Study of toxigenic Aspergillus in feed and impact of bovine breed on aflatoxins carryover in milk and urine in dairy cows: A case of Bulawayo, Zimbabwe

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Declaration

I, Nancy Nleya, declare that this dissertation is my own original work. It is submitted to the Faculty of Natural and Agricultural Sciences, Northwest University for the Doctorate Degree: Animal Health and has never been previously submitted to any other University or institution for degree purposes.

Signature: _____ Date_____/_____/2019

Dedication

To my parents, Mr Philip Nharo Dangwa (Late) and Mrs Julia Dangwa.

Thank you for laying the good foundation.

To my children, Rachel and Richard Nleya,

The sky is the limit.

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List of abbreviations

ACF- autocorrelation factor

ADM- *Aspergillus* differential medium

AF- aflatoxin

AFB_1-aflatoxin B_1

AFB_2- aflatoxin B_2

AFBO- AFB_1-8,9-exo-epoxide

AFG_1- aflatoxin G_1

AFG_2- aflatoxin B_2

AFM_1- aflatoxin M_1

AFM_2- aflatoxin M_2

ANOVA-One way analysis of variance

BLAST - Basic Local Alignment Search Tool

BSG- Brewer's spent grains

CN-concentrate

CYP- cytochrome P450

DCA- desiccated coconut agar

DHOMST- dihydro-O-methylsterigmatocystin

DHST-dihydrosterigmatocystin

DMST-demethylsterigmatocystin

DNA-deoxyrinonucleic acid

EAI- enzyme immunoassays

EC –European Community

ELISA- enzyme linked Immuno-sorbent assay

EU- European Union

FDA-Food and Drug Administration

GC-MS - gas chromatography- mass spectrometry

GR-grass

GST- Glutathione-S-transferase

HPLC-high performance liquid chromatography

HRP-Horseradish peroxidase

IAC -immunoaffinity column assays

IACR-International Agency on Cancer Research

ITS- internal transcribed spacer

LC-MS- liquid chromatography mass spectrometry

LC-MS/MS- liquid chromatography with tandem mass spectrometry

LLE-liquid-liquid extraction

MANOVA- multivariate analysis of variance

MRL- maximum residual limit

MR-mixed ration

NCBI -National Centre for Biotechnology and Information

NRDCA - neutral red desiccated coconut agar

OMST- O-methylsterigmatocystin

PCR- polymerase chain reaction

PDA- potato dextrose agar

PKA- palm kernel agar

rDNA-ribosomal ribonucleic acid

RIA -radioimmunoassay

SIIA -sequential injection immunoassay

SPE -solid-phase extraction

TAE- Tris Acetate-EDTA

TLC- thin layer chromatography

TMB- tetramethylbenzidine

UHT- ultra heat treatment

US-FDA- United States Food and Drug Administration

UV- ultraviolet

VER A- versicolorin A

VER B- versicolorin B

YES - yeast extract sucrose

β-CNRDCA- β-cyclodextrin neutral red desiccated coconut agar

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General Abstract

This thesis explored the biodiversity and phylogenetic relationships of aflatoxigenic *Aspergillus* isolated from different feeds used for feeding dairy cows under intensive, semi-intensive and extensive feeding systems in Zimbabwe during the dry and rainy seasons. It also looked at the relationship between AFB1 dietary intake and AFM_1 output in milk on cows fed with naturally contaminated feed on a daily basis. The work also involved the determination of effect of breed on aflatoxin carryover from feed into milk and urine. In Chapter 3, both morphological and molecular methods were used for the identification of aflatoxigenic speciess isolated from the different feed types. The bulk of of the feeds were contaminated by moulds from the *Aspergillus* genus with the potential of producing aflatoxins. The most contaminated feed was the mixed rations and most of the toxigenic strains were isolated during the rainy season. Phylogenetic trees constructed based on single gene were not able to distinguish the isolates to species level or group them into their respective sections. However gene concatenation was able to cluster the isolates into the individual clades as well as grouping them based on the time of isolation giving a true reflection of the evolution aspect of the isolates with time. In Chapter 4, quantification of aflatoxins in the feeds was done by high performance liquid chromatography which indicated the presence of all the four naturally occurring aflatoxins namely aflatoxin B_1, B_2, G_1 and G_2 in the feeds. The mixed rations had the highest average total aflatoxin concentration of 29.0µg/kg. Aflatoxin B_1, the most potent aflatoxin was present in all feeds with an average concentration of 9.0µg/kg which was above the European Union (EU) standard of 5.0µg/kg for lactating cows which the country adopted and grass had the lowest aflatoxin concentrations of 2.5µg/kg. Chapter 5 showed that AFM_1 was present in 70.6% of the milk samples with the bulk of of the samples coming from the dry season. Milk samples from the rainy season had a higher percentage (88%) of compliance to the EU limit of 0.05µg/L compared to the dry season which had 58% of the milk samples below the EU limit. Aflatoxin M_1 concentrations in urine were between 0 and 2.36µg/L with samples from the dry season having higher concentrations than those from the rainy season. Although most of the milk samples complied with the EU and FDA limits, the consumers are still at the risk of having chronic aflatoxicosis. In Chapter 6, regression analysis showed that the concentration of AFM_1 in milk on any given day is governed by the AFB_1 concentration in the cow's diet on that particular day and that AFM_1 carryover from the previous day usually remains in the system from the previous day(s). This is because 3-4 days are required for the AFM_1 to get cleared from the system; however, this does not happen in a real life situation as the cows feed on naturally contaminated feed daily. Therefore there is a cumulative effect of the milk aflatoxin on a daily basis depending on the amount of AFB_1 in the feed. Multivariate analysis of variance and correlation analysis showed that the effect of diet on aflatoxin carryover into milk diet is significant but breed does not have any significant effect of AFM_1 in milk. Repeated measures one way ANOVA also showed that breed had no effect on the amount of AFM_1 secreted into milk. Findings from this study showed that feeds used by the farmers are contaminated by aflatoxigenic *Aspergillus*. Daily consumption of the contaminated feeds by the dairy cows results in the release of the aflatoxins in milk on a daily basis posing a threat to the population. The association of breed on both AFM_1 secretion into milk and excretion into urine was insignificant whereas diet effect was significant at 0.05% significance level.

Chapter 1

General Introduction

Milk and milk products play a major role in the human diet as many people especially children, include these products in their diets (Queiroz *et al.*, 2012). Milk is a good source of energy, protein, micronutrients and some biologically active molecules with polyvalent roles in immune function, nutrient transport and absorption (Dror & Allen, 2014; Pereira, 2014). However, milk and its products may also be a source of natural food contaminants that may cause undesirable health effects (Colak *et al.*, 2007; Girma *et al.*, 2014). Most of the reported illnesses associated with consumption of dairy products are related to bacterial contamination but some of the cases are linked to metabolites produced by fungi such as aflatoxins (Oluwafemi & Lawal, 2015).

Mould contamination of food and feeds is of great concern worldwide as some of the moulds are capable of producing mycotoxins in the food matrices (Davari *et al.*, 2015). Mycotoxins are fungal secondary metabolites that can have damaging effects on human and animal health (Fakruddin *et al.*, 2015; Sudini *et al.*, 2015). Mycotoxin producing moulds that normally contaminate food and feeds include *Fusarium, Penicillium* and *Aspergillus* (Ogbuewu, 2011; Ahmed *et al.*, 2017). These fungi usually contaminate agricultural commodities in the tropics especially cereal grains, nuts and oil seeds (Abdel-Hadi *et al.*, 2011; Denli, 2015; Guchi, 2015; Kumar *et al.*, 2016). Cattle mixed rations (MR) are usually produced by combining cereals and plant by-products like seed cake mixed with green fodder which may be contaminated with mycotoxin producing moulds (Gelven, 2010; Gónzalez-Pereyra *et al.*, 2012).

The *Aspergillus* is the predominant genus that has been associated with contamination of compound feed (Dutta & Das, 2001; Zulkifli & Zakaria, 2017). The presence of *Aspergillus* in the feeds does not only affect the nutritional quality of the feed but may also result in the production of aflatoxins (Pirestani & Toghyani, 2010; Pleadin, 2015; Zulkifli & Zakaria, 2017). Aflatoxins are highly toxic and carcinogenic compounds that can cause disease in livestock and humans (Arapcheska *et al.*, 2015). Aflatoxins of great importance are aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2, AFM_1 and AFM_2 (Dors *et al.*, 2011; Makun *et al.*, 2012) as they have been proven to be highly toxic and carcinogenic with AFB_1 being the most potent of them all (Sudini *et al.*, 2015; Ahmed *et al.*, 2017).

Ecological and environmental conditions play an important role in the production of mycotoxins (Afsah-Hejri *et al.*, 2013). The differences in the geographical and climatic conditions determine the fungal species contaminating the food and feed commodities (Jallow, 2015; Patel *et al.*, 2015; Songsermsakul, 2015). Mycotoxin contamination of foods and feeds is more common in warm and humid climates (Ramesh *et al.*, 2013; Patel *et al.*, 2015) which are mainly experienced in the tropical and subtropical regions. In addition some agricultural practices such as poor harvesting methods, insufficient drying of the produce and improper storage can promote fungal growth with subsequent mycotoxin production (Makun *et al.*, 2012).

Feeding livestock with aflatoxin contaminated feed reduces animal productivity, affect the health of the animals through immune system suppression, and may even result in the death of the animals (Fakruddin *et al.*, 2015; Atherstone *et al.*, 2016). Consumption of AFB_1 contaminated diet by breastfeeding mothers and lactating animals result in the secretion of its hydroxylated metabolite AFM_1 in milk (Ketney *et al.*, 2014; Davari *et al.*, 2015), thus aflatoxin is carried over into animal food products intended for human consumption thereby exposing humans to aflatoxin contamination (Sarica *et al.*, 2015; Becker-Algeri *et al.*, 2016a; Kumar *et al.*, 2017).

In extensive farming, grazing makes up a large portion of the diet and the intake of rations is limited to a smaller percentage of the total feed intake. Herds are taken out from the village to nearby communal ranges in the summer grazing season. Crop residues, weeds, wheat and barley stovers are alternative sources of animal feed (Tajkarimi *et al.*, 2008). During the grazing period, especially during the rainy season, these animals also feed on mouldy crops on the farms which could probably be the source of aflatoxin contamination in milk (Oluwafemi & Lawal, 2015). In contrast, intensive dairy cattle operation may use up to 70% of daily feed as mixed rations (Fink-Gremmels, 2008a). Feed components used in these feeds are often contaminated with aflatoxins, which are released into milk following their ingestion (Aslam & Wynn, 2015; Křížová *et al.*, 2016).

Previous studies have shown that aflatoxin carry over in dairy cows range from 1–2 % of the ingested aflatoxin B_1 (AFB_1) for low-yielding cows and up to approximately 6 % for high-yielding cows (Britzi *et al.*, 2013). The percentage carryover is affected by milk yield, stage of lactation, animal species and the health of the mammary alveolar cell membrane (Masoero *et*

al., 2007; Britzi *et al.*, 2013). Geographical location and weather have also been shown to have effect on the carryover rate (Hernández-Martínez & Navarro-Blasco, 2015)

Aflatoxin production is species specific with *A flavus, A parasiticus* and *A nomius* having been mainly associated with aflatoxin production in compound feeds (Rodrigues *et al.*, 2007; Silva *et al.*, 2015; Bharose *et al.*, 2017; Faria *et al.*, 2017; Kumar *et al.*, 2017; Sineque *et al.*, 2017). However some species such as *A niger, A fumigatus* and *A terreus* have also shown some aflatoxin producing potential. *Aspergilli* are ubiquitous in nature and the toxigenic species and non-toxigenic species are much similar to each other morphologically making their accurate identification to species level very difficult (Ehrlich *et al.*, 2014; Iheanacho *et al.*, 2014; Sudini *et al.*, 2015; Ahmed *et al.*, 2017; Zulkifli & Zakaria, 2017). Therefore there is a need for accurate identification and characterization of *Aspergillus* species of major significance with regards to aflatoxin production in order to come up with possible prevention strategies of controlling and reducing human and animal exposure to aflatoxin contaminated foods (Fakruddin *et al.*, 2015).

Differentiation of toxigenic and non-toxigenic *Aspergillus* mainly uses polyphasic method (Almoammar *et al.*, 2013; Sudini *et al.*, 2015). Cultural methods involve growing the moulds on solid media followed by selecting isolates based on colony morphology and microscopy (Henry *et al.*, 2000). This is time consuming and laborious, therefore some other methods for the detection of aflatoxin production in *Aspergillus* isolates have been developed. These methods use media that have some additives that enhance aflatoxin production which can be easily visualised directly or as blue or green fluorescence under ultraviolet (UV) radiation at 365nm (Fente *et al.*, 2001; Midorikawa *et al.*, 2008). However there are also limitations in these methods such as insensitivities and misidentification of compounds during visual determination under UV radiation as some of the fluorescence can be unclear (Suzuki & Iwahashi, 2016). Molecular methods have been developed for rapid differentiation of aflatoxigenic species from non-toxigenic species. These involve detection of aflatoxin genes and their amplification through the polymerase chain reaction (PCR). Therefore a polyphasic approach is needed for precise identification of aflatoxigenic species for both research and extenuation. However, cultural method and microscopy still remains the commonly used methods for initial identification of *Aspergillus* (Kamili & Ganai, 2012*).*

Since aflatoxins have been shown to be very toxic and harmful to animals and humans at very low concentrations, there is need for researchers to develop sensitive and precise methods for

aflatoxin detection and quantification (Rahmani *et al.*, 2009; Kumar *et al.*, 2016) to make sure that the population is safe from aflatoxin contamination. Detection of aflatoxins is based on their ability to fluoresce under ultraviolet (UV) light. The B series fluoresce blue whereas the G series fluoresce green (Dhanasekaran *et al.*, 2011; Yu & Ehrlich, 2011). Chromatography is the main technique used in detection of aflatoxins with thin layer chromatography (TLC) being one of the oldest detection method. The most commonly used chromatographic methods are high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LCMS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Immuno-based analytical methods like the enzyme linked Immuno-sorbent assay (ELISA) has also been frequently used for rapid detection and quantification of aflatoxins (Rahmani *et al.*, 2009).

Problem Statement

Aflatoxin contamination of food and feed has gained global significance as a result of its deleterious effects on human as well as animal health (Rajarajan *et al.*, 2013). Dairy foods though commonly considered as balanced and nutritive foods (Pereira, 2014) have also been implicated as sources of mycotoxins in human diet (Oluwafemi & Lawal, 2015). Previous research have indicated that cows fed on aflatoxin B_1 contaminated feed excrete aflatoxin M_1 into their milk thereby making milk a risk factor of human exposure to aflatoxins (Yitbarek & Tamir, 2014). Incidences of mycotoxin contamination in feed and the ingredients used in feed formulation have been reported (Ji *et al.*, 2016; Pinotti *et al.*, 2016). Previous reports have shown that aflatoxin carry over in dairy cows ranged from 1–2 % of the ingested aflatoxin B_1 (AFB_1) for low-yielding cows and up to approximately 6 % for high-yielding cows (Britzi *et al.*, 2013). Some authors have reported on limited research on AFM_1 by African countries (Mwanza *et al.*, 2013; Flores-Flores *et al.*, 2015). Moreover, the reclassification of AFM_1 into Group 1 carcinogen by the International Agency on Cancer Research, (IACR) in 2002 (Giovati *et al.*, 2015; Sarica *et al.*, 2015; Campagnollo *et al.*, 2016) makes its presence in milk a major public health concern especially for developing countries hence the need to monitor and keep the concentration of aflatoxin M_1 in milk within safe levels. It has also been shown that improper handling and storage of animal feeds lead to contamination by toxigenic strains of the *Aspergillus* genus (Zaki *et al.*, 2012; Becker-Algeri *et al.*, 2016a). Therefore it is important to identify the common toxigenic moulds present in the feeds and the aflatoxins they produce as there is very limited information on the biodiversity and extent of toxigenic *Aspergillus* contamination of stock feeds in Zimbabwe. Fungal contamination in grains and feed as well as

mycotoxin control therefore remains a challenge. There is also very limited information on aflatoxin carryover in milk and other biological samples present in pure breeds and cross breeds. There is need to enquire out if the breed of the cow has an influence on the mycotoxin carryover and other biological samples. Therefore this study is aimed at addressing these issues so that we can be able to advise farmers on the breeds to purchase with regards to mycotoxin control.

Aim

The aim of the study was to investigate the extent and incidence of mould and aflatoxin contamination of feed and subsequent excretion of AFM_1 in the milk and urine by different breeds of dairy cows under different feeding systems in Zimbabwe.

Objectives

The objectives of the study were to:

1. Characterise and evaluate the toxigenecity of *Aspergillus* species isolated from animal feed
2. Ascertain if phylogenetic relationship existed among the *Aspergillus* isolates from the different feeds.
3. Determine the level of aflatoxin contamination in feed from different areas.
4. Evaluate the level of aflatoxin M_1 contamination of milk and urine from dairy cow bred under different feeding systems.
5. Establish if there was a correlation between dietary intake of aflatoxins and aflatoxin M_1 (AFM_1) metabolite excretion into milk and urine by dairy cows in Zimbabwe.
6. To determine the influence of breed on aflatoxin carryover in milk.

Chapter 2

Literature Review

2.1 Mycotoxins

Milk contains numerous nutrients required for growth and maintenance of good health (Becker-Algeri *et al.*, 2016a; Younis *et al.*, 2016) especially in children but can also be a source of contaminants such as microbial cells and their metabolites that can cause diseases in humans (Campagnollo *et al.*, 2016; Younis *et al.*, 2016). The presence of fungal metabolites especially mycotoxins in milk and its products is undesirable as people of all age groups, especially children, consume these products in their daily diets worldwide (Campagnollo *et al.*, 2016; Nile *et al.*, 2016).

Mycotoxins are naturally occurring low molecular weight secondary metabolites of moulds which can be found in various food matrices and have toxic effects on human and animal health (Pleadin, 2015; Hove *et al.*, 2016; Tola & Kebede, 2016; Smith *et al.*, 2017). These moulds infect plants at various stages of production namely pre-harvest, during harvest and postharvest (Tola & Kebede, 2016). Over 400 mycotoxins have been identified, however the number still remains irresolute as more fungal metabolites are still to be identified (Fink-Gremmels, 2008b). Mycotoxins of importance in agriculture include aflatoxins, zearalenone, fumonisins, ochratoxins and trichothecenes (Ibáñez-Vea *et al.*, 2012). They are produced mainly by moulds belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium* (Sultana & Hanif, 2009; Datsugwai *et al.*, 2013; Aiko & Mehta, 2015). Humans and animals are exposed to mycotoxins through consumption of contaminated food and feed respectively (Alonso *et al.*, 2013). Toxicological syndromes associated with the ingestion of mycotoxins are called mycotoxicosis and can be acute or chronic with symptoms ranging from gastroenteritis to cancers depending on the quantity of the toxin ingested respectively (Pleadin, 2015; Ketney *et al.*, 2017). Health problems associated with mycotoxin poisoning include cancer, immunosuppression and impaired growth (Tola & Kebede, 2016). Research in mycotoxins has mainly focused on the mycotoxins that are involved in human carcinogenesis such as aflatoxins, fumonisins and ochratoxins (Fink-Gremmels, 2008b; Bosco & Mollea, 2012). Aflatoxins, produced by moulds from the genus *Aspergillus* are the most poisonous of all the mycotoxins (Jonathan *et al.*, 2016) as they are highly carcinogenic and mutagenic (Abedi & Talebi, 2006; Patel *et al.*, 2015). They have also been associated with several disease conditions such as tissue necrosis and hepatic

cirrhosis cancers. Aflatoxins have also been considered as an important sanitary problem since human exposure to this mycotoxin is not only through consumption of contaminated food but can also be through exposure to air and dust containing the toxin (Lizárraga-Paulín *et al.*, 2011; Carvajal-Moreno, 2015).

2.2 Aflatoxins

Aflatoxins are a group of difuranocoumarins (Figure 2.1.) produced in a polyketide pathway (Klich, 2007; Carvajal-Moreno, 2015; Bellio *et al.*, 2016) by *Aspergillus* species belonging to subgenera *Circumdati* sections *Flavi, Ochraceorosei* and *Nidulatans* (Baranyi *et al.*, 2013; Varga *et al.*, 2015; Campagnollo *et al.*, 2016). *Aspergillus flavus*, *A parasiticus* and *A nomius* from section *Flavi* are the main species associated with aflatoxin production although research has shown that other species are also able to produce aflatoxins (Piotrowska *et al.*, 2013; Monson *et al.*, 2015; Gherbawy *et al.*, 2016). Aflatoxins are the most toxic and studied mycotoxins since their discovery in the early 1960s (Perrone *et al.*, 2014; Jallow, 2015) when they were identified as the causative agent of the 'Turkey-X-disease' which caused the death of a hundred thousands of birds in England after consumption of groundnut meal contaminated with *A flavus* (Aiko & Mehta, 2015; Patel *et al.*, 2015; Atherstone *et al.*, 2016).

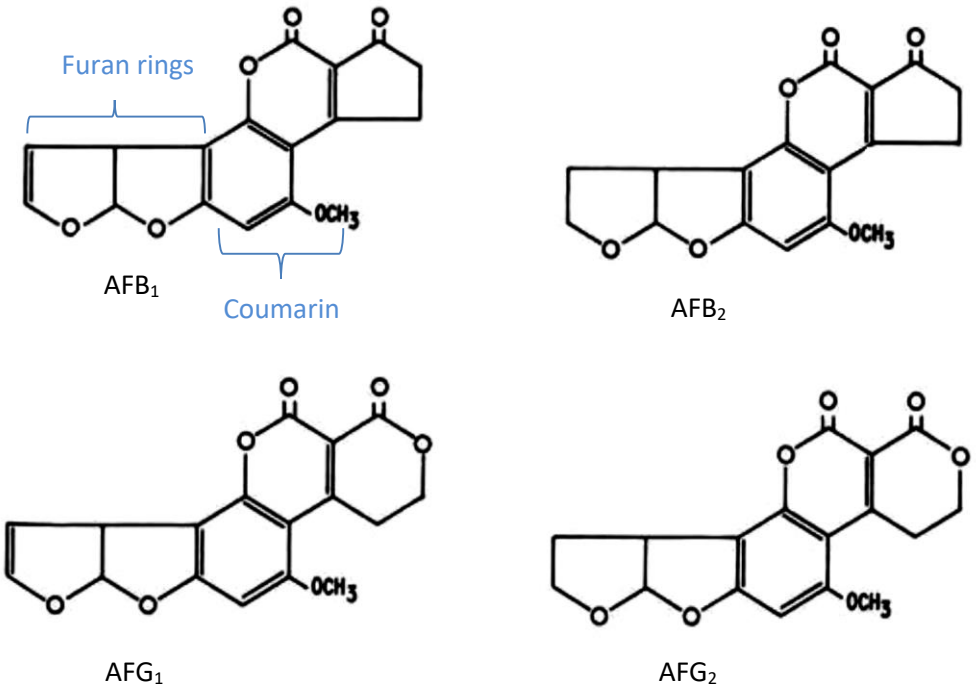

Figure 2.1. Structure of aflatoxins B_1, B_2, G_1 and G_2 (Jalili, 2016).

There are more than 20 aflatoxins that have been identified. The most important are aflatoxin (AF) B_1, B_2, G_1, G_2, M_1 and M_2 (Filazi & Sireli, 2013; Mosbah *et al.*, 2017) as they have toxic,

carcinogenic and immunosuppressive properties posing health complications to both humans and animals (Baranyi *et al.*, 2013). Aflatoxins (B and G) are named based on their fluorescence under UV light (blue or green) at 365 nm (Baranyi *et al.*, 2013; Arapcheska *et al.*, 2015; Reid *et al.*, 2016). AFM_1 and AFM_2 are hydroxylated biotransformation products of AFB_1 and AFB_2 respectively. AFM was first isolated from milk hence the name aflatoxin M (Yu *et al.*, 2004). However research has also shown that AFM_1 and AFM_2 can also be produced by *A flavus* and *A parasiticus* (Filazi & Sireli, 2013; Giovati *et al.*, 2015; Montaro *et al.*, 2016). Figure 2.2 shows the potent aflatoxins and their hydroxylated derivatives. The severity of the toxicity of the aflatoxins is in the order : $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Carvajal-Moreno, 2015; Kumar *et al.*, 2017). International Agency for Research of Cancer (IARC) have classified AFB_1 and AFM_1 as class 1A carcinogens (IARC, 2012). AFB_1 is the most potent and major aflatoxin produced by all toxigenic strains thus occurring more frequently compared to the other aflatoxins (Baranyi *et al.*, 2013; Gherbawy *et al.*, 2015; Bellio *et al.*, 2016). It has hepatotoxic, teratogenic and mutagenic properties. In addition, it can also cause the following conditions in mammals; hepatitis, hemorrhage, oedema, immunosuppression and hepatic carcinoma (Datsugwai *et al.*, 2013). Aflatoxins are stable at high processing temperatures with melting temperatures ranging from 237°C to 320°C resulting in very insignificant changes occurring during cooking and other severe heat food processing technologies (Carvajal-Moreno, 2015; Gurav & Medhe, 2018).

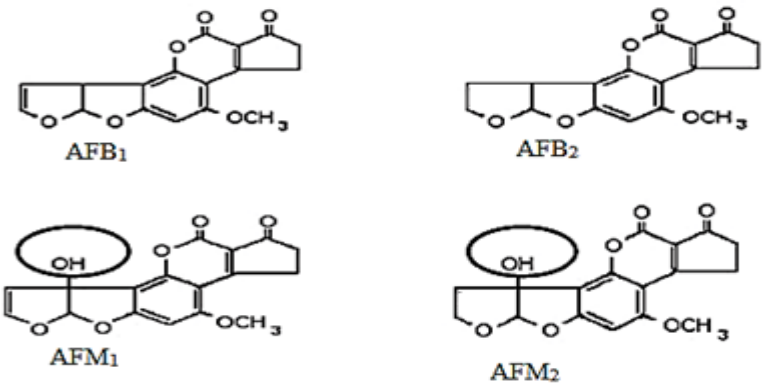

Figure 2.2. Structures of AFB_1, AFB_2 and their hydroxylated biotransformation metabolites , AFM_1 and AFM_2 respectively (Dhanasekaran *et al.*, 2011).

2.3 The genus *Aspergillus*

Aspergillus is the genus name of moulds that belongs to a group of filamentous Deutromycetes that reproduce asexually (Bennett, 2010). They were first described by Micheli in 1729 (Prakash & Jha, 2014; Samson *et al.*, 2014). The most striking feature of the Aspergilli is their spore-bearing structure called the conidia head which resembles the aspergillum (Klich, 2007; Bennett, 2010; Houbraken *et al.*, 2014). Figure 2.3 shows *Aspegillus* conidiophores. During development and differentiation, some of the mycelial cells develop into a 'foot cell' produce a single erect hyphal branch perpendicular to the long axis of the cell culminating into the conidiophore. The vesicles at the apex of the conidiophore give rise to a layer(s) of cells called phialides which produce conidia or conidiospores (Refai *et al.*, 2014).

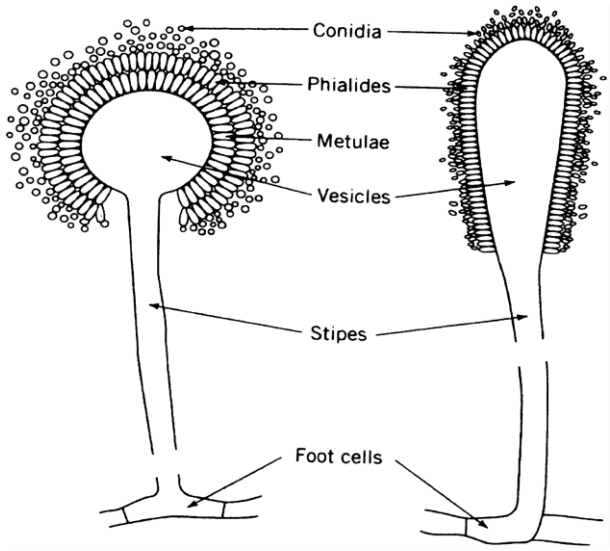

Figure 2.3. *Aspergillus* conidiophores (Klich, 2009)

The genus has been divided into four subgenera based on polyphasic taxonomy namely ; *Aspergillus, Circumdati*, *Fumigati* and *Nidulantes* (Figure 2.4) which are further divided into 20 sections (Houbraken *et al.*, 2014; Samson *et al.*, 2014; Jurjević *et al.*, 2015; Giusiano *et al.*, 2017).

Most mycotoxin producing species belong to sub-genus *Circumdati* (Frisvad & Samson, 2000). This sub-genus is further divided into seven sections (Bennett, 2010; Jurjević *et al.*, 2015; Nyongesa *et al.*, 2015) namely; *Flavi, Fumigati, Nigri, Circumdati, Clavati, Nidulantes* and *Candidi* (Nyongesa *et al.*, 2015). Section *Flavi* is the most important of them all as it contains the main aflatoxin producing species ; *A flavus, A parasiticus* and *A nomius* (Frisvad & Samson, 2000; De Valk *et al.*, 2008; Passone *et al.*, 2010; Soares *et al.*, 2012) .

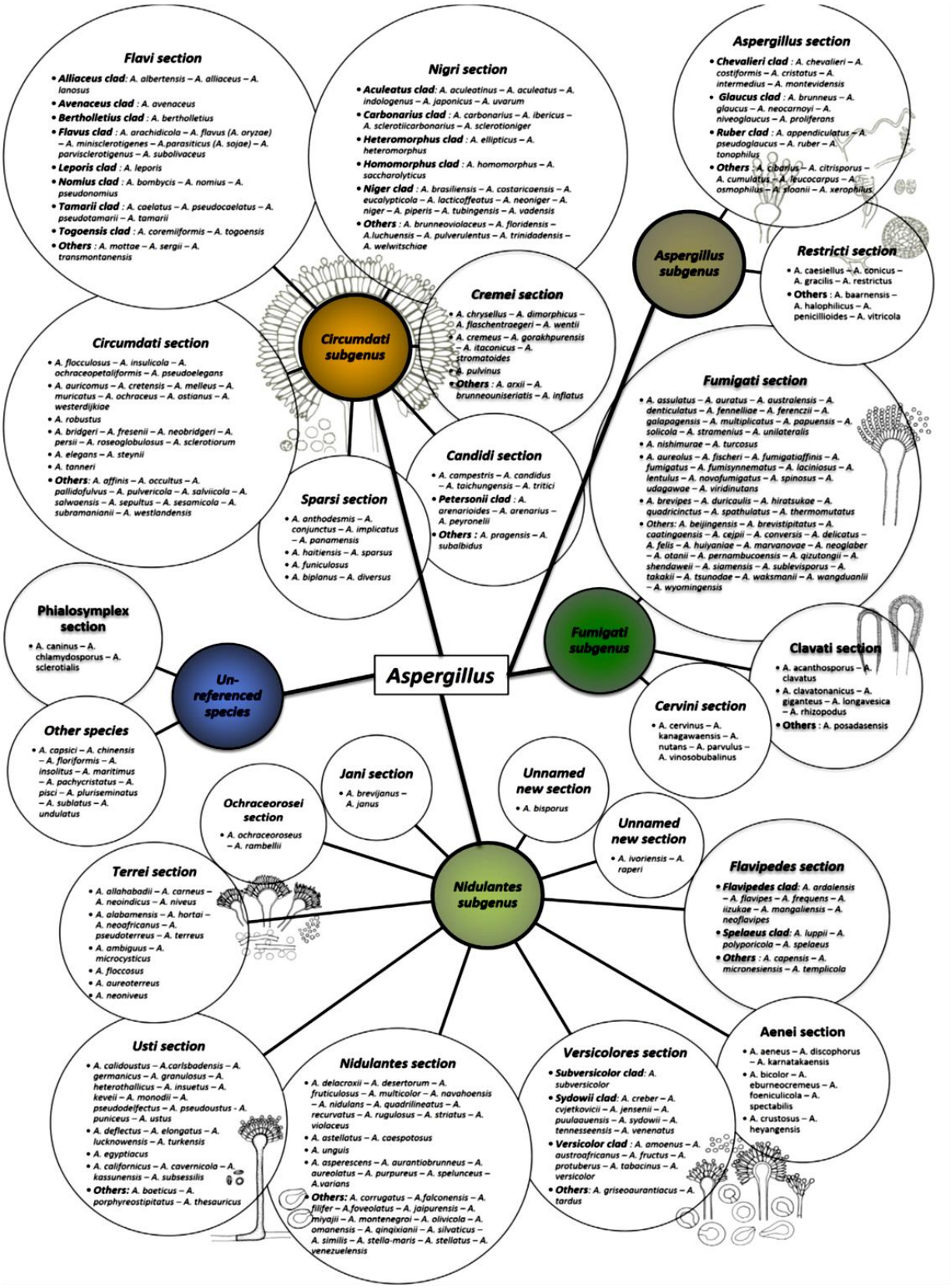
Aspergillus
Flavi section
• Alliaceus clad: A. albertensis – A. alliaceus – A. lanosus
• Avenaceus clad : A. avenaceus
• Bertholletius clad : A. bertholletius
• Flavus clad : A. arachidicola – A. flavus (A. oryzae) – A. minisclerotigenes – A.parasiticus (A. sojae) – A. parvisclerotigenus – A. subolivaceus
• Leporis clad : A. leporis
• Nomius clad : A. bombycis – A. nomius – A. pseudonomius
• Tamarii clad : A. caelatus – A. pseudocaelatus – A. pseudotamarii – A. tamarii
• Togoensis clad : A. coremiiformis – A. togoensis
• Others : A. mottae – A. sergii – A. transmontanensis
Nigri section
• Aculeatus clad: A. aculeatinus – A. aculeatus – A. indologenus – A. japonicus – A. uvarum
• Carbonarius clad : A. carbonarius – A. ibericus – A. sclerotiicarbonarius – A. sclerotioniger
• Heteromorphus clad : A. ellipticus – A. heteromorphus
• Homomorphus clad : A. homomorphus – A. saccharolyticus
• Niger clad : A. brasiliensis – A. costaricaensis – A. eucalypticola – A. lacticoffeatus – A. neoniger – A. niger – A. piperis – A. tubingensis – A. vadensis
• Others : A. brunneoviolaceus – A. floridensis – A.luchuensis – A. pulverulentus – A. trinidadensis – A. welwitschiae
Aspergillus section
• Chevalieri clad : A. chevalieri – A. costiformis – A. cristatus – A. intermedius – A. montevidensis
• Glaucus clad : A. brunneus – A. glaucus – A. neocarnoyi – A. niveoglaucus – A. proliferans
• Ruber clad : A. appendiculatus – A. pseudoglaucus – A. ruber – A. tonophilus
• Others : A. cibarius – A. citrisporus – A. cumulatus – A. leucocarpus – A. osmophilus – A. sloanii – A. xerophilus
Aspergillus subgenus
Restricti section
• A. caesiellus – A. conicus – A. gracilis – A. restrictus
• Others : A. baarnensis – A. halophilicus – A. penicillioides – A. vitricola
Circumdati subgenus
Cremei section
• A. chrysellus – A. dimorphicus – A. flaschentraegeri – A. wentii
• A. cremeus – A. gorakhpurensis – A. itaconicus – A. stromatoides
• A. pulvinus
• Others : A. arxii – A. brunneouniseriatis – A. inflatus
Circumdati section
• A. flocculosus – A. insulicola – A. ochraceopetaliformis – A. pseudoelegans
• A. auricomus – A. cretensis – A. melleus – A. muricatus – A. ochraceus – A. ostianus – A. westerdijkiae
• A. robustus
• A. bridgeri – A. fresenii – A. neobridgeri – A. persii – A. roseoglobulosus – A. sclerotiorum
• A. elegans – A. steynii
• A. tanneri
• Others: A. affinis – A. occultus – A. pallidofulvus – A. pulvericola – A. salviicola – A. salwaensis – A. sepultus – A. sesamicola – A. subramanianii – A. westlandensis
Candidi section
• A. campestris – A. candidus – A. taichungensis – A. tritici
• Petersonii clad : A. arenarioides – A. arenarius – A. peyronelii
• Others : A. progensis – A. subalbidus
Sparsi section
• A. anthodesmis – A. conjunctus – A. implicatus – A. panamensis
• A. haitiensis – A. sparsus
• A. funiculosus
• A. biplanus – A. diversus
Fumigati subgenus
Fumigati section
• A. assulatus – A. auratus – A. australensis – A. denticulatus – A. fennelliae – A. ferenczii – A. galapagensis – A. multiplicatus – A. papuensis – A. solicola – A. stramenius – A. unilateralis
• A. nishimurae – A. turcosus
• A. aureolus – A. fischeri – A. fumigatiaffinis – A. fumigatus – A. fumisynnematus – A. laciniosus – A. lentulus – A. novofumigatus – A. spinosus – A. udagawae – A. viridinutans
• A. brevipes – A. duricaulis – A. hiratsukae – A. quadricinctus – A. spathulatus – A. thermomutatus
• Others: A. beijingensis – A. brevistipitatus – A. caatingaensis – A. cejpii – A. conversis – A. delicatus – A. felis – A. huiyaniae – A. marvanovae – A. neoglaber – A. otanii – A. pernambucoensis – A. qizutongii – A. shendaweii – A. siamensis – A. sublevisporus – A. takakii – A. tsunodae – A. waksmanii – A. wangduanlii – A. wyomingensis
Clavati section
• A. acanthosporus – A. clavatus
• A. clavatonanicus – A. giganteus – A. longavesica – A. rhizopodus
• Others : A. posadasensis
Cervini section
• A. cervinus – A. kanagawaensis – A. nutans – A. parvulus – A. vinosobubalinus
Un-referenced species
Phialosymplex section
• A. caninus – A. chlamydosporus – A. sclerotialis
Other species
• A. capsici – A. chinensis – A. floriformis – A. insolitus – A. maritimus – A. pachycristatus – A. pisci – A. pluriseminatus – A. sublatus – A. undulatus
Nidulantes subgenus
Jani section
• A. brevijanus – A. janus
Unnamed new section
• A. bisporus
Ochraceorosei section
• A. ochraceoroseus – A. rambellii
Unnamed new section
• A. ivoriensis – A. raperi
Terrei section
• A. allahabadii – A. carneus – A. neoindicus – A. niveus
• A. alabamensis – A. hortai – A. neoafricanus – A. pseudoterreus – A. terreus
• A. ambiguus – A. microcysticus
• A. floccosus
• A. aureoterreus
• A. neoniveus
Flavipedes section
• Flavipedes clad: A. ardalensis – A. flavipes – A. frequens – A. iizukae – A. mangaliensis – A. neoflavipes
• Spelaeus clad: A. luppii – A. polyporicola – A. spelaeus
• Others : A. capensis – A. micronesiensis – A. templicola
Aenei section
• A. aeneus – A. discophorus – A. karnatakaensis
• A. bicolor – A. eburneocremeus – A. foeniculicola – A. spectabilis
• A. crustosus – A. heyangensis
Versicolores section
• Subversicolor clad: A. subversicolor
• Sydowii clad: A. creber – A. cvjetkovicii – A. jensenii – A. puulaauensis – A. sydowii – A. tennesseensis – A. venenatus
• Versicolor clad : A. amoenus – A. austroafricanus – A. fructus – A. protuberus – A. tabacinus – A. versicolor
• Others: A. griseoaurantiacus – A. tardus
Nidulantes section
• A. delacroxii – A. desertorum – A. fruticulosus – A. multicolor – A. navahoensis – A. nidulans – A. quadrilineatus – A. recurvatus – A. rugulosus – A. striatus – A. violaceus
• A. astellatus – A. caespotosus
• A. unguis
• A. asperescens – A. aurantiobrunneus – A. aureolatus – A. purpureus – A. spelunceus – A.varians
• Others: A. corrugatus – A.falconensis – A. filifer – A.foveolatus – A. jaipurensis – A. miyajii – A. montenegroi – A. olivicola – A. omanensis – A. qinqixianii – A. silvaticus – A. similis – A. stella-maris – A. stellatus – A. venezuelensis
Usti section
• A. calidoustus – A.carlsbadensis – A. germanicus – A. granulosus – A. heterothallicus – A. insuetus – A. keveii – A. monodii – A. pseudodelfectus – A. pseudoustus - A. puniceus – A. ustus
• A. deflectus – A. elongatus – A. lucknowensis – A. turkensis
• A. egyptiacus
• A. californicus – A. cavernicola – A. kassunensis – A. subsessilis
• Others: A. baeticus – A. porphyreostipitatus – A. thesauricus

Figure 2.4. *Aspergillus* classification according to subgenera and sections (Gautier *et al.*, 2016).

Most species in this section are similar to *A flavus* morphologically (Klich, 2007) making it difficult to differentiate the species. It is therefore important to know the level of mould infection and accurately identify and distinguish the potential aflatoxin producers from the non-producers as a way of determination of food quality. Well informed decisions about the shelf life of the product can also be accurately made (Passone *et al.*, 2010; Soares *et al.*, 2012).

The initial step in the identification and characterisation of members of the genus *Aspergillus* uses morphological features (Samson *et al.*, 2011; Kamili & Ganai, 2012; Houbraken *et al.*, 2014) based on the protocols of Raper and Fennell (1965), Klich (2002) and Pitt and Hocking (2009). This involves the culturing of the moulds on solid media and observing macro morphological characteristics such as colour of conidia, mycelia, colony diameter and colony reverse colour (Sudini *et al.*, 2015; Thathana *et al.*, 2017). This is followed by microscopy where features such as the morphology of cleistothecia, ascospores, vesicles and conidia are observed as well as the size are measured (Thathana *et al.*, 2017). This is time consuming and laborious and requires well trained mycologists (Henry *et al.*, 2000; Passone *et al.*, 2010). Moreover there is a possibility of misclassification of the organisms since their morphological characters can be highly variable depending on the media and culture conditions (Geiser *et al.*, 2007; Gherbawy *et al.*, 2016) . Therefore molecular methods which are rapid and sensitive have been developed.

Proposal by Schoch *et al.* (2012) led to ribosomal DNA (rDNA) internal transcribed spacer region (ITS1-5.8S-ITS2) being the universally accepted barcode for fungal identification (Peterson, 2012; Gautier *et al.*, 2016). However, the ITS region lacks polymorphism for the genus *Aspergillus* (Frisvad & Samson, 2000; Peterson, 2012) therefore additional markers such as the β-tubulin and calmodulin genes have been identified to complement the ITS in the identification of the isolates (Gautier *et al.*, 2016). Furthermore, use of one gene for the construction of phylogenetic trees usually result in some unresolved branches, this has led to the concept of gene concatenation where several genes are joined together to give one supergene sequence (Krimitzas *et al.*, 2013). Phylogenetic trees obtained this way have managed to solve some uncertainties associated with the use of single gene sequence.

2.4 Aflatoxin producing *Aspergillus*

Not all *Aspergillus* species produce aflatoxins (Atanda *et al.*, 2006; Degola *et al.*, 2007; Yazdani *et al.*, 2010). Aflatoxin producing species cannot easily be differentiated from non

aflatoxigenic species as they are morphologically similar. It is therefore important to differentiate the aflatoxigenic species from the nonaflatoxigenic species in order to ascertain the risk of contamination and be able to come up with aflatoxin management strategies (Mehl *et al.*, 2012).

Conventional ways of identifying aflatoxin producers is based on culturing of the moulds on media followed by extraction of the aflatoxins from the media using organic solvents and detection by TLC (Lin & Dianese, 1976). This is, however, laborious and time consuming and therefore media that induce aflatoxin production were developed. Bothast and Fennell (1974) developed the *Aspergillus* differential medium (ADM) which results in positive isolates producing yellow colouration on the reverse side of the colony for aflatoxin producing isolates. However, non aflatoxin producers also produced the yellow pigmentation (Saito & Machida, 1999). Lin and Dianese (1976) formulated a coconut based medium which also produced yellow colouration on the reverse side of toxigenic isolates and also showed fluorescence of agar under UV light. Palm kernel agar (PKA) was formulated by Atanda *et al.* (2006) which also showed yellow colouration on the reverse side for aflatoxin producers within a shorter time frame when compared to the coconut based agar. Moreover the pink background of the PKA makes visibility of the fluorescence clearer as compared to the white background of the coconut media. A neutral red was thereafter added to improve desiccated coconut agar (DCA) in order to give a similar contrasting pink background of PKA thus making visibility of fluorescence clearer since PKA has a short shelf life (Atanda *et al.*, 2011). Addition of β-cyclodextrin to Czapek agar, Sabaroud agar, Yeast extract sucrose agar and aflatoxin-producing ability APA medium by Fente *et al.* (2001) showed that the fluorescence of the aflatoxins become enhanced making it easy to identify aflatoxigenic strains under UV light after 3 days of incubation (Stark, 2009). Using both fluorescence of agar and yellow pigmentation of fungal colonies as ways of identification of aflatoxigenic strains are not reliable as some non-aflatoxigenic *Aspergillus* species tend to fluoresce under UV light (Stark, 2009; Sudini *et al.*, 2015) and were also capable of showing yellow pigmentation (Atanda *et al.*, 2011). Moreover, there may be misidentification of compounds during visual determination under UV radiation as some of the fluorescence can be unclear (Suzuki & Iwahashi, 2016). Saito and Machida (1999) also demonstrated the use of ammonia vapour on isolates grown on yeast extract sucrose (YES) agar for the identification of aflatoxigenic strains which may show pink to red coloration on the reverse side of their colonies. Validation of TLC or HPLC has also shown that these methods are not reliable as they can produce false negatives

and positives (Yazdani *et al.*, 2010). Using one method for the identification of toxigenic and non-toxigenic strains is therefore not reliable (Sudini *et al.*, 2015). There is therefore need to combine several methods for proper differentiation.

Molecular methods have been developed for rapid differentiation of aflatoxigenic species from non-toxigenic species. These involve detection of aflatoxin genes and their amplification through the polymerase chain reaction (PCR). Out of the 25 genes in the aflatoxin biosynthetic pathway, 2 regulatory genes (*aflR* and *aflS*) and 3 structural genes (*aflD, aflM* and *aflP*) have been identified as important in the production of aflatoxins (OBrian *et al.*, 2007; Abdel-Hadi *et al.*, 2011; Mejía-Teniente *et al.*, 2011; Baranyi *et al.*, 2013; Zhi *et al.*, 2013; Verheecke *et al.*, 2015).These genes code for the key enzymes in aflatoxin biosynthesis (Davari *et al.*, 2015) hence their use in molecular identification of aflatoxin producing strains (Baranyi *et al.*, 2013; Ibrahim *et al.*, 2016).

2.5 Aflatoxin biosynthesis

Biosynthesis of aflatoxin involve numerous elements that include the aflatoxin biosynthesis gene cluster, various genes, enzymes, and some regulatory components (Zhi *et al.*, 2013). The aflatoxin biosynthesis gene cluster is represented by a 70kilobase (kb) sequence consisting of 25 structural genes (Yu *et al.*, 2004; Bhatnagar *et al.*, 2006; Klich, 2007) as shown in Figure 2.5a. The genes are named according to their substrates or enzymatic functions (Yu *et al.*, 2004). Aflatoxin biosynthesis follows the pathway from acetyl CoA to aflatoxins in the sequence; acetate → polyketide → anthraquinones → xanthones → aflatoxins (Yu *et al.*, 2004) through oxidation-reduction reactions (Baranyi *et al.*, 2013) with norsolinic acid (NOR) being the first stable intermediate in the pathway (Figure 2.5b). There are also other genes outside the gene cluster that have an effect on aflatoxin production (Zhi *et al.*, 2013). Environmental factors such as drought and heat and humidity also have an effect in aflatoxin production (Milani, 2013; Fountain *et al.*, 2014; Becker-Algeri *et al.*, 2016a). Therefore aflatoxin contamination is a problem usually associated with the tropical and subtropical regions of the world where such weather conditions prevail (Baranyi *et al.*, 2013; Gurav & Medhe, 2018).

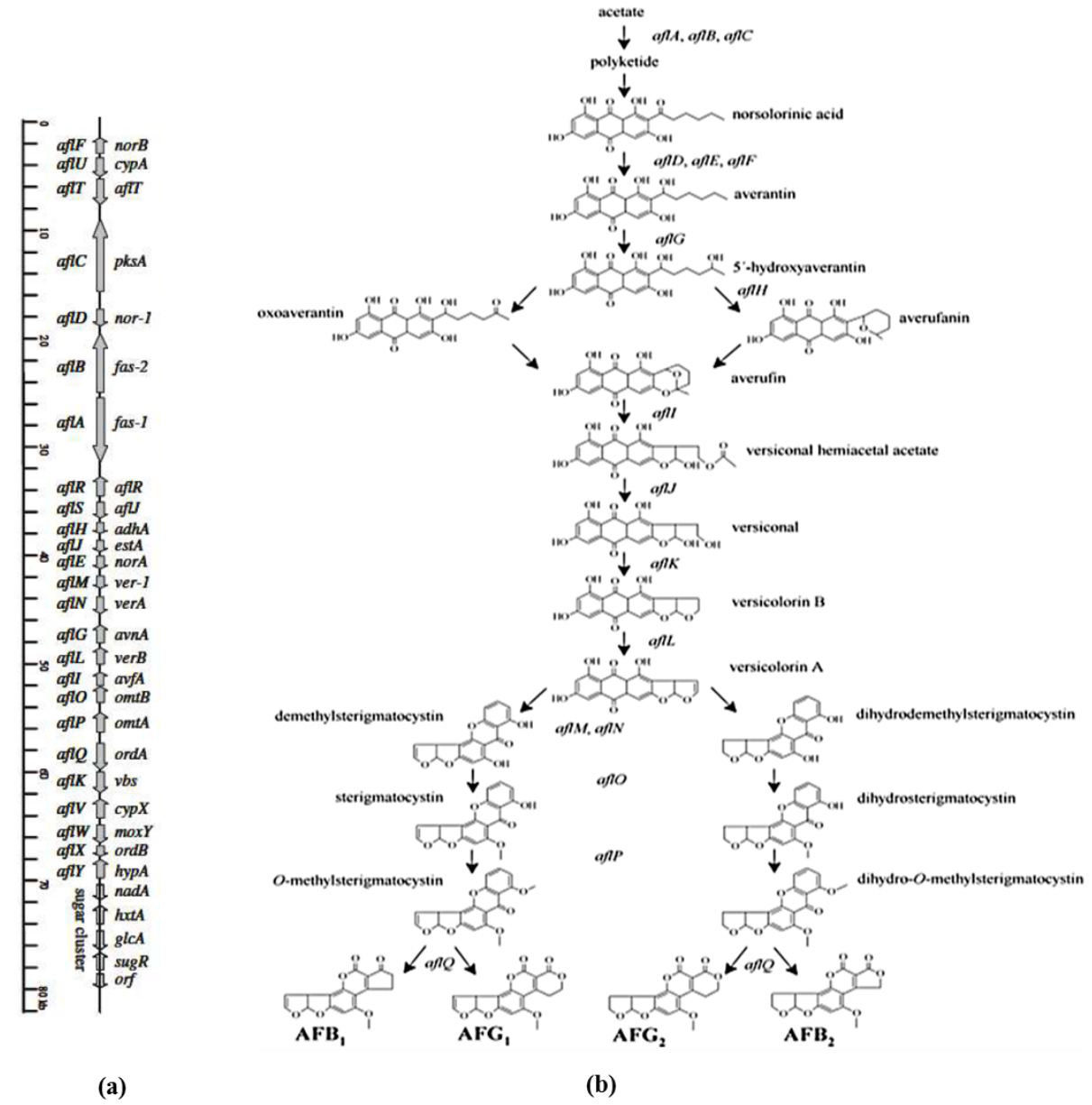

Figure 2.5. Aflatoxin biosynthesis gene cluster (a), the arrows shows the direction of gene transcription. The old gene names are on the right side of the arrows and the new gene names on the left. (b) is the aflatoxin biosynthetic pathway and the genes encoding the enzyme involved (Yu *et al.*, 2004; Bhatnagar *et al.*, 2006; Šimončicová *et al.*, 2017) modified.

2.6 Aflatoxin biotransformation

Following their ingestion, aflatoxins are absorbed by the small intestines and taken to the liver where their catabolism takes place. Aflatoxin catabolism takes place in two phases with the first phase involving reductive, oxidative and hydrolysis reaction. Oxidative reactions are mainly catalysed by cytochrome P450 enzymes (CYP), microsomal mono-oxygenases and alcohol dehydrogenases (Jouany *et al.*, 2009). CYPs convert AFB_1 to AFB_1-8,9-exo-epoxide (AFBO) and 8,9-endo-epoxide (Figure 2.6). AFB_1-8,9-exo-epoxide is the carcinogenic

metabolite of AFB_1 (Diaz & Murcia, 2011; Ketney *et al.*, 2014) which will either bind to proteins and cause acute toxicity (aflatoxicosis) or to DNA and induce liver cancer (Ogodo & Ugbogu, 2016). Epoxide-hydrolases, aldehyde-reductases and ketone-reductases are responsible for reduction reactions. Hydrolysis reactions are catalysed by the numerous non-specific esterases and amidases found in the tissue fluids of the animals (Jouany *et al.*, 2009). Hydroxylation of AFB_1 results in the formation of AFM_1, AFQ_1 and AFB_{2a} (Diaz & Murcia, 2011). AFQ_1 and AFB_{2a} can be described as the detoxified forms of AFB_1 whereas AFM_1 retains some cytotoxicity and toxigenecity which are comparable to AFB_1 (Bbosa *et al.*, 2013b; Giovati *et al.*, 2015). The second phase involves conjugation of the metabolites from the first phase making them more soluble so that they can be easily excreted through the urinary system (Diaz & Murcia, 2011; Ketney *et al.*, 2014). Glutathione-S-transferase (GST) an enzyme found in humans and animals is able to detoxify the 8,9-epoxide through conjugation with glutathione and excreted in urine (Jouany *et al.*, 2009). Other conjugating enzymes include; glucuronosyl-transferases, sulpho-transferases, methyl-transferases, amino acyl-transferases and N-acetyl-transferases. However humans have lower concentrations of GST than ruminants making them more susceptible to the toxic effect of AFB_1 (Jouany *et al.*, 2009). Aflatoxin B_1 can also be bio-transformed into AFM_1, AFQ_1 and aflatoxicol. Aflatoxicol is biologically inactive and is excreted through the urinary system without further modification. Aflatoxin M_1 either conjugated with glucuronic acid and enters the biliary system and excreted in faeces or it enters into the circulatory system and excreted in the urine or is secreted into milk (Maleki *et al.*, 2015).

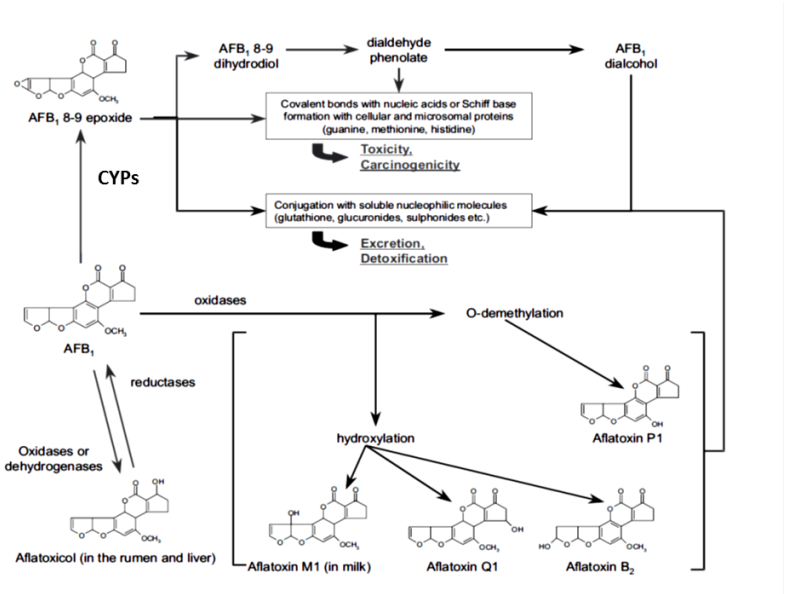

Figure 2.6. Metabolism of AFB_1 in the liver (Yiannikouris & Jouany, 2002).

In ruminants, initial degradation of AFB_1 takes place in the rumen by ruminal microbiota to give aflatoxicol, AFM_1 and several other hydroxylated metabolites (Gallo *et al.*, 2015). However, not all AFB_1 is metabolised in the rumen, some will leave the rumen unaltered and get to the liver where they are activated into the epoxide or hydroxylated into a less toxic metabolite AFM_1 (Fink-Gremmels, 2008a). Studies have shown that the carry-over rate of AFB_1 from feed to AFM_1 in milk ranges from 1-2% in low yielding cows up to 6% in high yielding cows (Gelven, 2010; Churchill *et al.*, 2016). Although AFM_1 is 90% less toxic than AFB_1 (Iqbal *et al.*, 2015; Hof, 2016) it still retains the carcinogenicity having the ability to cause DNA damage, gene mutation, chromosomal anomalies, and cell transformation (Sarica *et al.*, 2015; Nile *et al.*, 2016; Womack *et al.*, 2016). This has resulted in the IACR classifying AFM_1 into a class1 carcinogen though it had been previously put in group 2B (Var & Kabak, 2009).

2.7 Aflatoxin toxicity

Poisoning caused by mycotoxin ingestion is known as mycotoxicosis (Datsugwai *et al.*, 2013; Gallo *et al.*, 2015). This is characterised by symptoms that manifest differently depending on the test system, dose and duration of exposure (Qazi & Fayyaz, 2006). Aflatoxins have been shown to be lethal to animals and animal cells in culture when administered acutely in sufficiently large doses, causing histological changes in animals when smaller doses were administered sub-acutely (Dhanasekaran *et al.*, 2011). The largest and most severe case of aflatoxin poisoning recorded was in 2004 in Kenya which left 317 people hospitalised and 125 dead (Wagacha & Muthomi, 2008).

2.7.1 Humans

The toxic and carcinogenic effects of aflatoxin B_1 are intimately linked to both the rate of activation and the rate of detoxification at the primary and secondary levels of metabolism (Bosco & Mollea, 2012). Aflatoxin toxicity referred to as aflatoxicosis (Diaz & Murcia, 2011) and can be classified as acute or chronic. Acute toxicity is characterised by a rapid onset of toxic response due to ingestion of a high dose of the toxin whereas chronic toxicity results in cancers and other irreversible effects due to low-dose exposure to the toxin over a long period of time (Afsah-Hejri *et al.*, 2013; Carvajal-Moreno, 2015; Sarica *et al.*, 2015). Symptoms of acute toxicity include jaundice, diarrhoea, depression, low-grade fever, anorexia and liver damage. In severe cases death can occur. In humans acute aflatoxicosis is characterised by vomiting, high fever, highly coloured urine, tremors, convulsion, cerebral oedema, coma, elevated serum transaminases, hypoglycaemia, and fatty degeneration in the liver and kidneys

(Bosco & Mollea, 2012). Aflatoxins have also been associated with kwashiorkor and marasmus in most children in sub-Saharan Africa (Jallow, 2015).

2.7.2 Animals

The effects of aflatoxins in animals are diverse depending on the animal species, amount of toxin consumed, health status, age, nutritional status of the as well as the duration of the exposure (Jouany *et al.*, 2009; Afsah-Hejri *et al.*, 2013; Atherstone *et al.*, 2016). Acute aflatoxicosis in animals is characterised by depression, anorexia, weight loss, disease, gastrointestinal bleeding, pulmonary oedema and liver damage. Symptoms of moderate to prolonged exposure to aflatoxins may result in reduced feed intake and nutrient absorption by the animals and this will result in a decrease in weight of the animals. A decline in feed consumption and productivity which may include a reduction in milk production in dairy cattle (Fink-Gremmels, 2008a) as well as suppressed immune function thereby making animals susceptible to infections (Queiroz *et al.*, 2012; Bbosa *et al.*, 2013b; Senerwa *et al.*, 2016).

2.8 Aflatoxin legislation

Although there had been several outbreaks of mycotoxicosis in many countries, the disease remained neglected for so many years. It was only after the Turkey-X-disease that regulations imposing maximum limits of aflatoxins in foods and feeds (Mazumder & Sasmal, 2001; Fountain *et al.*, 2014) were instituted. Since mycotoxins are natural contaminants which are difficult to eliminate from human and animal diets (Giovati *et al.*, 2015), tolerance levels have been set so as to reduce the risk of exposure. However these tolerance levels differ with countries (Klich, 2007) depending on the developmental level and the economic situation of the country (Var & Kabak, 2009).

The maximum residual limit (MRL) of AFM_1 in milk for most countries ranges from 0-1.0µg/l (Xiong *et al.*, 2013) with the Commission of the European Community and the Codex Alimentarius Commission having the lowest tolerable limit of 0.05µg/l in raw milk (Giovati *et al.*, 2015; Campagnollo *et al.*, 2016; Hof, 2016; Ketney *et al.*, 2017) which has also been adopted by many African countries (Makau *et al.*, 2016). The United States Food and Drug Administration (US-FDA) has established AFM_1 levels of 0.5µg/l in milk (Bellio *et al.*, 2016; Campagnollo *et al.*, 2016). Since the effects of aflatoxins are more pronounced in children, a limit of 0.025µg/l has been set for milk intended for feeding children (Bellio *et al.*, 2016; Ketney *et al.*, 2017). To avoid carry-over, a maximum residual limit (MRL) of AFB_1 in feed

of lactating cows have also been set, ranging from 4µg/kg (European Community) to 10µg/kg (China) and 20µg/kg (USA) (Giovati *et al.*, 2015; Ketney *et al.*, 2017).

Most developing countries lack strict regulatory measures as the legislation is only applied to commodities intended for export. They are also limited when it comes to the detection and monitoring of aflatoxins in food because they lag behind in terms of technological advancement (Mehl & Cotty, 2013; Giovati *et al.*, 2015), therefore they are the most affected with most cases of outbreaks. In 1971 Zimbabwe adopted maximum limit of 25µg/kg for food intended for human consumption which was later revised downward in 1990 to 20µg/kg (Siwela & Nziramasanga, 1999). However, the monitoring of food commodities is not controlled.

2.9 Aflatoxins in dairy feeds

Ruminant diet is made up of several components which include cereal grains, forages, concentrates, preserved feed (silage, hay and straw) and pressed cakes from oil seeds such as sunflower, groundnut, cotton seed and soybean. Mycotoxin presence in the feedstuffs can have negative effects in animal husbandry leading to reduced productivity (Denli, 2015). It has been suggested that ruminants are less susceptible to mycotoxins poisoning due the their rumen microbiota which convert the toxins into less toxic or biologically inactive metabolites thereby protecting the animals (Akande *et al.*, 2006; Fink-Gremmels, 2008a; Afsah-Hejri *et al.*, 2013). However, ruminal digestion is not very effective for some mycotoxins such as aflatoxins (Jouany *et al.*, 2009; Becker-Algeri *et al.*, 2016a; Flores-Flores & González-Peñas, 2018) as they tend to disturb the microbial ecosystem of the rumen inhibiting some of the microorganisms at AFB_1 concentrations of less than 10µg/ml (Jouany *et al.*, 2009). As a result the aflatoxins will leave the rumen unaltered and are transferred into the animal tissues and fluids (Flores-Flores & González-Peñas, 2018). Studies have shown that feeding lactating cows with AFB_1 contaminated feed results in its bio-transformation into AFM_1 which is subsequently secreted into milk (Janković *et al.*, 2009). Humans are at the risk of aflatoxin poisoning through the consumption of contaminated food products like meat and milk and dairy products (Hassanin, 1993; Akande *et al.*, 2006; Gallo *et al.*, 2015; Atherstone *et al.*, 2016; Ji *et al.*, 2016).

2.10 Aflatoxins in milk

The presence of aflatoxins in milk is mainly due to the consumption of AFB_1 contaminated feed by lactating cows resulting in carry-over as AFM_1 (Campagnollo *et al.*, 2016; Ketney *et al.*, 2017). Aflatoxin M_1 in milk and its by-products is a worldwide concern because of its effects on those who consume it in large quantities especially the children, who are also more

susceptible to the adverse effects of mycotoxins (Elsayed & El-Fatah, 2015; Giovati *et al.*, 2015; Obade *et al.*, 2015; Campagnollo *et al.*, 2016; Ketney *et al.*, 2017). Data from several studies show that aflatoxin carry-over as AFM_1 into milk of dairy cows has been observed to range from 0.3% to 6.2% with the higher carry-over rates in the high yielding cows (Var & Kabak, 2009; Britzi *et al.*, 2013; Giovati *et al.*, 2015). Several factors such as breed, lactation stage, health status of the animal, milking time, feed intake and season of the year affect the occurrence of AFM_1 in milk (Ketney *et al.*, 2017). Usually AFM_1 is detected 12-24 hours after ingestion of contaminated feed and is usually cleared in the system 24 hours after withdrawal of aflatoxin contaminated diet (Campagnollo *et al.*, 2016; Besufekad *et al.*, 2018).

Like any other aflatoxins, AFM_1 is very stable and is not really affected by milk processing technologies such as pasteurisation, ultra heat treatment (UHT) and fermentation (Panahi *et al.*, 2011; Elsayed & El-Fatah, 2015; Becker-Algeri *et al.*, 2016a; Womack *et al.*, 2016). Unlike AFB_1, AFM_1 does not need to be converted to an epoxide for it to exert its toxic effect (Giovati *et al.*, 2015).

2.11 Aflatoxin detection and analysis methods

Aflatoxins and other mycotoxin toxicity can occur at very low concentrations and most countries continue lowering the acceptable limits, therefore robust methods for their detection that are sensitive, precise and reliable are needed for the detection with the highest degree of accuracy (Rahmani *et al.*, 2009; Bellio *et al.*, 2016; Nile *et al.*, 2016). The methods commonly employed in the identification and quantification of aflatoxins are chromatography and immunochemical assays (Rahmani *et al.*, 2009; Bellio *et al.*, 2016). Immunochemical methods are used mainly for rapid detection of mycotoxins in food or feed matrices (Bellio *et al.*, 2016) and do not require much technical expertise thus are suitable for screening (Nile *et al.*, 2016; Ketney *et al.*, 2017). On the other hand chromatographic methods require extensive sample preparation and well-trained personnel therefore are mainly used for confirmation of results obtained from screening (Arapcheska *et al.*, 2015; Obade *et al.*, 2015; Bellio *et al.*, 2016; Nile *et al.*, 2016; Ketney *et al.*, 2017). In addition, immunochemical methods tend to be specific for one mycotoxin or a group of related mycotoxins whereas chromatographic methods can detect several toxins whose structures are not even related resulting in multitoxin detection (Atanda *et al.*, 2013; Ketney *et al.*, 2017). Among the chromatographic methods are the thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV or fluorescence detection (FD), and enzyme immunoassays (EIAs) (Rahmani *et al.*, 2009). Chromatography coupled with mass spectrometry, liquid chromatography- mass spectrometry

(LC-MS), liquid chromatography with tandem mass spectrometry (LC-MS/MS) and gas chromatography- mass spectrometry (GC-MS), have also gained popularity in the analysis of mycotoxins (Ketney *et al.*, 2017). However for a long period of time, HPLC became the main method for mycotoxin analysis (Pirestani & Toghyani, 2010) but has gradually been replaced by LC-MS/MS method.. The commonly used immunochemical techniques are enzyme linked immunosorbent assay (ELISA), immunoaffinity column assays (IAC), sequential injection immunoassay (SIIA), and radioimmunoassay (RIA) (Obade *et al.*, 2015; Bellio *et al.*, 2016)

The heterogeneous nature and physical properties of aflatoxins are factors that affect the extraction process, they therefore need to be considered during analysis (Rahmani *et al.*, 2009). Aflatoxin extraction can be done through liquid-liquid extraction (LLE) which uses two immiscible liquids or solid-phase extraction (SPE) using a solid and a liquid phase. For some extraction methods, a clean-up step is required for analyte enrichment and removal of substances that may interfere with the detection of the analyte (Rahmani *et al.*, 2009). Immunochemical methods and LC-MS/MS do not require this clean-up step. Extract clean-up can be done through liquid-liquid partitioning, SPE, use of immunoaffinity columns (IAC).

It has been demonstrated that molecular methods based on DNA sequencing in combination with traditional methods that use phenotypic features provide the most accurate and reliable means of characterising members of *Aspergillus* genus to species level (Davolos *et al.*, 2012). Therefore in this study morphological, analytical and molecular methods were used for the determination of species diversity, phylogenetic relationships and aflatoxin producing potential of *Aspergillus* species present in dairy feeds. For the quantification of aflatoxins in the feeds, HPLC with IAC clean-up was used whereas for milk and urine HPLC with IAC clean-up and ELISA were used.

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Chapter 3

Isolation and characterisation of aflatoxigenic *Aspergillus* species from dairy feeds from Bulawayo

Abstract

Aflatoxin contamination of foods and feeds as a result of mould contamination has gained global attention since aflatoxins pose negative effects on the health of animals. In this study , aflatoxigenic *Aspergillus* isolates from 95 feeds consisting of feed concentrates (CN), mixed rations (MR), brewers spent grain (BSG) and grass (GR) used in feeding dairy cows under intensive, semi-intensive and extensive feeding systems in peri-urban Bulawayo, Zimbabwe were characterised using morphological and molecular methods. Presumptive *Aspergillus* isolation was done through serial plate dilution method followed by identification using colony colour and morphology. Pure cultures were obtained through the single spore technique. Molecular characterisation of the isolates was done through the amplification of the internal transcribed spacer regions of the DNA, ITS1 and ITS4 through the polymerase chain reaction (PCR). This was followed by sequencing and blasting using the Basic Local Alignment Search Tool (BLAST). Species identification was based on the best score of similarity to sequences already deposited in the gene bank of the National Centre for Biotechnology and Information (NCBI), (≥99% similarity). A polyphasic approach was used in the determination of the isolates' aflatoxin production potential. Yeast extract sucrose (YES) agar; desiccated coconut agar (DCA); desiccated coconut agar with β-cyclodextrin; neutral red desiccated coconut agar (NRDCA) and neutral red desiccated coconut agar with β-cyclodextrin were used in the morphological methods. For molecular identification of aflatoxin producers, four major genes in the aflatoxin biosynthetic pathway, *aflD, aflM, aflP, aflJ* and *aflR* were amplified through PCR. The PCR products were then electrophoresed on 1% agarose gel stained with ethidium bromide to show presence or absence of gene. For phylogenetic and molecular evolutionary analysis of the isolates, single gene Neighbour joining and maximum likelihood and concatenated trees were constructed using MEGA X following amplification and sequencing of β-tubulin, calmodulin and ITS. A total of 199 presumptive isolates were obtained and 140 showed 99-100% similarity to the following species: *A niger, A awamori, A tubingensis, A flavus, A nomius, A oryzae, A parasiticus, A fumigatus, A, foetidius, A chavalieri, A sydowii, A brasiliensis, A cristatum, A ochraceus, A welwitschiae, A phoenicis, A parvisclerotigenus* and *A japonicas*. These species belonged to the following sections: *Nigri* (39%), *Flavi* (33%), *Fumigati* (27%), and *Circumdati* (1%). A total of 44 isolates were identified as potential aflatoxin producers using both morphological and molecular methods. The bulk of these isolates were from feeds used in the intensive and semi-intensive feeding farming systems with the mixed ration being the one mostly contaminated by the toxigenic *Aspergillus*. Analysis of the presence of the two regulatory genes, *aflR* and *aflJ* showed that *aflJ* was the major gene in the isolates as it was detected in 29% of the isolates whereas only 14% of the isolates possessed the *aflR* gene. Evolutionary analysis of the isolates also showed the low power of discrimination in the use of single genes in phylogenetic tree construction therefore concatenation is recommended for phylogenetic analysis. Findings from this study shows that feeds used for feeding dairy cows in peri-urban Bulawayo were contaminated by moulds from the *Aspergillus* genus with the potential of producing aflatoxins. The occurrence of toxigenic species was high in intensive and semi-intensive feeding systems due to higher usage of mixed rations which contain cotton seed cake, a waste product of oil extraction as the major ingredient. Therefore there is a need

for monitoring of ingredients used in feed formulations and the need to avoid the use of contaminated feed constituents in formulating the mixed rations as a strategy of reducing contamination of feeds.

3.1 Introduction

The most common mould contaminants of agricultural commodities worldwide belong to the *Aspergillus* genus namely, *A. flavus* and *A. parasiticus* (Dutta & Das, 2001; Ghiasian & Maghsood, 2011; Ibrahim *et al.*, 2016). These two species together with *A. nomius* are well known aflatoxin producers (Rodrigues *et al.*, 2011; Monson *et al.*, 2015). Aflatoxin contamination of food and feeds has gained global attention due to their negative effects on the health of both humans and animals (Fink-Grernmels, 1999; Zain, 2011; Arapcheska *et al.*, 2015; Kumar *et al.*, 2017). Animal feeds are made from agricultural commodities which include cereal grains and stovers, oil seed cakes, as well as silage which are likely to be contaminated with moulds belonging to the *Aspergillus* genus which is capable of producing aflatoxins (Pleadin, 2015; Morrison *et al.*, 2017).

Consumption of aflatoxin contaminated food and feeds lead to toxicological effects collectively known as aflatoxicosis (Bennett *et al.*, 2007; Daniel *et al.*, 2011; Nurul Adilah & Mohd Redzwan, 2017). Aflatoxicosis can be classified as acute or chronic depending on the amount of toxin ingested (Ogodo & Ugbogu, 2016). The major mycotoxins produced by *Aspergillus* species are aflatoxin (AF) B_1, B_2, G_1 and G_2 (Kumar *et al.*, 2017) named depending on their fluorescence (blue or green) under ultraviolet (UV) light at 350mn (Reiter *et al.*, 2009; Dors *et al.*, 2011; Bbosa *et al.*, 2013a). AFB_1 is the most potent of them all having carcinogenic properties and has been implicated as a causative agent of human hepatocarcinoma (Liu & Wu, 2010; Baranyi *et al.*, 2013; Patel *et al.*, 2015). Upon their ingestion, aflatoxins are absorbed and transported to the liver where their bio-transformation and detoxification takes place through the action of cytochrome P450 enzymes (Diaz *et al.*, 2010; Bbosa *et al.*, 2013b; Dohnal *et al.*, 2014). In mammals, AFB_1 is bio-transformed into its hydroxylated metabolite aflatoxin M_1 (AFM_1) which is secreted mainly in the milk (Britzi *et al.*, 2013; Giovati *et al.*, 2015) although it can also be found in urine and faeces. Milk therefore becomes a route through which aflatoxins can be introduced into humans.

Members of *Aspergillus* species are ubiquitous and can grow in a variety of food products under diverse environmental conditions. Several studies have reported cases of stock feeds to be contaminated by the genus *Aspergillus*. The presence of *Aspergillu*s species in food and feeds does not always mean there is aflatoxin contamination since not all *Aspergillus* are aflatoxin producers. Moreover, aflatoxin production only takes place under hot and humid atmosphere and improper storage conditions (Ibrahim *et al.*, 2016).

Due to the similarities in the morphological features of the species, it becomes difficult identify members of the *Aspergillus* genus to species level using a single method (Moslem *et al.*, 2010; Zulkifli & Zakaria, 2017). This has led to researchers using a polyphasic approach in the identification and characterisation of *Aspergillus* species (Silva *et al.*, 2015). Morphological methods involve inoculating the isolates on media known to support growth of *Aspergillus* followed by looking at the colony morphology and colour of spores/conidia as well as other phenotypic features (Hinrikson *et al.*, 2005). This is followed by microscopy where spore size and morphology as well as sclerotia can be observed and used for characterisation. This process is tedious and time consuming (Henry *et al.*, 2000). In some cases the conidia may fail to develop making the identification process difficult (Hinrikson *et al.*, 2005). Therefore nucleic acid based methods also known as molecular methods have been developed which look at specific markers peculiar to certain species. The ITS region has become the primary bar code for fungal identification (Schoch *et al.*, 2012) due to its sensitivity and high copy number per genome (Henry *et al.*, 2000). However, its specificity has become questionable and is now restricted to biodiversity studies. Other markers like the β-tubulin and calmodulin are being used for identification of *Aspergillus* to species level and phylogenetic studies (Samson *et al.*, 2014).

Aflatoxin production potential of an isolate is mainly shown by presence of fluorescence under UV light. However, fluorescence of colonies can be misleading as some nontoxigenic strains can also fluoresce. Addition of components that enhance aflatoxin production in the media is another method of differentiating aflatoxins producers from non aflatoxin producers but has also shown possibilities of having false negatives and false positives. The amplification of major genes in the aflatoxin biosynthetic pathway has gained much attention from researchers in determination of the aflatoxigenicity of isolates. Therefore in this study morphological, biochemical and molecular methods were used to identify, characterise and determine the aflatoxigenic potential of the isolates from dairy feeds.

3.1.1 Aim

Isolation and characterisation of aflatoxigenic *Aspergillus* from dairy feeds in Bulawayo.

3.1.2 Objectives

1. To isolate and characterise *Aspergillus* species from dairy feeds using morphological features.
2. To determine the biodiversity of *Aspergillus* species in dairy feeds by amplification and sequencing of the ITS region.
3. To determine the phylogenetic relatedness of the *Aspergillus* isolates using ITS, β-tubulin and calmodulin genes.

3.2 Methodology

3.2.1 Sampling

Sampling of feed was undertaken in Bulawayo, Zimbabwe (Figure 3.1). Bulawayo is the second-largest city in Zimbabwe after the capital Harare covering an area of 1,707 km² and an altitude of 1,358 m above sea the level. It is in agro-ecological zone IV characterised by low and erratic rainfall (400-600 mm/annum) and mean annual temperature range of 20-30°C (Chinogaramombe *et al*., 2008). This zone is a semi-extensive region suitable for farm systems based on livestock and resistant fodder crops, forestry, wildlife/tourism. Three seasons are recognised in Zimbabwe namely: (1) a hot wet season from mid- November to March (summer); (2) a cold dry season from April to July (winter), and a hot dry season from August to mid-November (spring). The coordinates of the sampling sites are shown in Appendix I.

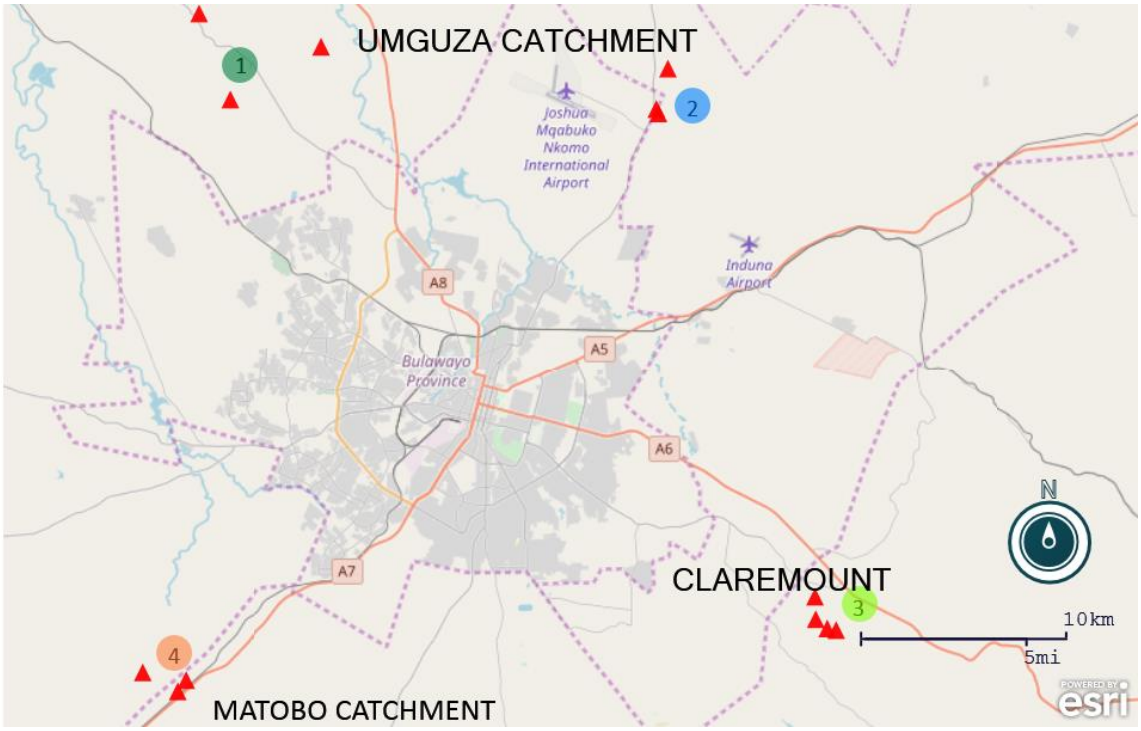

Figure 3.1. Map of Bulawayo, Zimabwe showing the sample collection areas 1, 2, 3 and 4.

3.2.2 Sample collection

Feed samples were collected during hot dry season (August – October 2016) and rainy season (January – March 2017). Collection of the samples was done in sterile polythene zip lock bags

after thoroughly mixing the feed portion to be given to the cows on that particular day (Kang'ethe & Lang'a, 2009) and transported in cooler boxes to the lab where they were ground to a fine powder using IKA® M20 universal batch mill (Germany) and stored in the freezer at -20°C until time for analysis (Beukovic *et al.*, 2015).

3.2.3 Isolation and cultural identification of *Aspergillus* isolates

Fungal isolation from feed samples was carried out by the dilution-plate method. Briefly, 1g of each feed sample was mixed with 9 ml of sterile distilled water and vortexed by hand inversion. Decimal dilutions of the samples was made and 100µl of each dilution was spread plated onto potato dextrose agar (PDA) supplemented with chloramphenicol (100µg/ml). The plates were then incubated at 25±2°C for 3-7 days. Identification of *Aspergillus* isolates was based on their colony morphology and spore colours (Almoammar *et al.*, 2013; Mostafa & Amer, 2013). Pure cultures were obtained through the single spore technique (Almoammar *et al.*, 2013) where sterile extra fine forceps were used to pick a single spore from the colony and transfer onto a new PDA plate (Choi et al., 1999), followed by incubation at 25±2°C.

3.2.4 Molecular identification of isolates

3.2.4.1 DNA extraction

Presumptive *Aspergillus* isolates were grown on PDA for 72 hours at 25±2°C followed by scrapping of the mycelia and spores which were used for DNA extraction (Gontia-Mishra *et al.*, 2014). Extraction of DNA was undertaken by use of a Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA). Briefly, 750µl of lysis solution was added to the mycelia and spores followed by cell disruption carried out using a genie disruptor (Scientific Industries Inc., USA). The lysed mixture was centrifuged at 10 000 rpm and supernatant filtered and collected. Separation of the DNA from the rest of the cell contents was done using the DNA binding solution. The solution was centrifuged at 10 000 rpm. The supernatant was discarded and the DNA pellet re-suspended in 100 µl of elution buffer. The quality of the DNA was determined using Nano drop where the absorbance ratio of 260/280 and 260/230 were measured. The extracted DNA was stored at -20 °C until time for analysis (Egbuta *et al.*, 2015).

3.2.4.2 Polymerase Chain Reaction (PCR)

Molecular identification of the isolates was undertaken by the PCR amplification of three molecular markers namely; ITS, β-tubulin and calmodulin by PCR. Internal transcribed spacer regions of the DNA (ITS1-5.8S-ITS2) primers, ITS 1 (5′-CTTGGT CAT TTA GAG GAA

GTA A-3′) (forward) and ITS 4 (5′-TCC TCC GCT TAT TGA TATGC-3′) (reverse) were used (Roca *et al.*, 2003; Pinto *et al.*, 2012) following the method of Egbuta *et al.* (2015) with some modifications. The PCR reactions were carried out in a Bio-Rad T100™ thermocycler (Singapore). The PCR reaction had 12.5µl of master mix (One*Taq*®Quick-Load, Biolabs Inc, New England), 0.5 µl of each 25µM primer (Inqaba Biotech, South Africa), 5µl of the extracted DNA, and enough nuclease free water to produce a final volume of a 25µl. Cycling conditions were as follows: Pre-dwelling at 95°C for 3 min, 35 cycles denaturation at 95°C for 1 min, annealing at 58°C for 45 sec, extension at 72°C for 1 min 30 seconds, post-dwelling at 72°C for 10 min. (Egbuta *et al*., 2015). β-tubulin primers Bt_2a (5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′) (forward) and Bt_2b (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (reverse) by Glass and Donaldson (1995) were used following method of (Nouripour-Sisakht *et al.*, 2015) with some modifications as follows; The PCR reaction had 12.5µl of master mix (One*Taq*®Quick-Load, Biolabs Inc, New England), 0.5 µl of each 25µM primer (Inqaba Biotech, South Africa), 5µl of the extracted DNA, and enough nuclease free water to produce a final volume of a 25µl. using the following PCR conditions: Initial de-naturation for 5 min at 94,8°C, followed by 35 cycles of amplification, consisting of denaturation for 30 sec at 94,8°C, annealing for 30 sec at 58,8°C, and extension for 1 min at 72,8°C, followed by an ultimate extension step at 72,8°C for 10 min.

For calmodulin genes, amplifications were carried out using primers CMD5 (5′-CCG AGT ACA AGG ARG CCT TC-3′) (forward) and CMD6 (5′-CCG ATR GAG GTC ATR ACG TGG-3′) by Hong *et al.* (2005) following the method by Sabino *et al.* (2014). The PCR reaction had 12.5µl of master mix (One*Taq*®Quick-Load, Biolabs Inc, New England), 0.5 µl of each 25µM primer (Inqaba Biotech, South Africa), 5µl of the extracted DNA, and enough nuclease free water to produce a final volume of a 25µl. Amplifications were carried out as follows; an initial denaturation at 95ºC for 10 min, followed by 38 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min, followed by a final extension step of 72ºC for 7 min.

3.2.4.3 Identification of the *Aspergillus* isolates

The PCR products were electrophoresed on a 1.0% agarose gel containing 0.5 mg/mL ethidium bromide for about one hour (80volts; 400 A) in 1×TAE (Tris Acetate-EDTA) buffer. The gel was visualised using the gel documentation system (Bio-Rad Molecular Image® Gel Doc™ XR+ with Image Lab™ software, USA). The amplicons were sent for sequencing at Inqaba Biotech, South Africa. Editing of the amplicons sequences was undertaken using Finch TV software version 1.4.0 (Frickmann *et al.*, 2015). Similarity of amplicons sequences were

compared with those already deposited in the gene bank of the National Centre for Biotechnology and Information (NCBI) through the Basic Local Alignment Search Tool (Magnani *et al.*, 2005; Castrillo *et al.*, 2012; Zarrin & Erfaninejad, 2016). Species identification was based on the best score (≥99% similarity) (Gajjar *et al.*, 2013; Frickmann *et al.*, 2015; Rossi-Tamisier *et al.*, 2015; Beye *et al.*, 2017).

3.2.4.4 Biodiversity of *Aspergillus* isolates from the feeds

Edited DNA sequences were displayed using the BioEdit sequence alignment editor together with sequences of other *Aspergillus* species belonging to *Flavi* section and outside it from the GenBank databases (Gallo *et al.*, 2012; Bıyık *et al.*, 2016) as outliers. The sequence data was aligned with the ClustalW multiple sequence alignment (Gallo *et al.*, 2012). Neighbour joining and maximum likelihood trees were constructed using MEGA X 10.0.4 for phylogenetic and molecular evolutionary analysis (Gallo *et al.*, 2012; Adegboye & Babalola, 2013).

3.2.5 Screening isolates for aflatoxin production

3.2.5.1 Cultural and biochemical identification of aflatoxin producers

Morphological identification of aflatoxin production by the isolates was determined by inoculating on several media which included yeast extract sucrose (YES) agar; desiccated coconut agar (DCA); desiccated coconut agar with β-cyclodextrin; neutral red desiccated coconut agar (NRDCA) and neutral red desiccated coconut agar with β-cyclodextrin. Table 1 shows the ability of the isolates to produce aflatoxins.

Table 1. Media for cultural identification of aflatoxin producers and expected results.

Medium	Composition/litre	Aflatoxin production	Reference
YES	15g yeast extract, 150g sucrose 15g agar	Pink to red colouration on the underside of colony after addition of ammonia.	(Yazdani *et al.*, 2010; Almoammar *et al.*, 2013)
NRDCA	200g desiccated coconut 1.5g agar 3ml neutral red dye	Yellow ring on the underside of colony. -Flourescent ring around the colony under UV light (365nm).	(Atanda *et al.*, 2011)

NRDCA with β-cyclodextrin	200g desiccated coconut 1.5g agar 3ml neutral red dye 3g β-cyclodextrin	Yellow ring on the underside of colony. -Flourescent ring around the colony under UV light (365nm).	(Adetunji *et al*., 2018)

3.2.5.2 Molecular identification of aflatoxin producers

Genes *aflD, aflM, aflP,* and *aflR* were amplified by PCR using the primer pairs and conditions listed in Table 2, (Rashid *et al.*, 2008; Gallo *et al.*, 2012; Abd El-Aziz *et al.*, 2015; Fakruddin *et al.*, 2015). PCR was performed in 25 μL containing 12.5 μL of 2× PCR mix (One*Taq*®Quick-Load, Biolabs Inc, New England), 0.5 µl of each 25µM primer (Inqaba Biotech, South Africa), 5µl of the extracted DNA, and adequate nuclease free water to produce a final volume of a 25µl. PCR products were electrophoresed on 1% agarose gel and ethidium bromide staining and visualised using the gel documentation system.

Table 2. Primer sequences and PCR conditions used for the detection of aflatoxigenic *Aspergillus* isolates from dairy feeds.

Prime pair	Target Gene	Primer sequence (5′ → 3′)	PCR conditions							Reference
			1	2	3	4	5	6	Product size	
Nor-1F	*aflD*	ACCGCTACGCCGGCACTCTCGGCAC	94°C:10mins	94°C:1min	65°C:1min	72°C:2mins	33	72°C:mins	400 bp	
Nor-1R		GTT GGCCGCCAGCTTCGACACTCCG								
Ver-1F	*aflM*	GCCGCAGGCCGCGGAGAAAGTGGT	95°C:4mins	95°C:1min	58°C:1min	72°C:30sec	30	72°C:10mins	600 bp	(Rashid *et al.*, 2008; Abd El-Aziz *et al.*, 2015)
Ver-1R		GGGGATATACTCCCGCGACACAGCC								
Omt-1F	*aflP*	GTGGACGGACCTAGTCCGACATCAC	94°C:5mins	94°C:1min	75°C:2mins	72°C:2mins	33	72°C:10mins	797 bp	
Omt-1R		GTCGGCGCCACGCACTGGGTTGGGG								
aflR-1F	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	95°C:4mins	94°C1min	60°C:1min	72°C:30sec	30	72°C:10mins	1000 bp	
aflR-1R		GTCGGCGCCACGCACTGGGTTGGGG								
AflS-1F	*aflJ*	TGAATCCGTACCCTTTGAGG	95°C:10mins	95°C:50sec	58°C:50sec	72°C:2mins	30	72°C:5mins	684 bp	(Gallo *et al.*, 2012; Fakruddin *et al.*, 2015)
AflS-2R		GGAATGGGATGGAGATGAGA								

1- Pre-dwelling
2- Denaturation
3- Annealing
4- Extension
5- Cycles
6- Post-dwelling

3.2.6 Evolutionary relationships of the aflatoxin producing *Aspergillus* isolates

Single gene and concatenated genes phylogenetic trees were constructed using the Neighbour Joining analysis (Saitou & Nei, 1987) and node reliability was assessed using 1000 bootstrap replications . The sequences were aligned using Clustal W multiple alignment in Bioedit and joined using Seaview software 4.7. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Analysis was carried out by use of MEGA X.

3.3 Results

3.3.1 Cultural and morphological identification of isolates.

A total of 95 feed samples were obtained from 13 farms (47 from rainy season and 48 from dry season). These consisted of dairy feed concentrates (CN), mixed ration (MR), brewers spent grain (BSG) and grass (GR). A total of 123 presumptive isolates were also obtained from the dry season samples and 76 isolates from the rainy season. Based on colony morphology and spore colours, the isolates were grouped into two main subtypes namely *Circumdati* and *Fumigati.* They were further grouped into four sections based on colour of their spores, blue *(Fumigati*), green (*Flavi*), yellow (*Circumdati*) and black (*Nigri*) as shown in Figure 3.2.

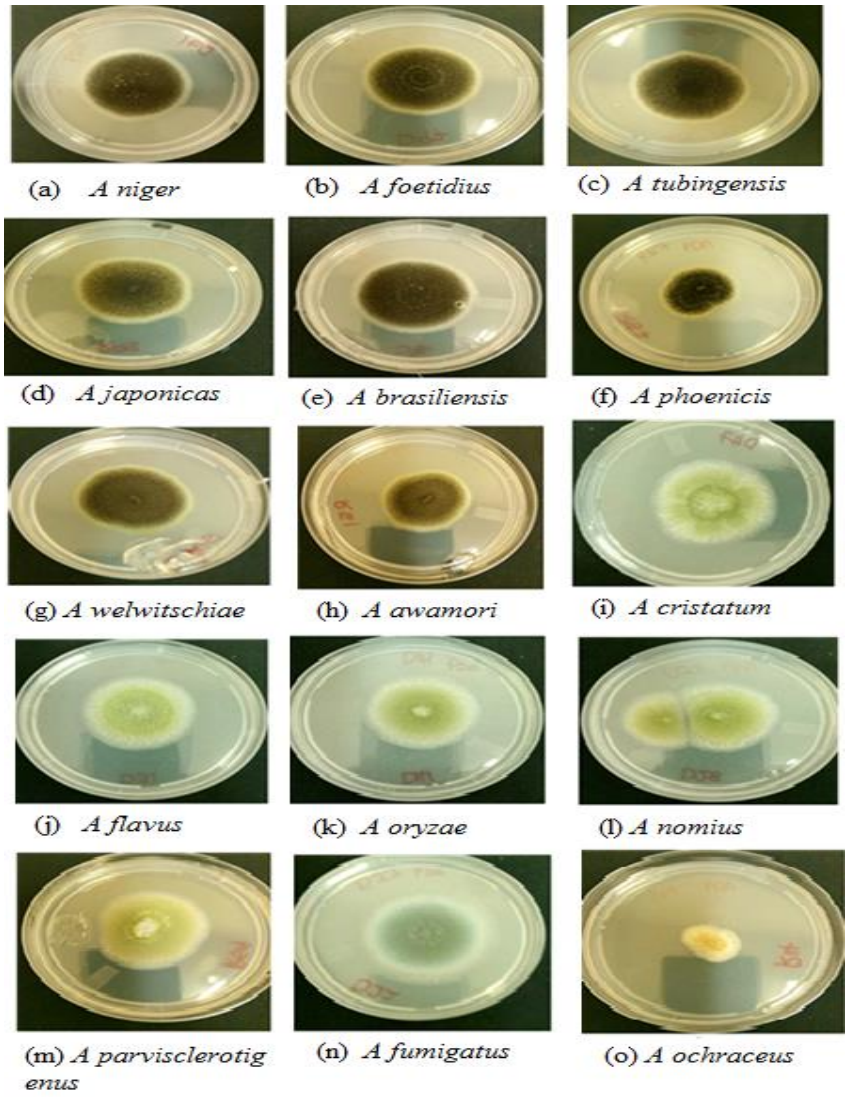

Figure 3.2. Colony morphologies of presumptive isolates growing on PDA after 3 days at 28°C. The isolates belonged to four sections namely, *Nigri, Flavi, Fumigati,* and *Circumdati* (Gautier *et al.*, 2016). Section *Nigri* is characterised by isolates by black colonies (a-h), section *Flavi*, green colonies (i-m), whereas section *Fumigati* had blue colonies (n) and section *Circumdati*, yellow colonies (o).

3.3.2 Molecular identification and characterisation of the isolates.

3.3.2.1 Polymerase Chain Reaction (PCR)

Amplification of the ITS region produced a 600bp product in all the isolates as shown in Figure 3.3.

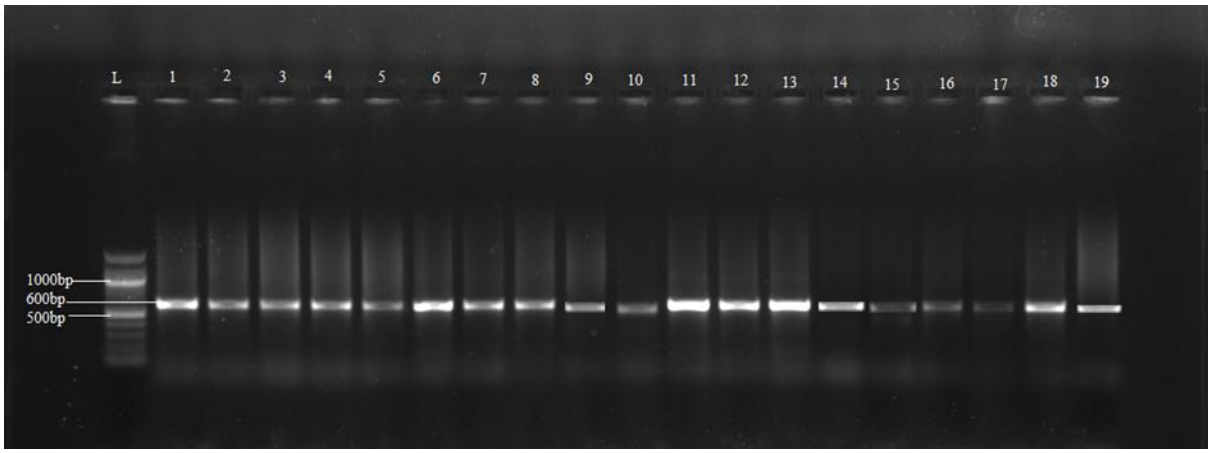

Figure 3.3. Gel electrophoresis of PCR products using ITS1/ITS4 primers and DNA from the morphologically presumed *Aspergillus* isolates. L is the 100bp ladder whereas numbers 1-19 denotes the different isolates. The band was present for all the isolates.

Amplification of the β-tubulin gene produced a 550bp product as shown in Figure 3.4.

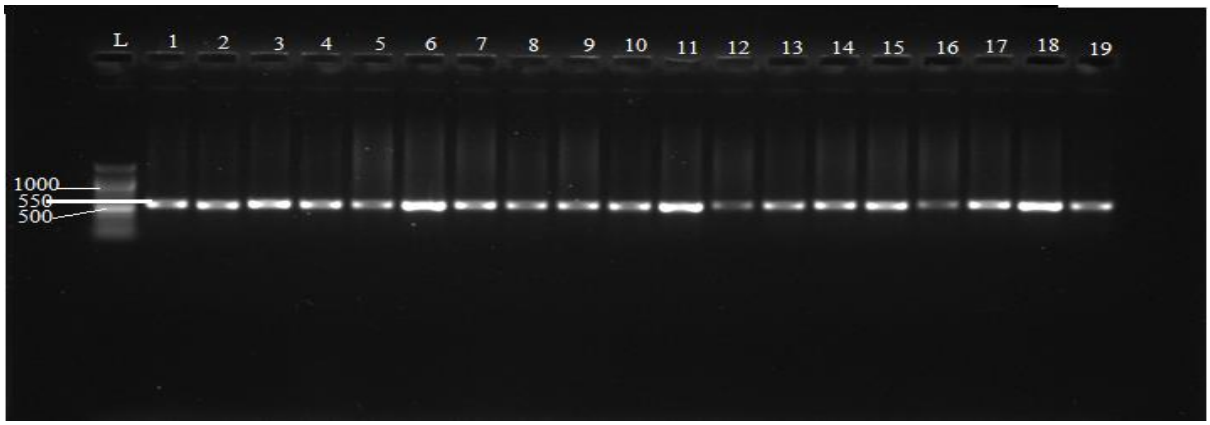

Figure 3.4. Gel electrophoresis of PCR products using Bt2a/Bt2b primers and DNA from the morphologically presumed *Aspergillus* isolates. L is the 100bp ladder whereas numbers 1-19 denotes the different isolates. A 550bp product was obtained from all the isolates.

Amplification of the calmodulin gene produced a 580bp product as shown in Figure 3.5.

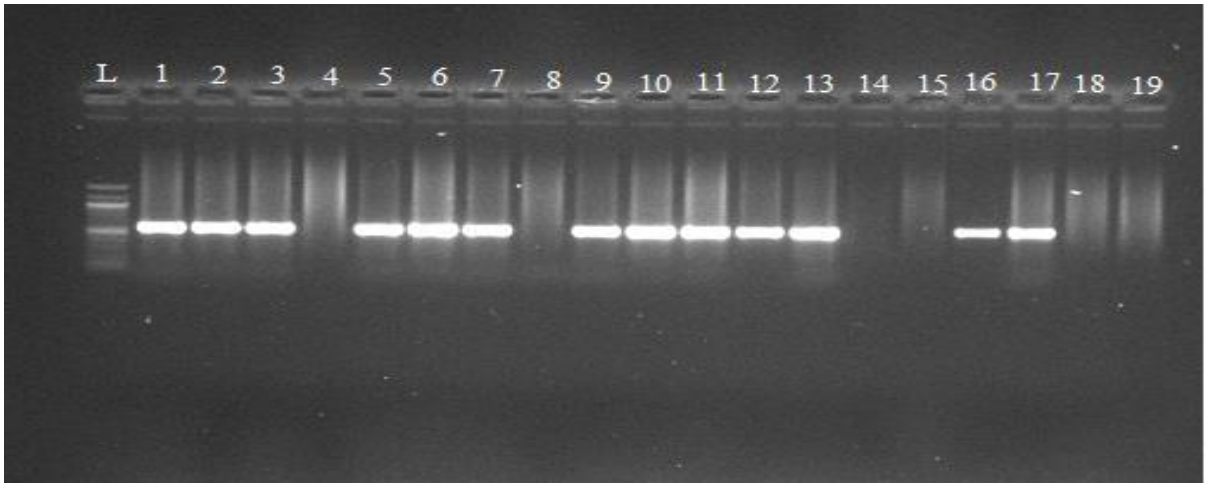

Figure 3.5. Gel electrophoresis of PCR products using CMD5/CMD6 primers and DNA from the morphologically presumed *Aspergillus* isolates. L is the 1.5kb ladder whereas numbers 1-19 denotes the different isolates.

BLAST results for the ITS region showed that 82 isolates from the dry season had 99-100% similarity to the following species; *A niger, A awamori, A tubingensis, A flavus, A nomius, A oryzae, A parasiticus, A fumigatus, A, foetidius, A chavalieri, A sydowii, A brasiliensis* and *A cristatum.* From the rainy season, 58 isolates showed 99-100% similarity to the following species; *A niger, A ochraceus, A flavus, A fumigatus, A tubingensis, A oryzae, A welwitschiae, A phoenicis, A awamori, A parvisclerotigenus, A japonicas* and *A nomius.* The isolates were given genbank accession numbers MG659595 to MG659694 and MH270529 to MH270615 (see Appendix II).

The isolates were grouped according to their sections and distribution across the feeds which showed that the occurrence frequency was in the following order; *Nigri > Flavi > Fumigatus > Circumdati (*Figure 3.6). Section *Nigri* had the highest prevalence of 39%.

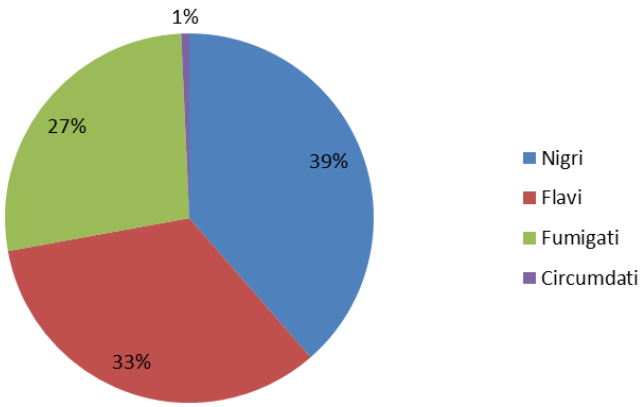

Figure 3.6. Percentage distribution of *Aspergilli* in Bulawayo feeds..

3.3.2.2 Phylogenetic analysis

The phylogenetic trees constructed from ITS, calmodulin and β-tubulin genes showed different clustering patterns as in Figures (3.7 -3.9).

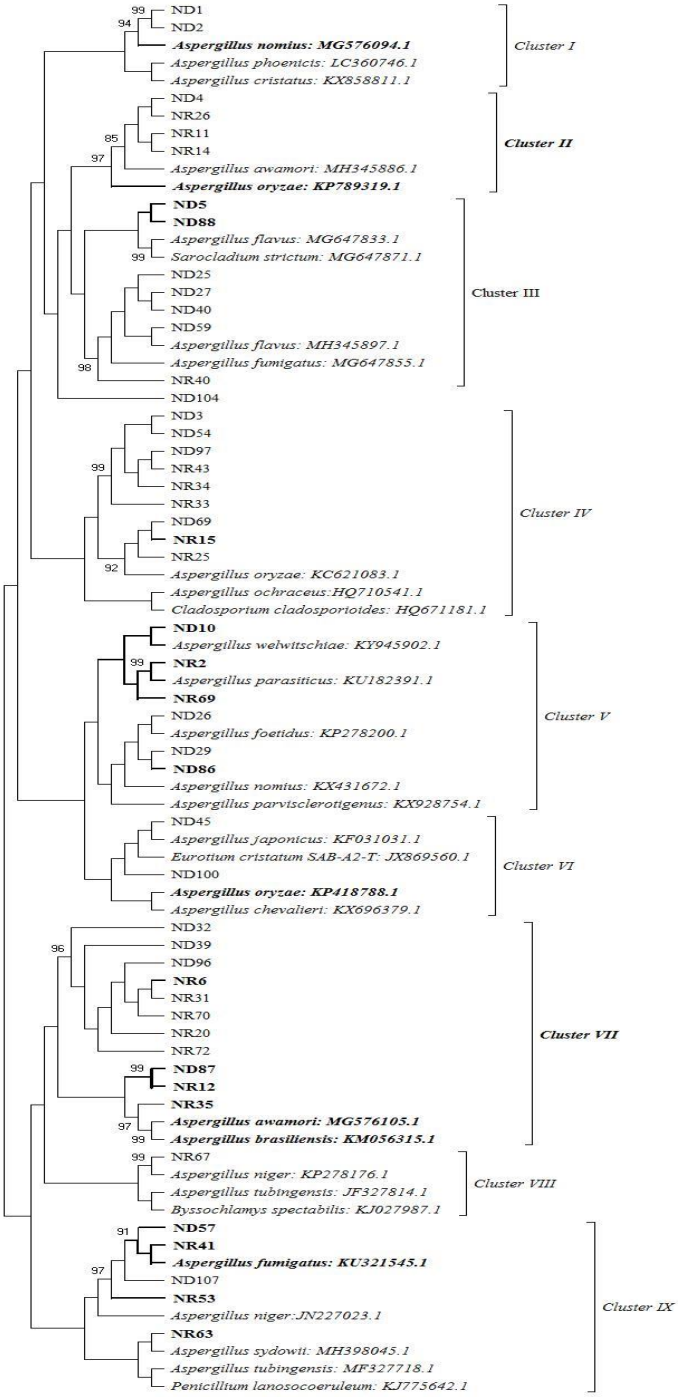

Figure 3.7. ITS phylogenetic tree , showing evolutionary relationships of *Aspergillus* isolates and other closely related species from the GenBank. Isolates from the dry season are prefixed ND whereas those from the rainy season NR. The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987) with

1000 bootstrap replicates (Felsenstein, 1985). Values greater than 50% bootstrap replicates are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

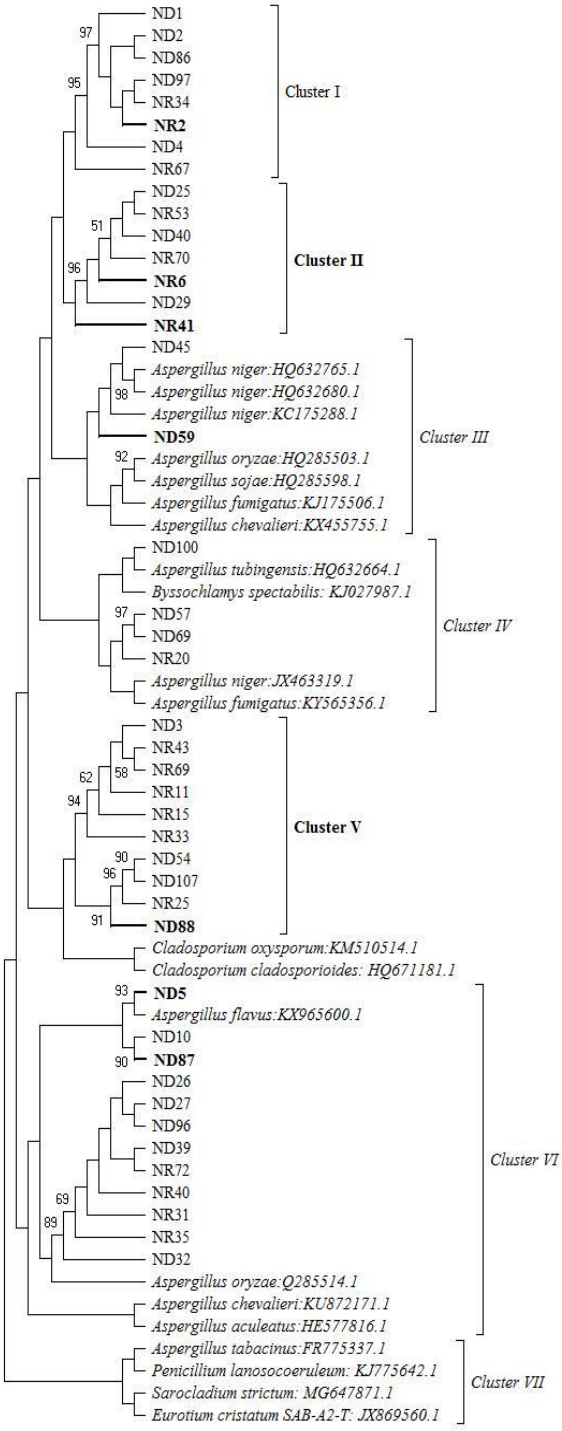

Figure 3.8. β-tubulin (*benA*) gene phylogenetic tree , showing evolutionary relationships of *Aspergillus* isolates and other closely related species from the GenBank. Isolates from the dry season were prefixed ND whereas those from the rainy season NR. The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985). Values greater than 50% bootstrap replicates were

shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

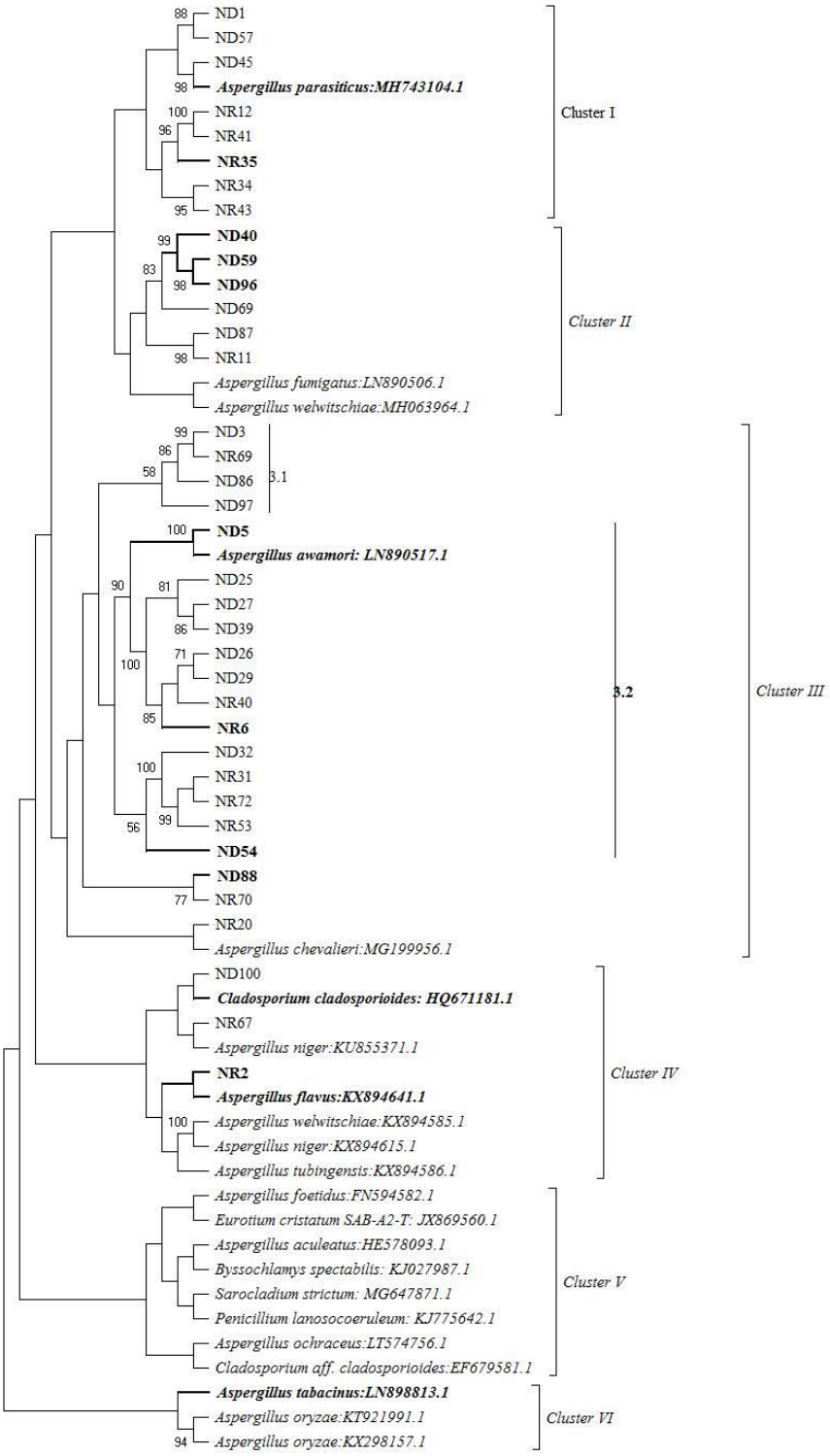

Figure 3.9. Calmodulin (*CaM*) gene phylogenetic tree, showing evolutionary relationships of *Aspergillus* isolates and other closely related species from the GenBank. Isolates from the dry season wereprefixed ND whereas those from the rainy season NR. The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985). Values greater than 50% bootstrap replicates were shown next to the branches. The evolutionary distances were computed using the

Maximum Composite Likelihood method (Tamura *et al.*, 2004) Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

3.3.3 Identification of aflatoxin producing isolates

3.3.3.1 Morphological identification of aflatoxigenic isolates

Ammonia vapour tests

The ability of an isolate to produce aflatoxins after addition of ammonia vapour was shown by production of pink to red colouration on the underside of a colony growing on YES as shown in Figure 3.10.

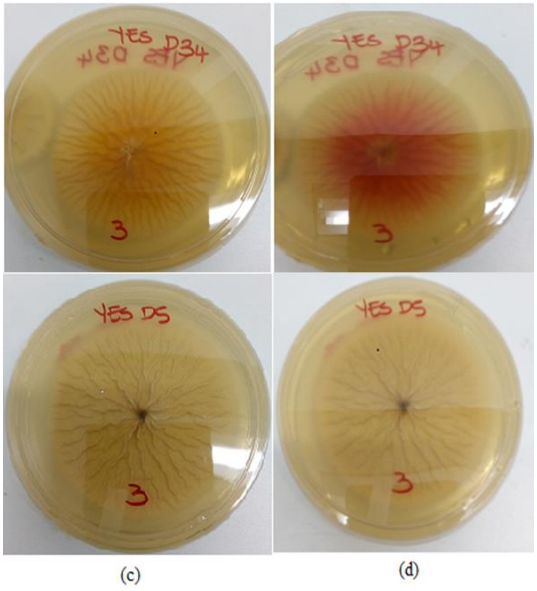

Figure 3.10. Ammonia vapour reaction on YES agar. Showing isolate D34 before (a) and after (b) reaction with ammonia vapour after incubation for 3 days at 28°C. The reddish colouration (b) indicates that isolate is a potential aflatoxin producer, whereas (c) and (d) shows results for isolate D5 before and after addition of ammonia vapour respectively. Absence of colour change indicates that the isolate is a non aflatoxin producer.

Neutral red desiccated coconut agar

Aflatoxin production was confirmed by the presence of a blue/green fluorescence ring at the reverse side of the plate and an orange ring at the observe side under ultraviolet light 365nm as shown in Figure 3.11.

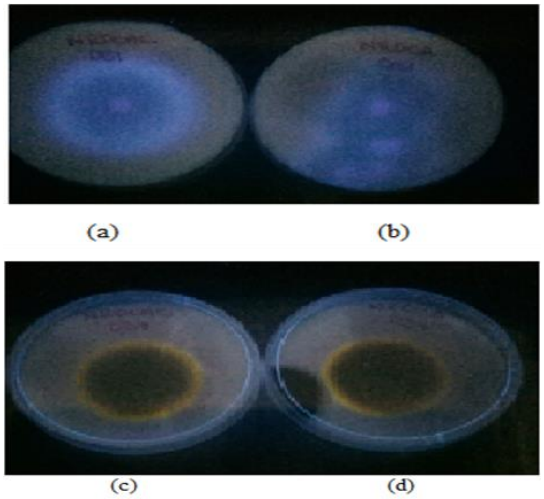

Figure 3.11. Potential aflatoxin producing isolates on β-CNRDCA (a and c) and NRDCA (b and d) at 28°C after 3 days of incubation. β-cyclodextrin enhanced the intensity of the rings as shown in (a and c).

3.3.3.2 Molecular identification of aflatoxigenic isolates

Numbers of isolates showing the presence of the major genes in the aflatoxin biosynthetic pathway expressed as a percentage are shown in Table 3.

Table 3. Distribution of the aflatoxin biosynthetic pathway genes.

Gene	Isolates possessing genes (%)
aflR	14
aflJ	29
aflD	53
aflM	13
aflP	32

Amplification of the five major genes in the aflatoxin biosynthetic pathway namely *aflD, aflM, aflP, aflR* and *aflJ* produced amplicons of the following sizes 400, 600, 797, 1000 and 684 bases respectively as shown in Figures 3.12 – 3.16.

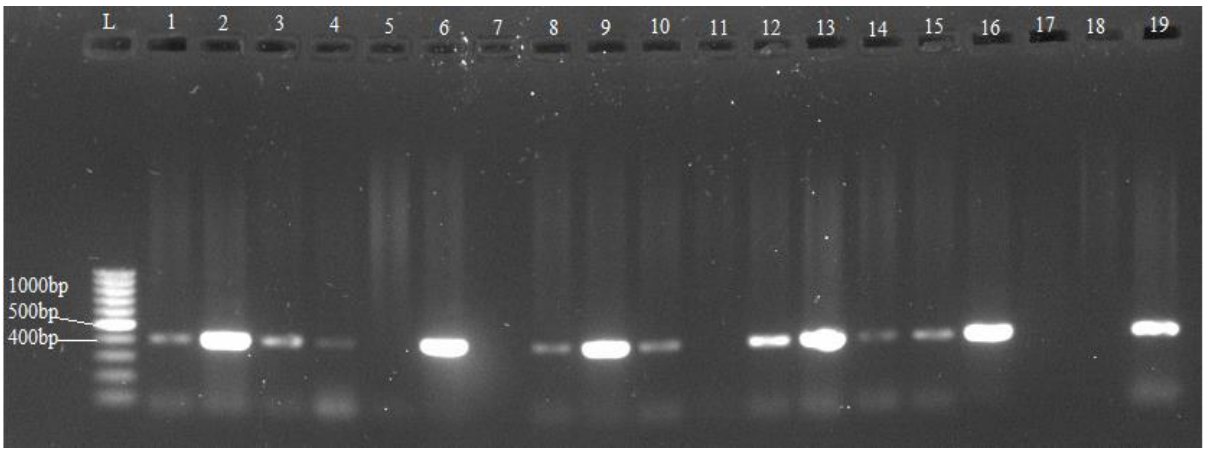

Figure 3.12. Gel electrophoresis of PCR products for *nor* (*aflD*) gene. A band of 400bp is present in isolates that have the gene. L is the 1.5kb ladder whereas numbers 1-19 denotes the isolates NR29, NR31, NR32, NR33, NR34, NR35, NR37, NR38, NR40, NR41, NR43, NR44, NR46, NR47, NR49, NR50, NR51, NR54 and NR57 respectively. NR34, NR37, NR43, NR51 and NR54 showed the absence of the gene.

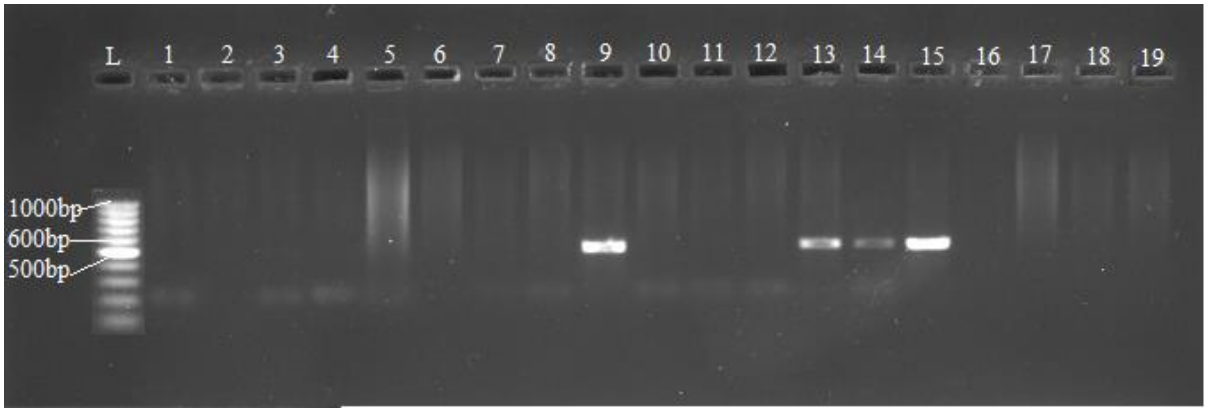

Figure 3.13. Gel electrophoresis of PCR products for *ver* (*aflM*) gene. The presence of the genes in the isolates is shown by a 600bp band. L is the 100bp ladder whereas numbers 1-19 denotes the isolates NR29, NR31, NR32, NR33, NR34, NR35, NR37, NR38, NR40, NR41, NR43, NR44, NR46, NR47, NR49, NR50, NR51, NR54 and NR57 respectively. Only NR40, NR46, NR49 and NR50 showed the presence of the gene.

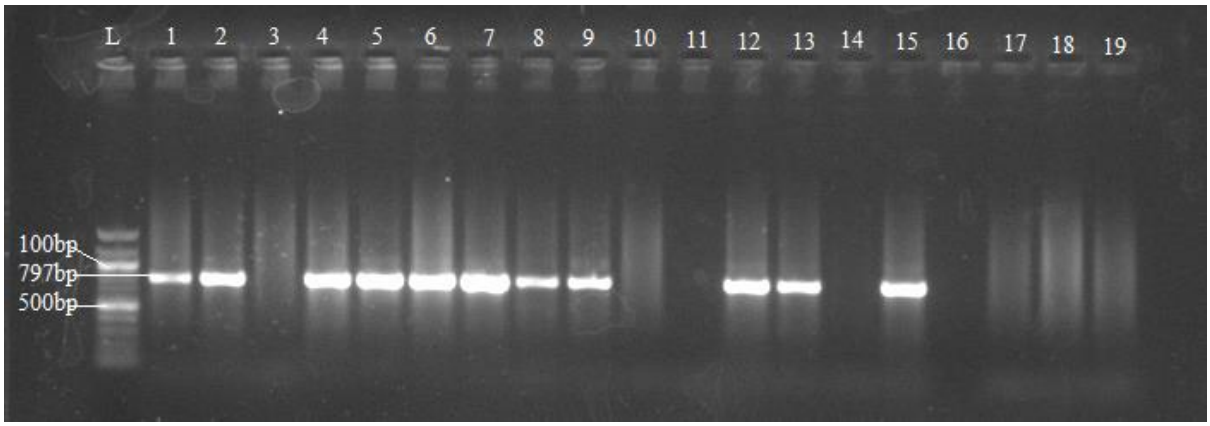

Figure 3.14. Gel electrophoresis of PCR products for *omt* (*aflP*) gene. The presence of the genes in the isolates is shown by a 797bp band. L is the 100bp ladder whereas numbers 1-19 denotes the isolates ND29, ND30, ND31, ND32, ND33, ND34, ND35, ND36, ND37, ND38, ND39, ND40, ND41, ND45, ND51, ND52, ND52, ND55 and ND56 respectively. Isolates ND31, ND38, ND3, ND45, ND52, ND54, ND55 and ND56 showed the absence of the gene.

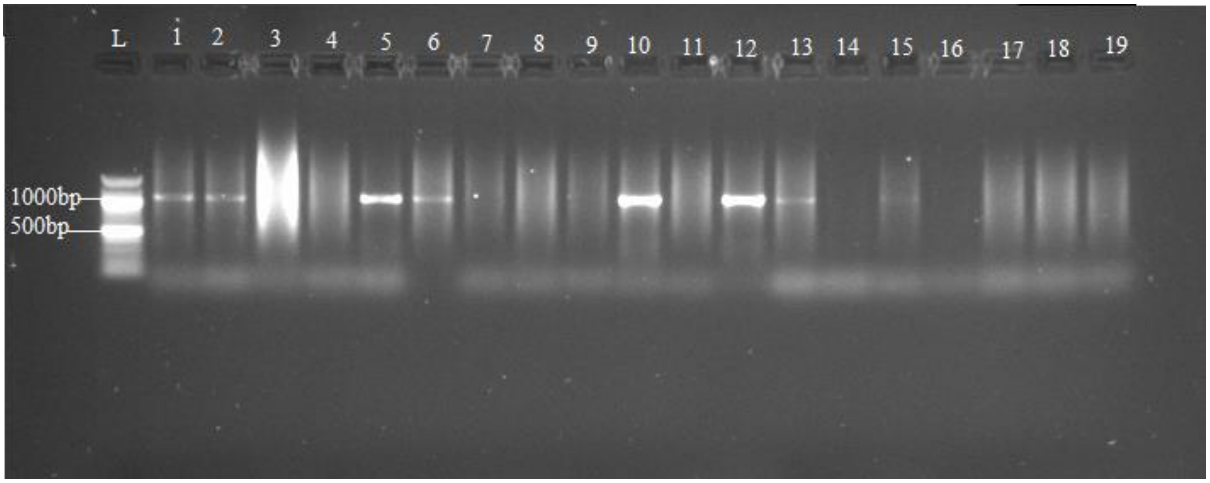

Figure 3.15. Gel electrophoresis of PCR products for *aflR* gene, a regulatory gene in the aflatoxin biosynthetic pathway. The presence of the genes in the isolates is shown by a 1000bp band. L is the 100bp ladder whereas numbers 1-19 denotes the isolates ND29, ND30, ND31, ND32, ND33, ND34, ND35, ND36, ND37, ND38, ND39, ND40, ND41, ND45, ND51, ND52, ND52, ND55 and ND56 respectively. The presence of the bands was shown by isolates ND29, ND30, ND33, ND34, ND38, ND40, ND41 and ND51.

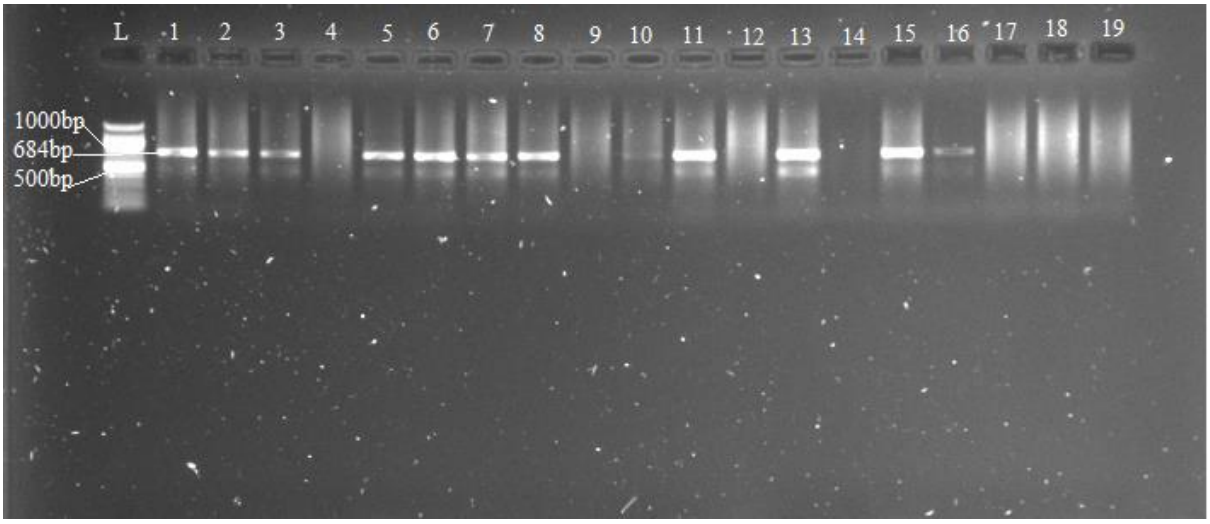

Figure 3.16. Gel electrophoresis of PCR products for *aflJ*, another gene a regulatory gene in the aflatoxin biosynthetic pathway. The presence of the genes in the isolates is shown by a 1000bp band. L is the 100bp ladder whereas numbers 1-19 denotes the isolates ND29, ND30, ND31, ND32, ND33, ND34, ND35, ND36, ND37,

ND38, ND39, ND40, ND41, ND45, ND51, ND52, ND52, ND55 and ND56 respectively. The presence of the bands was shown by isolates ND29, ND30, ND31, ND33, ND34, ND35, ND36, ND38, ND39, ND40, ND41, ND51 and ND52.

3.3.4 Geographical distribution of aflatoxigenic isolates.

A total of 44 isolates were identified as potential aflatoxin producers using both morphological and molecular methods (see Appendix I). Claremount area had the highest incidence of aflatoxigenic isolates and Matobo catchment area had the lowest incidence whereas there was no difference between area 1 and 2 as shown in Figure 3.17. The distribution of the toxigenic isolates in the feeds is shown in figure 3.18 where the highest incidence (45.5%) was in the mixed ration and least in BSG.

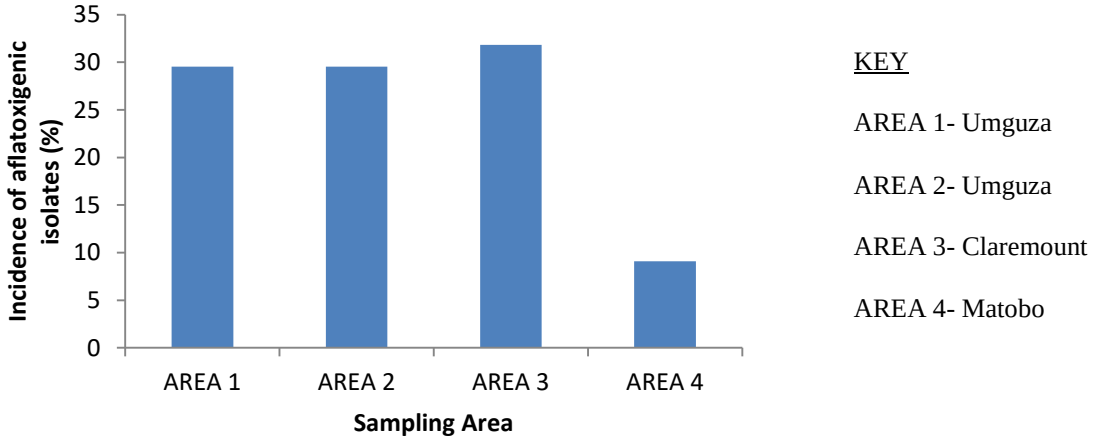

Figure 3.17. Distribution of aflatoxigenic *Aspergillus* species by area.

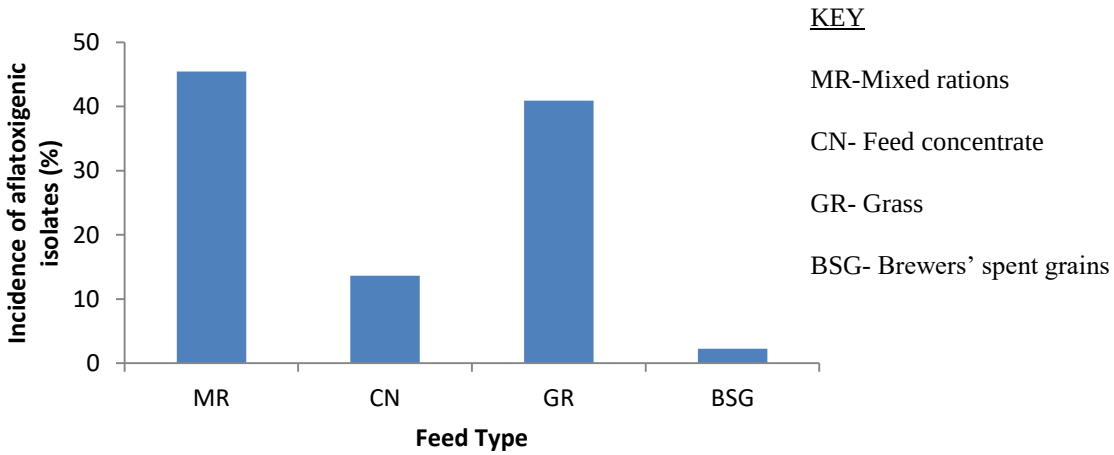

Figure 3.18. Occurence of aflatoxigenic *Aspergillus* strains in the different types of feeds. The mixed ration harboured most of the aflatoxigenic strains and BSG the least.

3.3.5 Phylogenetic analysis of the aflatoxigenic isolates

The aflatoxin producers belonged to the following species; *A. flavus* (61.3%), *A. oryzae* (18.2%), *A. fumigatus* (9.1%), *A. parasiticus* (4.5%), *A. nomius* (4.5%) and *A. parviscelotigenus* (2.3%) (Appendix II). Phylogenetic trees showing the evolutionary relationship of the aflatoxigenic isolates based on the ITS region, β-tubulin and calmodulin genes are shown in Figure 3.19. ITS grouped the isolates into five clusters whereas β-tubulin and calmodulin grouped them into four.

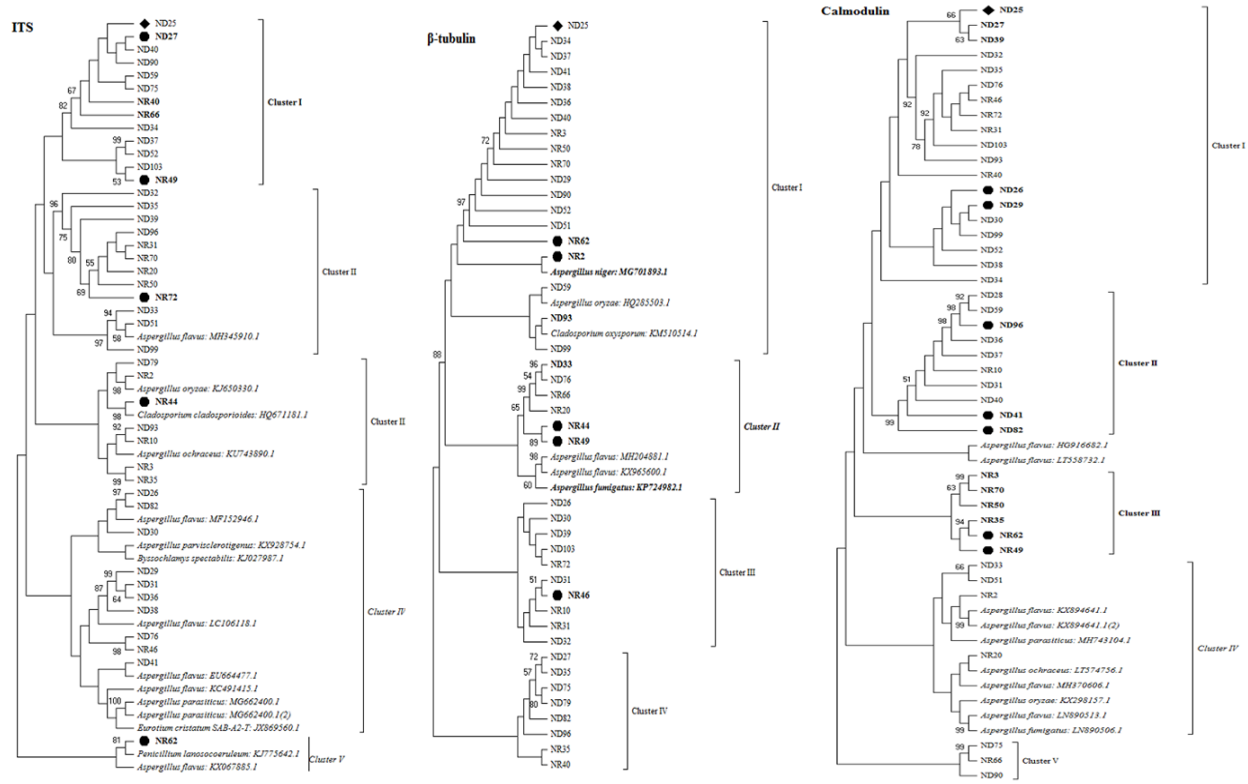

Figure 3.19. Single gene phylogenetic trees showing the evolutionary history of the isolates using the Neighbour-Joining method (Saitou & Nei, 1987) based on (a) the ITS region bootstrap on, (b) β-tubulin and (c) calmodulin genes. Taxa analysis was done using 1000 bootstrap replicates (Felsenstein, 1985). Branches shown had bootstrap replicates 0f 50% and above. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Maximum Composite Likelihood method was used to compute the evolutionary (Tamura et al., 2004). All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Concatenation of the genes was in the order ITS-β-tubulin- calmodulin and the phylogenetic tree obtained grouped the isolates into four clusters. The tree was able to group the isolates

according to seasons of sampling. Isolates from the rainy season were prefixed (NR) and those from the dry season (ND) as shown in Figure 3.20.

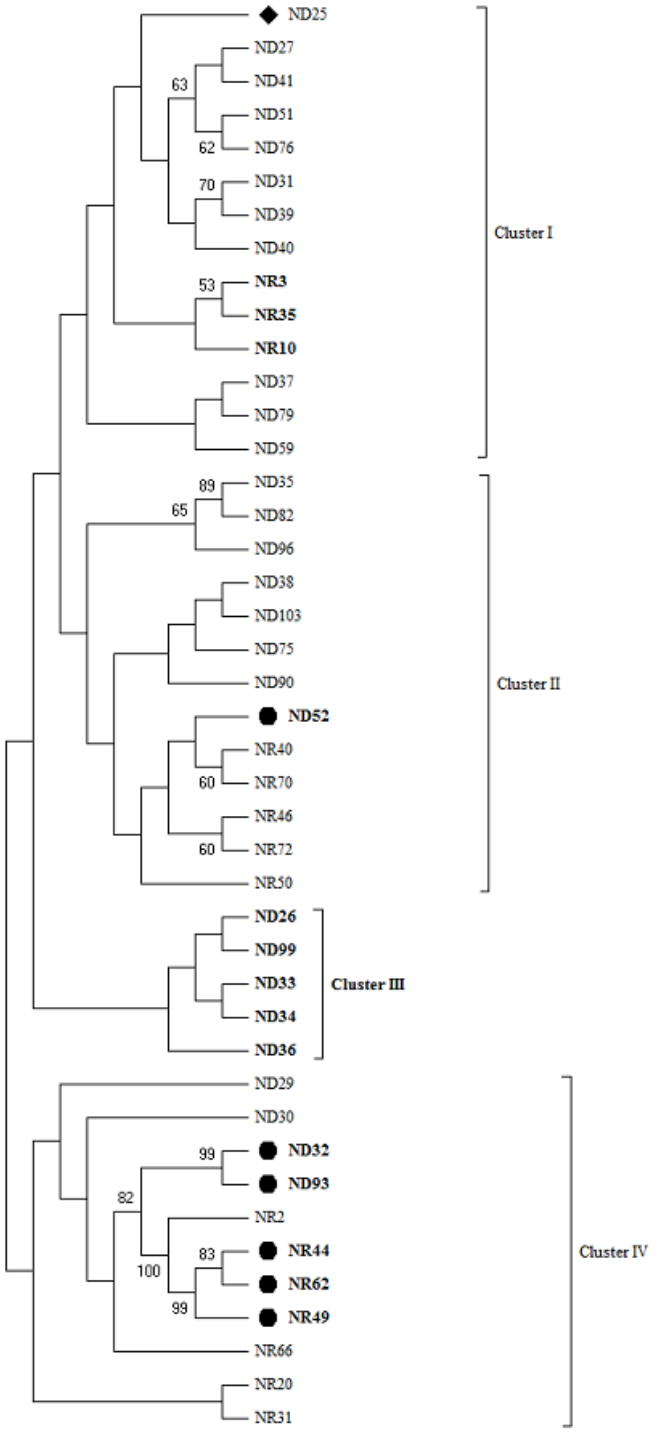

Figure 3.20. Concatenated tree (ITS, β-tubulin and calmodulin) showing the evolutionary relationship of the aflatoxigenic isolates. The evolutionary history of the isolates using the Neighbour-Joining method (Saitou & Nei, 1987). Branches shown had bootstrap replicates 0f 50% and above. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Maximum Composite Likelihood method was used to compute the evolutionary (Tamura et

al., 2004). All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

3.4 Discussion

The strong correlation between presence of moulds in feed and mycotoxin contamination has triggered research on understanding the diversity of *Aspergillus* species in feeds and feedstuffs (Variane *et al.*, 2018). Studies have shown that a wide range of *Aspergillus* species and some outside the *Flavi* section that have the potential to produce aflatoxins from feed and feed ingredients.

In order to understand the diversity of the *Aspergillus* isolates from the feeds, phylogenetic analysis of the ITS region, β-tubulin (*benA*) and calmodulin (*CaM*) genes were carried out. This is because the use of only one marker for evolutionary analysis may not give a true reflection of the history of the whole genome resulting in wrong conclusions. .

The single gene analysis showed that the isolates were clustered into either *Nigri* or *Flavi* sections. However, it could not group section *Fumigati* in their own cluster. The evolutionary tree based on ITS produced 9 clusters which grouped the isolates into either black or green *Aspergilli* (Figure 3.7). In these clusters one or two isolates were wrongly placed for example isolates ND57, NR41 and NR53 were clustered with black *Aspergilli* whereas they had been identified as *A. fumigatus* (ND57 and NR41) and *A. flavus* (NR53) by cultural methods. Isolates ND87, NR6 and NR12 also identified as *A. fumigatus* were grouped in cluster VII which mainly consisted of isolates from section *Flavi*. Another *A. fumigatus* isolate (ND88) and other black isolates, ND5 and ND104 were found in cluster III, which had isolates mainly belonging to *Flavi* section. *Aspergillus oryzae* (NR15) was also wrongly grouped with black *Aspergilli* in cluster IV. Cluster V had isolates from all sections being grouped making it very difficult to understand the evolutionary history of these isolates.

The tree based on β-tubulin gene had only 8 isolates that were wrongly grouped (Figure 3.8). Isolate NR2 (*A. ochraceus*), ND59 and NR41 (*A. flavus*) and NR15 (*A. oryzae*) were clustered with black *Aspergilli*. *A. fumigatus* could not also be clustered on their own as they were either placed in the green or black clusters. Cluster IV could not be defined as it consisted of isolates from all the four sections.

Classification based on calmodulin gene (Figure 3.9) also had an undefined cluster I. Isolates from *Flavi* section ND40, ND59 and ND96 (*A. flavus*, *A. flavus* and *A. oryzae* respectively) were placed in cluster II which was rooted to parent belonging to the black and blue *Aspergilli*.

Also classification of isolates from section *Fumigati* was a challenge as they were clustered with isolates from section *Flavi* in cluster III (NR6 and ND88).

The clustering of the isolates was independent from the area of sampling because some isolates were isolated from farms more than 20 kilometres apart. However, for this could be explained in terms of the type of feed that the isolates came from. For example, isolate NR26, NR11 and NR14 are all black *Aspergilli* in cluster II of the ITS phylogenetic tree (Figure 3.7). All were isolated from feed concentrates but from different farms during the same season. This suggests that the feed ingredients used in the production of the concentrate were a source of the fungi and possibly the farmers had bought their feed from the same batch.

Classification based on ITS could not discriminate the isolates according to their groups as there were several that were placed in the wrong clusters. Initially, the use of the ITS region as a universal bar code for fungal classification was supported by many scientists but later it was found that this marker did not work well for some genera such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma* (Raja *et al.*, 2017) which may explain why the isolates could not be grouped according to their groups effectively. However, for β-tubulin only 8 isolates were misgrouped. This concurred with findings of Krimitzas *et al.* (2013) who were able to group members of *Aspergillus* Nigri section into their respective subgenera and further divided them into clades using β-tubulin gene. However, Hubka and Kolarik (2012) described the discriminatory power of β-tubulin being stronger in grouping members of section *Nigri* and this explained why most of the wrongly grouped isolates were placed in the back *Aspergilli* clusters.

It is well known that not all *Aspergillus* species produce aflatoxins (Mohamed *et al.*, 2017), therefore there was a need to further characterise the isolates based on their aflatoxin producing potential. This was undertaken through morphological, biochemical and molecular methods. The morphological methods were combined with biochemical methods as the media used had components that enhanced production of aflatoxins. For maximum aflatoxin production metabolisable sugar, high nitrogen composition and a temperature of 25°C to 30°C are required (Mohamed *et al.*, 2017). These conditions were met by culturing the isolates at 28°C on YES agar which had high sugar content and yeast extracts. Aflatoxin production by the isolate was then confirmed by use of ammonia vapour which resulted in a pink to red colouration on the underside of the plate as shown in Figure 3.10.

Another medium for aflatoxigenic *Aspergillus* is the desiccated coconut agar (DCA) and aflatoxin producers are identified by fluorescence on the underside of the plate under ultraviolet light (Davis *et al.*, 1987) and yellow pigmentation (Lin & Dianese, 1976). Atanda *et al.* (2011) reported that the white background of DCA sometimes interferes with the fluorescence making it difficult to separate aflatoxin producers from non-producers. He therefore incorporated neutral red dye into the DCA to give neutral red desiccated coconut agar (NRDCA) which would give a pink background making it easy to detect aflatoxin producing fungi and visualise the toxins by the fluorescence they exhibit. Use of enhancing agents such as β- cyclodextrin to improve fluorescence of aflatoxins have been reported (Abbas *et al.*, 2004; Stark, 2009; Degola *et al.*, 2012) in the detection of aflatoxin producing fungi on culture media. For this study NRDCA was supplemented with β- cyclodextrin (β-CNRDCA) and used along with NRDCA (Figure 3.11).

Identification of aflatoxigenic isolates by morphological methods is not reliable as there are chances of producing false negatives and false positives (Sudini *et al.*, 2015) as it has previously been shown that some non-aflatoxigenic species are capable of producing yellow pigmentation and fluorescing under UV light (Stark, 2009; Atanda *et al.*, 2011). Isolate NR17 did show some fluorescence on both NRDCA and β-CNRDCAC but had negative results on YES medium. Molecular analysis of this isolate indicated absence of either of the regulatory genes. So identification of this isolate based on the use of coconut based media would have classified it as an aflatoxin producer whilst it was not. Thus it is important to classify organisms based on combined methods. In this study, molecular methods were also used for identification of aflatoxigenic fungi and five major genes in the aflatoxin biosynthetic pathway namely *aflR*, *aflJ*, *aflD*, *aflM* and *aflP* were used, the first two being regulatory genes (Baranyi *et al.*, 2013) and the rest structural genes (Davari *et al.*, 2015). The roles of these genes are shown in Figure 1.5. Majority of the isolates showed the presence of *aflD* gene (53%) which codes for an enzyme that convert norsolorinic acid, the first stable intermediate in the aflatoxin biosynthetic pathway, to averantin (Cleveland *et al.*, 2009; Abdel-Hadi *et al.*, 2011; Yu, 2012).

The conversion of versicolorin A (VER A) to demethylsterigmatocystin (DMST) and versicolorin B(VER B) to demethyldihydrosterigmatocystin (DMDHST) which is carried out by the product of *aflM* gene (Yu, 2012) occurred in the pathway. The presence of the gene was exhibited by 13% of the isolates. The last important step in the conversion of involves the ability of *aflP* gene to convert sterigmatocystin (ST) to O-methylsterigmatocystin (OMST) and dihydrosterigmatocystin (DHST) to dihydro-O-methylsterigmatocystin (DHOMST). The

absence of the gene always results in the isolate producing strigmatocystin not aflatoxin. This gene was present in 32% of the isolates. The pathway was initially said to be regulated by *aflR* which encodes for transcriptional activators for the structural genes. However, after the discovery of *aflJ* it was noted that *aflR* gene did not solely regulate the biosynthesis of aflatoxins but its activities were affected by *aflJ* (Cleveland *et al.*, 2009). The presence of *aflR* was low in the isolates (14%), however *aflJ* had a much higher percentage (29%) and was detected in some isolates that were negative for *aflR* (Appendix II). This may mean that regulation of aflatoxin production by the isolates used in this study was under the influence of *aflJ*. In the overall determination of the potential aflatoxin production of the isolates the isolates had to possess either of the regulatory genes *aflR* and *aflJ* as well as *aflP* is required for the final synthesis of aflatoxins (Latha *et al.*, 2008).

A total of 44 isolates showed aflatoxin producing potential (see Appendix II) and most of them were isolated from farms under the intensive and semi-intensive feeding farming systems. Thus the sampling area with the least incidence of aflatoxigenic fungi was area 4 (Figures 3.17). The evolutionary analysis of the isolates also showed that the discriminating power of ITS is very low as shown by the phylogenetic tree of the individual genes. The ITS placed isolates belonging to section *Fumigati* (NR62 and NR49) in the *A. flavus* cluster. Isolate ND2 was identified as *A. nomius* through NCBI blast search. On the other hand, the same isolate was placed in the *A. flavus* cluster by the use of other two genes. This shows that even though sequence based approaches in identification of fungi offers better chances of distinguishing closely related species, no DNA marker has exhibited the potential to identify *Aspergillus* species without ambiguity (Nouripour-Sisakht *et al.*, 2017). β-tubulin and calmodulin were able to discriminate *A. fumigatus* from the *A. flavus* and grouped them in their own cluster. The grouping of the isolates was not area specific as we could find isolates from all the four sampling areas in a cluster. This could have been due to the fact that majority of the isolates were from concentrates and mixed rations whose ingredient come from diverse areas thus there were no isolates that could be distinguished by their area of origin. The concatenated tree (Figure 3.20) had more resolving power than the single genes. It managed to place the *A. fumigatus* isolates in their own cluster (NR44, NR49 and NR62). Furthermore the concatenated tree grouped the isolates according to seasons. Previous studies concatenation of genes would group the *Aspergilli* into their specific clades. This study in addition to clustering the isolates into the individual clades was also able to group them based on the time of isolation giving a true reflection of the evolution aspect of the isolates with time. The same tree was also able to group some isolates from farms in the same area 3 under semi intensive farming. The isolates

were from grass, mixed rations and feed concentrate suggesting the possibility of escape of spores into the atmosphere from the formulated feed and eventually landing on the grass. However, in all the four trees, isolate ND25 was distinguished from the other isolates because it was a non-toxigenic strain.

Section *Flavi* had an occurrence frequency of 33% across the feeds. This is a cause for concern as this section is well known for its aflatoxin producing species *A. flavus* (Rodrigues *et al.*, 2007). *Aspergillus flavus* constituted 72% of the isolates in this section and 64% of these isolates showed the potential to produce aflatoxins using both cultural and molecular methods. *Aspergillus oryzae* has not been associated with aflatoxin production and had been used in the production of fermented foods (Chang *et al.*, 2006). However, 6 out of 9 *A. oryzae* isolates NR35, NR66, ND29, ND35, ND39 and ND96 exhibited aflatoxin production ability whilst two were identified as potential producers (NR57 and NR70) whereas only one isolate, NR15 as a non aflatoxin producer (Appendix II). This becomes a threat to the food industry if species normally used for food production are also producing aflatoxins. *A. fumigatus* are not also known for aflatoxin production, they are often associated with aspergillosis in humans and animals. Nonetheless, in this study, five *A. fumigatus* isolates showed potential to produce aflatoxins (NR17, NR44, NR49, NR51 and NR62) . El-Naghy *et al.* (1991) also reported isolation of an *A. fumigatus* isolates which exhibited aflatoxin production potential from cotton seeds in Egypt.

3.5 Conclusion

Findings from this study showed that feeds used for feeding dairy cows by farmers in peri-urban Bulawayo were contaminated by *Aspergillus* species with the potential of producing aflatoxins. The most contaminated feed was the mixed rations and is usually made by mixing mainly waste products of oil extraction from seeds. Cotton seed cake was the main waste product used by most farmers for the mixed ration. In Zimbabwe, regulatory limits have only been set for foods intended for human consumption and poultry feeds without regards for animal feeds. Farmers and feed producers as well as policy makers in Zimbabwe should come up with strategies for reducing contamination in feeds. This could be done through strict monitoring of ingredients used for feed formulation and by avoiding the use of contaminated feedstuffs for formulating mixed rations.

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Chapter 4

Determination of aflatoxins in feeds from dairy farms

Abstract

The study aimed at assessing the level of aflatoxin contamination in feeds used by Bulawayo peri-urban farmers to feed their dairy cows. A total of 96 feeds from 13 farms were collected and analysed. The study showed that 57% of the farmers were practising semi-intensive farming, followed by extensive farming (36%) and 14% intensive farming. The farmers also supplemented their animal feeds with mixed rations, concentrated feed, grass and brewers spent grains. Mixed ration was the most commonly used feed by the farmers. Analysis of the feeds by high performance liquid chromatography (HPLC) showed the presence of all the four naturally occurring aflatoxins namely aflatoxin B_1, B_2, G_1 and G_2. Total aflatoxin concentration of the feeds ranged from 0-250.9µg/kg. The mixed rations had the highest aflatoxin concentration of 29.0µg/kg. Aflatoxin B_1, the most potent aflatoxin was the predominant aflatoxin across all feeds with an average concentration of 9.0µg/kg and highest concentration of 149.6µg/kg in mixed rations. This was also above the EU limit of 5.0µg/kg for lactating cows. The responses from farm workers to the questionnaire on aflatoxins showed that most of them do not have basic knowledge of aflatoxins. Therefore there is need to educate farmers, personnel and the general public on the importance of aflatoxin prevention and monitoring in human foods and livestock feeds.

4.1 Introduction

Animal feeds are at risk of mycotoxin contamination as moulds are ubiquitous and they can contaminate food and feedstuffs ingredients pre-harvest, during harvest and post-harvest (Sultana & Hanif, 2009; Bryden, 2012; Peng *et al.*, 2018). The sources of the individual components used in the formulation of dairy feeds are quite diverse ranging from cereals, cereal products, oil seeds as well as hay and forages (Fink-Gremmels, 2008a; Sultana & Hanif, 2009). Also the high cost of feed has led to the addition of stale bread, kitchen and bakery wastes to the feed. Furthermore scarcity of protein sources for animal feeds has led to the use of alternative protein sources such as brewers spent grains (BSG), (Li *et al.*, 2014). These waste products are usually tainted with fungus and may be a contributing factor in mycotoxins production in cattle feed. The presence of aflatoxins in animal feedstuffs becomes a potential health hazard to both animals and humans (Danesh Mesgaran *et al.*, 2013) due to their toxic nature. The toxic effects of aflatoxins in ruminants include liver damage, diminished growth efficiency, diminished milk production and quality and impaired resistance to infectious diseases (Queiroz *et al.*, 2012; Bbosa *et al.*, 2013b; Danesh Mesgaran *et al.*, 2013).

In dairy farming, depending on the farming system adopted, the diet consists of concentrates, alternative protein sources as well as forage hence the animals are exposed to more than one type of mycotoxins (Fink-Gremmels, 2008). Although there are more than 20 aflatoxins known, only four of these occur naturally namely; aflatoxin (AF) B_1, B_2, G_1 and G_2 based on their fluorescence under UV light (blue or green) (Baranyi *et al.*, 2013; Arapcheska *et al.*, 2015; Reid *et al.*, 2016). The most abundant aflatoxin in cow feeds and rations is aflatoxin B_1 and is also the most potent of them all (Tajkarimi *et al.*, 2011; Bräse *et al.*, 2013).

Animals differ in their sensitivity to mycotoxin toxicity (Denli, 2015) with ruminants being more resistant as compared to the monogastrics (Jouany *et al.*, 2009) mainly because they have microorganisms in their rumen which play significant roles in the deactivation and degradation of the aflatoxins as well as alteration of the binding of the aflatoxins to some essential nutrients (Afsah-Hejri *et al.*, 2013; Gallo *et al.*, 2015). However, aflatoxins are poorly degraded by ruminants as most of the rumen microbiota are inhibited by AFB_1 concentration of 10µg/ml ((Jouany *et al.*, 2009). The aflatoxins will get to the bioconversion sites of nutrients and xenobiotics like the intestinal epithelium, liver and kidneys unaltered (Jouany *et al.*, 2009). In the liver, AFB_1 is bio-transformed to AFM_1 which enters the circulatory system or is conjugated to glucuronic acid. The conjugated AFM_1 is excreted through the biliary system and the one in circulation may be excreted in urine and milk. It has been shown that AFM_1

retains some carcinogenic activity resulting in its reclassification by IARC as a group 1 carcinogen (Janković *et al.*, 2009; Ketney *et al.*, 2014; Sarica *et al.*, 2015). Consumption of AFB_1 contaminated feed by lactating cows result in its being metabolised to AFM_1 which is subsequently secreted into milk thereby making milk a source of aflatoxin contamination in humans. In this study the extent of aflatoxin contamination of feeds used in different feeding systems adopted by dairy farmers was assessed.

4.1.1Aim

The aim of the study was to detect and quantify aflatoxins in feeds used by dairy farmers in Bulawayo southern of Zimbabwe.

4.1.2 Objective

To quantify the level of aflatoxin contamination of feeds used by dairy farmers by different analytical methods

4.2 Methodology

4.2.1 Data collection

Snowball convenience sampling method was used to identify farmers to participate in the research. This involved the identification of farmers who had the desired breed of cows who

then gave information on farmers in the area with the same breed of cows. Identified participants were approached and those willing to participate were recruited (Naderifar *et al*., 2017; Etikan *et al*., 2016). Questionnaires were used to get information from the farmers. The information required from the farmers included the following; plot size (acres), number of cattle owned by the farmers, number of cows that were being milked, age, breed, lactation stage, milking method, volume of milk produced on the farm per day, volume of milk produced by each cow per day, number of milking per day, amount of feed given to each cow per day and also knowledge of aflatoxins (Appendix III).

4.2.2 Sample collection

A total of 96 feed samples were collected which consisted of dairy feed concentrates (CN), mixed ration (MR), brewers spent grain (BSG) and grass (GR) were collected from 13 farms during the dry season (August- October 2016) and the rainy season (January-March 2017). Collection of the samples was done in sterile polythene zip lock bags after thoroughly mixing the feed portion to be given to the cows on that particular day (Kang'ethe & Lang'a, 2009) and transported in cooler boxes to the lab where they were ground to a fine powder using IKA® M20 universal batch mill (Germany) and stored in the freezer at -20°C until time for analysis (Beukovic *et al*., 2015).4.2.3 Sample preparation for HPLC analysis

Aflatoxins from feeds were extracted using the immune-affinity extraction method using Easi-Extract® aflatoxin immunoaffinity columns (R-Biopharm Rhone Limited, Glasglow G20 OXA, Scotland). Extraction was carried out according to the manufacture's protocol with some modifications as follows; a portion of 50g of the sample was mixed with 5g of sodium chloride (NaCl) in a laboratory blender followed by 100ml of methanol: water (80:20 v/v) and blended for 5 min. The mixture was filtered through a fluted filter paper (Whatman No.1) into a clean vessel. A volume of 2ml of the filtrate was then diluted with 14ml phosphate buffer saline (PBS) solution and passed through an immune-affinity column. The column was washed with 20ml of PBS and the aflatoxins finally eluted with 1ml methanol (LiChrosolv®, Merk, Germany) into a glass cuvette and diluted with 1ml of distilled water and stored at -20°C prior to analysis. Aflatoxins B_1, B_2, G_1 and G_2 standards (Trilogy Analytical Laboratory, Washington, USA) were diluted using acetonitrile (LiChrosolv®, Merk, Germany) to give the following concentrations; 5×10^{-6}, 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}mg/ml. Aflatoxin detection and quantification was done using HPLC (Shimadzu FCV-20H2) with operation conditions as given in the KOBRA® cell instruction manual as follows; derivatisation using KOBRA ® cell at 100µA setting, with an analytical column Intersil ODS-3V 5µm,4.6mm×150mm equipped

with a C18 4 × 3 mm^2 i.d. security guard cartridge (Phenomenex, Torrance, CA, U.S.A.)., Mobile phase was modified from the recommended water: methanol (60:40) to a working condition of (55:45) with 119mg/litre of potassium bromide (KBr) and 1ml/litre of 65% nitric acid added at a flow rate of 1.0ml/minute, Fluorescence detector set at 362nm for excitation and emission 425nm(AFB_1 and B_2) and 455nm (AFG_1 and G_2). Injector was an auto sampler which injected 100µl of sample and elution of the aflatoxins was in the order (AF) G_2, G_1, B_2 and B_1.

Calibration curves for each aflatoxin, AF (B_1), B_2, G_1 and G_2 were constructed using standard solutions which were diluted with acetonitrile to give the following concentrations; 0.005, 0.05, 0.5, 5, 50µg/kg. The limit of detection for all the standards was 0.005µg/kg. The linearity of the standard curves was determined using correlation regression (r^2). A curve with good linearity will have an r^2 value close to 1. Aflatoxin concentration of the samples was calculated by measuring the area of the peak and then interpolating from the standard curve.

4.3 Results

4.3.1 Farmer Survey

A total of 14 farmers participated in this study. Responses of the farmers (Appendix III) showed that their average farm sizes were over 8.5 hectares and most of them were milking cows ranging between 20 and 250 per day and a few had less than 10 cows. The cows that were being milked were 25 months old and above and the common breeds were the Jersey, Holstein and crossbreed (Holstein /Jersey) across all milking stages. Majority of the farmers milked by hand and got a volume of 100 to over 200liters of milk per farm per day with each cow giving an average of 6-10 litres. Most of the farmers who took part in the study were practising semi-intensive, extensive and intensive farming (Figure 4.1).

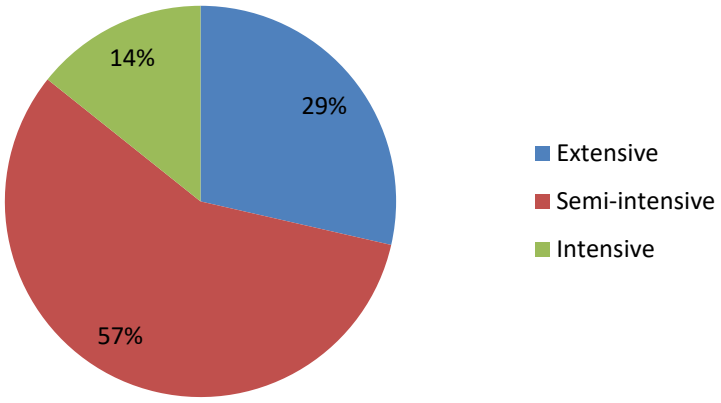

Figure 4.1. Farming systems adopted by dairy farmers in Bulawayo peri-urban, showing that most the farmers practise semi-intensive farming.

The cows were mainly fed with concentrates, mixed rations, brewers' spent grain and grass ranging from 6-10kg per animal per day. Only 36% of the farmers had some knowledge of aflatoxins (see Appendix III). The most utilised feed was mixed-ration as shown by Figure 4.2.

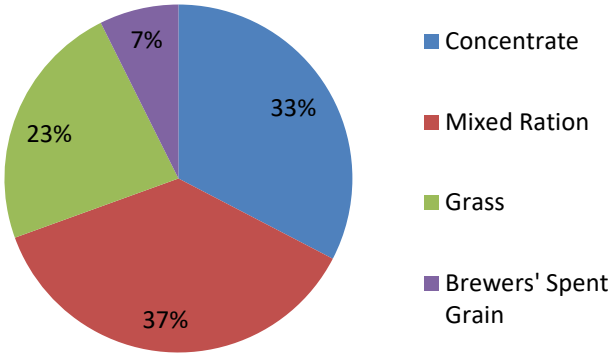

Figure 4.2. Percentage utilisation of feed types by dairy farmers in peri-urban Bulawayo showing that the most common feed used by the farmers is the mixed ration.

4.3.2 Analysis of aflatoxins

HPLC analysis of aflatoxins showed the presence of all the major types of aflatoxins AF; (B_1), B_2, G_1 and G_2 and this is indicated by the peaks in the chromatograms as shown in Figure 4.3. The calibration curves had good linearity for the different aflatoxins with r^2 values of 1 (Appendix IV). Total aflatoxin concentration in the feeds ranged from 0- 250.9µg/kg. The individual and total aflatoxin concentrations of the samples are shown in (Appendix V)

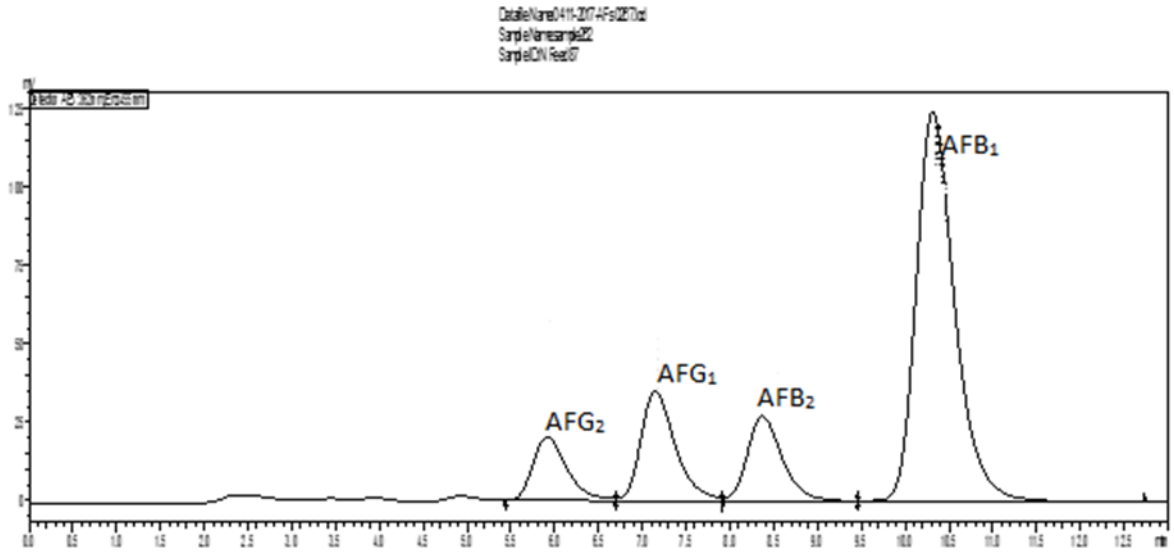

Figure 4.3. Representative chromatogram showing peaks of major aflatoxins in animal feeds.

4.3.2 Aflatoxin distribution in feeds

The mixed rations had the highest total AF concentrations with an average concentration of 29.8µg/kg and grass had the lowest concentrations as shown in Figure 4.4. One way analysis

of variance (ANOVA) (Table 4.) gave a p value of 0.043, meaning that at 95% confidence level ($p < 0.05$) there was enough evidence to conclude that there was a significant difference in the total mean concentration of aflatoxins across the feeds. However looking at MR and CN (Table 5.), $p = 0.766$ there was no significant difference in the total aflatoxin concentrations.

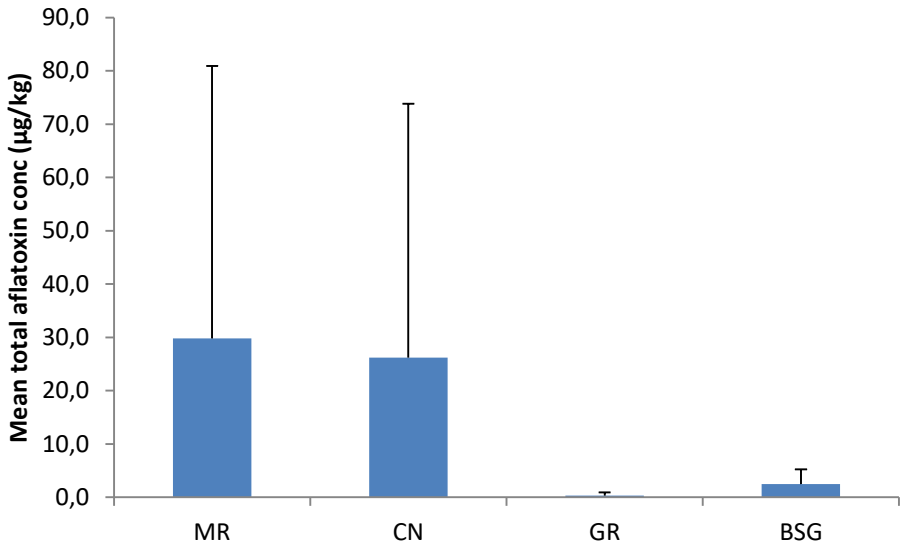

Figure 4.4. Average total aflatoxin concentrations in the feeds. Mixed ration had the highest contamination

Table 4. One way ANOVA for all feed types.

ANOVA					
Total AF conc (ug/kg)					
	Sum of Squares	**df**	**Mean Square**	**F**	**p value**
Between Groups	14860.674	3	4953.558	2.832	**0.043**
Within Groups	159185.082	91	1749.287		
Total	174045.756	94			

A p value < 0.05 indicated that there was a significant difference in the levels of aflatoxin contamination of the different types of feeds used for feeding the dairy cows.

Table 5. One way ANOVA between the mixed ration and feed concentrates.

ANOVA

Total AF conc (µg/kg)					
	Sum of Squares	**df**	**Mean Square**	**F**	**p value**
Between Groups	218.928	1	218.928	0.089	**0.766**
Within Groups	159133.265	65	2448.204		
Total	159352.193	66			

A *p* value > 0.05 indicated that there was no significant difference in the level of aflatoxin contamination

The distribution of aflatoxins in the feeds showed that AFB_1 was the most common aflatoxin across the feeds (Figure 4.5). However there were variations with individual feeds as shown in Figure 4.6 (a-d).

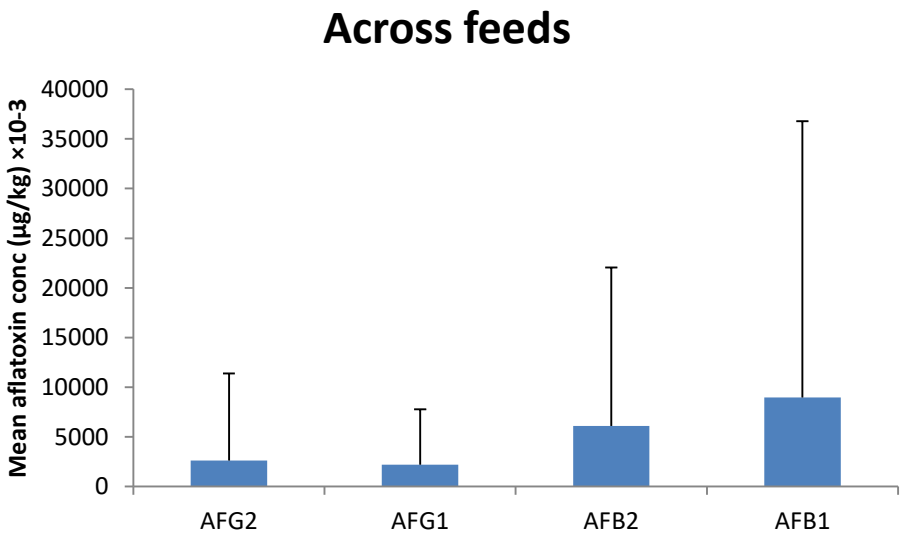

Figure 4.5. Distribution of aflatoxins across all feed types.

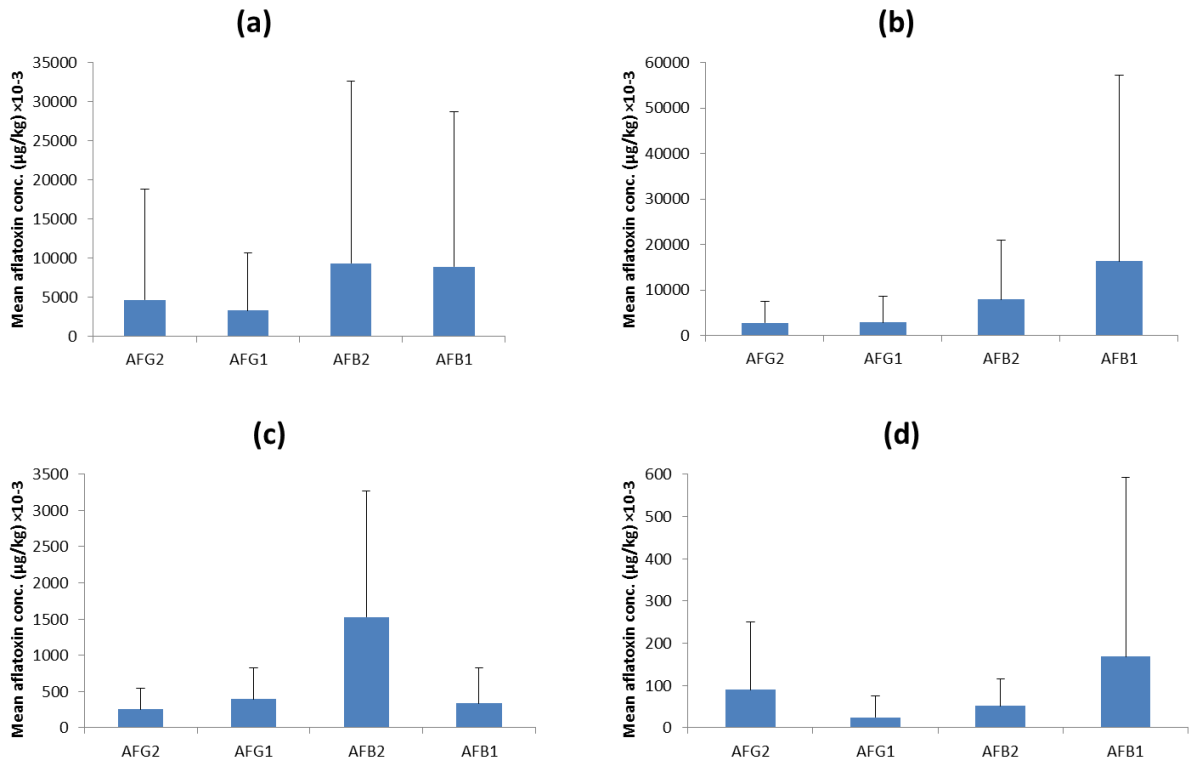

Figure 4.6. Distribution of aflatoxins in the feeds , (a) feed concentrates, (b) mixed rations, (c) brewers' spent grains and (d) grass. AFB_1 was the dominant aflatoxin in mixed rations and grass whereas for concentrates and brewers' spent grains AFB_2 was the predominating aflatoxin.

Looking at the distribution of total aflatoxins across the different farming systems, Figure 4.7 shows that the semi intensive system had the highest aflatoxin concentration with an average of 21.6µg/kg. One way ANOVA (Table 6) however indicated that there was no significant difference in the mean aflatoxin concentration of the feeds from semi-intensive and intensive farming systems as p= 0.937 which was greater than p value of 0.05 at 95% confidence level.

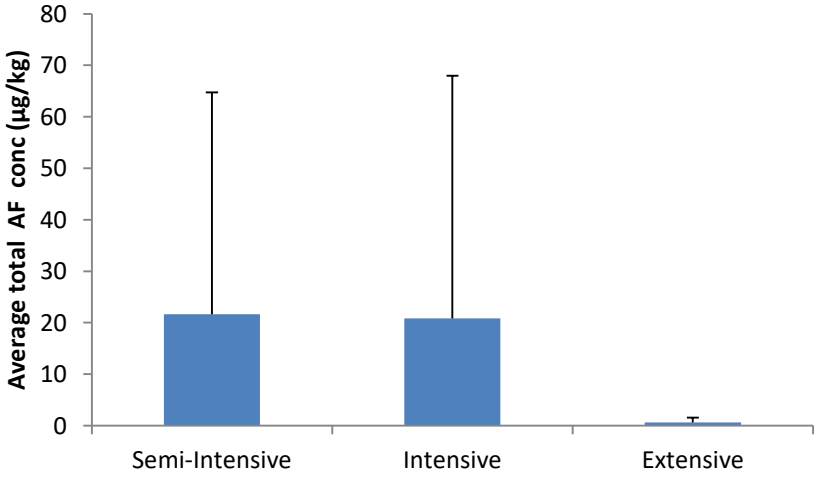

Figure 4.7 Distribution of aflatoxins across the farming systems in Bulawayo showing that the semi-intensive farming has more aflatoxin concentrations in their feeds.

Table 6. One way ANOVA results comparing the semi-intensive and intensive farming systems.
A *p* value >0.05 indicated that there was no significant difference in the level of aflatoxin concentration.

ANOVA					
Total AF conc (µg/kg)					
	Sum of Squares	**df**	**Mean Square**	**F**	**p value**
Between Groups	12.581	1	12.581	0.006	**0.937**
Within Groups	171580.359	87	1972.188		
Total	171592.940	88			

Distribution of AFB_1 in the feeds in dry and rainy season is shown in Figure 4.8 and ANOVA analysis showed that there was a significant difference in AFB_1 concentrations in the different seasons (Table 7.).

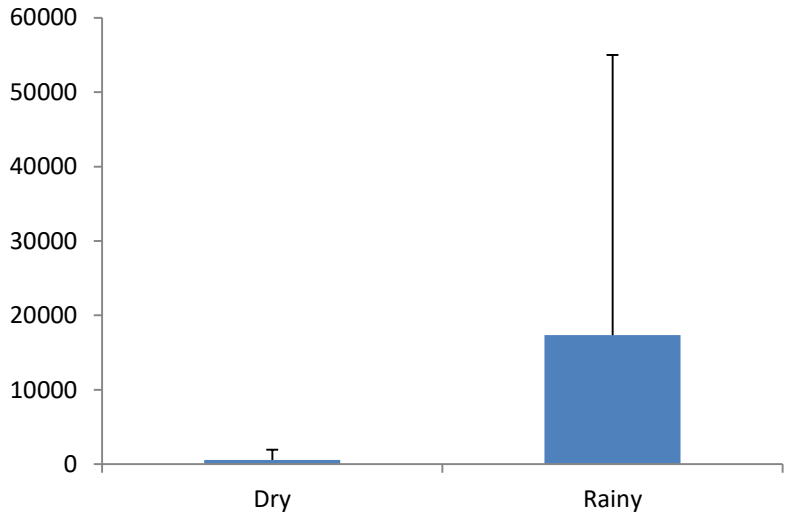

Figure 4.8 Seasonal variation in the distribution of AFB_1. Samples from the rainy season had higher concentration of AFB_1 as compared to dry season samples.

Table 7. One way ANOVA results for dry season and the rainy season.

ANOVA					
AFB_1 conc (µg/kg) $\times 10^{-3}$					
	Sum of Squares	**df**	**Mean Square**	**F**	**p value**
Between Groups	6747185610.100	1	6747185610.100	9.500	**0.003**
Within Groups	66758340020.045	94	710195106.596		
Total	73505525630.144	95			

A *p* value <0.05 indicated that there was a significant difference in the level of AFB_1 contamination of feeds during the rainy season.

4.4 Discussion

Feed quality is of great importance in animal husbandry as it affects both animal health and productivity (Raju *et al.*, 2016). Consumption of aflatoxin contaminated feeds by dairy cows

may result in the aflatoxins occuring in milk posing health risks to humans (Afsah-Hejri *et al.*, 2013). Studies have shown that some feedstuffs used in formulating animal feeds can become infected by aflatoxin producing fungi (Gizachew *et al.*, 2016). Worldwide researchers have been analysing dairy feed for aflatoxin contaminaion and have reported various findings with most feeds exceeding the regulatory limits (Sumantri *et al.*, 2012; Ismail *et al.*, 2017; Kang'ethe *et al.*, 2017; Changwa *et al.*, 2018).

This study also showed that 96% of feeds used in feeding dairy cows in peri-urban Bulawayo were contaminated with at least one of the naturally occurring aflatoxins. The results also indicated that 21% of the samples analysed had total aflatoxin levels above the regulatory limit of 20µg/kg set by international governing for animal feeds. This concurs with the findings of Reddy and Salleh (2010) who reported that 22.5% of wheat and barley samplesthey analysed had aflatoxin concentartions above this regulatory limit. Zimbabwe reviewed the AFB_1 regulatory limit to 20µg/kg in 1990 (Siwela & Nziramasanga, 1999) for food intended for human consumption. However, there is no regulatory limits for animal feeds (Mazumder & Sasmal, 2001).

The feeds that are used in feeding dairy cows by farmers in peri-urban Bulawayo included feed concentrates, mixed rations, grass and brewer's spent grains. This is in accordance with the requirements of the diets of dairy cows which should consist of components that provide protein and energy and also roughages (Driehuis *et al.*, 2008). In this study the protein and energy was supplied by the concentrates, mixed ration and the brewer's spent grains whereas the roughage was provided by the hay stored at the farm or fresh grass in the grazing land.

Mixed rations are considered a whole meal for the cow as they contain basically all the nutriens that are found in forages and concentrates. Formulation of a mixed ration involves combining forages, by-products of other processes such as whole cottonseed or cottonseed cake, grains, protein source, minerals and vitamins (Amaral-Phillips *et al.*, 2002). The mixed rations from this study had the highest aflatoxin conccentrations and corroborated the work of Mozafari *et al*. (2017) with an average of 29.0µg/kg. ANOVA also showed that at 95% confidence level there was a significant difference in the mean aflatoxin concentration of the feeds. The diversity of the components used could have been potential sources of aflatoxigenic fungi which resulted in contamination of the feed types with aflatoxins. Gizachew *et al.* (2016) also reported high aflatoxin concentration in noug cake, a product of oil processing industry used in feeding

dairy cows. Cotton seed was the most utilised feed ingredient for mixed rations by the farmers who participated in this study. However, Chohan *et al.* (2016) reported that feed concentrates had the highest aflatoxin concentration followed by mixed rations in their study on aflatoxin contamnation of diferrent feeds and feed ingredients used to feed dairy cows in Pakistan.

Grass samples had the least aflatoxin concentrations with an average total aflatoxin concentration of 2.5µg/kg and 169 $\times 10^{-3}$ µg/kg of AFB_1. This corroborates the findings of Gizachew *et al.* (2016) who also reported that grass had the least contaminated feed. However, the minimum AFB_1 concentration of 7µg/kg reported by them was higher than the value of 169 $\times 10^{-3}$ µg/kg reported in this s study. Sassahara *et al.* (2005) analysed feedstuffs supplied to dairy cows in North of Paraná state, Brazil and did not detect any aflatoxins in the silage samples. Driehuis *et al.* (2008) also reported the absence of aflatoxins in silage samples used to feed dairy cows in Netherlands. These findings suggest that grass in the form of silage or pastures is not necessarily prone to fungal infections which may result in aflatoxin production. In this study however most of the aflatoxigenic strains were isolated from the grass but it was the feed with the least aflatoxin concentration. Gonzalez Pereyra *et al.* (2011) highlited that the presence of aflatoxigenic fungi on a subatrate does not mean that the toxin is present in that particular food/feed matrix, but there is a risk of toxin production if the environmental conditions become favourable for aflatoxin producton. Nonetheless, detection of aflatoxins in a sample means the substrate has been contaminated by toxigenic species which could either be present or absent at the time of sampling. This was the case wih the feed concentrates which had aflatoxin concentrations higher than the grass samples but fewer toxigenic strains.

The most dominant aflatoxin across all feeds was AFB_1 with an average concentration of 9.0µg/kg and was detected in all the samples that tested positive for aflatoxin contamination. This is above the EU 5µg/kg set for lactating cows. Udom *et al.* (2012) and Gizachew *et al.* (2016) also reported AFB_1 concentrations of 8.75µg/kg and 7-419 µg/kg in the cattle feed concentrates and dairy feed samples respectively which exceeded the EU regulatory limit. The high levels of AFB_1 in most samples could be attributed to the fact that it was the most common and prevalent aflatoxin in most food matrices (Wacoo *et al.*, 2014; Patel *et al.*, 2015). Moreover, some authors have indicated that most toxigenic *Aspergillus* strains produce AFB_1 therefore it occurs more frequently compared to other aflatoxins (Baranyi *et al.*, 2013; Gherbawy *et al.*, 2015; Bellio *et al.*, 2016). Hernández-Martínez and Navarro-Blasco (2015) reported that AFB_1 was predominant in the rainy season which was also the case with findings

from this study (Figure 4.8). These results are also in agreement with the findings by Chohan *et al.* (2016) who also reported high concentrations of AFB_1 during the rainy season. For aflatoxin production, high temperatures and high humidity is required and these conditions prevail during the rainy season.

However, for brewers spent grains (BSG), AFB_2 was the prdominant aflatoxin. The BSG are a product of beer brewing industry (Aghabeigi *et al.*, 2013) and has been found to be of valuable use in the feedstock industry mainly because it is affordable and available throughout the year (Mussatto, 2014). The brewers' spent grains used in this study were from opaque beer. The presence of aflatoxins in beer production has been associated with contaminated malt. Malt production inolves inceasing the moisture content of the grains to allow partal germination of the grains. Aflatoxigenic fungi are known to contaminate cereal grains which are also used in the beer production processes (Tangni & Larondelle, 2002). If the malt is not properly dried and stored, fungal growth may be promoted resulting in the producton of aflatoxins. Previous report on the fate of mycotoxins during beer fermentation process showed that recovery of AFB_2 in BSG was higher as compared to other aflatoxins (Inoue *et al.*, 2013). Deepak *et al.* (2015) showed that AFB_2 is able to adsorb onto yeast cells during fermentation. The yeast cells and the grain particles that are removed through filtration and are collectively known as brewers' spent grains. This could be the possible reason why AFB_2 levels were higher in BSG samples. However, Gonzalez Pereyra *et al.* (2011) did not detect any AFB_2 in barley malt and brewers' spent grains from Argentina breweries. Aflatoxin B_1 has been reported as the most common aflatoxin occurring naturally in feedtuffs, but for this study it was not the case for BSG as the concentration of AFB_2 was higher than that of AFB_1.

This study also showed that brewers' spent grains a known source of nitrogen and roughage and grass had aflatoxin contamination within the regulatory limits making them safer when compared to the concentrates and mixed ration. However nutritional composition of the grass will not meet the dietary demands of the cows.

4.5 Conclusion

Detection of aflatoxins in the feed samples used for this study is a source of concern as indicates the possibility of transfer into the milk by dairy cows. Although the concentration of most samples were within the acceptable limit for total aflatoxins, it was noted that concentrations of AFB_1 the most potent of them were above the regulatory limits. Moreover, t

AFB_1 can be carried over into milk as its hydroxylated metabolite, AFM_1 making milk a route through which humans are exposed to aflatoxins. High prevalence of AFB_1 during the rainy season could be an indication of poor storage of the feeds which may result in increased moisture content resulting in ploriferation of aflatoxin producing *Aspergillus*. Therefore there is a need to educate the farmers and their personnel on the importance of proper feed storage facilities in order to control contamintion of the feeds.

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Chapter 5

Occurrence of aflatoxin M_1 in milk and urine of dairy cows under different feeding systems in from farms around Bulawayo farms, Zimbabwe.

Abstract

The aim of this study was to assess the level of aflatoxin M_1 contamination of milk and urine samples from cows under three different farming systems; intensive, semi-intensive and extensive, from farmers around Bulawayo, Zimbabwe. Samples were analysed using enzyme linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC). Majority of the milk samples (70.6%) tested positive for aflatoxin M_1 and the range of contamination was between 0 and 0.57µg/L with most of the samples (91%) coming from the dry season. Only one sample from the dry season had a concentration above both the European Union (EU) limit of 0.05µg.L and the Food and Drug Administration of the United States (FDA) limit of and 0.5µg/L respectively. Of the positive samples from the dry season, 58% had concentrations below the EU limit. However, during the rainy season 88% of the samples complied with the EU regulatory limit and none where above the FDA limit. Samples from semi-intensive farming system were the most contaminated during the dry season whereas for the rainy season the most contaminated samples came from the intensive farming season. The extensive farming system exhibited the least contamination. Detection of AFM_1 in milk samples by HPLC showed similar results although there were variations in the quantity of aflatoxins. AFM_1 concentrations in urine were between 0 and 2.36µg/L with samples from the dry season having a higher concentration than those from the rainy season. The presence of AFM_1 in both milk and urine indicated the exposure of the cows to aflatoxin B_1 contaminated feed. Even though most of the milk samples were within the regulatory limits, the consumers are still at the risk of suffering from chronic aflatoxicosis which includes cancers and other chronic disorders like congenital malformations as well mutagenic disorders due to continual consumption of AFM_1 contaminated milk. Therefore there is need to monitor and control aflatoxin B_1 contamination of feed in order to curb its carryover into milk.

5.1 Introduction

Mycotoxins, secondary metabolites of several fungal species, have become a potential threat to public health as they cause an array of disorders (Hernández-Martínez & Navarro-Blasco, 2015). Aflatoxins are the most toxic and well-studied mycotoxins produced by some species of the *Aspergillus* genus. The naturally occurring aflatoxins AF(B_1), B_2, G_1 and G_2 have been detected in several animal feeds and feedstuffs worldwide (Rodrigues & Naehrer, 2012;

Schatzmayr & Streit, 2013). Aflatoxin B_1 is the most potent and known to cause hepatocarcinoma as well as suppressing the immune system (Rahimirad *et al.,* 2014). Hydroxylation of the ingested AFB_1 by mammals can result in the formation of AFM_1 which can be detected in milk and urine (Janković *et al.,* 2009). The secretion of AFM_1 into milk of the lactating cows usually occurs between 12-24 hours after the ingestion of contaminated feed by the animal (Alshannaq & Yu, 2017; Balina *et al.,* 2018). The percentage carryover of AFB_1 into milk as AFM_1 ranges from 1- 6% in low and high yielding cows (Britzi *et al.,* 2013; Bellio *et al.,* 2016). A correlation of AFM_1 in urine and AFB_1 in the food/feed ingested for both humans and animals has also been established (Jager *et al.,* 2014)

Although AFM_1 is less toxic than AFB_1, it still exhibits some carcinogenicity and has therefore been classified as a group 1 human carcinogen (Masoero *et al.,* 2007; Mwanza *et al.,* 2013). Studies that have been conducted globally have indicated presence of AFM_1 in milk and milk products (Yitbarek & Tamir, 2014; Aiko & Mehta, 2015; Becker-Algeri *et al.,* 2016b). Milk is the main source of nutrients for children and also consumed by adults (Negash, 2018; Rodríguez-Carrasco *et al.,* 2018) therefore humans can be exposed to aflatoxin poisoning through the consumption of milk. This high global rate of milk consumption has led to regulatory measures being put in place in order to reduce the risks of being exposed to AFM_1 (Martins *et al.,* 2007; Vagef & Mahmoudi, 2013). The food and drug administration (FDA) set a limit of 0.5ng/l, however the European Unions and South Africa have a lower limit to 0.05µg/l (Mwanza *et al.,* 2013) although no scientific information was used to standardise this legal limit (Galvano *et al.*, 1996a) . The United States of America adopted the FDA limit of 0.5µg/l (Bellio *et al.,* 2016; Negash, 2018). However, most African countries do not have set limits for AFM_1 in milk (Mwanza *et al.,* 2013; Misihairabgwi *et al.,* 2017). In sub-Saharan Africa there is limited information on the occurrence of AFM_1 in milk samples as there is no data available from most of the countries with the exception of South Africa (Misihairabgwi *et al.,* 2017). In Zimbabwe, a study by Choga *et al.* (2016) reported the presence of AFM_1 in milk samples that were collected from small scale and commercial farmers around Harare which had levels above the EU (0.05µg/l) and FDA (0.5ng/l) regulatory limits. The limited aflatoxin research in sub-Saharan Africa is mainly due to lack of resources ranging from equipment, funding and expertise Moreover, the legislation is relaxed and does not exist at all in some countries to enforce the regulatory limits (Misihairabgwi *et al.,* 2017).

Immunochemical and chromatography are the main methods used in the analysis of AFM_1 (Bellio *et al.,* 2016). In particular, enzyme-linked immunosorbent assays (ELISA), thin layer

chromatography (TLC) and high performance liquid chromatography (HPLC) are the most common. This study ELISA and HPLC were the methods that were used for AFM_1 detection and quantification.

5.1.1Aim

The aim of the study was to quantify the level of aflatoxin M_1 (AFM_1) contamination of milk and urine samples from dairy cows from Bulawayo, Zimbabwe.

5.1.2 Objectives

1. To quantify the level of aflatoxin M1 contamination of milk and urine samples from dairy cows from Bulawayo, Zimbabwe by ELISA and HPLC methods.

2. To compare the two methods of analysis.

5.2 Methodology

5.2.1 Sampling

5.2.1.1 Milk

Milk samples were collected from cows during milking operations in the morning (4-5am) for 5 days for three consecutive months during the dry (August, September and October) and rainy seasons (January, February and March). Sample collection was done using sterile screw capped bottles and transported in iced cool boxes to the laboratory as reported by (Yosef *et al*., 2014). Upon arrival at the laboratory, samples were frozen at -20°C until they were analysed as reported by (Bilandžić *et al*., 2014) within 3 months of collection (Kang'ethe and Lang'a, 2009).

5.2.1.2 Urine

Urine samples were also collected during the milking operations in sterile bottles and transported in cooler boxes to the laboratory and stored at -20°C until analysis.

5.2.2 Aflatoxin extraction and quantification

5.2.2.1 Milk

ELISA

The determination of AFM_1 in the milk samples by the ELISA method was undertaken by use of reagents in a RIDASCREEN® Aflatoxin M_1, R-Biopharm AG, Darmstadt, Germany kit following the manufacturer's instructions. The test procedure involved coating of the microtitre plate with 100µl of antibody solution and incubating for 15 min in the dark at 20 - 25°C. This was followed by washing the wells three times using 250µl washing buffer (10 mM phosphate buffer pH 7.4; 0.05 % Tween 20) per wash. Aliquots of 100µl standards solutions of concentrations 0, 0.005, 0.01, 0.02, 0.04 and 0.08µg/L and the prepared skim milk samples were then added to the microtitre plate and incubated for 30 min away from light at 20 - 25°C. After 30 min, the plates were emptied and washed three times with the washing buffer. A volume of 100µl of the conjugate solution was added to each well followed by incubation at 20 - 25°C for 15 min in the dark. The wells were re-washed using the washing buffer followed by addition of 100µl of the substrate/chromogen (peroxidase/ tetramethylbenzidine (TMB)) and incubated for 15 min at 20 - 25°C. The reaction was terminated by addition of 100µl of the stop solution (1N sulphuric acid) to give a yellow colour and absorbance was read at 450nm within 15 min of the addition of the stop solution using a plate reader (Spectramax M2, Molecular devices, TUV Rheinland North America Inc, US).

HPLC

Aflatoxin M_1 extraction from the milk was carried out following the method of Mohamadi Sani *et al.* (2015). One hundred millilitres of the frozen sample was thawed and warmed to 37°C in a water bath followed by centrifugation at 6000 rpm for 10 min using a bench top centrifuge (Centurion 1000 series, Centurion Scientific Ltd, West Sussex, UK). The fat layer was removed and 50ml of the skim milk passed through Whatman No.1 filter paper. The filtrate was then passed through immunoaffinity columns (AFLAPREP® M WIDE, R-Biopharm, Rhone Ltd, Glasgow G20 OXA, Scotland) by gravity as per manufacturer's instruction. The column was washed with 20ml of phosphate buffered saline and AFM_1 eluted with 1ml of methanol (LiChrosolv®, Merk, Germany) into a glass cuvette and diluted with 1ml of distilled

water and then stored at -20°C prior to HPLC analysis. Aflatoxin M_1 standard (Trilogy Analytical Laboratory, Washington, USA) was diluted using acetonitrile (LiChrosolv®, Merk, Germany) to give the following concentrations; 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-1} µg/ml. Aflatoxin detection and quantification was by HPLC (Shimadzu FCV-20H2) with operation conditions as given in the KOBRA® cell instruction manual as follows; derivatisation with KOBRA ® cell at 100µA setting, analytical column Intersil ODS-3V 5µm,4.6mm×150mm equipped with a C18 4 × 3 mm^2 i.d. security guard cartridge (Phenomenex, Torrance, CA, U.S.A.). Mobile phase was modified from the recommended water: methanol (60:40) to a working condition of (55:45) with 119mg/litre of potassium bromide (KBr) and 1ml/litre of 65% nitric acid added at a flow rate of 1.0ml/min, The fluorescence detector was set at 350nm for excitation and emission 450nm. The injector was an auto sampler which injected 100µl of sample. A standard curve was constructed by plotting the area of the peak against the concentration of the standards. Sample concentration was determined by interpolating from the standard curve.

5.2.2.2 Urine

Detection and quantification of AFM_1 in urine was done by ELISA only using the urine aflatoxin kit (Helica Biosystem Inc, Santa Ana, CA 92704, USA). The urine samples were thawed and brought to room temperature (20 - 25°C). Debris was removed from the samples by filtering with Whatman No. 1 filter paper. A dilution of 1:20 (sample: distilled water) was carried out for both standards and samples (50µl:950µl). The wells of the microtitre plate were already coated with the antibody. A volume of 100µl of the diluted standards of concentrations 0.15, 0.40, 0.80, 1.50 and 4µg/L and samples were mixed with 200µl of the assay buffer. New pipettes for each sample were used to transfer the contents from the mixing wells into the corresponding antibody coated wells of a separate microtitre plate. The reactions were incubated at room temperature (20 - 25°C) for 1h. The contents in the wells were discarded and the plate washed three times using PB-Tween buffer. Horseradish peroxidase (HRP) conjugate was added to the wells and incubated for 15 min followed by washing with the wash buffer three times. Aliquots of 120µl of the substrate tetramethylbenzidine (TMB) were added to the wells which were kept away from light and incubated for 15min at room temperature (23±2°C). The reaction was stopped by adding 120µl of the stop solution and absorbance read at 450nm within 15 min of addition of the stop solution.

Dose-response curves were constructed using readings from the known standard by plotting absorbance against concentration of the standards. The concentrations of the samples were determined by interpolation from the standard curve.

5.3 Results

5.3.1 Comparison of HPLC and ELISA

Analysis of the milk samples using the two methods showed that there was no significant difference in the two methods used for detection of AFM_1 in the samples. However, the values from ELISA were lower than those of HPLC as shown in Table 8. Some samples in which AFM_1 was not detected by ELISA were positive for HPLC.

Table 8. Comparison of HPLC and ELISA methods in AFM_1 analysis.

Parameter	HPLC AFM_1 conc (µg/L)	ELISA AFM_1 conc (µg/L)
Minimum	0	0
Maximum	0.568	0.564
Average	0.062	0.050
Standard Deviation	0.091	0.067

5.3.2 Analysis of AFM_1 in milk by HPLC

The plot of peak area against AFM_1 standard concentration showed a calibration curve with a correlation (r^2) value of 0.9997 (Figure 5.1). There was a good linearity of the standards range used. The presence of AFM_1 was indicated by a peak with retention time of 5.35 min as shown in Figure 5.1. However, some samples showed several peaks (Figure 5.2) which may suggest multitoxin occurrence in these samples.

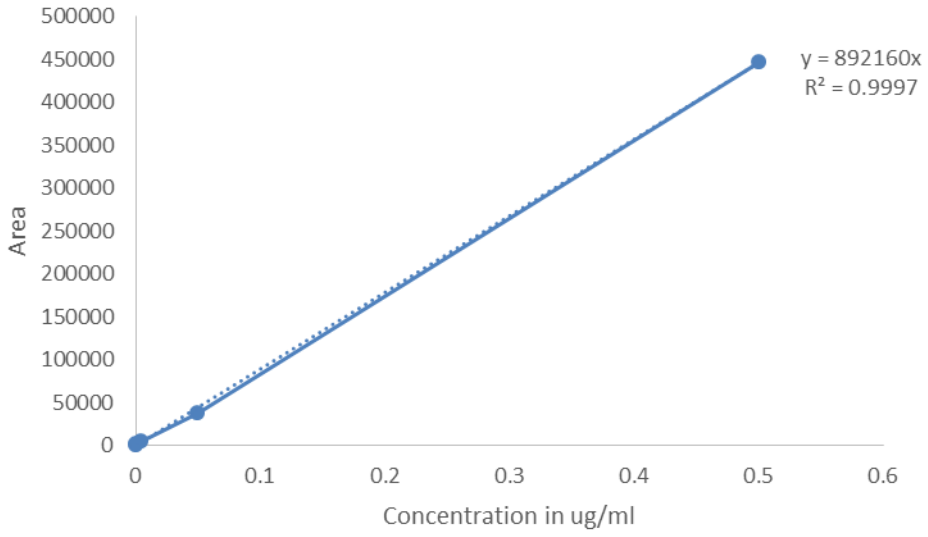

Figure 5.1. Calibration curve for AFM_1 standards by HPLC analysis.

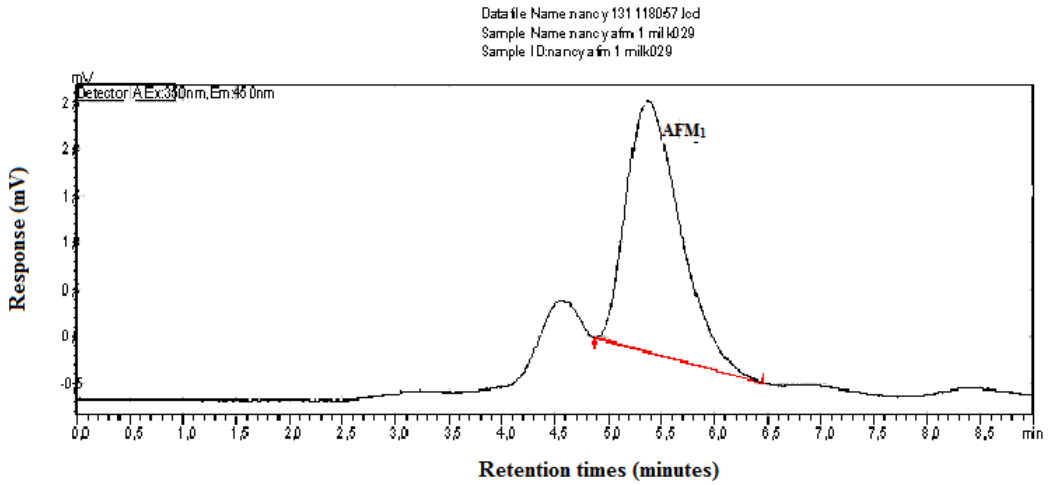

Figure 5.2. Representative chromatogram for a milk sample with retention time of 5.35 min signifying the presence of AFM_1.

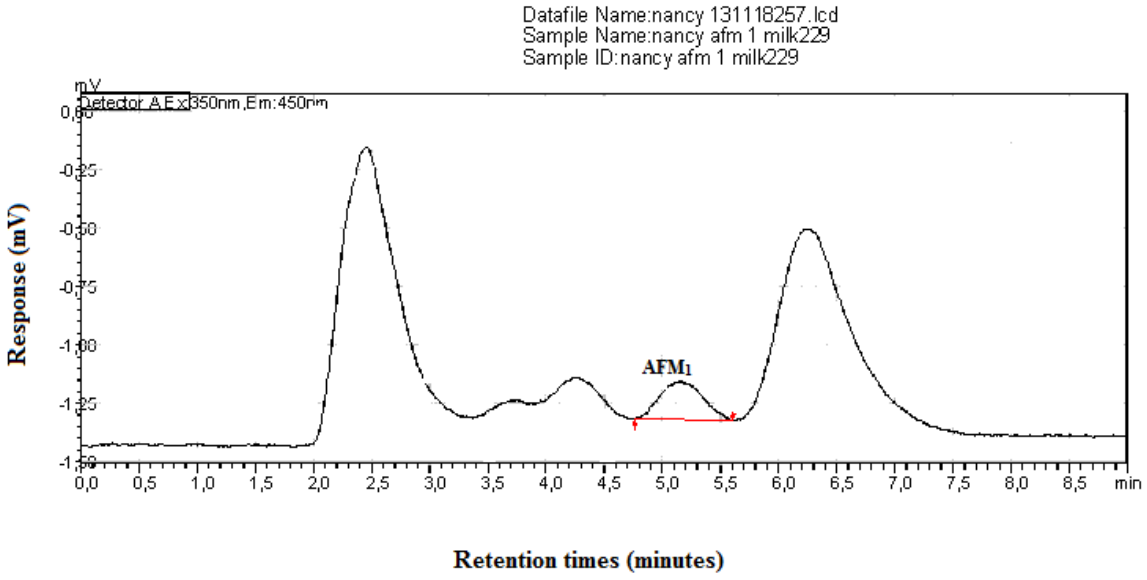

Figure 5.3. Chromatogram showing presence of other metabolites other than AFM_1 in a milk sample indicated by the presence of several peaks.

Analysis of the samples showed 91% testing positive for AFM_1 with samples from the dry season ranging from with concentrations ranging from non-detected - 0.57µg/L. A total of 58% of the positive samples had AFM_1 concentration less than the EU limit of 0.05µg/L whereas only 1 sample was above the FDA limit of 0.5µg/L. From the rainy season samples, 53% of the samples tested positive and 88% of the positive samples had concentrations below EU limit. None of the samples from the rainy season were above the FDA limit. Samples from the intensive farming system had the highest AFM_1 concentrations for both seasons with an average concentration of 0.1µg/L for the dry season and 0.05µg/L for the rainy season as shown

in Figure 5.4. One way ANOVA for comparison of means gave *p* value = 0 indicating a significant difference in the average concentrations of AFM_1 in milk from the three farming system.

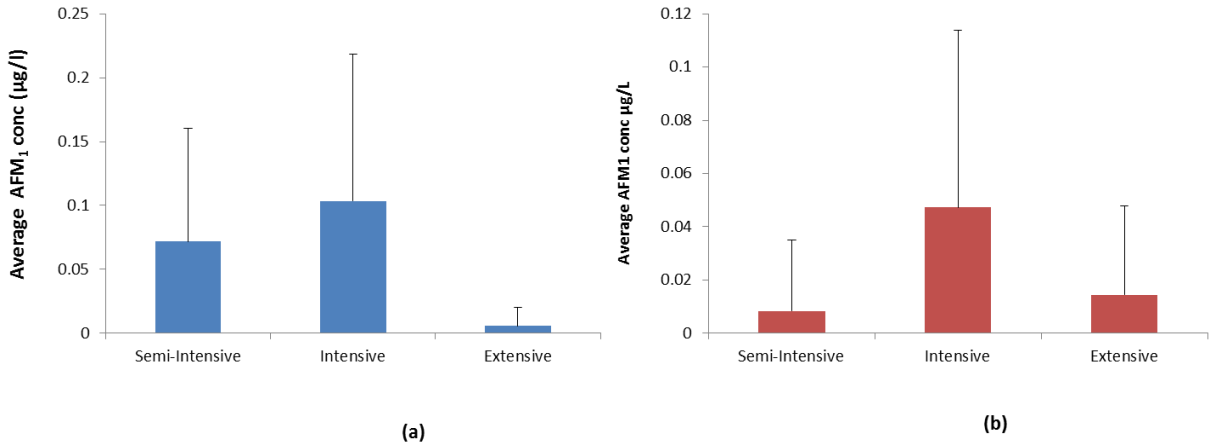

Figure 5.4. AFM_1 concentration of milk samples from different feeding systems during the (a) dry season and (b) rainy season showing highest average concentration in the intensive farming system.

Table 9. Dunnett's T3 multiple comparisons for the three farming systems in the dry season.

Multiple Comparisons						
Dependent Variable: AFM_1 conc ((µg/l)						
Dunnett T3						
(I) Farming System	**(J) Farming System**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
Semi-Intensive	Intensive	-0.03144	0.02025	0.330	-0.0813	0.0184
	Extensive	0.06606*	0.00859	0.000	0.0453	0.0868
Intensive	Semi-Intensive	0.03144	0.02025	0.330	-0.0184	0.0813
	Extensive	0.09750*	0.01858	0.000	0.0512	0.1438
Extensive	Semi-Intensive	-0.06606*	0.00859	0.000	-0.0868	-0.0453
	Intensive	-0.09750*	0.01858	0.000	-0.1438	-0.0512
*. The mean difference is significant at the 0.05 level.						

Dunnett's multiple comparison test (Table 9) showed that there was a significant difference in AFM_1 concentrations in milk from the semi-intensive and the extensive farming systems as well as milk from intensive and extensive farming systems. However, there was no significant difference in AFM_1 concentrations in milk from the extensive and semi-intensive system during the dry season. During the rainy season AFM_1 concentration was highest in the intensive farming system (Figure 5.4b) with significant differences between the extensive farming and intensive farming as well as semi-intensive farming and intensive farming. However, there was no significant difference between milk samples from the semi-intensive and extensive farming systems (Table 10).

Table 10. Dunnett's T3 multiple comparisons for the three farming systems in the rainy season.

Multiple Comparisons						
Dependent Variable: Average AFM_1 conc (µg/L)						
Dunnett T3						
(I) Farming System	**(J) Farming System**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
Semi-Intensive	Intensive	-0.03884*	0.00835	**0.000**	-0.0592	-0.0185
	Extensive	-0.00612	0.00583	**0.649**	-0.0205	0.0082
Intensive	Semi-Intensive	0.03884*	0.00835	0.000	0.0185	0.0592
	Extensive	0.03272*	0.00961	**0.003**	0.0094	0.0560
Extensive	Semi-Intensive	0.00612	0.00583	0.649	-0.0082	0.0205
	Intensive	-0.03272*	0.00961	0.003	-0.0560	-0.0094
***. The mean difference is significant at the 0.05 level.**						

5.3.3 Urine analysis

Urinary AFM_1 levels ranged from 0 – 2.4µg/L with a mean concentration of 0.337µg/L. The semi-intensive farming system had the highest concentration with an average of 0.39µg/L. However, for the rainy season AFM_1 concentration in the urine ranged from 0 – 1.07µg/L. The intensive system had the highest AFM_1 concentration with an average of 0.23µg/L as shown in Figure 5.5.

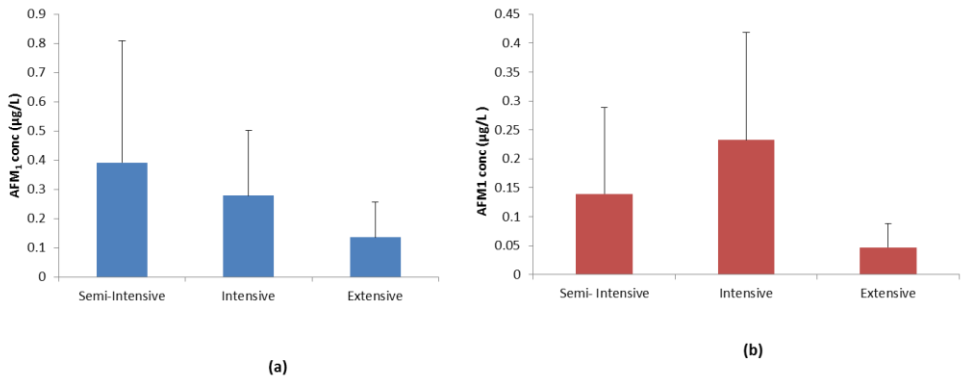

Figure 5.5. Showing average AFM_1 concentration in urine of cows from different farming systems during the (a) dry and (b) rainy seasons.

5.4 Discussion

The presence of AFM_1 in milk and other dairy products is undesirable as it poses health risks to the consumers especially the children because the greater part of their diet consists of milk (Sohrabi & Gharahkoli, 2016).

The study showed that the presence of AFM_1 (range 0-0.5) in milk samples from Bulawayo peri-urban dairy farmers. Thirty percent of combined season samples exceeded the EU limit of 0.05µg/L and 0.3% exceeded the FDA limit of 0.5µg/L. A higher percentage of contamination was noticed during the dry season compared to the rainy season where 42 and 12% of the samples exceeded the EU limit respectively. Choga *et al.* (2016) analysed milk samples from commercial and small-scale farmers around Harare, Zimbabwe and reported contamination in 79.2% of the samples as against the present study of 70.6%. All the samples from Harare had concentrations above the EU of 0.05 µg/L. Tajkarimi *et al.* (2008) worked on raw milk samples from dairy farmers and milk collection centres in Iran. They reported that 23% of milk samples from winter and summer seasons had a concentration >0.05µg/L.. A study done by Kang'ethe and Lang'a (2009) on milk samples from Kenya reported that 35% of the positive samples exceeded the EU limit and 2% exceeded the FDA limit. The most contaminated sample had an AFM_1 concentration of 0.68µg/L as against a value 0.57µg/L obtained in this study. Mwanza (2016) reported that 90.6% and 62.1% of cow milk samples from two rural areas of the Limpopo province of South Africa had AFM_1 levels of 0.092 and 0.073µg/l which was higher than the 0.05µg/l EU limit. In Tanzania work done by Mohammed *et al.* (2016) had all the positive cow milk samples having concentrations above the EU limit. However, some researchers working on cow milk samples from European countries reported on 100% of their samples having concentrations of less than the 0.05 µg/L (Visciano *et al.*, 2015; Polovinski Horvatović *et al.*, 2016)

Tajkarimi *et al.* (2008) and Choga *et al.* (2016) also reported that higher concentrations of AFM_1 were observed during the dry season as compared to the rainy season. It was also established from this study that during the dry season milk from the semi-intensive farming system had higher AFM_1 concentrations compared to other systems and during the rainy season the intensive farming system had the highest AFM_1 concentrations. These results are similar to those of Choga *et al.* (2016) who reported that most contaminated samples of commercial farmers occurred during the rainy season. Cows from the semi-intensive farming systems are normally fed with mixed rations during the dry season which hasbeen noted by most researchers to have high AFB_1 concentrations (Chohan *et al.*, 2016). AFB_1 is then carried over into the milk by the dairy cows upon consumption. During the rainy season, cows from the

semi-intensive and extensive farming systems have a greater portion of their diet being the fresh forages from the grazing pastures and a small fraction of the feed hence low concentrations of AFM_1 in their milk. Overall milk from the extensive farming system had the least AFM_1 concentration, most probably due the fact that they entirely depend on pastures compared to the other systems which incorporate compound feeds in the diet of the cows. These results are similar to the findings by Fallah *et al.* (2011) who reported on low concentrations of AFM_1 in milk from goats and sheep compared to milk from the cows due to the fact that for goats and sheep their diet is pasture based.

The fact that contamination rates of most of the samples were below the regulatory limit does not mean that the milk consumers are safe from the toxicological effects of aflatoxins. This is mainly because most milk processing technologies like heat treatment and fermentation have not been shown to have noticeable effect in reducing the AFM_1 levels in milk. Therefore the consumers are still exposed to chronic aflatoxicosis which is associated with consumption of low doses of the toxin. This may result in cancers as it has been shown that even if AFM_1 is the transformed form of AFB_1 that can be easily excreted from the body, it still retains some carcinogenicity.

Most research of mycotoxins in milk has been focussing only on AFM_1, however results from this study also showed that AFM_1 may not be the only fungal metabolite present in the milk. This was highlighted by several peaks that were detected in some of the milk samples (Figure 5.3). Multitoxin occurrence in dairy feeds has been reported and this may indicate that not only aflatoxins are carried over into the milk. In a review of mycotoxin carryover into milk, Fink-Gremmels (2008a) indicated that some researchers have reported on carryover of some mycotoxins other than AFM_1 into milk. Only Switzerland and Argentina are the only countries that have regulatory limits for other mycotoxins found in milk in addition to AFM_1 (Galvano *et al.*, 1996a).

Aflatoxin M_1 can also be excreted through the urinary system. This study showed the presence of AFM_1 in urine samples. The concentrations ranged from 0- 2.36µg/L. The trend was also the same as for milk where higher concentrations were noticed in the dry season and lower concentrations in the rainy season. Information on AFM_1 in urine from cows and humans is scarce except where it is used as a biomarker for aflatoxin exposure in humans.

A greater percentage (70.6%) of milk samples from farmers around Bulawayo was contaminated with AFM_1 and 30% of these positive samples had concentrations above the EU limit. Although most of the samples complied with the regulatory limits, the consumers are still at the risk of having chronic aflatoxicosis. The best way of controlling occurrence of AFM_1 in milk is to feed the cows with aflatoxin free feed. Fungal contamination of feeds is inevitable but can be minimised by having good storage facilities that do not result in increase in the moisture content which often trigger aflatoxin production by the moulds. The legislation should also be strict on the quality of the feed being supplied to the farmers and ensure the aflatoxin concentrations are within the stipulated maximum residual limits.

5.5 Conclusion

Findings from this study showed that the bulk of the milk samples analysed were contaminated with AFM_1 in low amounts. Higher contamination rates were however observed during the dry season compared to the rainy season. This is mainly because the cows were fed with feed supplements due to unavailability of pastures during the dry season. The presence of AFM_1 in both milk and urine is an indicator that the feeds given to the cows were contaminated with AFB_1. Although most of the samples complied with the limits, the consumers are still at the risk of having chronic aflatoxicosis. Therefore there is need for strict measures on prevention of aflatoxin contamination in feed and monitoring aflatoxin contamination of feeds and milk.

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Chapter 6

Association of bovine breed and aflatoxin carryover from aflatoxin B_1-naturally contaminated feed into aflatoxin M_1 in milk and urine of dairy cows.

Abstract

The relationship of breed and AFM_1 carryover in milk and urine of cows fed on naturally contaminated feeds were modelled using general linear models based on Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and multiple analysis of variance (MANOVA). Correlation analysis revealed a strong Pearson or Spearman's association between milk and urine. The AFM_1 concentration in the milk per given time was explained by the AFB_1 concentration in the diet of the cow and AFM_1 remaining in the cow's system from the previous feed treatment. Thus, AFM_1 in the cow's milk in real life situation when they are fed daily on AFB_1 naturally

contaminated feed does not get cleared in the system and therefore tend to be a cumulative effect with time. However, the cumulative effect over time was not significant in urine when compared to milk. Multivariate analysis of variance of breed effect on secretion of AFM_1 into milk was not significant ($p > 0.05$), since all breeds had the same levels of AFM_1 in their milk and urine. A positive correlation between AFM_1 concentration in milk and urine of the cows fed on the naturally contaminated feed was observed. There was significant evidence in ANCOVA that aflatoxin carryover in both milk and urine was affected by diet and not by breed ($p < 0.005$). The level of AFM_1 contamination in urine is therefore a potential indicator for bio-monitoring short-term exposure of cows to aflatoxin-contaminated feed. On the other hand, AFM_1 in milk is a potential long-term indicator of bio-monitoring exposure of the cows to aflatoxin-contaminated feed. This will assist farmers in the management of aflatoxin contamination at the farm-level.

6.1 Introduction

The presence of aflatoxins in animal feed stuffs exposes dairy cows to aflatoxin B_1 (AFB_1) which can be carried over into milk and excreta. AFB_1 is the most toxic of all the aflatoxins and has been classified as Group 1 carcinogen by the International Agency of Research on Cancer (IARC, 2012). Upon ingestion by ruminants, AFB_1 is partially metabolised by the rumen microflora into aflatoxicol while some is absorbed in the digestive system and carried to the liver where it is bio-transformed by the hepatic microsomal cells into soluble less toxic metabolites. Among these metabolites is aflatoxin M_1 (AFM_1) which is the hydroxylated form of AFB_1 that is either excreted from the body in the faeces via the biliary system after conjugation to glucuronic acid or remains in the circulatory system where it is eventually secreted into the milk and urine (Fink-Gremmels, 2008a). Toxicology tests done on laboratory

animals showed that AFM_1 has effects similar to those of AFB_1 though at a lower magnitude (Galvano *et al.*, 1996b; Janković *et al.*, 2009; Sumantri *et al.*, 2012). The presence of AFM_1 in milk and milk products has gained so much attention worldwide resulting in its classification as a Group 1 carcinogen by the IARC. Moreover, several food governing bodies have set regulatory limits for this toxin.

The presence of AFM_1 in milk from dairy cows is often associated with consumption of AFB_1 contaminated feed. The amount of AFM_1 excreted into the milk as a percentage of the AFB_1 consumed by the cow usually ranges between 1 and 3% of the amount of AFB_1 consumed , however in some animals, values as high as 6% have been obtained (Janković *et al.*, 2009; Churchill *et al.*, 2016). Several factors influence the rate of transfer of AFB_1 into the milk aflatoxin including the rate of feed consumption, the health status of the cow, bio-transformation and milk production as well as the species of the cow among others (Fink-Gremmels, 2008a; Völkel *et al.*, 2011; Britzi *et al.*, 2013). It is important to know the carry-over rates of the dairy cows as it helps in the determination of the regulatory limits of AFB_1 in feeds by different countries (Britzi *et al.*, 2013).

In this study the effect of bovine breed on the rate of aflatoxin carry over was assessed on Holstein, Jersey and a crossbreed (Holstein/Jersey) cows from dairy farms around Bulawayo.

6.1.1 Aim

To determine the association of bovine breed and aflatoxin carryover in milk and urine from cows.

6.1.2 Objective

1. To establish a relationship between dietary intake of aflatoxins and aflatoxin metabolite (AFM_1) secreted into milk and excreted in urine of dairy cows.
2. To determine the influence of breed on aflatoxin carryover in milk.

6.2 Methodology

Milk samples were collected from cows during milking operations in the morning (4-5am) for 5 days for three consecutive months during the dry (August, September and October) and rainy seasons (January, February and March). A total of 14 cows from 4 different farms were used for this study. Analysis of AFB_1 in the feed was carried by HPLC (Chapter 4.2.3) and AFM_1 in milk and urine was undertaken using HPLC and ELISA respectively (Chapter 5.2.2.1 and

5.2.2.2). Aflatoxin M_1 carry-over in milk and urine was calculated as a percentage of the AFB_1 consumed in the feed (Masoero *et al.*, 2007). Multiple linear regression analysis was carried out using log transformed results to determine the effect of AFB_1 dietary intake (diet) and breed on AFM_1 secretion in milk and urine using R software (version 3.5.2). The assumptions of normality of errors, homogeneity of error variance, independence of errors and linearity or additivity of the general linear models were diagnosed before the analysis. Orthogonality was investigated using ANOVA (type I) and ANOVA (type II) by comparing the sequential sum of squares and adjusted sum of squares, respectively. If data is orthogonal the sequential sum of squares should be the same. The autocorrelation factor (ACF) plot was used for analysis of independence of variables (Osborne & Waters, 2002).

For correlation analysis, depending on data normality, Pearson or Spearman's coefficients were used (Mukaka, 2012). Pearson's coefficient is a parametric test and assumes both variables are normally distributed. Wherever normality was violated the Spearman's coefficient was used. The models showing significance were then selected to come up with mathematical expressions that can be used for prediction of AFM_1 concentrations in milk or urine per given time.

6.3 Results

6.3.1 Meeting the assumptions for linear modelling of aflatoxin B_1 concentration in diets (feeds) and breeds to the aflatoxin M_1 concentration in milk and urine.

All the response variables were log transformed and the assumption of normality of error was met in Figures 6.1 and 6.2 showing the Q-Q normal probability plots for diet and breed effect respectively. Table 11 shows the *p* values of the Shapiro-Wilk tests where $p > 0.05$ indicated that data was normal. It is clear from Figures 6.3 and 6.4 that residuals versus fitted values and ACF plots respectively that the linearity assumption and independence of residuals were met. The homoscedasticity of the residuals was also met from Figure 6.3 since residuals and fitted values are uncorrelated with an even or constant variance. The assumption that the covariate (previous month's AFM_1 concentration in milk) was not affected by the current month's diet (treatment) was not violated since the covariate was measured before the treatment.

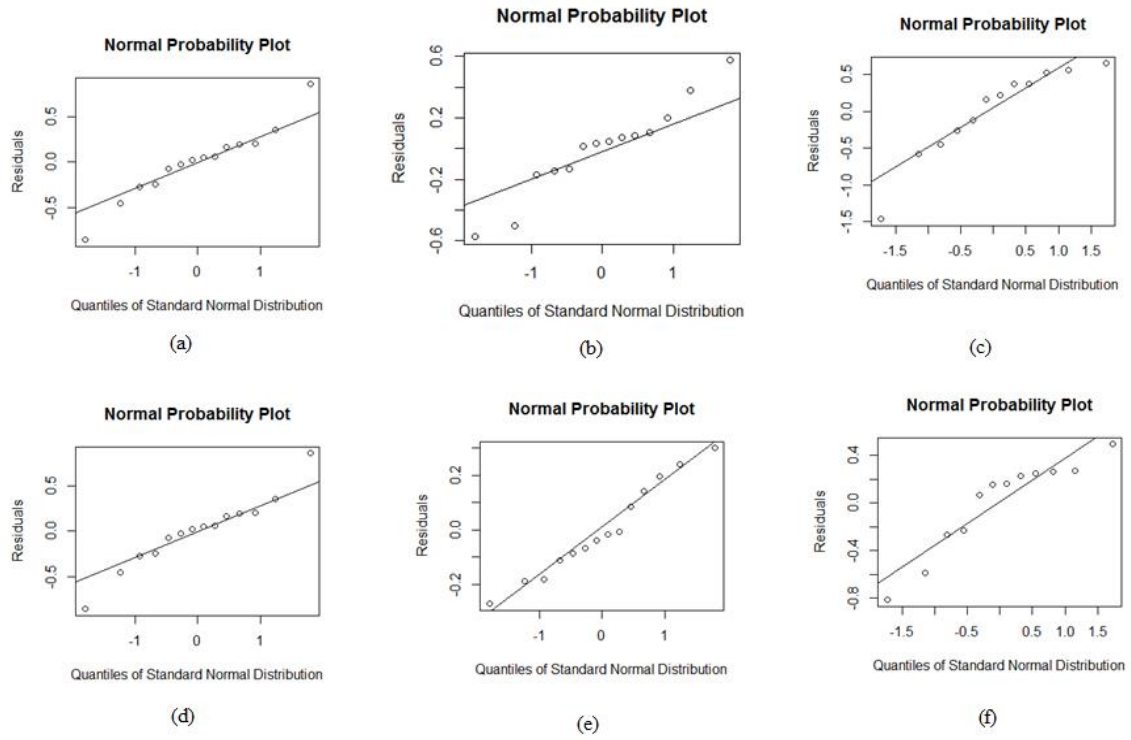

Figure 6.1. Q-Q plots for diet effect (a) August milk, (b) September milk, (c) October milk, (d) August urine, (e) September urine and (f) October urine showing normal distribution of data with the data points lying on a straight diagonal line.

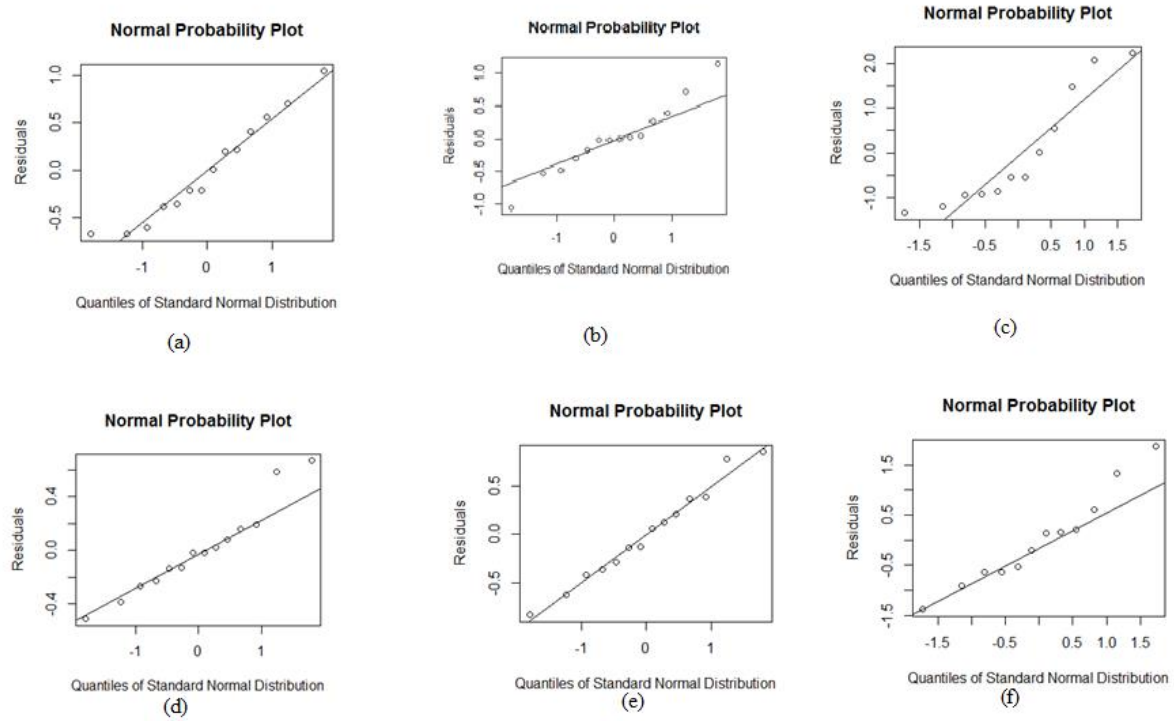

Figure 6.2. Q-Q plots for breed effect (a) August milk, (b) September milk, (c) October milk, (d) August urine, (e) September urine and (f) October urine showing normal distribution of data with the data points lying on a straight diagonal line.

Table 11. Shapiro Wilk test for data normality

Month	**Shapiro Wilk (*p*—value)**			
	Diet on milk	**Diet on urine**	**Breed on milk**	**Breed on urine**
August	0.561	0.643	0.561	0.643
September	0.959	0.091	0.959	0.091
October	0.195	0.044	0.195	0.044

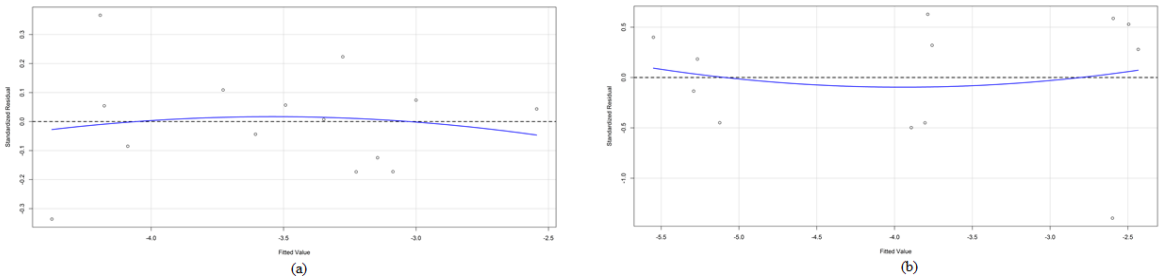

Figure 6.3. Residual error vs fitted values plot showing that the selected models (a) September and (b) October met the llinearity and independence of residuals assumption. The points are scattered not following any pattern meeting the homoscedasticity assumption.

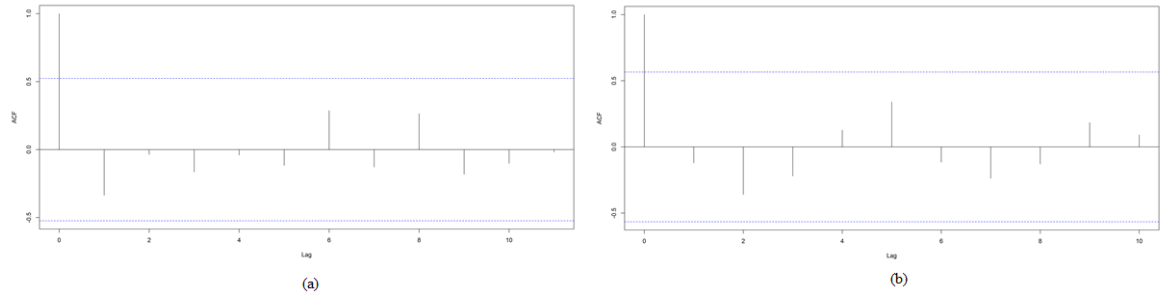

Figure 6.4. Autocorrelation factor plot for (a) September and (b) October models showing that llinearity and independence of residuals assumption were met with most values lying on the zero line.

6.3.2 Breed Effect

Repeated measures one way ANOVA showed that the breed effect on the amount of AFM_1 secreted into milk for the three months is not statistically significant (p-value > 0.05, Table 12). Box plots medians were asymmetric with overlaps (Figure 6.5).

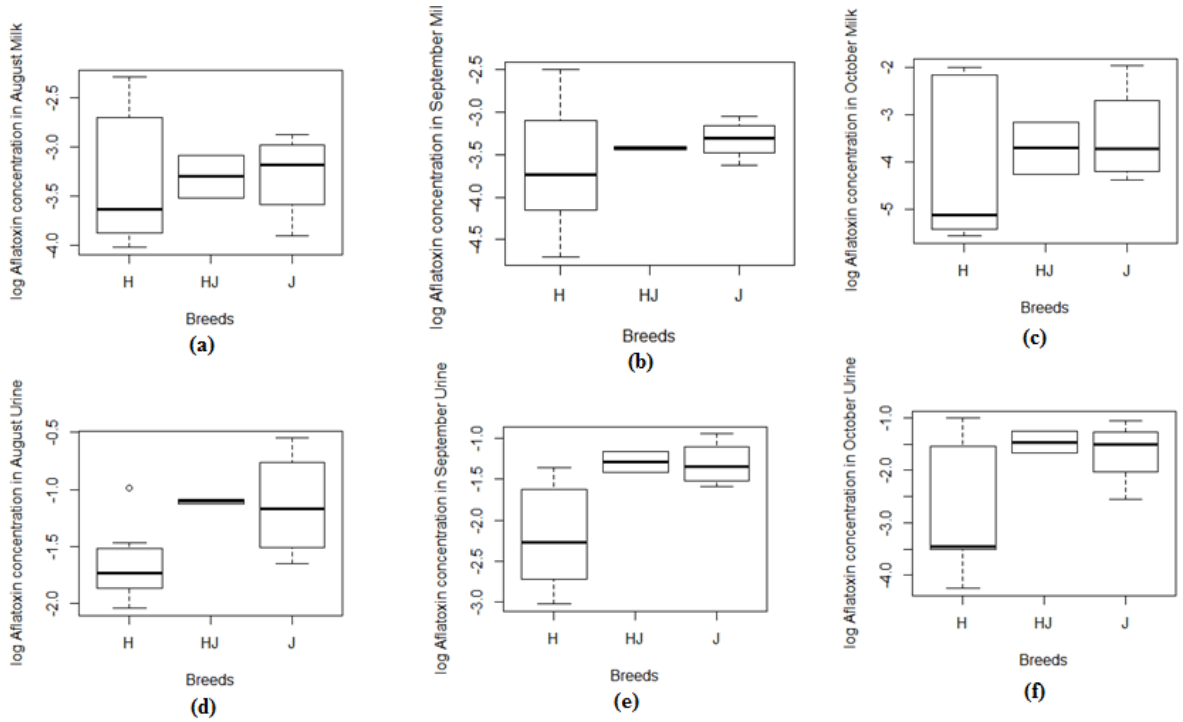

Figure 6.5. Variation of AFM_1 concentration in samples based on breed. August milk (a), September milk (b), October milk (c) August urine (d), September urine (e) and October urine (f). For milk the plots are suggesting that the three breeds are the same (a-c) whereas for urine breed H is different from breeds HJ and J.

The adjusted R^2 values for all the three months showed that the breed effect alone cannot explain variability in the concentrations of AFM_1 in the milk. However, the covariate representing the AFM_1 levels of the previous month improved the breed effect in September ($F_{(3,10)} = 10.13$, $p < 0.05$, $R^2 = 0.678$) and October ($F_{(3,8)} = 6.266$ $p < 0.05$, $R^2 = 0.590$) as detailed in Table 12. This suggests that AFM_1 carryover in the cow's milk secretory system has an effect on the quantity of AFM_1 in the milk for the next day.

Table 12. ANOVA analysis results for effect of breed on AFM_1 in milk.

Month	***p* value (*without covariate*)**	***p* value (*with covariate*)**	**Adjusted R^2 value (*without covariate*)**	**Adjusted R^2 value (*with covariate*)**
August	0.987	N/A	-0.179	N/A
September	0.654	$2.242\times10^{-3**}$	-0.094	0.678
October	0.690	0.0170^{*}	-0.125	0.590
Significance codes: At the 0.05 level. ***highly significant, **very significant, *significant.				

MANOVA of the three breeds shows that they were e not significantly different in their secretion of AFM_1 in milk ($p > 0.05$).

On the other hand, the breed effect on the AFM_1 excretion in the urine (Table 13)were significant for September ($F_{(2,11)} = 4.881$, $p < 0.05$, $R^2 = 0.374$). .

Table 13. ANOVA analysis results for effect of breed on AFM_1 in urine.

Month	*p* value (*without covariate*)	*p* value (*with covariate*)	Adjusted R^2 value (*without covariate*)	Adjusted R^2 value (*with covariate*)
August	0.058	N/A	0.296	N/A
September	0.030*	0.052	0.374	0.379
October	0.151	0.003**	0.197	0.743
Significance codes: At the 0.05 level. ***highly significant, **very significant, *significant.				

The covariate improved the influence of the breed effect on levels of AFM_1 in urine in October ($F_{(3,8)} = 11.57$, $p < 0.05$, $R^2 = 0.743$). Therefore, the breed effect was taken to represent random effects in the linear modelling of AFM_1 concentrations in cow milk and urine.

6.3.3 Diet effect on AFM_1 concentration in milk.

Basic descriptive statistics and visual inspection of the models suggested that the logarithmic aflatoxin concentrations differed between diets. Box plots medians were symmetric with no overlaps (Figure 6.6).

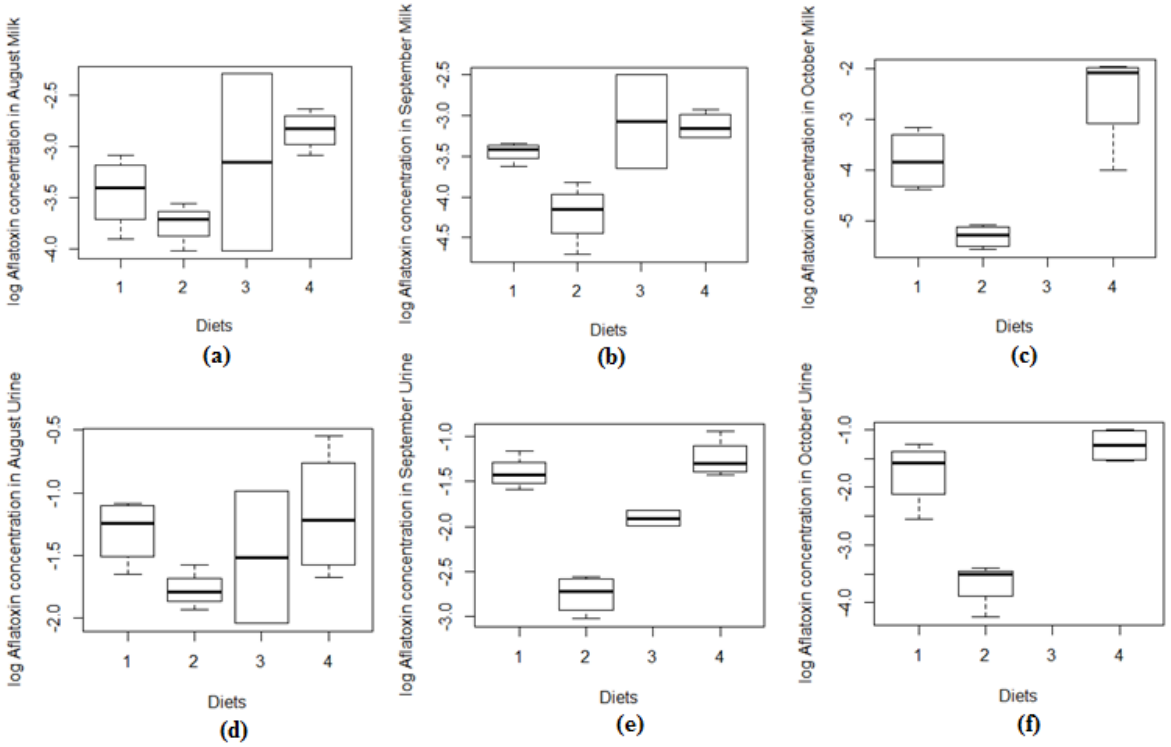

Figure 6.6. Variation of AFM_1 concentration in samples based on diets. August milk (a), September milk (b), October milk (c) August urine (d), September urine (e) and October urine (f). Diet 2 had the least aflatoxin B_1 concentrations and diet 4 had the highest.

In August, diet had no effect on the amount of AFM_1 secreted in milk ($F_{(3,10)} = 2.865$, $p > 0.05$, $R^2 = 0.30$) (Table 14). Moreover, only 30% of the variation in AFM_1 in milk could be explained by the diet effect indicating a weak relationship with high scatter. However, for September

and October there was enough evidence supporting that diet contributed significantly to the amount of AFM_1 secreted in the milk.

Table 14. ANOVA analysis results for effect of diet on AFM_1 in milk

Month/Diet	***p* value (*without covariate*)**	***p* value** (*with covariate*)	**Adjusted R^2 value (*without covariate*)**	**Adjusted R^2 value** (*with covariate*)
August	0.090	N/A	0.30	N/A
September	$4.814\times10^{-3**}$	$1.511\times10^{-4***}$	0.623	0.857
October	$9.118\times10^{-4***}$	$3.954\times10^{-3**}$	0.742	0.719
Significance codes: At the 0.05 level. ***highly significant, **very significant, *significant.				

From the output of adjusted and sequential sums of squares, the experiment is orthogonal implying that the covariates (previous month's AFM_1 concentration in milk) are not affected by the diet effect of the current month's diet effect.

From the correlation plots (Figure 6.7), it can be deduced that there was an association between aflatoxin concentration in September and that of August and also between October and September. There was a significant, positive correlation ($F_{(4,9)} = 20.54$, $p < 0.05$, $R^2 = 0.857$) for September . The same effect was seen for AFM_1 in the October milk when additive effect of October feed and September milk was taken into consideration, $F_{(3,8)} = 10.36$, $p < 0.05$, $R^2 = 0.719$ (Table 14). This suggested that the amount of AFM_1 in the milk per given time was explained by AFB_1 concentration in the diet of the cow and AFM_1 reminants in the cow's system from the previous feed. Thus AFM_1 in the cows is real life situation when they are constantly feeding on AFB_1 naturally contaminated feed, it does not get cleared in the system and there tend to be a cumulative effect with time. Therefore AFM_1 in milk can be used as a long-term indicator of bio-monitoring exposure of the cows to aflatoxins through feed.

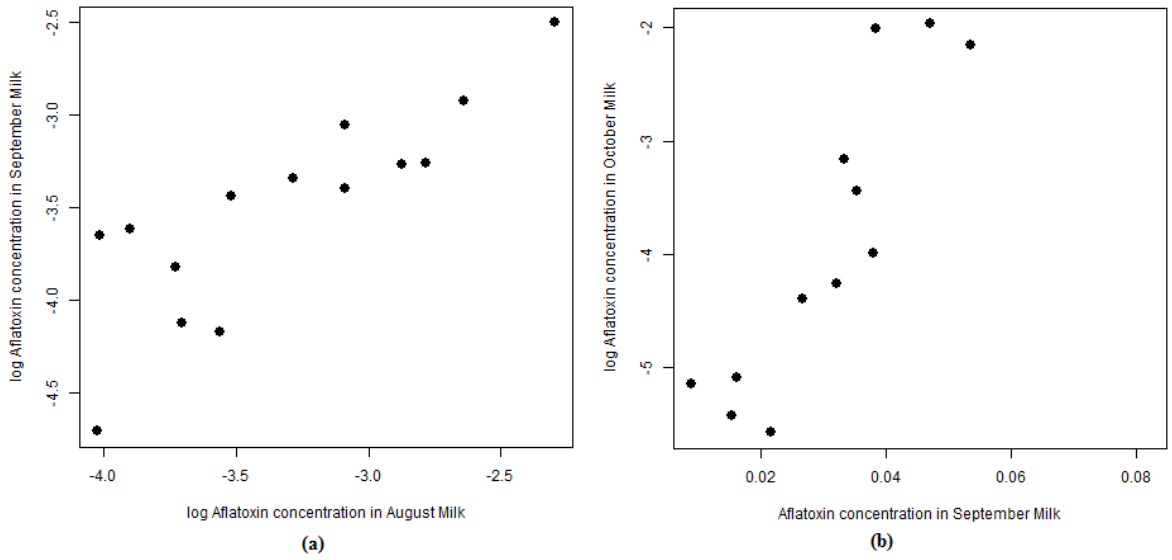

Figure 6.7 The relationship of covariate and treatment on AFM_1 concentration in milk. Showing the relationship between: (a) aflatoxin concentration in covariate August milk and aflatoxin concentration in treatment September milk; and (b) aflatoxin concentration in covariate September milk and aflatoxin concentration in treatment October milk.

According to the MANOVA test, there was adequate evidence that there was a significant difference in the diets received by the cows from the different farms.

For urine excretion, diet had no effect on the AFM_1 concentration in the urine for the month of August ($F_{(3,10)} = 1.706$, $p > 0.05$, $R^2 = 0.228$) (Table 15).

Table 15. ANOVA analysis results for effect of diet on AFM_1 in urine.

Month/Diet	***p* value (*without covariate*)**	***p* value (*with covariate*)**	**Adjusted R^2 value (*without covariate*)**	**Adjusted R^2 value (*with covariate*)**
August	0.228	N/A	0.140	N/A
September	$2.987\times10^{-6***}$	$1.656\times10^{-5***}$	0.916	0.913
October	$6.153\times10^{-5***}$	$3.997\times10^{-4***}$	0.858	0.843
Significance codes: At the 0.05 level,*highly significant, ** very significant, *significant.**				

When the covariate effect of AFM_1 in urine from August and September 2016 were taken into consideration along AFM_1 concentration in urine for September and October 2016 respectively, the R^2 values dropped (Table 15) indicating that the additive effect was less in urine as compared to milk. This suggests that AFM_1 in milk can be used as an indicator for bio-monitoring short-term exposure of cows to aflatoxins though feeds. MANOVA also indicated that there were no statistically significant differences in the AFB_1 concentrations in the diets received by the animals with $p > 0.05$.

6.3.4 Correlation analysis

Multivariate analysis showed that breed had no effect on aflatoxin concentration in milk but diet had a significant effect. The correlation of urine and milk from the cows that had received diet with aflatoxins showed that there was no correlation for August, however strong positive correlation was observed for September and October (Figure 6.8). Table 16 shows the corresponding correlation coefficients.

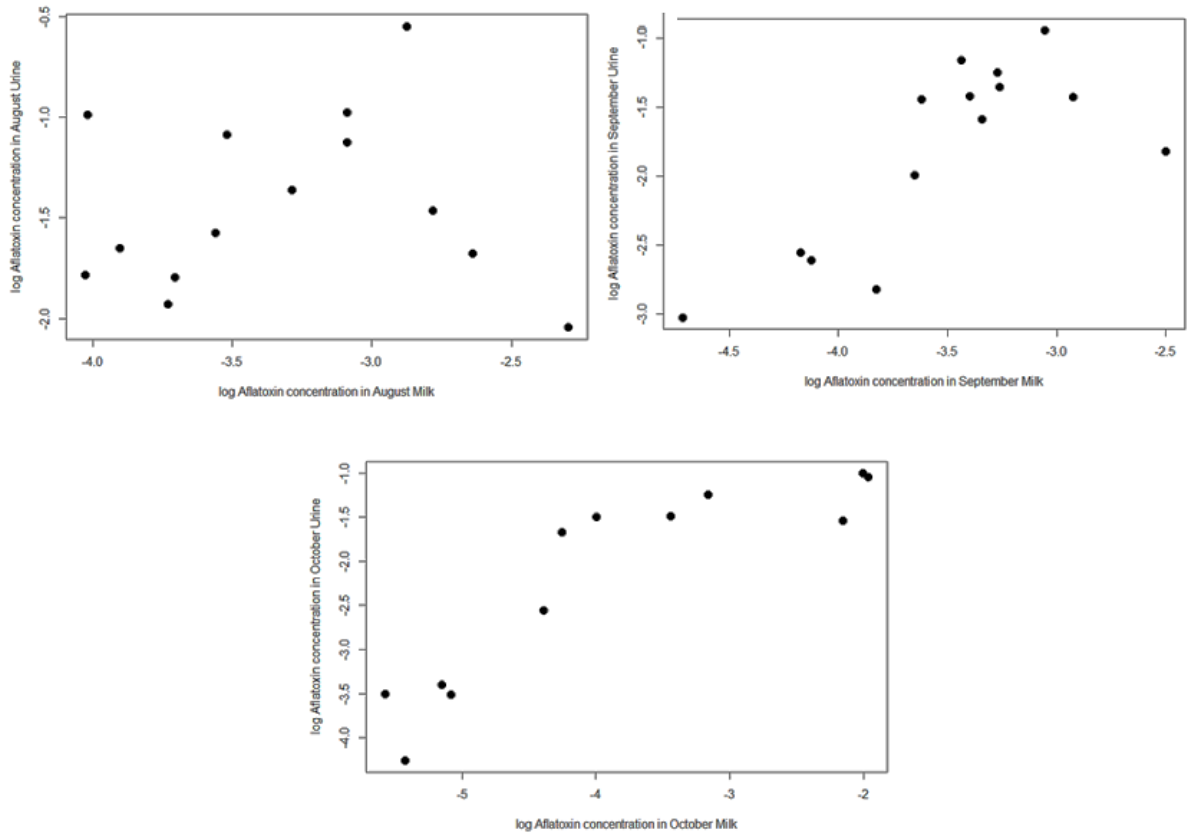

Figure 6.8. Correlation of AFM_1 in milk and urine. (a) no correlation was observed in August, (b) moderate positive correlation for September and (c) strong positive correlation for October

Table 16. Relationship of AFM_1 in urine and milk from cows receiving aflatoxin contaminated diets.

Month	Shapiro Wilk	Correlation used	Correlation coefficient	p-value	Significance
August	0.562	Pearson	0.044	0.881	insignificant
September	0.171	Pearson	0.757	0.002^{**}	significant
October	0.044	Spearman	0.916	$2.843\times10^{-5***}$	significant
Significance codes: At the 0.05 level. ***highly significant, **very significant, *significant.					

For August, normality was not violated (Shapiro test $p > 0.05$). Pearson's product-moment correlation, r =0.044 which is close to zero indicated that there was no relationship between AFM_1 concentrations in milk and urine for the month of August. In September, normality of data was not violated so Pearson's correlation was used and gave $r = 0.757$, a strong positive correlation between AFM_1 in milk and urine. Thus as AFM_1 concentration in milk increased there was also an increase in urine concentration. A highly significant positive Spearman's correlation $r_s = 0.916$ was also obtained for October.

September linear model for the effect of diet on AFM_1 concentration in milk was formulated using the general linear model equation. Effect of diet after taking into consideration AFM_1 concentration in August milk was modelled to give explanatory and predictive power of AFM_1 in milk for the month of September.

Model building and variable selection followed the basic equation of general linear models;

$\hat{y}_i = \hat{\mu} + \hat{\alpha}_i + \hat{\beta}x$

Where, in September

$\hat{y}_i$ is the estimated log AFM_1 concentration in September milk (log.WTSEPM) for the i^{th} DIET.

$\hat{\mu}$ is the estimated overall mean log. WTSEPM.

$\hat{\alpha}_i$ is the estimated effect of the i^{th} DIET.

$\hat{\beta}$ is the estimated coefficient for the linear regression of $\hat{y}_i$ on log.AFM_1 concentration in August milk (log.WTAUGM).

$$\text{log.WTSEPM} = -1.32103 + 0.61694 * \text{log.WTAUGM} + \begin{bmatrix} 0 & if\ DIET\ 1 \\ -0.57030 & if\ DIET\ 2 \\ 0.19335 & if\ DIET\ 3 \\ -0.05065 & if\ DIET\ 4 \end{bmatrix}$$

AFM_1 concentration in September urine therefore showed that the effect of diet was more significant when compared to the breed effect with $R^2 = 0.933$ and $p = 2.539 \times 10^{-5}$. Analysis of variance (ANOVA) for the relationship AFM_1 concentration in September urine and diet with breed as a covariate can be explained by the following equation;

$$\text{log.WTSEPU} = -1.80976 + \begin{bmatrix} 0 & if\ BREED\ H \\ 0.51894 & if\ BREED\ HJ \\ 0.29376 & if\ BREED\ J \end{bmatrix} + \begin{bmatrix} 0 & if\ DIET\ 1 \\ -0.94330 & if\ DIET\ 2 \\ -0.09578 & if\ DIET\ 3 \\ 0.41847 & if\ DIET\ 4 \end{bmatrix}$$

For October milk, it was shown that the effect of the covariate was significant ($p<0.05$. and $R^2= 0.719$). Prediction of AFM_1 concentration in the October milk was explained by the following equation;

$$\text{log.WTOCTM} = -2.1497 + 0.4816 * \text{log.WTSEPM} + \begin{bmatrix} 0 & if\ DIET\ 1 \\ -1.1318 & if\ DIET\ 2 \\ NA & if\ DIET\ 3 \\ 1.1251 & if\ DIET\ 4 \end{bmatrix}$$

For October urine AFM_1, inclusion of breed as a covariate once diet improved the model giving $R^2 = 0.852$ and $p < 0.05$. The concentration of AFM_1 for October was predicted using the following equation.

$$\log.WTOCTU = -2.0253059 + \begin{bmatrix} 0 & if\ BREED\ H \\ 0.5658611 & if\ BREED\ HJ \\ 0.0009399 & if\ BREED\ J \end{bmatrix} + \begin{bmatrix} 0 & if\ DIET\ 1 \\ -1.6430734 & if\ DIET\ 2 \\ NA & if\ DIET\ 3 \\ 0.7511145 & if\ DIET\ 4 \end{bmatrix}$$

6.4 Discussion

Feeds and their ingredients can be contaminated with aflatoxins resulting in the cows fed on such feed subsequently secreting AFM_1 in their milk and urine (Xiong *et al.*, 2015). Variation in the AFM_1 concentrations in milk depend on the type of feed, milk yield, lactation stage and the type of feed consumed by the cows but not all the ingested AFB_1 present in the feed is converted into milk AFM_1 (Masoero *et al.*, 2007; Becker-Algeri *et al.*, 2016b). A greater percentage of AFM_1 is excreted through the biliary pathway in faeces as well as in urine (Gallo, 2008; Britzi *et al.*, 2013). Other factors include health status of the cow, liver bio-transformation capacity and rate of ingestion as well as species among others.

From this study, the carryover average at 6.7% ranged from 0- 22% for milk while the urine carryover range was 0-63% and averaged at 34.4% range. These results were much higher than previously reported carryover rates (0.3 - 6.2 %) in milk (Masoero *et al.*, 2007; Britzi *et al.*, 2013). However, statistical analysis of the relationship between AFB_1 concentrations in the feeds and AFM_1 concentration in milk showed that there is a cumulative effect as the cows were fed with naturally contaminated feed on a daily basis. The previously reported carryover

rates focussed on cows that were given feed of known concentration which were monitored until AFM_1 cleared from their system. However, this study presents a field on-farm investigation that is farmer centred. Cumulative AFM_1 excretion in urine of 19% and 53% in rat faeces has been reported (Gallo, 2008) after having received an intravenous dose unlike in this study where they took the AFB_1 orally resulting in more aflatoxins being absorbed through the digestive system. Dalezios *et al.* (1973) reported a 40 and 42% AFM_1 cumulative excretion by male Rhesus monkeys in urine and faeces respectively. Some authors have indicated that carryover rates for cows being large ruminants are usually higher compared to small ruminants (Ronchi *et al.*, 2005; Battacone *et al.*, 2012) corroborating findings from this current study .

The effect of breed on the rate of AFB_1 biotransformation into AFM_1 was not significant $p>0.05$.The covariate effect which suggested cumulative secretion of AFM_1 in milk was significant with $p<0.05$. Breed effect was also not statistically significant for AFM_1 excretion into urine and the effect of the covariate was also not significant. MANOVA also showed that there was no significant differences in the way the three breeds secrete AFM_1 into milk suggesting that the breeds were the same. Therefore breed tends to have a random effect which does not really explain the presence of AFM_1 in both milk and urine.

However, there was significant evidence in ANCOVA in this study, that aflatoxin carryover in both milk and urine was affected by diet and not by breed ($p < 0.005$). This corroborates the findings of Frobish *et al.* (1986) who stated that the amount of AFB_1 converted into AFM_1 is affected by the level of aflatoxin contamination in the feed.

AFM_1 had a higher resident time in milk compared to urine as shown by an improved adjusted R^2 value for milk and a decrease in the adjusted R^2 in urine when the covariate was taken into account. This may be due to the fact that the cow had no control on the removal of milk from its udder but relies on the milking time determined by the farmer. This may result in a cumulative effect on the concentration of AFM_1 in milk. On the other hand, the farmer had no control on the release of urine by the cow. Several authors have reported different frequencies of urination by cows ranging from 2-19 times a day to 11-31 times a day (Vaughan, 2014; Edwards *et al.*, 2015). MANOVA had $p > 0.05$ indicating that there were no significant differences in the AFB_1 concentrations in the diets received by the animals meaning the concentration of urinary AFM_1 in the urine of the cows receiving the different diets was the same after inclusion of the covariate. This suggested that by the time urine was collected, most of the AFM_1 had been lost through urination and the effect of dietary AFB_1 on urinary AFM_1 became insignificant. Therefore AFM_1 concentration in urine can only be used as an indicator

in the monitoring of short term dietary AFB_1 exposure whereas AFM_1 concentration in milk suffice as a long term indicator.

6.5 Conclusion

Findings from this study have shown that breed is a random factor and cannot explain the presence of AFM_1 in both milk and urine. However, diet is a fixed factor that can explain the presence of AFM_1 in both milk and urine. For AFM_1 to be detected in milk and urine the cow must have consumed AFB_1 contaminated feed. Variation in the AFM_1 concentrations is affected by other biological factors which are not breed. These may include rate of digestion by the individual animals, health status of the cow among others. It was also shown that higher residence time of milk in the cow due to the fact that the farmer decides when to milk the cow, results in a cumulative effect on the concentration of AFM_1 in milk. However for urine excretion the farmer has no control, thus it was not possible for AFM_1 to accumulate in the urine as the cows had higher urination frequencies. Thus AFM_1 contamination in milk can be used to show long term exposure to contaminated feed whereas AFM_1 in urine can be only be used as an indicator of short term exposure to aflatoxin contaminated feed.

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Chapter 7

General discussion and conclusions

This research reports for the first time biodiversity of the genus *Aspergillus* and aflatoxin contamination of dairy feeds u in Bulawayo, Zimbabwe. The findings showed the presence of four sections of the *Aspergilli* with the predominating section being section *Nigri*. The report also showed that gene concatenation is a better tool in the analysis of evolutionary phylogenetics as it was possible to have isolates from the same season being clustered together. However, the area of sampling had no effect on the clustering of the isolates as it was possible to find isolates from all four areas examined in one cluster. This could be due to the fact that most of the toxigenic isolates came from the feed concentrates and mixed rations. The feeds were formulated from ingredients of several suppliers; therefore the farmers could have probably bought the feed concentrates or ingredients from the same suppliers. In addition, *AflJ* gene was the most prominent regulatory gene in the isolates from Bulawayo, Zimbabwe. Analysis of this gene and how it can be regulated can be useful in the control of aflatoxigenic

Aspergillus species found in Zimbabwe thereby circumventing the problems associated with aflatoxin contamination of food commodities.

Majority of the feed samples were contaminated by aflatoxins and 21% of the samples exceeded the regulatory limits. Mixed rations which were the most commonly used feed types by the farmers had the highest concentrations of aflatoxins due to the diverse ingredients used in their formulations indicating the need for quality control in the choice of the components to be used in formulating feed types. Most of the contaminated samples were from the rainy season; this indicates the importance of moisture control in the development of aflatoxins as the environment tends to be humid during the rainy seasons. It is therefore important that proper drying and storage of both feed ingredients as well as the feed be done. Higher percentage of milk contamination was noted in the dry season, this is the time when the animals are fed mainly on formulated feeds as pastures available are not sufficient. Although contamination of the milk samples were within the stipulated regulatory limits, the population is exposed to chronic aflatoxicosis which usually manifests in the form of cancers and other health related effects. Multitoxin occurrence in both feed and milk is also an indicator to the regulatory bodies to come up with standard limits for other mycotoxins other than the major ones.

Statistics analyses indicated that the breeds had no effect on the secretion and excretion of AFM_1 into milk and urine respectively. However, the effect of diet was significant thus diet is significant in the contamination of milk and urine by aflatoxins. There was cumulative secretion of AFM_1 into milk as against urine. These findings will help scientist in the development of rapid test kits for AFM_1 in milk and urine which can be used by farmers in the detection of short-term and long-term exposure of their animals to aflatoxin contaminated feed.. This will go a long way in helping aflatoxin management at farm level.

7.1 Limitations and recommendations

The analysis of association of bovine breed on aflatoxin carryover was farmer centred with the farmer making the decisions in the selection of which animals to include in the study. This resulted in cows in different lactation stages and uneven distribution of the breeds. A controlled study with equal numbers of the different breeds in the same lactation stage is recommended for the determination of random factors that may affect aflatoxin carryover into milk. Moreover labelling of the aflatoxins will also help in the follow up of the metabolites in the milk. Farmers are encouraged to send their workers for workshops that educate them on effects of aflatoxin

and aflatoxin management as a way of reducing contamination of feed and milk. This will help the personnel in appreciating proper storage of feeds which eliminates moisture pick up by the feeds thus reducing mould contamination and subsequent aflatoxin production.

7.2 Conferences and Publications

Poster presentation

Nleya, N., Ngoma, L. & Mwanza, M. 2018. Biodiversity of *Aspergillus* species and aflatoxin contamination of dairy feeds from farms around Bulawayo, Zimbabwe. The World Mycotoxin Forum – 10th Conference 12-14 March, 2018, Amsterdam, The Netherlands.

Publications

Nleya, N., Adetunji, M. & Mwanza, M. 2018. Current Status of Mycotoxin Contamination of Food Commodities in Zimbabwe. *Toxins*, 10(5):

DOI: http//www.doi.org/10.3390/toxins10050089

Book Chapter

Nleya, N., Ngoma, L. & Mwanza, M. 2019. Aflatoxin Occurrence in Dairy Feeds: A Case of Bulawayo, Zimbabwe. Aflatoxin B_1 Occurrence, Detection and Toxicological Effects. IntechOpen.

DOI: 10.5772/intechopen.88582

Appendices

APPENDIX I. GPS coordinates for the farms sites.

Farm	Lattitude	Longitude
1	-20.280686	28.44128
2	-20.275702	28.445221
3	-20.272304	28.424747
4	-19.995222	28.508857
5	-20.238809	28.741986
6	-20.24878	28.742352
7	-20.25287	28.747754
8	-20.253577	28.751954
9	-20.024748	28.668077
10	-20.02284	28.667102
11	-20.00492	28.672576
12	-20.018589	28.466064
13	-19.980495	28.451336

APPENDIX II. Cultural and molecular characterisation of Aspergillus species from the feeds.

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued)

Isolate	YES	β -CNRDCA		NRDCA		*nor (aflD)*	*ver (aflM)*	*omt (aflP)*	*aflR*	*aflJ*	Organism	Aflatoxin production	Accession number
	Pink colour	Yellow ring	Flourescnce	Yellow ring	Flourescnce						Based on ITS sequences		
NR32	-	-	-	-	-	+	-	+	-	-	*Aspergillus fumigatus*	NP	MH270529
NR33	+	+	+	+	+	+	-	-	-	-	*Aspergillus niger*	PP	MH270530
NR34	+	+	-	- grey	-	+	+	+	+	+	*Aspergillus flavus*	P	MH270531
NR35	+	+	-	- grey	-	+	-	+	+	+	*Aspergillus fumigatus*	P	MH270534
NR36	-	-	-	-	-	+	-	-	-	+	*Aspergillus fumigatus*	NP	MH270535
NR37	+	+	+	grey	+	-	-	+	-	-	*Aspergillus flavus*	PP	MH270538
NR38	-	-	-	-	-	+	-	-	-	+	*Aspergillus parvisclerotigenus*	NP	MH270539
NR39	-	-	-	-	-	-	-	+	-	-	*Aspergillus phoenicis*	NP	MH270567
NR12	-	+	+	-	+	+	-	-	-	-	*Aspergillus fumigatus*	NP	MH270540
NR40	-	-	-	- grey	-	+	+	+	+	+	*Aspergillus*	PP	MH270568
NR14	-	-	-	-	-	+	-	-	-	-	*Aspergillus tubingensis*	NP	MH270542
NR41	-	-	-	-	-	+	-	+	-	+	*Aspergillus fumigatus*	NP	MH270543
NR43	-	-	-	- grey	-	+	-	+	-	-	*Aspergillus flavus*	NP	MH270544
NR44	-	-	+	-	+	+	-	+	-	+	*Aspergillus fumigatus*	PP	MH270545
NR46	+	+	+	+	+	+	+	+	-	+	*Aspergillus flavus*	P	MH270546
NR47	+	+	+	grey	+	+	-	+	-	+	*Aspergillus flavus*	NP	MH270548
NR49	-	-	+	-	+	+	+	+	-	-	*Aspergillus phoenicis*	PP	MH270550
NR50	-	-	-	-	-	+	+	+	+	+	*Aspergillus flavus*	P	MH270552
NR51	-	-	+	-	+	+	-	+	+	+	*Aspergillus phoenicis*	PP	MH270553
NR52	-	-	-	-	-	+	-	-	-	-	*Aspergillus penicillioides*	NP	MH270554
NR53	-	-	-	-	-	-	-	-	-	-	*Aspergillus flavus*	NP	MH270555
NR54	-	-	+	-	+	+	-	-	-	-	*Aspergillus fumigatus*	NP	MH270556
NR57	+	+	+	+	+	+	-	-	-	-	*Aspergillus niger*	PP	MH270557
NR58	+	+	+	+	+	+	-	+	+	+	*Aspergillus flavus*	P	MH270559

NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued).

Isolate	**YES**	**B-CNRDCA**		**NRDCA**		***nor (aflD)***	***ver (aflM)***	***omt (aflP)***	***aflR***	***aflJ***	**Organism**	**Aflatoxin production**	**Accession number**
	Pink colour	**Yellow ring**	**Flourescnce**	**Yellow ring**	**Flourescnce**						**Based on ITS sequences**		
NR59	-	-	+	-	+	-	-	+	-	-	*Aspergillus fumigatus*	NP	MH270587
NR62	-	-	+	-	+	+	-	+	-	-	*Aspergillus fumigatus*	PP	MH270590
NR63	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MH270591
NR65	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MH270593
NR66	+	+	+	+	+	+	+	+	+	-	*Aspergillus oryzae*	P	MH270594
NR67	+	+	-	+	+	-	-	+	-	-	*Aspergillus japonicus*	NP	MH270595
NR68	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MH270596
NR69	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MH270597
NR70	+	+	-	+	-	-	+	+	-	-	*Aspergillus oryzae*	PP	MH270598
NR71	-	-	-	-	-	+	-	-	-	-	*Aspergillus fumigatus*	NP	MH270599
NR72	+	+	-	+	-	+	+	+	-	-	*Aspergillus nomius*	P	MH270600
NR73	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MH270601
NR74	-	-	-	-	-	+	-	-	-	-	*Aspergillus fumigatus*	NP	MH270602
NR75	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MH270603
NR76	-	-	+	-grey	+	+	-	-	-	-	*Aspergillus fumigatus*	NP	MH270604
ND99	+	+	+	+	+	+	+	+	+	+	*Aspergillus flavus*	P	MH270605
ND100	-	-	-	-	-	-	-	-	-	-	*Aspergillus chevalieri*	NP	MH270606
ND102	-	-	-	-	-	+	-	+	-	-	*Aspergillus fumigatus*	NP	MH270608
ND103	+	-	-	-	-	-	+	+	-	+	*Aspergillus flavus*	P	MH270609
ND104	-	-	-	-	-	-	-	-	-	-	*Aspergillus sydowii*	NP	MH270610
ND106	+	+	-	+	-	-	-	-	-	-	*Aspergillus flavus*	NP	MH270612
ND107	-	-	-	-	-	+	-	-	-	+	*Aspergillus brasiliensis*	NP	MH270613
ND109	-	-	-	-	-	-	-	-	-	-	*Aspergillus flavus*	NP	MH270615

NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued).

Isolate	**YES**	**β-CNRDCA**		**NRDCA**		***nor (aflD)***	***ver (aflM)***	***omt (aflP)***	***aflR***	***aflJ***	**Organism**	**Aflatoxin production**	**Accession number**
	Pink colour	**Yellow ring**	**Flourescnce**	**Yellow ring**	**Flourescnce**						**Based on ITS sequences**		
ND1	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659595
ND2	+	+	-	+	-	+	-	-	-	-	*Aspergillus tubingensis*	NP	MG659596
ND3	-	-	-	-	-	-	-	-	-	-	*Aspergillus awamori*	NP	MG659597
ND4	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659598
ND5	-	-	-	-	-	-	-	-	-	-	*Aspergillus tubingensis*	NP	MG659599
ND6	-	-	-	-	-	-	-	-	-	-	*Aspergillus awamori*	NP	MG659600
ND7	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659601
ND8	-	-	-	-	-	+	-	-	-	-	*Aspergillus tubingensis*	NP	MG659602
ND9	+	-	-	-	-	-	-	-	-	-	*Aspergillus tubingensis*	NP	MG659603
ND10	-	+	-	+	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659604
ND11	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659605
ND12	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659606
ND13	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659607
ND20	-	-	-	-	-	+	-	-	-	-	*Aspergillus fumigatus*	NP	MG659614
ND21	-	-	-	-	-	+	-	-	-	-	*Aspergillus fumigatus*	NP	MG659615
ND25	+	+	-	+	-	-	-	-	-	-	*Aspergillus flavus*	NP	MG659619
ND26	+	+	+	+	+	+	-	+	+	+	*Aspergillus flavus*	P	MG659620
ND27	+	+	+	+	+	+	+	+	+	+	*Aspergillus nomius*	P	MG659621
ND28	-	-	-	-	-	+	+	-	-	-	*Aspergillus flavus*	NP	MG659622
ND29	+	+	+	+	+	+	-	+	+	+	*Aspergillus oryzae*	P	MG659623
ND30	+	+	+	+	+	+	-	+	+	+	*Aspergillus flavus*	P	MG659624
ND31	+	+	+	+	+	+	+	-	-	+	*Aspergillus flavus*	P	MG659625
ND32	+	+	+	+	+	+	-	+	-	-	*Aspergillus parasiticus*	P	MG659626

NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued).

Isolate	**YES**	**β-CNRDCA**		**NRDCA**		***nor* (*aflD*)**	***ver* (*aflM*)**	***omt* (*aflP*)**	***aflR***	***aflJ***	**Organism**	**Aflatoxin production**	**Accession number**
	Pink colour	**Yellow ring**	**Flourescnce**	**Yellow ring**	**Flourescnce**						**Based on ITS sequences**		
ND33	+	+	+	+	+	+	+	+	+	+	*Aspergillus flavus*	P	MG659627
ND34	+	+	+	+	+	+	-	+	+	+	*Aspergillus flavus*	P	MG659628
ND35	+	+	+	+	+	+	-	+	-	+	*Aspergillus oryzae*	P	MG659629
ND36	+	+	+	+	-	-	-	+	-	-	*Aspergillus flavus*	P	MG659630
ND37	+	+	+	+	+	+	+	+	-	+	*Aspergillus flavus*	P	MG659631
ND38	+	+	+	+	+	+	+	-	+	+	*Aspergillus flavus*	P	MG659632
ND39	+	+	+	+	+	+	-	-	-	+	*Aspergillus oryzae*	P	MG659633
ND40	+	+	+	+	+	+	+	+	+	+	*Aspergillus flavus*	P	MG659634
ND41	+	+	+	+	+	+	+	+	+	+	*Aspergillus flavus*	P	MG659635
ND44	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659638
ND45	-	-	-	-	-	+	-	-	-	-	*Aspergillus cristatus*	NP	MG659639
ND51	+	+	+	+	+	+	-	+	-	+	*Aspergillus flavus*	P	MG659645
ND52	+	+	+	+	+	+	-	-	-	+	*Aspergillus flavus*	P	MG659646
ND54	-	-	-	-	-	+	-	-	-	-	*Aspergillus awamori*	NP	MG659648
ND55	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659649
ND56	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659650
ND57	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659651
ND58	-	-	-	-	-	+	-	-	-	+	*Aspergillus niger*	NP	MG659652
ND59	+	+	+	+	+	+	-	-	-	+	*Aspergillus flavus*	NP	MG659653
ND60	-	-	-	-	-	-	-	-	-	-	*Aspergillus awamori*	NP	MG659654
ND61	-	-	-	-	-	+	-	-	-	-	*Aspergillus fumigatus*	NP	MG659655
ND62	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659656
ND63	+	+	-	+	-	-	-	-	-	-	*Aspergillus flavus*	NP	MG659657

NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued).

Isolate	**YES**	**β-CNRDCA**		**NRDCA**		***nor (aflD)***	***ver (aflM)***	***omt (aflP)***	***aflR***	***aflJ***	**Organism**	**Aflatoxin production**	**Accession number**
	Pink colour	**Yellow ring**	**Flourescnce**	**Yellow ring**	**Flourescnce**						**Based on ITS sequences**		
ND64	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659658
ND65	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659659
ND67	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659661
ND68	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659662
ND69	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659663
ND71	-	-	-	-	-	+	-	-	-	-	*Aspergillus niger*	NP	MG659665
ND73	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659667
ND74	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659668
ND75	-	+	-	+	-	+	-	+	-	+	*Aspergillus flavus*	P	MG659669
ND76	-	+	-	+	-	+	-	+	-	-	*Aspergillus flavus*	P	MG659670
ND77	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659671
ND78	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659672
ND79	+	+	+	+	+	-	-	+	-	-	*Aspergillus flavus*	PP	MG659673
ND80	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659674
ND81	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659675
ND82	-	-	-	-	-	-	+	+	+	+	*Aspergillus flavus*	P	MG659676
ND83	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659677
ND84	-	-	-	-	-	-	-	-	-	-	*Aspergillus tubigensis*	NP	MG659678
ND85	-	-		-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659679
ND86	-	-	-	-	-	-	-	-	-	-	*Aspergillus foetidus*	NP	MG659680
ND87	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659681
ND88	-	-	-	-	-	-	-	-	-	-	*Aspergillus fumigatus*	NP	MG659682
ND89	-	-	-	-	-	-	-	-	-	+	*Aspergillus niger*	NP	MG659683

NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

Appendix II. Cultural and molecular characterisation of *Aspergillus* species from the feeds (continued).

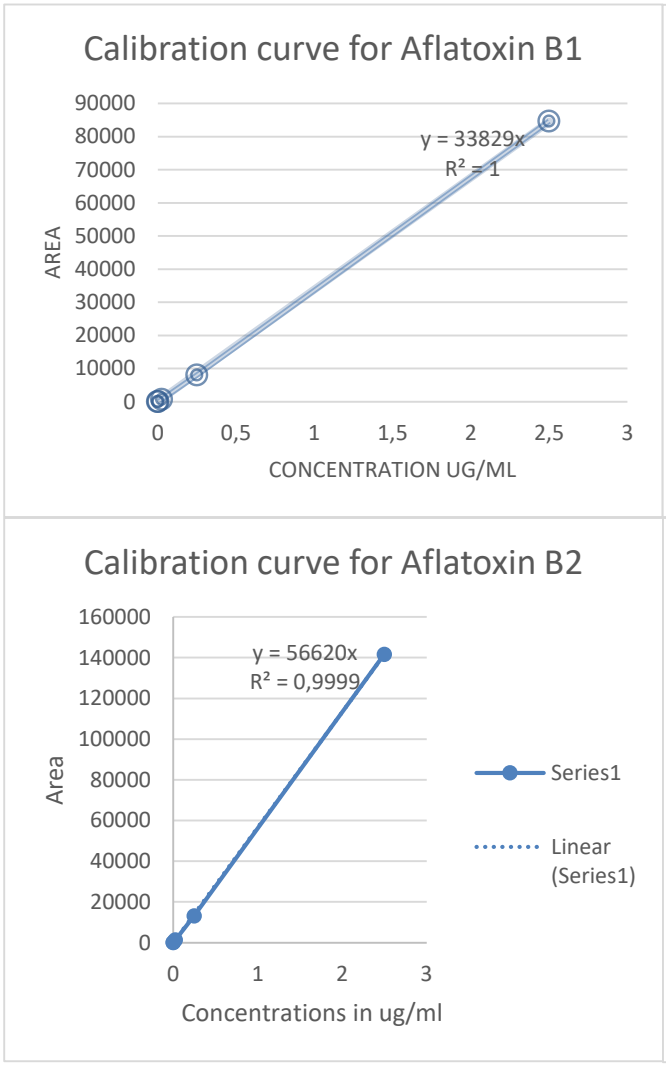
Isolate	**YES**	**β-CNRDCA**		**NRDCA**		***nor (aflD)***	***ver (aflM)***	***omt (aflP)***	***aflR***	***aflJ***	**Organism**	**Aflatoxin production**	**Accession number**
	Pink colour	**Yellow ring**	**Flourescnce**	**Yellow ring**	**Flourescnce**						**Based on ITS sequences**		
ND90	-	-	+	-	+	-	-	-	-	+	*Aspergillus flavus*	PP	MG659684
ND93	+	+	+	+	+	-	-	-	+	+	*Aspergillus parasiticus*	P	MG659687
ND94	-	-	-	-	-	-	-	-	-	-	*Aspergillus tubingensis*	NP	MG659688
ND96	+	+	+	+	+	-	+	+	+	+	*Aspergillus oryzae*	P	MG659690
ND97	-	-	-	-	-	-	-	-	-	-	*Aspergillus niger*	NP	MG659691
ND98	-	-	-	-	-	-	_	-	-	-	*Aspergillus flavus*	NP	MG659692

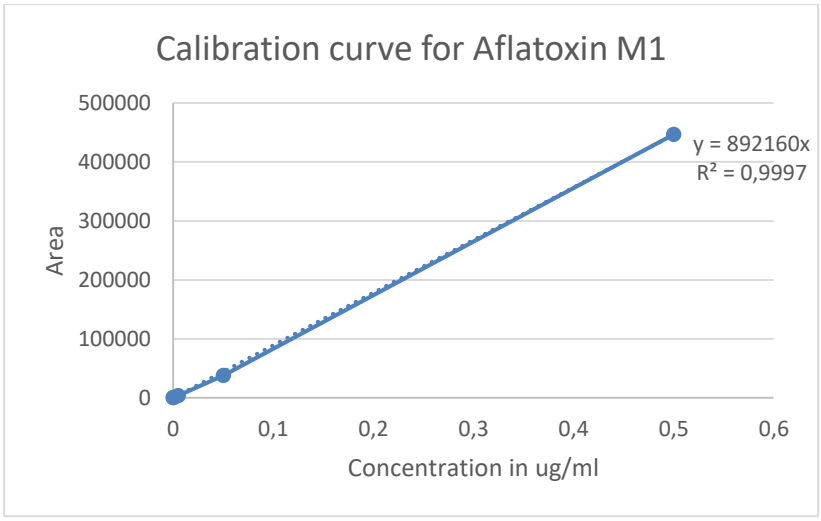
NR-isolates from the rainy season, ND-isolates from the dry season, YES-yeast extract sucrose agar, NRDCA-neutral red desiccated coconut agar, β-CNRDCA-neutral red desiccated coconut agar with β-cyclodextrin, P-aflatoxin producer, PP-potential aflatoxin producer, NP-non aflatoxin producer

APPENDIX III. Farmer survey responses

Farmer	Plot size (acres)	Herd size	Number of cows	Number milked	Age of cows	Breeds	Milking stage	Milking method	Milk volume/day (liters)	Milk volume/cow (litres)	Feed Type	Feed/day/animal (kg)	Aflatoxin knowledge	Milkings (per day)
SI-1	>21	>21	>21	>21	>25months	Jersey &Holstein	Mid	Hand	>21	6 t0 10	Commercial + grazing	0 t0 5	yes	2
SI-2	6 to 20	>21	11 to 20	1 to 10	>25months	Jersey &Holstein		Hand	>21	7 t0 10	Commercial + grazing	6 t0 10	no	2
I-1	>21	>21	>21	>21	>25months	Jersey &Holstein	Across all ages	Machine	>21	≥11	Silage+ Ration	≥11	yes	2
I-2	>21	>21	>21	>21	>25months	Holstein	Across all ages	Machine	>21	≥11	Commercial + grazing	≥11	yes	3
SI-3	>21	>21	>21	11 to 20		Jersey &Holstein	late	Hand		6 t0 10 & ≥11	Commercial + grazing		no	2
SI4	>21	>21	>21	11 to 20	>25months	Ayrshire/Jersey	Across all ages	Hand	>21	≥11	Commercial + grazing	≥11	yes	2
SI-5	6 to 20	1 to 10	1 to 10	2	16 24 months	Holstein	Early	Hand	11 to 20	6 t0 10	Commercial + grazing	6 t0 10	no	2
SI-6	>21	>21	11 to 20	11 to 20	>25months	Jersey &Holstein	late	Hand	>21	≥11	Commercial + grazing		no	2
I-3	>21	>21	>21	>21	>25months	Jersey &Holstein	Across all ages	Machine	>21	6 t0 10	Silage+ Ration	≥11	no	2
SI-7	>21	>21	>21	>21	>25months	Jersey &Holstein	Across all ages	Hand	>21	6 t0 10	Commercial + grazing	6 t0 10	no	2
E-1	>21	11 to 20	11 to 20	1 to 10	>25months	Jersey &Holstein	Early	Hand	>21	6 t0 10	Grazing		no	2
E-2	>21	>21	>21	1 to 10	10 t0 15 months	Thuli Brahman	late	Hand	11 to 20	0 to 5	Grazing		yes	1
E-3	>21	>21	11 to 20	1 to 10	>25months	Brahman	Mid	Hand	1 to 10	0 to 5	Grazing		no	1
E-4	>21	>21	>21	11 to 20		Brahman	late	Hand	>21	0 to 5	Grazing		no	1

APPENDIX IV. Calibration curves for aflatoxin standards.





APPENDIX V. Aflatoxin distribution in feeds.

		Aflatoxin concentration (µg/kg ×10^{-3})				Total aflatoxin concentration (µg/kg)
Sample ID	Feed type	AFG_2	AFG_1	AFB_2	AFB_1	Total Aflatoxin
F1	Mixed Ration	465	155	26	1139	1.8
F2	Feed Concentrate	715	2240	9960	49	13.0
F3	Mixed Ration	3868	252	2224	1390	7.7
F4	Mixed Ration	13099	9235	29033	53	51.4
F5	Feed Concentrate	1443	530	1289	7335	10.6
F6	Feed Concentrate	92	111	410	178	0.8
F7	Feed Concentrate	53	1372	5152	16	6.6
F8	Feed Concentrate	78876	38687	132795	564	250.9
F9	Feed Concentrate	978	5291	18998	12	25.3
F10	Feed Concentrate	93	1610	6015	70	7.8
F11	Mixed Ration	3498	17999	59244	82	80.8
F12	Mixed Ration	1085	1102	14640	6136	23.0
F13	Feed Concentrate	6505	9333	25363	80	41.3
F14	Feed Concentrate	1548	1274	5833	1262	9.9
F15	Feed Concentrate	4748	2122	7681	366	14.9
F16	Mixed Ration	1885	2000	5829	933	10.6
F17	Feed Concentrate	5536	3311	9137	48	18.0
F18	Feed Concentrate	33	72	294	54	0.5
F19	Feed Concentrate	4	531	1640	67	2.2
F20	Feed Concentrate	1053	6101	18566	384	26.1
F21	Feed Concentrate	6499	3076	9093	335	19.0
F22	Mixed Ration	13869	8789	28139	635	51.4
F23	Mixed Ration	4729	9438	34152	148	48.5
F24	Mixed Ration	17324	4975	16952	599	39.8
F25	Brewer's Spent Grain	22	230	238	52	0.5
F26	Feed Concentrate	19786	1469	5751	529	27.5
F27	Grass	370	0	18	369	0.8
F28	Grass	60	11	118	214	0.4
F29	Grass	18	27	38	21	0.1
F30	Feed Concentrate	74	171	883	196	1.3
F31	Grass	218	0	169	21	0.4
F32	Mixed Ration	4199	2965	9378	379	16.9
F33	Feed Concentrate	246	525	189	885	1.8
F34	Grass	15	50	48	46	0.2
F35	Grass	89	4	21	83	0.2
F36	Grass	38	23	16	34	0.1
F37	Mixed Ration	475	711	1951	651	3.8
F38	Mixed Ration	232	179	1121	41	1.6
F39	Mixed Ration	21	166	577	34	0.8
F40	Mixed Ration	271	76	35	174	0.6
F41	Mixed Ration	33	60	140	72	0.3

APPENDIX V. Aflatoxin distribution in feeds (cont).

		Aflatoxin concentration (µg/kg ×10^{-3})				Total aflatoxin concentration (µg/kg)
Sample ID	Feed type	AFG_2	AFG_1	AFB_2	AFB_1	Total Aflatoxin
F42	Mixed Ration	51	98	558	34	0.7
F43	Mixed Ration	189	97	416	134	0.8
F44	Mixed Ration	1237	595	2892	100	4.8
F45	Mixed Ration	511	116	417	164	1.2
F46	Brewer's Spent Grain	856	714	4573	671	6.8
F47	Brewer's Spent Grain	196	166	185	9	0.6
F48	Brewer's Spent Grain	292	1199	3057	1322	5.9
RF1	Feed Concentrate	299	181	7576	34174	42.2
RF2	Feed Concentrate	88	121	298	2361	2.9
RF3	Mixed Ration	7586	13514	16251	138678	176.0
RF4	Mixed Ration	1809	1253	1224	8083	12.4
RF5	Brewer's Spent Grain	59	0	55	182	0.3
RF6	Mixed Ration	15606	24040	13318	112650	165.6
RF7	Feed Concentrate	9875	17166	4715	47749	79.5
RF8	Feed Concentrate	268	2	283	1853	2.4
RF9	Feed Concentrate	349	520	5206	42044	48.1
RF10	Mixed Ration	383	588	346	2853	4.2
RF11	Mixed Ration	470	508	4198	107115	112.3
RF12	Mixed Ration	1598	152	24942	149589	176.3
RF13	Feed Concentrate	2130	1386	9004	84383	96.9
RF14	Mixed Ration	1599	1589	5017	875	9.1
RF15	Mixed Ration	11	28	61	404	0.5
RF16	Feed Concentrate	7	73	29	68	0.2
RF17	Feed Concentrate	89	213	1158	142	1.6
RF18	Mixed Ration	34	52	56	815	1.0
RF19	Feed Concentrate	2207	170	163	2115	4.7
RF20	Mixed Ration	315	12	4	488	0.8
RF21	Feed Concentrate	5019	6306	6112	48002	65.4
RF22	Feed Concentrate	83	256	464	3203	4.0
RF23	Mixed Ration	252	234	1156	23385	25.0
RF24	Feed Concentrate	390	1086	3276	542	5.3
RF25	Feed Concentrate	20	74	489	4944	5.5
RF26	Feed Concentrate	15	102	143	1454	1.7
RF27	Grass	23	0	7	7	0.0
RF28	Grass	20	0	31	92	0.1
RF29	Grass	16	5	85	30	0.1
RF30	Mixed Ration	7	6	195	2801	3.0
RF31	Mixed Ration	10	14	131	1845	2.0
RF32	Mixed Ration	134	103	38	486	0.8
RF33	Mixed Ration	203	46	273	2104	2.6
RF34	Mixed Ration	344	315	178	4743	5.6

APPENDIX V. Aflatoxin distribution in feeds (cont).

		Aflatoxin concentration (µg/kg ×10^{-3})				Total aflatoxin concentration (µg/kg)
Sample ID	Feed type	AFG_2	AFG_1	AFB_2	AFB_1	Total Aflatoxin
RF35	Brewer's Spent Grain	272	455	1935	0	2.7
RF36	Brewer's Spent Grain	109	14	645	106	0.9
RF37	Grass	0	0	0	0	0.0
RF38	Grass	21	9	10	44	0.1
RF39	Grass	9	0	33	37	0.1
RF40	Grass	271	76	35	174	0.6
RF41	Grass	3	82	101	143	0.3
RF42	Grass	659	0	127	249	1.0
RF43	Grass	20	0	0	54	0.1
RF44	Mixed Ration	9	5	17	0	0.0
RF45	Grass	15	5	29	10	0.1
RF46	Grass	1	36	5	65	0.1
RF47	Grass	3	1	13	2	0.0
RF48	Grass	0	0	0	0	0.0