.3)
5	2 mg AFB_1/kg feed; 9 g oxihumate/kg feed	172.4[a] (2.4)	637.1[b] (12.5)	1104.2[bc] (28.9)	1480.2[bc] (40.9)
6	2 mg AFB_1/kg feed; 12 g oxihumate/kg feed	167.8[a] (2.4)	659.6[b] (12.5)	1178.8[c] (28.9)	1571.2[c] (40.9)
7	2 mg AFB_1/kg feed; 15 g oxihumate/kg feed	173.3[a] (2.7)	646.0[b] (14.0)	1159.2[cd] (32.3)	1539.3[cd] (45.8)
8	2 mg AFB_1/kg feed; 18 g oxihumate/kg feed	166.7[a] (2.3)	627.9[b] (12.3)	1093.9[bd] (28.4)	1455.8[bd] (40.3)

[a-d] Values within a column with no common superscripts are significantly different ($P<0.05$)

The effect of increasing levels of oxihumate on the body weights of birds fed 2 mg AFB_1/kg feed is presented in Table 4.5. Body weight from day 21 onwards was significantly ($P<0.05$) reduced in broilers receiving aflatoxin in their diets. Oxihumate had a slight but not significant positive effect on body weight at day 21. At days 35 and 42 all concentration levels of oxihumate improved the

growth of birds fed aflatoxin-contaminated diets. This ameliorating effect was, however, only significant at a concentration of 12 and 15 g/kg. No significant difference in growth performance between the different oxihumate levels could be detected. The final body weight of the different treatment groups is schematically illustrated in Figure 4.1.

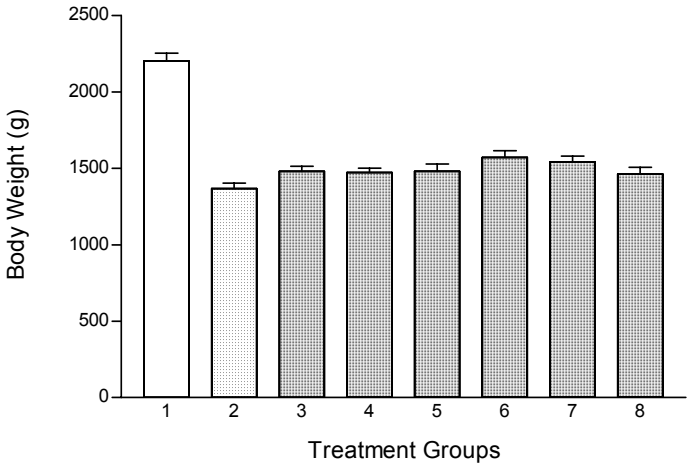

Figure 4.1. Effect of different levels of oxihumate[1] on the 42-day body weight of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

Haematocrit

Consumption of aflatoxin-contaminated feed reduced the haematocrit significantly ($P<0.05$) from 31.9% to 27.2%. An oxihumate concentration of 3.5 g/kg aflatoxin-contaminated feed, improved haematocrit levels of the broilers significantly to 29% (Table 4.6). The haematocrit levels of the different treatments are schematically illustrated in Figure 4.2.

Serum albumin

AFB_1 at a concentration of 2 mg/kg feed had a significantly ($P<0.05$) lowering effect on serum albumin levels of the birds. Oxihumate improved the serum albumin levels and this improvement was significant at levels of 9 and 12 g oxihumate/kg feed (Table 4.6). The serum albumin levels of the different treatments are schematically illustrated in Figure 4.3.

Liver Lesions

The inclusion of 2 mg AFB_1/kg in the diet of broilers caused a significant increase in the quantity and severity of liver lesions in the birds ($P<0.05$). Although the inclusion of oxihumate showed some protective effects at certain concentrations, none of these was significant (Table 4.6). Figure 4.4 illustrates the effect that AFB_1 had on the livers of broilers.

Mortality

No significant differences in mortality rates were noted among the different treatments in Experiment 1 (Table 4.6).

Table 4.6. Haematocrit, serum albumin levels, liver lesions and mortalities of birds receiving different levels of oxihumate and 2 mg aflatoxin B_1/kg feed (AFB_1) (± standard error of the mean)

Group	Treatment	Haematocrit (%)	Serum Albumin (g/L)	Liver Lesions	Mortality (mean per pen)
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed	31.92^{a} (0.57)	13.91^{a} (0.41)	2.76^{a} (0.33)	1.0^{a} (0.84)
2	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed	27.21^{b} (0.59)	6.30^{b} (0.42)	7.58^{b} (0.35)	2.0^{a} (0.84)
3	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed	29.03^{c} (0.65)	7.03^{bc} (0.45)	7.36^{b} (0.38)	3.3^{a} (0.84)
4	2 mg AFB_1/kg feed; 6 g oxihumate/kg feed	27.69^{bc} (0.57)	7.09^{bc} (0.41)	8.66^{c} (0.32)	1.0^{a} (0.84)
5	2 mg AFB_1/kg feed; 9 g oxihumate/kg feed	28.21^{bc} (0.56)	7.79^{c} (0.39)	7.57^{b} (0.36)	1.3^{a} (0.84)
6	2 mg AFB_1/kg feed; 12 g oxihumate/kg feed	27.58^{bc} (0.60)	7.77^{c} (0.41)	6.97^{b} (0.34)	1.3^{a} (0.84)
7	2 mg AFB_1/kg feed; 15 g oxihumate/kg feed	27.35^{bc} (0.63)	7.45^{bc} (0.45)	6.64^{b} (0.39)	3.0^{a} (0.84)
8	2 mg AFB_1/kg feed; 18 g oxihumate/kg feed	28.44^{bc} (0.57)	7.38^{bc} (0.41)	6.85^{b} (0.33)	1.0^{a} (0.84)
	R^2	0.28	0.59	0.58	0.35

[a-c] Values within a column with no common superscripts are significantly different ($P<0.05$)

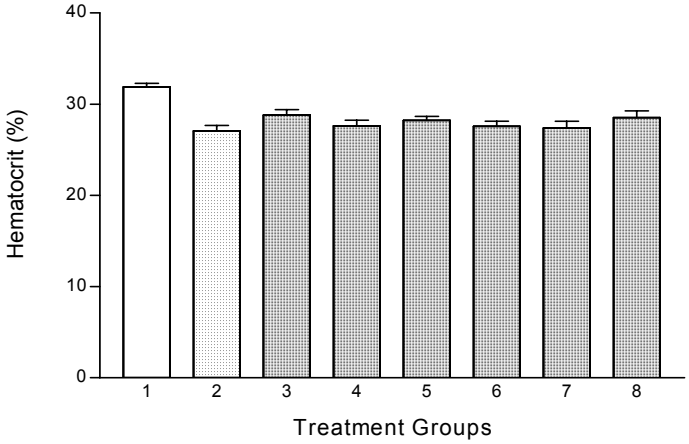

Figure 4.2. Effect of different levels of oxihumate[1] on haematocrit (%) of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

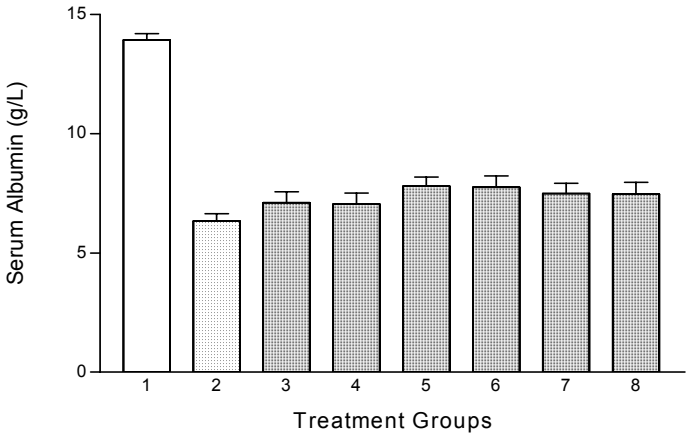

Figure 4.3. Effect of different levels of oxihumate[1] on serum albumin levels of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

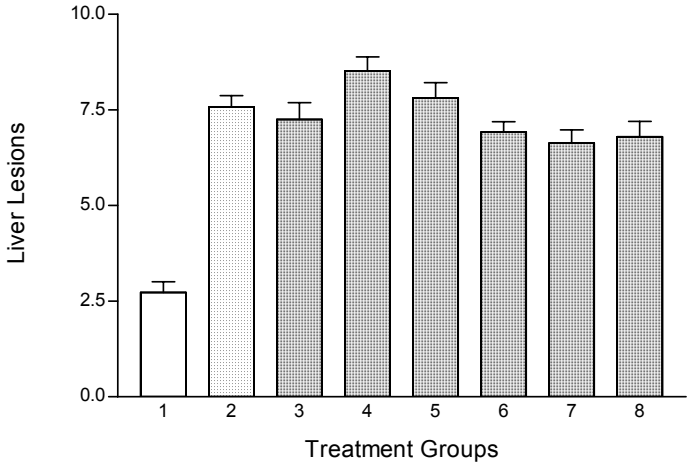

Figure 4.4. Effect of different levels of oxihumate[1] on liver lesions of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

Relative liver, heart and stomach weight

Table 4.7 shows data from Experiment 1 indicating a significant increase ($P<0.05$) in relative organ weights in broilers that received AFB_1 in their diets. The effect of aflatoxin ingestion on liver weight was especially severe with a two-fold increase in relative weight. The inclusion of oxihumate did not alleviate the response of this organ to aflatoxin (Figure 4.5). The aflatoxin-induced stomach enlargement in the birds showed some improvement with oxihumate supplementation in the contaminated diets, with especially significant differences notably at the 3.5, 6 and 12 g oxihumate/kg feed concentrations (Figure 4.6). An oxihumate level of 3.5 and 12 g/kg feed protected the hearts of birds feeding on aflatoxin-contaminated diets from enlarging, as compared to the control group (Figure 4.7).

Table 4.7. Liver, heart and stomach weight as percentage of body weight of birds receiving different levels of oxihumate and 2 mg aflatoxin B_1/kg feed (AFB_1) (± standard error of the mean)

Group	Treatment	Relative Liver Weight	Relative Heart Weight	Relative Stomach Weight
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed	2.28^{a} (0.17)	0.62^{a} (0.02)	2.88^{a} (0.08)
2	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed	4.41^{bc} (0.18)	0.70^{b} (0.02)	3.67^{b} (0.09)
3	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed	4.93^{c} (0.21)	0.65^{ab} (0.03)	3.19^{c} (0.10)
4	2 mg AFB_1/kg feed; 6 g oxihumate/kg feed	4.89^{cd} (0.17)	0.71^{b} (0.02)	3.42^{cd} (0.08)
5	2 mg AFB_1/kg feed; 9 g oxihumate/kg feed	4.49^{bc} (0.17)	0.70^{b} (0.02)	3.62^{bd} (0.08)
6	2 mg AFB_1/kg feed; 12 g oxihumate/kg feed	4.33^{b} (0.17)	0.67^{ab} (0.02)	3.32^{c} (0.08)
7	2 mg AFB_1/kg feed; 15 g oxihumate/kg feed	4.34^{b} (0.19)	0.70^{b} (0.02)	3.46^{bc} (0.09)
8	2 mg AFB_1/kg feed; 18 g oxihumate/kg feed	4.26^{b} (0.17)	0.68^{b} (0.02)	3.59^{bd} (0.08)
	R^2	0.51	0.12	0.32

[a-d] Values within a column with no common superscripts are significantly different ($P<0.05$)

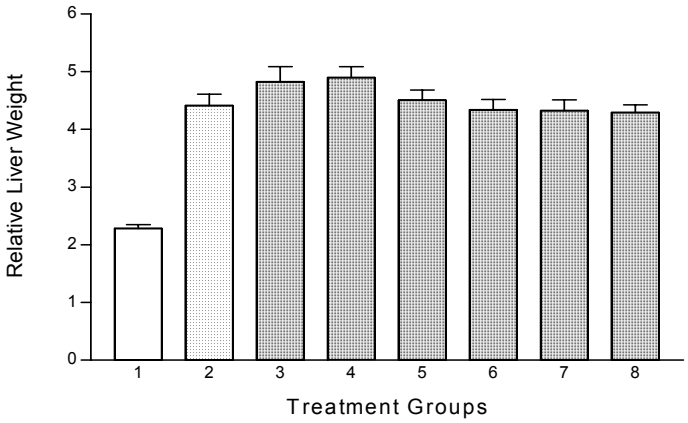

Figure 4.5. Effect of different levels of oxihumate[1] on the liver weight, expressed as a percentage of body weight, of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

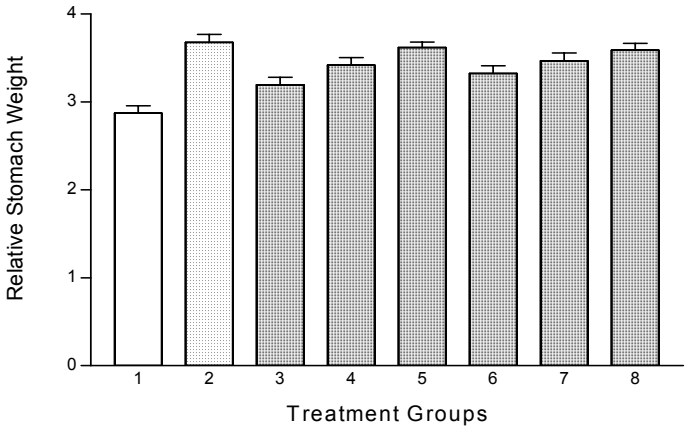

Figure 4.6. Effect of different levels of oxihumate[1] on the stomach weight, expressed as a percentage of body weight, of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

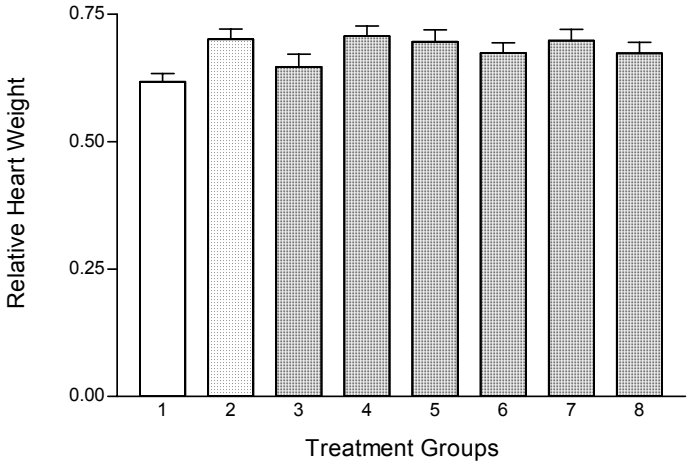

Figure 4.7. Effect of different levels of oxihumate[1] on the heart weight, expressed as a percentage of body weight, of birds fed 2 mg aflatoxin B_1/kg feed (AFB_1) (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 2: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed
Group 3: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed
Group 4: 2 mg AFB_1/kg feed; 6 g oxihumate/kg feed
Group 5: 2 mg AFB_1/kg feed; 9 g oxihumate/kg feed
Group 6: 2 mg AFB_1/kg feed; 12 g oxihumate/kg feed
Group 7: 2 mg AFB_1/kg feed; 15 g oxihumate/kg feed
Group 8: 2 mg AFB_1/kg feed; 18 g oxihumate/kg feed

Experiment 2: Toxicity of oxihumate in broilers

The results of Experiment 1 indicated that a concentration of 3.5 g oxihumate/kg feed ameliorated some of the toxic effects of AFB_1 in broilers. Higher levels of inclusion did not proof to be more effective and the 3.5 g oxihumate/kg feed level was therefore chosen for all further experiments.

The absence of any significant differences in the body weight and serum profile data of Experiment 2, (Table 4.8 and 4.9), together with the two mortalities experienced per treatment, suggest that oxihumate at 3.5 g/kg in feed has no apparent toxic effect on broilers.

Table 4.8. The effect of oxihumate (3.5 g/kg feed) on the body weight (g) of broiler chickens (± standard error of the mean)[1]

Treatment	Day 7	Day 14	Day 21	Day 28	Day 35
0 g oxihumate/kg feed	147.1 (3.04)	360.5 (8.94)	760.8 (19.20)	1224.5 (30.19)	1743.5 (20.53)
3.5 g oxihumate/kg feed	148.0 (2.89)	360.9 (8.50)	758.7 (18.26)	1230.6 (28.72)	1781.2 (19.53)

[1] Values between treatments within a column did not differ significantly (P>0.05)

Table 4.9. The effect of oxihumate (3.5 g/kg feed) on the serum profile and anti-New Castle Disease Virus (NDV) antibody titre (± standard error of the mean)[1]

Treatment	Serum Globulin (g/L)	Serum Albumin (g/L)	Serum δ-Glutamyl-transferase (g/L)	Serum Creatine Kinase (IU/L)	Total Serum Protein (g/L)	Anti-NDV Antibody Titre
0 g oxihumate /kg feed	15.51 (0.41)	16.50 (0.35)	9.82 (1.25)	2554.5 (380.2)	32.15 (0.46)	1616 (414)
3.5 g oxihumate /kg feed	16.28 (0.50)	17.85 (0.37)	8.40 (1.19)	2551.3 (400.7)	33.85 (0.49)	2181 (414)
R^2	0.53	0.37	0.18	0.42	0.72	0.13

[1] Values between treatments within a column did not differ significantly (P>0.05)

Experiment 3: The effect of oxihumate on aflatoxicosis in broilers

Body Weight

Data presented in Table 4.10 show the effect of dietary treatment on body weight. A concentration of 1 mg AFB_1/kg feed did not affect the body weight and thus growth of the broilers up to 42 days of age. However, the 2 mg AFB_1/kg feed level depressed growth with significantly lower body

weights evident from an age of 21 days, two weeks after intake of the contaminated feed started. This reduction in weight gain continued throughout the study. The decrease in body weight gain caused by the addition of 2 mg AFB_1/kg feed was diminished at 42 days of age by the addition of 3.5 g oxihumate/kg to the diet (Figure 4.8). The Mycosorb™ treatment did not have any positive effect on body weight when compared with the birds that received 2 mg AFB_1/kg feed only.

Haematocrit

According to the data presented in Table 4.11, 1 mg AFB_1/kg of the diet did not affect haematocrit levels of the broilers. Haematocrit was, however, significantly reduced by the 2 mg AFB_1/kg feed treatment. Adding oxihumate to this diet protected the birds to such an extent that no effect of the aflatoxin could be detected on the haematocrit levels when compared with the controls. Mycosorb™ did not improve the haematocrit of the contaminated birds. The haematocrit levels of the different treatments are schematically illustrated in Figure 4.9.

Liver Lesions

The livers from chicks fed the aflatoxin diets were enlarged and pale in appearance compared with those of the control broilers. Aflatoxin ingestion by broilers had a pronounced dosage-response effect on the quantity and severity of hepatic lesions with a lesion value of 1.75 for the controls, 4.13 for the broilers that received 1 mg AFB_1/kg feed and 9.60 for the 2 mg AFB_1/kg feed treatment group (Table 4.11). Supplementation of oxihumate improved the liver lesion value of the latter significantly to 6.30. Mycosorb™ treatment of the contaminated diets did not show any positive effect on the liver lesions values. Figure 4.10 illustrates the effect of oxihumate and Mycosorb™ on liver lesions in aflatoxin fed broilers.

Mortalities

Although the average mortalities per pen increased as aflatoxin concentration in the feed increased, these differences were not significant (Table 4.11).

Table 4.10. Body weight (g) of birds receiving 3.5 g oxihumate or Mycosorb™/kg aflatoxin B_1 (AFB_1) contaminated diet (1 or 2 mg AFB_1/kg feed) (± standard error of the mean)

Group	Treatment	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	153.2[a] (1.8)	379.9[a] (4.8)	723.7[a] (10.4)	1137.3[a] (18.0)	1630.3[a] (26.4)	2194.1[a] (38.9)
2	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	155.4[a] (1.8)	382.3[a] (4.9)	717.0[ac] (10.6)	1143.1[a] (18.3)	1646.5[a] (26.8)	2227.1[a] (39.5)
3	1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	157.0[a] (1.8)	386.7[a] (5.1)	723.0[a] (10.9)	1146.9[a] (18.8)	1641.8[a] (27.5)	2226.2[a] (40.6)
4	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	157.9[a] (1.8)	384.8[a] (4.9)	725.2[a] (10.6)	1127.2[a] (18.1)	1602.1[a] (26.6)	2123.1[a] (39.2)
5	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	158.0[a] (1.9)	383.8[a] (5.1)	689.0[bc] (11.1)	995.2[b] (19.0)	1317.2[b] (27.9)	1692.3[b] (41.2)
6	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	157.6[a] (1.8)	380.1[a] (5.0)	669.0[b] (10.8)	983.4[b] (18.6)	1361.2[b] (27.3)	1820.9[c] (40.3)
7	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	156.8[a] (1.8)	381.5[a] (5.0)	681.3[b] (10.9)	992.9[b] (18.7)	1339.0[b] (27.4)	1727.4[bc] (40.4)

[a-c] Values within a column with no common superscripts are significantly different ($P<0.05$)

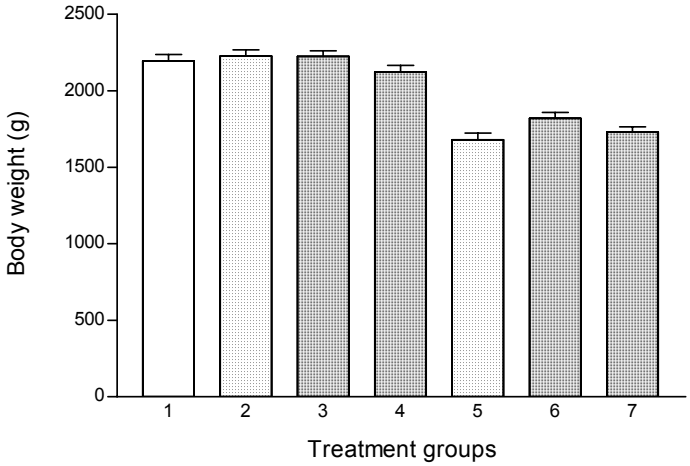

Figure 4.8. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on the 42 day body weight of birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

Relative organ weights

Data presented in Table 4.12 and illustrated in Figures 4.11 – 4.13 show the effects of dietary treatment on relative organ weights. Feed contamination at 1 mg AFB_1/kg had no effect on the relative weight of the liver and heart but it did, however, cause a significant increase in stomach weight. Both oxihumate and Mycosorb™ addition to the feed inhibited these effects so that there were no significant differences when compared to the controls. Levels of 2 mg AFB_1/kg in the feed increased the relative weight of the livers, hearts and stomachs of the birds significantly ($P<0.05$). The enlargement of the liver was especially severe, which was almost 75% heavier than the control. Oxihumate, but not Mycosorb™, in the feed significantly ($P<0.05$) reduce the effects that 2 mg AFB_1/kg feed had on the liver and heart weights. Both oxihumate and Mycosorb™ had a slight but not significant protective effect on stomach weight.

Table 4.11. Haematocrit, liver lesions and mortalities of birds receiving 3.5 g oxihumate or Mycosorb™/kg aflatoxin B_1 (AFB_1) contaminated diet (1 or 2 mg AFB_1/kg feed) (± standard error of the mean)

Group	Treatment	Haematocrit (%)	Liver Lesions[1]	Mortality (mean per pen)
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	31.58[a] (0.52)	1.75[a] (0.67)	0.25[a] (0.59)
2	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	32.03[a] (0.52)	4.13[b] (0.71)	0.75[a] (0.59)
3	1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	30.83[a] (0.52)	3.79[b] (0.69)	1.25[a] (0.59)
4	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	31.98[a] (0.52)	3.85[b] (0.67)	0.50[a] (0.59)
5	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	28.97[b] (0.46)	9.60[c] (0.67)	1.75[a] (0.59)
6	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	30.88[a] (0.52)	6.30[d] (0.67)	1.25[a] (0.59)
7	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	28.81[b] (0.52)	9.85[c] (0.67)	1.25[a] (0.59)
	R^2	0.31	0.57	0.18

[1] Mean numeric value expressing both presence and degree of severity of liver lesions

[a-d] Values within a column with no common superscripts are significantly different ($P<0.05$)

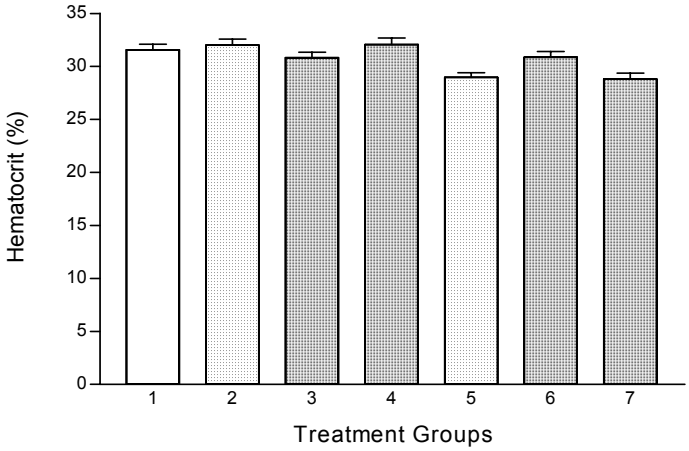

Figure 4.9. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on haematocrit (%) of birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

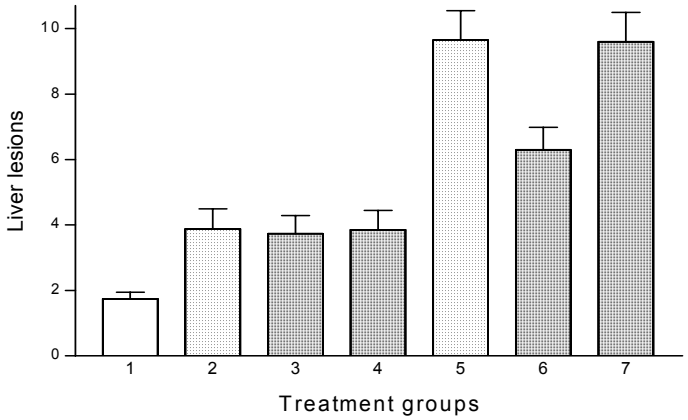

Figure 4.10. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on liver lesions in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
[2] Mean numeric value expressing both presence and degree of severity of liver lesions

Table 4.12. Liver, heart and stomach weight as percentage of body weight of birds receiving 3.5g/kg of either oxihumate or Mycosorb™ in an aflatoxin B_1 (AFB_1) contaminated diet (1 or 2 mg/kg) (± standard error of the mean)

Group	Treatment	Relative Liver Weight	Relative Heart Weight	Relative Stomach Weight
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	2.44[a] (0.20)	0.622[a] (0.02)	3.00[a] (0.11)
2	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	2.31[a] (0.20)	0.591[a] (0.02)	3.43[bc] (0.11)
3	1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	2.27[a] (0.20)	0.595[a] (0.02)	3.15[ab] (0.11)
4	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	2.41[a] (0.20)	0.598[a] (0.02)	3.31[abc] (0.11)
5	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	4.25[b] (0.20)	0.689[b] (0.02)	3.77[d] (0.11)
6	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	3.08[c] (0.20)	0.631[a] (0.02)	3.53[cd] (0.11)
7	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	4.08[b] (0.20)	0.639[ab] (0.02)	3.46[cd] (0.11)
	R^2	0.53	0.28	0.34

[a-d] Values within a column with no common superscripts are significantly different ($P<0.05$)

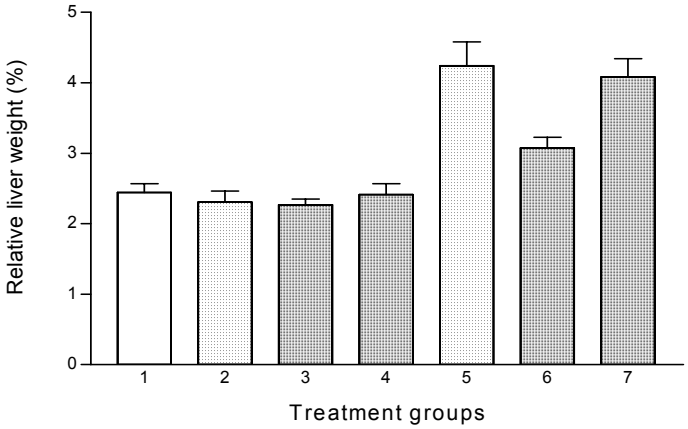

Figure 4.11. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on liver weight, expressed as a percentage of body weight, in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

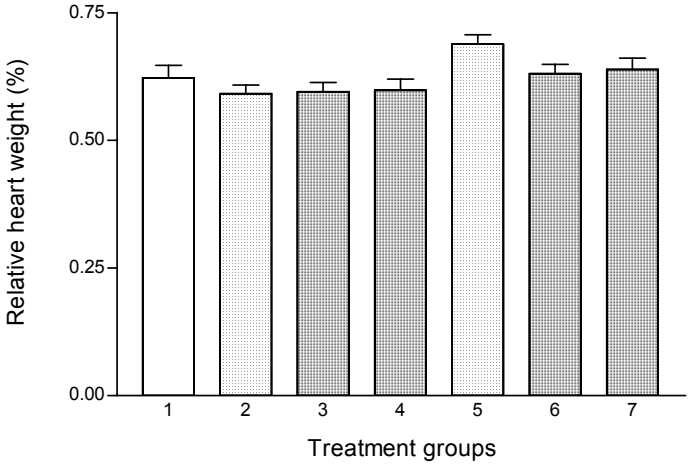

Figure 4.12. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on heart weight, expressed as a percentage of body weight, in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

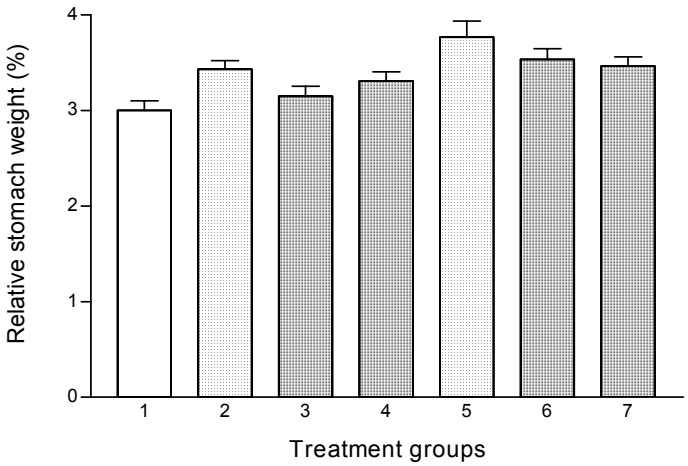

Figure 4.13. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on stomach weight, expressed as a percentage of body weight, in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

Serum profile

The effects that dietary treatments had on serum levels of albumin and total protein and δ-glutamyl transferase and aspartate amino transferase activities are presented in Table 4.13 and illustrated in Figures 4.14 – 4.17. AFB_1, at a concentration of 1 mg/kg feed, significantly decreased serum levels of albumin and total protein. The addition of oxihumate and Mycosorb™ did not reduce the response to the toxin in this case. The concentrations of serum albumin, total protein and δ-glutamyl transferase were reduced for the chicks consuming diets with 2 mg of AFB_1/kg. Significant protection was provided by oxihumate from the effect on serum albumin with the values being higher than for the chicks consuming the diets with AFB_1 alone or with Mycosorb™. Serum aspartate amino transferase activity was not affected by aflatoxin intoxication.

Table 4.13. Serum profile of birds receiving 3.5 g oxihumate or Mycosorb™/kg aflatoxin B_1 (AFB_1) contaminated diet (1 or 2 mg AFB_1/kg feed) (± standard error of the mean)

Group	Treatment	Serum Albumin (g/L)	Total Serum Protein (g/L)	Serum δ-Glutamyl Transferase (g/L)	Serum Aspartate Amino Transferase (IU/L)
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	15.19^{a} (0.44)	31.30^{a} (1.01)	9.66^{a} (0.84)	97.4^{ad} (2.99)
2	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	11.60^{b} (0.44)	27.13^{bc} (1.01)	7.78^{ab} (0.84)	102.5^{acd} (2.99)
3	1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	11.39^{b} (0.44)	26.22^{bc} (1.01)	7.79^{ab} (0.84)	106.5^{bc} (3.01)
4	1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	11.76^{b} (0.44)	27.36^{b} (1.01)	7.72^{abc} (0.84)	104.0^{ac} (2.99)
5	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	8.01^{c} (0.44)	24.44^{cd} (1.01)	5.94^{bc} (0.84)	94.3^{de} (2.99)
6	2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed	9.67^{d} (0.44)	23.19^{d} (0.99)	5.55^{bc} (0.83)	97.8^{ad} (2.95)
7	2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed	7.32^{c} (0.45)	19.31^{e} (1.03)	5.36^{c} (0.86)	88.8^{e} (3.06)
	R^2	0.56	0.36	0.21	0.17

[a-e] Values within a column with no common superscripts are significantly different ($P<0.05$)

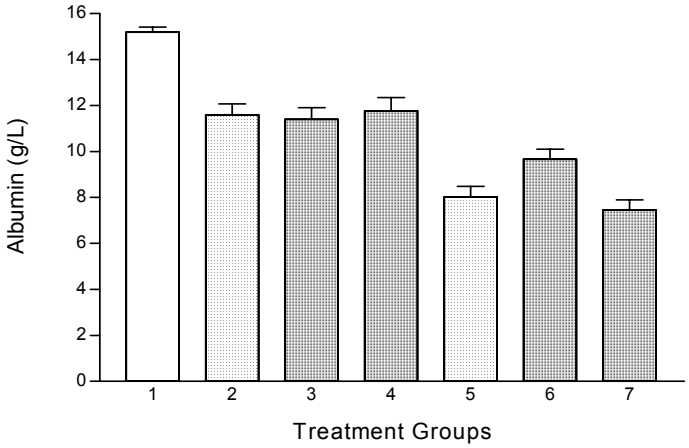

Figure 4.14. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on serum albumin levels in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

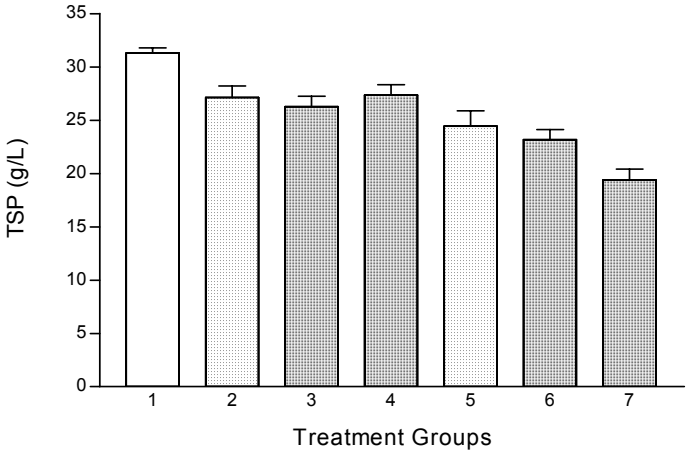

Figure 4.15. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on total serum protein (TSP) levels in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

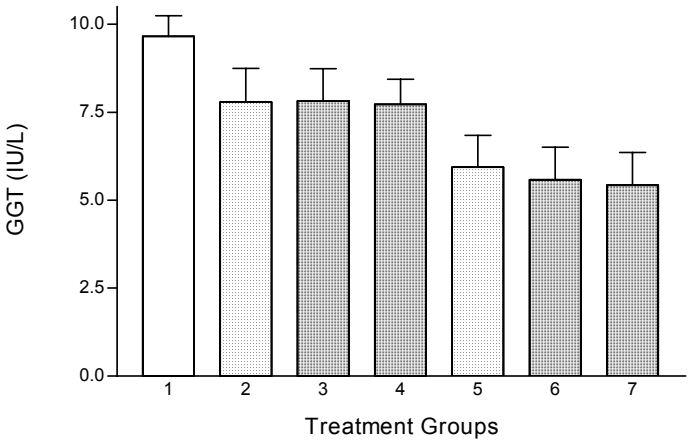

Figure 4.16. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on serum δ-glutamyl transferase (GGT) activities in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

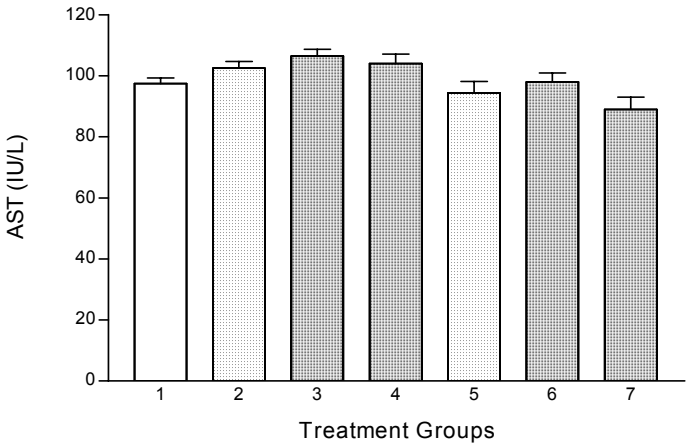

Figure 4.17. Effect of 3.5 g oxihumate and Mycosorb™/kg feed on serum aspartate amino transferase (AST) activities in birds fed either 1 or 2 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 2: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 3: 1 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 4: 1 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed
Group 5: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 6: 2 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; 0 g Mycosorb™/kg feed
Group 7: 2 mg AFB_1/kg feed; 0 g oxihumate/kg feed; 3.5 g Mycosorb™/kg feed

Experiment 4: The effect of oxihumate, alone or in combination with elevated dietary levels of antioxidants on aflatoxicosis

Body Weight

The data in Table 4.14 show the effect of oxihumate and Mycosorb™, alone and in combination with elevated dietary levels of antioxidants on body weight of broilers exposed to 3 mg AFB_1/kg feed. The aflatoxin-contaminated feed caused a reduction in body weight gain from the first week after commencement of treatments up to the end of the trial at 42 days of age (Figure 4.18). Supplementation of oxihumate at standard antioxidant levels elevated body weight gain significantly at both 35 and 42 days of age, when compared to the broilers that received aflatoxin alone. The high antioxidant treatments and added Mycosorb™ to the diets did not ameliorate the effect of the aflatoxin on body weight gain of the birds.

Serum Profile

AFB_1 at 3 mg/kg feed caused a severe reduction in the serum albumin and δ-glutamyl transferase levels. The addition of oxihumate, with and without elevated antioxidant levels, increased the concentration of albumin in the serum significantly. Serum creatine kinase activity in broilers proved to be insensitive to the presence of 3 mg AFB_1/kg feed. These results are presented in Table 4.15 and illustrated in Figures 4.19-4.21.

Haematocrit

According to the data presented in Table 4.15 and Figure 4.22, the haematocrit levels of broilers exposed to 3 mg AFB_1/kg feed decreased significantly from 31.1% (control) to 23.3%. A high antioxidant level in the aflatoxin-contaminated diet increased the haematocrit, but this was not significant. Oxihumate and oxihumate in combination with high antioxidant concentrations protected the birds significantly against reductions in haematocrit, with levels of 26.9% and 29.5%, respectively. The difference in haematocrit between the control group and the group that received aflatoxin together with oxihumate and high antioxidant levels was not significant. Mycosorb™ treatment did not show any protective characteristics against the detrimental effects of aflatoxicosis on haematocrit level.

Table 4.14. The effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants on body weight (g) of broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1) (± standard error of the mean)

Group	Treatment	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	160.8^{a} (1.7)	402.7^{ab} (6.4)	799.5^{ab} (11.9)	1326.9^{a} (18.9)	2038.2^{a} (23.7)	2588.6^{a} (36.6)
2	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	161.8^{a} (1.8)	408.7^{a} (7.0)	815.3^{a} (13.1)	1374.8^{a} (20.8)	2115.0^{b} (26.1)	2688.2^{a} (40.1)
3	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	155.5^{a} (4.0)	402.7^{ab} (6.6)	801.5^{ab} (12.2)	1325.6^{a} (19.4)	2082.4^{abc} (24.4)	2626.6^{a} (37.5)
4	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	156.5^{a} (2.3)	389.7^{bc} (6.5)	779.0^{b} (12.1)	1320.7^{a} (19.2)	2030.8^{ac} (24.1)	2608.0^{a} (37.1)
5	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	154.2^{a} (2.2)	376.6^{cd} (7.1)	638.7^{c} (13.2)	886.5^{b} (21.0)	1227.5^{df} (26.4)	1546.3^{b} (40.6)
6	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	158.4^{a} (2.4)	377.9^{cd} (7.8)	631.9^{cd} (14.5)	848.7^{b} (23.0)	1156.7^{d} (28.9)	1461.9^{b} (44.5)
7	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	154.5^{a} (2.7)	364.6^{def} (6.9)	624.1^{cd} (12.9)	897.7^{b} (20.5)	1304.6^{e} (25.8)	1676.5^{c} (39.7)
8	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	153.4^{a} (2.8)	355.6^{e} (6.9)	595.4^{d} (12.9)	841.1^{b} (20.5)	1207.4^{df} (25.8)	1561.1^{b} (39.7)
9	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 3.5 g Mycosorb™/kg feed	160.5^{a} (2.7)	382.1^{cf} (6.8)	628.7^{cd} (12.6)	884.1^{b} (20.1)	1243.1^{ef} (25.2)	1570.8^{bc} (38.8)

[1] standard antioxidant levels
[2] 4 times standard antioxidant levels + 120 mg Vit C/kg feed
[a-f] Values within a column with no common superscripts are significantly different ($P<0.05$)

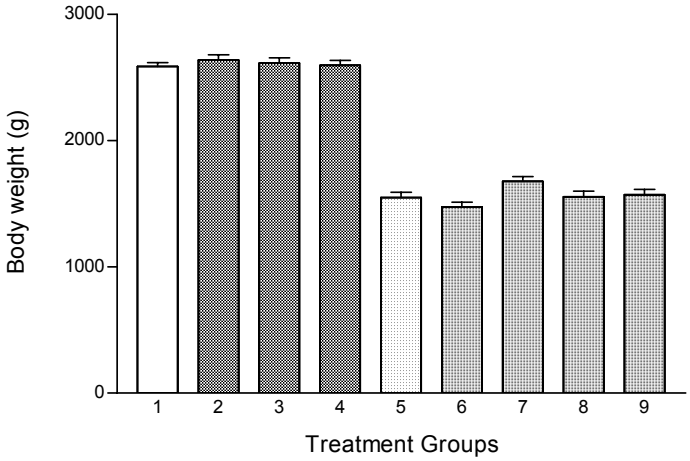

Figure 4.18. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on final body weight (g) of broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

Anti-NDV Antibody Titre

Results presented in Table 4.15 show that aflatoxin ingestion did not inhibit the formation of anti-NDV antibody titres in reaction to exposure to NDV antigens after vaccination. The groups that received oxihumate and high antioxidant levels in their feed, with or without aflatoxin, produced more anti-NDV antibodies than the control. This increased antibody production was significant in aflatoxin treated broilers that received elevated antioxidants, without and in combination with oxihumate (Figure 4.23).

Liver Lesions

Histological examination of the livers of the broilers revealed normal histological pictures for the control group and lesions typical of aflatoxicosis in all aflatoxin treated birds (Table 4.16). None of the treatments was effective in preventing the formation of hepatic lesions as a result of aflatoxicosis (Figure 4.24).

Table 4.15. The effect of 3.5 g oxihumate/kg feed, in combination with elevated levels of antioxidants on the serum profile, haematocrit levels and anti-NDV antibody titres of broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1) (± standard error of the mean)

Group	Treatment	Serum Albumin (g/L)	Serum δ-Glutamyl Transferase (IU/L)	Serum Creatine Kinase (IU/L)	Haema-tocrit (%)	Anti-NDV Antibody Titre
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	15.94^{a} (0.28)	7.76^{a} (0.60)	2136^{a} (220)	31.1^{ab} (0.82)	1672^{a} (388)
2	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	16.58^{a} (0.30)	7.63^{a} (0.65)	2505^{a} (239)	32.4^{a} (0.79)	1876^{a} (388)
3	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	16.15^{a} (0.29)	8.29^{ab} (0.62)	2341^{a} (230)	31.7^{a} (0.79)	2301^{ab} (388)
4	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	16.54^{a} (0.31)	9.64^{b} (0.68)	2154^{a} (249)	31.4^{ab} (0.79)	2370^{ab} (380)
5	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	8.39^{b} (0.29)	3.33^{c} (0.63)	2041^{a} (231)	23.3^{ce} (0.79)	1265^{a} (486)
6	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	8.31^{b} (0.31)	2.92^{cd} (0.68)	2143^{a} (249)	26.4^{de} (0.79)	3252^{bd} (486)
7	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	9.52^{c} (0.32)	2.59^{cd} (0.70)	2511^{a} (257)	26.9^{d} (0.79)	1738^{a} (445)
8	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	9.32^{c} (0.33)	1.85^{cd} (0.70)	2049^{a} (258)	29.5^{b} (0.79)	3584^{cd} (445)
9	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 3.5 g Mycosorb™/kg feed	8.19^{b} (0.30)	1.18^{d} (0.66)	2114^{a} (241)	24.6^{e} (0.79)	1623^{a} (486)
	R^2	0.86	0.51	0.15	0.61	0.21

[1] standard antioxidant levels; [2] 4 times standard antioxidant levels + 120 mg Vit C/kg feed

[a-e] Values within a column with no common superscripts are significantly different ($P<0.05$)

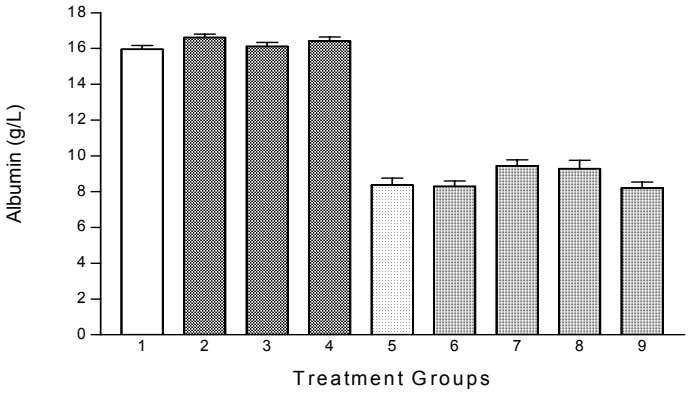

Figure 4.19. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on serum albumin levels in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

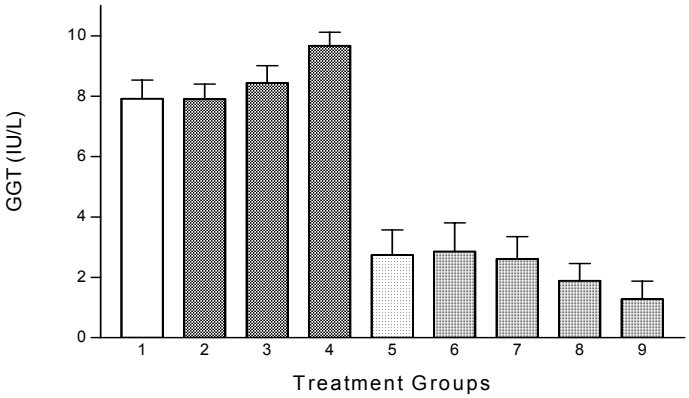

Figure 4.20. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on serum δ-glutamyl transferase (GGT) in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

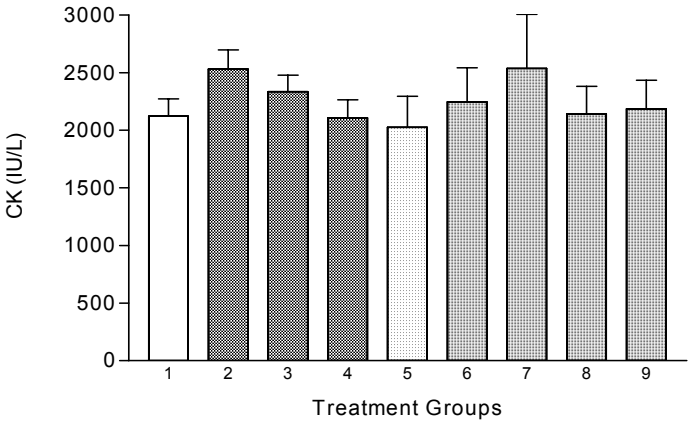

Figure 4.21. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on serum creatine kinase (CK) in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

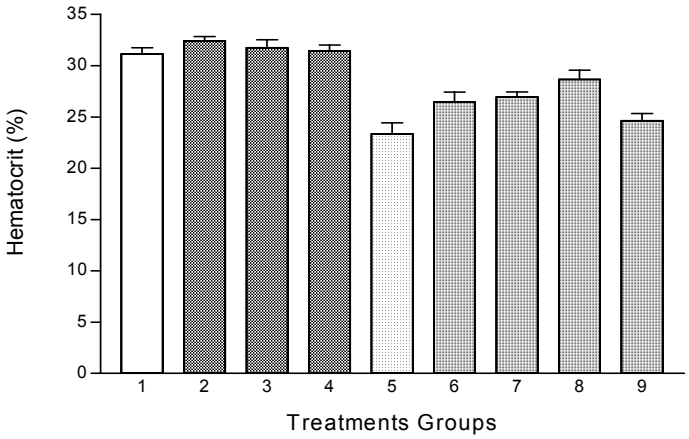

Figure 4.22. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on the haematocrit in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

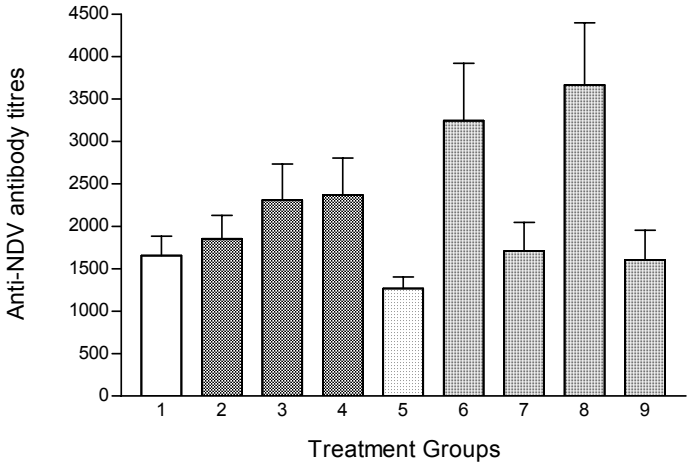

Figure 4.23. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on anti-New Castle Disease Virus (NDV) antibody production in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

Relative organ weights

AFB_1 at a level of 3 mg/kg feed caused severe enlargement of the liver, heart and stomach of intoxicated broilers (Table 4.16). Results revealed that the liver is especially sensitive to aflatoxin exposure as the livers of aflatoxin treated birds (4.69%) were double the relative weight of the control group (2.30%). Results indicated that the addition of oxihumate, but not Mycosorb™ or high antioxidant levels, to the aflatoxin-contaminated diet could significantly diminish this effect on the liver. The heart and stomach weights of the group receiving aflatoxin together with oxihumate were slightly but not significantly lower than the heart and stomach weights of the aflatoxin treated broilers. These data are illustrated in Figures 4.25 – 4.27.

Mortalities

Data presented in Table 4.16 show that a dietary intake of 3 mg AFB_1/kg feed caused a significant increase in mortality, compared to the control group. The addition of oxihumate and Mycosorb™ to the contaminated feed helped to reduce the rate of mortality.

Table 4.16. The effect of 3.5 g oxihumate/kg feed, in combination with elevated levels of antioxidants on the liver, heart and stomach weight, as a percentage of body weight, and liver lesions of broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1) (± standard error of the mean)

Group	Treatment	Relative Liver Weight	Relative Heart Weight	Relative Stomach Weight	Liver Lesions	Mortality (mean per pen)
1	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	2.30^{a} (0.30)	0.55^{ab} (0.03)	2.69^{a} (0.14)	5.00^{a} (0.63)	0.67^{a} (0.83)
2	0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	2.60^{a} (0.30)	0.51^{b} (0.03)	2.70^{a} (0.14)	6.78^{ab} (0.68)	2.67^{ab} (0.83)
3	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	2.24^{a} (0.30)	0.61^{adef} (0.03)	2.63^{a} (0.14)	6.83^{b} (0.63)	1.00^{a} (0.83)
4	0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	2.38^{a} (0.30)	0.56^{abe} (0.03)	2.77^{a} (0.14)	7.06^{b} (0.60)	1.00^{a} (0.83)
5	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	4.69^{b} (0.25)	0.72^{cg} (0.03)	3.53^{b} (0.12)	10.05^{ce} (0.53)	3.00^{ab} (0.83)
6	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	4.87^{b} (0.25)	0.74^{c} (0.03)	3.43^{b} (0.12)	12.40^{df} (0.53)	4.33^{b} (0.83)
7	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; Standard antioxidant[1] 0 g Mycosorb™/kg feed	3.96^{c} (0.25)	0.66^{fg} (0.03)	3.57^{b} (0.12)	13.04^{f} (0.55)	2.67^{ab} (0.83)
8	3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; High antioxidant[2] 0 g Mycosorb™/kg feed	4.72^{b} (0.25)	0.65^{efg} (0.03)	3.45^{b} (0.12)	11.93^{df} (0.53)	2.67^{ab} (0.83)
9	3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; Standard antioxidant[1] 3.5 g Mycosorb™/kg feed	4.29^{bc} (0.25)	0.78^{c} (0.03)	3.31^{b} (0.12)	11.46^{de} (0.53)	2.00^{ab} (0.83)
	R^2	0.66	0.55	0.56	0.77	0.47

[1] standard antioxidant levels; [2] 4 times standard antioxidant levels + 120 mg Vit C/kg feed
[a-e] Values within a column with no common superscripts are significantly different ($P<0.05$)

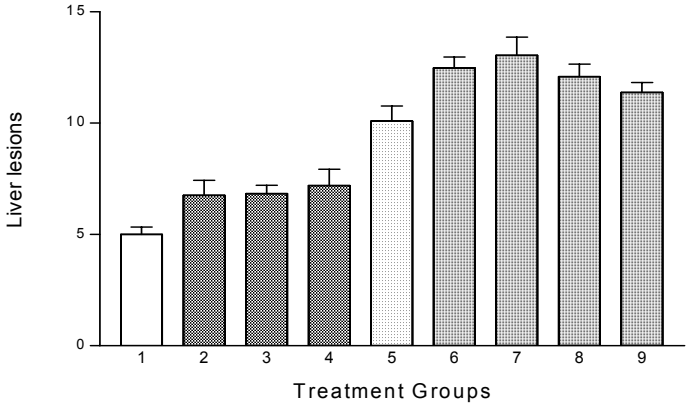

Figure 4.24. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on liver lesions in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of the mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

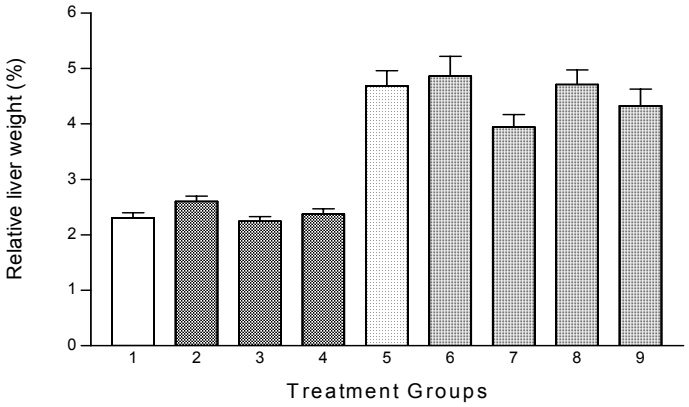

Figure 4.25. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on liver weight, expressed as percentage of body weight, in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

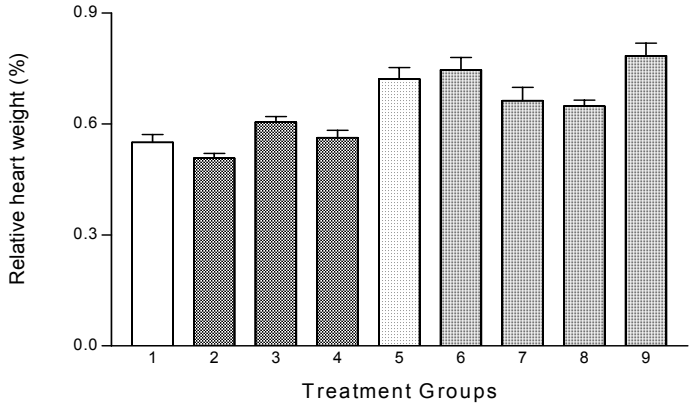

Figure 4.26. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on heart weight, expressed as percentage of body weight, in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

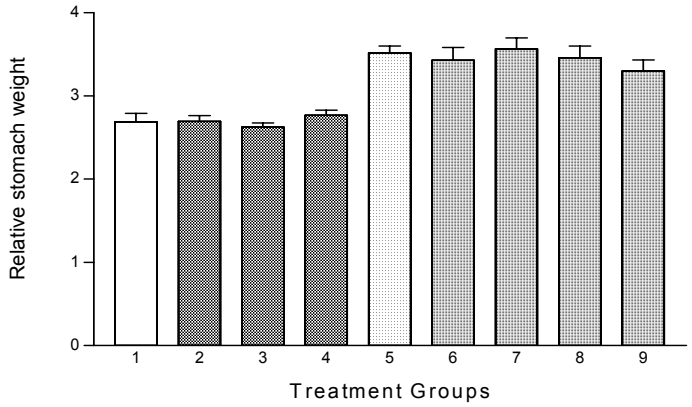

Figure 4.27. Effect of 3.5 g oxihumate/kg feed, alone or in combination with elevated levels of antioxidants, and Mycosorb™ on stomach weight, expressed as percentage of body weight, in broilers exposed to 3 mg aflatoxin B_1/kg feed (AFB_1)[1] (bars indicate standard error of mean)

[1]Group 1: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 2: 0 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 3: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 4: 0 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 5: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 6: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 7: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; standard antioxidant levels; 0 g Mycosorb™/kg feed
Group 8: 3 mg AFB_1/kg feed; 3.5 g oxihumate/kg feed; high antioxidant levels; 0 g Mycosorb™/kg feed
Group 9: 3 mg AFB_1/kg feed; 0 g oxihumate/kg feed; standard antioxidant levels; 3.5 g Mycosorb™/kg feed

Discussion

Mycotoxins are a cause of concern in the poultry industry due to health problems in the flocks and potential economic losses (Bailey *et al*., 1998). Consequently, poultry producers are in need of aids and methods to assist them in the protection of their flocks against these toxins. Industry efforts to protect their animals from the effects of mycotoxicosis have created an entrance point into the market for many different products. The efficacy of various relatively inert materials in diminishing the deleterious effects of toxins such as aflatoxin, as demonstrated in previous studies, is of great interest (Kubena *et al*, 1990a,b). Data from the study of Bailey *et al*. (1998) show that there is great variability in the efficacy of adsorbents *in vivo*, even though the compounds may show great potential for binding to the toxins *in vitro*. This observation emphasizes the importance for industry scrutiny of new products, purported to ameliorate the effects of mycotoxins in poultry, to ensure that sound scientific principles have been applied in the evaluation of these products (Dale, 1998).

The *in vivo* efficacy of oxihumate as an aflatoxin binder was evaluated in the present study after results obtained during *in vitro* binding studies showed that it has a high mycotoxin adsorption capacity and therefore definite potential for lessening the effects of aflatoxicosis in farm animals.

Results of the first experiment showed that high levels of oxihumate are not necessarily more effective in diminishing the effects of aflatoxin in broilers than the lower levels used. It was therefore decided that, for economical reasons in commercial practice, a low level of 3.5g oxihumate/kg feed would be used for the duration of the trial. Data from the toxicity experiment demonstrated that 3.5g oxihumate/kg feed is not detrimental for the health of broiler chickens as no significant changes were shown in body weight gain, haematocrit, serum profile (albumin, globulin, total protein, γ-glutamyltransferase and creatine kinase), anti-NDV antibody titre and mortality rate.

In this study aflatoxin was found to decrease body weight gain of broilers fed diets contaminated with AFB_1 at levels of 2 and 3 mg/kg feed. This supports the results of various researchers who found that aflatoxin ingestion inhibits growth in chickens (Ledoux *et al*., 1999; Miazzo *et al*., 2000). AFB_1 at a concentration of 1 mg/kg feed had no effect on body weight. Raju & Devegowda (2000) found that 0.3 mg AFB_1/kg feed decreased the body weight gain in broilers significantly. This is, however, one of a few studies where a concentration below 2.5 mg AFB_1/kg feed was used and

caused a negative effect on body weight. Oxihumate was significantly effective in diminishing the adverse effects caused by AFB_1 on body weight. In the present study, no trend towards any improvement in weight gain was observed when the chicks consumed diets containing aflatoxin plus Mycosorb^{TM}, contrary to the report of Stanley *et al*. (1993).

The stunted growth associated with aflatoxin intoxication may be a secondary effect as the liver is considered to be the main target organ for aflatoxicosis (Coulombe, 1994). The toxic metabolites of aflatoxin bind to nucleic acids and nucleoproteins, essential to cellular viability, and result in an excessive build-up of hepatic lipids, with enlargement of the liver, proliferation of bile duct epithelium (Adav & Godinwar, 1997), necrosis (Kichou & Walser, 1994) and hepatocellular carcinoma (Hamilton, 1978; Van Rensburg *et al*. 1985; Peers *et al*., 1987; Bammler *et al*., 2000). In poultry, the relative weight of the liver is increased by aflatoxin ingestion more than that of any other organ (Huff *et al*., 1986; Kubena *et al*, 1990a). The present data indicate that the relative weights for the livers were significantly increased for the chicks consuming diets contaminated with 2 and 3 mg AFB_1/kg. The livers of these chicks appeared also to be friable and pale yellow as a result of fat accumulation in the cytoplasm of the hepatocytes. In some instances the livers were up to twice the weight of those of the controls. Oxihumate showed some significant protective effects with respect to liver damage, as indicated by liver enlargement and colour. Microscopically, the lesions caused by aflatoxin treatment were severe, but oxihumate reduced the formation of lesions significantly.

The feed contaminated with 1 mg AFB_1/kg had no effect on the relative weight of the liver and heart but it did cause a significant increase in stomach weight, indicating a possible higher sensitivity of the stomach to aflatoxin contamination than the other organs. Mycotoxins have been known to irritate the proventriculus and gizzard of the gastrointestinal tract, thus causing an increase in the relative weight of these organs (Huff & Doerr, 1981). The addition of oxihumate inhibited this effect at the contamination level of 1 mg AFB_1/kg and reduced the enlargement of the stomach at 2 and 3 mg AFB_1/kg feed. Oxihumate prevented the enlargement of the heart at 2 mg AFB_1/kg feed.

Verma & Raval (1991) revealed a concentration dependent increase in the rate of haemolysis, indicating AFB_1–induced cytotoxicity, which could be due to lipid peroxidation of plasma membrane, permeability alterations and cell lysis. Haematocrit levels were reduced during this

study in the chickens receiving rations contaminated with 2 and 3 mg AFB_1/kg. Oxihumate supplementation to these diets improved the haematocrit significantly, probably as a result of effective adsorption in the gut to reduce the amount of aflatoxin absorption by the body. The higher haematocrit levels noted after the addition of both oxihumate and high antioxidant levels in diets contaminated with 3 mg AFB_1/kg could be a result of a combination of lower aflatoxin exposure and antioxidant protection of erythrocyte plasma membranes against lipid peroxidation.

Various authors found a rise in serum activity of creatine kinase in chickens after aflatoxin ingestion, possibly because of tissue damage and a resulting leakage of enzymes into the blood (Bailey *et al*., 1998; Kubena *et al*., 1998). Tissue damage may be caused by plasma membrane lipid peroxidation and disturbances of intracellular calcium homeostasis, compromising mitochondrial function, cytoskeletal organization and ultimately activation of degradative processes and thus cellular injury. No effect on serum creatine kinase activity could be detected during this study, which supports the findings of studies such as Kubena *et al*. (1990a), where a concentration of 3.5 mg aflatoxin/kg feed failed to increase serum creatine kinase levels.

The observed reduction in serum concentration of total protein and albumin in all groups fed aflatoxin indicates impaired protein synthesis (Tung *et al*., 1975) resulting from the hepatotoxicity seen in aflatoxicosis (Bailey *et al*., 1998). Albumin and total protein levels in the serum proved to be sensitive indicators of aflatoxicosis in broilers, as significant decreases were already observed at 1 mg AFB_1/kg feed, a level which was too low for body weight gain to be affected. The decrease in serum albumin levels in broilers that consumed AFB_1 contaminated diets with added oxihumate was less profound when compared to the group that received aflatoxin alone. With continued exposure intra-hepatic biliary epithelial hyperplasia occurred as an attempt to regenerate the hepatic parenchyma when the parenchymal cells themselves have lost their capacity. Such hepatobiliary hyperplasia resulted in a significant increase of alanine aminotransferase, γ-glutamyl transferase and total bilirubin (Zaky *et al*., 1998). Serum γ-glutamyl transferase enzyme activity is a sensitive indicator of liver disease, whether the disorder involves liver inflammation, lesions or obstruction to the biliary tract (Kubena *et al*., 1990a, b). In the present study serum γ-glutamyl transferase activity was significantly decreased by the aflatoxin ingestion. This observation is contrary to the results of several studies where an increase in γ-glutamyl transferase levels in the serum was reported (Kubena *et al*, 1990a, b). Aflatoxin contaminated diets in this study did not affect the serum aspartate aminotransferase activity significantly, in contrast to the findings of Huff *et al*. (1992)

where aflatoxin caused decreased activity of aspartate aminotransferase. Abo-Norag *et al*. (1995) did not find aflatoxin to have an effect on serum activity of γ-glutamyl transferase and γ-glutamyl transferase.

Several reports showed that the immune system is a highly sensitive indicator of aflatoxicosis in poultry (Giambrone *et al*., 1985b). Aflatoxin-induced immunosuppression may be manifested as depressed cellular and humoral immune reactions (Çelik *et al*., 2000b). Aflatoxin causes excessive degradation of antibodies by accelerating lysosomal enzyme activities in chicken liver and skeletal muscles (Tung *et al*., 1970). Boulton *et al*. (1982) found that layer-breeders fed a diet containing 0.5 mg aflatoxin/kg showed a significant decrease in haemaglutination-inhibition antibody titres over a 12-week period, after a single Newcastle disease vaccination. However, Giambrone *et al*. (1985b) could not find an effect of aflatoxin on antibody synthesis or on thymic or bursal development. The results in this study demonstrate only a slight, but not significant, effect of aflatoxin on anti-NDV antibody titres. The addition of high levels of antioxidants to the aflatoxin contaminated diets produced very high anti-NDV antibody titres. This is an interesting observation, as the same stimulatory effect was not detected with the high antioxidant diets without aflatoxin.

The results from this study indicate that aflatoxin significantly reduced body weight and affected overall broiler health and performance. Oxihumate was effective in diminishing the growth inhibitory effects of aflatoxin and there was apparent protection noted for some of the organ, haematological and serum biochemical changes associated with aflatoxin toxicity. Oxihumate proved to be much more effective in the amelioration of aflatoxicosis in broilers than the commercially available mycotoxin binder, Mycosorb^{TM}. This is contradictory to the reports of studies where turkeys (Savage *et al*., 1995; Smith *et al*., 2000) and broilers (Raju & Devegowda, 2000) were fed mycotoxin-contaminated diets and Mycosorb^{TM} had promoted growth and feed intake of affected birds. The protective effect of oxihumate appears to involve sequestration of aflatoxin in the gastrointestinal tract and a reduction in bioavailability of aflatoxin. These results suggest that oxihumate can alleviate some of the toxic effects of aflatoxin in growing broilers, and when used with other sound mycotoxin management practices, may prove beneficial in the preventative management of aflatoxin-contaminated feedstuffs for poultry.

CHAPTER 5

In vitro effect of oxihumate on mitogen-induced proliferation of aflatoxin-treated lymphocytes

Abstract

At low levels of exposure, several mycotoxins typically act as immunosuppressive agents and can increase disease susceptibility. The immune system is a highly sensitive indicator of aflatoxicosis in poultry. The lymphocyte proliferation assay is used in immunological research particularly to evaluate the functional capabilities of T lymphocytes. Proliferation of lymphoid cells can be stimulated *in vitro* to synthesize DNA and undergo blastogenic transformation upon exposure to mitogens.

Humic acids are substances widely present in natural materials as a product of chemical and biological transformations of animal and plant residues. The humic acids in peat, which have been well known for its therapeutic properties already in ancient times, caused stimulation of lymphatic system cells, thymus activity, neutrophil function and phagocytic activity of granulocytes. A South African company developed an effective large-scale regeneration process for very pure and high quality humic acids, called oxihumic acids, from coal. The main objective of this study was to elucidate the effect of aflatoxin and oxihumate, alone or in combination, on stimulated broiler lymphocyte proliferation using the MTT colorimetric assay. No protective effect of oxihumate against the inhibitory effect of AFB_1 on chicken lymphocyte proliferation was found.

Introduction

Mycotoxins are a structurally diverse group of secondary metabolites produced by different genera of fungi. These toxins are implicated in several animal and human toxicoses. Several mycotoxins have been shown to suppress immune responses and cause immunomodulation in domestic animals. The immune system is an important defensive mechanism against invading organisms and concomitant infectious diseases. At low levels of exposure, these mycotoxins typically act as immunosuppressive agents and can increase disease susceptibility. However, depending on the dose, timing and route of exposure, mycotoxins can also be immunostimulatory. Mycotoxins can affect both cell-mediated and humoral immune compartments (Meky *et al.*, 2001).

Reports showed that the immune system is a highly sensitive indicator of aflatoxicosis in poultry (Giambrone *et al*., 1985b) affecting both cellular and humoral immune reactions (Giambrone *et al*., 1978). AFB_1 inhibited lymphoblastogenesis in tests that relied on mitogen-stimulated uptake of [^{3}H]thymidine into various lymphocyte populations (Coulombe, 1994). A significant depression was found in T cell-dependent functions of splenic lymphocytes isolated from CD-1 mice, treated *in vivo* with up to 0.7 mg AFB_1/kg feed for 2 weeks (Reddy & Sharma, 1987). These workers also found a similar T cell-specific toxicity in a later study (Reddy & Sharma, 1989). In addition, they discovered that the natural killer cell function of the peripheral blood lymphocytes was affected by AFB_1 treatment. Total lymphocyte and T lymphocyte counts were significantly lower in chicks fed 0.3 and 1 mg AFB_1/kg feed (Ghosh *et al*., 1990; Ghosh *et al*., 1991) and 2.5 mg total aflatoxin/kg feed (Çelik *et al*., 2000b).

The lymphocyte proliferation assay is used in immunological research particularly to evaluate the functional capabilities of T lymphocytes (Gogal *et al*., 1997). Proliferation of lymphoid cells can be stimulated *in vitro* to synthesize DNA and the cells undergo blastogenic transformation upon exposure to mitogens or antibodies against cell surface receptors. Lymphocyte transformation has been used to evaluate chicken lymphocyte function in normal birds, in genetically characterized birds and in birds after virus infections and after stimulation with bacterial antigens (Barta *et al*., 1992). A number of assays has been used to evaluate lymphocyte proliferation. One method involves the capacity of cells to incorporate a radioactive substrate, tritiated thymidine deoxyriboside (3H-Tdr) (Knudsen *et al*., 1974). Another method, now widely accepted as a safe and reliable alternative to radiometric testing, is a rapid colorimetric assay based on the capacity of the mitochondrial enzyme succinate-dehydrogenase to reduce the yellow tetrazolium salt of MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) to purple MTT formazan (Mosmann, 1983). In birds, the MTT assay has been applied successfully in studies of cytotoxicity using tumor cell lines (Qureshi *et al*., 1990), in measuring bactericidal activity of macrophages (Harmon *et al*., 1992) and evaluation of lymphocyte proliferation of chicken splenocytes (Bounous *et al*., 1992).

Humic substances are ubiquitous, and are found wherever matter is being decomposed, or has been transposed as in the case of sediments (Hayes *et al*., 1989). The humic acids in peat, which have been well known for its therapeutic properties already in ancient times, showed several characteristics such as anti-inflammatory, antiviral, oestrogenic and profibrinolytic activities

(Klöcking, 1994). Obminska-Domoradzka *et al*. (1993a and b) found that the administration of Tolpa Peat Preparation TPP, a mixture rich in humic substances, to immunologically mature mice causes functional stimulation of the lymphatic system cells. Jankowski *et al.* (1993) showed that the phagocytic activity of granulocytes was significantly stimulated after 6 months of treatment with TPP in 39 patients with recurrent respiratory tract infections. Riede *et al*. (1991) tested three different specimens of low-molecular humic substances (two naturally occurring humates and one synthetically prepared humate) on neutrophil function. They were all capable of stimulating certain functions of human neutrophils, such as the respiratory reaction with hydrogen peroxide as the main product. It was suggested that the humic substances acted as signals to change dormant human neutrophils into activated cells. Jooné *et al*. (2003) found that oxihumate increased the *in vitro* proliferative response of phytohaemagglutinin-stimulated human lymphocytes, from a concentration of 20 µg oxihumate/ml and upwards. This response was even more significant in the lymphocytes from HIV-infected patients. Similar effects were observed *ex vivo* following administration of a dosage of 4 g oxihumate per day to HIV-positive individuals for two weeks. Stimulation of the proliferative response of lymphocytes by oxihumate is associated with an increased production of IL-2, as well as expression of the IL-2 receptor in the setting of decreased production of IL-10.

A South African company, Enerkom (Pty) Ltd, developed an effective large-scale regeneration process for humic acids from coal. This technology can economically regenerate large quantities of pure, high quality humic acids by reversing the process whereby coal was formed. Humic acids produced in this way are called oxihumic acids. Chemically oxihumic acids differ only marginally from humic acids obtained from other sources (Cloete *et al*., 1990; Dekker *et al*., 1990; Cronjé *et al*., 1991; Bergh *et al*., 1997).

The main objective of this study was to elucidate the effect of aflatoxin and oxihumate, alone or in combination, on stimulated broiler lymphocyte proliferation using the MTT colorimetric assay.

Materials and Methods

All procedures were carried out under sterile conditions. A series of preliminary lymphocyte assays were performed using 3 different mitogens (concanavalin A, pokeweed mitogen and

phytohaemagglutinin) at different concentrations and also varying concentrations of lymphocytes, oxihumate and AFB_1 to determine optimum conditions for this study (data not shown).

Lymphocyte isolation

Four healthy 6 week-old Ross broilers were bled from the jugular vein using heparinized tubes. Lymphocytes were isolated according to a technique adapted from the method described by Li (1998). Ficoll-sodium diatrizoate separation medium (Sigma-Aldrich, St.Louis, Missouri, USA) was prewarmed to room temperature. The blood was diluted with an equal volume of RPMI-1640 complete medium (RPMI-1640 medium + 2 mM L-glutamine + 100 U penicillin/mL + 100 mg streptomycin/mL; Sterilab). 10 mL of Ficoll-sodium diatrizoate separation medium was placed into 50 mL conical screw-cap tubes and 20 mL of diluted blood was slowly layered onto the top of the separation medium. The tubes were spun at 1800 rpm with no brake on for 25 minutes at room temperature. The lymphocyte layers were then harvested from each tube and washed three times with RPMI-1640 complete medium at 1000 rpm with brake on for 10 minutes. After discarding the supernatant, the tubes were filled with sterile, cold 0.8% ammoniumchloride solution to induce lyses of contaminating red cells. After 10 minutes the tubes were centrifuged for 10 minutes at 1000 rpm, supernatant discarded and washed 2 times with RPMI-1640 complete medium at 1000 rpm with brake on for 10 minutes. The cell pellets were suspended with RPMI-1640 complete medium. 10µl of cell suspension was mixed with 90µl of crystal violet 0.1% solution and cells were counted using a hemocytometer. Cell suspensions were adjusted to a final concentration of 4 x 10^6 cells/mL in RPMI-1640 complete medium.

Lymphocyte proliferation assay

The proliferation test was based on a method described by Mossmann (1983) and conducted in 96-well round-bottomed microtitre plates. Eight plates (2 plates for each of the 4 broilers) were each divided into 7 parts for the 7 different treatments shown in Table 5.1. Each well contained a total volume of 200 µl. The mitogen used for the stimulation of lymphocyte proliferation was concanavalin A (ConA) (Sigma-Aldrich). All media that were used in the wells and for the dilution of mitogen, oxihumate and aflatoxin B_1 (Sigma-Aldrich) contained 10% chicken serum (obtained from the same chicken as the specific lymphocytes) to give a final concentration of 5% of chicken serum in each well.

Table 5.1. Treatments used in an MTT colorimetric assay to determine the effect of aflatoxin and oxihumate, alone or in combination, on stimulated broiler lymphocyte proliferation

Group	Treatment
Control (Resting)	100 µL of cell suspension (2 x 10^6 cells/mL) 100 µL RPMI-1640 complete medium
Control (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 80 µL RPMI-1640 complete medium 20 µL ConA (20 µg/mL)
Oxihumate (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 60 µL RPMI-1640 complete medium 20 µL ConA (20µg/mL) 20µL oxihumate (50 µg/mL)
16 µg AFB_1/mL (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 60 µL RPMI-1640 complete medium 20 µL ConA (20 µg/mL) 20 µL aflatoxin B_1 (16 µg/mL)
8 µg AFB_1/mL (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 60µL RPMI-1640 complete medium 20µL ConA (20µg/mL) 20µL aflatoxin B_1 (8 µg/mL)
16µg AFB_1/mL + Oxihumate (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 40 µL RPMI-1640 complete medium 20 µL ConA (20µg/mL) 20µl aflatoxin B_1 (16µg/ml) 20µl oxihumate (50µg/ml)
8 µg AFB_1/mL + Oxihumate (Stimulated)	100 µL of cell suspension (2 x 10^6 cells/mL) 40 µL RPMI-1640 complete medium 20 µL ConA (20µg/mL) 20 µL aflatoxin B_1 (8µg/mL) 20 µL oxihumate (50µg/mL)

The lymphocytes were incubated in a humidified incubator with 5% CO_2 at 40°C for 72 hours. 20 µl of an MTT solution (5 mg MTT stain (Sigma-Alldrich)/mL phosphate buffered saline) was then added to each well. The cells were re-incubated for 4 hours and centrifuged at 2000 rpm for 10 minutes. The supernatant was removed from each well without disturbing the pellet and each pellet was washed with 150 µl phosphate buffered saline. Hundred µl DMSO was added to the wells and gently shaken for 1 hour. The colour intensity was measured spectrophotometrically with a Ceres 900 Bio-Tek EIA Microtitre Plate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) with a 540 nm filter. The absorbance values of each sample from the same treatment were averaged. A stimulation index (SI), calculated as the mean absorbance value of cells treated with ConA divided by the mean absorbance value of cells not treated with ConA, was determined for the different treatments.

Statistical analysis

An analysis of variance with the ANOVA model (Statistical Analysis Systems, 1994) was used to determine the significance between different treatment effects for the balanced data. Means and standard deviations were calculated. Significance of difference (5%) between means was determined by multiple comparisons using Tukey t-test (Samuels, 1989).

Results

The effect of AFB_1 and oxihumate, alone and in combination, on the proliferation of broiler lymphocytes is presented in Table 5.2. The mitogen, ConA, had a significant stimulating effect on the growth of chicken lymphocytes. When oxihumate was added to the stimulated cells, no significant effect was noted. Both 8 µg AFB_1/mL and 16 µg AFB_1/mL, alone and in combination with oxihumate, inhibited the proliferation of lymphocytes.

Table 5.2. The effect of aflatoxin and oxihumate, alone and in combination, on stimulated broiler lymphocyte proliferation

Treatment	Mean[1]	Standard Deviation
Control (Resting)	0.574[b]	0.079
Control (Stimulated)	1.169[a]	0.238
Oxihumate (Stimulated)	1.016[a]	0.155
16 µg AFB_1/mL (Stimulated)	0.248[c]	0.109
8 µg AFB_1/mL (Stimulated)	0.313[c]	0.142
16 µg AFB_1/mL + Oxihumate (Stimulated)	0.274[c]	0.110
8 µg AFB_1/mL + Oxihumate (Stimulated)	0.309[bc]	0.123
R^2	0.909	

[1] Results are given as absorbance values for unstimulated cell cultures and as a stimulation index (SI) for stimulated cell cultures

[a-c] Values within the column with no common superscript are significantly different ($P<0.05$)

Discussion

This study is the first *in vitro* study on the effects of humic acid on aflatoxin-treated chicken lymphocytes. It was revealed that *in vitro* exposure of broiler lymphocytes to AFB_1 inhibited their proliferation after ConA-stimulation severely, using the MTT assay. The immunosuppressive properties of AFB_1, particularly on cell-mediated immunity, have been demonstrated in various animal models (Sharma, 1993). AFB_1 inhibited lymphoblastogenesis in tests that relied on mitogen-stimulated uptake of [^{3}H]thymidine into various lymphocyte populations (Coulombe, 1994). However, in a study of Meky *et al*. (2001), AFB_1 had no effect on human cell proliferation at concentrations up to 10 µg/mL. According to Meky *et al*. (2001) the lack of effect in their study may possibly be attributed to an absence of bioactivation of AFB_1. Neal *et al*. (1998) found that AFB_1 was only cytotoxic in cells expressing transfected cytochrome P450's and according to Raucy & Ingelman-Sundberg (1999) the primary P450's involved in the activation of AFB_1, do not appear to be expressed in human lymphocytes. However, a counter argument for the need for metabolic activation of AFB_1 for immunosuppressive effects comes from observations in rat macrophages and human monocytes where effects were achieved without metabolic activation (Cusumano *et al*., 1990; Rossano *et al*., 1999). Also, an early study on human lymphocytes by Savel *et al*. (1970) reported reduced proliferation with 5 µg/mL AFB_1.

This study revealed no protective effect of oxihumate against the inhibitory effect of AFB_1 on chicken lymphocyte proliferation. This is contradictory to reports of Obminska-Domoradzka *et al*. (1993a, b) who found that the administration of Tolpa Peat Preparation TPP, a mixture rich in humic substances, to immunologically mature mice caused functional stimulation of the lymphatic system cells. Jooné *et al*. (2003) found that 20 µg oxihumate/ml increased the *in vitro* proliferative response of phytohaemagglutinin-stimulated human lymphocytes of both healthy and HIV-infected individuals. Similar effects were observed *ex vivo* following administration of a dosage of 4 g oxihumate per day to HIV-positive individuals for two weeks. Stimulation of the proliferative response of lymphocytes by oxihumate was associated with an increased production of IL-2, as well as expression of the IL-2 receptor in the setting of decreased production of IL-10.

Jankowski *et al.* (1993) showed that the phagocytic activity of granulocytes was significantly stimulated after 6 months of treatment with TPP in 39 patients with recurrent respiratory tract infections. Riede *et al*. (1991) tested three different specimens of low-molecular humic substances (two naturally occurring humates and one synthetically prepared humate) on neutrophil function. They were all capable of stimulating certain functions of human neutrophils, such as the respiratory reaction with hydrogen peroxide as the main product. It was suggested that the humic substances acted as signals to change dormant human neutrophils into activated cells.

The contradictory results from this study and previous reports on the stimulatory effect of humic acids on lymphocyte proliferation needs to be further investigated.

CHAPTER 6

Critical review and recommendations

Consumers have a right to expect that their food is not only adequate, but also safe and of good quality. In this regard, both the food industry and government have a responsibility to honour that right. To do so, food quality needs to be protected from the farm to the consumer. Consumers, the food industry, government ministries and international agencies all have important, interrelated roles in ensuring food quality and safety.

Control of food safety requires that there should be in place laws, regulations and standards related to food quality and safety, plus a system for food inspection and monitoring to ensure compliance. Industrialized countries have well-established systems to guarantee a reasonable level of safety and quality of foods consumed. Most developing countries, however, have rudimentary inspection and monitoring systems that need to be strengthened to have any significant effect. Poor countries often do not have the institutions or the personnel to ensure food safety and control, although most do have some legislation, standards and regulations on the books. To complicate the matter further, farmers, food processors and the public are not always conversant with the regulations. As a result, unsafe contaminated foods reach the consumer, putting the public at risk of illness. Steps to improve the quality and safety of food are important if people are to have good health and nutrition. Such steps will also benefit food trade because contaminated or unsafe food should not be traded either in internal markets or for export.

Larger, emerging developing countries, such as South Africa, should devote much more effort than at present to improve food safety. These developing countries are becoming highly urbanized and commercialized with increasing demands for larger amounts of foods, straining the food industry, which often results in the appearance of unsafe commodities on the local market.

A contaminant regularly found in foods and feeds, threatening the consumer's right to safe and healthy nutrition, is the fungal mycotoxins. These are produced by fungi growing on crops either in the field, at harvest, in storage or during the processing of feed. Mycotoxins are found globally and their negative impact on animal productivity and human health is widespread. Guidelines for acceptable levels of mycotoxins need to be conservatively low due to non-uniform distribution

within feed or food, uncertainties in sampling and analysis, the potential for multi-source contamination of food and the limited research information available. Any level of mycotoxin contamination carries a risk of production losses and a negative impact on health and it is impossible to define a safe level under laboratory conditions that will be accurate under field conditions.

A large percentage of grain has unacceptable high levels of mycotoxin contamination, and as a result the use of intervention mechanisms to maintain food safety is of increasing importance. Although the primary sources of mycotoxins in human diets are grains and vegetable-based foods, there is some transfer of mycotoxins from animal feed to animal products, and mycotoxin control in feeds are therefore not only critical for production profitability, but also for human safety. However, with the tightening of regulatory limits and more stringent control, the economic losses resulting from the loss of mycotoxin-contaminated agricultural commodities globally is phenomenal.

An effective method to neutralise mycotoxins in animal feeds will not only be a huge economic advantage for the agriculturalist, but will also increase the safety of food consumed by humans. Practical methods to detoxify mycotoxin-contaminated grain on a large scale and in a cost-effective manner are not currently available. The most recent and promising approach is the use of non-nutritive adsorptive materials, which bind the mycotoxin molecule, reducing their absorption from the gastrointestinal tract. Some of the 'ideal' features of such a mycotoxin adsorbent include the ability to absorb a wide range of mycotoxins, a low effective inclusion rate in the feed, no affinity for any nutrient and high stability over a wide pH range. The search for such an adsorbent is still continuing and should be widely supported by all role-players involved in food safety control. An effective adsorbent will be especially beneficial in developing countries with poor food regulation systems to be included routinely in animal feeds, minimizing the risk of mycotoxicosis.

Humic acids are known to adsorb heavy metals, herbicides, mutagens, monoaromatic compounds, polycyclic aromatic compounds, some minerals and bacterial DNA. It was, however, never before evaluated as a mycotoxin adsorbent. As the humic acid, oxihumate, was produced on large scale as a fertilizer and the manufacturers were prepared to invest into the development of new applications thereof, I seized the opportunity. Small opportunities are often the beginning of great enterprises and the challenge of developing a mycotoxin binder consequently led to the launch of this project.

Oxihumate showed a high affinity for AFB_1, zearalenone, ochratoxin A, ergosine, ergotamine, ergocornine, ergocryptine and ergocristine. This is an important finding as some commercially available binders decrease the toxicity of aflatoxin, but not other mycotoxins. During the management of a mycotoxin problem, the lack of a specific binder to reduce the toxicity of other mycotoxins and mycotoxin combinations could become very important. A truly effective binder should have the ability to adsorb a large number of chemically distinct mycotoxins, such as oxihumate proved to have under *in vitro* conditions.

Oxihumate showed adsorptions of approximately 10.3, 7.4 and 11.9 mg AFB_1/g and 1.2, 2.6 and 8.5 mg AFG_2/g oxihumate at pH 3, 5 and 7, respectively. Oxihumate is thus more effective at adsorbing AFB_1 than AFG_2. It will be interesting to obtain the Langmuir and Freundlich oxihumate adsorption isotherm parameters for the other mycotoxins. In this study, Mycosorb^{TM} demonstrated poor adsorption of AFB_1, with the highest adsorption shown as 204 µg/g Mycosorb^{TM} at pH 7. As various authors reported a high binding affinity of Mycosorb^{TM} for mycotoxins *in vitro*, differences in experimental conditions must exist, exerting an influence on the outcome of results.

Oxihumate did not bind feed molecules and, thus, all oxihumate particles mixed into mycotoxin-contaminated feed, should be available for the formation of an oxihumate-mycotoxin complex. The complex may, however, not be very stable as it was ruptured to a high degree in the presence of acetonitrile and acetone.

According to the *in vitro* results of this study, addition of oxihumate to the initial composition of feedstuffs should ameliorate aflatoxicosis efficiently by inducing the formation of an oxihumate-aflatoxin complex that prevents aflatoxin absorption from the gastrointestinal tract. *In vitro* binding tests may, however, not always be a reliable indicator of ability to bind a mycotoxin. Therefore, it is important that adsorbents be subjected to *in vivo* evaluation both with respect to efficacy and to determine if impaired nutrient utilisation occurs.

The results from the *in vivo* experiments indicate that aflatoxin significantly reduced body weight and affected overall broiler health and performance. Oxihumate was effective in diminishing the growth inhibitory effects of aflatoxin and there was apparent protection noted for some of the organ, haematological and serum biochemical changes associated with aflatoxin toxicity. Oxihumate

proved to be much more effective in the amelioration of aflatoxicosis in broilers than the commercially available mycotoxin binder, Mycosorb™. This, as in the *in vitro* experiments, questions the differences in experimental conditions of this study and other studies where Mycosorb™ did show effective binding of aflatoxin.

The failure of oxihumate to alter the *in vitro* effects of AFB_1 exposure on lymphocyte proliferation supports the idea that the protective effect of oxihumate appears to involve the sequestration of aflatoxin in the gastrointestinal tract and a reduction in bioavailability of aflatoxin rather than a therapeutic effect at cellular level.

The results of this study suggest that oxihumate could alleviate some of the toxic effects of aflatoxin in growing broilers, and when used with other sound mycotoxin management practices, might prove beneficial in the preventative management of aflatoxin-contaminated feedstuffs for poultry. In my personal opinion, the improvement in production observed during this specific study might, however, not be substantial enough to recommend oxihumate as a commercially feasible product and I would recommend that the economical viability must first be evaluated.

In view of the promising results of the *in vitro* binding study, future studies should address the ability of oxihumate to prevent the adverse effects of other mycotoxins than aflatoxin, in poultry but also in other farm animals. Several materials show a relative high binding ability for aflatoxin, but very little for other mycotoxins. There must furthermore be looked at certain characteristics of oxihumate such as particle size that might influence the ability to adsorb mycotoxins. Alternative uses for oxihumate could also be investigated, such as the removal of high concentrations of heavy metals from water and therapeutical application in human and veterinary medicine.