

**Efficacy of a Yeast Cell Wall-Derived Mycotoxin Adsorbent  
on Excretion of Aflatoxin B<sub>2</sub> in Weanling Rats**

by

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A Thesis

presented to

The University of Guelph

In partial fulfillment of requirements

for the degree of

Master of Science

in

Animal and Poultry Science (Toxicology)

Guelph, Ontario, Canada

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## ABSTRACT

### **EFFICACY OF A YEAST CELL WALL-DERIVED MYCOTOXIN ADSORBENT ON EXCRETION OF AFLATOXIN B<sub>2</sub> IN WEANLING RATS**

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Mycotoxins are natural contaminants of livestock feeds that can impair production efficiency. Specialized feed additives known as mycotoxin adsorbents may reduce absorption of mycotoxins in animals. Many additives are thought to use nonspecific binding to sequester mycotoxins in the lumen of the gastrointestinal tract, but there is a lack of *in vivo* mechanistic evidence. The current research sought to quantify the adsorptive capabilities of a yeast cell wall-derived mycotoxin adsorbent and confirm whether its mode of action is by adsorption of the mycotoxin. Using radioactive aflatoxin B<sub>2</sub>, it was found that there was a significant reduction in urine radioactivity when the adsorbent was present and observed over time. No other significant effects of diet were noticed in the liver, kidneys or muscle. Without feces and tissue data in agreement with urine, it was inconclusive whether this adsorbent reduced absorption of aflatoxin in rats.

## Acknowledgements

The author would like to thank OMAFRA and Alltech for funding this project as well as the Animal and Poultry Science department (University of Guelph), Dr. Smith and Maureen Crump for providing invaluable assistance and accommodations. The author would like to acknowledge the University of Guelph's Animal Care Committee for monitoring the rats used and ensuring that the animals used were treated humanely and with proper care.

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## 1. Literature Review

### 1.1 Mycotoxins

Mycotoxins are secondary metabolites produced by some species of fungi and pose a health risk to animals. Mycotoxins are produced in response to stressful environmental conditions such as drought. Fungi contaminate crops preharvest or during transport, storage and feed manufacturing (Binder, 2007). Mycotoxins have been categorized according to chemical structure and to species of originating fungi. Since hundreds of mycotoxins have been chemically identified, this review will focus on mycotoxins with documented effects on the health of livestock and humans.

#### 1.1.1 Mycotoxin production

Production of mycotoxins is dependent on environmental conditions and agricultural practices. Levels of moisture between 13% to 18% and temperatures between 20°C and 30°C can increase fungal growth rate and are common during growing seasons as well as during transport and storage (Muschen and Frank, 1994). Growth of fungi is enhanced in crops damaged by insects or through the harvesting process. Physical damage compromises barriers used to prevent entry of fungi and bacteria. Some crops are dried after harvest and this can cause cracking of the outer mechanical barriers (Santin, 2005).

The nature of mycotoxin contamination varies with geographical region. Monoculture (crops with limited genetic variability) and adverse environmental conditions combine to increase the likelihood of mycotoxin production. Balkan Endemic Nephropathy is a well-studied example of ochratoxin A contamination occurring frequently in Lithuania, Croatia and Bulgaria and leading to kidney damage in humans and animals (Creppy, 2002). The World Health

Organization (WHO) published a bulletin on mycotoxin contamination of crops and the incidence of disease in different geographical areas (Peraica et al., 1999). Human cases of mycotoxicosis were correlated with food contamination as well as the period and levels of exposure. Cases included the number of mortalities from toxin exposure and the region where cases were observed. India had the highest number of clinical cases and deaths attributed to aflatoxin-contaminated maize with two outbreaks causing at least 1391 cases and 203 deaths. A summary of cases of children with Reye syndrome found that in the Czech Republic, New Zealand and the United States, 40%-100% of the patients had liver samples with detectable levels of aflatoxin B<sub>1</sub>. The authors did note, however, that aflatoxin may not have been the sole causative factor for this condition. It can be concluded that mycotoxin exposure is not limited to developing countries as ochratoxin A has been detected in trace amounts in human blood samples in Canada, France and other European countries (Speijers et al., 1993). The frequency of disease and mortality arising from food-borne mycotoxins is much higher in developing countries and may be a result of poor farming and storage practices, inadequate regulation and monitoring of foodstuffs and insufficient medical care. It is important to note that global trade results in contaminated grains to move between regions thereby increasing the possibility of exposure regardless of location. This makes mycotoxin control a global problem.

#### 1.1.2 Mycotoxins commonly known to contaminate feed

Several major mycotoxins are summarized below. Of note are the feedstuffs which are typically contaminated, the mechanism of toxicity, bioavailability and kinetics of the mycotoxin as well as an overview of susceptible animals. An emphasis will be placed on aflatoxin which was studied in the experiments described later in this thesis.

#### 1.1.2.1 Ochratoxin A

Ochratoxin A (OTA) is structurally characterized by a chlorine group on carbon 5. This sets it apart from ochratoxin B which lacks a chlorine group but is otherwise identical. An ester group exists on Ochratoxin C in place of the carboxylic acid on OTA. Ochratoxin C has a chlorine group on carbon 5 similar to that found in OTA. The chlorine group is considered to be a contributing factor in ochratoxin toxicity as some cytochrome P450 enzymes will replace it with a hydroxyl group creating OTQ which undergoes redox cycling between a quinone and paraquinone, creating free radicals in the process (Josephy and Mannervik, 2006). Renal concentrating of OTA makes kidneys more susceptible to free radical damage and nephropathy. OTA is recognized as a nephrotoxin and group 2B carcinogen (possible carcinogen in humans) according to IARC (Santin, 2005). OTA is a common contaminant of commodities such as coffee, cocoa and grapes (Amézqueta et al., 2009), OTA can cause pathologies in humans and animals. Animals sensitive to OTA include rats, pigs and chickens which suffer reduced growth, nephropathy and reduction in hematocrit (ratio of red blood cells to total cell volume). Nephropathy can reduce growth and general health leading to poorer feed efficiency and increased costs of production. Exposure to ochratoxins can also reduce sperm quality which can reduce the success of breeding programs (Smith et al., 2005).

#### 1.1.2.2 Ergot

Ergot alkaloids are a group of mycotoxins which have lysergic acid as a common structure. It is thought that the historical condition St. Anthony's fire was caused by cereals and flour contaminated with ergot alkaloids (Binder, 2007). Stricter government regulations have reduced the risk of ergotism in humans but this has not been extended to animal feeds. Livestock

are at risk of developing ergotism with clinical signs including gangrene, ataxia and abortions. Co-contamination of feedstuffs with ergot and other mycotoxins under natural circumstances complicates exact definition of a toxicological profile (Binder, 2007). Several studies summarized by the European Food Safety Authority (2012) indicated that ergot alkaloids often used in pharmaceutical products had a No Observable Adverse Effect Level (NOAEL) range of 0.22-0.60 mg/kg bw/ day in rats.

#### 1.1.2.3 *Fusarium* mycotoxins

Various *Fusarium* spp. produce mycotoxins which are largely grouped by similarity of chemical structure or by the genus of fungi from which they are synthesized. Fusaric acid, fumonisin, zearalenone and the subgroup of trichothecenes are all part of this group. The majority of *Fusarium* mycotoxins have effects on the central nervous system (Riley and Pestka, 2005).

An early identified and well characterized member of the trichothecenes, T-2 toxin, can cause skin irritation and immunosuppression. A less toxic trichothecene, deoxynivalenol (vomitoxin, DON) can cause ulceration in the gastrointestinal system resulting in reduced feed intake (Riley and Pestka, 2005). By causing immunosuppression and reduced appetite, feed contaminated with these mycotoxins can result in reduced production efficiency, growth and profitability of livestock production systems.

Zearalenone is a *Fusarium* mycotoxin known for its structural and endocrinological similarity to estradiol (Ramos et al., 1996). Pigs are the species most sensitive to this mycotoxin which is often a contaminant of corn and other cereals. Symptoms of zearalenone toxicity include feminisation and decreased sperm count in males as well as vulvovaginitis, abortions and

infertility in females (Ramos et al., 1996). This toxin does not affect animal growth rates but does pose a risk to reproductive efficiency.

#### 1.1.2.4 Aflatoxin

Aflatoxin was identified in the mid-20<sup>th</sup> century and was named after *Aspergillus flavus*. It has four common chemical structural forms including B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> as well as several metabolic products. The B and G classifications are derived from the color of UV fluorescence in which one form is detected green and the other blue. Aflatoxin B<sub>1</sub> is significantly more toxic than aflatoxin B<sub>2</sub>. Aflatoxins are enzymically synthesised in fungi from sterigmatocystin. Altering or inhibiting key enzymes in fungi can reduce aflatoxin production or cause the less toxic aflatoxin B<sub>2</sub> to be synthesized instead of aflatoxin B<sub>1</sub> (Dutton et al., 1985). Like other mycotoxins, aflatoxin is of greatest concern when it has contaminated crops which can be ingested by livestock or humans. Aflatoxin metabolism has been well-documented in several animal models (Newberne and Butler, 1969).

While some industrial workers can inhale fungal spores and mycotoxins, aflatoxins are predominantly a dietary risk. Kumagai (1989) determined the absorptive pathway of aflatoxin by compartmentalizing the gastric lumen into segments and monitoring absorption. Once ingested, aflatoxin is absorbed across the intestinal mucosa, particularly in the duodenum, and then transported via the hepatic portal vein to the liver (Kumagai, 1989). Forrester et al. (1990) reported that the cytochrome P450s (CYP) involved in hepatic aflatoxin biotransformation include the CYP 1A, 2B, 2C, 3A and 4B families. The CYP 3A3 and 3A4 families were observed to form a dihydrodiol product which is a more stable form of the reactive epoxide. In these studies production of both a quinone metabolite as well as an epoxide were observed. The

involvement of CYP1A2 and CYP3A4 in biotransformation is strengthened by the findings that the CYP1A2/3A4 inhibitory drug oltipraz reduced aflatoxin B<sub>1</sub> metabolism (Langouet et al., 1995). After phase I metabolism, aflatoxin metabolites are conjugated by several phase II enzymes including glutathione transferase (GST) (Manson et al., 1997). The glutathione-aflatoxin conjugate has a higher solubility in water although excretion is still achieved mainly through bile in rats with as much as 60% of an acute aflatoxin dose being recovered in the first 24h and only 20% being recovered in the urine over the same period. After 24h, 10% was recovered in liver with remaining 10% was distributed throughout the carcass (Wogan et al., 1967). Other investigators have established a biological half-life for aflatoxin B<sub>1</sub> of 96h when orally administered (Coulombe and Sharma, 1985). These long excretion periods may be due in part to enterohepatic recirculation or a large initial excretion followed by a prolonged excretion of metabolites over several days. Constant exposure to contaminated feed could create a near steady state of exposure and elimination or bioaccumulation with high exposure.

Aflatoxins are predominantly liver toxins and are classified as Class I human carcinogens according to the IARC (Binder, 2007). This is in part due to the hydrophobicity ( $\log K_{ow}=1.23$ , higher octanol:water coefficient indicates hydrophobicity) of the molecule, first-pass metabolism, and the localization of cytochrome P450s which bioactivate the toxin. It is hypothesized that this is due to the double bond between carbons 8 and 9 in aflatoxin B<sub>1</sub>. As mentioned previously, synthesis of the double bond is catalyzed by CYP3A4 and some other isoforms to create a reactive epoxide which undergoes heterolytic ring opening creating free radicals (Wong and Hsieh, 1980). Free radicals lead to oxidative stress characterized by changes in protein structure, DNA damage and destruction of lipid bilayers. The challenged cell either undergoes apoptosis or necrosis eliciting an inflammatory response. The initial damage is

localized to the centrilobular hepatocytes which have the highest levels of P450s and the lowest activity of phase II enzymes (Klassenn, 2008).

The sensitivity of species to aflatoxicosis is, therefore, determined by the cytochrome P450s expressed by that species as well as the ability to effectively conjugate aflatoxins to glutathione. Mice are less sensitive than rats because they lack particular P450 isoforms. With respect to livestock, aflatoxin B<sub>1</sub> has been shown to reduce growth in pigs when feed contamination was greater than 500 µg/kg (Bonomi et al., 1992). With contamination as low as 280 µg/kg, immune suppression has been noted in swine (Smith et al., 2005) which could possibly lead to higher incidence of disease and reduced profitability (Van Heugten et al., 1994). Ruminants metabolize aflatoxin in slightly different ways with aflatoxicol being a microbial metabolite formed in the rumen and aflatoxin M<sub>1</sub> and M<sub>2</sub> being products produced in the liver. These changes in metabolism are not sufficient to completely protect ruminants from aflatoxins (Jouany and Diaz, 2005). Aflatoxin M<sub>1</sub> and M<sub>2</sub> are still toxic and are found in milk together with aflatoxin Q and aflatoxicol. Acute exposure of cattle to feed contaminated with 300µg/kg and 100µg/kg aflatoxin B<sub>1</sub> did not result in any observable adverse effects although liver weight increased (Garret et al., 1968). Studies not using naturally contaminated feeds may not simulate field conditions as combinations of mycotoxins are usually present in feedstuffs (Jouany and Diaz, 2005).

Special care is needed in controlling aflatoxin contamination in feedstuffs for the poultry industry. Ducks and other birds are very susceptible to aflatoxins. Acute exposure with as little as 340 µg/kg can be lethal to ducks (Leeson et al., 1995). In a comparative analysis, ducks metabolized twice as much aflatoxin into the epoxide form when compared to chickens (Lozano and Diaz, 2006). Chickens are still quite susceptible to aflatoxicosis and suffer from reduced

vitamin D synthesis in addition to the other effects. This leads to reduced growth, increased bruising and leg weakness with contamination as low as 14 µg/kg (Devegowda and Murthy, 2005; Tung et al., 1971).

## 1.2 Policies regarding allowable mycotoxin concentrations in feeds and foods

Mycotoxin contamination can affect growth, reproduction and animal health through various mechanisms as described above. Many countries have set contamination limits for specific mycotoxins in domestic and imported crops. Egmond et al. (2007) reported that 100 countries had regulations or guidelines for aflatoxins, trichothecenes, fumonisins, ergot alkaloids ochratoxin A and others. Mycotoxin regulation is also now entrusted to international agencies such as the FAO/WHO. Internationally harmonized regulations are intended to facilitate trade.

Limitations on contamination are based on risk assessments for mycotoxins. These assessments require multiple animal models and often are corrected for uncertainty between species, gender and environment. Regulations and limits to mycotoxin exposure are influenced by numerous factors as described by Egmond and Jonker (2004) including:

- Availability of toxicological data
- Availability of data on the occurrence of a given mycotoxin in different commodities
- Knowledge of the distribution of mycotoxin concentration in a storage facility in source feeds
- Availability of analytical methods
- National legislation
- Need for adequate food supplies

It is important to note that consumption habits play an important role in creating limitations in feedstuffs. Staple feeds are consumed in greater amounts and contamination below a limit for one species may be too high for another species only because the latter species consumes more of this potentially contaminated commodity. Different producers use different feed formulas with different storage conditions and different geographical regions produce different feed grains.

Much of the available data on mycotoxin toxicity is from studies using single mycotoxins as opposed to co-contamination with multiple mycotoxins which is typical of natural contamination. A wide margin of safety may be required because of potential co-contamination (Egmond et al., 2007).

Canada has set regulations for maximum aflatoxin contamination in feedstuffs at 20 µg/kg. Canada currently has guidelines in place only for Fusarium toxins including deoxynivalenol and HT-2 toxin which differ from the more official regulations. The guidelines limit contamination by deoxynivalenol to 1-5 mg/kg (depending on species) and HT-2 toxin to 0.1 mg/kg for non-dairy cattle and poultry. Regulations in the U.S. are specific for aflatoxins. Maximum limits for all foods are set to 20 µg/kg while regulations are less strict for some ingredients intended for animals (100-300 µg/kg for corn intended for beef cattle, swine and poultry and 200 µg/kg for swine of 45 kg bw or more). Milk for human consumption has a limit of aflatoxin at 0.5 µg/kg in the U.S. to possibly account for the consumption habits and age of children (CFIA, 2009; Egmond and Jonker, 2004). Adverse effects of feed-borne aflatoxin are not typically seen until contamination is in excess of 200µg/kg. Toxicity usually occurs above the limit set in the U.S. and Canada for crops (20-200 µg/kg). These limits are within the detection range of conventional analytical techniques.

### 1.3 Contamination monitoring

Where countries and organizations limit mycotoxin contamination in feedstuffs, monitoring is required for locally produced feedstuffs as well as for imports. Fungal growth may not always be visible without a microscope and mycotoxins may exist even when no fungi are present. Advanced detection techniques and protocols are, therefore, necessary. Common analytical techniques are described below with the advantages and disadvantages for each.

Mycotoxin analysis needs to be regularly performed on batches and shipments of crops at different stages of the production cycle as contamination is variable. Mycotoxins can be produced at any stage from production to consumption. Hazard Assessment and Critical Control Points (HACCP) plans are designed to fit particular operations to determine the points at which mycotoxin analysis should be done.

Some considerations must be taken into account when testing for mycotoxins. According to Binder (2007), sampling, sample preparation and the analytical procedure itself need to be addressed. In a given shipment of feed, highly contaminated zones can be unequally distributed within areas with little or no contamination. The feed must be tested from multiple locations to get an overall view of contamination in the shipment. Due to time constraints and labor required, large quantities of products cannot be completely tested so multiple and randomized sampling and subsampling is used. Sample preparation refers to the method by which the feedstuffs are ground, sub sampled and extracted. When analyzing for a particular mycotoxin, extraction removes other confounding materials which may interfere with detection. Extraction methods are well documented for common mycotoxins and standard operating protocols have been published. Extraction methods vary based on the chemical properties of mycotoxins, the analytical technique being used and the matrix being extracted. Extraction techniques include column

chromatography, liquid-liquid extraction, solid phase extraction columns, immuno-affinity columns and multifunctional clean-up columns (Binder, 2007). These extraction techniques attempt to isolate a specific mycotoxin based on chemical or structural characteristics. Immuno-affinity columns use fixed antibodies which prevent the elution of the desired mycotoxins (Trucksess et al., 1983). Some analyses require simpler extraction such as in the detection of deoxynivalenol in maize (Tran and Smith, 2011). Older methods such as immunoaffinity and solid-phase extraction are much more time consuming requiring more steps than the more recent column techniques and often do not provide better recovery percentage. Methods such as immunoaffinity column preparation, however, can be specific for individual mycotoxins and groups of mycotoxins depending on the antibodies used (Binder 2007).

Some procedures account for conjugated (masked) mycotoxins which will be discussed later. The analytical technique used depends on the intent of the analyst. Quick detection assays such as ELISA may not have the level of precision as more analytical procedures such as HPLC and MS but can be used to monitor a large number of samples in a short period of time.

Two immunosorbent assays are commonly used in mycotoxin analysis: enzyme linked immunosorbent assay (ELISA) and radio immunoassay (RIA). These assays work on the principle of mycotoxin-antibody interactions. In the case of ELISA, samples are placed in microplate wells where antibodies adhere to the walls and trap mycotoxins in the sample. Enzymes conjugated to the antibody or a mycotoxin are used to detect the mycotoxin in samples and the level of contamination. Direct ELISA methods have the primary polyclonal or monoclonal antibody tagged with a peroxidase which enables a colour change to indicate the presence or absence of mycotoxins. Indirect ELISA uses a primary antibody to bind the

mycotoxin and a secondary antibody with a peroxidase attached which binds the primary antibody. Indirect ELISA is more popular and can allow for more flexibility in detection.

Radio immunoassays use radiolabelled mycotoxin and an antibody specific against that antigen. A known quantity of antibody and radiolabelled mycotoxin are mixed and bind. Unlabelled mycotoxin from the sample being tested displaces the radiolabelled mycotoxin proportional to concentration. The radioactivity of the sample is inversely proportional to the concentration of mycotoxin in the sample. It is important to note that both ELISA and RIA require calibration curves to standardize the output.

New ELISA kits need to be validated against High Pressure Liquid Chromatography (HPLC) or Gas Chromatography (GC) which are more rigorous analytical methods. These methods are much slower but do not rely on antibodies which are prone to a matrix effect where non-mycotoxin molecules bind the antibody creating a false reading. The accuracy and precision of GC and HPLC methods are enough for them to be used as benchmarks for other analytical techniques and for discovering new mycotoxins. HPLC separates mycotoxins based on physical and chemical properties so that particular mycotoxins elute at particular times. GC functions in much the same way but uses gas as the carrier instead of liquid and size or polarity exclusion as the means of separating the contents of the sample. Both HPLC and GC can be used to find multiple mycotoxins or multiple forms of mycotoxins in the same sample (Martos et al., 2010). Proper set up is required to ensure that there is good resolution between peaks so individual mycotoxins can be identified. Various detectors can be used with HPLC and GC including UV-vis, fluorescence, and mass spectrometry. HPLC and GC can be coupled with mass spectrometry to identify unknown materials in a sample that could be conjugated (masked) mycotoxins or mycotoxin metabolites which may not have been detected by immunosorbent assays. Mass

spectrometry is a type of detector which analyzes chemicals by determining their mass to charge ratios and the mass to charge ratios of breakdown products. The decomposition of a chemical has a profile which can be compared to a library of known chemicals so that an unknown mycotoxin could be identified and quantified. Unknown mycotoxins and mycotoxins possessing additional functional groups can be identified by examining decomposition profiles and piecing the original chemical back together. This process usually requires an internal standard.

#### 1.4 Challenges in detecting mycotoxins

There is no ideal mycotoxin screening method as each method is limited by either time constraints, false positives/negatives or scope of detection. Binder (2007) reported that modern methods can account for up to 82% of the contamination in a product using random sampling and subsampling. There is still a possibility that contamination can be overlooked. Depending on storage procedures, it is also possible for fungal growth to occur after testing and while being stored or during transport. This error can be corrected for if proper Hazard Analysis and Critical Control Point (HACCP) procedures are in place.

Masked mycotoxins can be similar to the parent mycotoxin but bound to a functional group such as a sugar chain or, concealed in a large protein. Masked mycotoxins may have properties different enough that they cannot be detected by immunosorbent assays (epitope is concealed) or chromatography/spectroscopy (change in structure can change elution time and breakdown products). Methods exist to deconjugate masked mycotoxins. Tran and Smith (2011) used trifluoromethylsulfonic acid to remove sugar or other moieties from deoxynivalenol to improve ELISA detection of the toxin in corn and wheat. Sulyok et al. (2006) created a library for the purpose of screening feed samples for 39 mycotoxins simultaneously via liquid

chromatography with electrospray ionization and tandem mass spectrometry. This specific library is more likely to detect common mycotoxins as well as some masked mycotoxins and can be expanded in the future. Some materials can imitate or interfere with ELISA procedures leading to false positives. Typically, a sample with contamination is later screened by HPLC or GC to confirm that the result is correct. Some sampling methods may also lead to false negatives.

Isotope-labelled mycotoxins have been used extensively in research to monitor mycotoxins as they are metabolized in organisms. Isotopes have the same chemical characteristics as parent compounds but are not abundant in nature. Stable isotopes can be detected using mass spectrometry as the  $m/z$  ratio of a given molecule or ion is altered. Unstable isotopes emit radiation which can be detected using scintillation counters in a manner similar to radioimmunoassays. Both techniques have their limitations. Stable isotopes are ideally used as internal standards and using them as tracers would require a considerable amount of isolation and clean-up of biological samples. Radioactive tracers are easier to detect and do not require extraction, however, they often emit weakly and can be obscured by quenching.

### 1.5 Strategies for controlling mycotoxin contamination

Mycotoxin contamination can occur both pre- and post-harvest. Hazard Analysis and Critical Control Point (HACCP) plans commonly employ management at all of these levels to ensure a reduction in the exposure of animals and humans. HACCP is designed to address production protocols and to follow international guidelines. HACCP plans use combinations of strategies for controlling mycotoxin contamination and these are listed below.

### 1.5.1 Pre-Harvest

Fungal growth is the initial step in mycotoxin contamination. Chemical fungicides, insecticides, resistant plant varieties and proper growing methods can all reduce fungal growth. Insecticides and insect resistant plant varieties reduce the damage caused by insects to the protective mechanical barriers while fungicides and the use of resistant plant varieties either kill or inhibit the growth of fungi. Proper irrigation, fertilization, weed control, crop rotation and favourable climatic conditions can reduce stress on the crop allowing the plant to combat fungi more effectively. Favourable weather conditions can also reduce fungal growth. It is possible to genetically modify crops or use selective breeding to make them more resistant. The cost of implementation and current popular opinion, however, may make producers hesitant to use this approach. Bio-competitive but non-toxicogenic fungi may also be used to compete with mycotoxin-producing fungal species (Smith and Girish, 2012).

### 1.5.2 Post-Harvest

Mycotoxins can still be produced post-harvest. Contamination can be reduced by controlling humidity and temperature during storage and transport. Preservatives and separation of damaged kernels reduce opportunities for fungal growth. Mycotoxins synthesized pre-harvest, however, cannot be controlled by these methods (Binder, 2007). Preventing fungal spores from growing and producing mycotoxins can be achieved through chemical agents or irradiation of the feed (Smith and Girish, 2012). There are several physical methods such as sorting crops by particle size and dehulling which can also reduce mycotoxin contamination. Redistributing feedstuffs to species which are less sensitive and blending contaminated feedstuffs with safe feed to dilute the mycotoxins can reduce the hazard. Chemical treatments with acids, bases,

ammonia and ozone have been used to inactivate mycotoxins previous to feeding. Some of these treatments are still used (Kolossova et al., 2009). Fungal spores can be destroyed using irradiation chemical or thermal inactivation to reduce mycotoxin synthesis post-harvest (CAST, 2003).

### 1.5.3 Ingested Mycotoxins

Once the animal had been fed contaminated feed, exposure must be handled by preventative treatments which reduce the amount of mycotoxin absorbed across the intestinal mucosa or by therapeutic treatments that ameliorate the effects of mycotoxins post-absorption.

#### 1.5.3.1 Preventative Additives

Preventative additives must adhere to certain guidelines as outlined by Sinha (1998).

Preventative feed additives should:

- Effectively remove, destroy or inactivate a mycotoxin.
- Not be toxic, carcinogenic or mutagenic or cause other materials in the feed to be toxic, carcinogenic or mutagenic.
- Not alter nutritional value or feed acceptability (palatability).
- Be economically and technologically feasible so product cost and distribution is not significantly changed.

Mycotoxins are chemically quite stable but can still be destroyed via chemical or biochemical processes as noted by Binder (2007) and CAST (2003). Rumen microflora have been shown to break epoxide rings and otherwise deactivate mycotoxins *in vitro* and *in vivo* (Swanson et al., 1988). This is a strategy that has been incorporated into some commercial feed additives.

Mycotoxins can also be inactivated by inclusion of probiotics such as species of *eubacterium* which modify deoxynivalenol into a less toxic form (Smith and Girish, 2012). Specific enzymes such as epoxidase and lactonase were noted to decrease the effects of aflatoxin B<sub>1</sub> on broilers but failed to have an effect on deoxynivalenol and zearalenone (Smith and Girish, 2012). The options for chemically destroying fungi or mycotoxins may not be able to fulfill all of the above criteria especially a lack of toxicity.

A common approach to handling contaminated feeds is to add adsorbents which bind mycotoxins and prevent them from being absorbed at the level of the digestive tract. Adsorbents are typically large molecular weight polymers which are non-nutritive, non-digestible, non-fermentable and inexpensive to produce. Examples include activated charcoal, silicates, yeast cell wall extracts, chlorophyll-based products or combinations of the above. Mycotoxin adsorbents sequester mycotoxins through non-specific interactions with highly branched chains and binding pockets.

Activated charcoal is one of the earliest used mycotoxin adsorbents and can be used preventatively and therapeutically. Due to its low specificity and large surface area, activated charcoal can quickly remove toxins in the intestinal lumen after the onset of symptoms and will reduce the development of further symptoms (Diaz and Smith, 2005). A study by Bonna et al. (1991) showed a reduction in mortality and liver lesions in minks exposed to 34 or 102 µg/kg aflatoxin B<sub>1</sub> with dietary inclusion of 1% activated charcoal (w/w). Galvano et al. (1996) employed two different types of activated charcoal to reduce aflatoxin carryover from feed to milk of dairy cows. Only one of the two products was successful which implies that not all activated charcoal products have equal efficacy in this regard. Mixed results in efficacy were also observed for T-2 toxin depending on exposure duration with activated charcoal having a

more pronounced effect in response to large acute exposures (Edrington et al., 1997; Fricke RF and Jorge J, 1990). Ochratoxin A has not been shown to be well adsorbed by activated charcoal (Diaz and Smith, 2005). The lack of specificity also lowers efficacy as it is prone to adsorbing multiple compounds in the intestinal lumen including nutrients. Activated charcoal is best used, therefore, in cases of acute mycotoxicoses.

A broad group of minerals which have shown efficacy in binding mycotoxins are the silicates. Silicates can be categorized as phyllosilicates and tectosilicates (Smith and Diaz, 2005). Phyllosilicates include bentonite, kaolinite and illite clays. Tectosilicates include zeolite clays. Bentonites can be composed of a variety of cations and form gels in aqueous solution. These products have been shown to bind aflatoxin B<sub>1</sub> *in vitro* and reduce the effect of aflatoxicoses *in vivo* (Ramos and Hernandez, 1996; Lindemann et al., 1993). Zeolites are composed of SiO<sub>4</sub> or AlO<sub>4</sub> which interlock to create a negatively charged active site unlike the positively charged active sites of bentonites. Certain configurations of zeolites form channels which can allow penetration by large molecules. It has been shown that zeolites can sequester aflatoxin B<sub>1</sub> *in vitro* and can limit the negative effects of 2.5 µg/g of aflatoxin B<sub>1</sub> in broiler chickens with as little as 1% inclusion of the clay (Schiedeler, 1993). Zeolite chemical composition is variable and binding efficacy is not always consistent. Hydrated sodium calcium aluminosilicates are derived from zeolites and has been extensively studied. HSCAS have been used as anti-caking agents in feedstuffs but have shown ability to sequester aflatoxin B<sub>1</sub> (Diaz and Smith, 2005). Diaz and Smith (2005) summarised findings from several studies which indicated that HSCAS was unable to sequester or prevent the toxic effects of deoxynivalenol, ochratoxin A or T-2 toxin. Another study investigated the effects of a zeolite-based mycotoxin adsorbent in combination with an encapsulated bacterium fed to broiler chickens and their ability to inactivate T-2 toxin and

ochratoxin A (Garcia et al., 2003). The study looked at mycotoxin adsorption *in vitro* using ELISA analysis, weight gain and organ weights *in vivo* to determine if the adsorbents were reducing mycotoxicoses. The *in vitro* test indicated both adsorbents bound ochratoxin A well and T-2 toxin poorly. The *in vivo* study showed that there was no significant effect of either adsorbent on weight gain after 21 days of feeding.

Chlorophyll is a pigment found in plants and plays a role in photosynthesis. It is possible for this compound to be turned into a water soluble polymer known as chlorophyllin. Arimoto-Kobayashi et al. (1997) noted that chlorophyllin in combination with a polyglucosamine can form noncovalent bonds with polycyclic compounds due to the porphyrin-like structure of chlorophyllin and the planar structure of aflatoxin B<sub>1</sub>.

Yeast cell walls, in particular from *Saccharomyces cerevisiae*, have highly branched glucomannan polymers which contain a mix of positive and negative charges that may adsorb mycotoxins electrostatically. Research has indicated that these materials ameliorate the effects of mycotoxins both *in vitro* and *in vivo*. Leung et al. (2007) observed the effects of a yeast cell wall product in mature beagle dogs consuming *Fusarium* contaminated diets. These diets were naturally contaminated with multiple mycotoxins including zearalenone, fusaric acid and deoxynivalenol. The naturally contaminated diet with 0.2% inclusion of adsorbent was found to have higher levels of mycotoxins than the contaminated diet without adsorbent. Based on animal weight, the animals in both contaminated diet groups received similar exposure to *Fusarium* mycotoxins. The study monitored feed intake which is commonly reduced in animals exposed to deoxynivalenol and fusaric acid. While the contaminated diet reduced feed intake in the test animals, there was no significant preventative effect of the mycotoxin adsorbent. A recent study by Firmin et al., (2011) used a yeast cell wall-derived mycotoxin adsorbent to prevent aflatoxin

B<sub>1</sub> toxicosis in ewes. The ewes were fed either a control diet or a diet supplemented with the yeast cell wall adsorbent. Aflatoxin-contaminated wheat was fed via a gelatin capsule. The ewes were acutely exposed to aflatoxin for 3 days with a 3 day collection period and then chronically exposed for 21 days with a 3 day collection period. Ewes were housed in metabolic cages while urine, feces and feed intake and milk production were recorded. Aflatoxin was extracted and analyzed using HPLC with fluorescence detection. Biological indicators of aflatoxicosis did not indicate an effect of the adsorbent. At the same time, the study showed that there was a significant effect of diet on fecal excretion of aflatoxin B<sub>1</sub> in the first 24h of collection (last day of exposure) for both the acute and chronic periods. Recovered aflatoxin B<sub>1</sub> was significantly higher in the diet containing the yeast cell wall product indicating that absorption from the intestinal tract was reduced. Urine analyses indicated only a numerical effect of diet (Firmin et al., 2011). In a study by Swamy et al. (2002), grain naturally contaminated with *Fusarium* mycotoxins were given to pigs in combination with a yeast cell wall mycotoxin adsorbent. The mycotoxin adsorbent was only able to prevent some of the neurochemical changes induced by *Fusarium* mycotoxins. Including the yeast cell wall additive prevented reduced blood concentrations of IgA and IgM caused by the feeding of contaminated grain (Swamy et al., 2002).

These studies demonstrated that *in vitro* and *in vivo* experiments may not always correlate well and *in vitro* studies may not be good predictors of *in vivo* efficacy (Garcia et al., 2003; Rotter et al., 1989; Dwyer et al., 1997; Diaz et al., 2004). *In vivo* research does not address the mode of action of mycotoxin adsorbents. There is also variation in efficacy within the classes of adsorbents as each are manufactured differently and may vary in chemical and physical structure. Several types of silicates could be combined to make one product just as different

strains of yeast would produce relatively different amounts of yeast cell wall components. General comments on efficacy can be made for specific products but extrapolations to different but similar products should be done with caution. For these reasons, mechanistic studies are necessary.

#### 1.5.3.2 Therapeutic treatments

Lipid-soluble mycotoxins can undergo enterohepatic recirculation where they are excreted into the intestinal lumen then reabsorbed. Mycotoxin adsorbents can reduce this recirculation by binding the parent mycotoxin and any metabolites upon re-entry into the intestinal lumen. Once contaminated feed has been ingested and the mycotoxins have been absorbed into the blood stream, mycotoxin adsorbents and other deactivating agents are only effective before absorption or after excretion. Some feed additives used to prevent mycotoxicoses have been shown to have therapeutic effects as noted in the previous section. If a mycotoxin has been absorbed into the bloodstream, therapeutic treatments have the greatest potential to minimize harmful effects on target tissues.

Many mycotoxins exert effects on cells by altering the oxidative status by adduction or creation of reactive oxidative species. DNA adduction and lipid peroxidation can be caused directly or indirectly by free radicals produced by mycotoxins. Decreasing the levels of oxidative stress is an effective means by which cellular damage can be controlled. Therapeutic treatments can increase cellular antioxidative enzyme activities or substrate concentrations or can be antioxidants in their own right. Modification of the diet can also alter oxidative status by regulating enzyme activity.

Vitamins A, C and E all have antioxidative properties and have been shown to ameliorate the effects of mycotoxicoses as determined using biomarkers. Vitamin E stabilizes free radicals by donating a hydrogen atom from the phenolic ring. Peroxidization of lipids can be reduced by this mechanism. Vitamin E can donate a single electron and not become a free radical itself due to stabilization by the phenolic group of the molecule (Smith and Girish, 2012). Vitamin A and vitamin C act in a similar way to scavenge free radicals and reduce damage to cellular membranes.

Selenium is a component of glutathione peroxidase, an enzyme which uses two glutathione molecules to convert hydrogen peroxide into water. Hydrogen peroxide damages cell membranes and is formed when two hydroxyl radicals react with each other. Studies have used selenium-enhanced yeast in combination with vitamin E to reduce oxidative stress in mycotoxin-challenged rats (Yu et al., 2009). Other potential treatments for mycotoxin-induced oxidative stress include chlorophyll, carotenoids and synthetic antioxidants such as butylated hydroxytoluene. Changes to diet can, therefore, have an indirect effect on oxidative status in the body.

Coffey et al. (1987) evaluated the effect of dietary protein and fat on feed utilization in pigs challenged with aflatoxin B<sub>1</sub>. Feed utilization was improved when 5% of the diet was fat. Inclusion of fat or increased protein in the diet was also able to maintain cholesterol reduction in the plasma when pigs were challenged with a diet containing 132 µg/kg AFB<sub>1</sub>. It was proposed that increases in dietary protein reduce the incidence of aflatoxicosis by altering the activity of cytochrome P450 isoforms involved in aflatoxin biotransformation. Cytochrome P450s are needed to reduce the toxicity of aflatoxin (in conjunction with glutathione) and are upregulated by increasing protein levels. Aflatoxin B<sub>1</sub> is metabolized by P450 CYP3A4 to create a reactive

epoxide. This can potentially lead to an increase in carcinogenesis if exposure to aflatoxin B<sub>1</sub> is prolonged. When treating acute aflatoxicosis, therefore, dietary protein levels should be increased while chronic aflatoxicosis can be addressed by lowering dietary protein levels (Smith and Girish, 2012). Specific amino acids such as methionine and lysine also have effects on neurotransmitters which control intestinal active transporters responsible for uptake of materials (Coffey, 1989). Dietary methionine supplementation, moreover, increases cellular glutathione concentrations as it is bioavailable and a precursor of cysteine which is an essential component of glutathione.

#### 1.6 Regulations regarding mycotoxin adsorbents

Current regulations limit specific health claims on feed additives. The Canadian Food Inspection Agency states that yeast cell wall products are feed additives and can have label claims of being sources of glucomannans (CFIA section 3.23). Health related claims are not currently permissible on labels for yeast cell wall products, moreover, though claims such as “improved growth” or “improved feed efficiency” are permissible. Any claim which relates to the alleviation of symptoms or the removal of disease falls within the Food and Drug Act and classifies that additive as a drug. Research which has focused on mycotoxin adsorbents and whether they alleviate symptoms of mycotoxicoses would support the position that the product tested is a drug. A feed additive classified as a drug must follow strict guidelines and is subject to use only if prescribed. This is not acceptable assuming the hypothesized mode of action of mycotoxin adsorbents is correct. Biomarkers and indicators used for testing mycotoxin adsorbents should be able to quantify mycotoxin absorption or adsorption and not rely on parameters such as feed intake or disease state.

The United States Food and Drug Administration (FDA) has not approved any mycotoxin adsorbents as feed additives. Products which are in the market use different claims (e.g. anti-caking agents) and are considered to be Generally Regarded As Safe (GRAS) for those roles. The FDA has yet to approve any feed additive as a mycotoxin binder or establish any mycotoxin binder as GRAS (FDA, 2009).

The European Union has not yet approved any feed additive as a mycotoxin binder or adsorbent. Kolossova et al. (2009) stressed that there has been a lack of evidence regarding the binding ability of these products as well as the effect of these products on nutrient status.

Overall, mycotoxin adsorbents are not currently allowed to be sold as such in the U.S.A., E.U. or in Canada. Many products which have been tested as mycotoxin binders (silicates, clays, yeast cell wall products, activated charcoal) are allowed to be sold for other benefits.

## 2. Experimental Rationale

The current research was conducted with the objective of understanding the kinetics of aflatoxin B<sub>2</sub> *in vivo* following administration of a yeast cell wall mycotoxin adsorbent and how the yeast cell wall mycotoxin adsorbent influences intestinal uptake of aflatoxin B<sub>2</sub>. In addition, this research sought to determine the mode of action of yeast cell wall products in preventing mycotoxin absorption.

The mode of action of the yeast cell wall product is best determined by examining radioactive aflatoxin B<sub>2</sub> concentrations in feces, urine and tissues. Using these samples, aflatoxin B<sub>2</sub> can be traced from the time of initial administration to understand kinetics in a male weanling rat model and the impact of yeast cell wall extract on dietary absorption of aflatoxin B<sub>2</sub>. If the mode of action of the yeast cell wall extract on aflatoxin B<sub>2</sub> reduces levels of aflatoxin being

absorbed, then it can be surmised that the previous studies that showed a reduction in symptoms associated with aflatoxicosis were not caused by other physiological effects of the supplement, such as immunomodulation. It is important to note that this procedure does not differentiate between the parent compound and any metabolites because the tritium ring label is not removed from aflatoxin B<sub>2</sub> during metabolism. Alterations to aflatoxin B<sub>2</sub> by metabolism would be detected as if they were the parent compound.

Based on previous studies, it was hypothesized that the yeast cell wall product will reduce uptake of aflatoxin B<sub>2</sub> from the intestinal lumen and this reduction will be proportional to the level of dietary inclusion of the yeast cell wall extract. The effects of the yeast cell wall extract on mycotoxin absorption are expected to be reflected on the excretion rates of radioactivity.

This work is necessary to understand how yeast cell wall derived mycotoxin adsorbents act *in vivo* and will provide support for or against the use of this material as a mycotoxin adsorbent. There are additional implications for other materials derived from yeast cell wall and the specific claims that can be made regarding feed additive labels.

### 3. Pilot Trial

#### 3.1 Justification of aflatoxin B<sub>2</sub> as opposed to aflatoxin B<sub>1</sub>

The series of experiments in the current research used <sup>3</sup>H-8,9-aflatoxin B<sub>2</sub>. There is little structural difference between aflatoxins B<sub>1</sub> and B<sub>2</sub>. Aflatoxin B<sub>2</sub> occurs naturally and is less acutely toxic than aflatoxin B<sub>1</sub>. The current study did not seek any toxicological endpoints as the goal to observe how the adsorbent altered absorption and kinetics of the mycotoxin and not the

health status of the animals examined. This pilot trial was conducted to establish a protocol for maximizing dose recovery, optimizing dose and elucidating the mode of action of the adsorbent.

### 3.2 Introduction

The pilot trial was conducted over a 24h period to determine the effect of dietary inclusion levels of the yeast cell wall product and to validate the recovery of the radioactive label from the tissues, feces and urine. Procedures regarding the handling of animals and radioactivity were in accordance with the guidelines set out by the University of Guelph Animal Care Committee and the Canadian Nuclear Safety Commission.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Weanling, Sprague-Dawley rats were purchased from Charles River Canada, Ltd., (Saint Constant, QC). There were three replications with twenty male rats in each pilot trial. An additional eight rats were used to act as controls which received neither the yeast cell wall adsorbent, nor the radiolabelled aflatoxin. The total number of rats utilized in the pilot trials was 67. The diet used was LabDiet 5012 from Purina Mills Corp. (Brentwood, MO). A stock solution of  $^3\text{H}$ -8,9-aflatoxin B<sub>2</sub> was obtained from Sigma-Aldrich Chemical Corp. (Saint Louis, MO). The stock solution was 1 mCi/ml when obtained. The yeast cell wall product (Integral) was provided by Alltech Inc. (Nicholasville, KY).

Several reagents were required for sample analysis. Ethanol (70%) and hydrogen peroxide (30%) were both obtained from Ricca Chemical Co. (Arlington, TX). Glacial acetic acid was obtained from Acros Organics (Fair Lawn, NJ). Perchloric acid and Decon 75 were

obtained from Fisher Scientific (Fair Lawn, NJ). The tissue solubilizer (Protosol) was obtained from NEN Research Products (Boston, MA). Carbon dioxide was acquired from Linde Canada, Inc. (Mississauga, ON). The counting scintillant NBCS104 was obtained from Amersham Biosciences (Buckinghamshire, England).

### 3.3.2 Methods

Twenty Sprague-Dawley rats (~100 g at time of arrival with weight gains measured prior to dosing, ~27 to 32 days old) were randomly divided into two diet groups of ten for each of the three replicates of the pilot trial. Rats were individually housed in stainless steel metabolism cages for the duration of the trial. The cage floors were made of raised wire, which enabled feces and urine to be collected.

The rats in both groups were fed the same commercial diet but the modified diet group received the commercial diet supplemented with the yeast cell wall adsorbent. Over the course of the pilot trial, the inclusion level of the yeast cell wall adsorbent was 1, 2 and 5% w/w for the first, second and third replications of the trial, respectively. The commercial diet was provided from the manufacturer in pellets but was ground using a blender to make addition of the yeast cell wall product more homogenous. The yeast cell wall product was added to the ground rat diet and mixed mechanically for no less than 10 minutes. The feed was provided as a mash and no pelleting was done. Rats were fed the diets from arrival for seven days prior to the start of the trial. Feed and water were provided *ad libitum* in porcelain containers and glass bottles, respectively.

The rat diet and the yeast cell wall product were both sent to Analytical Services at the University of Guelph (Guelph, Ontario) for mycotoxin analysis. The multi-mycotoxin analysis procedure was conducted using a combination of ELISA and LC-MS/MS techniques.

In addition to the rats used above, a total of eight rats were fed the same control diet and were not dosed with radioactive tracer. These rats were used to ensure that the methods being used in this study were providing results that were not false positives or due to cross-contamination of samples. Four of these rats were used in the first replication, three in the second and one in the third replication as the researchers became comfortable with the procedures. The rats not dosed with radioactive tracer were housed at the same time and for the same period as the twenty rats that were dosed with the radioactive tracer.

Rats were subsequently weighed and dosed intragastrically with 34  $\mu\text{Ci}/\text{kg}$  bwt of  $^3\text{H}$ -8,9-aflatoxin B<sub>2</sub> using a stainless steel blunt nosed needle. The total volume of the gavage was approximately 400-500  $\mu\text{l}$  depending on rat weight. No additional non-radioactive aflatoxin was used.

Feces and urine were collected for 24 h following dosing. Feces samples were retained on a fine metal mesh screen while urine was collected in 50 ml plastic centrifuge tubes after passing through glass wool packing to remove particulate matter. At the time of collection, the sides of the funnel were washed with 20 ml of water to maximize the recovery of urine. Rats were sacrificed 24 h after dosing individually by placing them in a closed container filled with CO<sub>2</sub>. Liver, kidneys and femur muscle samples were collected, individually wrapped in parafilm and stored at -20°C until analyzed.

### 3.3.2.1 Sample processing and analysis

Analysis of fecal, urine and tissue samples was a modification of the procedures of Carson and Smith (1983). The methods were first validated using samples collected from a previous study. Several changes to reaction times and amounts of solvents used were made to allow for better digestion and reduced quenching in the samples. All samples from all tests and collection periods were analyzed in triplicate. Fecal samples were dried, weighed and ground before 100 mg subsamples were weighed out into 20 ml glass scintillation vials. Using a pipette, 1.0 ml H<sub>2</sub>O, 0.8 ml H<sub>2</sub>O<sub>2</sub>, and 0.4 ml HClO<sub>4</sub> were added to each vial. The samples were then placed in a 60°C oven (Isotemp 500, Fisher Scientific) for 24 h. After the vials and contents had cooled, 10 ml of scintillant (NBCS104) was added and the vials were left to for 12 h to adapt to the dark before being analyzed using a Beckman 6000 Liquid Scintillation Detector (Beckman Coulter, Brea, CA).

Urine samples were centrifuged at (3640 x g) and 1 ml aliquots of the supernatant were placed into 20 ml glass scintillation vials with 100 µl glacial acetic acid and 10ml of scintillant. The samples were left to adapt to the dark for 24 hours prior to analysis by scintillation counting.

Liver, kidney and muscle samples which were stored at -20°C were thawed, weighed and homogenized using a rotary homogenizer (Tissuemiser, Fisher Scientific, Fair Lawn, NJ). From each homogenate, 1.0 ml subsamples were pipetted into 20ml glass scintillation vials. To digest the tissues, 1.0ml of homogenate and 1.0 ml protosol were added and the vials were tightly capped and heated overnight at 60°C. After the samples cooled, 500 µl H<sub>2</sub>O<sub>2</sub> was added and the vials were re-capped while the reaction occurred. After allowing the reaction to occur overnight, 10 ml of scintillant was added and the samples were allowed to dark adapt overnight before analysis.

The scintillation detector measured each sample for 3 min and reported the average in disintegrations per minute (DPM) after having corrected for luminescence from non-radioactive sources and efficiency for  $^3\text{H}$ . The detector was set to scan energy the energy range from  $\ln(\text{keV}) = 0$  to 400.

This method is unable to detect any differences between the parent aflatoxin B<sub>2</sub> and its metabolites. The tritium label is located in a position which is not removed by metabolism. Detection of radioactivity in the feces may include the parent molecule which passed through the rat unabsorbed or unmetabolised as well as metabolites which were absorbed and later eliminated via the bile.

### 3.3.2.2 Statistical Analyses

Scintillation counting data was in disintegrations per minute (DPM) as opposed to mCi of radiolabelled aflatoxin. To compare the recovered radioactivity found in the urine, feces or tissue samples, the initial dose had to be in terms of DPM. Using the same oral gavage that was used to dose the rats, 500  $\mu\text{l}$  of the diluted dosing solution was transferred to a 20 ml scintillation vial where 10ml of scintillant was added and analyzed after dark adapting. This analysis was done in triplicate to determine the amount of dose in the diluted solution in DPM. Based on this value, the amount of radiation administered in DPM for each rat was calculated. Based on the calculations outlined below, the DPM of the recovered radioactivity in a particular sample was divided by the DPM administered to the rat and multiplied by 100 to get a percent recovery of dose. Feces collection at -24 h was used as a baseline to correct for any error in the scintillation counting.

DPM of initial dose = DPM in 0.5 ml of dosing solution / 0.5 ml dosing solution x dosage  
volume given to rats

$$\% \text{ dose recovered} = \text{DPM sample} / \text{DPM of initial dose} \times 100\%$$

DPM sample calculations

Where m refers to mass, vol refers to volume in ml, and DPM refers to disintegrations per minute.

$$\text{Feces} = \text{DPM}_{\text{obs}} / m_{\text{subsample}} \times m_{\text{sample}}$$

$$\text{Urine} = \text{DPM}_{\text{obs}} / \text{vol}_{\text{subsample}} \times \text{vol}_{\text{sample}}$$

$$\text{Liver} = \text{DPM}_{\text{obs}} / m_{\text{subsample}} \times m_{\text{liver}} \times (\text{vol}_{\text{homogenate}} / \text{vol}_{\text{subsample}})$$

$$\text{Kidney} = \text{DPM}_{\text{obs}} / \text{vol}_{\text{subsample}} \times \text{vol}_{\text{homogenate}}$$

Note: The mass of the kidneys were not relevant for these calculations as both were homogenized entirely.

$$\% \text{ dose/g Muscle} = (\text{DPM}_{\text{obs}} / \text{mass}_{\text{sample}}) (\text{DPM}_{\text{dose}} / \text{mass}_{\text{rat}}) \times 100\%$$

Note: Because the total amount of rat muscle could not be established, the recovered dose needed to be reported in terms of dose per unit mass.

The recovered doses observed in the control and modified diet groups were plotted against each other for the tissue, feces and urine samples collected in the pilot trials. Statistical analysis was done using SAS 9.2 (SAS Institute, Cary, NC). The mean percent recovered dose of the modified diet group was subtracted from the mean percent recovered dose of the control diet group and this difference was checked for significance using a Student's t-test. The model used had the assumptions that diet was a fixed effect and significance for this and all other trials was set at  $\alpha=0.05$  while  $\alpha=0.10$  denoted a trend.

### 3.4 Results

The composition of the diet used in these trials was outlined in table 3.1. The diet was tested for mycotoxin contamination and the results were presented in table 3.2. The levels of mycotoxin contamination that were detected in the feed and in the mycotoxin adsorbent were modest (aflatoxin: 3.3 $\mu$ g/kg in additive, deoxynivalenol: 0.066 $\mu$ g/g in feed. Diacetoxyscirpenol: 0.082 $\mu$ g/g in feed). The amount of contamination by aflatoxin in the yeast cell wall adsorbent was near the limit of detection, may have been a false positive and was otherwise too low to be of a concern to these trials. Between the high inclusion level of the mycotoxin adsorbent and the low levels of mycotoxins, interference between the adsorbent and the tritiated aflatoxin B<sub>2</sub> were not likely as there would be more available space to bind than the total amount of aflatoxin in the feed and dose. Further, the level of exposure of these mycotoxins was not high enough to have an effect on the rats within the time frame of the study.

For each sample type collected, there was sufficient radioactivity to let the signal be resolved from the background during the detection. The percent recovered radioactive tracer dose for the feces samples collected from the rats in both diet groups was outlined in table 3.4. The numerical difference in percent recovered dose for feces between the control group and the modified diet group showed that the modified diet had 2.80% and 2.18% more recovered dose than the control diet group for inclusion levels of 2% and 5% yeast cell wall product. With only 1% inclusion, the control group had higher levels of recovered dose by 2.04%. The percent recovered dose was mostly consistent among replications and the recovered dose in the control rats was mirrored in the rats fed the modified diet. There was no significant difference between the two diets for the feces samples in any of the pilot trial runs. No trend with respect to inclusion level of the yeast cell wall adsorbent was noted.

The urine data for the pilot trial showed a significant effect of diet in only the replicate where 1% inclusion of the yeast cell wall material was used (1.78% decrease when adsorbent was present in the diet,  $p=0.014$ ). In the trials using 2% and 5% inclusion, a numerical difference of 2.646% (trial 2) and 2.6% (trial 3) was observed with the control rats having higher levels of recovered dose in all three trials. Tissue samples analyzed for the pilot trial showed no significant effect of diet on residual radioactivity. Kidney values were not significantly different between diet groups. Liver analysis indicated that there was no significant effect of diet comparing the modified diet group and the control diet group. The residual dose overall was very low and did not exceed 2% of the initial dose for any of the tissue samples collected.

### 3.5 Discussion

In the pilot trial, eight rats were fed the control diet and did not receive any radioactive aflatoxin and were considered to be in the blank group as opposed to the control or modified groups. The analysis of the feces, tissue and urine of these rats established that the radioactivity found in dosed rats was due to the radioactive tracer and not a consequence of the sample preparation process or characteristics of the samples themselves.

Data from the 24 h pilot trial did not clearly show an effect of adsorbent on radioactivity in the feces samples. The urine of rats fed the control diet showed a significantly higher level of radioactivity compared to the urine of rats fed a diet with a 1% inclusion of the yeast cell wall adsorbent at the end of the 24 h collection period. The difference was not significant for the urine of rats fed diets with 2% and 5% inclusion of the yeast cell wall product when compared to controls. Rats fed all three dietary inclusion levels had numerically lower levels of radioactivity relative to controls. This is to be expected as the adsorbent should function by reducing the

absorption of aflatoxin thus a reduction in urinary elimination. This indicated some possibility of a diet effect which the current design did not have power or methodology to detect such as an insufficient timeline or wide sampling windows. A power analysis could be conducted to determine the exact number of animals required to detect a noticeable change. It is possible that there was not enough adsorption by the yeast cell wall product caused by either a lack of affinity to aflatoxin B<sub>2</sub> or a greater affinity between the yeast cell wall product and another material. Alternatively, individual variability with only 10 rats per treatment may have been too great to detect a significant difference.

Fecal aflatoxin was expected to increase with adsorption of aflatoxin and subsequent reduction of aflatoxin absorption. The feces data, however, showed no significant effect of adsorbent. This could also be explained by the above hypothesis. Lack of a biologically noticeable effect could have been a result of quenching – a process where the radiation energy is absorbed by another molecule thus preventing the scintillant from fluorescing (Moore, 1981). Quenching can be corrected for by internal quench curves used in scintillation counters. Other chemicals can also emit energy which the detector can pick up and read as a false positive despite correcting for it in the design. This type of variation was kept at a minimum by optimizing the digestion and counting procedure using old samples prior to the trials. The variation between sub-samples was low enough that excessive quenching and sample variability seemed unlikely. It is likely, however, that there was simply no effect of diet for this exposure period, exposure level and inclusion levels of the adsorbent.

After the initial exposure and 24 h collection period, tissue samples were collected and analyzed for radioactivity. Low levels of radioactivity were detected in the tissues ranging from 1.64% to 3.73% in muscle, 1.4% to 2.5% in liver and 0.08% to 0.1. Aflatoxin tends to sequester

in the liver and it has been reported that the amount retained after 100 h is 6.5% of the initial dose (Wong and Hsieh, 1980). The current study recovered 1.4% to 2.5% of the initial dose in liver 24 h after dosing which roughly coincided with, but was less than, literature reports. The value determined by Wong and Hsieh of 6.5% after 100 h, however, is not relevant to the current study as intravenous injections of aflatoxin B<sub>1</sub> and were used. The difference in recovered dose of aflatoxin not significantly altered by the three levels of the adsorbent. Given the amount of radioactive aflatoxin to which the rats were exposed and the length of the collection period, any differences between the two diets (with adsorbent or without adsorbent) may have been negligible. It is possible that by extending the time period following exposure to the radioactive tracer would increase the amount of sequestered aflatoxin in the liver and thus better demonstrate an adsorbent effect.

While the kidney and muscle tissues accumulated some radioactivity, these are not toxic target organs for aflatoxin. The primary toxic target organ is the liver. The lack of a significant difference between the two diets would be expected as the differences were small. Aflatoxin can accumulate in tissues throughout the body. Without whole carcass analysis, the total amount of dose recovered will not equal the amount administered. Longer exposure periods or higher exposure levels would be needed to confirm if there is no effect of diet.

Complications in sample collection and preparation may have led to a smaller difference between the two diet groups, however, this seemed unlikely as variation within the subsamples and the variation within each treatment group was low. The methods used, moreover, were validated by Carson and Smith, 1983. It was hypothesized that the yeast cell wall product was able to increase elimination of the tracer dose in the feces within the 24 h period. According to Wong and Hsieh (1980), 35% of aflatoxin is excreted via feces and 15-20% in the urine in the

first 24 h. Small changes which can happen early in the 24 h period could be missed based on the experimental design in the pilot trial such as differences in excretion rate which only happen very early in the 24 h period. The yeast cell wall adsorbent increasing the rate and ultimately the quantity (by reducing urinary excretion) of fecal elimination of the aflatoxin could be explained mechanistically by it binding the mycotoxin enough to limit absorption and enterohepatic recirculation. If this was correct, total fecal radioactivity would be equivalent between treatment and control groups at the end of the 24h period but more excretion would occur sooner after dosing in the modified diet group compared to the control group.

### 3.6 Conclusions

The pilot trial was successful in determining proper dosage methods of the radioactive aflatoxin tracer for the rats. The collection and sample analysis methods were also found to be appropriate. Larger initial doses of aflatoxin B<sub>2</sub> may have demonstrated a significant effect of diet. It was noted that there was some inconsistency in the feces data which was likely a result of the complex mixture of materials and possible quenching sources in the sample. The pilot trial, moreover, did not demonstrate a mode of action of the mycotoxin adsorbent. It was supposed that any differences may have been masked by the short 24 h length of the collection periods. The kinetics-based trial described in chapter 4 addresses this issue.

### 3.7 Tables

Table 3.1 Composition of rat chow

Nutrient	Amount in ration	Nutrient	Amount in ration
<b>Protein, %</b>	<b>23.2</b>	<b>Fat (ether extract), %</b>	<b>5.0</b>
Arginine, %	1.39	<b>Fat (acid hydrolysis), %</b>	<b>5.8</b>
Cystine, %	0.31	Cholesterol, ppm	170
Glycine, %	1.11	Linoleic Acid, %	2.25
Histidine, %	0.57	Linolenic Acid, %	0.25
Isoleucine, %	1.12	Arachidonic Acid, %	<0.01
Leucine, %	1.85	Omega-3 Fatty Acids, %	0.32
Lysine, %	1.35	Total Saturated Fatty Acids, %	0.96
Methionine, %	0.67	Total monounsaturated Fatty Acids, %	1.04
Phenylalanine, %	1.04		
Tyrosine, %	0.68	<b>Fibre (crude), %</b>	<b>3.8</b>
Threonine, %	0.87	Neutral Detergent Fibre, %	1371
Tryptophan, %	0.29	Acid Detergent Fibre, %	4.9
Valine, %	1.15		
Serine, %	1.19	<b>Nitrogen-Free Extract, %</b>	<b>51.2</b>
Aspartic Acid, %	2.70	Starch, %	39.5
Glutamic Acid, %	4.54	Glucose, %	0.29
Alanine, %	1.38	Fructose, %	0.34
Proline, %	1.54	Sucrose, %	3.38
Taurine, %	0.02	Lactose, %	0.00
<b>Mineals Ash, %</b>	<b>6.6</b>	<b>Total Digestible Nutrients, %</b>	<b>76.6</b>
Calcium, %	0.95	<b>Gross Energy, kcal/gm</b>	<b>4.14</b>
Phosphorous, %	0.74	<b>Physiological Fuel Value, kcal/gm</b>	<b>3.43</b>
Phosphorous (non-phytate), %	0.46	<b>Metabolizable Energy, kcal/gm</b>	<b>3.10</b>
Potassium, %	1.09		
Magnesium, %	0.20	<b>Vitamins</b>	
Sulfur, %	0.33	Carotene, ppm	1.9
Sodium, %	0.28	Vitamin K (as menadione), ppm	1.2
Chlorine, %	0.52	Thiamin HCl, ppm	12
Fluorine, ppm	25	Riboflavin, ppm	4.6
Iron, ppm	310	Niacin, ppm	81
Zinc, ppm	82	Pantothenic Acid, ppm	12
Manganese, ppm	75	Choline Chloride, ppm	1900

Table 3.1 Composition of rat chow continued

Nutrient	Amount in ration	Nutrient	Amount in ration
<b>Minerals Ash, % (cont'd)</b>		<b>Vitamins (cont'd)</b>	
Copper, ppm	12	Vitamin E, IU/kg	35
Cobalt, ppm	0.65	Ascorbic Acid, mg/gm	NA
Iodine, ppm	1.1	Folic Acid, ppm	1.0
Chromium, ppm	1.4	Puridoxine, ppm	6.5
Selenium, ppm	0.30	Biotin, ppm	0.3
		Vitamin B12, mcg/kg	50
<b>Energy Provided by</b>		Vitamin A, IU/gm	12
Protein, %	27.068	Vitamin D, IU/kg	3.3
Fat (ether extract), %	13.244		
Carbohydrates, %	59.688		

Table 3.2 Mycotoxin analysis for rat chow and yeast cell wall adsorbent

Source Material	Mycotoxin	Contamination	Limit of Detection
Yeast Cell Wall	Aflatoxin	3.3 µg/kg	1.0 µg/kg
	Nivalenol	<MDL	0.12 µg/g
	Deoxynivalenol	<MDL	0.06 µg/g
	Fusarenan-X	<MDL	0.11 µg/g
	15-Acetyldeoxynivalenol	<MDL	0.05 µg/g
	3-Acetyldeoxynivalenol	<MDL	0.05 µg/g
	Neosolanlol	<MDL	0.07 µg/g
	Diacetoxyscirpenol	<MDL	0.06 µg/g
	HT-2 Toxin	<MDL	0.04 µg/g
	T-2 Toxin	<MDL	0.06 µg/g
Rat Diet	Aflatoxin	<MDL	
	Nivalenol	<MDL	0.12 µg/g
	Deoxynivalenol	0.066 µg/g	0.06 µg/g
	Fusarenan-X	<MDL	0.11 µg/g
	15-Acetyldeoxynivalenol	<MDL	0.05 µg/g
	3-Acetyldeoxynivalenol	<MDL	0.05 µg/g
	Neosolanlol	<MDL	0.07 µg/g
	Diacetoxyscirpenol	0.082 µg/g	0.06 µg/g
	HT-2 Toxin	<MDL	0.04 µg/g
	T-2 Toxin	<MDL	0.06 µg/g

<MDL indicated that levels of the toxin were less than the method detection level

Table 3.3 Percent recovery of urinary <sup>3</sup>H-8,9-aflatoxin B<sub>2</sub> after 24 h exposure – pilot trials

Trial number	Diet Type (% inclusion)	Percent recovered dose ( SE)	Difference (Control – modified)	p-value
1	Control	17.51 ± 0.54	1.78	0.014*
	Modified (1% adsorbent)	15.73 ± 0.36		
2	Control	31.35 ± 0.35	2.646	0.38
	Modified (2% adsorbent)	28.71 ± 0.84		
3	Control	21.62 ± 0.34	0.56	0.671
	Modified (5% adsorbent)	21.06 ± 0.19		

Significant difference (p<0.05) between diet groups denoted with \*

Table 3.4 Percent recovery of fecal <sup>3</sup>H-8,9-aflatoxin B<sub>2</sub> after 24 h exposure – pilot trials

Trial number	Diet Type (% inclusion)	Percent recovered dose ( SE)	Difference (Control – modified)	p-value
1	Control	22.10 ± 1.29	-2.04	0.24
	Modified (1% adsorbent)	24.15 ± 1.08		
2	Control	31.46 ± 2.42	2.80	0.37
	Modified (2% adsorbent)	28.66 ± 1.86		
3	Control	33.36 ± 0.38	2.18	0.709
	Modified (5% adsorbent)	31.18 ± 0.43		

Table 3.5 Percent recovery of selected tissue 3H-8,9-aflatoxin B2 24 h after exposure

Tissue	Trial number	Diet Type (% inclusion)	Percent recovered dose ( SE)	Difference (Control – modified)	p-value
Liver	1	Control	2.449 ± 0.0481	0.0711	0.6626
		Modified (1% adsorbent)	2.519 ± 0.1422		
	2	Control	2.116 ± 0.1538	0.1892	0.3994
		Modified (2% adsorbent)	1.926 ± 0.1548		
	3	Control	1.407 ± 1.0390	0.0049	0.9722
		Modified (5% adsorbent)	1.402 ± 0.7660		
Kidney	1	Control	0.0841 ± 0.006	0.0034	0.73
		Modified (1% adsorbent)	0.0875 ± 0.008		
	2	Control	0.1050 ± 0.00875	0.0041	0.74
		Modified (2% adsorbent)	0.1009 ± 0.00843		
	3	Control	0.0810 ± 0.0661	0.00927	0.14
		Modified (5% adsorbent)	0.0902 ± 0.0721		
Muscle (expressed in % dose/g)	1	Control	1.775 ± 0.1104	0.045	0.416
		Modified (1% adsorbent)	1.820 ± 0.1771		
	2	Control	3.729 ± 0.4849	0.2355	0.7142
		Modified (2% adsorbent)	3.494 ± 0.4067		
	3	Control	1.644 ± 0.0728	-0.0695	0.5521
		Modified (5% adsorbent)	1.714 ± 0.0885		

## 4. Kinetics trial

### 4.1 Experimental Justification

Based on the pilot trial, it was concluded that dietary effects may have been masked by the single collection period 24 h after dosing. The kinetic studies included one dietary inclusion level of the yeast cell wall adsorbent based on the highest inclusion level from the pilot trial while radioactivity in feces and urine were determined at intervals to better detect changes in mycotoxin adsorption and excretion over time. The increased number of collection periods would increase the amount of data yielded per rat and give a better evaluation of the mode of action of the adsorbent on aflatoxin B<sub>2</sub>.

### 4.2 Introduction

In the current study, the pilot trial data showed a recovery between 20% and 30% of the initial dose in the feces within the first 24 h and 15% and 30% within the urine. These data agreed with literature findings. The difference between the two experimental groups, however, was not significant enough to establish a mode of action for the adsorbent. Kinetics studies have been used by other researchers to monitor the clearance rate of materials over time (Wong and Hsieh, 1979). These studies can be used to compare two treatments or diets and the effects they have on the kinetics of a given toxin, such as aflatoxin (Firmin et al., 2011). As stated in section 4.1, a kinetics study would be able to monitor the change in excreted radioactivity over a given period of time. The added data provided by each animal would increase the statistical strength of the experiment and allow for detecting changes between the two diets more accurately.

A consistent clearance rate for aflatoxin has not been determined in the literature, though the majority of excretion occurs in the first 48 hours after dosing (Wong and Hsieh, 1979).

Collection over four days would be sufficient to monitor the rate of toxin excretion and would provide multiple data points to obtain an effective curve which can be used to calculate the rate of excretion. Practicality dictated that collection should occur at -24, 6, 12, 18, 24, 48, and 72 h after the initial dose with a follow-up trial using collections points which have been shifted in time by 3 hours. The mycotoxin will be absorbed from the lumen of the digestive tract while some aflatoxin will pass through the digestive tract unabsorbed. Excretion of the absorbed mycotoxin will be primarily through fecal elimination (due to its higher lipid solubility) and urinary excretion. The shift is useful for noticing any sudden changes in the excretion of radioactivity that may have been overlooked in the other collection period.

## 4.3 Materials and Methods

### 4.3.1 Materials

The materials used in these trials were the same as in the pilot trial and described in section 3.3.1.

### 4.3.2 Methods

Two replications of this trial were conducted using identical experimental designs with the exception of sample collection times which will be described later. Twenty Sprague-Dawley rats (~100 g at time of arrival) were randomly assigned into one of two diet groups. Ten were fed a diet modified with 5% (w/w) of the yeast cell wall adsorbent while the control rats used the same diet, unmodified.. The rats had *ad libitum* access to this feed and to water for the duration of the trial. After 10 days of feeding, the rats were administered a dose of  $^3\text{H}$ -8,9-aflatoxin B<sub>2</sub> via oral gavage using the same dosage and method as in the pilot trial.

#### 4.3.2.1 Sample Collection

Feces and urine were collected at -24, 6, 12, 18, 24, 48 and 72 h after dosing in the first study and at 3, 9, 15, 21, 27, 51 and 75 h post dosing in the second study. At the last collection times (72 and 75 h), the rats were sacrificed and liver, kidney and muscle samples were excised using the method described in the pilot trial. The methods used to collect and analyze feces and urine samples were the same as described in chapter 3.

#### 4.3.2.2 Statistical analysis

Tissue samples were compared as before using the same model and assumptions as in the pilot trial. Due to the change in experimental design with respect to time, the urine and feces samples were analyzed using repeated measures ANOVA where the recovered dose was the response variable, the rats were random effects and diet was a fixed effect. Repeated measures ANOVA accounted for the sampling intervals which were conducted over time. The model could be described by:

$$\text{Recovered dose} = \mu + \text{diet} + \text{time} + \text{diet}*\text{time} + \varepsilon$$

Where  $\mu$  is the true mean of the recovered dose for a particular sample type and  $\varepsilon$  is the experimental error. Subsamples were averaged prior to the analysis and not accounted for by the model. Given that the variation within the subsamples was low, this was deemed to be permissible.

#### 4.3.2.4 Graphical Analysis

For the feces and urine data from the kinetics-based trial, the response of each diet group for a given time point were averaged and plotted. In addition, cumulative excretion or

elimination of aflatoxin was plotted as a function of time. The area under the curve was calculated to determine the rate of excretion or elimination for urine and feces samples.

#### 4.4 Results

##### 4.4.1 Tables

The feces and urine samples for both iterations of this trial are summarized in tables 4.1 and 4.2, respectively.

##### 4.4.2 Tissue Residual Radioactivity

The recovered dose in the liver, kidneys and muscle samples is summarized in table 4.3, which includes the percent dose recovered, the numerical difference between the two diet groups and the statistical significance of the difference. The liver samples had higher radioactivity in the control diet group (trial 1: control = 1.183%, modified = 1.040; trial 2: control = 1.014%, modified = 0.9057%). The kidney samples had higher radioactivity in the control group (trial 1: control = 0.0691%, modified = 0.0666; trial 2: control = 0.0726%, modified = 0.0719%). The control diet had lower radioactivity in the muscle samples for the first trial (control = 1.115%, modified = 1.187%). There was no significant effect of dietary adsorbent on residual dose remaining in liver at the conclusion of the trials. Numerically, there was a higher level (p-values: 0.11 and 0.16) in the livers of the rats fed the control diet. There was similarly no significant effect of diet on residual radioactivity in kidneys or muscle. Numerically, there was slightly more radioactivity in the kidneys of control rats.

#### 4.4.3 Residual Radioactivity In Urine

Aflatoxin detected in urine for both iterations of this trial are summarized in table 4.1. Radioactivity recovered in urine for the first trial showed a trend in the effect of diet ( $p=0.083$ ). The second replication with the 3h shift in collection points shows a significant effect of diet ( $p=0.011$ ). In both cases, the dose recovered in the urine was consistently higher at every collection time for rats fed the control diet compared to those fed the adsorbent-modified diet. The amount of dose recovered varied with respect to time of collection. Over the 3-day collection period, the cumulative percent of the initial dose which was recovered ranged from 19% to 27%. The first replication which had collections up to 72 h post-dosing had lower levels of recovered dose compared to the second replication where urine was collected up until 75h post-dosing.

#### 4.4.4 Residual Radioactivity In Feces

Radioactivity in fecal samples ranged from 41.5% to 44.6% of the dose in the first replication in which feces were collected over 72 h. In the second replication, which lasted 75h, the dose recovered in the feces ranged from 37.6% to 38.8%. In both replications, the recovered dose of radioactivity in the feces was found to be numerically higher in rats fed the control diet compared to the rats fed the diet modified with the yeast cell wall product. There was no significant effect of diet in either replication.

## 4.5 Discussion

### 4.5.1 Effect of adsorbent on urinary aflatoxin excretion

For both replications of the kinetics trial, the amount of radioactivity recovered in the urine of rats fed the control diet was higher than in the samples of the rats fed the diet modified with adsorbent implying a reduction in aflatoxin B<sub>2</sub> absorption. By measuring multiple points in time, a significant effect of diet ( $p < 0.05$ ) in the second replication and a trend ( $p < 0.1$ ) in the first replication were observed. Urine measurements alone are not sufficient to conclude that the yeast cell wall adsorbent decreased the amount of radioactive aflatoxin by reducing intestinal absorption though it does infer that there was less aflatoxin available in the body to be excreted in urine. In the work conducted by Firmin et al. (2011) on ewes, a similar experimental design was used and found the effect of diet had a trend ( $p < 0.1$ ) in the urine. Firmin et al. noted a 40% reduction in aflatoxin B<sub>1</sub> in the urine when the diet contained a yeast cell wall product compared to the control diet. This effect was only noticeable after the feed additive had been administered for 21 days but not noticeable in the 3 day feeding trial. The trends in the current study were similar to the study by the work by Firmin et al. though the percent difference between the two diets was not similar. Some differences existed between these studies, most notable, the species, duration and mode of administration.

Total recovery of radioactivity in urine ranged from 19% to 27% of the initial dose over the 72 to 75 h period. Coulombe and Sharma (1984), using Wistar rats as a model, recovered 15% of the initial dose in the urine 23 days after the last aflatoxin dose was administered. Similarly, Wong and Hsieh (1979) recovered 19% of the initial dose in the urine after 4 days of collection. The recovered dose in the urine of the current study was higher than what was found in the literature. This may be due to the rat model used, collection and analysis. Feces and urine

interaction was unavoidable given the design of the cages and some radioactivity may have transferred to the urine. The methods and materials used in the process of scintillation counting differed as well and the current study may have had higher sensitivity by using larger aliquots of urine.

The difference between the values in each replication may indicate a better sampling window is possible. The initial collection periods were set arbitrarily and allowed sufficient time to collect the samples. In the initial collection, the first collection point (6 h) was at least 10% of the administered dose. Compared to the first collection (3 h) of the second replication, the recovered radioactivity was above 6.5% of the administered dose indicating that the first replication missed the start of the increase. At the same time, the recovered dose was observed at 10% to 12% of the administered dose at both 6 h and 9 h after dosing. This implies that the peak excretion point may have been between 6 h and 9 h. Ideally, sampling should be done constantly to get the most accurate data possible but this is limited by time as well as the rate and amount at which urine and feces are excreted from rats. The two replications of this trial were enough to conclude that the overall rate of excretion was well represented. The data from both replications could not be combined due to the nature of the sampling. Each data point consisted of the sum of radioactivity excreted for a given 6h time period and combining data with different sampling periods would cause an overlap.

The area under the curve was calculated for each trial and used to determine the rate of excretion of aflatoxin in the urine. Overall, the modified diet exhibited a lower cumulative recovered dose compared to the control rats. The rates of excretion were higher in the modified diets ( $7.3 \times 10^{-4}$  %dose/h in trial 1,  $5.68 \times 10^{-4}$  %dose/h in trial 2) compared to the rates of excretion in the control diets ( $5.87 \times 10^{-4}$  %dose/h in trial 1,  $4.88 \times 10^{-4}$  %dose/h in trial 2). The actual

difference between the excretion rates in the modified diet and the control diet is small. It does not appear that the yeast cell wall product had a biologically significant effect on the urinary excretion of aflatoxin (specifically, aflatoxin B<sub>2</sub> metabolites, the major constituent of urinary aflatoxin excretions).

#### 4.5.2 Residual Fecal Radioactivity

There was no significant effect of diet on fecal radioactivity in either replication of the trial. The difference in recovered radioactivity between the diets did not show that either group had consistently higher levels compared to the other. Early fecal elimination in the first 24 h after dosing was higher in the rats fed the modified diet. The majority of aflatoxin is excreted in the bile. Early elimination could imply that the yeast cell wall adsorbent had reduced the transit time of aflatoxin, possibly by adsorption. The higher levels of aflatoxin recovered in the first 24 h could be attributed to a decrease in absorption of the parent aflatoxin while the aflatoxin recovered in the control rats from 48 h to 72 h could have been absorbed in the first 24 h and later eliminated as a metabolite. Lack of significance could be attributed to colour quenching and other interferences within the samples as stated in the feces analysis of the pilot trial. It was noted that the amount of radioactivity recovered in the feces was higher in the modified diet group during the first 12h after initial dosing in both replications. It is possible that the yeast cell wall adsorbent increased the rate of excretion of aflatoxin in the modified diet group but not by much and the control rats eventually excreted the same amount of aflatoxin by the end of the trial. Aflatoxin can undergo some enterohepatic recirculation (Wogan et al., 1967) but not enough to skew these results. This study did not account for the form of aflatoxin that was excreted and was unable to determine if it had been metabolised by the liver. It is possible that

the modified diet prevented aflatoxin from being absorbed and was excreted earlier in an unchanged state, while the control diet had no effect and aflatoxin which was excreted had been absorbed and then excreted as a metabolite.

Over the 72 h and 75 h collection periods, the total recovered radioactive dose ranged from 37% to 44% of the administered dose. In the first 24 h of collection, the recovered dose in the feces ranged from 36% to 39%. In work done by Coulombe and Sharma (1984), the percent of the initial aflatoxin dose recovered in the feces was 33% after 24 h of collection. The current study provided fecal radioactivity recovery similar to what was observed by Coulombe and Sharma. The current study's results did not match well with the findings of Wong and Hsieh (1979) who recovered 53% of the initial aflatoxin dose after a 72 h collection. After the 72 h and 75 h periods, it was noted that the rats had consumed some of the feces or had thrown some food into the collection funnel. This could have led to an overestimation of the mass of the feces collected or an absorption of urine which would have led to a transfer of radiation into the feces sample. With due diligence during sample collection, this error was reduced. These actions may have skewed the results in the longer studies and led to the lower recovery observed.

Overall, the current data suggested that the rate of elimination of aflatoxin from the rat is not affected by the yeast cell wall adsorbent over the time of this experiment. More detailed analysis would be required to differentiate aflatoxin from metabolites and would involve extracting aflatoxin and any metabolites from the feces and using mass spectrometry or HPLC to identify the aflatoxin type and amount found.

#### 4.5.3 Effect of adsorbent on tissue aflatoxin levels

There was no significant effect of diet on residual radioactivity in liver, kidney or muscle. Radioactivity recovered in the liver and kidneys was numerically higher in the rats receiving aflatoxin without an adsorbent. Possibly, due to the amount low level of aflatoxin dose recovered and the low sample size of rats (10 per group), there may have not been sufficient power to detect any significant difference.

The amount of recovered dose in the liver was not higher than that recovered during the pilot trial for the same tissue. Based on findings by Wong and Hsieh (1980), the amount of dose recovered in the liver after 100h would be 6% of the initial dose. The current study did not recover dosages that high. The research done by Wong and Hsieh involved intravenous administration of aflatoxin which would make comparisons with the current study difficult as their work did not account for absorption kinetics. Given that bioavailability by intravenous injections is higher than bioavailability by ingestion, it would be unlikely that the recovered dose in the current study would be equal to or higher than the recovered dose reported by Wong and Hsieh. Ultimately, this illustrates the importance of considering absorption in kinetics studies.

#### 4.5.4 Mode of action based on the current results

Given that the only dataset with a significant effect of diet was urine, the effect of the yeast cell wall adsorbent must be explained with said data in mind. The hypothesis which was originally being tested was based on previous research (Firmin et al., 2011; Leung et al., 2007) looking at yeast cell wall adsorbents binding mycotoxins and preventing them from being absorbed from the intestine into the blood stream. Under this hypothesis, it was expected that if the yeast cell wall product worked as an adsorbent, there would be an observable increase in

excreted radioactivity in the feces and a decrease in radioactivity in the urine in rats fed the modified diet. There would be lower levels of radioactivity recovered in the livers of the rats fed the modified diet, as well. Since the kidneys and muscle are not tissues where aflatoxin tends to accumulate, the lack of significant differences observed and the low recovery of dose was not unexpected. Without the feces or liver samples showing the expected significant effects of diet to support this hypothesis, the current work cannot decisively conclude that it is the mode of action of the yeast cell wall product, though only the urinary excretion value support this position.

It is also possible that the effects of the yeast cell wall product were indirect and the product did not bind the mycotoxin in the small intestine. The glucomannans in the product could have reduced uptake of the toxin by altering the ability for molecules to cross gastrointestinal epithelium. From a broader standpoint, the yeast cell wall product would have the same effect on the animal were this possible mode of action present. There is, however, a large amount of *in vitro* and *in vivo* work which has shown that adsorption of aflatoxin does occur by yeast glucomannans and other materials (Ramos and Hernandez, 1996; Firmin et al., 2011).

#### 4.5.5 Kinetic model and predictions

The percent recovered dose of the urine and feces samples were plotted in response to the collection time. A regression analysis was done for each sample and treatment combination with repeated measures being accounted for. The analysis looked at the entire 72h collection period as well as for just the first 24h period. The results for the determination of the excretion rates indicated very little change between the modified and control diets for both urine and feces samples. The rate of excretion or elimination of aflatoxin in the urine and feces over time were

outlined in table 4.4. Excretion of aflatoxin in the urine was displayed in figures 7.1, 7.2, with the cumulative excretion displayed in figures 7.3 and 7.4. Elimination of aflatoxin in the feces was displayed in figures 7.5, 7.6, with the cumulative elimination displayed in figures 7.7 and 7.8. The slope calculated for the urine data was very consistent between both diet types indicating that the mycotoxin adsorbent did not modify the rate of excretion. The differences noticed between the two diets were likely caused by changes in bioavailability of the dose in the rats. The rats fed the adsorbent-containing diet had absorbed a smaller dose compared to the control diet group and concurrently excreted less via the urine. The feces data was not significantly different for either replication of the kinetics trial. When the feces data was analyzed using just the first 24h of data the slope of the regression was upward. In the first 24h, the dose excreted by feces does not reach a maximum until approximately 12h after the initial dose. For the remaining 12h of the 24h period, the dose recovered remains higher than the recovered dose at 6h. For these reasons, the slope of the regression was positive. Regardless, no trends could be derived from these analyses. Currently, the lack of an effect or trend in the feces data reduces the confidence in the hypothesis that the mycotoxin adsorbent prevents uptake of aflatoxin B<sub>1</sub>.

#### 4.6 Conclusions

The kinetics-based trial was able to show an effect of diet in the collected urine samples. The regression analysis established that the difference between the two diets was due to differences in the amount of absorbed aflatoxin B<sub>2</sub> between each diet. Feces and tissue data were not able to support these findings, possibly due to the complexity of the samples themselves. The findings from the urine excretion data of this study suggest that the yeast cell wall product

reduces levels of aflatoxin in the urine. This effect may involve binding or otherwise preventing absorption of aflatoxin B<sub>2</sub> out of the intestinal lumen. Lack of feces and tissue data which agrees with the findings from the urine data prevents any conclusions from being made about the mode of action of the yeast cell wall product.

#### 4.7 Tables

Table 4.1 Percent recovered dose of radiolabelled aflatoxin B<sub>2</sub> in feces of rats fed control diet or diet modified with a yeast cell wall-derived product, measured over time

Replication	Diet type	Time intervals							p-value
		-24	6	12	18	24	48	72	
1	Control		1.331	20.540	8.716	6.198	6.351	1.204	0.2961
	Modified		2.590	22.236	9.617	4.649	4.407	1.204	
		3	9	15	21	27	51	75	
2	Control	0.0127	4.935	19.826	8.884	2.941	1.582	0.606	0.9249
	Modified	0.0075	6.873	19.262	7.051	2.312	1.507	0.607	

Table 4.2 Percent recovered dose of radiolabelled aflatoxin B<sub>2</sub> in urine of rats fed control diet or diet modified with a yeast cell wall-derived product, measured over time

Replication	Diet type	Time intervals							p-value
		-24	6	12	18	24	48	72	
1	Control		12.669	4.9779	1.660	1.301	2.025	1.032	0.083
	Modified		10.868	4.0578	1.103	0.785	1.433	0.759	
		3	9	15	21	27	51	75	
2	Control	6.813	12.306	4.1073	1.232	0.920	1.717	0.750	0.011
	Modified	6.606	10.207	3.194	1.083	0.772	0.936	0.664	

Table 4.3 Percent recovered dose of radiolabelled aflatoxin B2 in tissues of rats fed control diet or diet modified with a yeast cell wall-derived product, at (1) 72 h or (2) 75 h post dose

Tissue	Trial number	Diet Type (% inclusion)	Percent recovered dose ( SE)	Difference (Control – modified)	p-value
Liver	1	Control	1.183 ± 0.0761	0.1431	0.11
		Modified	1.040 ± 0.0381		
	2	Control	1.014 ± 0.0638	0.1083	0.16
		Modified	0.9057 ± 0.0379		
Kidney	1	Control	0.0691 ± 0.00488	0.00245	0.64
		Modified	0.0666 ± 0.00167		
	2	Control	0.0726 ± 0.00403	0.000694	0.8816
		Modified	0.0719 ± 0.00221		
Muscle	1	Control	1.115 ± 0.0810	-0.0714	0.4997
		Modified	1.187 ± 0.0646		
	2	Control	1.217 ± 0.0430	0.0881	0.2429
		Modified	1.129 ± 0.0589		

Table 4.4 Elimination and excretion rates for dose recovered as a percent of the administered dose based on the sampling model of feces and urine samples collected during kinetics trial

	Control	Modified
<b>Feces</b>		
Trial 1 elimination rate	3.13 x10 <sup>-4</sup> %dose/h	3.15 x10 <sup>-4</sup> %dose/h
Trial 2 elimination rate	3.44 x10 <sup>-4</sup> %dose/h	3.54 x10 <sup>-4</sup> %dose/h
<b>Urine</b>		
Trial 1 excretion rate	5.87 x10 <sup>-4</sup> %dose/h	7.31 x10 <sup>-4</sup> %dose/h
Trial 2 excretion rate	4.88 x10 <sup>-4</sup> %dose/h	5.68 x10 <sup>-4</sup> %dose/h

## 5. Overall Conclusions

The trials which were conducted in this work did determine that the amount of aflatoxin excreted in the urine of rats was lower when the yeast cell wall adsorbent was fed. An explanation for this was a reduction in absorption of aflatoxin across the intestinal mucosa. Without similar results in the feces or tissue, other modes of action can still be considered but seem unlikely based on work by other researchers. These results alone are not sufficient to confirm the hypothesis tested. Therefore, the experiments were not able to conclusively demonstrate the mode of action of the yeast cell wall product marketed as Integral using rats as an experimental model.

The effect which the yeast cell wall product did exert on the rats was minor. At the exposure level used, the changes in the percent recovered dose between the treatment groups and their respective controls would not justify inclusion of the feed additive. Higher exposure levels of aflatoxin, however, may have shown greater differences. In the current study, the amount of aflatoxin which could be used was restricted as the amount of radioactive aflatoxin was a limiting factor. Natural contamination of aflatoxins in feed has been reported to be as high as 347  $\mu\text{g}/\text{kg}$  with average contamination between 30 and 40  $\mu\text{g}/\text{kg}$  (Binder et al., 2007). The current study exposed rats to 34  $\mu\text{Ci}/\text{kg}$  bwt. The exact dosage of aflatoxin B<sub>2</sub> cannot be reported in micrograms as the radioactive tracer concentration was only reported in mCi, though, it is unlikely that the concentration of the tracer is similar to natural contamination levels. Using a radioactive tracer, it is not economically feasible to use exposure levels this high.

It is recommended that further study be conducted. The current work does demonstrate a mode of action which is related to a functional interaction between aflatoxin and the product as opposed to a mode of action which directly involves modifying the immune system or the incidence of disease.

## 6. Future Research

There are many opportunities for research which stem from this work. Among them include studies where the kinetics of the yeast cell wall product is more rigorously tested in rats or a similar study is conducted in livestock such as pigs or ducks. Each study has its own unique challenges, but, these can be addressed with proper experimental design. Using more rats or a different animal model would improve the power of the analysis. The difference in recovered dose noted in the current study was very small and would require either higher exposure levels of

the aflatoxin or more rats. Higher exposure levels have the added benefit of being closer to scenarios involving naturally contaminated crops. The current method used to detect aflatoxin would need to be changed if higher concentrations of the mycotoxin were used. Radio labelled compounds are both costly and hazardous. Larger exposures and the use of larger animals would require more extensive clean up protocols if tritiated aflatoxin were used. Stable isotopes could be used as an alternative. They are just as expensive but are easier to handle. Unmodified aflatoxin can be used but suffers from detection issues. Extraction techniques may not be able to recover all the dose and may not detect compounds modified after metabolism. After accounting for losses during extraction, isolation (possibly through an internal standard) and the additional steps needed for analysis, unmodified aflatoxin would be much cheaper and safer. With respect to the experimental design, chronic exposure periods using either multiple dosing or contaminated feed could reveal more apparent differences between the two diet types. Longer trials and more consistent dosing, moreover, give the possibility of blood sampling. Unlike feces, blood is easier for aflatoxin isolation and analysis. Levels of aflatoxin in blood could indicate absorption into the body and can be monitored regularly.

## 7. Figures

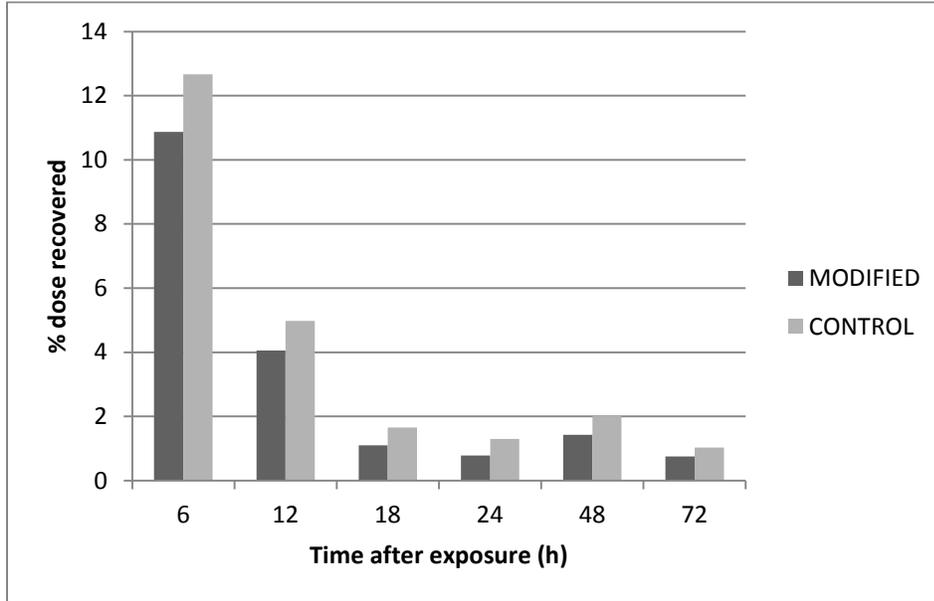


Figure 7.1 Urinary Excretion of 8,9-3H-aflatoxin B2 dose for 72 hours after initial exposure

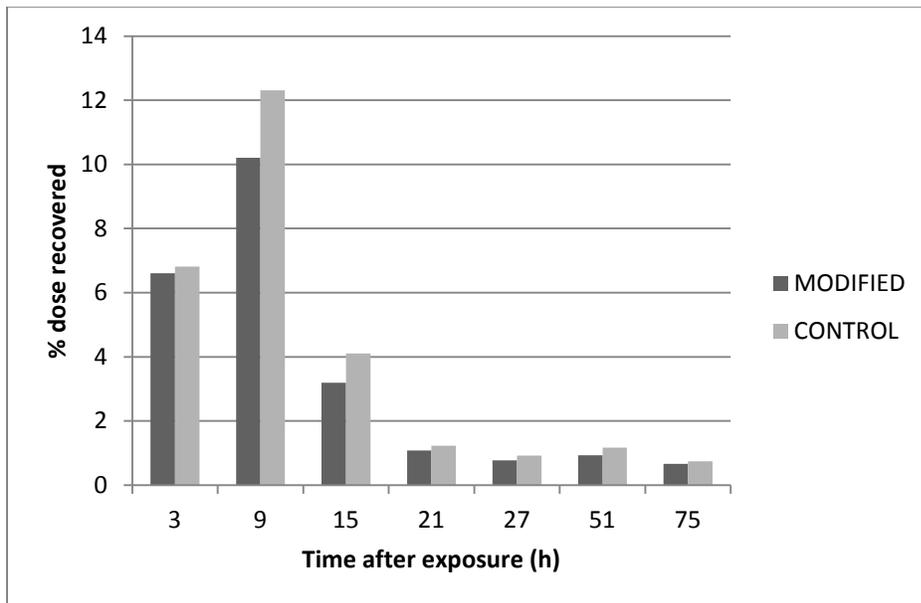


Figure 7.2 Urinary Excretion of 8,9-3H-aflatoxin B2 dose for 75 hours after initial exposure

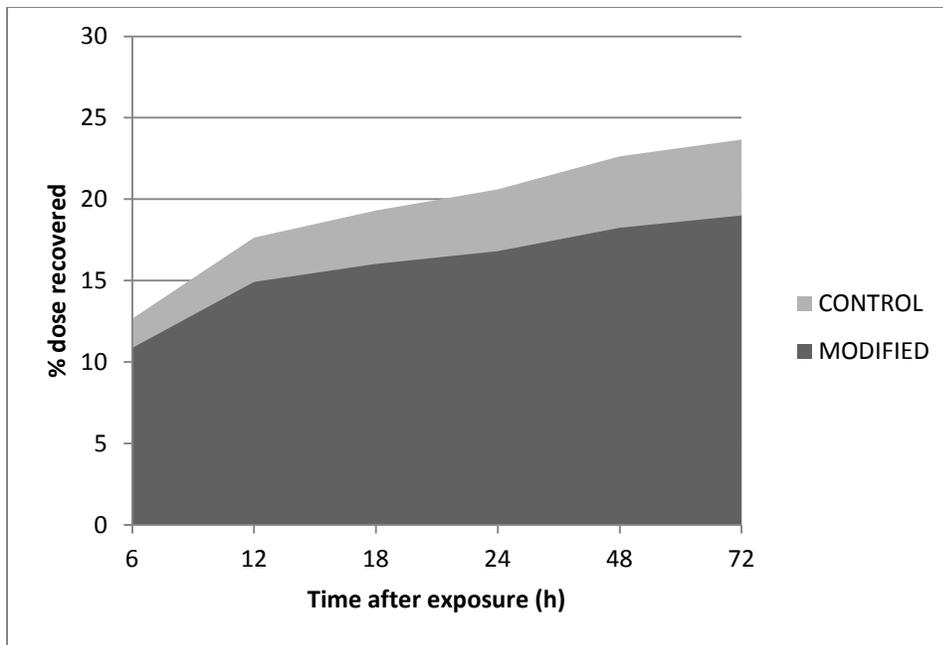


Figure 7.3 Cumulative Urinary Excretion of 8,9-3H-aflatoxin B2 for 72 hours after initial exposure

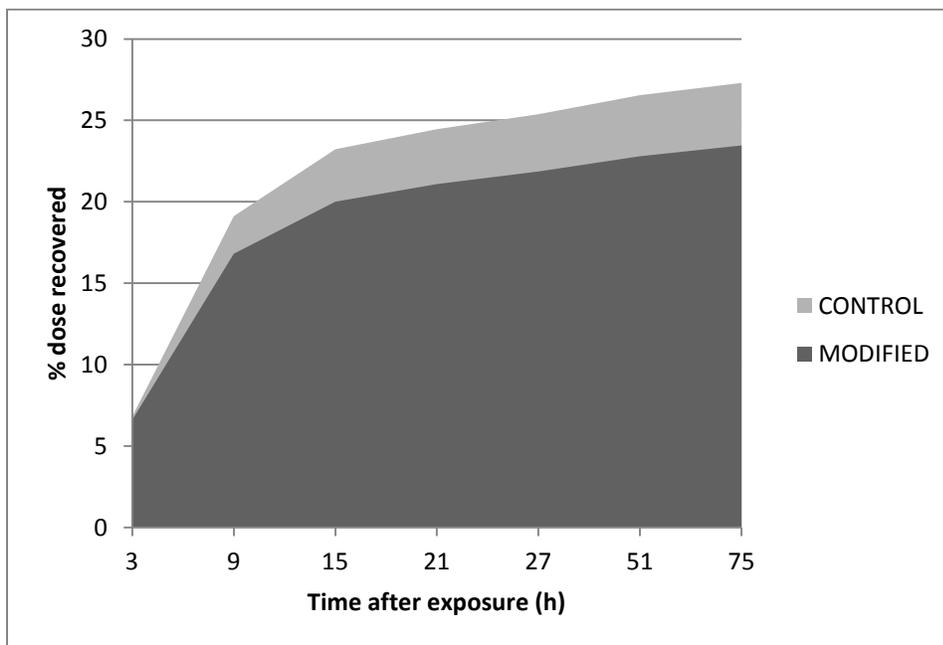


Figure 7.4 Cumulative Urinary Excretion of 8,9-3H-aflatoxin B2 for 75 hours after initial exposure

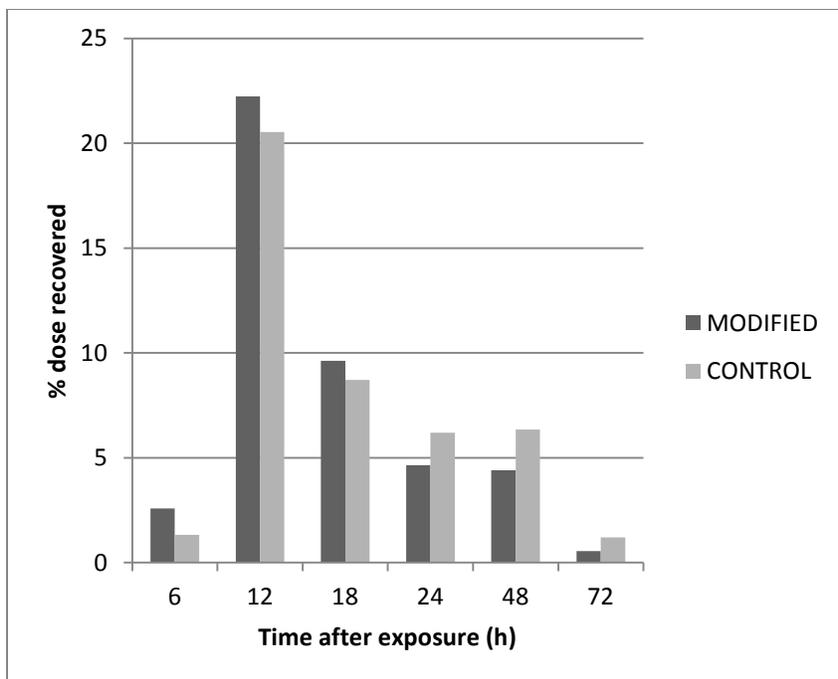


Figure 7.5 Fecal elimination of 8,9-3H-aflatoxin B2 dose for 72 hours after initial exposure

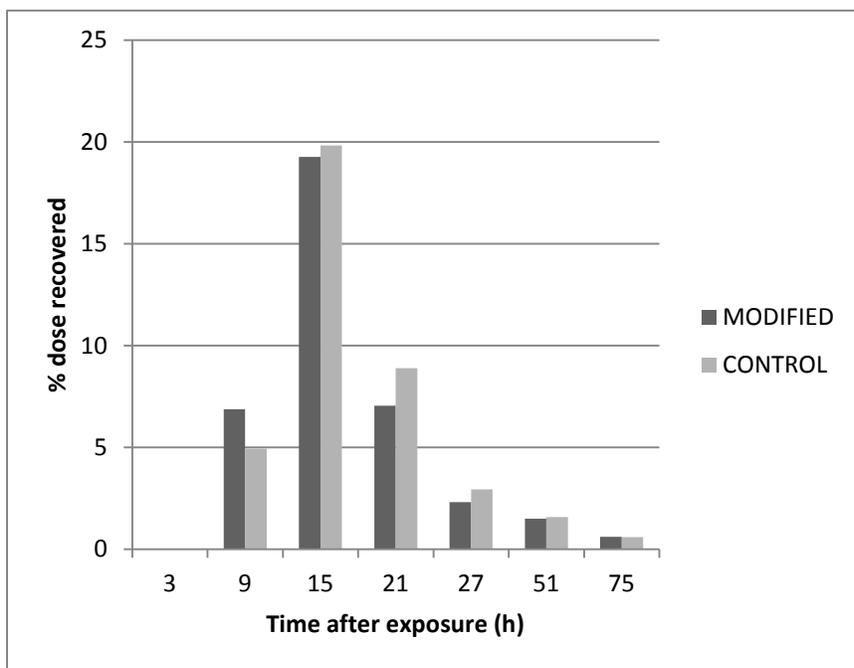


Figure 7.6 Fecal elimination of 8,9-3H-aflatoxin B2 dose for 75 hours after initial exposure

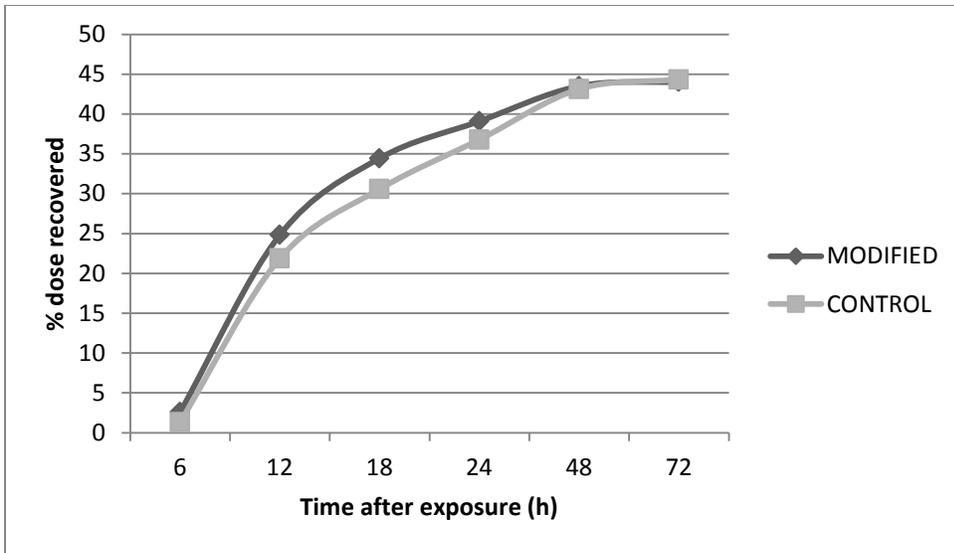


Figure 7.7 Cumulative elimination of 8,9-3H-aflatoxin B2 for 72 hours after initial exposure

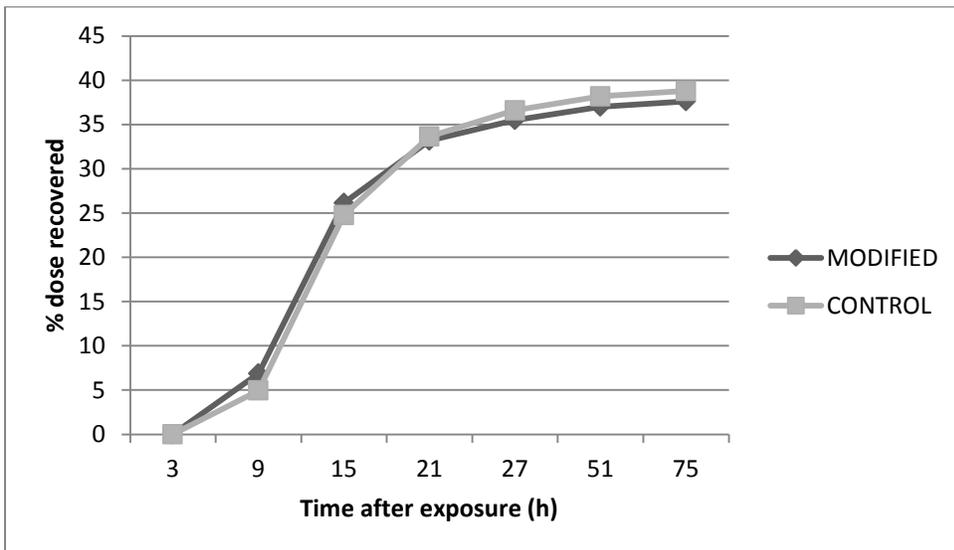


Figure 7.8 Cumulative elimination of 8,9-3H-aflatoxin B2 for 75 hours after initial exposure

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