THE EXPRESSION OF THE *NOR~1* GENE OF *ASPERGILLUS* SPP. AND AFLATOXIN PRODUCTION IN COMPOUND FEEDS FROM SOUTH AFRICA IN RELATION TO ANIMAL HEALTH DISORDERS

By

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DECLARATION

I hereby declare that this dissertation, which I herewith submit for the research qualification

MASTERS' DEGREE IN BIOMEDICAL TECHNOLOGY

to the University of Johannesburg, Department of Biomedical Technology, is, apart from the recognised assistance of my supervisor and co-supervisor, my own work and has not previously been submitted by me to another institution to obtain a research diploma or degree.

_____ on this ____ day of _____

(Candidate)

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_____ on this 18th day of November 2012

(Co-supervisor)

DEDICATION

Dedicated

To the Glory

Of the Glorified

And Glorifying

God the Father, God the Son and God the Holy Spirit.

For His saving Divine Grace at all the challenging stages of my academic life.

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ABSTRACT

Aflatoxins (AFs) are naturally occurring secondary metabolites produced principally by *Aspergillus flavus* and *Aspergillus parasiticus* in food and feed commodities worldwide. Contaminations of compound feeds by AFs do not only affect animal health, but the economy as well. It is for this purpose that a study was carried out to establish the quality of South African feeds with respect to AF-producing fungi, establish a correlation between levels of AFs and determinant gene (*nor-1*) responsible for producing these toxins. To this end, compound feeds (n=92) from various feed manufacturers in South Africa were sampled and analysed for aflatoxigenic fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) and *nor~1* genes using the conventional identification and real time-polymerize reaction (RT-PCR) methods, respectively. Data obtained revealed that 66.5 and 53.1% of samples were positive for *A. flavus* and *A. parasiticus*, respectively. Aflatoxins levels in similar samples were estimated by high performance liquid chromatography (HPLC) following an immune-affinity clean-up and multi mycotoxin extraction procedures. Accordingly, levels established ranged from 0.06 – 77.97 ppb (mean: 16.8 ppb) with feeds for poultry being the main contaminating substrate and no correlation (overall R^2=0.093) was established between the concentrations of AFs and those of *nor~1*. The cytotoxic effect of some selected AF extracts from these feeds on human lymphocyte cells was performed in comparison to that of AFB_1 standard. Data obtained from the cytotoxic assay revealed that cell viability was affected significantly (P<0.001) by both the dose and duration of exposure, which was much more noticeable when cells were exposed to AFB_1 standard than for individual extracts. In conclusion, even though none of the feeds analysed contained levels of AFs above regulatory limits established in South Africa, such feeds when consumed on a continuous basis may pose some serious health problems especially when AFs is found in co-contamination with such significant mycotoxins as ochratoxins (OTs) and fumonisins (FBs). Thus, the continuous need to limit AFs levels in feed commodities from South Africa is imperative.

Key words: *Aspergillus* species, aflatoxins, *nor-1* gene, compound feeds, cytotoxicity, health, South Africa.

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LIST OF ACRONYMS & ABBREVIATIONS

Spp.: Species

pg: Picogram

AFs: Aflatoxins

UV: Ultraviolet

a_w: Water activity

FBs: Fumonisins

OTs: Ochratoxins

US: United States

AF(s): Aflatoxin(s)

AFB_1: Aflatoxin B_1

AFB_2: Aflatoxin B_2

AFG_1: Aflatoxin G_1

AFG2: Aflatoxin G_2

AFM1: Aflatoxin M_1

AFM2: Aflatoxin M_2

C_t: Threshold cycle

R_f: Retardation factor

EU: European Union

DMI: Dry matter intake

LOD: Limit of detection

R.H.: Relative humidity

MEA: Malt extract agar

EC: European Commission

TDI: Tolerable daily intake

GIT: Gastrointestinal tract

PDA: Potato dextrose agar

UVR: Ultra violet radiation

DNA: Deoxyribonucleic acid

EST: Expressed Sequence Tag

WHO: World Health organisation

(p_w): Vapour pressure of pure water

NOR-1: None-derived orphan receptor

TEF-1: Translation elongation factor 1

EFSA: European Food Safety Authority

(p_s): Vapour pressure of water in a substrate

BLAST: Basic Local Alignment Search Tool

AFMA: Africa Feed Manufacturers' Association

RT-PCR: Real Time Polymerase chain Reaction

HPLC: High performance liquid chromatography

AFLP: Amplified fragment length polymorphisms

JECFA: Joint Expert Committee on Food Additives

NCBI: National Centre for Biotechnology Information

FAO: Food and Agriculture Organization of the United Nations

LASER: Light amplification by stimulated emission of radiation

LIST OF UNITS

L: Litre

g: Gram

M: Moles

V: Volume

%: Per cent

Hrs: Hours

<: Less than

µl: Microliter

bp: Base pair

Kg: Kilogram

Mins: Minutes

Mm: Millimetre

nm: Nanometre

>: Greater than

Secs: Seconds

µM: Micro molar

µm: Micrometre

˚C: Degree Celsius

ppb: Parts per billion

ppm: Parts per million

ng/g: Nano gram/gram

µg/g: Microgram/gram

µg/ml: Microgram/millilitre

µg/kg: Microgram/kilogram

CHAPTER ONE

INTRODUCTION

1.1 Problem statement

Aflatoxins, principally produced by *Aspergillus flavus* and *A. parasiticus* represent a group of potent mycotoxins (especially AFB_1) that contaminate food and feed commodities worldwide. Their biosynthetic determinant gene is *nor-1*. Animal exposure to significant amounts of these toxins, seriously affect animal health, but also leads to serious economic losses due to poor animal performance arising from reduced feed intake/utilization, weight loss, poor immune function, decreased reproduction and even death in severe circumstances (Njobeh *et al*., 2012). Aflatoxicosis, a general term referring to a disease in animal and man due to exposure to AFs is often reported in literature. The point in case is the recent outbreak of the disease in the Gauteng Province of South Africa that killed over 220 dogs and several others affected after consuming pet food contaminated with high doses of AFs (Arnot *et al.,* 2012). Several reasons can be advanced for this but more importantly, is the fact that there is lack of proper management strategies put in place to limit AFs contaminations. Limiting these contaminations can be achieved if there is continuous monitoring of feeds for AFs and the fungi responsible in producing them.

1.2 Justification

On-going studies in the research group (Food Environmental and Health, University of Johannesburg) have focused almost exclusively on growth of fungi, detection/ quantification of mycotoxins levels in foods/feeds. However, works in literature, in relation to AF and *nor-1* levels have been studied with focus on cereals but no work has been published that attempts to establish this relationship in compound feeds. It is for this reason that the study was carried out, to estimate and correlate the quantification of the AF load and its determinant gene in selected compound feeds from South Africa.

1.3 Background

Many species (about 185) belonging to the *Aspergillus* genera are known (Guarro *et al.*, 2010). They contaminate various agricultural commodities including compound feeds. Contamination by these fungal species is often accompanied by the production of mycotoxins. Mycotoxins are secondary metabolites produced by fungi of which about 300-400 of them have been detected and characterised so far (Magnolic *et al*., 1997; Fink-Gremmels, 1999; Hinton, 2000; Yiannikouris and Jouany, 2002; Turner *et al.*, 2009) with a

number of them yet to be discovered. Of the mycotoxins known, AFs are the most significant of all, probably because they are the most toxic. Aflatoxins are produced principally by *Aspergillus flavus* and *A. parasiticus* (Ali *et al.*, 2005; Lamanaka *et al*., 2007; Alcaide-Molina *et al*., 2009). However, other identified *Aspergillus* species (*A. nomius, A. bombycis, A. ochraceoroseus, A. australis, A. tamarii, A. pseudotamarii and A. oryzae*) have also been found to produce AFs. Amongst the AFs, AFB_1 is the most toxic and is a potent carcinogen, teratogen and mutagen (Anderson *et al.,* 1999; Mayer *et al*., 2003; Afriyie-Gyawu *et al*., 2008) and is also involved in immune suppression and reduced reproductivity (Virdi *et al*., 1989; Bingham *et al.*, 2003; Turkez and Geyikoglu, 2010). The toxic effects of AFs can be acute or chronic, depending on the level of the toxin in feeds and duration of exposure in animals fed AFs contaminated feed (Binder *et al*., 2007).

Production of AFs takes place during the secondary metabolic activity of aflatoxigenic fungi. This usually involves the expression of nor~1 transcript gene (Yu *et al.*, 2004a; David, 2009) which is the determinant gene in the anabolic process of AFs. *Nor~1* gene encodes a reductase that converts norsolorinic acid to averantin. Subsequent reactions and enzymes conversions lead to aflatoxin synthesis (Dutton, 1988). This could be either AFB_1 and AFG_1 or AFB_2 and AFG_2, depending on the existing branch point. *Aspergillus* fungal genomic DNA analysis in naturally contaminated agricultural commodities can be performed via TaqMan fluorescent probe technology (María *et al*., 2008; Abdin *et al*., 2010). Sensitivity of the test demonstrates that DNA amounts expressed, account for toxins production that can be detected via RT-PCR chain reaction assay. This technique has been reliable and absolute in the prediction of potential *Aspergillus* and aflatoxigenic risk in stored agricultural commodities including compound feeds. The molecular technique is suitable for rapid, automated and throughput analysis (Valsesia *et al.,* 2005; Degola *et al.*, 2007) in fungal identification. The principle behind this tool is based on the reaction with two specified primers which defines a target sequence and an additional internal probe that hybridizes between the primers. This increases the required specificity of a needed quantification reaction with an internal probe 5'-label with fluorescence dye (FAM) and a 3'-end ligating to a quencher (TAM) (Lo and Chan, 2006; Lee *et al*., 2006). The quencher reduces the fluorescence quantum yield of the dye owing to proximity. In a polymerase chain reaction, the hybridized probe is degraded by the 5'-3' activity of exonucleases of the TaqMan polymerase, releasing a fluorescent dye which may increase and hence, quantitatively ascertained (Lo and Chan, 2006; Brinda and Paul, 2011).

As the gene responsible for AF production can be quantitatively detected, so also can the toxins be detected and quantified via several methodologies that have been developed. Of these methods, the principal immunochemical based assay is the commonly used enzyme linked immune-sorbent assay (ELISA). Other methods of detection and quantification of AFs are based on electrochemical and optical principles (Hajian and Ensafi, 2009; Herzallah, 2009; Jin *et al*., 2009).

In evaluating mycotoxin contamination in feeds, it is imperative to identify fungal species responsible for producing them. In which case, the producing fungi can be identified and quantified morphologically. This can be time consuming and requires taxonomic skills. Hence, Gourama and Bullerman (1995) developed and described a rapid immunological approach. This approach has its drawback of lack of specificity, not differentiating between fungal species. Development by Woloshuk and Prieto (1998), characterized AF biosynthetic genes and by way of application of diagnostic PCR procedures, the detection of aflatoxinogenic fungi was made possible (Geisen, 1996). This only gave qualitative results, showing presence or absence of an aflatoxinogenic fungus (Mayer et al., 2003, Rahimi *et al.*, 2008) in agricultural products like maize, peanuts wheat, etc. but has not been found in literature on compound feeds in relation to AF load and AF fungal toxigenic gene presence to determine the quality of feeds.

1.4 Hypothesis

It was hypothesised that

(i) There is a correlation between levels of transcript gene (*nor~1*) and those of AFs in compound feeds as established in maize.

(ii) Feeds contaminated with high amounts of AFs are more cytotoxic than those with low AF content.

1.5 Aim of the study

The aim of conducting this study was to assess the quality of South African compound feeds with respect to aflatoxigenic producing fungi, the determinant gene nor-1 and AFs.

1.6 Objectives of the study

To achieve the aim (Section 1.3), the following objectives will be met:

(i) To screen and characterize AFs producing fungi in South African compound feeds.

(ii) To quantify *nor~1* transcript genes and AFs in these feeds.

(iii) To establish the correlation between nor~1 transcript genes and AFs levels in these feeds

(iv) To evaluate the toxicological effects of AFs extracts obtained from feeds on human lymphocyte cells.

CHAPTER TWO
LITERATURE REVIEW

2.1 *Introduction*

Compound feeds are feed types used in animal nutrition. They have the potential of being invaded by *Aspergillus* fungi, particularly *A. flavus* and *A. parasiticus* during pre-production or post-production of these feeds, thereby contaminating the feeds. The ability of these fungal species to survive in these feeds depends on the Eco physiological conditions suitable for growth, hence proliferation. As these fungi proliferate, biochemical and metabolic activities takes place within the feed of contamination, resulting to production of secondary metabolites in the feed, which can be toxic, particularly in the case of AFs. Biological effects of AF(s) are many and have been well elaborated depending on dosage, animal species and during animal exposure to the toxin. Reports of Njobeh *et al.* (2010), Makun *et al*. (2011) and Dutton *et al.* (2012) indicate that the metabolic activities of *Aspergillus* fungi yield AFs, which have been found in various agricultural commodities including compound feeds. This contamination results in poor quality feeds and consumption of such feeds results in a wide range of injurious effects on animals, reducing their health and performance leading to serious economic loses.

The presence of AFs in under developed and developing countries is increasingly recognised, owing to their high concentrations in agricultural commodities from these countries (Iqbal *et al*., 2011). Data on prevalence of AFs which causes aflatoxicosis (a disease caused by ingestion of feeds contaminated with AFs) in feeds in Africa is limited. There have been reports of AF contamination and the disease outbreak in human, from stapled agricultural products in Kenya (Probst *et al*., 2007) and in dogs in South Africa (Otto, 2011) due to AFs exposures. These incidences did arose quality check on animal feeds in relation to AF contamination.

2.2 Animal feeds

2.2.1 Definition and concepts

Feeds are commercially blended substances or materials for animals as part of their daily ration, which are designed to maximise animal productivity, either for meat, milk or egg production (McDonald *et al*., 1995, McDonald *et al*., 2002). These feeds are formulated to supply the animal's nutritional needs for their daily metabolic activities, particularly where animals are confined and not allowed to free range. Such nutritional requirements as defined by Klaus and Josephine (2007) include protein, energy, fats, minerals, vitamins and water. A variety of feed ingredients of plant

and animal origins are commonly used to compose balanced feeds that generally have high protein quality (Waldroup, 1999, Viljoen, 2003). Nutrients which are mainly for growth, tissue repairs, maintenance and reproduction, form the different components of animal feed as will be reviewed subsequently in this chapter.

The demand for animal feed remains exponential on a large scale (Connor *et al*., 2011, Jia, 2007). This is because livestock continues to be an integral part of the agricultural activities as animals are kept for various purposes. As such, different feed types are formulated for different classes of livestock. These feeds can be categorized into forages, fodders, silage and concentrate or compound feeds (Church, 1991; Coffey, 2008; Heuzé *et al*., 2012) and they all have the essential nutritional components required for livestock growth.

2.2.2 Nutritional compositions of animal feeds

There are six nutrients compositions in livestock feeds required by the animal. However, the amount of each nutrient varies from one animal species to another as well as the age of the animal, physiological status and the purpose for which the animal is kept (Njobeh, 2003; Scanes *et al*., 2004). For optimal growth and high productivity to be achieved, animal feeds must be formulated to provide the various nutrients at sufficient levels (Dimcho *et al*., 2005, Reddy and Krishna, 2009). The main nutritional classes in compound feeds are shown in Figure 2.1 and will be briefly reviewed subsequently.

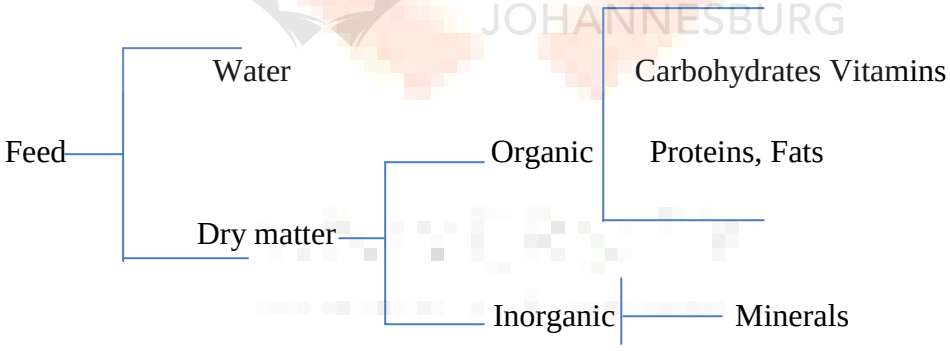


Figure: 2.1 Total nutritional composition of compound feeds

2.2.2.1 Carbohydrates

Carbohydrates are naturally occurring chemical compounds, constituting of carbon, hydrogen and oxygen. The main examples are sugars, starches, cellulose and hemi-celluloses. Functionally, these nutrients are energy providers that power muscular movement, provide a source for body heat and act as building blocks for other nutrients (NRC, 2001; Kumar *et al*., 2011). According to Wondra *et al*. (1995), simple carbohydrates (sugar and starches) are referred to as nitrogen free extracts and are mostly present in cereal grains. Complex carbohydrates (cellulose and hemicellulose) are difficult to digest and can be found mostly in roughages (Caroline *et al*., 2003). The digestive system of animals determines its carbohydrate utilization (Cantarel *et al*., 2012).

Simple stomached animals (mono-gastric) can rarely digest large quantities of fibre, but ruminant animals with four stomach compartments can eat and digest large amounts of fibrous materials (Wondra *et al*., 1995). This makes the carbohydrate ration of simple stomached animal to contain really a much less fibre content but more of cereal grains rather than forage and roughages, which is the reverse for ruminants.

2.2.2.2 Protein

Like carbohydrates, proteins contain carbon, hydrogen, oxygen, but with the addition of nitrogen and sulphur. Proteins are made-up of amino acids as building blocks which can be released when proteins are hydrolysed by enzymes, acids or alkalis (Merchen and Titgemeyer, 1992). There are over 200 amino acids isolated from biological materials (McNab and Boorman, 2002) but only 20 are commonly found as proteins components. Animals cannot synthesize certain amino acids (often referred to as essential amino acids) (Coffey, 2008) and hence these protiens must be included in their diets. There are 10 classes of such essential amino acids including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Lawrie and Ledward, 2006). Proteins are needed to replace dead body cells, to supply materials for body building and tissue repairs in animals. Thus, proteins play an important role as a basic structural unit. They are also needed for metabolism, hormone, antibody and DNA production in animals (Nelson and Cox, 2005).

2.3.2.3 Fats

Fats are also referred to as lipids or oils and are neutral compounds. They contain the same elements as for carbohydrates but contain more carbon and hydrogen atoms than oxygen (Riediger *et al*., 2009). Thus they have 2.25 times as much energy value as carbohydrates (Freeman, 2003; Micha and Mozaffarian, 2008). They are solids (fats) or liquids (oil), depending on their composition and surrounding temperature. They serve as source of energy, heat, insulation, cushioning (body protection), the basic structure of cellular membranes, hormones, and fat-soluble vitamins as their carriers and have an immune function. Fats are easily digested by animals but highly stored in the body as triglycerides than carbohydrates which are stored limited amount as glycogen (Merchen and Titgemeyer, 1992; Swiezewska and Danikiewicz, 2005). Sources of fats include soybean oil, corn oil, fish oil, and peanut oil and by-product fats for compound feed production.

2.2.2.4 Vitamins

They are required by animals as nutrients in small amounts (Becker, 2004) and are classified as water soluble (vitamins C and B) and fat soluble vitamins (vitamins A, D, E, and K). Water soluble vitamins are not readily stored and dissolve readily in water (Mortensen and Skibsted, 1997; Padayatty *et al*., 2003). They help in teeth and bone formation, prevent infections and to improve appetite, growth, reproduction and chemical reactions in the body. In animals, fat-soluble vitamins

are absorbed through the intestinal tract, vitamin A serves for healthy eyes, good conception rate, and disease resistance (Jacob *et al*., 2000), vitamin D serves for good bone development and mineral balance of the blood (Wintergerst *et al*., 2007), vitamin E serves for normal reproduction, muscle development and helps boost the immune system (De la Fuente *et al*., 1998), while vitamin K helps in blood clotting, preventing excessive bleeding during injuries.

2.2.2.5 Minerals

Minerals (e.g. calcium, phosphorous, magnesium, potassium, zinc, etc.) other than carbon, hydrogen, nitrogen and oxygen are present in nearly all organic molecules (Lindemann, 1996). They are needed in small amounts and may be available in livestock feeds as major or trace minerals, based on the nutritional requirements of the animal. They are often artificially added to animal feeds as supplements (salt lick). They provide materials for growth of bones, teeth, tissue, regulate chemical processes, aid in muscular activities, release energy for body heat, protein synthesis, oxygen transport, fluid and acid-base balance in body as well as enzyme activities (Dryden, 2008).

2.2.2.6 Water

Like all other feed nutritional constituents discussed above, water also constitutes a nutrient. It is an essential animal requirement for normal body metabolism. Animal feeds excluded of water is referred to as “dry matter” (Zonderland *et al*., 2004). This is a measure of the mass of the feed (or anything as generalised) when completely dried. Dry matter of animal feeds would be its solids, excluding water but including other feed chemical nutritional constituents (carbohydrates, fats, proteins, vitamins, and minerals). Carbohydrates, fats, and proteins make up 90% of the dry weight of a feed diet (Brown, 2007). Though livestock naturally consume water, the water content in feeds varies widely depending on the type of feed and its specification for formulation (Bender and Bender, 2005). By this, there is room for comparison between the level of a given nutrient on a dry matter basis and that required by the animal (Wattiaux, 2011). According to Buckmaster (1990), there are feeds of low-energy content but with high percentage of water. Animals eating such feeds have been shown to consume less dry matter and food energy (Mehmet *et al*., 2005).

2.3 Types of animal feeds produced in South Africa

Animal feeds can be classified into three broad categories (Figure 2.2) which include forages, fodders and compound feeds.

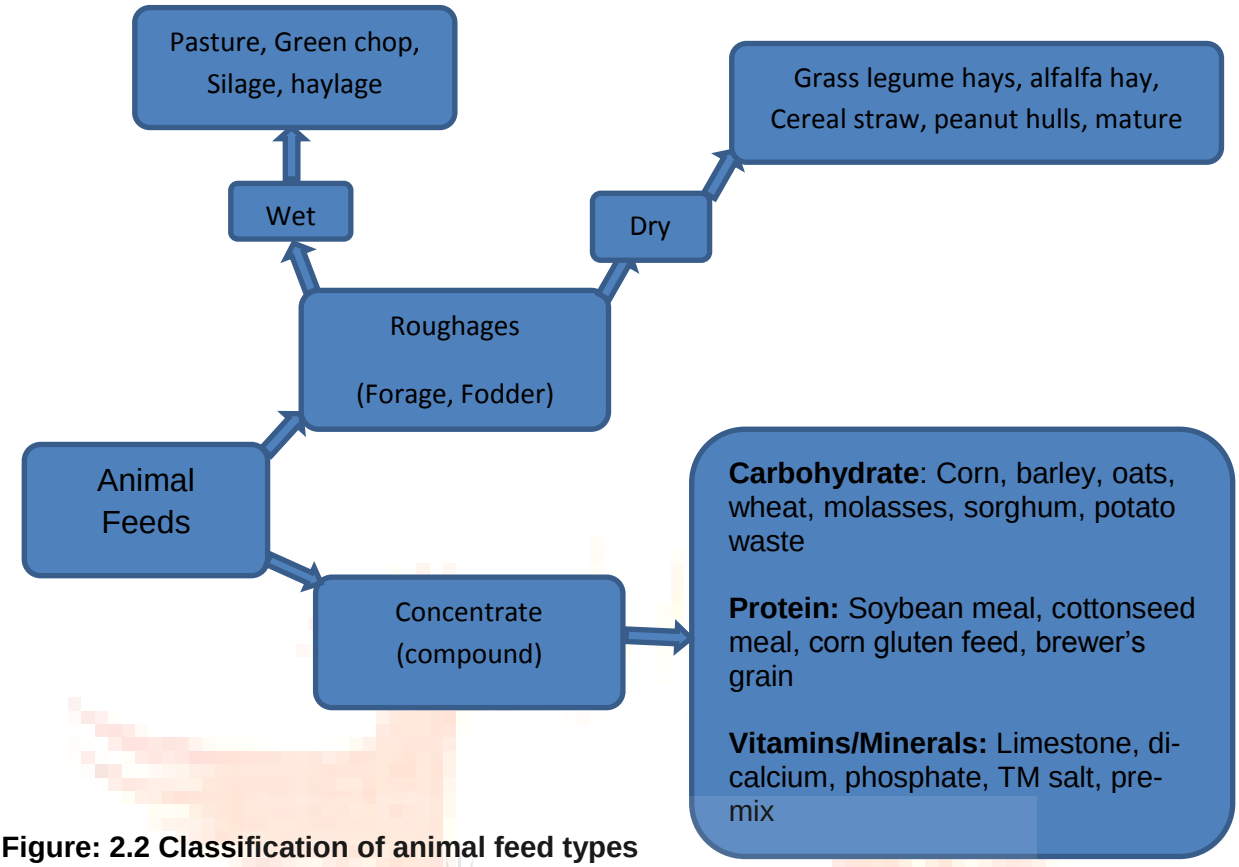


Figure: 2.2 Classification of animal feed types

2.3.1 Forages

These are plant materials grown in confined areas for livestock grazing (Fageria, 1997). They could be legumes, grasses, corn, oats, alfalfa and other edible plant parts. Livestock that are into foraging include but not limited to cattle, goats and sheep. The act of eating or grazing upon the plant matter is known as foraging. Management type of this grazing by livestock can be controlled or continuous (Shaffer, 2008). When controlled, the producer regulates forage availability and quality to the livestock. When continuous, the livestock have free selection of forage. There are a number of ecological effects derived from grazing (Jones, 2002) including fungal contamination. Occasionally, forages are exposed to fungal contamination with the appearance of infected spots on different parts of the plant. In continuous foraging management type, livestock are prone to such fungal diseases as *Aspergillosis* (Kradin and Mark, 2008). The most common forms which are allergic broncho pulmonary *aspergillosis,* pulmonary *aspergilloma* and invasive *aspergillosis* (Herbrecht *et al*., 2002). By way of time and normal eco-physiological conditions, these fungi attach the forage material and develop biochemical and metabolic activities. As these activities proceed, secondary metabolites (mycotoxins) are produced and in a case of certain *Aspergillus* contamination, AF is bound.

2.3.2 Fodder

The term fodder is used to describe plant parts given to animals after the plants have been harvested, which contrasts with forages (Jones *et al*., 1985). Fodders are used primarily to feed domesticated livestock. It is typically composed of plant matters such as hay, straw and grains (Jones, 1983). Meat and bone meal are occasionally mixed into fodder. This has been blamed for the spread of mad cow disease (Freudenrich and Craig, 2001). Dry weight analysis of fodder has been shown to have about 89% moisture content (Kumar *et al*., 2009). This characteristic makes this type of animal feed vulnerable to *Aspergillus* contamination and subsequent mycotoxin production.

2.3.3 Compound feeds

Compound feeds are commonly referred to as feedstuffs or animal feeds that are formulated from various raw materials (plants and/or animals) and fortified with additives to make up feeds also known as concentrate feeds (Chandrasekaran and Sundrain, 2001). These compound feeds are of high nutritional value unlike other feeds mentioned above. The target animal determines the specific requirements that determine for the final formulation of a particular feed (Thomas *et al*., 1988). It could be formulated depending on the purpose for raising such livestock, the age/species of livestock and the type of ingredients available. Compound feeds provide all the daily required nutrients which forms part of the supplements that only provide additional micronutrients (FAO/WHO, 2001). They play a role in providing concentrated sources of nutrients necessary for livestock production i.e. mono-gastric (example pig, poultry, etc.) and ruminants (example cattle, goat, etc.). Mono-gastric have limited capacity to digest fibre (Kohlmeier, 1990), thus, they require less roughage but diets of very high nutrient density and higher proportion of concentrate feed. Ruminants are able to digest fibrous feeds but are also provided with concentrate feeds especially during intensive system of livestock rearing i.e. production of milk or fattening period, with an aim to meat production (Hall *et al*., 2009).

Like forages and fodders, compound feeds are commercially produced and stored, as they are essential to farm animals' food chain. Nevertheless, contamination of these feeds by fungi is common and poses a big threat to international trade and to animal health and productivity.

2.4 Compound feed production

The beginning of industrial scale production of compound feeds can be traced back to the late 19th century (Weaver, 2007). Since then, feed production has increased significantly due to increased commercialization of the animal industry. The efficiency of feed utilization in a country such as South Africa depends on the type and quality of feeds produced (Reddy, 2001) which is based on the nutritional compositions and constituents (as discussed in Section 2.2.2 of this chapter) for feed formulation.

In South Africa, compound feed production on a large scale started in the 1930's during the big droughts and great depression (Dunn, 2011). From then, alternatives for feeding systems were developed. The use of offals / by-products of other industries came into place e.g. wheat bran; groundnut, offal and brewers grain. Feed mills were erected in close proximity to these sources and rail networks. A structured industrial body known as Balanced Feed Manufacturers' Association, overseeing feed production chain (Figure 2.3) and representing the feed industry's interests, was established. In 1988, this association was renamed; Animal Feed Manufacturer's Association (AFMA) to represent the feed producers including non-AFMA members in South Africa (Loutjie, 2011). Production was based on a calculation of livestock requirement in the country established in 2004 and was updated in 2006 (Dunn, 2011).

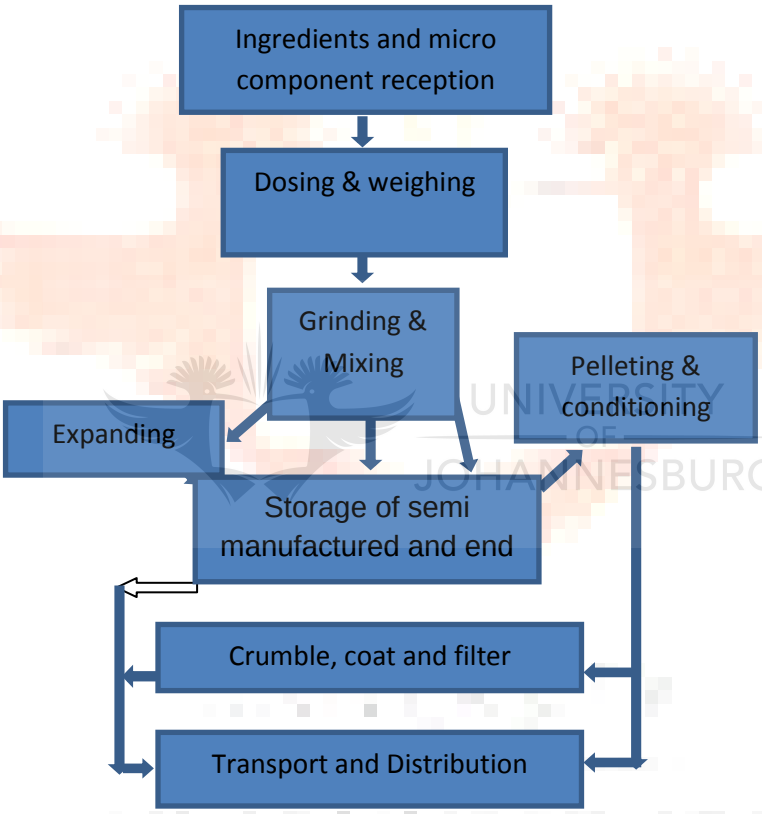


Figure: 2.3 Flow chart showing compound feed production chains.

Averagely, annual growth of commercial feed sales in South Africa, is about 3% and feed production per annum is over 8 million tonnes (Loutjie, 2011) as shown in Figure 2.4.

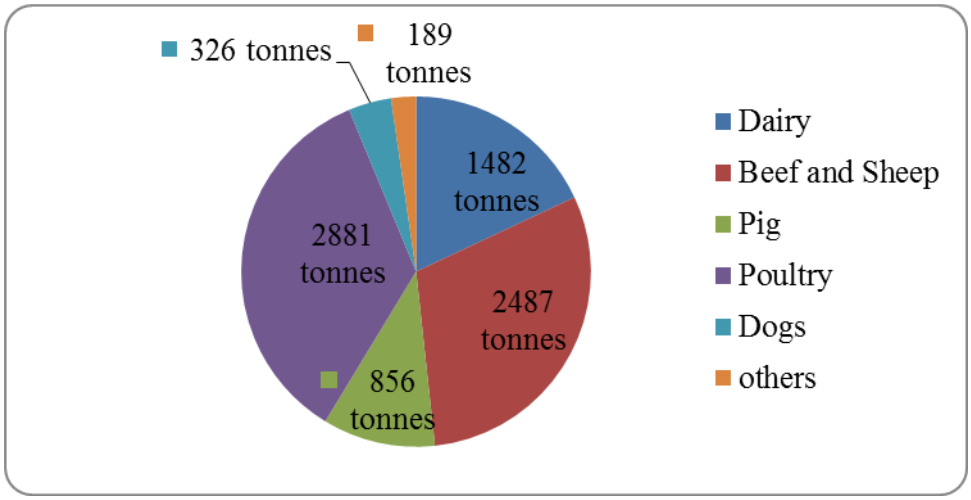


Figure 2.4 Pie chart representing the statistics on total feed production in South Africa, as updated in 2006

Production in the formal compound feed industry (AFMA members) gradually increased from 3.9 million tons in 1997/98 to 4 million tons in 2001/02, 4.3 million tons in 2004/05, and 4.7 million tons in 2006/07 with a gross turnover value of 8.3 billion Rand (Dunn, 2011). However, total national feed production as of last year is estimated at about 10.7 million tons per annum with a gross value calculated to be between 22-25 billion Rand (Loutjie, 2011).

The main ingredients used in the formulation of commercial compound feeds are cereal grains and legume seeds. These include corn, soybeans, sorghum, peanuts, cotton seed oats, barley and premixes. Premixes are composed of micro ingredients such as vitamins, minerals, chemical preservatives, antibiotics, fermentation products and other essential ingredients. These compound feeds can be manufactured by feed compounders as meal type, pellets and/or crumbles which give rise to different types of compound feeds with varying nutritional compositions.

2.5 Types of compound feeds produced in South Africa

Various compound feeds for different classes of livestock are produced in South Africa. This formulation involves the judicious use of feed ingredients (corn, soybeans, sorghum, oats, and barley) as in Table 2.1, with respect to the compound feeds of the present study. This is to supply in adequate amounts, nutrients required by the animal. Formulation of these feeds, in specific ratio or percentage composition of nutrients and premixes, makes up the types of animal compound feeds in South Africa, as specified by AFMA.

The different feed types include feed for cattle, poultry, pig and those that are classed as miscellaneousfeeds.

Table 2.1 Major components of compound feed samples collected from feed producers from South Africa (adapted from Njobeh *et al.*, 2012)

Feed Type	Nutrient components
Poultry	
Breeder	Maize, maize germ, soya oilcake, sunflower oilcake
Broiler	Maize, maize germ, soya oilcake, sunflower oilcake
Layer	Maize, maize germ, soya oilcake, sunflower oilcake
Cattle	
Dairy	Maize, maize germ, cottonseed, soya oilcake, sunflower oilcake
Calf grower	Maize, maize germ, cottonseed, sunflower oilcake, Lucerne meal
Finisher	Maize, maize germ, cottonseed, sunflower oilcake, Lucerne meal
Others	
Horse	Maize, maize germ, full fat soya, Lucerne meal
Pig	Maize, maize germ, soya oilcake, sunflower oilcake

2.5.1 Cattle feed

Feeds for cattle can be categorized into feed for dairy, beef cattle, calve, heifers, etc. However, dairy feed takes up the largest share of the cattle feed market (www.mediaclubsouthafrica.com, 2012). Cattle's feeding mostly involves roughages (forage and fodder). These materials are not sufficient in supplying the required nutrients especially for milk production and fattening (NRC, 1981). As such, it is of paramount importance to supplement them with concentrate feeds. In some cases, these roughages are chopped to small particle sizes and mixed with the concentrate feeds during formulation. Sometimes, more unusual ingredients are used which range from dried citrus fruit pulps to dried coffee residues, including nutritionally improved straw and feather meal (UKASTA, 1996, Barfe, 1997). Feeds for cattle are mostly comprised of cereals and by- products sourced from plants and animals (Ross, 2000). As a result of high feed cost, cattle feeds may be mixed with stale bread and bakery waste products (Sultana and Hanif, 2009). These waste products, commonly tainted with fungi, can contribute to mycotoxin synthesis in cattle feed. In hot and humid environment common as the case may be in some parts of South Africa, chances of important mycotoxins like AFs are more likely to be found in cattle feeds. An elaboration on contamination of cattle feeds and other feed types by fungi and mycotoxins will be provided in the subsequent section of this review.

2.5.2 Poultry feed

Poultry feeds include feed for chickens (layers and broilers), turkey, ducks, geese, quails and domestic fowl feeds. The feed intake of poultry is affected by the nutrient composition of the diet

(Mingan, 2009). According to Hy-Line Variety Brown Commercial Management Guide (2006-2008), the optimum nutrient composition and intake of feed for a particular type of poultry raised commercially depends on the commercial goals of the poultry establishments/enterprise. During the production process of these feeds, there are different risks concerning feed safety which could occur (Nørrung and Buncic, 2008). An example is the lubricating jelly used for machines, which could contaminate the product. Aside from this, there are other contamination possibilities like physical, microbiological and fungal contamination. As these feeds are processed and stored, they are highly susceptible to AFs contamination and some other mycotoxins (Mabett, 2004; Opara and Okoli, 2005). This may be as a result of various grains and root tuber based raw materials which may be infested with mycotoxigenic fungi that are used as components of poultry feeds (Okoli *et al*., 2007).

2.5.3 Pig feed

According to Brendemuhl and Myer (2009), feeding of pigs can be divided into three categories; feeding of the piglet**,** feeding of the growing pig up to slaughter and feeding of the boar and sow for reproduction. Gerbaldo *et al*. (2011), noted that diet based exclusively on a balanced commercial feed to obtain the best product is not always an economically viable alternative. This made the natural or traditional beer industry brewer's grain (by-product) useful as a feedstuff intended for swine. The susceptibility of pig feed to fungal contamination and mycotoxin production at a later stage is not always inevitable as the feed consists of susceptible crops such as cereal grains. These susceptible crops are the primary ingredients (corn, soybeans, sorghum, oats, and barley) for most animal feeds and often of sub-standard grade, predisposed to mycotoxin (AFs) contamination (Phakamile *et al*., 2008) and contains high moisture levels of about 80% (Johnston and Hawton, 2008) which favours *Aspergillus* aflatoxin producers fungal niche. Studies that were performed in Brazil showed the presence of the fungal flora as well as different mycotoxins in pig feed (Simas *et al*., 2007; Cavaglieri *et al*., 2009).

2.5.4 Miscellaneous feeds

Other types of compound feeds produced in South Africa have been classified as miscellaneous feeds according to AFMA. Such feeds include feeds for dogs, horse, fish and ostrich.

Feed for dogs are commonly referred to as dog food. They come in different forms ranging from (a) canned dog food which is usually moist with good tastes, easily digestible and has long shelf life (O'Grady, 2007); (*b*) semi moist merit, which is easy to store, typically tastes good and easily digestible (Bonham, 2007); and (c) dry food merit, which often presents storage problem to get stale and lose some nutritional value (Helgren, 2001). Most quality dog foods provide ample nutrition for dogs. The ingredients used in the formulation of dog food are derived from natural sources. Some of the more common natural ingredients include vitamins, minerals, flax, omega 3

oils mainly from fish is more suitable (Jones, 2009), amino acids of plants and animal related components such as herbs, colostrum, glucosamine and digestive enzymes. The availability of water and ingredient types make pet food susceptible to fungal and mycotoxin contaminations. Very few studies have focused on mycotoxins in dog food, which may be due to a lack of interest in their productivity (Basalan *et al*., 2004a).

In horse feed formulation, there are a large number of different feeds available and no two horses are alike (Sauvant *et al*., 2004). The amount and type of feed given will depend on the type of horse, age, weight, health, workload, climate and what is locally available. However, feed for horses needs to meet seven fundamental requirements (USDA, 1999) which include energy, mineral and vitamins, bulk and high fibre content, digestibility, safety, continues feeding and stimulation. Majority of horse feeds are made from corn, oats, other cereal grains and they are highly susceptible to aflatoxins contamination (Aller *et al*., 1981). Studies (Gunsen and Yaroglu, 2002; Basalan *et al*., 2004b; Buckley *et al*., 2007; Keller *et al*., 2007; Sacchi *et al*., 2009) have evaluated several horse feeds in terms of *Aspergillus* fungi and AFs contaminations and results have been positive in this respect and in terms of exceeded limit of 20 ppb present in some samples (FDA, 1989).

Fish feeds which can be in a plant or animal formulation (Landsvik, 2008), normally contains macro-nutrients, trace elements and vitamins which are paramount to keep fish in good health (Axelrod, 2007). About 80% of fish feed are exclusively prepared foods that are commonly produced in flakes, pellet or tablet form (Riche and Garling, 2003). They are an important part of modern commercial aquaculture. Modern fish feeds are made by grinding and mixing together ingredients such as fishmeal, vegetable proteins and binding agents such as wheat. Water is added and the resulting paste is extruded through holes in a metal plate (Landsvik, 2008). The nutrients composition make up of this feed have identified the presence of AFs and other mycotoxins (Bhatnagar *et al*., 2004).

Ostrich (ratites) husbandry is limited relative to other livestock production, including South Africa. Most of the times, its feed formulation poses a great challenge, because there is little scientifically based information on their nutrient requirements (Scheideler and Sell, 1997) and efficiency of nutrient utilization by ostriches. However, the knowledge of nutritional requirements for poultry and other closely related species have successfully developed feeding programs by the use of basic physiological and historical information available about ostriches. Because these feeds are produced from cereal grains and other agricultural products, they are prone to fungal contamination and mycotoxin production. Reports on contamination of ostrich feed by AFs and its fungi is very limited to the extent that several findings on *aspergillosis* in ostrich has been based on

cumulative historical findings since the end of the 19th century into the early 20th century (Khosravi *et al*., 2008).

Generally, feeds for animals are mostly produced on a commercial volume (tonnes) and stored for considerable period of time. Fungal contamination and proliferation in these feeds is considered one of the most common forms of spoilage as off-flavours and an unpalatable taste does occur leading to the production of a wide array of mycotoxins therein. This may constitute public health hazard as well as result in economic losses (Hassan *et al*., 2007; El-Ahl and Rasha, 2010) hence the need for proper storage and preservation.

2.6 *Feed storage and preservation*

Feeds are produced, stored and preserved for the purpose of being constantly available in the farm, since it may not be practicable to produce feeds on daily basis. It is then very imperative that all processes (Figure 2.2, above) to produce a finished animal feed are put under check and control, and even after production. Though fungi, its spores and secondary metabolites are unavoidable contaminants (USDA, 1999) in animal feeds, meaningful approach systems should be put in place to reduce if not, totally eliminate fungi and mycotoxins. Storage facilities both on and off-site must always be clean prior to intake of feed raw materials. “Dead” spots in these facilities (like a conveyor system) have the capacity to accumulate organic matter which may become colonized by toxigenic fungi, distributing spores into and all over fresh materials (Cruz and Diop, 1989). Fungal growth inhibitors should be used regularly to clean and treat facilities. Sieving should be used to clean out broken kernels and chaff that are prone to fungal colonization to prevent cross contamination (FAO/WHO, 2001). These measures should reduce contamination or at best prevent or eliminate it. During storage, recommended moisture levels for a specific feed type must be reached, as moisture greatly contributes to the activities of fungi. Generally, it is recommended that feeds of cereal origin are stored at moisture levels below 14% and those of legumes stored at moisture levels of 9 – 11 %. Reducing the risk of insect and rodent damage can also reduce the risk of fungal colonization and subsequent mycotoxins production (Edwards, 2004). Temperature control of storage structures is also an important consideration as the ideal temperature range for fungal growth is 25-30 °C. The recommended maximum for stored commodities being 18 °C (Chulze, 2010). With forced air systems, stock rotation, temperature control could be achieved within the storage facility. This however, may be expensive to maintain or not practical in a sense. Feeds stored in bags can be stacked on pallets, standing free of walls and ceilings, to be protected from damage by moisture (Manjo and Tridib , 2009).

As these feeds are manufactured and stored, production activities in South Africa have been and are still being regulated and guided by a government act on mycotoxin and an overall international

body which AFMA and non-AFMA have a working relationship with. There are technical committees that liaise in this respect towards good manufacturing practices according to certain guidelines and regulations.

2.7 *Regulations and guidelines of compound feed production in South Africa*

Compound feed production relative to mycotoxin content amongst others in South Africa is regulated by the Department of Agriculture and Department of Health in close collaboration with AFMA (Loutjie, 2011). However, AFMA still strictly adheres to EU regulations and guidelines for compound feeding, also taking into account those of the United States. There are some situations where following the EU and US regulation guidelines are not achievable. In such a situation, the matter is thoroughly studied by the AFMA technical committee and recommendations submitted to the Department of Agriculture for consideration and implementation. According to Loutjie (2011), successes and positive outcomes are achieved through this, confirming AFMA's commitment to maintaining a long-term objective to foster good co-operation and a positive working relation with this institutions.

Because large amounts of feeds are produced and stored for considerable period of time, they are very often prone to fungal contamination and subsequent mycotoxin production. It is thus imperative to provide a detailed review on fungal and mycotoxin contamination of feeds in the proceeding section.

2.8 *Fungi*

Fungi are a large group of spore-producing organisms, which feed on organic matters of plant or animal origin (Concise Oxford Dictionary, 2011). They have well defined membrane-bound nuclei with a number of chromosomes (Moss and Smith, 1985). Before, fungi were classified as plants but with the concept of living things being organized into large, basic groups called kingdoms, scientists learned that fungi show a closer relation to animals, but have unique and separate life forms. This has made fungi to be placed in their own Kingdom (Glazer and Nikaido, 2007).

2.8.1 Concepts of fungi colonization

Fungi are ubiquitous in the air from which they can have access to any organic habitat to infect and use them as food (Krings *et al*., 2007). With thread-like hyphae, they form a feeding network of mycelia and take up nutrients in soluble forms via their cell wall (Moss and Smith, 1985). Fungi can be a parasite or saprophyte of agricultural commodities enabling them to cause diseases at some points (Simberloff and Rejmánek, 2011). Reproduction by these fungi is either sexually or asexually (mostly of a typical fungus). However, many fungi imperfecti (have no sexual fruiting structure) are known. They spread as spores growing on solid or liquid surfaces as long as the

conditions of moisture and temperature are ideal (Kanaani *et al*., 2008). They can be classified into kingdom, phylum, class, order, genera and species.

2.8.2 *Aspergillus* genera and species

The *Aspergillus* exhibit immense ecological and metabolic differences (Perrone *et al*., 2007). They are filamentous cosmopolitan microbes that can be isolated from soil, plant/animal debris and indoor environment and reproduction is by cell division (Geiser, 2009). Members belonging to this genus of spore-bearing fungi appears like an *aspergillum* (a device used in the Catholic Church to sprinkle Holy water) in structure hence the name *aspergillus* as coined in 1729 by Micheli, P.A. (Asan, 2004). This structural similarity indicated a good fortune because *Aspergilli* have indeed been considered useful to humankind, as they have been used in commercial food processes (e.g. *A. niger)* and medicinal drugs (lovastatin; from *A. terreus* and Cilofungin; from *A. nidulans*) production. However, the genus has also been and is still a curse, as it invades agricultural commodities including animal feeds and subsequently produces secondary metabolites (Klich, 2002) which degrade the quality of these products resulting in serious economic losses to livestock production.

This fungal genus competes with *Penicillium* [300 members] and *Fusarium* [191 members] genera in the fungi floral world (Pitt and Hockings, 1997) as there are over one-hundred and eighty members that belong to this genus (Pitt *et al*., 2000). These include *Aspergillus fumigatus, Aspergillus parasiticus* and *Aspergillus flavus* which are most commonly isolated (Clipson, 2010). There are other species which are less common as opportunistic pathogens including *Aspergillus versicolor, Aspergillus ochraceus, Aspergillus terreus*, *Aspergillus oryzae, Aspergillus* (*Emericella*) *nidulans, Aspergillus japonicas, Aspergillus clavatus* and *Aspergillus niger* (Hoog, 2000, Samson *et al*., 2004, Clipson, 2010).

Several reports (Bossche *et al.*, 1988; Bennett and Klich, 1992; Powell *et al*., 1994; Smith, 1994; Njobeh *et al.*, 2010b) have in various aspects, addressed the economic and medical importance of *Aspergillus* genera. There are those that could be useful, but others are toxigenic (producing mycotoxins) or pathogenic (causing diseases) in animals either by way of their food/feed primary contamination (fungal growth on food/feed) or by way of their secondary metabolism (mycotoxin production). Of the 180 species known, about 60 are pathogenic causing diseases generally referred to as *aspergillosis* (Bozkurt. M.K. *et al.*, 2008). Others are used in commercial microbial fermentation (Thom and Church, 2001). *Aspergillus niger* perhaps has been widely used as a major source of citric acid, accounting for over 99% of global production of citric acid, including enzymes (Archer *et al*., 2008). Some other members of this fungal genus that produce ergot alkaloids, e.g., some isolates of *A. fumigatus* (Rao and Rao, 1975) can also be used to develop medications of natural products that could be used to treat diseases like Parkinson's disease and

migraine (Fenical *et al*., 2007). Members belonging to this fungal genera that are of problematic of pathological importance to animals are *Aspergillus fumigatus*, *A. flavus, A. parasiticus* and *A. clavatus* (Zirbes and Milla, 2008), while others are agricultural saprophytes or parasites, colonizing and causing degradation of many agricultural commodities.

2.8.3 Factors enhancing compound feed colonization by aflatoxigenic fungi and aflatoxins production

Reports of Santine (2005),Krnjaja *et al.* (2008) and Stanković *et al. (*2009) have indicated the susceptibility of compound feeds to aflatoxigenic fungal attack and colonization. This colonization and growth are generally enhanced by a variety of factors leading possibly to aflatoxins production. The most important factors that could promote growth of these fungi could be categorised into three (Figure 2.5). A brief review of how each of these factors enhances fungal growth and subsequent mycotoxin production in agricultural commodities including animal feeds is provided subsequently.

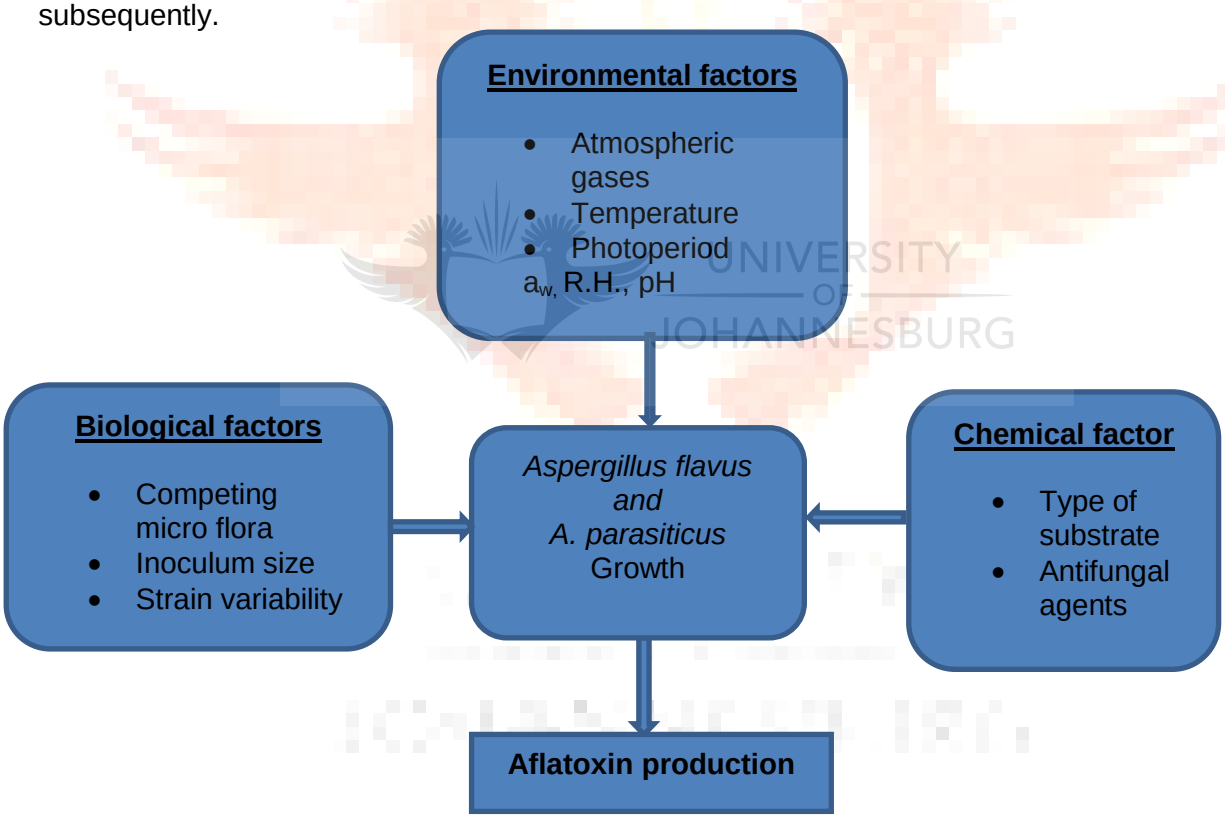


Figure 2.5 Factors influencing fungal growth and aflatoxin production

2.8.3.1 Environmental factors

The ability of members belonging to the *Aspergillus* genera to attack and colonize agricultural commodities is largely due to their relative versatility (Pardo *et al*., 2005). The capability of surviving under a wide range of temperatures is determined by the growth environment. The usual

temperature for colonization is in the range of 10-35°C (Mónica and Leda, 2002; Johnsson *et al*., 2008). This classifies them as mesophilic organisms. *Aspergillus flavus* and *Aspergillus parasiticus* growth is in most cases favoured by hot dry conditions. The optimum temperature for their growth is 37 °C (98.6 F) (Payne, 1998). Although fungus readily grows within the temperature ranges of 25-42 °C (77-108 F), and will grow at temperature ranges from 12-48°C (54-118 F), this high temperature contributes to their pathogenicity on animals (Richard and Payne, 2003).

Fungi require water for absorption of nutrients and metabolic activities. Water requirements for fungi are usually expressed as (a_w). Water activity is a key determinant for fungal growth as it predicts the growth of the fungi, since they require a certain amount of water to support growth. However, the “available" water, differs considerably depending on the feed type. Not only is available moist/wet substrate essential for colonization, but also water from the atmosphere (Carlile and Watkinson, 1996; Ramakrishna *et al*., 1993). A moisture requirement for their growth is relatively low as most species of *Aspergillus* grow at a 0.85 a_w or less (Miguel *et al*., 1986). According to Lstiburek (2002), when a_w is expressed as a percentage, it is equal to 1/100th the equilibrium relative humidity (RH). The (RH) expresses the amount of moisture in the air which can promote fungi growth, as a percentage of the total moisture the air can contain at a particular temperature. In most cases, fungi will start to make spores and grow when feed nutrients are exploited or when triggered by certain environmental factors like water. This usually occurs within 72 hrs under high moisture conditions.

Other important factors considered in influencing fungal colonization are pH and oxygen potentials. Information on the influence of pH on the colonization of *Aspergillus* is of limited value. This is because their secondary metabolism alters pH during their growth (Wheeler *et al*., 1991). *Aspergillus* species are more tolerant to alkaline than acidic conditions. Hydrogen ion concentration influence growth either indirectly by its effect on the availability of nutrients or directly by action on the cell surfaces (Wheeler *et al*., 1991). Feed spoilage aflatoxigenic fungi have an absolute requirement for oxygen. The total amount of oxygen available, rather than the oxygen tension (concentration of oxygen at specific pressure), determines growth (Mónica and Leda, 2002). The concentration of oxygen dissolved in the substrate has a much greater influence on *Aspergillus* fungal growth than atmospheric oxygen tension (Pitt and Hockings, 1997). The most oxygen demanding species will colonize the feed surface, while the less could be found inside the feed.

According to Pitt and Hocking (2009), light will increase or more commonly reduce the spread of these *Aspergillus* fungi in a natural environment. This effect may be due the photochemical destruction of components of the medium (Morin and Hastings, 1971) but in other instance it could

be a direct effect on metabolism. There have been demonstrations of pigment biosynthesis, mainly carotenoids (Gruszecki, 2004) as regards to this phenomenon of fungal growths.

2.8.3.2 Chemical factors

Fungi requires exogenous materials to form their biomass (Dighton, 2003). *Aspergillus* species can utilize different carbon sources for building of carbohydrates, lipids, nucleic acids and proteins, sourcing energy from sugars, alcohols, proteins and lipids (Solís-Pereira *et al*., 1993). The availability and type of chemical nutrients such as carbon and nitrogen source can enhance growth of these fungi.

Antifungal agents i.e. fungicides differ in chemical nature, properties and mechanism of action (Carlile and Watkinson, 1996). The correct use of fungicides to diminish mycoflora could lead to a reduction in the amount of mycotoxins produced. But according to Moss and Frank (1987), the use of sub-lethal concentrations could favour toxins production. It is also possible that the antifungal agent increase the synthesis of mycotoxins, without affecting fungal growth (Draughton and Ayres, 1978; 1982).

2.8.3.3 Biological factors

Aflatoxigenic fungi can attack and colonize agricultural commodities by certain biotic mechanisms. Mycoflora which is a fungal population within a specified environment and the simultaneous presence of different microorganisms such as bacteria or other fungi could influence *Aspergillus* growth (Velicer, 2003), hence AF production. Several microorganisms have been regarded as biological pest agents and fungi strain can serve as a determinant for fungal attack and colonization of agricultural commodities like animal feeds (Nampoothiri *et al*., 2004).

Aspergillus fungi can derive all of their energy requirement and materials for growth from a medium (substrate), through biochemical decomposition processes. A large number of substrates can influence fungal growth (Chang *et al.* 2004). Nevertheless, the nutrients required for growth must already be present in the growth medium. These fungal species grow well at relative substrate moisture levels of 50 to 75% (Pardo *et al*., 2005). An ideal substrate for their growth will usually contain enough nitrogen and carbohydrate for rapid growth (Job, 2004). Common bulk substrates for growth include: wood chips or sawdust, mulched hay, straw-bedded horse or poultry manure, corncobs, waste or recycled paper, coffee pulp or grounds, nut and seed hulls, cottonseed hulls, cocoa bean hulls, cottonseed meal, soybean meal, brewer's grain, ammonium nitrate, urea. From these substrates, fungi metabolize complex carbohydrates i.e. glucose, which is then transported through the mycelium as needed for growth and energy (Philip, 2006). The glucose is used as a main energy source and its concentration in the growth medium do not exceed 2% (Chang *et al.*

2004). Many mycological substrates have high carbohydrate levels which warrant rapid growth of fungi. However, repeated invasions of fungi on rich substrates frequently, can lead to loss of virulence and substrate degeneration.

The genotype and physiological adaptation of aflatoxigenic fungi to an environment could determine their growth (Bhatnager *et al*., 2008) and colonization. If an isolate does not produce AFs under given conditions, this does not justify any conclusion about its general ability to produce AFs. Any grouping into AFs "producers" and "non-producers" based on such data can be misleading (Mühlencoert *et al.*, 2004).

Insect pests, rodents, water and wind can serve as vectors for aflatoxigenic fungal colonization of feed (Farrar and Davis, 1991; Makun *et al*., 2012). Insects and rodents defecate and urinate on feed material thereby, increasing the moisture content in feed, which is another factor that encourages microbial contamination (Njobeh, 2003). Furthermore, they may also act as agents of fungal spore dissemination alongside wind and water resulting in cross contamination of feeds. This may be via transport of primary inoculums; move inoculum throughout the commodity; disseminate spores within the feed; and/or facilitate colonization or infection by injuring the foodstuff (Payne and Brown, 1998). Consequently, an infestation of insects predisposes the commodity to the attack of different species of fungi and subsequent production of mycotoxins (Farrar and Davis, 1991).

2.8.4 Identification of *Aspergillus flavus* and *parasiticus*

In the classification system of fungi, *A. flavus* and *A. parasiticus* belong to the same domain (*Eukarya*), kingdom (Fungi), phylum (*Ascomycota*), class (*Eurotiomycetes*), order (*Eurotiales*), family (*Trichocomaceae*), and genus (*Aspergillus*). They differ in species level, hence the names; *Aspergillus flavus, Aspergillus parasiticus* (Klich and Pitt, 1988). Earlier before now, *Aspergillus flavus* was known to be producing only AFB_1 and AFB_2, while *A. parasiticus* produces AFB_1, AFB_2 AFG_1 and AFG_2 (Horn *et al*., 1996). With recent studies (Cardwell, 2002; Cotty and Cardwell, 1999; Varga *et al.*, 2009), the question of which isolate produces what AFs is now rather complex. However, they both have different aflatoxigenic profile (El Khoury *et al*., 2011) based on DNA (*nor-1*) sequence and AFLP fingerprint analyses (Barros *et al*., 2007; Patterson, 2009).

Microbiological characteristic forms and structures of these species of fungi can be described based on two morphologies, i.e., macroscopic and microscopic morphologies. A full description of the macroscopic and microscopic features of *A. flavus* (Figure 2.6) and *A. parasiticus* (Figure 2.7) are provided by Klich (2002). Accordingly, the macroscopic characteristics of *A. flavus* grown on PDA at 25 °C for 7 days are that the conidia is olive to lime green with a cream reverse. The colony's texture is woolly to cottony, to somewhat granular. The sclerotia are not always present and when present, they are dark brown. The exudates when present are clear or pale in colour.

Microscopically, the hyphae of *Aspergillus flavus* are septate and hyaline in very close view. The conidia are globose to sub-globose, 3-6 µm in diameter with smooth to very finely roughen walls. The conidiophores are coarsely roughened, uncoloured, about 800 µm long x 15 – 20 µm wide, with globose to sub-globose vesicles of 20-45 µm in diameter. The metulae are about 8-10 x 5-7 µm in diameter, covering nearly the entire vesicle in biseriate species not uniseriate. Isolates that may remain uniseriate produce only phialides which are 8 - 12 x 3 – 4 µm in diameter, covering the vesicle.

Figure 2.6 Macroscopic veiw (*left*) and microscopic view (*right*) of *Aspergillus flavus* (Chang, 2009)

The macroscopic morphology of *A. parasiticus* colonies on PDA at 25 °C of incubation are effulgent lime green with a cream white reverse. Its texture is woolly to cottony to somewhat granular. They sclerotia are also not always present just like *A. flavus* but when present, are effulgent dark brown. The exudates are clear and effuse pale but also present only in some isolates. The microscopic hyphae of *Aspergillus parasiticus* are also septate and hyaline. They have globose to subglobose conidia, which are about 3 - 6 µm in diameter and are very rough. The conidial heads are radiate to loosely columnar with age. The conidiophores are closely roughened, uncoloured within about 800 µm long x 15-20 µm wide, with globose to subglobose vesicles of 20-45 µm in diameter. The metulae diameter is about 8-10 x 5-7 µm, covering nearly the entire vesicle in biseriate species not uniseriate. Isolates that may remain uniseriate produces only phialides which are 8-12 x 3-4 µm in diameter, covering the vesicle.

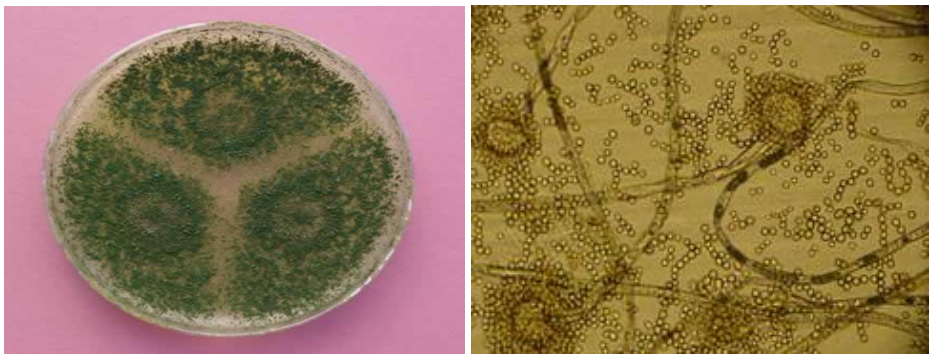


Figure 2.7 Macroscopic view (*left*) and microscopic view (*right*) of *Aspergillus parasiticus* (Chang, 2009)

Although both species have been considered to be strictly asexual and lack ability to undergo meiosis (Geiser *et al.*, 1996), a recent study of Horn *et al*. (2009) revealed that sexual reproduction of *A. flavus* occurs between compatible sex strains that belonged to different compatible vegetative groups. They can be easily distinguished phenotypically and genotypically by expert scrutiny aside from being separated by their morphology, mycotoxin profile and molecular characters. They cause the same diseases known as *aspergillosis* (Walsh *et al*., 2008; Patterson, 2009) and produce AFs.

2.9 *Aflatoxins*

The origin of the name aflatoxin is traced back to about 40 years ago after an outbreak of Turkey X disease in England, when it was originally extracted from *Aspergillus flavus,* hence the name a-fla-toxin, a type of mycotoxin (Klich *et al*., 2000). It is produced primarily by *A. flavus* and *A. parasiticus* (D'Mello and MacDonald, 1997; Williams *et al*., 2004; Richard, 2007). *Aspergillus flavus* generally produces only the B types and sometimes the mycotoxin, cyclopiazonic acid (CPA), while *A. parasiticus* produces both B and G types, but never CPA (Schroeder and Boller, 1973; Dorner *et al.*, 1984; Klich and Pitt, 1988; Pitt, 1993). However, from some recent reports (Pitt, 1993; IARC, 2002), other fungi belonging to the *Aspergillus* genera have been found to produce AFs (Table 2.1).

Table 2.2 Taxon of *Aspergillus* fungi species associated with aflatoxin production

***Aspergillus* species**	**AF(s) produced**	**References**
Aspergillus nomius	B and G	Saito *et al*. (1989), Pitt (1993), Ito *et al*. (2001)
Aspergillus bombycis	B and G	IARC (2002), Peterson *et al*. (2001)
Aspergillus ochraceoroseus	B and G	Frisvad (1997), Klich *et al*. (2000), Keller *et al*., (2000)
Aspergillus australis	B and G	IARC (2002)
Aspergillus tamarii	B	Ito *et al*. (2001)
Aspergillus pseudotamarii	B	Ito *et al*. (2001)
A. flavus var. parvisclerotigenus	B	Stubblefield *et al*. (1970)
Aspergillus flavus	B and G	Varga *et al*. (2009)
Aspergillus parasiticus	B and G	Cardwell (2002), Cotty and Cardwell (1999)

The ecophysiological factors that influence the production of AFs are somewhat similar to those that enhance fungal growth as previously discussed in Section 2.8.3, which results to series of chemistry that arise to the detection of these secondary metabolite (AF).

2.9.1 Chemistry of aflatoxins

There are 16 structurally related AFs characterized (Holtzapple *et al*., 1996; Bhatnagar *et al*., 2003; Yu *et al*., 2004b; Cervino *et al*., 2007) so far. However, only four major ones denoted as AFB_1, AFG_1, AFB_2 and AFG_2 that often contaminate agricultural commodities (Bennett, 1987; Bennett and Klich, 2003; Bhatnagar *et al*., 1993; Cleveland and Bhatnagar, 1991; Cleveland and Bhatnagar, 1992; Kelkar *et al*., 1997; Payne and Brown, 1998), pose a potential risk to livestock fed contaminated feeds. According to Vladimir (1985), 20 fluorescent spots were spotted on thin layer chromatography to detect aflatoxins. The thin layer chromatography (TLC) plate was exposed to UV light, showed two major components of blue and green fluorescing and designated aflatoxins B and G (Eaton and Groopman, 1994). Later it was recognised that the B and G comprised of other two components that differed in by by their decrease in retardation factors (R_f) on TLC and were designated as B_2 and G_2. From infrared and UV absorption spectrum, all four compounds were seen to be closely related in chemical composition and structure. This structural composition is made up of a lactone ring, a vinyl ether system, a methoxyl group and the absence of a free hydroxyl group (Kye-Simeon and Stefan, 2012).

A team led by Professor Büchi of the Massachusetts Institute of Technology elucidated the structures of aflatoxins B_1 and G_1, B_2 and G_2 (Figure 2.7). This elucidation relied upon

interpretation of ultraviolet, infrared, nuclear magnetic resonance and mass spectra. Chromatographic evidence showed an extract of *A. flavus* culture grown on crushed peanuts contained a component probably identical to a toxin found in cow milk. This toxin was designated as aflatoxin M (Ewaidah,1984) and was resolved to be AFM_1 and AFM_2 at the C.S.I.R. laboratories in Pretoria based on R_f values and colour (blue-violet fluorescence for M_1 and violet fluorescence for M_2). There have been two additional hydroxyl aflatoxins, one fluorescing blue and the other green, which was isolated from *A. flavus* cultures as well and were reported as of B_2a and G_2a. These names present interesting peculiarities.

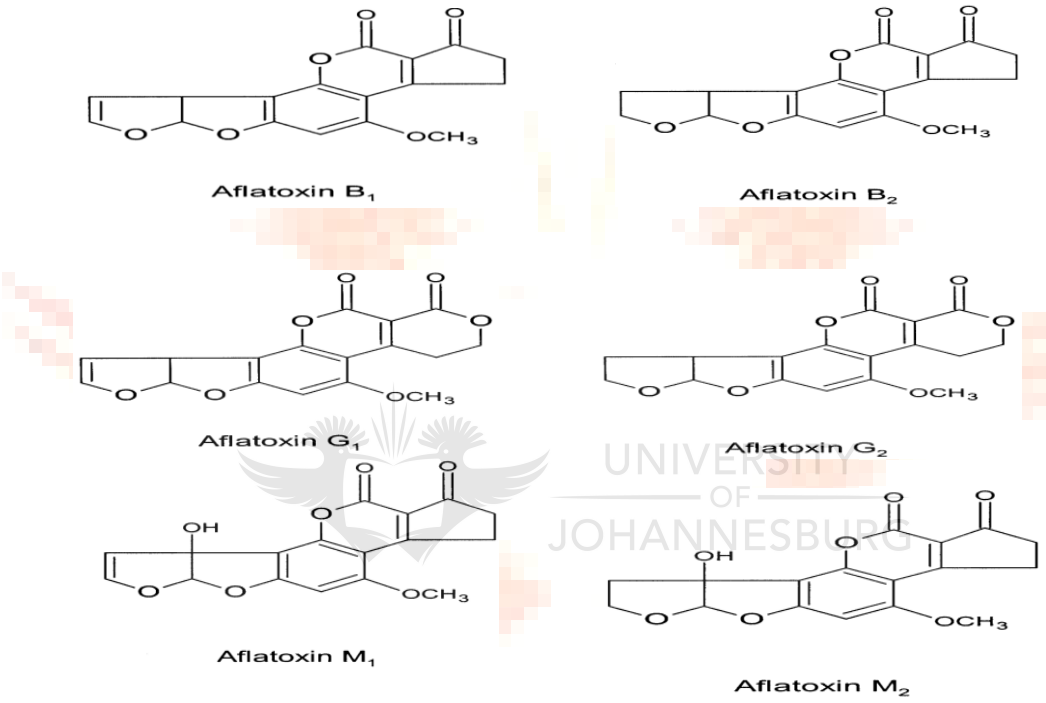


Figure 2.8 Chemical structures of aflatoxin B_1, B_2, G_1, G_2, M_1, and M_2

2.9.2 Biochemistry of aflatoxin production relative to the *NOR-1* gene

Aflatoxins are polyketide-derived secondary metabolites which are produced through the conversion path: acetate → polyketide → anthraquinones → xanthones → aflatoxins (Bennett *et al.*, 1980; Bennett and Christensen, 1983; Bhatnagar *et al*., 1991; Bhatnagar *et al*., 2003; Yabe, 2003; Yu *et al*., 2002). The conversion pathway involves the production of norsolorinic acid (NOR) through noranthrone. Noranthrone conversion to NOR is not well defined, but Trail *et al*. (1995) proposed it to occur through noranthrone oxidase, while Bhatnagar *et al*. (1992) proposed it via a mono oxygenase or it occurs spontaneously according to Dutton (1988).

The discovery of norsolorinic acid (NOR) made a major breakthrough in the biochemical pathway (Figure 2.9) of aflatoxin biosynthesis that led to the cloning of *nor-1* gene (Bennett *et al*., 1997; Bennett and Klich, 2003; Chang *et al*., 1992). The generic name was derived, just like those of many other genes in a biochemical pathway, based on the substrate converted by the gene product. *NOR-1* is the first stable precursor in the aflatoxin biosynthetic pathway (Dutton, 1988; Bennett *et al*., 1997; Schmidt-Heydt *et al*., 2009). The discovery of this precursor led to other fundamental AF intermediates that have been identified to establish the primary metabolites in the AF pathway. *NOR* provided the opportunity to isolate the first AF pathway gene i.e. *nor-1* that encodes a reductase for the conversion from *NOR* to eventually AFs (Hsieh *et al*., 1976; Chang *et al*., 2004; Chang, 2009).

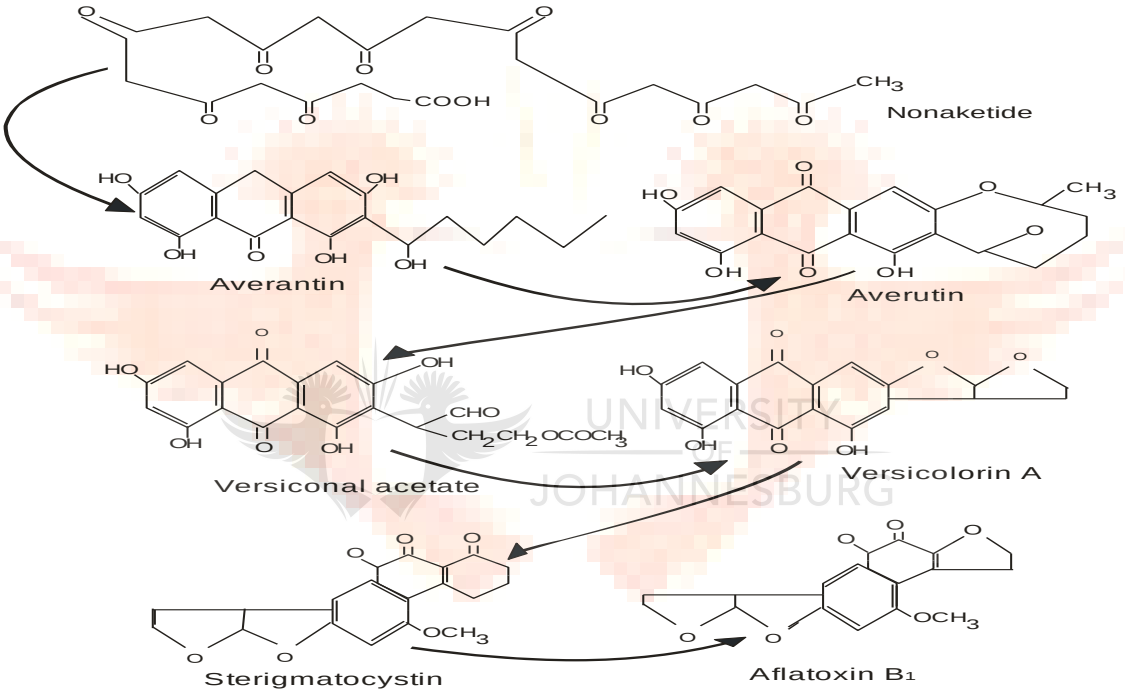


Figure 2.9 Biochemical pathway for aflatoxin B_1 (Dutton, 1988)

2.9.3 Biochemical pathway for aflatoxin production

At the molecular level, major biochemical steps and genetic components of AF biosynthesis have been elucidated only in the last two decades. Reports (Dutton, 1988; Minto and Townsend, 1997; Payne and Brown, 1998; Cary *et al*., 2000; Bhatnagar *et al*., 2002; Yu *et al*., 2004a) have described the biochemistry and genetics of AF formation to be a complicated process. It involves many levels of transcriptional and post-transcriptional control (Abbas *et al*., 2009; Chanda *et al*., 2009; Georgianna and Payne, 2009; Schmidt-Heydt *et al.*, 2009).

A study (Jiujiang *et al*., 2002) established that AFs are synthesized in two stages from malonyl CoA. The first being the formation of hexanoyl CoA as the starter unit to form a decaketide anthraquinone. A series of highly organized oxidation-reduction reactions then allow for the

formation of AFs (Dutton, 1988). The currently accepted scheme for AF biosynthesis is provide in Figure 2.9.

Hexanoly CoA precursor ⟶ malonyl CoA ----CoA + norsolorinic acid ⟶ averantin ⟶

Hydroxyaverantin ⟶ averufin ⟶ hydroxyversicolorone ⟶ versiconal hemiacetal acetate Versiconal ⟶ versicolorin B ⟶ versicolorin A ⟶ demethyl-sterigmatocystin Sterigmatocystin ⟶ O-methylsterigmatocystin ⟶ AFLATOXIN

Figure 2.10 Main bioconversion steps involved in aflatoxins synthesis that involve a set of genes coding for enzymes responsible for the metabolic steps (Jiujiang *et al.*, 2002)

The biochemical pathway for AF synthesis is described subsequently by Jiujiang *et al.* (2002). Accordingly, norsolorinic acid is derived from a polyketide pathway involving hexanoyl CoA and malonyl CoA resulting in an anthraquinone, norsolorinic acid. The nor-1 gene encodes a reductase in the conversion of norsolorinic acid to averantin. An avnAgene encodes a cytochrome P_{450} type monooxygenase involved in the conversion of averantin to hydroxyaverantin. The avfAgene encodes an oxidase involved in the conversion of averufin to versiconal hemiacetal acetate. The ver-1 and verAgenes encode dehydrogenases for the conversion of the versicolorins to demethyl-sterigmatocystin. The omtAgene encodes an O-methyltransferase for the conversion of sterigmatocystin to O-methylsterigmatocystin. The ordAgene encodes an oxidoreductase involved in the conversion from O-methylsterigmatocystin to AFB_1 and AFG_1. At the encoding of O-methyltransferase for the conversion of sterigmatocystin to O-methylsterigmatocystin, there exist a branch point that results to dihydrodemethylsterigmatocystin from demethylsterigmatocystin hence AFB_2 and AFG_2.

The conversion of NOR to AVN involves *Nor-1*, N*orA* and *NorB* (Bennett *et al*., 1997; Gallo *et al*., 2010; Jiujiang *et al*., 2012). This was demonstrated by Papa (1982) using NOR-accumulating mutants in *A. flavus* and by Bennett (1981) in *A parasiticus*. However, neither *nor*A or *norB* has a significant homology to the *nor-1* gene at either the DNA or amino acid level (Jiujiang *et al*., 2004; Passone *et al*., 2010). The conversion of NOR to AVN is catalysed/promoted by a reductase/dehydrogenase enzyme. The reaction is reversible depending on NADP(H) or NAD(H) (Bennett and Christensen, 1983; Bhatnagar *et al*., 1992; Dutton, 1988; Yabe *et al*., 1991). Cloning of the *nor-1* gene that complemented NOR-accumulating mutant of *A. parasiticus* was achieved by Chang (2009). This gene encoded a ketoreductase which can convert NOR to AVN (Skory *et al*., 1993; Trail *et al*., 1995). A possible allele of NOR reductase gene known as *norA* was cloned (Cary *et al*., 1996; Sardiñas *et al*., 2010). About 70% of the clone has a homology to aryl-alcohol dehydrogenase and is also involved in the conversion of NOR to AVN (Cary et al., 1999; Cary *et al*., 2000; Dyer *et al*., 2002).

A number of metabolic patterns that may provide alternate pathways to AFs and several specific enzyme activities associated with precursor conversions in the AF pathway have been elucidated (Dutton *et al*., 1990). As important AF pathway genes were cloned, a 75 kb aflatoxin pathway gene cluster which is very important in the biology of fungi was established in *A. parasiticus* and *A. flavus* (Yu *et al*., 1995). Studies (Bennett and Klich, 2003; Crawford *et al*., 2008; Chang *et al*., 1999; Ehrlich *et al*., 1999; Ehrlich and Yu, 2009; Yu *et al*., 2004b) have shown at least 27 enzymatic steps, starting from *nor*1 gene involved in bioconversion of AF intermediates to AFs.

Nor~1 gene is the determinant gene in the anabolic process involved in AFs production (Ehrlich and Yu, 2009) as it encodes a reductase that converts norsolorinic acid to averantin then subsequent reactions and enzymic conversions produce AFs. Significantly, progress has been made in elucidating the biosynthetic pathway, the pathway intermediates, genes, corresponding enzymes and regulatory mechanisms involved in the metabolic process of AF production. In present time, elucidation of genes involved in the biosynthetic process of AF production can be achieved using real time PCR.

2.9.4 *Real-time PCR* identification and quantification of *nor-1*

In the past, identification of fungi isolated from agricultural commodities has relied on the traditional morphological analysis. Recently, methods based on real-time PCR have attracted much attention (Brunner *et al*., 2009; Fredlund *et al*., 2010; Kulik, 2008; Yli-Mattila *et al*., 2008) for the enumeration of these fungi and their characteristic genes. The use of real-time PCR enumerates, identifies and quantifies *nor-1* present in a particular commodity. This molecular technique has been used in detecting fungi in foods and feeds (Shapira *et al.,* 1996; Somashekar *et al.*, 2004a,b; Price *et al*., 2005). Assays capable of detecting specific genes in aflatoxigenic fungi (*A. flavus* or *A. parasiticus*) involves defined characterization of PCR gene targets with substantial sequence variability (Niessen, 2008). Many genes with these characteristic features have been enumerated and now used in primers designs for the detection and quantification of genes (*nor-1)* involved in AF biosynthesis. Of these primers, the most frequently used are the intergenic spacer (ITS) 1 region rDNA, tri5 and translation elongation factor 1α gene (EF1α). These have, in studies of Henry *et al.* (2000), Patricia and Mark (2008), Prasanna *et al.* (2009), Maria *et al.* (2012) demonstrated to be both specific and provide accurate detection. Besides, probes and primers specificity as determinant factors to gene detection and quantification, essentially needed is a well-designed PCR setup for quantification. Absolute quantification method is achieved with real-time PCR approach. Its principle and technique have been based on a starting material of known concentration to be amplified.

In studies on gene quantification, the material for amplification is the DNA isolated from the mycelia of *Aspergillus flavus*/ *A. parasiticus* that was isolated and enumerated. With the

determination of OD_{260} of defined reference template and series of diluted amplification of known template concentrations, standard curve can be created (Dorak, 2006). Results generated are always used in the determination of the quantity of same target genes in samples of unknown concentrations and efficiency of the assay can be assessed. The expression of AF genes with phenotypic AF production in few studies (Geisen, 1996; Schmidt-Heydt *et al*., 2009) has been attempted under different environmental conditions.

Integration of correlation of ecophysiological conditions in relation to gene expression have also been studied (Jurado *et al*., 2008). To analyse *nor-1* gene content of a template gene, a standard curve is run simultaneously, in order to quantify the starting material using a threshold value (Georgianna, 2009). This has made it possible for the determination of the gene concentration as quantification is possible because the standard curve and the samples are run under the same conditions. Although, works in this light have been performed in some other agricultural products like maize (Mayer *et al.* 2003), coffee and peanuts (Passone *et al.* 2010) and wheat (Sardinas *et al.* 2011) and the resultant correlations found and justified, no such work has been done using compound feeds.

2.9.5 Aflatoxins occurrence and distribution in animal feeds

Occurrence of AF in animal feeds is worldwide, especially in sub-Saharan Africa including South Africa were, of the 1602 samples of feeds that constituted agricultural commodities submitted over a 10-year period for a commercial testing, 229 contained aflatoxins that varied from 1 to 500 ppb (Dutton and Kinsey, 1996). Distribution of AFs in agricultural commodities has been found in many geographically diverse regions of the world and has resulted in serious feed safety problems (Williams *et al*., 2004). The resultant health effect associated with AFs and its economic implications for the agricultural industry was first discovered centuries ago. Since then, AFs has been reported to contaminate a variety of compound feeds. Occurrences of such have been established and reported in recent case studies in South Africa (Table 2.2).

Table 2.3 Occurrence of aflatoxins associated with animal compound feed in South Africa

Feed type	Range (ppb)	Reference
Poultry	0.8±0.2 to 156±8	Mngadi *et al*. (2008)
Cattle	< 5 ±1.6 to 4946 ±21.3	Gordon (2005), Mwanza (2008)
Pig	2 ±0.1 to 200	Hannes (2009)
Dog	< 5±2.0 to 4946±19.0	Arnot *et al*. (2011)
Horse	0.8±0.2 to 156±8	Mngadi *et al*. (2008)

The occurrence of AF in compound feeds is considered as the most naturally occurring fungi metabolite, as they are termed as 'non-avoidable' contaminants by the United States Food and Drug Administration. Because of the contamination levels of AFs encountered in several animal feeds in South Africa at levels above regulated limits according to the National Department of Agriculture , this may likely pose some health effects and to the economy.

2.9.6 Effects of feeds contamination with aflatoxins

Aflatoxins represent a serious problem in feed safety in many parts of the world, especially in such developing countries as South Africa. Of the four major analogues of AFs, AFB_1 is known to be the most abundant and toxic. This class of toxins has been recognised as a major contaminant of feeds, being responsible for decreased feed quality resulting in mortality among livestock and humans (Murjani, 2003), with a consequential loss to the economy.

2.9.6.1 Socio-economic effects

The economic losses associated with AFs contamination of feeds includes but not limited to discarding of feeds/foods, trade rejections, loss of income, loss of livelihoods, health-care costs and veterinary expenses, cost of feed borne disease surveillance and monitoring (WHO, 2006). Data on the socio-economic impact of AFs in South Africa and Africa as a whole cannot be overemphasized and often underestimated. The Food and Drug Administration of Nigeria destroyed animal feeds worth more than US$ 200,000 as it contained large amounts of AFs (Anyanwu and Jukes, 1990). A World Bank study revealed that the European Union regulation on AFs costs nine African countries about US$ 750 million each year in exports of cereals, dried fruits and nuts (Diaz Rios and Jaffee, 2008). This was due to a standard harmonization of maximum acceptable levels of toxins in feeds by the European Union in 1997 that was implemented (Otsuki *et al.*, 2001a). According to Otsuki *et al*. (2001b), the new standard implemented, greatly affected African exports to Europe in the sum of US$ 400 million as estimated by the Department of International Development (DFID) in UK. Evaluation cost by DFID for surveillance and monitoring activities of AF in just one standard laboratory was at US$ 125, 000 per year, let alone the training cost for researchers, laboratory technicians and other personnel involved in quality control. However, capacity building is required to provide national authorities with the tools to evaluate losses and impact of AFs on trade, poverty and health.

The end effects in terms of health and the socio-economy cannot be overemphasized as contamination of feedstuffs by AFs alone constitute an annual losses of about $ 750 million in Africa (Goyal, 2003). This has attracted worldwide attention due to the significant losses associated with AFs impacts on national socio economic and animal health effects.

2.9.6.2 Health effect

Aflatoxins, mainly AFB_1 are recognized to be the most toxic class of mycotoxins known. This may be because they are the most studied class of mycotoxins (D'Mello and MacDonald, 1997). Consumption of feeds contaminated with AFs by animals may cause diseases generally known as aflatoxicosis which could be primary (AF disease occurring on animal due to direct consumption of contaminated feed) or secondary (AF disease susceptibility from animal products) as shown in Figure 2.11. This disease was indicated in dog food produced in Brakpan and Roodepoort, South Africa which caused the death of 220 dogs as a result of aflatoxicoses (Serrao, 2011) This was also the case in fishes, when feed contaminated with AFs was eaten by the fish (Cagauan *et al*., 2004). In a report released by the Pakistan Council for Scientific and Industrial Research (PCSIR) Karachi, 493 cattle died and 1,200 fell sick after consuming high concentration of AFs in their feed

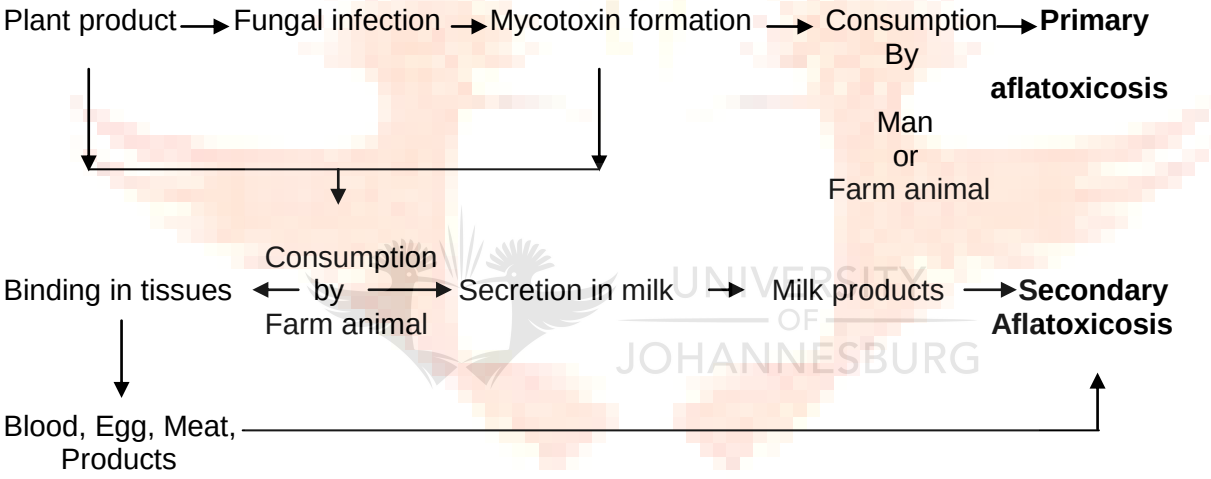


Figure 2.11 Food chain showing possible occurrence of 1^0 and 2^0 aflatoxicosis (Moss and Smith, 1985)

All species of animal are susceptible to aflatoxicosis; however, the severity of the disease may vary from one animal species to the other, age and the health status of the animal in question. The most susceptible are mostly sheep, cattle and poultry (Radostits *et al*., 2000). Beef and dairy cattle are more susceptible to aflatoxicosis than sheep or horses (Reagor, 1996). Younger animals of all species are more susceptible than mature animals (Kim Cassel *et al*., 2012). Less susceptibility can be experienced in pregnant and growing animals than young animals, however, more susceptible than mature ones (Cassel *et al*., 1988). Oral LD_{50} values of AFB_1 vary from 0.03 to 18 mg/kg body weight for most animal species (Jones *et al*., 1994). Incidences of this disease have been experienced in South Africa as seen in Table 2.3.

Table 2.4 Incidence of animal aflatoxicosis in South Africa

Animal affected	Biological effects	References
Dogs	Hepato-carcinogen	Otto (2011)
Dogs, cats	Hepato-carcinogen	Huston (2011); Barboza and Barrionuevo (2007)
Young pigs, dogs, calves, cattle monkey	Hepatotoxin and Hepato-carcinogen	Bryden *et al.* (2002)
Young pigs, dogs, calves, cattle, sheep, cats, monkey.	Hepatoxin and Hepato-carcinogen	*Van Halderen et al. (2000)*
Cattle	Hepatoxin and Hepato-carcinogen	Van Halderen *et al*. (1998)
Chicken	Reproductive problem, vaccine and drug failure, poor growth, deficiency of Vitamin A, death.	Bryden *et al.* (1980)

Consumption of feeds contaminated with AFs can exert toxicity in many ways as seen in Figure 2.12 as adapted from Wu (2010). Aflatoxin alters the integrity of intestine (Gong *et al*., 2008), modulate cytokines and protein expression (Felicia *et al*., 2011), resulting in stunted growth and immune suppression (Wu, 2010). Certain P450 enzymes such as CYP1A2, 3A4, 3A5 and 3A7 may transform AFs to aflatoxin-8-9-expoxide (DNA reactive form) (Felicia *et al*., 2011). As this binds to the liver where it is metabolized, it results in potential acute aflatoxicosis (Huston, 2011). Aflatoxin-induced hepatocellular carcinoma (liver cancer) may result as AF binds to DNA. In a case of humans, a synergistic effect between AFs and chronic infection with hepatitis B virus (HBV) results in significantly higher liver cancer risk as reported (Wu, 2010).

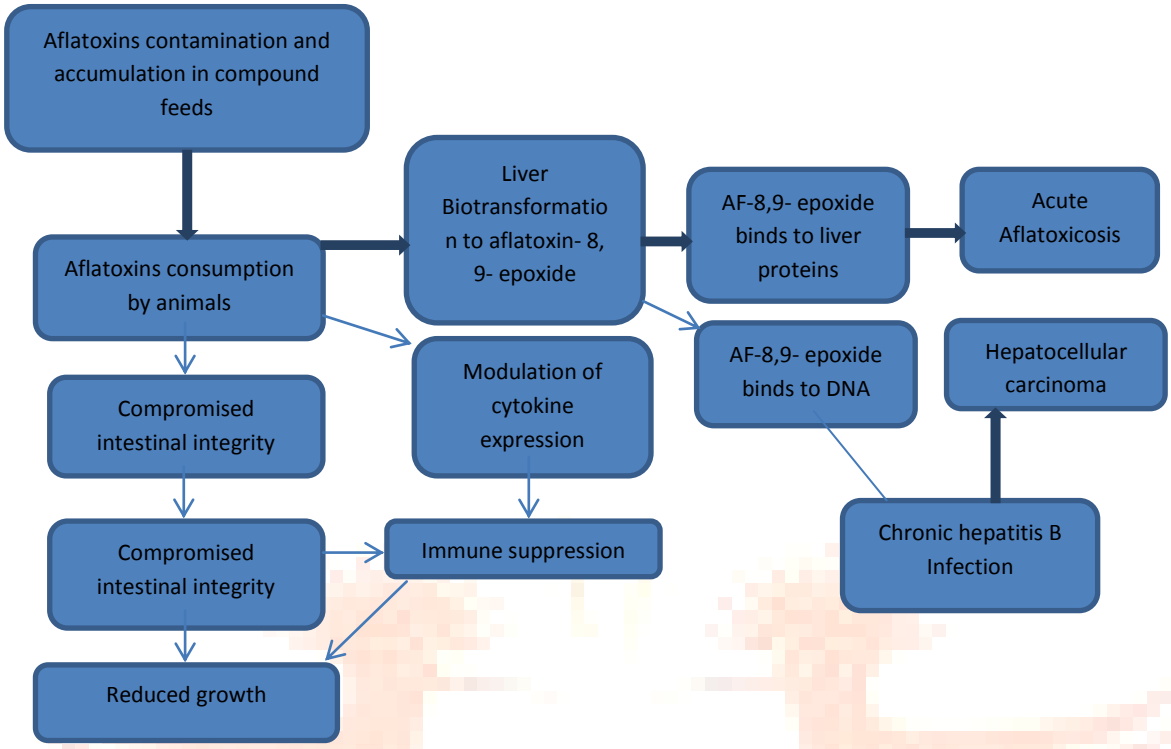


Figure 2.12 Aflatoxins and disease pathways in animals

Aflatoxicosis can be acute or chronic. Acute aflatoxicosis (effects of AF that result either from a single exposure or from multiple exposures in a short space of time usually within 24 hours or less than 14 days) occurs in animals (Janardan *et al*. 1976). It is characterized by haemorrhage, acute liver damage, oedema and death. A typical incidence which gave rise to the discovery of AFs was when more than 100,000 turkey poults died in the United Kingdom after consuming feeds contaminated with AF, hence the name “Turkey X disease”. Since then, other incidences have been recorded especially last year in South Africa (Table 2.3) and other parts of the world. An example in point for other countries is the 1981 outbreak of aflatoxicosis in Australia where several hundreds of calves fed peanut hay died (McKenize and Blaney, 1981). In 2007, in a Chinchilla farm in Argentina, several hundreds of animal died due to AF poisoning (González Pereyra *et al*., 2008).

Studies on animal health have shown that chronic exposure to AFs in animals can cause growth inhibition and immune suppression (Khlangwiset *et al*., 2011). Impaired reproductive efficiency, reduced feed conversion efficiency, increased mortality rates, reduced weight gain, anaemia and jaundice are all characterised by chronic aflatoxicosis. According to Anamika and Farid (2003) when animals consume sub-lethal quantities of AFs for a week or so, sub-acute, moderate to severe liver damage is achieved. A report of Janardan *et al*. (1976) indicated that carcinogenicity

related to AFB_1 has been observed in livestock, damaging the liver, swellings the gall bladder as immunosuppression is due to the reactivity of AFs with T-cells, decreasing vitamin K and phagocytic activities.

At a cellular level, AFB_1 inhibits nucleic acid synthesis because of their high affinity for polynucleotides and nucleic acids (Chu and Saffhill, 1983). They are easily attached to residues of guanine as adducts of nucleic acid (Stone *et al*., 1988). Aflatoxins decrease lipid metabolism, protein synthesis, mitochondrial respiration, causing an accumulation of lipids in the liver, hence a fatty liver due to impaired transport of lipids out of the liver after synthesis, leading to high faecal fat content (Anyanwu *et al*., 2007; Kirk *et al*., 2005).

The health and social implications associated with AFs over the last few decades have raised serious concerns as AF contamination and subsequent animal and human exposure to these toxins is devastating. This has led to the establishment of regulatory bodies as well as developing measures which could significantly minimize AF contamination and subsequent exposure to the toxins.

2.10 Control of AFs and possible strategies of prevention in South Africa

Aflatoxins are amongst the mycotoxins that have received the greatest attention and are addressed by the Animal Feed Manufacturers' Association (AFMA). It gave guidance to the management and production of high quality animal feeds in South Africa, for safe animal feeding and a "Code of practice was published in 2003 for the control of mycotoxins in feeds (Purchase, 2003). This code gives an overview on mycotoxins and guidelines so as to put up good practices for the control of AFs and other mycotoxins in the feed industry.

The National Mycotoxin Group supported and funded by the Maize Trust with participation by interested industries and research institutes are making more efforts in combating AFs (Viljoen, 2008) by assessing the levels in animal feeds which is paramount to evaluating feed safety and animal health. With respect to recommendations for effective AFs survey and implementation of legislation, regulatory bodies are strengthened to monitor AFs and other mycotoxins in feeds regularly. Just like in other countries of the world, South Africa has instituted legislations against AFs (Fellinger, 2006; Njobeh *et al*., 2010a). The South Africa dietary limit of AF tolerance in feeds for different livestock are in the range of 100 ppb, 50 ppb (Manegar *et al.,* 2010), 10 ppb (Caloni and Cortinovis, 2011), 20 ppb (Akinrinmade and Akinrinde, 2012) for cattle, poultry, horse and pig respectively. The maximum tolerable limit of AFs in animal feeds is from 5 to 300 ppb (Njobeh *et al*., 2010a). However, this is determined with respect to the feeds nutrients compositions. These maximum allowable limits may protect animals and humans from the danger of AFs, thus, must be

enforced. Hence, an effective surveillance and feed quality control units that should ensure all feeds for livestock are devoid of AF at harmful levels should be put in place.

Potential harmful effects associated with AFs could be averted by employing good practice regulations and standards to secure safe feeds. Two main international bodies are mainly involved in the development of feed/food standards and risk assessments. There is a joint collaboration between the World Health Organization (WHO) and the Food, Agriculture Organization of the United Nations (FAO) to which the South African Department of Agriculture and Department of Health in conjunction with AFMA operate towards prevention/control. Nevertheless, there exist the Expert Committee on Food Additives (JECFA) and European Food Safety Authority (EFSA) involved in performing risk assessments, in response to different feed safety matters in collaboration with experts within the field of concern. Results published as scientific opinions are upon which the EU bases its recommendations and legislation. The common occurrence nature of AF as an "unavoidable contaminant", have made it very difficult to combat and eliminate its effects in animals associated with consumption of contaminated feed. However, there are approaches followed to combat this effect during pre or post exposure which can help in eliminating, or at best reduce the presence of AFs in feeds and their associated effects thereof in animals. According to Mathers *et al*. (2007), overall disease burden (ODB) can be accessed via the concept of "disability-adjusted life year" (DALY). This concept could be applied in reducing AF in feed commodities (Makun *et al*., 2012). However, this is based upon regulations that governs the risk factors which must be stringent due to economic losses based on AFs because of not meeting up to recommended standards (Wu, 2010). To avoid these risks, it is much better to device ways of preventing or stopping the action and toxic effects of AFs rather than dealing with its aftermath. Hence, approaches such as the use of K49 (Suszkiw and USDA-ARS., 2012) which is a non-toxigenic (or atoxigenic) fungi because of its inability to produce aflatoxin; as a first-line of defence against AF contamination, use of Afla-Guard, a non-aflatoxigenic strains in agricultural environments could be considered, as it suppresses naturally occurring aflatoxigenic strains (Abbas *et al*., 2011) and is presently implemented by the International Institute of Tropical Agriculture (IITA), Ibadan-Nigeria. The use of genetically modified crops which are resistant to insect pests and diseases could also be introduced in agricultural environments (Wu, 2006). So also, the availability of amino acids such as lysine and vitamin C as constituents of feeds could go a long way to prevent or hamper AF activities since they both exhibit some protective actions against mycotoxins (Obidoa and Gugnani, 1992; Smith *et al*., 2000).

Effectively, proper reduction of moisture content in livestock feeds and their ingredients to a recommended moisture level, cleaning, removal of contaminated parts (Chiou *et al*., 1994) or sorting infected materials and controlling humidity during storage could be very effective in

lowering the level of contamination. Due to the relationship between *Aspergillus* growth and ecophysiological factors, there exist a strong reason to support the fact that altering factors could have an effect on AF contamination. Several reports (McMullen *et al*., 1997; Doohan *et al.*, 2003; Miller, 2008; FAO, 2008) lay emphasis on potential consequences of these determinant factors. Forecasting models are becoming an interesting tool to predict prevalence of AFs in response to these reports. In a report of FAO (2008), suggestion in the use of predictive modelling in addition to prevention, agricultural policies put in place and monitoring provide a means of reducing the incidence of AF. Proactive actions by changing agronomic practices at the onset of an outbreak are higher than applying fungicides on general basis (Miller, 2008), although this development of forecasting systems has proven to be a very complicated.

Traditionally, cats and dogs could be used to ward off rodents, birds and monkeys that may invade agricultural products and cross contaminate or expose them further to contamination. The use of fungicides, insecticides and pesticides as well, has been useful though it has lost popularity in recent times due to the increasing demand of producing agricultural commodities with no chemical residues. Modern studies do no longer favour this since it could elicit toxicity to consuming animals, hence the use of natural, safer and environmental mycocidal or fungicidal products. This include the use of *Lippia multiflora* leaf extracts which have been shown to have some fungistatic effect on *A. flavus* and *A. parasiticus* (Anjorin *et al*., 2008). Light amplification by stimulated emitted radiation i.e. Gamma irradiation (Ogbadu and Bassir, 1979; Ogbadu, 1988) can be used to lower toxicity of AF contaminated feeds and products, as it can serve for a long term storage process. A proactive management system of Hazard Analysis Critical Control Point (HACCP) (FAO/WHO, 2012) could be employed to maintain a safe feed through the analysis and control of biological, chemical and physical hazards along the production chain i.e. from raw materials, manufacturing, distribution and consumption is now a priceless tool for the control of notable mycotoxins such as AF.

2.11 Concluding remarks

Aflatoxins and their producers have been found to be problematic. This is an issue in terms of their role in influencing feed quality, animal health and the economy. To maintain good feed quality by way of minimizing their occurrence and deleterious effects, it is highly essential that issues relating to fungal contamination, proliferation and mycotoxin production are monitored and addressed on a regular basis so that immediate actions can be taken before situations become out of control. An attempt has been made in this chapter to look into these aspects mainly with the focus on AFs. In this regard, it is required that more accurate and modern analytical tools such as RT-PCR for the quantitative detection of toxigenic fungi and their toxins are employed. It is in this light that studies related to toxigenic fungi and their attendant mycotoxins in agricultural commodities are designed

and carried out. From these reviews, it can be seen that no work has been performed to quantitatively detect toxigenic fungi and the genes responsible for producing mycotoxins in feeds and correlating that to levels of mycotoxins therein but some works have reported on the subject when dealing with other commodities such as maize, wheat, etc. This work attempts to establish a correlation between *nor-1* gene levels and those of AFs in compound feeds.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials and Reagents

Chemicals and reagents used were from Merck & Sigma (Germany) and are of analytical grade, unless otherwise stated, and these include:

(a) *Fungal isolation and enumeration equipment and materials:* Olympus B061 compound microscope (Wirsam Scientific, S. Africa), Laboratory weighing balance (Precisa XB 120A, Swiss), Wire loop, Pipettes (Schott duran Laborglas), Medical flasks (Schott duran Laborglas), disposable sterile petri dishes (Merck), McCartney bottles with metal caps, wire baskets to hold McCartney bottles, Ringers tablet (MERCK KGaA), agar (for preparations kindly refer to Appendix I) which include; OAESA (Ohio Agricultural Experimental Station Agar), CYA (Czapek yeast extract agar), MEA (malt extract agar), PDA (Potato dextrose agar), Autoclave (Equitron^{R}), Incubator (Incotherm – Labotec) set at 30^{o}C, Water bath (Wirsam Scientific, S. Africa), streptomycin and chloramphenicol (Sigma, Aldrich), Microscope Standard 19 (470919-9902/06) equipped with an Axiocam MRC Camera Serial. No. 2 08 06 0245 and AxioVision Release 4.5 SP1 (03/2006) software (Zeiss, West Germany).

(b) Aflatoxins extraction, detection and quantification: Separating funnels fitted with stoppers (MERCK)**,** large test tubes (15 x 25cm)/boiling tubes, wash bottles, elastic bands, methanol (HPLC grade)**,** sodium chloride, nitric acid, potassium bromide, anhydrous sodium bicarbonate solution (saturated), acetonitrile, toluene**,** formic acid**,** Propan-2-ol, Ethyl acetate, dichloromethane, variable hot air drier (FENICI, 41512), TLC tank (Camag Ltd), Amber vials (MERCK), HPLC vials, UV box, rotary blade blender (Torrington, CT. USA), phosphate buffered saline tablets (Prod codes: RP202), microfibre filter paper (Whatmann N^{o} 113 (Prod codes: P66 and P67), aflaprep immunoaffinity columns (Prod code: AP01; R-Biopharm AG; Darmstadt, Germany), mobile phases, standards of aflatoxins (AFs) i.e. aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) (ARC, South Africa), 20 x 20 cm pre-coated aluminium backed silica gel G TLC plates Merck Art 5553, Aldrich), HPLC Spectra Physics SCM400 SYSTEM (Shimadzu Corporation, Kyoto, Japan) equipped with a LiChrospher 100 RP-18 column (250 mm x 4 mm i.d and 5 µm particle size) (Merck, Darmstadt, Germany), Waters Sentry^{TM} guard column and a fluorescent detector (Shimadzu Corporation, Kyoto, Japan), , thermostatically controlled hot

plate (University of Natal 198364), visking dialysis tubing (8/32) (Sigma), water pump with trap to supply vacuum, Virtis homogeniser (Sigma).

(c) Cytotoxicity assay equipment and materials: Tetrazolium salt [3-(4, 5-dimethylthiazol -2-yl) -2,5-diphenyltetrazolium bromide (MTT)], MTT Assay Kit (Sigma, St Louis, USA), histopaque 1077 (Sigma, St Louis, USA), complete tissue culture medium (RPMI-1640 supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin), humidified incubator set at 37^{o}C, 96-Well micro titre plates (Corning Cell Wells^{TM}, Corning, USA), Hank's Balanced Salt Solution (Adcock Ingram), ELISA microplate Reader (modello:A2; Rome, Italy), 15 ml vacutainers sterile needle and tube syringes, (Shalom SA), swab, centrifuge, dimethylsulphoxide (DMSO), 0.2% Trypan blue solution, Neubauer cover slips, sterile haemocytometer (Neubauer counting chamber), Pasteur pipettes, phase contrast light microscope, foetal calf serum (FCS), phytohaemagglutinin-p (PHA-p) (Sigma, Aldrich) penstrep-fungizone (Adcock Ingram), Phosphate buffer saline (pH 7.4), Trypan blue solution, fluorometer.

(d) DNA assay: Micro Amp reaction tubes, TaqMan environmental Master Mix 2.0, Rotor Gene Amp 6000 detection system (Corbett Life Science – R080762, Australia), nor probe and primers (QuantiFast pathogen PCR + IC kit, Qiagen, White Scientific), molecular grade water (nuclease free), tissue disruptor, SYBER green loading dye, Plant and fungi DNA® extraction Mini and Maxi Kits (White Scientific Qiagen product, S. Africa).

3.2 Experimental procedures

The experimental procedures followed in this study are illustrated in Figure 3.1.

Figure 3.1 Flow chart of experimental procedures

3.3 *Sampling and sample preparation*

A total of 92 samples of compound feeds (Table 3.1) which include poultry, cattle, pig and horse feeds were donated by different feed manufacturers in South Africa under the auspices of the South African Feed Manufacturer's Association (AFMA). Samples (about 500 g each) were collected following the standard sampling protocols, to give a representative sample which was then put in sealed plastic bags and taken to the laboratory of the Food, Environmental and Health Research Group, Faculty of Health Sciences of the University of Johannesburg. These were milled to powder and stored at -20^{o}C until analysed.

Table 3.1 Compound feeds from different feed manufacturers in South Africa analyzed

Compound feed type	Sample size	Total
Poultry feeds		
Layer	20	62
Broiler	28	
Breeder	14	
Cattle feeds		
Dairy	11	
Calf	8	25
Finisher	6	
Other feeds		
Horse	3	
Pig	2	5
Total	92	92

3.4 *Fungal screening and isolation*

A microbiological analytical procedure of with some modifications was used in this study and carried out under aseptic condition. Accordingly, 1 g of ground sample was weighed into a sterile test tube and suspended in 9 ml of sterile Ringer's solution and vortexed. The suspension (1 ml) was serially diluted in 9 ml of the Ringers' solution further to 10^{-6}. One ml from each dilution was cultured by pour plate technique on Ohio Agricultural Station agar (OAESA) and potato dextrose agar (PDA) and incubated for 5-7 days at 30^{o}C. Between 5th and 7th day in each plate, fungal colonies were counted macroscopically using a colony counter. Colonies forming units per gram (CFU/g) of sample was calculated and expressed in colony forming units per gram of sample (CFU/g) as:

$$\text{CFU/g} = \frac{\text{Number of colonies x reciprocal of the dilution factor}}{\text{Plating volume (1ml)}}$$

Isolates of *Aspergillus* spp. of interest (*A. flavus* & *A. parasiticus*) colonies were further sub-cultured on PDA, Czapek yeast agar (CYA) and malt extract agar (MEA) under aseptic conditions and incubated at 30^{o}C for 7 days. Pure colonies were harvested and stained with lacto phenol in cotton blue and viewed microscopically. The macro- and microscopic identifications of the species were done following the identification keys of Klich and Pitt (1988b) and Klich (2002). Isolates were sub-cultured on PDA for 7 days at 25 ^{0}Cand then stored at 4 ^{0}C for further analysis.

3.5 Fungal toxigenicity testing

To determine the toxigenic potentials of isolates from feeds in producing AFs, the fungal species were grown on solid YES agar (composition as in Appendix I) plates for 14 days. Plugs were cut at the centre of the colony on a Petri dish using a sterile plunger of about 4 mm in diameter. The agar plug was dissolved in DCM and 20 µl gently spotted for a few seconds onto a TLC plate, with distance of 1 cm between test samples, including AFs standards and 2.5 cm from the bottom line of the plate. This set up was placed into the TLC tank composition of toluene: ethyl acetate: formic acid in the ratio of 6:3:1 solvent system, allowed to dried and viewed under UV light of 365 nm.

3.6 Aflatoxin analysis

3.6.1 Extraction and clean-up of aflatoxins in feeds

Two methods were employed for the extraction of AFs in compound feed samples i.e. multi-mycotoxin extractions and immuno affinity column extractions.

3.6.1.1 Multi-mycotoxins extraction

Aflatoxin extraction following the multi-mycotoxins extraction procedure was done following that of Patterson and Robert (1979) with some modifications. Into a 250 ml flask containing 100 ml solution containing 4% w/v of potassium chloride and acetonitrile (1:9, v/v), 25 g of feed sample were added and placed on a shaker for 1 hour. The contents were filtered through a Whatman No.1 filter paper into a separating funnel. The extract was defatted twice using 25 ml iso-octane. Saturated sodium hydrogen carbonate solution made up to 50 ml with water (30 ml) was added and gently shaken and then 50 ml dichloromethane (DCM) again added and shaken vigorously. The lower layer was passed through a bed of anhydrous sodium sulphate (5-10 g) and the DCM fraction collected in a round bottom evaporating flask. The filtrate (DCM layer) in the evaporation flask was evaporated using a rotary vacuum evaporator. The dried extract was reconstituted with 3 ml of acetonitrile and the content carefully transferred into dialysis tubing, which was previously soaked in deionised water for 1 hour. The dialysis tube were then placed in a boiling tube containing 50 ml 30% acetone, sealed with a rubber banded foil and left overnight on a shaker. The dialysate was transferred into a separating funnel, the boiling tube washed with a small amount of 30% acetone and added to the dialysate. Mycotoxins were extracted thrice using 25 ml

DCM and each time, passing it through a bed of sodium sulphate into rotary round bottom flask. This was evaporated as previously described. The dried extract was reconstituted with 2 ml DCM and put in an amber vial, which was then placed on a heating block set at a temperature of 60^{o}C and dried under a stream of N_2 gas. This was retained as an AF fraction which was then stored at 0^{o}C until analysis.

3.6.1.2 Aflatoxin extraction and clean-up

Aflatoxins extraction from feed samples using an immuno-affinity column was achieved following an extraction and clean-up protocol described by Candlish *et al*. (1998) with modifications using the version PO7/V15/26.01.05 aflaprep kit. The milled sample (12.5 g) and 1 gram NaCl were weighed into a solvent resistant blender jar into which 62.5 ml HPLC grade methanol and distilled water (60:40, v/v) was added and blended for 60 secs. The extract was filtered and diluted with distilled H_2O (62.5 ml) which was well mixed by swirling. Sample extract (25 ml) was passed through a filter paper (Whatman No. 4) and 10 ml of the filtrate obtained (equivalent to 1 g of sample) was passed through an immuno-affinity column at a flow rate of 2-3 ml/min. After which, the immuno-affinity column was washed using 10 ml of phosphate buffered saline (PBS) at a flow rate of 5 ml/min. The analytes were then eluted (1 drop/sec) using 1 ml of HPLC grade methanol and collected in an amber vial. Back flushing was employed thrice with the eluent to ensure complete release of AF into the solution. The extract was dried using N_2 gas as previously described and stored at 0 ^{0}C until used for further analysis.

3.6.2 Thin layer chromatography

The TLC technique was carried out at room temperature according to modified method of Frisvad (1987) using two solvent mixtures of dichloromethane/ethyl acetate/2-propanol and Toluene/ethyl acetate/formic acid. A 10 x 10 aluminium backed silica gel TLC plate (cut from 20 x 20 plates, Merck) was used and 15 mm from the edge of the TLC plate was measured out. Dried extracts obtained both from the multi-mycotoxin extraction and the immuno-affinity extraction clean-up procedures were reconstituted in 200 µl of DCM and vortexed. The reconstituted extract (20 µl) was pipetted and spotted in 5 µl portions on about 15 mm from the edge of the plate, while drying at intervals with warm stream of air. A mixture of AFs standards (20 µl) was spotted alongside the sample spot. This allowed for concurrent two-dimensional chromatography of the sample extracts together with the AF standards (90 ^{0}C on each side). The TLC tank contained DEI (DCM/ethyl acetate/2-propanol; 90:5:5, v/v/v) as the solvent for the first run and after drying, the plates were put into the tank containing TEF (Toluene/ethyl acetate/ formic acid; 6:3:1, v/v/v) for the second run. The solvent migrated each time was for at least 750 mm from the application spot, removed and air dried for 3 mins. After the final run, plates were dried and without spraying examined under UV light (wavelength of 366 nm) and AFs spots were noted as they appeared as blue or green

spots under UV light. The AFs in the sample extracts were detected by comparing their retardation factor (R_f) values with those of AF standards. The R_f values were calculated using the formula:

$$R_f = \frac{\text{Distance from origin to the centre of the spot (cm)}}{\text{Distance from the origin to the solvent front (cm)}}$$

3.6.3 High performance liquid chromatography

Sample extracts were analysed for AFs by fluorescence detection following the HPLC method described by Ahsan *et al*. (2010.) with some modifications. The HPLC system used for this assay was a Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, SIL-20A auto sampler, RF-10AxL fluorescence detector, Kobra cell RID-10A refractive index detector and SPD-M20A photodiode array detector linked to an LC solutions version 1.22 Software Release. Extracts were redissolved in 1 ml CH_3OH, filtered through a 0.2 µm Millipore filter and filtrate used as analyte solution. Chromatographic separation of analytes and standards was performed by passing the analyte or standard through a Waters Sentry^{TM} guard column (Waters, Milford, USA) and a Symmetry column (250 x 4.6 mm i.d., 5 µm particle size). Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) without derivatization were detected using a fluorescent detector. The analysis was performed in an isocratic mode at a temperature of 30 ^{0}C with an excitation and emission wavelengths of 365 and 440 nm, respectively. The mobile phase pumped at a flow rate of 1.2 ml/min was water/acetonitrile/methanol (55:22.5:22.5, v/v/v). The injection volume of the analyte and standard toxins used was 20 µl. These chromatographic conditions gave good results. Calibration curve was obtained from a concentrations of standards, i.e., AFB_1 (2.5, 5 & 10 ppb), AFB_2 (0.5, 1 & 2 ppm), AFG_1 (2, 4 & 8 ppm) and AFG_2 (1, 2 & 4 ppm). The Peak areas and retention times of AF in sample extracts were used to determine their concentrations per sample, using the mathematical formula below, with respect to the limit of detection and limit of quantification.

$$[\text{Sample AF (ppb)}] = \frac{\text{Sample peak area x [standard (ppb of AF)]}}{\text{Standard curve area}}$$

Standard (ppb of AF) → Concentrations of AF standard

Sample AF (ppb) → Concentrations of AFs in the sample extract

Recoveries of AFs were determined by spiking 25 and 12.5 g of clean feed sample (does not contain any detectable amount of AF) for multi-mycotoxin and aflaprep immuno-affinity extraction and clean-up procedures, respectively, with known standard amounts of AFB_1, AFB2, AFG_1 and

AFG_2 standards i.e. 100, 5, 10 and 5 ppb, respectively. These spiked samples were thoroughly mixed and incubated at room temperature in a fume cupboard for at least an hour. Samples were prepared in triplicates and extraction for AF carried out as previously described.

3.7 Cytotoxicity Assay

Cytotoxicity testing was achieved using venous blood from a healthy male person, which was collected twice by vein puncture, using a 15 ml sterile syringe and immediately transferred into a 10 ml heparin tube. The preparation of the cells was according to methods of Hanelt *et al*. (1994). The blood was diluted with an equal volume of RPMI-1640. Ten ml of the mixture was layered onto 5 ml of histopaque in 15-ml polypropylene conical tubes and then centrifuged at 1,500 rpm for 30 mins at room temperature. The interface layer consisting of mononuclear cells was carefully removed using a sterile pipette and washed twice by centrifugation at 4°C. Viable and non-viable cells were determined using 20 µl of cell suspension, 80 µl of 0.2 % trypan blue solution in an Eppendorf tube and incubated for 2 mins at room temperature. Using a sterile Pasteur pipette, 10 µl of the trypan blue cell suspension mixture was then transferred using a haemocytometer chamber and covered with a cover glass slip. Cells were counted and coloured (blue) cells were considered dead, while cells excluding the dye (uncoloured cells), were viable cells and % cell viability was determined as:

% Cell viability = Viable cell counted/total number of cells) x 100

The concentration and number of cells were calculated using the formula:

Cell/ml = n/v x dilution factor (5) x 104

Where: n = number of cells counted Chambers

v = area of big squares counted x depth (0.1)

DF = dilution factor (10 µl of blood: 40 µl of Trypan blue) = 5

Mononuclear cells were transferred into a complete culture medium containing 1.5% L-glutamine, 10% foetal calf serum (FCS) and 1% penstrep (penicillin and streptomycin). Cells were seeded with methanol (used as a negative control) and AFB_1 (used as a positive control because it is the most toxic/occurring of all the aflatoxins known) and methanol with aflatoxins extracts into 96-wells culture micro plate and incubated with CO_2 at 37 ^oC for 48 hrs.

Cytotoxicity assay performed herein was a biological method in assessing the quality of compound feeds or to be precise, a confirmatory test to the data obtained via HPLC. In this regard, with respect to the negative control (MeOH) used, the effect of sample extracts (which were randomly chosen based on highest, medium and lowest AF concentration detected) obtained from feeds on

metabolic activity of lymphocytes was assessed *in vitro* by 3, 4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT) assay according to the method of Meky *et al*. (2001). This method assesses the ability of cells to convert MTT to formazan crystals. 3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (50 mg) was dissolved in 10 ml of 0.14 M phosphate buffered saline (PBS) (pH of 7.4) and filtered through Whatman No.1 filter paper. At the end of the culture period, 25 µl of 5 mg/ml MTT solution was added into each cell culture in the 96 wells culture plate and thoroughly shaken. The mixture was incubated for 2 hrs at 37 °C and thereafter, 50 µl DMSO was added and further incubated for 4 hrs to solubilize the formazan crystals formed. A microplate reader was used to measure optical density (OD) values set at wavelength of 620 nm. The percentage of viable cells realised after the assay was mathematically determined as thus:

% Cell viability = [Mean OD values of treated cells ***/*** Mean OD values of Control] x 100%

3.8 Molecular studies on compound feeds

The molecular studies on compound feeds were carried out following different microbiological analyses as described below.

3.8.1 DNA extraction

3.8.1.1 Fungal strains

Isolates of pure fungal strains (from feed samples) for DNA extraction were sub-cultured on YES broth medium and incubated for 7 days at 25 ^{o}C. The extraction of DNA was performed using a DNA extraction Mini kit according to a modified method originally described by the manufacturer (Fredlund *et al*., 2008). Mycelia of a pure fungal strain (100 mg) were harvested by filtration into a collection microtube, disrupted for 1 min using a tissue disruptor and 400 µl of Qiagen buffer AP1 and 4 µl RNase A (100 µg/ml) were added. This was vortexed and incubated for 10 mins at 65 ^{o}C with tube inversion 2-3 times during incubation. Buffer AP2 (130 µl) was added and mix with further incubation for 5 mins on ice. The lysate was pipetted into a QIAshredder Mini spin column in a 2 ml collection tube and centrifuged for 2 mins at 20,000 x g (14, 000 rpm). The flow-through was transferred into a new tube without disturbing the pellet formed. One and half of buffer AP3/E was added and mixed by pipetting. The mixture (650 µl) was transferred into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged for 1 min at 8000 rpm (6000 x g). The flow through was discarded, repeating the process with the remaining sample. The spin column was placed into a new 2 ml collection tube with the addition of 500 µl Qiagen buffer AW. This was centrifuged for 1 min at a revolution 8000 rpm and the flow-through was discarded. Again, 500 µl buffer AW was added and centrifuged for 2 mins at 14, 000 rpm. The spin column was removed from the collection tube carefully and was transferred into a new 2 ml micro-centrifuge tube. Qiagen buffer AE (100 µl) was used for elution of DNA, which was incubated for 5 mins at room temperature and

centrifuged for 1 min at 8000 rpm. This step was repeated for ensure complete elution and purification. The purified DNA was stored at -20 ^{0}C until further analysis.

3.8.1.2 Compound feed

For the extraction of DNA from compound feeds (n= 30), DNeasy Plant Maxi Kit (Qiagen) was used. One gram of sample was weighed and transferred into a micro reaction tube and cells disrupted using a Tissue disruptor for 1 min. Spin column procedure was carried out as described by the manufacturer. Preheated (65^{0}C) buffer AP1 (5 ml) and 10 µl RNase A were added, vortexed and incubated for 10 mins at 65^{0}C, following 2-3 times inversions during incubation. Buffer AP2 (1.8 ml) was added, mixed and the mixture was incubated for 10 min on ice. This was centrifuged at 5000 x g for 5 mins. The supernatant was decanted into a QIAshredder Maxi spin column in a 50 ml collection tube and centrifuged at 5000 x g for 5 mins. The flow-through was transferred without disturbing the pellet, into a new 50 ml tube with the addition of 1.5 ml volume of buffer AP3/E (Qiagen) which was mixed immediately by vortexing. A maximum volume of 15 ml of sample was transferred into a DNeasy Maxi spin column in a 50 ml collection tube and centrifuged at 5000 x g for 5 mins. The flow-through was discarded and 12 ml buffer AW was added and centrifuged for 10 mins at 5000 x g while discarding the flow-through. The spin column was transferred into a new 2 ml tube, adding 1 ml buffer AE to elute DNA. This was incubated for 5 mins at room temperature, centrifuged at 5000 x g and this step was repeated three times further. The resultant DNA solution was stored at -20^{0}C, until used for the real-time PCR experiments.

3.8.2 Agarose Gel DNA Electrophoresis

Agarose gel DNA electrophoresis was performed according to the modified method of Stephen and Lewis (2001). Two grams of agarose (BioRad agarose, Qiagen) was prepared in 98 ml 1x TAE (Tris/Acetate/EDTA) buffer to give a 2% solution and heated to boiling point in a water bath. The solution was allowed to cool to 60 ^{o}C prior the addition of 3 µl ethidium bromide (Et Br) (10 mg/l in water to a final concentration of 0.5 um/ml) and thoroughly mixed. Combs were positioned 0.5 -1.0 mm above plate to permit formation of wells when the agarose solidified. Air bubbles were avoided during this process between or under the teeth of the combs. A Pasteur pipette was used to seal the glass plate with little amounts of agarose solution. At the setting of seals, the gel was poured in the glass plate. The comb and tape were removed after 30 to 40 mins when the gel had completely hardened at room temperature. The gel was placed in an electrophoresis tank and electrophoresis buffer added in enough quantity (about 1 mm of depth) to cover the gel and wells tops so they were submerged. PCR products (2 µl) were slowly loaded into the wells using a micropipette, avoiding any mix up between wells. The lid of the tank was closed after ensuring that the PCR products are correctly positioned with respect to the anode and cathode, as DNA migrated only towards the anode. A voltage of 5 V/cm was applied to the gel for the electrophoresis run and

PCR product was viewed using the Vacutec Gel documentation system and product size confirmed by comparison to the Middle Range Fast Ruler.

3.8.3 Real-time PCR

A Rotor Gene Amp 6000 detection system (Corbett Life Science – R080762, Australia) was used to perform the real-time PCR cycle reactions. Both the primers and internal probes used in the reaction were suggested by the Primer Express 1.0 software. According to published sequence of Trail *et al*. (1994), the primer/probe set used had the following nucleotide sequence: nortaq-1, 5ˈ-GTCCAAGCAACAGGCCAAGT-3ˈ; nortaq-2, 5ˈ-TCGTGCATGTTGGTGATGGT-3ˈ; nor probe, 5ˈ-TGTCTTGATCGGCGCCCG- 3ˈ enclosing an amplicon of 66 bp from nucleotide 782 to 847. FAM labelled nor-1 probe (QuantiFast pathogen PCR + IC kit, Qiagen, Whitehead Scientific) was used for PCR as suggested by the manufacturer. Individual reactions contained 2.5 µl of the DNA sample solution which was mixed with 5 µl master mix, 3.5 µl of the primers i.e. nortaq-1 (1.75 µl), nortaq-2 (1.75 µl) each, 0.5 µl probe (0.5 nM) and 13.5 µl nuclease free water to make up a reaction volume of 25 µl. The PCR was performed in Micro- Amp reaction tubes placed in a 36-well rack of the GeneAmp 5700R Sequence Detection RT-PCR System. Incubation proceeded for 2 mins at 50 ^{o}C to allow for cleavage of uracil-Nglycosylase. AmpliTaq Gold activation was done by incubating for 10 min at 95 ^{O}C. The following temperature range i.e. 95^{o}C for 20 secs, 55^{o}C for 20 secs and 72 ^{o}C for 30 secs were used for the 35 PCR cycles.

3.8.4 Standard curve quality control for real-time PCR

In order to complete the assay efficiency enough to provide accurate results for a quality of RT-PCR quantification, a standard curve was generated. For this reason, standard curves were analysed and generated using reference DNA (*nor-1*) of known concentration extracted from fungal mycelium, before analysing those of unknown concentrations extracted from feed samples. A serial 5x 10 fold dilutions (10^{-1} to 10^{-6}) from aliquots of DNA of known concentration were prepared using Taqman method of Selma *et al.* (2008), with gene specific primers and probes and the best dilution curve generated was chosen for onward analysis. Dilutions were analysed in duplicates on a 36-well plates Rotor Gene Amp 6000 detection System and standard curves generated by the RG-6000 system software (Rotor-Gene 1.7.94). Slope of the y-intercept and R^2 values were noted and efficiency was determined from the slope of the regression line by the formula:

$$Efficiency = \left(10^{\left(-\frac{1}{slope}\right)}\right) - 1$$

3.8.5 Specificity of primers, probes and validation of reaction system

The specificity of primers, probes and validity of reaction system for the real time PCR was assayed to test for the sensitivity of the genomic DNA in the samples and that of fungal species (*Aspergillus oryzae, Aspergillus ochraceus*, *Aspergillus niger*, *Fusarium verticillioides*, *Fusarium proliferatum*, *Penicillium citrinum*) isolated in similar compound feeds. This was done in triplicates with a negative control template (all reaction components without DNA) and positive control template (all reaction components without sample extracted DNA but with 2.5 µl of the internal control DNA (ICD) and internal control assay (ICA). Results indicated the sensitivity, specificity and validity of the PCR primers/probe experimental reaction setup.

3.9 Statistical Analysis

Mean values of the cytological assay were derived using a one-way analysis of variance (ANOVA). Least significant difference was compared by a pairwise multiple comparism procedure of Holm-Sidak method. Linear regression and linear correlation analysis using SigmaStat 3.5 for Window (Systat Inc., 2006a) was done to estabilsh the coefficient of linear regression and linear correlation at 95% interval for the cell viabilty and AF dosage, and AF concentration and DNA concentration respectively. SigmaPlot for Windows Version 10.0 (Systat Inc., 2006b) was used to graphically represent the cytological data. With a $\leq$0.05 level of probability, mean values among treatment groups were deemed to be different.

CHAPTER FOUR
RESULTS

4.1 *Introduction*

The common fungi genera contaminating animal compound feeds in South Africa are in the species of the *Fusarium*, *Penicillium* and *Aspergillus* genus. The predominant species in the *Aspergillus* genus are *Aspergillus flavus* and *A. parasiticus* as they contribute to the deterioration of compound feeds, animal health, and hence human health. They proliferate in their growth and production of AFs which exhibit high level of disease pathogenicity in its diverse forms. Their toxigenic characteristics in terms of AF production, cytotoxicity and gene presences were investigated using the methods presented in Chapter 3, and the results obtained are presented.

4.2 *Aspergillus flavus and A. parasiticus contamination*

The occurrence and contamination levels of *A. flavus* and *A. parasiticus* in the various selected types of compound feeds were assessed and data summarised in Figure 4.1 and Table 4.1. Figure 4.1 shows typical colonies of these species of fungi isolated in samples. Overall data indicate that 67.5 and 51.1% of feed samples were found to be contaminated with *A. flavus* and *A. parasiticus,* respectively. Accordingly, poultry feed had the highest contamination mean level of 5.7×10^5 CFU/g when compared to cattle (mean: 4.0×10^6) or pig (mean: 2.7×10^4CFU/g) feed, meanwhile, the lowest contamination of fungi was observed in horse feed.

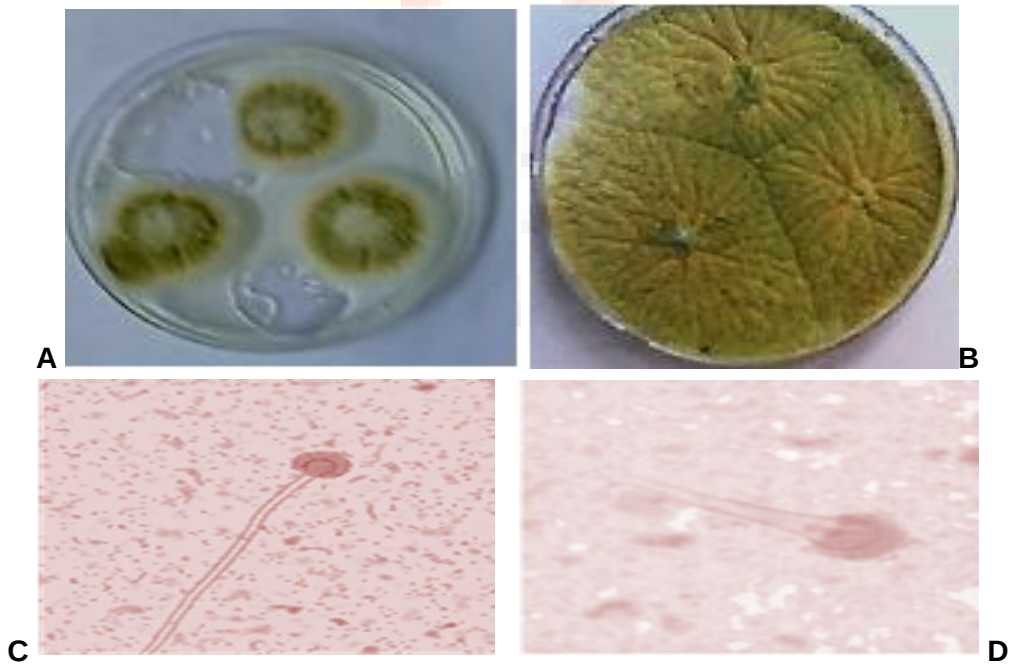


Figure 4.1 Macroscopic (A & B) and microscopic (C & D) views of 6 day-old isolates of *Aspergillus flavus (A & C)* and *Aspergillus parasiticus (B & D) grown on* PDA

In terms of total incidence rate of contamination, these fungal species were recovered from 74.8% of poultry feed samples, i.e. 60.5% of feeds for cattle and in horse and pig feeds, 4.6 and 3.0% contained these fungi (Table 4.1).

Table 4.1 Population of *Aspergillus* spp (CFU/g) of compound feeds South Africa

Feed types	N[a]	N[b]	Contamination level (CFU/g)
Poultry	62		
Layer	20	16 (80%)	4.0×10^6
Broiler	28	21 (75%)	5.7×10^5
Breeder	14	9 (64%)	3.4×10^5
Cattle	25		
Dairy	11	7 (63.6%)	4.0×10^4
Calf	8	4 (50%)	3.8×10^5
Finisher	6	4 (66.6%)	3.5×10^5
Others			
Horse	3	1 (33.5%)	2.0×10^3
Pig	2	1 (50%)	2.7×10^4

Dilution ranged from 10^{-1} - 10^{-6}
N[a]: Total number of feed type analysed
N[b]: Positive/percentage contaminations
[a]CFU/g: Colony forming unit per gram of sample

4.3 Aflatoxigenic fungi and AFs contamination in feeds

4.3.1 Detection of aflatoxigenic fungi and aflatoxins contamination in feeds by thin layer Chromatography

The presence of aflatoxigenic fungi and AFs extracted from feeds using immuno-affinity extraction and clean-up columns was determined via TLC. The calculated R_f values of the standards were 0.68, 0.63, 0.57 and 0.54 for aflatoxins B_1, B_2, G_1 and G_2, respectively. In comparison to these, toxigenic potentials of *A. flavus* and *A. parasiticus* in producing their attendant mycotoxins (AFs) using the agar plug TLC method was performed based on the fluorescence spots generated and data indicated that 68% of isolated species were found to be toxigenic in producing AFs i.e. *A. flavus* produced AFB_1 and AFB_2 and *A. parasiticus* produced AFB_1, AFB_2, AFG_1 and AFG_2. A qualitative separation and detection of AFs in extracts from feeds was also conducted. R_F values for aflatoxin B_1, B_2, G_1 and G_2 were respectively determined as 0.47, 0.43, 0.38 and 0.36. Following that, TLC data indicated that some extracts from feed samples showed some positive results i.e. blue and green fluorescence as presented in Figure 4.2.

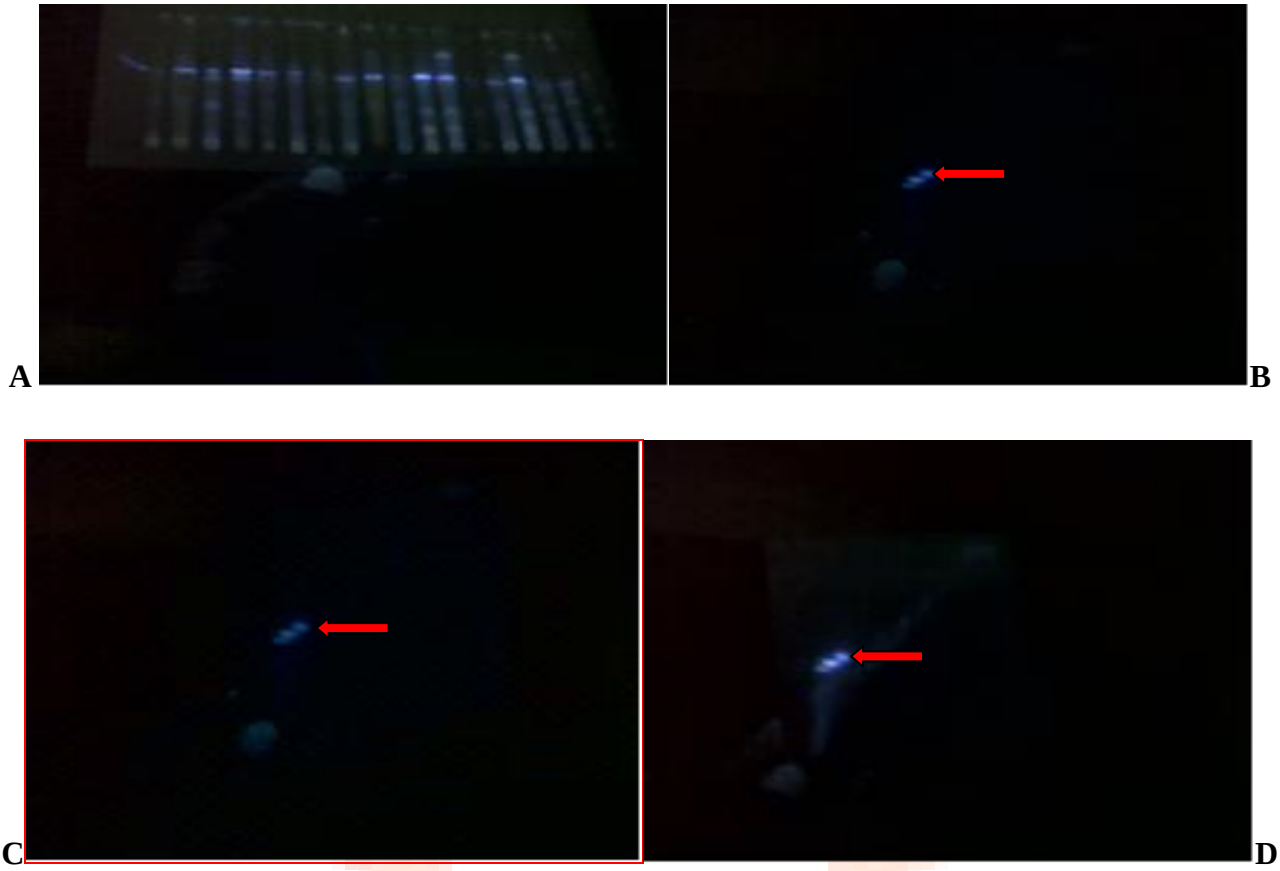


Figure 4.2 One (A) and two (B-D) dimensional thin layer chromatography plates showing AFs spots generated by feeds sample extracts (A, B, C) and aflatoxins standard (D) viewed under UV light; aflatoxin B_1 is indicated by an arrow

Accordingly, AFs were found in feeds as presented on Table 4.2 with poultry and cattle feed samples found to contain the toxins at a much higher occurrence rates of 77 and 54%, respectively, than observed in feeds for pig (5%) and horse (3%).

Table 4.2 Incidence (%) of aflatoxins in compound feed from South Africa as determined by thin layer chromatography.

Feed Type	AFB_1 (%)	AFG_1 (%)	AFB_2 (%)	AFG_2 (%)
Poultry feeds				
Layer	++++ 43	++ 19	++ 10	+ 3
Broiler	+++ 37	+ 21	8	+ 3
Breeder	+++ 40	+ 21	+ 7	- 0
Cattle feeds				
Dairy	++++ 57	++ 13	+ 4	- 0
Calf	++ 41	+ 20	+ 6	- 0
Finisher	+++ 35	++ 9	+ 4	- 0
Other feeds				
Pig	+ 4	+ 1	- 0	- 0
Horse	+ 2	- 0	- 0	- 0

Not present (-); present (+); intensity of fluorescence of the toxin in increasing order (+, ++, +++ and ++++)

4.3.2 High performance liquid chromatography detection and quantification of Aflatoxins in compound feeds from South Africa

Validation of the TLC results and the determination of the concentrations of AFs in South African feeds were achieved by HPLC analysis from immuno- affinity column extracts. This was based on the peak area of chromatograms of AFs standards (Figure 4.3) in comparison to those of extracts (Figure 4.4) AFs as presented.

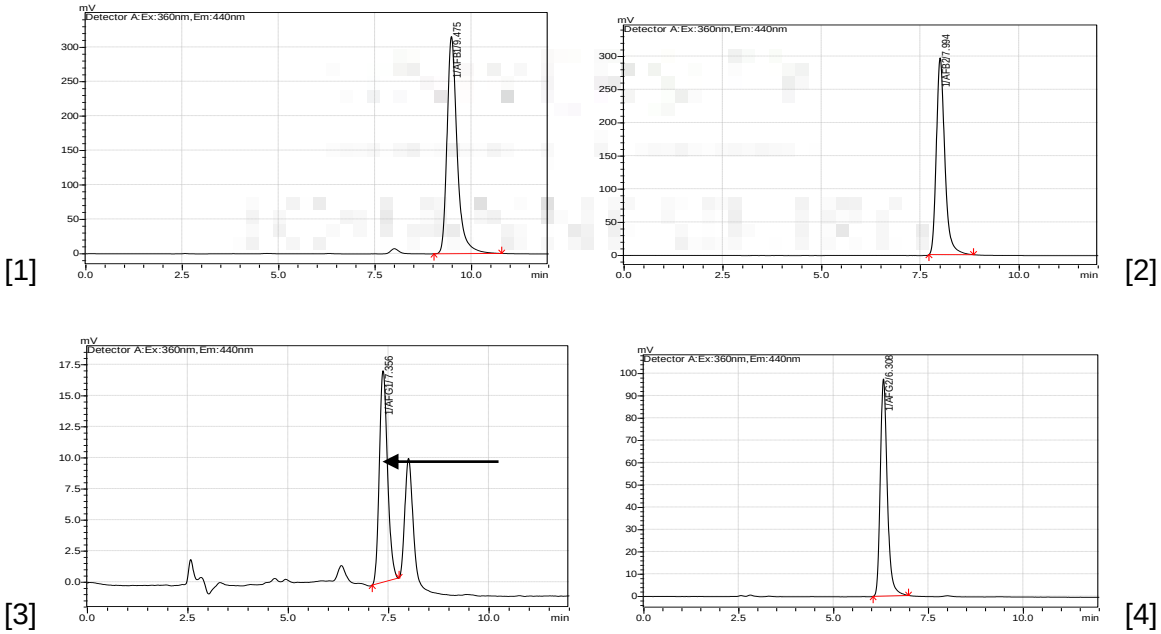
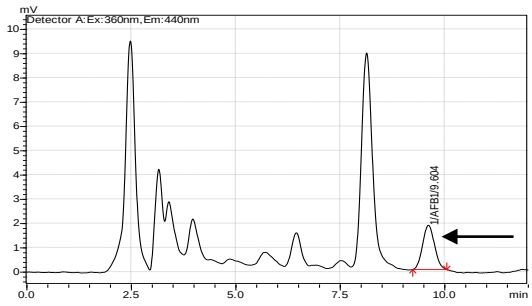
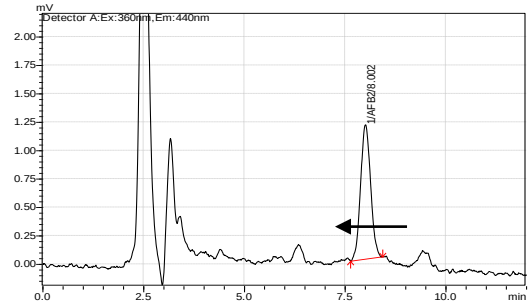
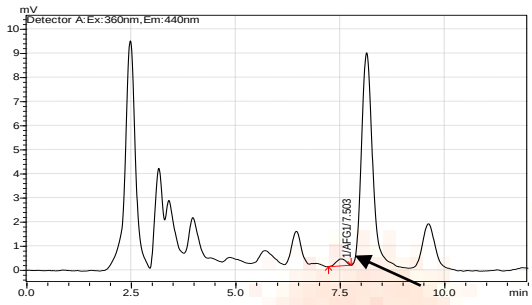


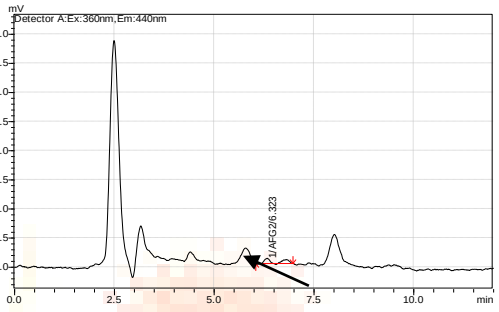
Figure 4.3 Chromatograms of aflatoxins standards; [1]: AFB_1, [2]: AFB_2, [3]: AFG_1, [4]: AFG_2 observed under high performance liquid chromatography

[1] 



[2]

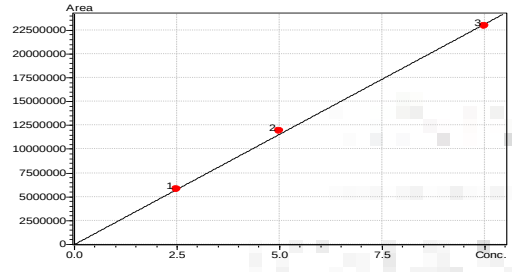
[3] 

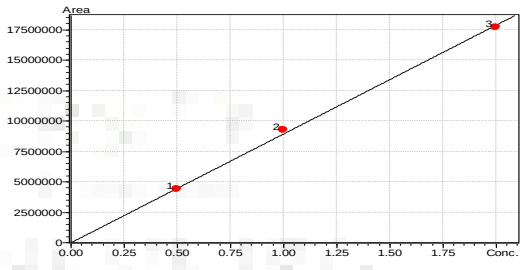


[4]

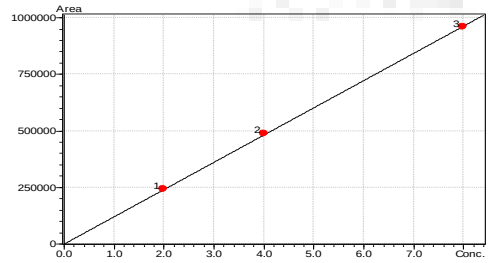
Figure 4.4 Chromatograms of aflatoxins in compound feed extracts from South Africa showing presence of aflatoxins ([1]:AFB_1, [2]:AFB_2, [3]:AFG_1 & [4]:AFG_2)

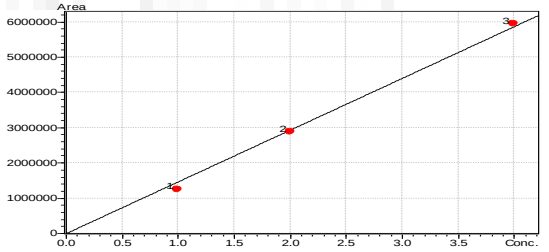
The experimental calibration curves (Figure 4.5) were obtained with known concentrations of standards and the equations describing calibrated curves; were Y= 2312588x, 8938199x, 120465x and 1466470x for standards of AFB_1, AFB_2, AFG_1 and AFG_2, respectively, (where x is the peak area of chromatogram and Y is the AF concentration) with correlation coefficient values of (R^2) of $\geq$ 0.9993 obtained and all at the y-intercept of zero showing a linearity of the method used.

[1] 



[2]

[3] 



[4]

Figure 4.5 Calibration curves of aflatoxins standards [1] AFB_1, [2] AFB_2, [3] AFG_1 and [4] AFG_2 generated via HPLC

The limit of detection (LOD) of AFs was higher for HPLC as mores samples (73%) were positive for AFs than for TLC (55%) with AFs recoveries as follows: AFB_1 in samples was recovered at a rate of 98.7 ± 1.0% (mean ± SD); AFB_2 at 99.5 ± 0.8%, AFG_1 at 98.1 ± 0.7% and AFG_2 at 96.9 ± 0.9%. Data on the incidence and levels of AFs contamination (not adjusted based on recovery) are summarized in Table 4.3. Overall, poultry feeds were the most contaminated feeds with AFs being recovered in 62% of samples analyzed when compared to cattle (57%), meanwhile pig (5%) and horse (3%) feeds were the least contaminated substrates. The highest contamination levels of AFs were found in poultry feeds ranging from 0.005 – 77 ppb (mean: 21.7 ppb), while much lower levels were estimated in cattle feeds (mean: 9.1 ppb; range: 0.007 to 18.27 ppb), with horse and pig feeds having the least mean contamination levels that varied between 0.005 and 0.25 ppb (max. 0.7 ppb).

Data obtained in this study revealed that 22.7 % of contaminated samples had AF levels that exceeded the South Africa regulatory limit (10 ppb for chicks, calves and piglets, 20 ppb for poultry and swine, 5 and 50 ppb, respectively for dairy and beef cattle) and 10 ppb acceptable limits of the EU especially for AFB_1.

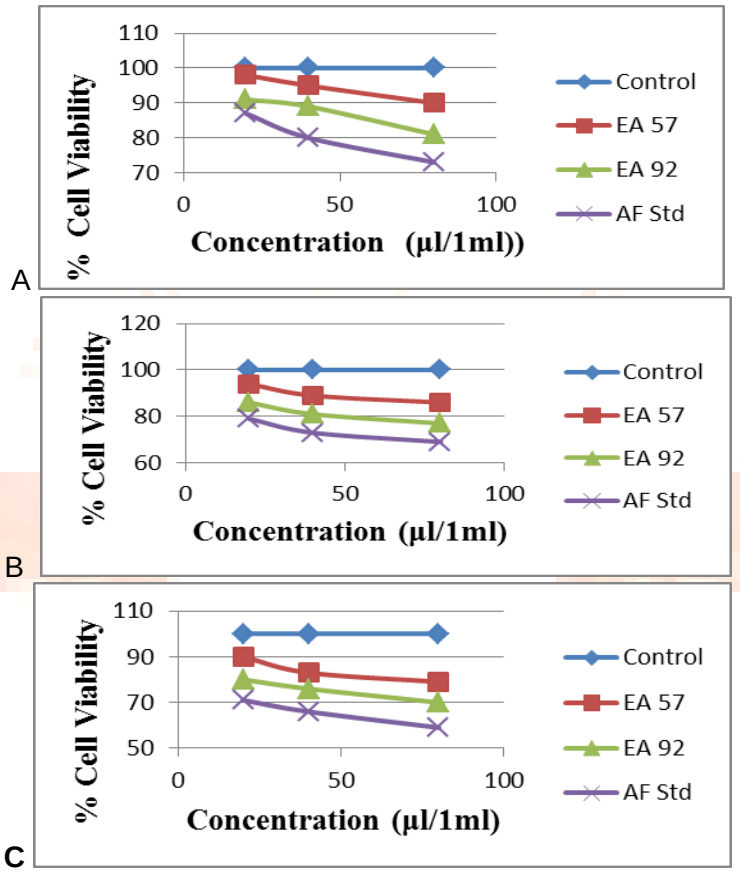
Table 4.3 Estimates of aflatoxins (ppb) in animal feeds from South Africa obtained by HPLC

	AFB_1				AFG_1			AFB_2			AFG_2		
Feed	**N[a]**	**N[b]**	**Range[c]**	**Mean[d]**	**N[b]**	**Range[c]**	**Mean[d]**	**N[b]**	**Range[c]**	**Mean[d]**	**N[b]**	**Range[c]**	**Mean[d]**
Poultry													
Broiler	28	20	1.2 - 72.0	21.7±0.5	17	3.8 - 48.2	16.8 ±0.1	8	1.5 -10.1	3.7 ±0.2	1	-	-
Layer	20	13	3.6 - 8.6	2.2 ±0.7	12	3.3 - 5.1	2.8 ±0.3	8	0.01 - 3.0	1.08±0.5	1	-	-
Breeder	14	9	3.0 - 7.4	1.8 ±0.2	7	0.5 - 5.1	1.3 ±0.5	5	2.8 - 3.3	1.1 ±0.3	-	-	-
Cattle													
Dairy	11	8	0.8 - 4.5	1.0 ±0.5	7	3.0 - 3.5	3.1 ±0.3	7	0.2 -1.1	2.6 ±0.5	-	-	-
Calf	8	5	0.5 - 4.5	1.1 ±0.8	5	0.7 - 4.9	1.5 ±0.2	3	0.2 -1.0	1.3 ±0.4	-	-	-
Finisher	6	5	0.2 - 2.3	0.7 ±0.6	4	0.5 - 3.1	1.0 ±0.6	4	0.1 -1.8	0.6 ±0.4	-	-	-
Others													
Horse	3	1	-	0.7 ±0.4	0	-	-	0	-	-	-	-	-
Pig	2	1	-	0.5 ±0.3	0	-	-	0	-	-	-	-	-

[a]Number of samples analyzed. [b]Number of samples positive with AF , [c, d] Range and mean levels of AF contents for positive samples

4.4 *Toxicity of AFs containing feed extracts*

Aflatoxin (AFB_1) standard at concentration levels of 20, 40 and 80 µl/ml as well as AFs containing feed extracts at different concentration levels were tested *in vitro* to evaluate their effects on the viability of human lymphocytes after 24, 48 and 72 hrs. of exposure. This was performed to confirm the estimated levels of AFs established via HPLC and also as a biological means of evaluating the safety of compound feeds from South Africa. Data obtained are presented in Figures 4.6A to 4.6C.



AFB_1 std (aflatoxin standard), EA 57 & EA 92 (feed sample codes)

Fig 4.6 Toxic effect of aflatoxin standard and aflatoxins extracts from compound feed samples on human lymphocytes over a 24 hrs (A), 48 hrs (B) and 72hrs (C) of exposure

According to data, it was observed that viability of cells treated with no aflatoxin standard nor extracts, used as a control was 99.8% after 24 hrs. of incubation. The viability of cells after 24 hrs. of exposure was strongly influenced by the concentration of the toxin with AFB_1 standard and sample extracts with high AF levels causing a higher reduction in cell viability when compared to the control or those of extracts with lower AF contents. Cell viability decreased significantly ($P<0.001$) over time because of continued exposure of AF. As a result of significant aflatoxin standard and aflatoxin extracts -induced-cell mortality with dose increment and time, the cytotoxic effect of AF standard on the lymphocyte cells was observed to be toxic at all levels with reference to percentage cells viability (mean + SD) of 87 ± 2.01%, 79 ± 1.09% and 72 ± 1.50% found after

24 hrs. of exposure at different dilution concentrations (Fig 4.6). A further reduction in cell viability was obtained after 48 and 72 hrs. of exposure under the same conditions.

4.5 Molecular Analysis

4.5.1 Gel Electrophoresis

The molecular size of DNA obtained after extraction was determined by gel electrophoresis. This is the most important information derived from the agarose gel and the usual reason for running a gel. The gel electrophoresis allowed for the separation and visualization (Figure 4.7) of DNA fragments from *Aspergillus parasiticus* and *A. flavus*. As found, molecular sizes of the DNA of fungal species were estimated by the fluorescence intensity and comparison of the distance travelled with that of the molecular weight of marker standard as measured. However, the data indicate that DNA fragment for IV than III have a relatively similar molecular size though both are relatively not that large in terms of concentration as seen in the position of bands distance covered.

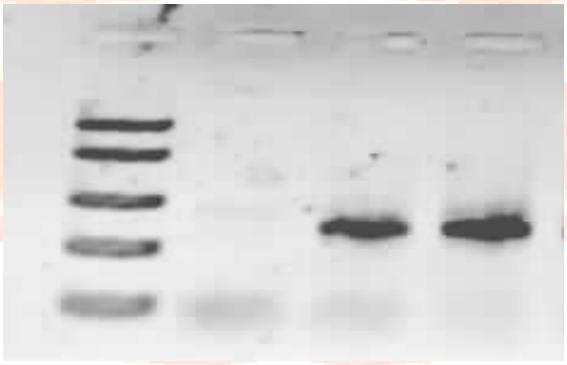


Figure 4.7 Gel electrophoresis photo showing DNA portions of *Aspergillus* isolates. [i]: 5 bands molecular weight marker with different melting points,[ii]: Negative control (water), [iii]: *Aspergillus parasiticus,* [iv]: *Aspergillus flavus*

4.5.2 Real time PCR

From the standard curve generated (Figure 4.8), a slope of approximately -3.361 was deduced, representing an efficiency range of 0.987 (approximately 99%). This result infers that dilution series are accurate and master mix components' concentrations were optimal since the spacing between each amplification curve based on the serial dilutions was approximately 3.36 cycles.

Template degradation was noted and quantification sensitivity did not decrease since the y-intercept of 32.9 was not too high and in addition, the R^2 value of 0.9955 indicates that there were no bad precisions in creating serial dilutions.

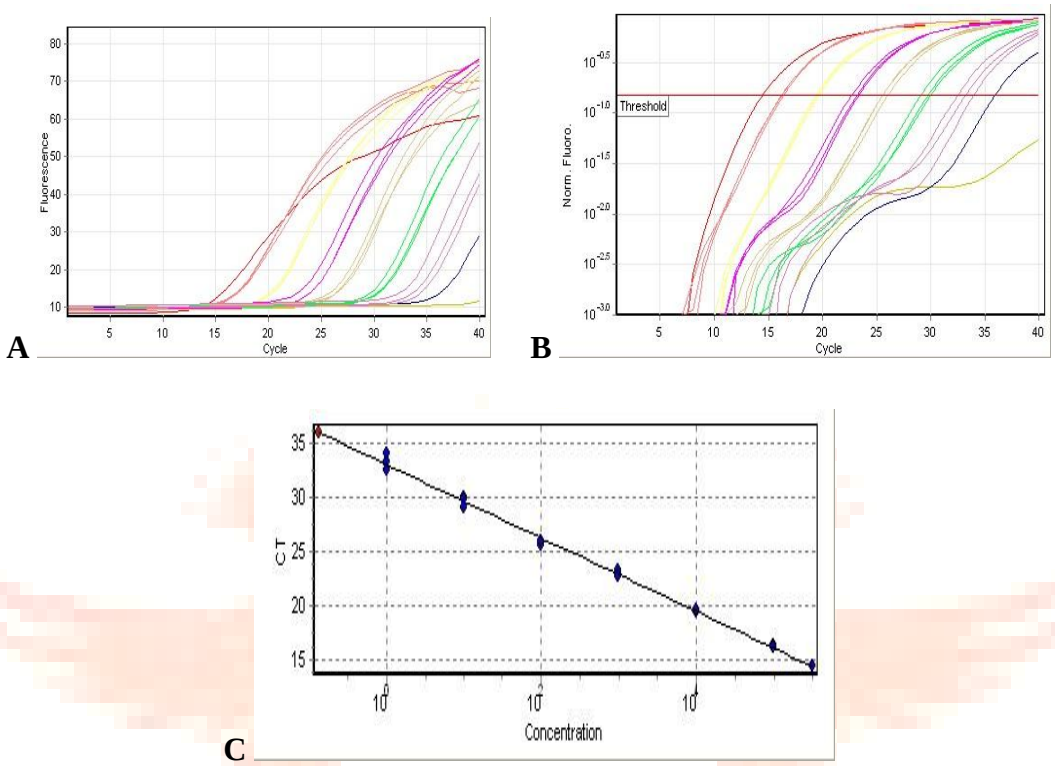


Figure 4.8 Amplification plot [A: raw data cycling A (Green), B: quantitation data cycling A] and [C] corresponding quantitation standard curve generated on real-time PCR analysis

A clear relationship between initial DNA concentration and changes in fluorescence showed an amplification curve with known template concentrations. DNA isolated from the fungi suspension in a media broth, in a range of 2.5×10^3 to 2.5×10^7 CFU/g reaction showed progressively lower threshold (C_t) values. Accordingly, with the observed data, mean percentage recovery for DNA isolated was 94.7±16.4% (n=3). Data indicated an inverse linear correlation between DNA/pg and Ct with slope (mean = -2.8) and r values similar in three independent assays (mean = 0.99), indicating the high linearity of RT-PCR system. With these results, the primer/probe system used (nortaq-1, nortaq-2, norprobe) appeared sensitive and accurate for detection of the nor-1 fragments extracted from the compound feeds samples.

4.5.3 Specificity of primers and probe reaction system

RT PCR was performed for the determination of reaction specificity, using different species of environmental fungi strains isolated from feeds. Primers and probes used in the reaction system showed high specificity (Table 4.5) as expected for aflatoxigenic fungi characterized using *nor-1* gene and a negative result was found for other fungi that do not contain the *nor-1* gene.

Table 4.5 Specificity of reaction as shown with DNA of different fungi strains

Fungi strains	RT PCR reaction with *nor-1* primers & probe
Aspergillus flavus	+
Aspergillus parasiticus	+
Aspergillus ochraceus	-
Aspergillus niger	-
Fusarium verticillioides	-
Fusarium proliferatum	-
Penicillium citrinum	-

4.5.4 Correlation of aflatoxin level to *nor-1* gene

The incidence of AFs recovered livestock feed samples was 78.4%. However, the concentrations of DNA varied (P>0.05) amongst the feeds. This variation was also a characteristic feature of the *nor -1* gene detected and quantified in the sample feeds. The picogram (pg*)* DNA/mg feed were correlated with AF. The correlation coefficient (r) determined for the target DNA gene and AF levels in the feeds type was within the range of -0.021 to 0.70 for the different feed samples (Table 4.6) based on picogram DNA/mg feed.

Table 4.6 Aflatoxin load in correlation to *nor-1* gene concentration in sampled animal feeds from South Africa

Sample feed types	AF (ppb)	DNA (pg/mg)±SD
Poultry feeds (n = 62)		
Broiler (n= 06/28)		
EA 003	5.0	19.3 ±1.1
EA 034	30.0	05.3 ±3.2
EA 040	1.8	00.2 ±0.1
EA 055	33.0	45.7 ±3.02
EA 087	17.3	150 ±27.9
EA 088	72.0	24.8 ±1.0
Layer (n = 5/20)		
EA 001	1.2	25.1 ±18.2
EA 011	3.6	7.6 ±6.1
EA 022	7.0	110.1 ±5.1
EA 030	8.6	159.2 ±3.8
EA 089	4.8	20.9 ±1.4
Breeder (n = 4/14)		
EA 010	4.8	0.9 ±0.3
EA 055	5.5	2.6 ±6.5
EA 060	7.2	2.7 ±0.9
EA 090	7.4	8.4 ±2.6
Cattle feeds (n=25)		
Dairy (n = 5/11)		
EA 064	3.1	3.9 ±1.4
EA 066	1.9	11.3 ±1.0
EA 068	1.7	4.8 ±1.2
EA 070	3.1	8.7 ±5.6
EA 091	4.5	17.5 ±2.9
Calf grower (n = 4/8)		
EA 072	2.4	0.1 ±0.0
EA 074	2.2	20.6 ±4.5
EA 076	4.5	4.5 ±2.8
EA 078	2.6	2.5 ±0.9
Finisher (n = 2/6)		
EA 079	2.3	5.9 ±3.0
EA 083	2.0	0.3 ±0.2
Other feeds (n = 5)		
Horse (n = 3/3)		
EA 013	0.0	nd
EA 085	1.0	2.3 ±0.9
EA 086	0.0	5.1 ±2.5
Pig (n = 2/2)		
EA 014	0.0	2.0 ±1.4
EA 017	0.7	0.6 ±2.4

*S.D: Standard deviation, *nd: not detected

*AFB_1 was used in the correlation with the genomic DNA as is it the most occurring;

*DNA (pg/mg) was average of three replicates and mean ± SD *n: number of samples analyzed and total is 30

*overall R^2=-0.093

CHAPTER FIVE
DISCUSSION

5.1 *Aspergillus flavus and Aspergillus parasiticus contaminations in livestock feed*

Aspergillus flavus and *A. parasiticus* are fungal species that are ubiquitous in nature. They both grow on a large number of substrates like livestock feeds, particularly under high moisture conditions and other favourable ecophysiological conditions. These two fungi in particular, have been isolated from a wide range of livestock feeds, especially those of cereal and nut origins (Saleemi *et al*., 2010). They are considered amongst the most important pathogenic fungi that contaminant livestock feeds universally (Ghiasian and Maghsood, 2011). There have been several reports (Owino *et al*., 2008; Akande *et al.*, 2006; Bennett and Klich, 2003; Dutton and Kinsey, 1996; Dutton and Westlake, 1985) from South Africa on contamination of agricultural commodities by *Aspergillus flavus* and *A. parasiticus*. In the present study, 77.2% of the 92 compound feeds analyzed were contaminated with *Aspergillus flavus* and or A. *parasiticus.* Although, 131 isolates were recovered from the analysed feed samples, *Aspergillus flavus* was more dominant (67.5%) than *A. parasiticus* (51.1%). With reports from South Africa (Passone *et al*., 2012; Somai and Belewa, 2011) and those from other regions of the world (Ige *et al*., 2012; Pitt and Hocking, 2006; Razzaghi-Abyaneh *et al*., 2006), these two species of *Aspergillus* are the most widely studied because of their importance in livestock feeds not only due to their pathogenic potentials in causing a wide array of diseases in plants and animals (*Aspergillosis*) but because they produce the most significant mycotoxins, AFs. A variety of other fungi belonging to the *Aspergillus, Penicillium,* and *Fusarium* species were also recovered as contaminants of feeds in this study as they have previously been isolated from livestock feeds (Logrieco *et al*., 2003). Samples under study were sampled from stored products. *Aspergillus* species has most frequently been isolated from feed commodities kept under storage conditions (Pitt and Ailsa, 2009) i.e. a_w of between 0.8 to 0.9 (Flannigan and Miller, 2001) with a wide temperatures range of 19-35 °C (Parra and Magan, 2004). These conditions favour *Aspergillus flavus* and A. *parasiticus* to outcompete other fungi in stored products particularly in humid and hot climate regions like South Africa.

Focusing in this present study, the identification of *A. flavus* and *A. parasiticus*, their morphology/phylogeny as isolated and identified the livestock feed samples, were studied in respect to the data from literature. Levels of contamination in horse and pig feeds were very low when compared with that of poultry and cattle feeds. This may be related to type of feed ingredients and their percentage inclusion levels during feed formulation and possibly those used in formulating cattle or chicken feed are susceptible to contamination by these two species. However, this is inconclusive based on the fact that a very small sample size for horse and pig

feeds was used in this study. The high incidence of *A. flavus* and *A. parasiticus* as observed in this study is in concordance with data reported by Mngadi *et al*. (2008) for another set of feed in South Africa. These were also reported in similar works of Dalcero *et al*. (1997), Oliveira *et al.* (2006), Rosa *et al*., (2006), Osho *et al.* (2007),Banu and Muthumary (2010) and Ouattara–Sourabie *et al.* (2012) in some other countries. The two species of *Aspergillus* isolated in present study ranked according to their isolation frequency. *Aspergillus. flavus* was in abundance and more commonly recovered from samples than *A. parasiticus*. *A. flavus* is reported to be more widely distributed and have a higher occurrence frequency in food commodities when compared to *A. parasiticus* (Njobeh *et al*., 2009; Copetti *et al*., 2011) although they both occur more frequently in foods than other fungal strains. In Brazil (Simas *et al*., 2007a), Argentina (Dalcero *et al*., 1998), Spain (Bragulat *et al*., 1995, Abarca *et al*., 1994), high frequency of isolation have been recorded for these *Aspergillus* species occurring in livestock feeds. Also, in Ghana (Kpodo *et al*., 2000), Italy (Giorni *et al*., 2007), Pakistan (Shah *et al*., 2008) and Algeria (Riba *et al.*, 2008), high frequency was recorded, especially in livestock feeds formulated from cereals. This may be due to the fact that different cereals make up a major ingredient of livestock feeds, since the mycoflora of cereals is a reflection of *Aspergillus* fungal contamination in livestock feeds. And according to Accensi *et al*. (2004), highest frequency of fungal contamination have also been recorded in Spain in livestock feeds. These contamination occurrences of *Aspergillus* species including that in this present study may be due to high temperature tolerance (Battilani *et al*., 2003) and high humidity (Amadi and Adeniyi, 2009) in their morphological and biochemical growths in these regions.

In this study, it was possible to identify aflatoxigenic fungi by looking at fluorescing spots representing AFB_1, AFB_2, AFG_1 and or AFG_2 under UV. Over 68% strains of *A. flavus* and *A. parasiticus* isolated from feeds were toxigenic in producing attendant AFs. In the literature, it has been reported that close to half of *A. flavus* isolated are aflatoxigenic and produces only the type B AF, while *A. parasiticus* produces both type B and G (Al-Hmoud, 2012; Copetti *et al*., 2011) as observed in this study. However, further confirmations of the aflatoxigenic potential of these fungal species were established using RT-PCR by isolating and quantifying *nor-1* gene responsible in producing AF.

Nevertheless, it is important to note that, there existed multiple contaminations of livestock feeds by different *Aspergillus* genera, including *Fusarium* and *Penicillium* fungi. However, feed contamination by AFs as reported herein were on the basis of aflatoxigenicity of *Aspergillus flavus* and *Aspergillus parasiticus* in the production of these toxins.

5.2 *Aflatoxin contamination*

In this study, TLC was used to screen for the presence of AFs in compound feeds which correlates that with HPLC data and provided estimated levels of four AFs (AFB_1, AFG_1, AFB_2 and AFG_2) in

samples. Thin layer chromatography data in the study showed the presence of AFs, though of very small number of samples contained AFG_2. The limit of detection (LOD) of AFs was higher for HPLC as mores samples (73%) were positive for AFs than for TLC (55%). There were a few of the samples that were negative under TLC analysis but were positive, though in very low concentration of (0.06 ppb) when analysed by HPLC. This could be attributed to the fact that the LOD of AFs, using HPLC methods is much sensitive than TLC, which detects higher concentrations. The level of AFs in the livestock feeds based on the HPLC results in the present study varied from 0.06 to 77.97 ppb. This may be based on the levels and types of feed ingredients used in formulating such feeds. These results are in line with those of Fraga *et al.* (2007), Campos *et al.* (2008), Anonymous (2008), Lanier *et al*. (2009) and Saleemi (2010) in livestock feeds, with AFB_1 being the most abundant of the AFs recovered.

The detection of AF in feeds analysed herein was not surprising since several of similar samples also contained aflatoxigenic fungi which may be correlated with the AF feed contamination. Presence of AFs in livestock feeds, and the continues consumption of such feeds by animals, may reduce livestock health and performance (Hanif *et al*., 2008; Campos *et al*., 2008; Binder *et al*., 2007). The negative effects on livestock due to such exposures have been that of cancer, reproductive problems, vaccine, drug failure, poor growth, deficiency of vitamin A and death in severe circumstance. For example, aflatoxicosis among dogs recently witnessed in the country last year, in which more than 220 dogs died due to pet food poisoning with high levels of AFs (Arnot *et al*., 2012). Therefore, it is very crucial that effective measures are adopted which include regulatory practices and routine analysis must always be performed to ascertain the AF contamination pattern in feeds and in worse case scenarios, to effectively reduce or at best, eliminate (if possible) the propagation of aflatoxins, hence ameliorating the health disorders they may cause thereby, improving livestock productivity.

5.3 *Cytotoxic effect of aflatoxin on cell viability of human lymphocytes cells*

In the present study, the cytotoxic effect of feed extracts containing AF on cell viability of human lymphocytes cells in comparison to that of standard AFB_1 was performed *in vitro*. *In vitro* cytotoxicity testing was initially describe by Jelinek (1977) on chick embryos toxicity screening test (CHEST) against toxic potentials of different chemicals. However, in the present study, human lymphocytes were used. Nevertheless, there are other reports (Vesela *et al.* 1983; Jelinek *et al*. 1985; Vesely *et al*., 1992; Javed *et al.,* 1993, Henry and Wyatt, 2001; Peterka *et al*., 2002 and Sehata *et al*., 2004) that determined toxic effects of chemical against lymphocytes cells, which included antitumor drugs, antipyretics, antibiotics, antibacterial, and major mycotoxins such as ZEA, TH, OT, FB, AF, patulin and ergot.

The toxico-pathological potential of AF, both standard and extracts from the different positive compound feeds showed that AFB_1 standard had higher toxicity on lymphocyte cells in terms of decreased cell viability, with further decrease observed over time of exposure. Overall data revealed that AFB_1 standard was the most toxic when exposed to cells; the lowest percentage cell viability was recorded when compared to feed extracts whose exposures also reduced cell viability but at lower levels. Data also indicated that cell viability on human lymphocytes was considerably reduced with increase in dosage concentration levels from 20, 40, 80 µl/ml as well as due to duration of exposure (24, 48 and 72 hrs.) This agreed with the reports of Bünger *et al*. (2004) and Meky *et al.* (2001) in which cell mortality increased with increasing AF levels and duration of exposure.

Aflatoxin has been linked to the increased immunosuppression probably resulting in many cases of aflatoxicosis in animals reported in South Africa (Pitout and Schabort, 1973; Moreau, 1979; Bastianello *et al*., 1987; van Halderen *et al*., 1989; Van Halderen *et al.*, 1998; Van Halderen *et al.*, 2000; Otto, 2011; Bamford, 2011; Arnot *et al*., 2012) and other parts of the world (Ketterer *et al.,* 1982; Patterson, 1983; Ramos and Hernández, 1997; Dhama *et al.*, 2007; Thomas *et al.*, 2010; Akinrinmade and Akinrinde, 2012). This occur would when animals ingest feeds/feed products containing high levels of AFs (Lee *et al*., 2004). The mechanism of action of AFs involves DNA binding and inhibition of nucleic acid synthesis (Stark, 1980; Tiwari *et al*., 1986; Somashekar *et al.*, 2004b; El Khoury *et al*., 2011) which may result in aflatoxicosis in animals with vague initial presentations (Dixon *et al*., 2008). Loss of lymphocyte cells initiated by aflatoxin may cause immunosuppression, since cell mortality is the initial step of immunomodulation in animal species (Forsell *et al*., 1985), hence favouring infections. It is known, that T lymphocytes play a pivotal role in the immune system by being responsible for immune response (Størmer and Lea, 1995) acting as a natural defence mechanism against host invasion of diseases. This may lead to a gradual but harmful onset of a disease condition. Some signs and symptoms due to such secondary infections are lethargy, anorexia and jaundice (Aller, 1981; Akande, 2006; David, 2009). Sometimes signs such as hematochezia, melena and hematemesis (Tedesco *et al.*, 2004) are present including widespread petechiae and ecchymoses or mucosa (Galvano *et al*., 2001). Some animals may present peripheral oedema or ascites, polyuria and polydipsia (Eraslan *et al*., 2005, Wilson, 2010) in the progressive stages of the disease. Though cases of deaths caused by AF related diseases are high, liver failure is usually a common phenomenon.

5.4 *Molecular aspects of selected fungi with nor-1 gene*

Identification of fungi by molecular means is considered the most reliable over conventional method. Though it is expensive, labour and time intensive, yet have become the most common tool for rapid identification of *Aspergillus flavus, Aspergillus parasiticus* and other types of environmental fungi (Borman *et al*., 2008). *Aspergillus flavus* and *Aspergillus parasiticus* isolated

from compound feeds were identified both on morphological and molecular bases. Both of these species of fungi are morphologically and molecularly similar, however, they can be identified further as a variety of closely related species (Perrone *et al*., 2007). Suggestions made by Martinez-Culebras and Ramon (2007) on phylogenetic analysis can be adopted in developing a relationship between closely related species of fungi like these. These two fungi initially identified morphologically appeared as the same species by molecular methods. This suggests precise identification looking at the presence of *nor-1* gene in these strains as closely related species in terms of DNA molecular size (600 kpb) which agrees with other reports (Mohankumar *et a*l., 2010; Godet and Munaut, 2010).

This *nor-1* gene of interest is a PCR based protocol which agrees with the study of González-Salgado *et al*. (2011), on highly sensitive PCR-based detection method specific for aflatoxigenic *Aspergillus* species and the efficiency determined was within the acceptable efficiency range of 80 - 100% for a real time PCR analysis (Stephen *et al*., 2009; Ran *et al*., 2012; Robert *et al*., 2012). This may be according to Dorak (2006a), due to the fact that more reaction cycles are required to detect the same amount of starting material (nor-1) reported by Abdel-Hadi *et al*. (2010) and Varga *et al*. (2011) as the first gene in the AF biosynthesis pathway in the fungal species under study on the production of AFs.

5.5 *Correlation of aflatoxin load in selected positive feed sample with nor-1 levels*

RT-PCR has been employed in the analysis of some commodities. The present study is the first performed not only to identify AF producing fungi i.e*. Aspergillus flavus* and *A. parasiticus*, but to also compare the influence of relative quantification of gene in relation to AF load in livestock feeds. This was based on *nor-1* gene levels in correlation to that of estimated AF levels in similar feeds. This provided information that relates molecular characteristics to morphological parameters which influence aflatoxin production by aflatoxigenic fungi. *Nor-1* gene, when compared to aflatoxin load, is a constant constitutive expression of the gene (Mayer *et al*., 2003) in the metabolic phases of the aflatoxigenic fungal species. In this study, there was no statistical correlation between aflatoxin load and the *nor-1* gene expression levels. The reason for such an observation cannot be explained with certainty, but it is possible that other extractable compounds obtained during DNA extraction may interfere RT-PCR analysis (Rossen *et al*., 1992). It may also suggest that even though *nor-1* gene was present in the feeds, it may or may not have been expressed as the case maybe, to produce AF.

The study of Passone *et al*. (2010), established a correlation between *nor-1* gene expression and CFUs of these *Aspergillus* species (R^2=0.613, P<0.0001) in naturally stored agricultural. However,

that study was not based on AF load, but CFU. Another study (Bagnara *et al.,* 2000) determined copy numbers of *nor-1* gene which were poorly correleted.

Furthermore, it is possible that propagules of *Aspergillus flavus* and *A. parasiticus* would contain the *nor-1* gene, but the presence of this gene may not entail AF production. Schmidt-Heydt *et al*. (2007) also examined *Penicillium verrucosum* populations, ochratoxin A (OTA) and the OTA polyketide synthesis gene (*otapks*Pv) expression in feeds and showed a good correlation between the *otapks*Pv expression and OTA production but this was paralleled by CFUs of *P. verrucosum*.

In the present study, there were samples (3/30) that did not contain *nor-1* but however were contaminated of AFs. This may point to the fact that there was no permissible detection because of incomplete induction of the gene and gene presence/expression may vary with respect to physiological conditions as viewed by Scherm *et al*. (2005). It could also be that some other genes i.e. *aflR* and *aflQ* present in some strains of *A. flavus* and *A. parasiticus* involved in AF biosynthetic pathway (Scherm *et al*., 2005) or other genes present in other less common AF producing fungi (*A. normius, A. bombycis*, and *A. pseudotamarii*) may account for this observation reported herein. This however, needs to be studied further because, the determination of *nor-1* gene expression in relation to AF production/load in livestock feeds by the real-time PCR system is possible as has been observed in other food commodities. As a model for livestock feed matrix, a real-time reverse transcription-PCR system to establish AFs in feeds is necessary, so as to completely characterize the mycobiota of livestock feeds. This presents a useful tool for further study, to determine of correlations between *nor-1 e*xpression and aflatoxin load by all AF producers in feeds. As such, help in predicting the potential risk of AF production in livestock feed based on *nor-1* gene levels alone, which may be an initial step towards improving feed safety.

CHAPTER SIX
CONCLUSION

The present study was carried out to isolate and enumerate aflatoxigenic *Aspergillus* fungi i.e. *A. flavus* and *A. parasiticus* from various compound feeds from South Africa, detect and quantify AF and the determinant gene (*nor-1*) involved in aflatoxin biosynthesis. The cytotoxic effect of AF extracts from feeds on human lymphocytes in comparison to that exhibited by AFB_1 was established. The detection and quantification of AF and the determinant gene (*nor-1*) was for the purpose of establishing the correlation between the various data on feeds relating to AF production i.e. make out a correlative quantification of the toxin via HPLC and the gene responsible for its production via RT PCR. In achieving these, livestock feed samples were taken and analysed. From the results obtained, *A. flavus* (most abundant and frequently occurring) and *A. parasiticus* were found to be the main feed contaminants, with a higher proportion of them being toxigenic in producing their attendant AFs. These two fungal species are supposedly responsible for the presence of AF in these feeds and in some cases (mainly in some poultry and cattle feeds), levels of the toxins found were considered unsafe for animal consumption especially as such feeds do contain such significant mycotoxins as FB, OTA, etc. as established. This was further confirmed when the cytotoxic effect of extracts of increasing concentrations of toxins decreased the viability of lymphocyte cells which further decreased with duration of exposure. Not only can animal health be affected, animal performance and international trade can as well be compromised since feeds from South Africa are exported to neighbouring countries.

The lack of of no direct relative correlation between the *nor-1* gene levels and those of AFs present in feed samples is in contrast to what has been observed when dealing with other agricultural commodities. Whatever the case may be, it is of paramount importance that control and preventives measures should be upheld both in legislation, act and practice, to ascertain the quality of livestock feeds towards breeding healthy livestock, to promote their health and enhance animal performance.

In conclusion, data obtained and reported herein showed that there is a need to adopt therefore, effective regulatory measures to control AFs and or other mycotoxin contamination of feeds in South Africa, while establishing surveillance programs on a continuous basis to safeguard public health and avoid the recurrence of aflatoxicosis as experienced in the previous years, especially that of year 2011 in the country.

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APPENDICES

Appendix I

1.0 Media preparations

All media preparation used in this study were prepared following standard preparation procedures under antiseptic conditions in the Food, Environmental and Health Research Group Laboratory, Faculty of Health Sciences, University of Johannesburg, South Africa.

1.1 Potato dextrose agar (PDA) (Nelson *et al*., 1983)

Fungi in raw materials, yeast and finished products can be isolated, cultivated and enumerated via a medium of potato dextrose agar. Due to its colour production, it is highly suitable for *Aspergillus* growth. Preparation of this media was achieved by dissolving 39 g PDA powder (Merck & Sigma) in 1 L of distilled H_2O. This was autoclaved at 121 ^{0}C for 15 minutes and cooled to 50 ^{0}C. This media (20 ml) was poured into Petri dishes; 8 ml of sterile? 1% chloramphenicol and 1% streptomycin were added to suppress bacterial growth during culturing.

1.2 Czapek Yeast Extract Agar (CYA) (Klich, 2002)

Preparation was achieved by dissolving 1 g of K_2HPO_4, 5 g of yeast extract agar, 30 g sucrose, 15 g of agar powder and 10 ml of Czapek concentrate in 1000 ml of distilled water. This was autoclaved at 121 ^{0}C for 15 mins and cooled to at 50 ^{0}C and 8 ml each, of prepared 1% chloramphenicol, 1% streptomycin were added. 20 ml was poured into Petri dishes for inoculation.

1.3 Malt Extract Agar – MEA

It was prepared by dissolving 20 g of malt extract powder, 1 g of peptone, 20 g of glucose and 20 g of agar in 1000 ml of distilled water and was autoclaved at 121 ^{0}C for 15 mins. After cooling to 50 ^{0}C, 8 ml each of sterile 1% chloramphenicol, 1% streptomycin were added and mixed by shaking. 20 ml was poured into each Petri dish. This media is very suitable for the isolation of *Penicillium* and *Aspergillus*.

1.4 Ohio Agricultural Experimental Station Agar – OAESA

Ohio Agricultural Experimental Station Agar is highly recommended for isolation of soil fungi. It was prepared by dissolving 5 g $C_6H_2O_6$, 2 g yeast extract, 1 g $NaNO_2$, 0.5 g $MgSO_4$, 1 g KH_2PO_4, 1 g ox bile, 1 g CH_3CH_2COONA and 20 g nutrient agar in 900 ml distilled. This was made up to 1000 ml. It was then autoclaved at 121 ^{0}C for 15 minutes and cooled to 50 ^{0}C in a water bath. 8 ml each of sterile 1% chloramphenicol and 1% streptomycin solutions was added and mixed.

1.5 Ringer's solution preparation

Prepared by dissolving 2 Ringer's solution tablets in 1 L of distilled water and autoclaved at 121 ^{0}C for 15 minutes.

1.6 Antibiotics preparation

1% each of streptomycin and chloramphenicol were prepared by dissolving 1 g each of streptomycin in 100 ml of sterile distilled water. The solution was sterilized by passing through a 0.22 μm sterile filter paper before use.

Appendix II

Identification of *Aspergillus flavus* and *parasiticus* isolated from feed samples, based upon synoptic key

Identification parameters	*Aspergillus flavus*	*Aspergillus parasiticus*
Macroscopy (Colonies Characteristics)		
Color observation	Yellowish green. Becomes green with maturation.	Green. Becomes dark green with maturation.
Petri dish reversed color observation	Cream to yellow	Cream yellow
Diameter (cm)	4 - 5 cm	3 - 4 cm
Microscopy (Slide Culture)		
Head	Radiating columnar	Globose radiating
Stipe/Hyphae	Long, non-septate	Long, rough stipe
Vesicle	Dome shaped fertile from surface	Loosely globose, fertile on surface
Phialides	Small in shape	Broad neck
Metulae	Present	Absent
Conidia	Globose to subglobose in shape	Globose

Appendix III

Raw Data for MTT Cytotoxicity Assay in Triplicates
Table 4.5a Toxicity on Human Lymphocytes Cells after 24 hrs

	% Cell Viability ±SD		
Sample	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	91±2.1	89±0.9	81±5.0
EA 57	98±0.7	95±1.3	90±2.7
EA 83	96±1.1	90±3.3	87±1.5
EA 26	97±1.1	91±4.0	89±2.0
EA 91	98±3.4	90±2.8	86±2.9
EA 70	98±0.3	93±2.2	91±0.5
EA 88	94±2.1	91±3.0	89±3.9
EA 78	99±5.3	96±2.7	90±1.1
EA 21	95±2.1	91±4.2	89±0.9
EA 34	97±4.4	92±3.0	85±2.7
AF Std	87±2.1	80±0.9	73±1.3

EA = Sample Code, AF Std = Aflatoxin standard, SD= Stanard Deviation

Table 4.5b Toxicity on Human Lymphocytes Cells after 48 hrs

	% Cell Viability ±SD		
Sample	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	86±0.7	81±1.1	77±2.0
EA 57	94±2.1	89±2.8	86±2.8
EA 83	94±1.3	89±0.9	82±1.2
EA 26	89±1.1	83±2.0	79±1.9
EA 91	92±0.9	87±2.3	81±3.5
EA 70	95±3.3	88±4.8	83±3.0
EA 88	90±5.3	84±1.1	80±1.1
EA 78	87±0.9	82±2.5	79±2.7
EA 21	91±2.7	87±3.1	81±3.2
EA 34	90±1.5	85±2.5	79±2.9
AF Std	79±1.3	73±2.0	69±2.2

EA = Sample Code, AF Std = Aflatoxin standard, SD= Stanard Deviation

Table 4.5c Toxicity on Human Lymphocytes Cells after 72 hrs

	% Cell Viability ±SD		
Sample	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	80±3.7	76±0.7	70±2.5
EA 57	90±2.2	83±1.9	79±4.1
EA 83	89±4.1	81±2.0	77±2.8
EA 26	82±2.5	79±2.0	72±3.3
EA 91	88±2.1	81±2.5	75±2.1
EA 70	87±3.0	83±4.1	78±3.5
EA 88	87±2.8	80±3.6	73±0.8
EA 78	84±2.2	79±0.9	76±2.4
EA 21	89±3.0	84±2.1	77±1.2
EA 34	85±2.7	79±2.0	71±3.7
AF Std	71±0.9	66±0.6	59±0.5

EA = Sample Code, AF Std = Aflatoxin standard, SD= Stanard Deviation

Appendix IV

Raw data on aflatoxins analysed in compound feed samples from South Africa on high performance liquid chromatography

Sample No.	AFB_1	AFB_2	AFG_1	AFG_2	Total AFS
EA01	9.621696	2.364647	757.0848	0.18777	769.2589
EA22	0.109021	0.0006629	11.27561	0.001571	11.39283
EA13	0.00522	0.002813	3.79143	0	3.800176
EA34	15.95577	0.9538	41.51707	4.39E-06	58.42635
EA55	0.12101	0.0538	167.6054	0	167.7803
EA16	1.004937	0.351467	116.4838	0.02317	117.8634
EA07	0.038246	0.027762	93.72607	0.001238	93.79331
EA28	0.129418	0.038242	2.321756	0.010882	2.500297
EA35	1.004937	0.351467	116.4838	0.02317	117.8634
EA20	0.001515	0.00063	0.220022	0	0.222167
EA77	0.000997	0	0.427407	0	0..428404
EA61	0.040128	0.026026	2.447015	0	2.513161
EA53	0.019805	0	39.14766	0.001613	39.16908
EA78	0.162308	0.047633	50.69109	0	50.90103
EA50	0.00096	0.000879	0.517282	0	0.519121
EA24	0.004179	0.001552	14.88386	2.28E-05	14.88962
EA40	26.30739	0	3193.306	0	3219..614
EA56	2.774265	0.935378	99.89615	0	103.6058
EA09	0.012242	0	1.269306	0	1.281548
EA21	0.791457	0	243.9793	0	244.7708
EA11	0.002334	0.000657	0.819958	7.94E-05	0.823029
EA33	0.000976	0	0	0	0.000976
EA79	0.000896	0	0	0	0.000896
EA90	0.000925	0.00585	2.862951	0	2.869726
EA89	0.834129	0.352072	54.46382	0.017606	55.66763

EA27	0.113913	0.2278	43.26047	0.009463	43.61165
EA92	0.005079	0.003064	6.762967	0.001317	6.772427
EA61	0.22009	0.028541	0.914055	0.002035	0.966639
EA88	0.000176	0.003376	0	0.001493	0.005045
EA65	0	0	0	0	0
EA81	0.007499	0.002773	17.40562	1.15E-06	17.41589
EA44	0.00567	0.001151	5.816639	0	5.823819
EA30	0.020827	0.005399	0.2888373	0.003131	0.31773
EA91	0.064229	0.021311	1.098665	0.006663	1.190869
EA03	0.021971	0.008069	0.005916	0.003324	0.03928
EA15	0.023248	0.00538	0	0.001814	0.030441
EA19	0.004951	0.001143	0	0.002011	0.008105
EA71	0.0014	0.00017666	0.18175	0.002559	0.185885
EA48	0.132193	0.037721	0	0.008543	0.178457
EA59	0.005036	0.001233	10.60802	0	10.61429
EA78	0.007102	0.000346	13.05856	4.61E-05	13.06605
EA09	0.626396	0.156522	534.1505	0.002444	534.9358
EA34	0.002906	0.004167	2.783852	0.000172	2.791097
EA54	0.000599	0.000307	0.06274	0	0.063646
EA19	0.073813	0.14975	0	0	0.088788
EA29	0.003606	0	0.23863	0	0.242236
EA37	0.009999	0	0.8188625	0	0.828624
EA41	0	0.000389	0	6.080E-06	0.000395
EA17	0.00000399	0.000691	0	0	0.00109
EA29	0.050843	0,002538	0	0.007962	0.061343
EA63	0.032298	0.01411	0	0.002163	0.048572
EA14	0.000224	0	121.6014	0.008158	121.6098

EA10	0.005481	0.000256	1.676327	0.001554	1.683619